

(19) 日本国特許庁(JP)

## (12) 公表特許公報(A)

(11) 特許出願公表番号

特表2004-501605

(P2004-501605A)

(43) 公表日 平成16年1月22日(2004.1.22)

(51) Int. Cl. <sup>7</sup>	F I	テーマコード (参考)
C 1 2 N 15/09	C 1 2 N 15/00	4 B 0 2 4
A 6 1 K 38/00	A 6 1 K 39/395	4 B 0 6 3
A 6 1 K 39/395	A 6 1 P 1/00	4 B 0 6 5
A 6 1 P 1/00	A 6 1 P 11/00	4 C 0 8 4
A 6 1 P 11/00	A 6 1 P 15/00	4 C 0 8 5
	審査請求 未請求 予備審査請求 有 (全 159 頁)	最終頁に続く

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(87) 国際公開日	平成13年8月23日 (2001.8.23)		
(31) 優先権主張番号	09/506,079		
(32) 優先日	平成12年2月16日 (2000.2.16)		
(33) 優先権主張国	米国 (US)		

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(54) 【発明の名称】 HER-2 に結合するアンタゴニスト

## (57) 【要約】

HER-2 を過剰発現するガンを治療するための薬理的組成物であって、(a) 配列 I D 番号 1 または配列 I D 番号 1 2 の配列から取られた約 50 ~ 79 個のアミノ酸を含み、HER-2 の細胞外領域 (ECD) に少なくとも  $10^8$  のアフィニティで結合する単離ポリペプチド、(b) 配列 I D 番号 2 または配列 I D 番号 1 3 の配列から取られた約 300 ~ 419 個のアミノ酸を含み、C 末端の 79 個のアミノ酸が存在し、少なくとも 3 つの N 結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、(c) HER-2 の ECD と結合するモノクローナル抗体、(d) これらの組み合わせ、からなるグループの中から選択した薬剤と、薬理的に受容可能な基剤とを含むが、上記薬剤がモノクローナル抗体のみからなることはありえないという薬理的組成物が開示されている。予後予測方法および診断方法も開示されている。

## 【特許請求の範囲】

## 【請求項 1】

配列 I D 番号 1 または配列 I D 番号 1 2 の配列から取られた約 5 0 ~ 7 9 個のアミノ酸を含み、H E R - 2 の細胞外領域 E C D に少なくとも  $10^8$  のアフィニティで結合する単離ポリペプチド。

## 【請求項 2】

長さがアミノ酸約 6 9 ~ 7 9 個である、請求項 1 に記載の単離ポリペプチド。

## 【請求項 3】

H E R - 2 の E C D 上の部位のうち、ハーセプチン（登録商標）（H E R - 2 の E C D と結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体）の結合部位とは異なる部位に結合する、請求項 1 に記載の単離ポリペプチド。

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## 【請求項 4】

発現したときに、配列 I D 番号 1 または配列 I D 番号 1 2 の配列から取られた約 5 0 ~ 7 9 個のアミノ酸を含み、H E R - 2 の細胞外領域 E C D に少なくとも  $10^8$  のアフィニティで結合するポリペプチドをコードしている単離 D N A 配列。

## 【請求項 5】

長さがアミノ酸約 6 9 ~ 7 9 個である、発現したときに請求項 4 のポリペプチドをコードしている単離 D N A 配列。

## 【請求項 6】

H E R - 2 の E C D 上の部位のうち、ハーセプチン（登録商標）の結合部位とは異なる部位に結合する、請求項 4 に記載の単離 D N A 配列。

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## 【請求項 7】

発現したときに、配列 I D 番号 1 の配列から取られた約 5 0 ~ 7 9 個のアミノ酸を含み、H E R - 2 の細胞外領域 E C D に少なくとも  $10^8$  のアフィニティで結合するポリペプチドをコードしている D N A 配列を有する発現ベクターを含む、トランスフェクションされた細胞。

## 【請求項 8】

配列 I D 番号 2 または配列 I D 番号 1 3 の配列から取られた約 3 0 0 ~ 4 1 9 個のアミノ酸を含み、C 末端の 7 9 個のアミノ酸が存在し、少なくとも 3 つの N 結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド。

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## 【請求項 9】

長さがアミノ酸約 3 5 0 ~ 4 1 9 個で、N 結合型グリコシル化部位が 4 つ存在している、請求項 8 に記載のグリコシル化された単離ポリペプチド。

## 【請求項 10】

H E R - 2 の E C D 上の部位のうち、ハーセプチン（登録商標）の結合部位とは異なる部位に結合する、請求項 8 のグリコシル化された単離ポリペプチド。

## 【請求項 11】

発現したときに、配列 I D 番号 2 または配列 I D 番号 1 3 の配列から取られた約 8 0 ~ 4 1 9 個のアミノ酸を含み、C 末端の 7 9 個のアミノ酸が存在し、少なくとも 3 つの N 結合型グリコシル化部位が存在している、グリコシル化されたポリペプチドをコードしている単離 D N A 配列。

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## 【請求項 12】

長さがアミノ酸約 3 5 0 ~ 4 1 9 個で、N 結合型グリコシル化部位が 4 つ存在している、発現したときに請求項 11 に記載のポリペプチドをコードしている単離 D N A 配列。

## 【請求項 13】

配列 I D 番号 2 または配列 I D 番号 1 3 の配列から取られた約 8 0 ~ 4 1 9 個のアミノ酸を含み、C 末端の 7 9 個のアミノ酸が存在し、少なくとも 3 つの N 結合型グリコシル化部位が存在している、ポリペプチドをコードしている D N A 配列を有する発現ベクターを含む、トランスフェクションされた細胞。

## 【請求項 14】

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HER-2の過剰発現を特徴とする固形ガンを治療するためにHER-2の細胞外領域(ECD)と結合する薬剤を投与する操作を含む方法であって、この薬剤を、(a)配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも10<sup>8</sup>のアフィニティで結合する単離ポリペプチド、(b)配列ID番号2または配列ID番号13の配列から取られた約80~419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、(c)HER-2のECDと結合するモノクローナル抗体、(d)これらの組み合わせ、からなるグループの中から選択するが、この薬剤がモノクローナル抗体のみからなるものではない、上記方法。

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【請求項15】

HER-2を過剰発現する固形ガンを、乳ガン、肺小細胞ガン、卵巣ガン、大腸ガンからなるグループの中から選択する、請求項14に記載の方法。

【請求項16】

上記薬剤が、配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドである、請求項14に記載の方法。

【請求項17】

上記薬剤が、配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合するモノクローナル抗体の組み合わせである、請求項16に記載の方法。

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【請求項18】

HER-2を過剰発現する固形ガンを治療するための薬理的組成物であって、(a)配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも10<sup>8</sup>のアフィニティで結合する単離ポリペプチド、(b)配列ID番号2または配列ID番号13の配列から取られた約80~419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、(c)HER-2のECDと結合するモノクローナル抗体、(d)これらの組み合わせ、からなるグループの中から選択した薬剤と、薬理的に受容可能な基剤とを含むが、上記薬剤がモノクローナル抗体のみからなるものではない、上記薬理的組成物。

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【請求項19】

上記薬剤が、配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドである、HER-2を過剰発現する固形ガンを治療するための請求項18に記載の薬理的組成物。

【請求項20】

上記薬剤が、配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合するモノクローナル抗体の組み合わせである、HER-2を過剰発現する固形ガンを治療するための請求項19に記載の薬理的組成物。

【請求項21】

HER-2の過剰発現を特徴とする固形ガン組織に治療薬を到達させる方法であって、この治療薬を、配列ID番号1または配列ID番号12の配列から取られた約50~79個のアミノ酸を含み、HER-2の細胞外領域(ECD)に少なくとも10<sup>8</sup>のアフィニティで結合する単離ポリペプチドに付着させる操作を含む方法。

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【請求項22】

長さがアミノ酸約69~79個である、固形ガン組織に治療薬を到達させる請求項21に記載の方法。

【請求項23】

上記単離ポリペプチドが、HER-2のECD上の部位のうち、ハーセプチン(登録商標)の結合部位とは異なる部位に結合する、固形ガン組織に治療薬を到達させる請求項21

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に記載の方法。

【請求項 24】

HER-2を過剰発現するガンの患者におけるガン治療の予後を予測する方法であって、  
(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、  
絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択したから体液サン  
プルを取得し、(b)ELISA、免疫沈降法、免疫組織化学法、ウエスタンブロット解  
析法からなるグループの中から選択して抗p68HER-2抗体に基づくアッセイを行な  
うことにより、発現したp68HER-2の量を測定する操作を含む方法。

【請求項 25】

体液中のp185HER-2のECDの量を測定する操作をさらに含む、HER-2を過剰発現するガンの治療の予後を予測する請求項24に記載の方法。 10

【請求項 26】

p68HER-2とp185HER-2の量の比を測定する操作をさらに含み、p185HER-2に対するp68HER-2の比の値が大きいほど患者の予後が優れている、HER-2を過剰発現するガンの治療の予後を予測する請求項25に記載の方法。

【請求項 27】

ガン患者の治療、予後予測、または診断を行なう方法であって、

(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、  
絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サン  
プルを取得し、 20

(b)配列同定アッセイにより、この体液サンプル中にECD III aが変異したタン  
パク質またはDNA配列、すなわちHER-2のイントロン8が変異したDNA配列が存  
在しているかどうかを判定し、

(c)歴史データベースを用いて、このECD III aが変異したタンパク質またはD  
NA配列、すなわちHER-2のイントロン8が変異したDNA配列の存在を、ガンの治  
療および診断と関連づける操作を含む方法。

【請求項 28】

上記配列同定アッセイを、DNAシーケンシング、PCRアッセイ、ELISA免疫ア  
ッセイ、イムノアッセイ、ハイブリダイゼーション・アッセイ、これらの組み合わせ、か  
らなるグループの中から選択する、請求項27に記載の診断方法。 30

【請求項 29】

体液中のp185HER-2のECDの量を測定する操作をさらに含む、請求項27に記載  
の診断方法。

【請求項 30】

ガン患者の治療、予後予測、または診断を行なう方法であって、

(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、  
絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サン  
プルを取得し、

(b)配列同定アッセイにより、この体液サンプル中にHER-2のイントロンが変異し  
たDNA配列が存在しているかどうかを判定し、 40

(c)歴史データベースを用いて、このHER-2のイントロンが変異したDNA配列の  
存在またはその量を、ガンの治療および診断と関連づける操作を含む方法。

【請求項 31】

上記配列同定アッセイを、DNAシーケンシング、PCRアッセイ、ハイブリダイゼー  
ション・アッセイ、これらの組み合わせ、からなるグループの中から選択する、請求項3  
0に記載の診断方法。

【請求項 32】

体液中のp185HER-2のECDの量を測定する操作をさらに含む、請求項30に記載  
の診断方法。

【請求項 33】

ガン患者の治療、予後予測、または診断を行なう方法であって、

(a) 患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サンプルを取得し、

(b) E L I S A、免疫沈降法、免疫組織化学法、ウエスタンブロット解析法からなるグループの中から選択して抗 p 6 8 H E R - 2 抗体に基づくアッセイを行なうことにより、この体液サンプル中に p 6 8 H E R - 2 の E C D I I I a 変異体が存在しているかどうかを判定し、

(c) 歴史データベースを用いて、この p 6 8 H E R - 2 の E C D I I I a 変異体の存在またはその量を、ガンの治療および診断と関連づける操作を含む方法。

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【請求項 3 4】

上記配列同定アッセイを、DNA シークエンシング、PCR アッセイ、E L I S A 免疫アッセイ、ハイブリダイゼーション・アッセイ、これらの組み合わせ、からなるグループの中から選択する、請求項 3 3 に記載の診断方法。

【請求項 3 5】

体液中の p 1 8 5 H E R - 2 の E C D の量を測定する操作をさらに含む、請求項 3 3 に記載の診断方法。

【請求項 3 6】

p 6 8 H E R - 2 と p 1 8 5 H E R - 2 の E C D の量の比を測定する操作をさらに含む、請求項 3 5 に記載の診断方法。

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【請求項 3 7】

配列 I D 番号 1、配列 I D 番号 2、配列 I D 番号 1 2、配列 I D 番号 1 3 のいずれかで表わされる配列の E C D I I I a 変異体に対して特異的な抗体。

【請求項 3 8】

p 6 8 H E R - 2 の E C D I I I a 変異体 3 に対して特異的な抗体。

【請求項 3 9】

(a) 配列 I D 番号 1、配列 I D 番号 2、配列 I D 番号 1 2、配列 I D 番号 1 3 のいずれかで表わされる配列の E C D I I I a 変異体に特異的に結合するモノクローナル抗体またはその抗原結合断片と；

(b) ステップ (a) の抗体の結合を検出できる検出可能な標識とを含む診断キット。

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【請求項 4 0】

上記標識を、酵素、放射性標識、発色団、化学発光タグ、蛍光発光体からなるグループの中から選択する、請求項 3 6 に記載の診断キット。

【発明の詳細な説明】

【発明の属する技術分野】

【0001】本発明は、HER-2 に結合するアンタゴニストを提供する。さらに詳細には、イントロンが保持されていることによって HER-2 受容体と結合する HER-2 の新奇なアンタゴニスト・ポリペプチドが生まれる。

【0002】この仕事は、国防総省 (DOD) の乳ガン研究計画からの助成を受けた。アメリカ合衆国政府は、この発明に関して所定の権利を有する。

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関連出願の相互参照

【0003】本出願は、HER-2 に結合するアンタゴニストという名称で 1999 年 1 月 20 日に提出されたアメリカ合衆国特許出願シリアル番号第 09 / 234, 208 号の一部継続出願である。

【従来技術】

【0004】HER-2 / neu (erbB-2) ガン遺伝子は、受容体様チロシンキナーゼ (RTK) をコードしている。この RTK は、ヒトの何種類かのガン (Hynes と Stem、Biochim. et Biophys. Acta、1198 巻、165 ~ 184 ページ、1994 年；Dougall 他、Oncogene、第 9 巻、2109 ~ 2123 ページ、1994 年) および哺乳類の発生 (Lee 他、Nature、第 378

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巻、394～398ページ、1995年)においてある役割を果たしているというので精力的に研究されている。HER-2タンパク質の配列は、クローニングされたcDNAが、胎盤(Coussens他、Science、第230巻、1132～1139ページ、1985年)および胃ガン細胞系(ヤマモト他、Nature、第319巻、230～234ページ、1986年)からの上皮成長因子受容体(EGFR)のmRNAと相同性を有することから決定された。HER-2のmRNAは、約4.5kbであることがわかっており(Coussens他、Science、第230巻、1132～1139ページ、1985年;ヤマモト他、Nature、第319巻、230～234ページ、1986年)、ヒトの正常組織および悪性組織において185kDaの膜貫通糖タンパク質(p185HER-2)をコードしている(HynesとStem、Biochim. et Biophys. Acta、1198巻、165～184ページ、1994年; Dougall他、Oncogene、第9巻、2109～2123ページ、1994年)。HER-2遺伝子の機能は、主として、4.5kbの転写産物に対応するcDNAをトランスフェクションした細胞内で発現させることによって、185kDaタンパク質産物の構造ならびに生化学的特性から調べられている。p185HER-2は、大きな細胞外領域と、膜貫通領域と、チロシンキナーゼ活性を有する細胞内領域とからなる(HynesとStem、Biochim. et Biophys. Acta、1198巻、165～184ページ、1994年; Dougall他、Oncogene、第9巻、2109～2123ページ、1994年)。p185HER-2を過剰発現させると、培養した細胞の形質転換が起こる(DiFiore他、Science、第237巻、178～182ページ、1987年; Hudziak他、Proc. Natl. Acad. Sci. USA、第84巻、7159～7163ページ、1987年)。また、p185HER-2の過剰発現には、臨床上、乳ガンと卵巣ガンの急激な進行が伴う(Slamon他、Science、第235巻、177～182ページ、1987年; Slamon他、Science、第244巻、707～712ページ、1989年)。p185HER-2は、EGFRと相同性が極めて大きい。しかしp185HER-2に対して高アフィニティで直接結合するリガンドは、まだ同定されていない。しかも、HER-2のシグナル伝達活性は、EGFRファミリーの他のリガンド結合メンバーとのヘテロダイマー化によるらしい(CarrawayとCantley、Cell、第78巻、5～8ページ、1994年; Earp他、Breast Cancer Res. Treat.、第35巻、115～132ページ、1995年; Qian他、Oncogene、第10巻、211～219ページ、1995年)。

**【0005】**HERファミリーのRTKの細胞外領域いくつかの領域を含むさまざまなタンパク質は、完全長受容体のタンパク質分解処理によって(LinとClinton、Oncogene、第6巻、639～643ページ、1991年; Zabrecky他、J. Biol. Chem.、第266巻、1716～1720ページ、1991年; Pupa他、Oncogene、第8巻、2917～2923ページ、1993年; Vecchi他、J. Biol. Chem.、第271巻、18989～18995ページ、1996年; VecchiとCarpenter、J. Cell. Biol.、第139巻、995～1003ページ、1997年)、また選択的RNA処理によって(Petch他、Mol. Cell. Biol.、第10巻、2973～2982ページ、1990年; Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年; LeeとMaible、Oncogene、第16巻、3243～3252ページ、1998年)産生される。p185HER-2の細胞外領域は、培養した乳ガン細胞のタンパク質分解によって与えられる(Petch他、Mol. Cell. Biol.、第10巻、2973～2982ページ、1990年; Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年; LeeとMaible、Oncogene、第16巻、3243～3252ページ、1998年)。また、このp185HER-2の細胞外領域は、ある種のガン患者の血清中に見いだされるため(Leitzel他、J. Clin. Oncol.、第10巻、143

6～1443ページ、1992年)、乳ガンの転移の血清マーカーとして使えるであろう(Leitzel他、J. Clin. Oncol.、第10巻、1436～1443ページ、1992年)。さらにこのp185HER-2の細胞外領域は、HER-2リッチな腫瘍を免疫系による監視から逃れさせている可能性もある(Baselga他、J. Clin. Oncol.、第14巻、737～744ページ、1996年; Brodowicz他、Int. J. Cancer、第73巻、875～879ページ、1997年)。

【0006】HER-2の細胞外領域の断片は、イントロン内のポリアデニル化シグナルを利用して産生される新奇な2.3kbの転写産物でもある(Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年)。この新たな転写産物は、胃ガン細胞系MKN7において最初に同定され(ヤマモト他、Nature、第319巻、230～234ページ、1986年; Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年)、この受容体断片は、これらガン細胞から分泌されるのではなく、むしろ核周辺の細胞質に位置していた(Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年)。しかしこの細胞外領域ポリペプチド断片には特別な治療上、診断上、研究上の用途が見つかっていない。選択的スプライシングによって産生されるEGFRの細胞外領域断片(Petch他、Mol. Cell. Biol.、第10巻、2973～2982ページ、1990年)は、分泌されて、リガンドに結合する性質とダイマー化する性質を示し(Basu他、Mol. Cell. Biol.、第9巻、671～677ページ、1989年)、受容体の機能に対してドミナント・ネガティブな効果を及ぼす可能性がある(Basu他、Mol. Cell. Biol.、第9巻、671～677ページ、1989年; Flickinger他、Mol. Cell. Biol.、第12巻、883～893ページ、1992年)。

【0007】したがって、従来から、細胞のHER-2に結合する分子、中でもHER-2に対するヒト化抗体(ハーセプチン(登録商標))が結合するのとは異なる部位に結合する分子を見つけることが必要とされている。そのような分子は、HER-2を過剰発現するさまざまなガンに対する治療薬として有効であろう。

【課題を解決するための手段】

【0008】本発明は、配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも $10^8$ のアフィニティで結合する単離ポリペプチドを提供する。この単離ポリペプチドは、長さがアミノ酸約69～79個であることが好ましい。この単離ポリペプチドは、HER-2のECD上の部位のうち、ハーセプチン(登録商標)(HER-2のECDと結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体)の結合部位とは異なる部位に結合することが好ましい。

【0009】本発明は、さらに、発現したときに、配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも $10^8$ のアフィニティで結合するポリペプチドをコードしている単離DNA配列を提供する。この単離ポリペプチドは、長さがアミノ酸約69～79個であることが好ましい。この単離ポリペプチドは、HER-2のECD上の部位のうち、ハーセプチン(登録商標)(HER-2のECDと結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体)の結合部位とは異なる部位に結合することが好ましい。本発明は、さらに、発現したときに、配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも $10^8$ のアフィニティで結合するポリペプチドをコードするDNA配列を有する発現ベクターを含む、トランスフェクションされた細胞を提供する。

【0010】本発明は、さらに、配列ID番号2の配列から取られた約80～419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチドを提供する。この単離ポリペプチドは、長さがアミノ酸約350～419個で、N結合型グリコシル化部位が4つ存在していることが好ましい。この単離ポリペプチドは、HER-2のECD上の部位

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のうち、ハーセプチン（登録商標）（HER-2のECDと結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体）の結合部位とは異なる部位に結合することが好ましい。

【0011】本発明は、さらに、配列ID番号2の配列から取られた約80～419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化されたポリペプチドをコードする単離DNA配列を提供する。この単離ポリペプチドは、長さがアミノ酸約350～419個で、N結合型グリコシル化部位が4つ存在していることが好ましい。本発明は、さらに、配列ID番号2の配列から取られた約80～419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化されたポリペプチドをコードするDNA配列を有する発現ベクターを含む、トランスフェクションされた細胞を提供する。

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【0012】本発明は、HER-2の過剰発現を特徴とする固形ガンを治療するためにHER-2の細胞外領域（ECD）と結合する薬剤を投与する操作を含む方法であって、この薬剤を、（a）配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも $10^8$ のアフィニティで結合する単離ポリペプチド、（b）配列ID番号2の配列から取られた約80～419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、（c）HER-2のECDと結合するモノクローナル抗体、（d）これらの組み合わせ、からなるグループの中から選択するが、この薬剤がモノクローナル抗体のみからなることはありえないという方法を提供する。HER-2を過剰発現する固形ガンは、乳ガン、肺小細胞ガン、卵巣ガン、大腸ガンからなるグループの中から選択することが好ましい。上記薬剤は、配列ID番号1の配列から取られた約50～79個のアミノ酸を含む単離ポリペプチドであることが好ましい。さらに好ましいのは、この薬剤が、配列ID番号1の配列から取られた約50～79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合するモノクローナル抗体の組み合わせになっていることである。

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【0013】本発明は、さらに、HER-2を過剰発現するガンを治療するための薬理的組成物であって、（a）配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域（ECD）に少なくとも $10^8$ のアフィニティで結合する単離ポリペプチド、（b）配列ID番号2の配列から取られた約80～419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、（c）HER-2のECDと結合するモノクローナル抗体、（d）これらの組み合わせ、からなるグループの中から選択した薬剤と、薬理的に受容可能な基剤とを含むが、上記薬剤がモノクローナル抗体のみからなることはありえないという薬理的組成物を提供する。上記薬剤は、配列ID番号1の配列から取られた約50～79個のアミノ酸を含む単離ポリペプチドであることが好ましい。さらに好ましいのは、この薬剤が、配列ID番号1の配列から取られた約50～79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合するモノクローナル抗体の組み合わせになっていることである。

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【0014】本発明は、さらに、HER-2の過剰発現を特徴とする固形ガン細胞に治療薬を到達させる方法であって、この治療薬を、配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域ECDに少なくとも $10^8$ のアフィニティで結合する単離ポリペプチドに付着させる操作を含む方法を提供する。この単離ポリペプチドは、長さがアミノ酸約69～79個であることが好ましい。この単離ポリペプチドは、HER-2のECD上の部位のうち、ハーセプチン（登録商標）（HER-2のECDと結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体）の結合部位とは異なる部位に結合することが好ましい。

【0015】本発明は、さらに、HER-2を過剰発現するガンの患者におけるガン治療の予後を予測する方法であって、（a）患者の血液、血清、尿、リンパ液、唾液、腫瘍組

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織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サンプルを取得し、(b) ELISA、免疫沈降法、免疫組織化学法、ウエスタンブロット解析法からなるグループの中から選択して抗 p 6 8 H E R - 2 抗体をベースとしたアッセイを行なうことにより、発現した p 6 8 H E R - 2 の量を測定する操作を含む方法を提供する。ガン治療の予後を予測する方法は、体液中の p 1 8 5 H E R - 2 の E C D の量を測定し、p 6 8 H E R - 2 と p 1 8 5 H E R - 2 の量の比を決定する操作をさらに含むことが好ましい。

【0016】本発明は、さらに、ガン患者の治療、予後予測、または診断を行なう方法であって、(a) 患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サンプルを取得し、(b) 配列同定アッセイにより、この体液サンプル中に特定の E C D I I I a 変異配列が存在しているかどうかを判定し、(c) 歴史データベースを用いて、この E C D I I I a 変異配列の存在を、ガンの治療および診断と関連づける操作を含む方法を提供する。配列同定アッセイは、DNAシーケンシング、PCRアッセイ、ELISA免疫アッセイ、イムノアッセイ、ハイブリダイゼーション・アッセイ、これらの組み合わせ、からなるグループの中から選択することが好ましい。

【0017】本発明は、さらに、ガン患者の治療、予後予測、または診断を行なう方法であって、(a) 患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択した体液サンプルを取得し、(b) DNAシーケンシング、PCRアッセイ、ELISA免疫アッセイ、イムノアッセイ、ハイブリダイゼーション・アッセイ、これらの組み合わせ、からなるグループの中から選択して抗 p 6 8 H E R - 2 抗体をベースとしたアッセイを行なうことにより、この体液サンプル中に p 6 8 H E R - 2 の E C D I I I a 変異体が存在しているかどうかを判定し、(c) 歴史データベースを用いて、この E C D I I I a 変異配列の存在またはその量を、ガンの治療および診断と関連づける操作を含む方法を提供する。

【0018】本発明は、さらに、ガン患者の治療、予後予測、または診断を行なう上記の方法が、体液サンプル中の p 1 8 5 H E R - 2 の E C D の量を測定する操作をさらに含む方法を提供する。

【0019】本発明は、さらに、ガン患者の治療、予後予測、または診断を行なう上記の方法が、体液サンプル中の p 1 8 5 H E R - 2 の E C D の量を測定し、p 1 8 5 H E R - 2 と特定の p 6 8 H E R - 2 の E C D I I I a 変異体の量の比を決定する操作をさらに含む方法を提供する。

【0020】本発明は、さらに、以下に示す配列 I D 番号 1 または配列 I D 番号 2 で表わされる配列の E C D I I I a 変異体に対して特異的な抗体を提供する。

#### 【発明の実施の形態】

【0021】本発明は、イントロン 8 であると同定された 2 7 4 b p の挿入体を有する、H E R - 2 の新たな 4 . 8 k b の m R N A が初めて発見されたことに基づいている。保持されているこのイントロンはイン・フレームであり、7 9 個のアミノ酸 [ 配列 I D 番号 1 ] をコードした後、ヌクレオチド配列 2 3 6 位に終止コドンを含む。この新たな m R N A からは、膜貫通領域と細胞内領域を欠いている H E R - 2 タンパク質断片が予測される。このタンパク質断片は、4 1 9 個のアミノ酸 [ 配列 I D 番号 2 ] を含む。4 1 9 個のアミノ酸は、p 1 8 5 H E R - 2 の N 末端と一致する 3 4 0 個の残基と、C 末端の新奇な 7 9 個の残基 [ 配列 I D 番号 1 ] からなる。C 末端の新奇な 7 9 個のアミノ酸残基 [ 配列 I D 番号 1 ] に対して特異的な抗体、または p 1 8 5 H E R - 2 の N 末端に対して特異的な抗体を用い、6 8 k D a のタンパク質産物を同定した [ 配列 I D 番号 2 ]。この 6 8 k D a のタンパク質は、新奇な H E R - 2 転写産物であり、細胞抽出物の中や、いくつかの細胞系からの細胞外マトリックスの中に見いだされる。この新奇な転写産物の発現は、非発ガン性のヒト胚性腎臓細胞系で最大であった。

【0022】ここに提示した結果は、H E R - 2 の新奇な m R N A の発現を示している。

この mRNA は、274 個多いヌクレオチドを含んでおり、それはおそらくイントロン 8 であろう。この知見に合致するように、約 4.8 kb の新奇な転写産物が、ヒト胎児の腎臓組織とヒト胚性腎臓細胞系 HEK 293 で検出された。さらに、2.6 kb の転写産物が、挿入された配列に対して、すなわち HER-2 の ECD に対して特異的なプローブを用いたノーザンブロット解析によりヒト胎児の肝臓組織で検出された (図 2)。この 2.6 kb というサイズは、挿入配列が HER-2 の 2.3 kb の mRNA 断片 (ヤマモト他、Nature、第 319 巻、230~234 ページ、1986 年; Scott 他、Mol. Cell. Biol.、第 13 巻、2247~2257 ページ、1993 年) に含まれている場合に予想されるサイズである。挿入された配列には終止コドンが含まれているため、p185 HER-2 タンパク質の 340 番目の残基の位置に、ECD IIIa と表記する新奇な 79 個のアミノ酸延長部が含まれることが予測される。したがってこの予測されるタンパク質は、p185 HER-2 の膜貫通領域と細胞内領域を欠いているが、細胞外領域のサブドメイン I と II を含んでいる。予想されるように、p185 HER-2 の N 末端配列と、新奇な配列を含むことで生まれる C 末端延長部とを含む分泌タンパク質が検出された (図 3 と図 5)。ECD IIIa タンパク質は 68 kDa であることがわかった。これは、p185 HER-2 のサブドメイン I と II に見られる 5 つの N 結合型グリコシル化部位がグリコシル化される場合に新奇な転写産物によってコードされるタンパク質として予想されるサイズとほぼ同じである (Stern 他、Mol. Cell. Biol.、第 6 巻、1729~1740 ページ、1986 年)。

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【0023】ここに提示したデータは、p68 HER-2 が p185 HER-2 に特異的に結合することを示している。p185 HER-2 との会合は、p68 HER-2 の N 末端のサブドメイン I と II よりは、プロリン・リッチな新奇な ECD IIIa 領域によって生じている可能性がある。インビトロでの欠失突然変異誘発によって生まれる HER-2 の ECD もサブドメイン I と II を備えているが、より近接した状態にされるのでなければ p185 HER-2 の細胞外領域とは会合しない (Tzahar 他、EMBO J.、第 16 巻、4938~4950 ページ、1997 年; O'Rourke 他、Proc. Natl. Acad. Sci. USA、第 94 巻、3250~3255 ページ、1997 年; Fitzpatrick 他、FEBS Letters、第 431 巻、102~106 ページ、1998 年)。しかし新奇な ECD IIIa ペプチドは、p185 HER-2 および、p185 HER-2 を過剰発現する形質転換された 17-3-1 細胞と高アフィニティ (nM の濃度) で結合する (図 5)。ECD IIIa ペプチドが 17-3-1 細胞に好んで結合するというのは、分泌された p68 HER-2 が p185 HER-2 の細胞外領域と細胞表面で相互作用することを示している。したがって、p68 HER-2 とその断片は、HER-2 遺伝子によってコードされる自然発生の HER-2 結合タンパク質であるように思われる。EGFR ファミリーのリガンド (Groenen 他、Growth Factors、第 11 巻、235~257 ページ、1994 年) とは異なり、p68 HER-2 は EGF 相同領域を欠いており、p185 HER という受容体そのものの最初の 340 個のアミノ酸を含んでいる。

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【0024】以前に推定された HER-2 のリガンドは、EGFR ファミリーのメンバーとヘテロダイマーの形態でのみ p185 HER-2 と間接的に会合することがわかった (Heldin と Ostman、Cytokine Growth Factor Rev.、第 7 巻、33~40 ページ、1996 年)。ECD IIIa は共通の受容体を通じて p185 HER-2 と間接的に結合することが可能であるが、表面活性剤で溶解させた p185 HER-2 は不動化された ECD IIIa ペプチドによって特異的かつ効果的に“引き落とす”ため、こんなことは起こりそうにない (図 5B)。

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【0025】哺乳類の EGFR ファミリーのメンバーに対する自然または人工のすべてのリガンドにとって、結合するというのは、受容体のダイマー化およびチロシンリン酸化を促進することと強く結びついている (Hynes と Stem、Biochim. et Biophys. Acta、1198 巻、165~184 ページ、1994 年; Dougal 他、Oncogene、第 9 巻、2109~2123 ページ、1994 年; Gro

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enen他、Growth Factors、第11巻、235~257ページ、1994年)。p68HER-2もECD I I I aペプチドもp185HER-2と結合するにもかかわらず、p185HER-2を活性化させないことがわかった。活性化は、p185HER-2のチロシンリン酸化の程度が異なる2つの別々の細胞系、すなわち形質転換された17-3-1細胞と、SKOV-3卵巣ガン細胞において評価した。さらに、p185HER-2がダイマーの形態になることによって増加するインビトロでの自己リン酸化活性(Dougal1他、Oncogene、第9巻、2109~2123ページ、1994年; Lin他、J. Cell. Biochem.、第49巻、290~295ページ、1992年)は、p68HER-2とECD I I I aにいずれによっても大きくならなかった。同様に、ショウジョウバエのEGF受容体の細胞外阻害剤であり、クラスIのRTKのアンタゴニストとして知られている唯一のものであるアルゴス・タンパク質は、この受容体のチロシンリン酸化を促進しなかった(Schweitzer他、Nature、第376巻、699~702ページ、1995年)。同様に、タイ2RTKの天然のアンタゴニストであるアンジオポエチン-2は、内皮受容体と結合したが、その受容体を活性化することはなかった(Maisonpiere他、Science、第277巻、55~60ページ、1997年)。

【0026】理論に囚われないとすると、p68HER-2がp185HER-2と結合するがp185HER-2を活性化させないのであるから、p68HER-2がp185HER-2のダイマー化を阻止している可能性がある。類推により、RTKへの結合が促進されるようなHER-2のECDを作ったところ、このHER-2のECDは、リン酸基転移と受容体活性化に必要な生産性のあるダイマーの形成を阻止し、ドメイン・ネガティブな効果を示すことになった(O'Rourke他、Proc. Natl. Acad. Sci. USA、第94巻、3250~3255ページ、1997年)。可溶性p68HER-2は、HER-2のECDとは違ってp185HER-2への強い結合力を示したが、ECDのサブドメインIとIIをやはり含んでいる。サブドメインIは、p185HER-2をヘテロマー複合体にするのに必要な、低アフィニティで何とでも結合するリガンド結合部位である可能性があるため(Tzahar他、EMBO J.、第16巻、4938~4950ページ、1997年)、p68HER-2がこの部位をブロックし、p185HER-2がダイマーになるのを妨げている可能性がある。また、p68HER-2はp185HER-2に結合するためのまだ特性が同定されていないリガンドと競合する可能性もある。ヒト胎児の肝臓および腎臓においてp68HER-2が組織特異的に発現することによって、これら器官の発達中にp185HER-2が占める程度が変わる可能性がある。しかも、HER-2遺伝子の増幅に伴ってガン細胞中でp68HER-2よりもp185HER-2が過剰に発現するということが(図3)が、p68HER-2などの結合タンパク質の効果を上回るような選択的圧力によって起こる可能性がある。したがって、p68HER-2は、p185HER-2の活性化を妨げる可能性のある天然のp185HER-2結合タンパク質の最初の例である。

#### 薬理的組成物

【0027】本発明は、さらに、HER-2を過剰発現する固形ガンを治療するための薬理的組成物であって、(a)配列ID番号1の配列から取られた約50~79個のアミノ酸を含み、HER-2の細胞外領域(ECD)に少なくとも $10^8$ のアフィニティで結合する単離ポリペプチド、(b)配列ID番号2の配列から取られた約300~419個のアミノ酸を含み、C末端の79個のアミノ酸が存在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、(c)HER-2のECDと結合するモノクローナル抗体、(d)これらの組み合わせ、からなるグループの中から選択した薬剤と、薬理的に受容可能な基剤とを含むが、上記薬剤がモノクローナル抗体のみからなることはありえないという薬理的組成物を提供する。上記薬剤は、配列ID番号1の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドであることが好ましい。さらに好ましいのは、この薬剤が、配列ID番号1の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合す

るモノクローナル抗体の組み合わせになっていることである。

【0028】本発明のポリペプチドおよび/またはモノクローナル抗体の一方または両方を含む本発明の薬理的組成物は、そのまま（複合体または組み合わせ）で、または、適切な基剤および添加剤と混合した薬理的組成物として、患者に投与することができる。本発明のポリペプチドは、静脈内注射または点滴、腹腔内注射、皮下注射、筋肉内注射などの非経口的な方法で投与することができる。本発明のポリペプチドは、基剤と添加剤を加え、錠剤、ピル、カプセル、液体、ゲル、シロップ、スラリー、分散液などの適切な製剤にして、経口的に、または直腸から投与することができる。本発明のポリペプチドは、皮膚パッチなどの方法で局所的に投与することにより、活性成分のレベルが全身で一定となるようにすることができる。本発明のポリペプチドは、局部に使用するクリーム、皮膚または粘膜のパッチ、皮膚または粘膜の表面に局所的に付与するのに適切な液体またはゲルにされる。本発明のポリペプチドは、HER-2の過剰発現を特徴とするガンの局所的治療または全身治療のため、吸入器を用いて気道に投与することができる。

【0029】本発明で使用する本発明のポリペプチドの用量は、この明細書に記載されている内容から当業者が決定することができる。本発明のポリペプチドは、（投与経路と、活性成分の薬物動態に依存する）本発明のポリペプチドの効果的な用量と、製剤に応じた投与経路（すなわち経口、非経口、局所的、吸入など）に適した薬理的基剤および添加剤を含むことになる。本発明の活性なポリペプチドは、混合、溶解、粒子化、糖衣形成、乳剤化、カプセル化、トラップ、凍結乾燥といった方法で混合して薬理的製剤にする。非経口投与する薬理的組成物は、水溶性にした本発明のポリペプチドの水溶液を含んで 20  
いる。さらに、本発明のポリペプチドの分散液は、油性注射分散液として調製することができる。適切な親油性溶媒または賦形剤としては、ゴマ油などの不揮発性油、オレイン酸エチルやトリグリセライドなどの合成脂肪酸エステル、リポソームなどが挙げられる。水溶性注射分散液は、この分散液の粘性を高める物質、例えばナトリウムカルボキシメチルセルロース、ソルビトール、デキストランなど含むことができる。分散液は、より濃縮された溶液にできるよう、複合体または組み合わせの溶解性を高めるための安定化剤や薬剤を含んでいてもよい。

【0030】経口投与する薬理的組成物は、活性成分を固体の添加剤と組み合わせることにより得られる。固体の添加剤としては、糖（例えばラクトース、ショ糖、マンニトール、ソルビトール）、セルロース調製物（例えばデンプン、メチルセルロース、ヒドロキシプロピルメチルセルロース、ナトリウムカルボキシメチルセルロース）、ゼラチン、ガム、ポリビニルピロリドンが挙げられる。さらに、分解剤や安定化剤を添加することもできる。

#### p 68 と C 末端領域の 79 個のアミノ酸の合成方法

【0031】ポリペプチドの合成は、ペプチド合成装置を、製造業者のペプチド合成指示書に従って使用することによりアミノ酸を順次つなげるといふ、多数ある標準的なポリペプチド合成法による。アミノ酸の数が100未満の短いポリペプチドの場合には、アミノ酸を順次つなげるといふポリペプチド合成法が最適である。さらに、異種ポリペプチドは、標準的な組み換えDNA技術を用いて形質転換した細胞で発現させることができる。すなわち、原核細胞または真核細胞のいずれかを形質転換し、発現に適した増殖培地を用意し、使用する細胞のタイプとその細胞の発現特性に応じてその培地または細胞内物質のいずれかから本発明のポリペプチドを精製する。

#### p 68、C 末端領域の 79 個のアミノ酸、またはこれらの組み合わせを用いたガンの治療法

【0032】本発明は、HER-2またはHER-2変異体（実施例8を参照のこと）の過剰発現を特徴とする固形ガンを治療するためにHER-2の細胞外領域（ECD）と結合する薬剤を投与する操作を含む方法であって、この薬剤を、（a）配列ID番号1の配列から取られた約50～79個のアミノ酸を含み、HER-2の細胞外領域（ECD）に少なくとも10<sup>8</sup>のアフィニティで結合する単離ポリペプチド、（b）配列ID番号2の配列から取られた約300～419個のアミノ酸を含み、C末端の79個のアミノ酸が存 50

在し、少なくとも3つのN結合型グリコシル化部位が存在している、グリコシル化された単離ポリペプチド、(c)HER-2のECDと結合するモノクローナル抗体、(d)これらの組み合わせ、からなるグループの中から選択するが、この薬剤がモノクローナル抗体のみからなることはありえないという方法を提供する。HER-2を過剰発現する固形ガンは、乳ガン、肺小細胞ガン、卵巣ガン、前立腺ガン、胃ガン、子宮頸ガン、食道ガン、大腸ガンからなるグループの中から選択することが好ましい。上記薬剤は、配列ID番号1の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドであることが好ましい。より好ましいのは、上記薬剤が、配列ID番号1の配列から取られた約50~79個のアミノ酸を含む単離ポリペプチドと、HER-2のECDと結合するモノクローナル抗体の組み合わせである。

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【0033】この明細書に記載したp68HER-2ポリペプチドは、HER-2と結合し、キナーゼ領域を通じてシグナル伝達を妨げることが見いだされた。理論に囚われないとすると、新奇なECD III a領域はp185HER-2との特異的結合に関与し、その結果としてp68ECD III aと相互作用することでp185HER-2のダイマー化を阻止し、続いてシグナル伝達を阻止する。したがってp68HER-2はHER-2のアンタゴニストとして機能し、シグナル伝達に不可欠なダイマー化を阻止することでシグナル伝達を阻止する。つまり、HER-2のアンタゴニストとしてのp68HER-2の機能は、この明細書に記載したアミノ酸79個のポリペプチドや、HER-2のECDと結合するモノクローナル抗体などの結合剤の機能とは異なっている。本発明の方法では、HER-2を過剰に発現する腫瘍内で腫瘍細胞に対してp68HER-2が選択的圧力を加えることによってこの腫瘍細胞の成長を抑制する。同様に、結合剤であるHER-2のアンタゴニストも、HER-2を過剰に発現する腫瘍内で腫瘍細胞に対して選択的圧力を加えてリガンドがHER-2のECDと結合することを阻止し、ダイマー化する可能性が生まれる前すでにシグナル伝達を阻止することにより、この腫瘍細胞の成長を抑制する。

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#### C末端領域の79個のアミノ酸の標的到達分子としての利用

【0034】本発明は、さらに、HER-2の過剰発現を特徴とする固形ガン組織に治療薬を到達させる方法であって、この治療薬を、配列ID番号1の配列から取られた約50~79個のアミノ酸を含み、HER-2の細胞外領域(ECD)に少なくとも $10^8$ のアフィニティで結合する単離ポリペプチドに付着させる操作を含む方法を提供する。この単離ポリペプチドは、長さがアミノ酸約69~79個であることが好ましい。この単離ポリペプチドは、HER-2のECD上の部位のうち、ハーセプチン(登録商標)(HER-2のECDと結合してガンの治療に用いられる、市販のヒト化モノクローナル抗体)の結合部位とは異なる部位に結合することが好ましい。アミノ酸79個のこのポリペプチド[配列ID番号1]は、HER-2のECDに対して驚くほど高いアフィニティで結合する特性を示すことがわかった。さらに、そのような結合部位は、市販のヒト化モノクローナル抗体(ハーセプチン(登録商標))の結合部位とは異なっていて、このヒト化モノクローナル抗体の影響を受けない。したがって、この高い結合アフィニティにより、アミノ酸79個のこのポリペプチドが、HER-2を発現する腫瘍細胞への標的到達分子として機能する。

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#### 診断/予後予測の薬剤としての抗p68抗体

【0035】p68HER-2のグリコシル化されたECD III a変異体3ポリペプチド(後出の表Iを参照のこと)を発現させ、抗体産生用の抗原として使用した。中でも、p68HER-2に対して特異的な抗体を、ポリヒスチジン・タグを有する精製ECD III a変異体3ペプチドをウサギに注射することによって調製した。このペプチドは、イントロンがコードしている新奇なC末端領域すなわちp68HER-2と同じものであり、この領域が、高いアフィニティでp185HER-2に結合する。単離されたポリクローナル抗体により、ECD III aペプチドまたはp68HER-2のpM量の高い特異性で検出した(図3と図5を参照のこと)。したがってp68HER-2に対して特異的な抗体は、体液中および腫瘍組織中のp68HER-2を、ELISA、免疫沈降

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法、免疫組織化学法、ウエスタンブロット解析法などの診断技術を利用して検出するための診断用薬剤として有用である。

【0036】ECD III aの1つまたはそれ以上のエピトープ、すなわちp68HER-2のエピトープ、またはペプチド断片を特異的に認識し、したがってECD III a変異体相互の違いを区別する抗体も、本発明に含まれる。そのような抗体としては、ポリクローナル抗体、モノクローナル抗体(mAbs)、ヒト化抗体、キメラ抗体、一本鎖抗体、Fab断片、F(ab')<sub>2</sub>断片、Fab発現ライブラリーによって産生された抗体、抗イディオタイプ(抗Id)抗体、これらのうちの任意のものエピトープ結合断片が挙げられるが、これだけに限定されるわけではない。本発明の抗体は、例えば、生物サンプル中のp68HER-2の特定のECD III a変異体を検出するのに使用でき、したがって、患者のサンプルまたは組織サンプルに特定の变異体が存在しているかどうかや、特定の变異体の量が異常であるかどうかを調べることで、診断または予後予測の方法の一部として利用できる。

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【0037】このような抗体は、テスト化合物が、特定のp68HER-2変異体の発現および/または活性に対して及ぼす効果を評価するための化合物スクリーニング法と合わせて利用することもできる。さらに、このような抗体は、この明細書に記載したガン治療法と合わせて利用することができる。

【0038】抗体産生のためには、例えばポリヒスチジン・タグを有するECD III a変異体ポリペプチド、ECD III a変異体ポリペプチドの断片、ECD III a変異体の機能的等価物、ECD III a領域の突然変異体を注射することによってさまざまな宿主動物に免疫を確立するとよい。このような宿主動物の具体例をほんの少しだけ挙げるならば、ウサギ、マウス、ハムスター、ラットであるが、これだけに限定されるわけではない。宿主の種が何であるかに応じ、さまざまなアジュバントを用いて免疫応答を高めることができる。アジュバントとしては、フロイント(完全、不完全)アジュバント、水酸化アルミニウムなどの無機ゲル、リソレシチンなどの表面活性物質、ポリオール、ポリアニオン、ペプチド、油性乳剤、スカシガイのヘモシアニン、ジニトロフェノール、役に立つ可能性のあるヒトのアジュバント(BCG(カルメット-ゲランの細菌)など)、コリネバクテリア(*Corynebacterium parvum*)などが挙げられるが、これだけに限定されるわけではない。

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【0039】ポリクローナル抗体は、免疫確立した動物の血清に由来する異種混合の抗体群である。モノクローナル抗体は、特定の抗原に対する均一な抗体群であり、培養した継代細胞系によって抗体分子を産生させることのできる任意の方法で得られる。方法としては、ケーラーとミルシュタインのハイブリドーマ法(Nature、第256巻、495~497ページ、1975年;アメリカ合衆国特許第4,376,110号)、ヒトB細胞ハイブリドーマ法(Kosbor他、Immunology Today、第4巻、72ページ、1983年;Cole他、Proc. Natl. Acad. Sci. USA、第80巻、2026~2030ページ、1983年)、EBV-ハイブリドーマ法(Cole他、『モノクローナル抗体とガン治療』、アラン R. リス社、77~96ページ、1985年)が挙げられるが、これだけに限定されるわけではない。このような抗体は、IgG、IgM、IgE、IgA、IgDというクラスのうちのどの免疫グロブリンでもよく、そのどのサブクラスでもよい。ハイブリドーマ産生mAbは、インビトロでもインビボでも培養することができる。インビボで大きな力価のmAbを産生させるというのが、現在のところ最も好ましい産生方法である。

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【0040】さらに、“キメラ抗体”を産生させるために開発された方法(Morrisson他、Proc. Natl. Acad. Sci. USA、第81巻、6851~6855ページ、1984年;Neuberger他、Nature、第312巻、604~608ページ、1984年;タケダ他、Nature、第314巻、452~454ページ、1984年)、すなわち、適切な抗原特異性を有するマウス抗体分子からの遺伝子と、適切な生物活性を有するヒト抗体分子からの遺伝子をスプライスするという方法を利用することができる。キメラ抗体は、さまざまな部分がそれぞれ異なる動物種に由来する

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構成の分子であり、例えば、ネズミの mAb に由来する可変領域と、ヒト免疫グロブリンの定常領域とを有する抗体（ヒト化抗体）がそうである。

【0041】また、一本鎖抗体の産生方法（アメリカ合衆国特許第4,946,778号；Bird、Science、第242巻、423～426ページ、1988年；Huston他、Proc. Natl. Acad. Sci. USA、第85巻、5879～5883ページ、1988年；Ward他、Nature、第334巻、544～546ページ、1989年）を改変してECD I I I a変異遺伝子産物に対する一本鎖抗体を産生させることもできる。一本鎖抗体は、Fv領域のH鎖とL鎖の断片をアミノ酸架橋を通じて結合させることによって形成されて一本鎖のポリペプチドになる。

【0042】特定のエピトープを認識する抗体断片は、公知の方法で産生させることができる。例えば、そのような断片としては、抗体分子をペプシンで消化させて得られるF(ab')<sub>2</sub>断片、F(ab')<sub>2</sub>断片のジスルフィド結合を還元することにより得られるFab断片が挙げられるが、これだけに限定されるわけではない。また、Fab発現ライブラリー（Huse他、Science、第246巻、1275～1281ページ、1989年）を構成して、望む特異性を有するモノクローナルFab断片を迅速かつ容易に同定することもできる。

【0043】特定のECD I I I a変異体に対する抗体は、さらに、当業者に周知の方法を用いて、ECD I I I a変異体を“真似る”抗イディオタイプ抗体を産生させるのに用いることができる（GreenspanとBona、FASEB J、第7巻（5）、437～444ページ、1993年；Nissinoff、J. Immunol.、第147巻、2429～2438ページ、1991年）。例えばECD I I I a変異体に結合し、p68HER-2がHER-2受容体に結合するのを競合的に抑制する抗体を用いると、ECD I I I a変異体を“真似し”、したがってHER-2受容体に結合してこのHER-2受容体を中性化する抗イディオタイプ抗体を産生させることができる。このような中性化抗イディオタイプ、またはこのような抗イディオタイプのFab断片は、ガン治療の投薬計画において利用することができる。

【0044】また、特定のECD I I I a変異体に対する抗体で、ECD I I I a変異体の活性のアゴニストまたはアンタゴニストとして作用しうる抗体を産生させることができる。このような抗体は、ECD I I I a変異体と結合し、p185HER-2受容体を介したシグナル伝達に対するp68HER-2の活性を変化させる。このような抗体は、特定のガンの治療および/または腫瘍の分化の調節に特に役立つ可能性がある。したがって、本発明は、さらに、HER-2を過剰発現するガンの治療の予後を予測する方法であって、(a)血液、血清、尿、リンパ液、唾液、腫瘍組織、これらの組み合わせ、からなるグループの中から選択した体液サンプルを取得し、(b)ELISA、免疫沈降法、免疫組織化学法、ウエスタンブロット解析法からなるグループの中から選択して抗p68HER-2抗体をベースとしたアッセイを行なうことにより、発現したp68HER-2の量を測定する操作を含む方法を提供する。ガン治療の予後を予測するこの方法は、体液中のp185HER-2のECDの量を測定し、p68HER-2とp185HER-2の量の比を決定する操作をさらに含むことが好ましい。p185HER-2に対するp68HER-2の比の値が大きいほど患者の予後が優れている。

診断/予後予測の薬剤としてのECD I I I a領域変異体

【0045】実施例11（後出）は、ヒトのイントロン8の配列が、プロリン・リッチかつ多型であることを示している。別々の15人から採取したゲノムDNAをシーケンシングした結果、HER-2のイントロン8に可変配列領域が10箇所あることが同定された。図8の配列ID番号10と表Iを参照のこと。図8は、イントロン8の最も一般的なヌクレオチド配列とそれに対応するポリペプチド配列を示している。この領域は、10通りの多型（配列ID番号10の中には文字W（2箇所）、Y（3箇所）、R、N、M、S（2箇所）で示し、図8には“X”で示してある）を含んでおり、その結果として保存されないアミノ酸置換が起こっている（表Iの注を参照のこと）。例えば、ヌクレオチド配列の161位に多型（G C）があると（図8；表I）、配列ID番号1のアミノ酸残基

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# 54 の位置、または配列 ID 番号 2 の残基 # 394 の位置でアルギニン (R) がプロリン (P) に代わるはずである。図 8 または配列 ID 番号 10 の 1 位で示される N 末端のグリシン (G) は、“ハースタチン”配列のアミノ酸残基 341 に対応する (Doherty 他、Proc. Natl. Acad. Sci. USA、第 96 巻、10、869~10、874 ページ、1999 年)。図 1 (A) に示したヌクレオチド配列 (Doherty 他、Proc. Natl. Acad. Sci. USA、第 96 巻、10、869~10、874 ページ、1999 年) は、図 8 に示した最も一般的であることがわかった配列と比べてアミノ酸残基 # 6 と # 73 の位置のアミノ酸が異なる多型になっている。

【0046】この結果は、ヒトの集団において、イントロン 8 がコードされている領域にいくつかのバリエーションが存在していて、ECD III a を含むタンパク質変異体相互の間で生化学的特性や生物学的特性が異なる可能性のあることを示している。ヒトは遺伝的に、特に、2 つの変異体に関してヘテロ接合になっているか、所定の 1 つの変異体に関してホモ接合になっているか、1 つの二重変異体に関してホモ接合になっているかの可能性がある。腫瘍の進行にせよ最適の治療にせよ、ある一人の人に現われる変異体が何であるかによって異なる可能性がある。

【0047】この違いは、予後予測と診断のどちらにも役立つ。本発明は、ECD III a を含むポリペプチドが HER-2 受容体に強固に結合でき、したがってこの HER-2 受容体をアンタゴニスト化できることを示している。このような高アフィニティで特異的な相互作用は、ECD III a を含むポリペプチドの特殊な一次構造、二次構造、三次構造に依存する。ECD III a 領域はプロリン・リッチであり、従来からよく知られていることとして、所定のタンパク質のプロリン・リッチ配列中にあるプロリン残基またはそれ以外の残基の保存されない置換は、このタンパク質の二次構造と三次構造に大きな影響を及ぼしうるといえることがある。したがって、本発明の多型は、(図 8 に示した)最も一般的な構造と比べて構造特性、生化学的特性、生物学的特性が大きく異なっているであろう。ECD III a 変異体タンパク質相互の間の構造上の違いとしては、例えば、サイズ、電気陰性度、抗原性の違いが挙げられる。ECD III a 変異体相互の間の生物学的特性の違いは、例えば、細胞分泌の程度、HER-2 受容体の変化の性質および/または程度、薬物動態 (例えば血清半減期、排出特性)、タンパク質分解に対する抵抗力、N 結合型グリコシル化のパターンなどに見られるであろう。これら生物学的性質の違いが今度は腫瘍の進行を変化させることが予想され、したがって最適な治療のプロトコルを変化させることが予想される。そこで、ある人が特定の 1 つまたは複数の ECD III a 変異体を有することがわかっていると (例えば所定の 1 つの変異体に関してヘテロ接合である人、または表 I の変異体 11 のように化合物変異体を有する人など)、そのことだけで、特定のガンになりやすさの予測ができる可能性がある。

【0048】ECD III a 領域の遺伝的違いが明らかになるということは、ある人が有する特定の ECD III a 変異体の性質を、その人の遺伝子診断を試みる前に配列同定アッセイによって確認できるはずであることを意味する。解析は、患者の体液に由来する任意のゲノム DNA について行なうことができる。体液は、一般には大人または子どもの血液サンプルであるが、その代わりとして、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織が可能である。ハイブリダイゼーションや増幅アッセイなどの標準的な遺伝子診断法を利用できると考えられる。特定の ECD III a 変異体配列を検出するのに、生物サンプルのハイブリダイゼーションまたは増幅アッセイにおいて、DNA と RNA のいずれかを用いることができる。このような配列同定アッセイとしては、サザンブロット解析法、ノーザンブロット解析法、一本鎖構造多形性分析、インサイチュ・ハイブリダイゼーション・アッセイ、ポリメラーゼ連鎖反応 (“PCR”) 解析法などが挙げられるが、これだけに限定されるわけではない。このような解析法により、ECD III a 変異体配列の発現の定量的側面と定性的側面の両方を明らかにできよう。こうした側面としては、例えば、点突然変異および/または遺伝子発現の活性化または不活性化が挙げられる。標準的なインサイチュ・ハイブリダイゼーション法を利用すると、所定の組織内でどの細胞が特定の ECD III a 変異体配列を発現しているか

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に関する情報が得られる可能性がある。

【0049】ECD III a変異体の核酸分子を検出する診断法は、解析する細胞種または組織に由来する核酸を、特定のECD III a変異体に対して特異的な、標識された1つまたはそれ以上の核酸試薬すなわちプローブと接触させて培養する操作を含んでいる。さらに好ましいのは、PCRまたは逆転写PCRを利用してECD III a領域内の核酸の違いを同定することである。PCR反応の条件は、増幅された産物の収量と特異性が最適であり、さらに、標準的なゲル電気泳動法を利用して解析できる長さを有する増幅産物が生成するように選択せねばならない。このような反応条件は当業者には周知であり、重要な反応パラメータとしては、例えば、オリゴヌクレオチド・プライマーの長さ、ヌクレオチド配列、アニーリングして伸長させるステップの温度、反応時間が挙げられる。PCR反応の後、PCR産物は、ヘテロ二本鎖検出、RNAアーゼAを利用したRNA-DNAハイブリッドの開裂、一本鎖構造多型、変性用勾配ゲル電気泳動などの方法で解析することができる。

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【0050】さらに、特定のECD III a配列変異体が制限部位を付加したり除去したりするとか、特定の制限断片のサイズを有意に変化させてしまっていることが知られている場合には、制限断片長多型(“RFLP”)解析に基づいたプロトコルが適切である可能性がある。

【0051】ECD III a変異体は、患者由来の体液に対して配列同定アッセイを行なうことにより、発現レベルで解析することもできる。体液は、一般には大人または子どもの血液サンプルであるが、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織などでもよい。発現を解析するためのよく知られている配列同定アッセイとしては、mRNAをベースとした方法、例えばノーザンブロット法、(関係するcDNAに由来する核酸プローブを利用した)インサイチュ・ハイブリダイゼーション法、(St-Jacques他、Endocrinology、第134巻、2645~2657ページ、1994年に記載されている)定量的PCR法が挙げられるが、これだけに限定されるわけではない。

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【0052】興味の対象であるECD III a変異体に対して特異的な抗体を利用することを含む、ポリペプチドをベースとした方法(例えばウエスタンブロット解析があるが、これだけに限定されるわけではない)も、上に説明したように、利用することができる。これらの方法により、所定のECD III a変異体の発現量を少なくとも正と負の制御に関して定量することができる。さまざまなECD III aのエピトープまたは変異体に対して特異的な種々のモノクローナル抗体を利用して、特定のECD III a変異体を発現している細胞サンプルまたは組織サンプルを迅速にスクリーニングすること、またはECD III a変異体ポリペプチドのレベルを定量することができると好ましい。ECD III a変異体ペプチド分子を定量的または定性的に検出するための好ましい診断法としては、例えば、特定のECD III aを含むペプチドが、抗ECD III a変異体に対して特異的な抗体との相互作用によって検出されるというイムノアッセイが挙げられる。これは、例えば、蛍光標識した抗体を用いる蛍光抗体法を、軽いミクロのフローサイトメトリー検出または蛍光定量検出と組み合わせることによって実現できる。本発明で有用な抗体(またはその断片)は、さらに、蛍光抗体法または免疫電子顕微鏡法などにおけるように組織構造を知るのに利用して、ECD III aを含むペプチドを元々ある場所で検出することもできる。このような方法を利用すると、特定のECD III aを含むポリペプチドの存在だけでなく、検査している組織内のその分布を検出することができる。

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【0053】ECD III a変異体ポリペプチドのイムノアッセイには、ECD III aを含むペプチドの同定が可能な検出可能標識をした抗体の存在下で培養した生物サンプル(例えば具体例を列挙した上記の体液)を培養し、結合した抗体を従来技術で周知の多数ある方法のうちの任意の方法で検出する操作が含まれていることが好ましい。生物サンプルは、ニトロセルロースなどの固相支持体または担体や、可溶性タンパク質、細胞、細胞粒子を固定することのできる他の固相支持体と接触させて、その上に固定するとよい。

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固相支持体は、抗 E C D I I I a 変異体に特異的な検出可能標識抗体で処理した後、適切な緩衝溶液で洗浄する。次に、緩衝溶液を用いて固相支持体の 2 回目の洗浄を行ない、結合していない抗体を除去する。続いて、固相支持体上に結合している標識の量を従来法で検出する。

【0054】別の方法として、抗 E C D I I I a 変異体に対して特異的な抗体は、酵素イムノアッセイすなわち固相酵素免疫検定法（“E L I S A”）で使用する酵素に結合させることによって、検出可能な標識をすることができる。抗体と結合する酵素が適切な基板、好ましくは発光性基板と反応して化学基が生成されるため、それを例えば分光光度計、蛍光測定手段、目視などによって検出することができる。抗体に対する検出可能な標識として使用可能な酵素としては、マレイン酸デヒドロゲナーゼ、スタフィロコッカル・ヌクレアーゼ、 $\alpha$ -5-ステロイド・イソメラーゼ、酵母のアルコール・デヒドロゲナーゼ、 $\alpha$ -グリセロリン酸デヒドロゲナーゼ、トリオースリン酸イソメラーゼ、西洋ワサビのペルオキシダーゼ、アルカリホスファターゼ、アスパラギナーゼ、グルコース・オキシダーゼ、 $\beta$ -ガラクトシダーゼ、リボヌクレアーゼ、ウレアーゼ、カタラーゼ、グルコース-6-リン酸デヒドロゲナーゼ、グルコアミラーゼ、アセチルコリンエステラーゼが挙げられるが、これだけに限定されるわけではない。

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【0055】検出は、酵素に対する発光性基板を用いる熱量測定法により実現することができる。検出は、目視により酵素反応の程度を適切な基準と比較することによって行なうこともできる。検出は、多彩な他のイムノアッセイ法のうちの任意の方法を用いて実現することもできる。例えば、抗体または抗体断片に放射性標識することにより、放射線免疫検定法（R I A）を利用して E C D I I I a を含むペプチドを検出することが可能である。放射性同位体は、 $\beta$ 線カウンタ、シンチレーション・カウンタなどの手段により、あるいは放射能写真撮影により検出することができる。

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【0056】抗体を蛍光化合物で標識することも可能である。蛍光標識した抗体を適切な波長の光に曝露すると、蛍光のためにその抗体の存在が検出できる。最もよく使われる蛍光標識化合物としては、フルオレセイン・イソチオシアナート、ローダミン、フィコエリトリン、フィコシアニン、アロフィコシアニン、*o*-フタルアルデヒド、フルオレスカミンがある。

【0057】抗体に対しては、 $^{152}\text{Eu}$  や他のランタノイド系列の蛍光発光金属を用いて検出可能な標識をすることもできる。これらの金属は、ジエチレントリアミン五酢酸（D T P A）、エチレンジアミン四酢酸（E D T A）などの金属キレート化剤を用いて抗体に付着させることができる。

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【0058】抗体は、化学発光化合物と結合させることによって検出可能な標識をすることもできる。化学発光体をタグとして有する抗体の存在は、化学反応中に発生する蛍光の存在を検出することによって明らかにされる。特に有用な標識用の化学発光化合物の具体例としては、ルミノール、イソルミノール、サーマティックな（*theromatic*）アクリジニウム・エステル、イミダゾール、アクリジニウム塩、シュウ酸エステルがある。同様に、生物発光化合物を用いて本発明の抗体に標識することもできる。生物発光は、生物系で見つかった化学発光の一種であり、生体内で触媒タンパク質が化学発光反応の効率を増大させている。生物発光タンパク質の存在は、蛍光の存在を検出することによって明らかにされる。標識の目的で重要な生物発光化合物は、ルシフェリン、ルシフェラーゼ、エクオリンである。

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【0059】抗 E C D I I I a 変異体に対して特異的な所定のロットの抗体の結合活性は、周知の方法で測定することができる。当業者であれば、定法を利用することによりそれぞれの測定におけるアッセイの操作条件や最適条件を決定できるはずである。

【0060】したがって、本発明は、プロリン・リッチな E C D I I I a 領域内に複数の可変配列位置が予想外に発見されたこと、また特定の E C D I I I a 変異体に対して特異的な抗体が予想外に発見されたことで、予後予測および診断に関する重要な情報とアッセイを提供する。

【0061】したがって、本発明は、さらに、H E R - 2 を過剰に発現しているガン患者

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のガン治療の予後を予測する方法であって、(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択したから体液サンプルを取得し、(b)ELISA、免疫沈降法、免疫組織化学法、ウエスタンブロット解析法からなるグループの中から選択して抗p68HER-2抗体をベースとしたアッセイを行なうことにより、発現したp68HER-2の量を測定する操作を含む方法を提供する。ガン治療の予後を予測するこの方法は、体液中のp185HER-2のECDの量を測定し、p68HER-2とp185HER-2の量の比を決定する操作をさらに含むことが好ましい。p185HER-2に対するp68HER-2の比の値が大きいほど患者の予後が優れている。ガン治療の予後を予測するこの方法は、特定のどのECD III a変異体が存在しているかを明らかにし、さまざまなECD III aタンパク質変異体の個々の生化学的特性と生物学的特性を考慮してガンの治療を最適化する操作をさらに含むことが好ましい。

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【0062】本発明は、さらに、ガン患者のガンの治療、予後予測、または診断を行なう方法であって、(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択したから体液サンプルを取得し、(b)配列同定アッセイにより、この体液サンプル中に特定のECD III a変異体配列が存在しているかどうかを判定し、(c)歴史データベースを用いて、このECD III a変異体配列の存在をガンの治療および診断と関連づける操作を含む方法を提供する。上記の配列同定アッセイは、DNAシーケンシング、PCRアッセイ、ELISA免疫アッセイ、イムノアッセイ、ハイブリダイゼーション・アッセイ、これらの組み合わせ、からなるグループの中から選択することが好ましい。

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【0063】本発明は、さらに、患者のガンの治療、予後予測、または診断を行なう方法であって、(a)患者の血液、血清、尿、リンパ液、唾液、腫瘍組織、胎盤組織、臍帯組織、羊水、絨毛膜絨毛組織、これらの組み合わせ、からなるグループの中から選択したから体液サンプルを取得し、(b)ELISA、免疫沈降法、免疫組織化学法、ウエスタンブロット解析法からなるグループの中から選択した、抗p68HER-2抗体に基づくアッセイにより、この体液サンプル中にp68HER-2のECD III a変異体が存在しているかどうかを判定し、(c)歴史データベースを用いて、このp68HER-2のECD III a変異体の存在またはその量をガンの治療および診断と関連づける操作を含む方法を提供する。

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【0064】本発明は、さらに、ガンの治療、予後予測、または診断を行なう上記の方法が、体液サンプル中のp185HER-2のECDの量を測定する操作をさらに含む方法を提供する。

【0065】本発明は、さらに、ガンの治療、予後予測、または診断を行なう上記の方法が、体液中のp185HER-2のECDの量を測定し、p185HER-2のECDと特定のp68HER-2のECD III a変異体の量の比を測定する操作をさらに含む方法を提供する。

【0066】本発明は、さらに、後に示す配列ID番号1または配列ID番号2の配列のECD III a変異体に対して特異的な抗体を提供する。

#### 治療薬としてのp68HER-2

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【0067】理論に囚われないとすると、p68HER-2またはECD III aペプチドが、細胞表面でp185HER-2に結合することにより、HER-2を過剰に発現している腫瘍細胞の成長を抑制しているように思われる。この仮説の検証を、p68HER-2の存在下および不在下で基質とは独立な細胞の成長をテストすることにより行なった。そのとき用いた細胞は、p185HER-2の過剰発現の程度に応じて悪性腫瘍が成長するが、p68HER-2はほとんど存在しないか検出できない細胞である。腫瘍の細胞毒性を予測するモデルとして、柔らかい寒天における、基質とは独立な細胞の成長を用いた。これは、形質転換活性を調べるための一般的な予測方法であり、細胞が腫瘍またはガンを発生させる能力を反映している(DiFiore他、Science、第237巻、178~182ページ、1987年; Hudziak他、Proc. Natl. Ac

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ad. Sci. USA、第84巻、7159～7163ページ、1987年；Baasner他、Oncogene、第13巻、901～911ページ、1996年）。

【0068】柔らかい寒天における、基質とは独立な細胞の成長にp68HER-2が及ぼす効果を、SKOV-3ガン細胞と、HER-2をトランスフェクションした17-3-1細胞を用いて測定した。両方の細胞とも腫瘍生成能力があり、p185HER-2を過剰発現している。これら細胞を、p68HER-2の存在下および不在下でウシ胎仔血清を補充した培地に分散させ、湿潤な培養器の中で21日間培養した。50個を超える細胞を含むコロニーの数を数えることにより、基質とは独立な細胞の成長を定量化した。図7は、p68HER-2の存在下で、SKOV-3細胞と17-3-1細胞の両方に関し、基質とは独立な成長が数分の一に抑制されたことを示している。したがって、これらのデータは、p68HER-2が単に細胞毒性だけを有するのではなく、細胞毒性に加え、おそらくアポトーシス誘導性も有することを示している。

#### 【実施例1】

【0069】この実施例では、細胞外領域(ECD)をコードしている配列中のHER-2のmRNAの多様性を、ポリメラーゼ連鎖反応(PCR)を利用して調べた実験の結果を示す。HER-2遺伝子が8倍に増幅されている卵巣ガン細胞系(Tyson他、Am. J. Obstet. Gynecol.、第165巻、640～646ページ、1991年)である、(アメリカ基準培養株コレクション(ロックヴィル、メリーランド州)が、10%のウシ胎仔血清と0.05%のゲンタマイシンを補充したDMEM中に維持している)SKOV-3細胞からのcDNAライブラリーを、エキソン1(Tal他、Mol. Cell. Biol.、第7巻、2597～2601ページ、1987年)に対して特異的でヌクレオチド142～161と一致する順プライマーと、エキソン9(Scott他、Mol. Cell. Biol.、第13巻、2247～2257ページ、1993年)のヌクレオチド1265～1286に相補的な逆プライマーとを用いて調べた。要するに、SKOV-3のcDNAライブラリーは、オリジン・テクノロジーズ社(ロックヴィル、メリーランド州)から提供してもらるか、SKOV-3細胞から抽出したRNAから調製するかした。トリリジェント(モレキュラー・リサーチ・センター社、シンシナチ、オハイオ州)を製造業者のプロトコルに従って用いることで15cmのプレート上に80%の集密度で成長したSKOV-3細胞からRNAを抽出し、全RNAを得た。RNAは、逆転写およびcDNAライブラリー構成のために10mMのトリスEDTA、pH8.0に再び分散させるか、リボヌクレアーゼ保護アッセイ(RPA)のためにRNAハイブリダイゼーション用緩衝溶液(80%のホルムアルデヒド、40mMのPIPES、4mMのNaCl、1mMのEDTA、pH7.5)に再び分散させるかした。RNAの濃度は、分光光度計でOD<sub>260</sub>における値を測定した。mRNA抽出キット(オリゴテックス、キアジェン社)を用いてポリA<sup>+</sup>mRNAを全RNAから選択した。

【0070】サザンブロット法によってHER-2に特異的であることがわかった約1420bpの産物は、以前に報告されたcDNA配列(Cousens他、Science、第230巻、1132～1139ページ、1985年)から予想される1144bpというサイズよりも約270bp大きかった。要するに、サザンブロット法によって真空下において0.4MのNaOH中のアガロース・ゲル(バイオ・ラド・モデル785ヴァキューム・プロッター)から核酸を遺伝子スクリーン・プラス・ハイブリダイゼーション・トランスファー膜(ネン・リサーチ・プロダクツ社、ボストン、マサチューセッツ州)に移した。UV-ストラターリンカー(ストラタジーン社、ラ・ジョラ、カリフォルニア州)の中でUV架橋させることにより核酸を膜に固定し、その膜をハイブリダイゼーション用緩衝溶液(50%のホルムアルデヒド、5×SSC、1%のSDS、10mg/mlのニシンの精子DNA)中で42℃にて2時間にわたってブロックした。ランダム・プライムDNA標識キット(ベーリンガー・マンハイム社)を用い、膜を、( -<sup>32</sup>P ) dCTP(ネン・ライフ・サイエンス社)で標識したECD IIIIaのcDNAからの220bpのKpn-HincII断片107cpmとともに、ハイブリダイゼーション用緩衝溶液中で42℃にて16時間にわたってハイブリダイズさせた。

【0071】鋳型を、2.5 mMのMgCl<sub>2</sub>、5 μgの各プライマー、200 μMのdNTPを含む1×ハイ・フィデリティPCR緩衝溶液とともに、拡張ハイ・フィデリティPCRシステム(ベーリンガー・マンハイム社)を用いてパーキン・エルマー遺伝子増幅PCRシステム2400(パーキン・エルマー・シータス社、エメリーヴィル、カリフォルニア州)の中で増幅した。すべてのプライマーは、GIBCO BRL社(ライフ・テクノロジー社)から得られた。ヌクレオチド残基とアミノ酸残基の番号は、Cousens他が報告しているHER-2のcDNA配列(Cousens他、Science、第230巻、1132~1139ページ、1985年)に従っている。HER-2の細胞外領域を標的とし、順プライマー(A)(5'-TGAGCACCAATGGGAGCTGGC-3'[配列ID番号3])と、逆プライマー(B)(5'-TCCGGCAGAAATGCCAGGCTCC-3'[配列ID番号4])を用いてSKOV-3のcDNAライブラリー(オリジン・テクノロジー社)から増幅した。順プライマーは、HER-2のcDNAのヌクレオチド(nt)142~161と一致していて開始コドン(下線部)の周囲に広がっており、逆プライマーは、HER-2のエキソン配列のnt1265~1286と相補的になっている。サイクリング・パラメータは、94 で30秒間; 58 で45秒間; 68 で3分間を30サイクルである。ゲノムDNAからの(ECD I I I aと表記する)新奇的な配列の周囲に広がっている領域は、(Bond他、FEBS Letters、第367巻、61~66ページ、1995年)が記載しているようにして調製したDNAについて、HER-2のエキソンに対して特異的な配列のnt1131~1152と一致する順プライマー(C)(5'-AACACAGCGGTGTGAGAAAGTGC-3'[配列ID番号5])と逆プライマー(B)[配列ID番号4]を用いて増幅した。サイクリング・パラメータは、94 で30秒間; 62 で30秒間; 72 で60秒間を25サイクルである。

【0072】逆転写ポリメラーゼ連鎖反応(RT-PCR)を利用して、ECD I I I a配列を含むmRNAの構造を調べた。まず最初に鎖状cDNAを、0.5 μgのオリゴ-dTで処理した5 μgのRNAを用いて逆転写した(Bond他、FEBS Letters、第367巻、61~66ページ、1995年)。ECD I I I a挿入体とそれに隣接する5' HER-2エキソン配列を増幅するため、上記の順プライマー(A)と、3' ECD I I I aに対して特異的な配列に相補的な逆プライマー(D)(5'-ATACCGGGACAGGTC AACAGC-3'[配列ID番号6])を用いた。サイクリング・パラメータは、94 で30秒間; 60 で40秒間; 68 で2分間を30サイクルである。

【0073】ECD I I I a挿入体とそれに隣接する3' HER-2エキソンに対して特異的な配列の増幅を、順プライマー(E)(5'-TCTGGGTACCCACTCACTGC-3'[配列ID番号7])と、逆プライマー(F)(5'-TTCACACTGGCACGTTCCAGACC-3'[配列ID番号8])を用いて行なった。順プライマーは、5' ECD I I I aに対して特異的な配列と一致していてKpn1制限部位を含んでおり、逆プライマーは、HER-2のエキソン配列のnt3898~3919と相補的で、終止コドン(下線部)のまわりに広がっている。サイクリング・パラメータは、94 で30秒間; 60 で40秒間; 68 で5分間を30サイクルである。

【0074】PCR産物をサブクロニングしてヌクレオチド配列を決定した。

【0075】その結果は、正常なHER-2をコードしている配列が、5'プライマー配列から始まって中断することなくヌクレオチド1171まで続いていることを示していた。そしてこの位置で、274個のヌクレオチドからなる挿入体が見つかり、その後には予想されるコード配列が続き、その中に3'プライマーが含まれていた。予想されるタンパク質産物を解析した結果、274個のヌクレオチドからなる挿入体が、残基340から始まる既知のHER-2タンパク質の延長部をコードしており(Cousens他、Science、第230巻、1132~1139ページ、1985年)、アミノ酸79個後にイン・フレーム終止コドンが存在していることを示していた(図1)。挿入されたヌクレオチドとそのヌクレオチドから予想されるアミノ酸配列を遺伝子バンクの配列と比較し

たところ、相同な配列は見つからなかった。分散している配列の5'接合部と3'接合部を調べたところ、共通するスプライス・ドナー部位とスプライス・アクセプター部位が明らかになり (SharpとBurge、Cell、第91巻、875~879ページ、1997年)、挿入配列の3'末端近傍に、ピリミジン領域と、分岐点となる可能性のあるアデニン残基が含まれていることがわかった (図1)。したがって、この挿入配列はイントロンらしい。

【0076】挿入配列によってコードされる新奇な79個のアミノ酸として予想されるアミノ酸配列 [配列ID番号1] を調べると、共通のN結合型グリコシル化部位があることと、19%という高い割合でプロリンが含まれていることがわかる (図1)。挿入配列は、p185HER-2配列の細胞外領域中のサブドメインIIとIIIの間に位置しているため (Lax他、Mol. Cell. Biol.、第8巻、1831~1834ページ、1988年)、ECD III aと表記した。この挿入配列は隣接する5'HER-2エキソン配列と236ntにわたってイン・フレームであり、その中に終止コドンが含まれている。

#### 【実施例2】

【0077】この実施例では、ゲノム内でHER-2のエキソンと隣接しているECD III aの特性を明らかにする実験の結果を示す。ECD III a配列が存在する領域におけるHER-2遺伝子の構造を調べるため、ヌクレオチド763~785と一致する順プライマーと、HER-2のcDNAのヌクレオチド1265~1286と相補的な逆プライマーを用いて、ヒトゲノムDNAについてPCRを行なった。増幅産物は、エキソン5 (Tal他、Mol. Cell. Biol.、第7巻、2597~2601ページ、1987年) からECD III a配列の3'に近接するエキソンまで広がっていることが予想された。イントロンの数とサイズは、PCR産物のサイズ、制限消化解析、増幅産物の部分配列の解析に基づいて推定した。

【0078】次に、HER-2のエキソンに特異的で挿入体に直接接するプライマーを用いてヒトゲノムDNAを調べ、ECD III a配列に直接接している配列を決定した。約430bpの産物を、正常なヒトゲノムDNAおよび3種類のガン細胞系SCOV-3、SKBR-3、BT474から抽出したゲノムDNAから増幅した。これらすべてのガン細胞系においてHER-2遺伝子が増幅され (Kraus他、EMBO J.、第6巻、605~610ページ、1987年)、cDNAにECD III aを発現していることがわかった。PCR産物がHER-2であることが、実施例1に記載した方法を利用したサザンブロット解析によって確認された。ヌクレオチド配列を解析したところ、ヒトゲノムDNAからのPCR産物がECD III a挿入体を含んでおり、その両側がHER-2をコードしている既知の配列に接していることがわかった。突然変異または再配置は見られなかった。これらのデータは、ECD III a配列が、完全な状態で保持されたイントロンであることを表わしており、イントロン4の後に増幅された産物のサイズと、相同なEGFR遺伝子およびHER-2遺伝子のイントロン8の位置 (LeeとMaille、Oncogene、第16巻、3243~3252ページ、1998年) とに基づくと、このイントロンがイントロン8であるらしいことを示している。

#### 【実施例3】

【0079】この実施例では、ECD III aが、HER-2のmRNAのコード配列内に保持されている唯一のイントロンであることを示す。ECD III a挿入配列を含むmRNAの中に別のイントロンが含まれているかどうかを明らかにするため、逆転写ポリメラーゼ連鎖反応 (RT-PCR) を行なった。まず最初に、5'HER-2のcDNA配列の142~161と一致して開始コドンを含む順プライマーと、3'ECD III a配列と相補的な逆プライマーを、SKBR-3とSKOV-3のcDNAに対して用いた。1.3kbの産物が増幅された。これは、この産物がイントロン8以外のイントロンを含んでいない場合に予想されるサイズである。次に、5'ECD III a配列と一致した順プライマーと、3'HER-2のcDNA配列のヌクレオチド3898~3919と相補的でp185HER-2の終止コドンを含む逆プライマーとを用い、3'H

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ER-2コード配列を増幅した。2.9 kbの産物が増幅された。これは、この産物が別のイントロンを含んでいない場合にHER-2のcDNAから予想されるサイズである。

【0080】制限消化解析とヌクレオチドのシーケンシングによって5' (1.3 kb)と3' (2.9 kb)の増幅産物の両方のキャラクタリゼーションを行なったところ、別のイントロンは保持されていないことがわかった。イントロン配列が含まれているときに増幅される産物のサイズを決定するため、ゲノムDNAをPCR反応の鋳型として用いた。その結果、5'コード配列に対しては約10 kbの産物、3'コード配列に対しては約5 kbの産物が得られた。これらの結果は、5'非翻訳領域(5'UTR)と3'非翻訳領域(3'UTR)のサイズが以前に報告されている約4.5 kbのHER-2のcDNA(Coussens他、Science、第230巻、1132~1139ページ、1985年)と同じであるならば、274 bpのイントロンが保持されていることで生まれるHER-2の新奇な転写産物の予想サイズが約4.8 kbとなることを示している。

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#### 【実施例4】

【0081】この実施例では、ECD III a配列を含むタンパク質の発現を示す。新奇な配列がタンパク質産物に翻訳されるかどうかを調べるため、ECD III a配列を細菌内でポリヒスチジン・タグを有するペプチドとして発現させ、このペプチドをニッケル・アフィニティ・クロマトグラフィにより精製し、この精製ペプチドに対する抗血清を得た。要するに、プライマーEと、ECD III a挿入配列の3'末端と相補的な逆プライマーとを用いてSKOV-3のcDNAライブラリーからECD III aを増幅することによって細菌の発現ベクターを調製した。逆プライマーはBamH1制限部位配列を含んでおり、(実施例1と2に記載した)RPAにおいて鋳型を構成するのに用いたプライマーと同じであった。約280 bpというPCR増幅産物をKpn1とBamH1で消化させ、ゲル(キアエックスII、キアジェン社、チャッツワース、カリフォルニア州)で精製し、pET30aベクターにクローニングした。このベクターは、発現したタンパク質のアミノ末端の位置に6個のヒスチジン・タグをコードしている(ノヴァジェン社、マディソン、ウィスコンシン州)。得られたpET-ECD III a発現ベクターは、細菌株BL21の形質転換に使用した。

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【0082】ECD III aタンパク質産物を発現させるため、pET-ECD III a発現ベクターで形質転換したBL21細胞をLB培養液の中で30 µg/mlのカナマイシンとともに37 °Cにて4時間にわたって成長させた。0.1 mMのIPTGで3時間にわたって発現を誘導し、回収した細胞を超音波処理して溶解させ、次に39,000 × gで20分間遠心分離した。室温で60分間にわたって攪拌しながら上澄みをNi-NTAアガロース樹脂(キアジェン社)に吸収させた。この樹脂を10倍容積の洗浄用緩衝溶液(10 mMのトリスpH 7.9と300 mMのNaCl)で洗浄し、次に、50 mMのイミダゾールを加えた10倍容積の洗浄用緩衝溶液で洗浄した。ヒスチジン・タグを有するECD III aタンパク質を、250 mMのイミダゾールを加えた洗浄用緩衝溶液中に溶離させた。ヒスチジン・タグを有するこのタンパク質は、ゲルをクーマッシー・ブルーで染色することにより、約90%の純度であると推定された。このタンパク質を用いて抗体を産生させ、その抗体のキャラクタリゼーションを行なった。

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【0083】要するに、コカリコ・バイオリジカルズ社(リームズタウン、ペンシルヴェニア州)に依頼し、2匹のウサギにポリヒスチジン・タグを有する精製ECD III aペプチド(以下に記載)を注射することにより抗ECD III a抗血清を生産してもらった。p185 HER-2のアミノ酸残基151~165と一致するペプチドに対するポリクローナル抗neu(N)を作った(LinとClinton、Oncogene、第6巻、639~643ページ、1991年)。p185 HER-2のカルボキシ末端の最後の15残基と一致するペプチドに対するポリクローナル抗neu(N)を作った(Lin他、Mol. Cell. Endocrin.、第69巻、111~119ページ、1990年)。免疫を確立した2匹のウサギからの抗血清をキャラクタリゼーションしたところ、精製ECD III aペプチドと反応する高力価の抗体が含まれていることがわかった。

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【0084】ウエスタンブロット解析により、cDNA中に新奇な配列を発現したSKBR-3細胞が、抗ECD III a抗体と反応するタンパク質を産生させたかどうかを調べた。細胞抽出物および細胞外マトリックスからの68kDaのタンパク質は、2匹のウサギからの抗ECD III a抗体を少なくとも20,000倍に希釈したものと反応したが、免疫確立前の血清とは反応しなかった。新奇な転写産物のcDNA配列(図1)を調べたところ、p185HER-2のN末端配列中の5つの共通N結合型グリコシル化部位すべてがグリコシル化されている場合には、65~70kDaの分泌タンパク質産物が予測されることがわかった(Stern他、Mol. Cell. Biol.、第6巻、1729~1740ページ、1986年)。

【0085】68kDaのECD III aタンパク質[配列ID番号2]は、HER-2の新奇なmRNAの翻訳産物であり、そのN末端の残基は、p185HER-2のN末端の340個の残基と一致してはいなくてはならない。そこで、SKBR-3細胞からの細胞抽出物を、HER-2のN末端配列である抗neu(N)(LinとClinton、Oncogene、第6巻、639~643ページ、1991年)に対する抗ペプチド抗体、または抗ECD III aを用いて免疫沈降させ、得られた免疫複合体を両方の抗体を用いてウエスタンブロット解析法で調べた。要するに、3~5μlの抗血清を、M-RIPA緩衝溶液(1%のノニデットP-40、50mMのトリスpH7.4、0.1%のデオキシコール酸ナトリウム、150mMのNaCl、1mMのPMSF、1%のアプロチニン)中に調製した細胞溶離物からのタンパク質2mgに添加した。なお細胞溶離物は、遠心分離で核をあらかじめ除去した。(Lin他、Mol. Cell. Endocrin.、第69巻、111~119ページ、1990年)に記載されているようにして、免疫沈降を、4にて攪拌しながら2時間にわたって行なった。免疫複合体は、攪拌しながら4にて1時間培養することによりプロテインGセファロース(ファルマシア社)に結合したので遠心分離で回収し、M-RIPAで4回洗浄した。タンパク質は、95で2分間にわたってSDS-PAGE用サンプル緩衝溶液中で培養することにより免疫複合体から放出され、それを7.5%のゲル中でSDS-PAGEにより分離させた(ミニプロティーンII電気泳動細胞、バイオ-ラド社)。

【0086】SDS-PAGEの後、ウエスタンブロット解析を行なった。半乾燥トランスファー・ユニット(バイオ-ラド社)を用い、電気泳動により、25mMのトリスpH8.3、192mMのグリシン、50mMのNaCl、20%のメタノールで平衡させたゲル(厚さ0.75mm)1つにつき15Vの電圧で20分間にわたってタンパク質をニトロセルロース膜(トランス-ブロット、バイオ-ラド社)上にブロットした。この膜を、5%の脱脂粉乳を用いて25にて1時間にわたってブロックした。次に、ブロットを一次抗体とともに培養し、TBS-トゥイーン(0.05%のトゥイーンを含むトリス緩衝溶液)で15分間ずつ2回にわたって洗浄し、5分間ずつ4回にわたって洗浄し、ヤギの抗ウサギ二次抗体とともに40分間培養し、西洋ワサビのペルオキシダーゼ(バイオ-ラド社)と結合させ、TBS-トゥイーン中に1:10,000の割合で希釈した。膜は、二次抗体とともに培養した後に上記のようにして洗浄し、化学発光試薬(ピアース社)と反応させ、コダック社のX-OMAT BLUフィルムに曝露した。

【0087】免疫沈降とウエスタンブロット解析で抗ECD III aを用いたときには、予想通りp68HER-2が検出された。免疫沈降で抗ECD III aを用い、ウエスタンブロット解析におけるプローブが抗neu(N)のときには、68kDaのタンパク質が検出された。これは、p68ECD III aがp185HER-2のN末端配列を含んでいることを示している。さらに、抗neu(N)がp68HER-2を沈降させた。そのことは、抗ECD III a抗体をプローブとして調べることにより検出された。これらの結果は、p68HER-2がECD III aとHER-2のN末端配列の両方を含んでいることを示している。

【0088】他のいくつかの細胞系におけるp68ECD III aの発現を調べた。cDNAにECD III a配列を含むガン細胞系(BT474、SKOV-3)もp68HER-2を含んでいた。調べたいくつかの細胞系のうち、正常なヒト胚の腎臓細胞に由

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来するHEK293細胞が、細胞抽出物中および細胞外マトリックス中で最高レベルのp68ECD III aを発現し、SKBR-3細胞よりも約5~10倍多い量だった。p185HER-2の過剰発現を調べたガン細胞系(SKBR-3、SKOV-3、BT474)と比較すると、HEK293はp185HER-2の含有量が約20分の1であった。したがって、p185HER-2に対するp68HER-2の割合は、HEK293細胞においては、調べたガン細胞系の少なくとも100倍であった。特にHEK293抽出物において明らかなp68HER-2および約120kDaのタンパク質との反応は、抗血清を、配列特異的な反応性を示す精製ECD III aペプチドとともに予備培養することによって停止させた。この大きなタンパク質はp68HER-2のダイマーである可能性がある。したがってp68HER-2が発現していくつかのガン細胞系から分泌され、HEK293中では5~10倍のレベルになる。

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#### 【実施例5】

【0089】この実施例では、ECD III aイントロン配列を含む新奇なHER-2転写産物の発現を示す。RT-PCR解析の結果は、ECD III a配列が挿入されていなければ正常なサイズであるはずのHER-2のmRNAにECD III a配列が挿入されたことを示していた。このデータは、約4.8kbの新奇な転写産物があることを示唆する。ECD III aの新奇な転写産物のサイズと発現を調べるため、ECD III aに対して特異的なプローブを用いてノーザンブロット解析を行なった。要するに、ECD III a挿入体の全配列にまたがっていて、この挿入体に隣接する5'HER-2エキソン配列を含む389bpの配列をPCRで増幅することにより、アンチセンスRNAプローブを合成するための鋳型をSKOV-3のcDNAから構成した。HER-2のcDNA配列とnt1131~1152が一致する順プライマーC[配列ID番号5]と、3'BamH1制限エンドヌクレアーゼ部位を含み、ECD III a配列の3'スプライス部位近傍の配列と相補的な逆プライマー(5'-GCACGGATCCATAGCA GACTGAGGAGG-3'[配列ID番号9])を用いてPCRを行なった。次に、PCR産物をBamH1で消化させ、375bpの断片を遊離させ、pBluescript SK(ストラタジーン社)にクローニングした。m13順プライマーと逆プライマーを用い、このプラスミドをヴォラム・インスティテュート・コア・シークエンシング・ファシリティ(ポートランド、オレゴン州)にてシークエンシングした。ECD III a配列全体と、挿入体に隣接した5'HER-2エキソン配列の87ntとに相補的なアンチセンスRNAプローブを、(<sup>32</sup>P)CTP、T7RNAポリメラーゼと、T7/SP6リポプローブ合成システム(プロメガ社、マディソン、ウィスコンシン州)とを用いて1μgの直線状鋳型から転写した。このプローブは、ECD III aおよびそれと隣接するHER-2エキソン配列を含むmRNAとハイブリダイズするとき、370ntの断片を保護し、完全にスプライスされたHER-2のmRNAとハイブリダイズするとき、87ntの断片を保護することが予想された。

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【0090】RNAハイブリッドを調製するため、30μgのRNAを約50,000cpmのアンチセンスRNAプローブと48℃にて16時間にわたってハイブリダイズさせた。このRNAハイブリッドを、40μg/mlのRNAアーゼA(ベーリンガー・マンハイム社)および2μg/mlのRNAアーゼT1(ライフ・テクノロジーズ社)を用い、250mMのNaCl、5mMのEDTA、10mMのトリスpH7.5からなる溶液中で、37℃にて30分間にわたって消化させた。プロテイナーゼK(100μg)(ライフ・テクノロジーズ社)を含む20μlの10%SDSを添加して消化を停止させた。サンプルを酸性フェノール(pH4.5;ライフ・テクノロジーズ社)とクロロホルムで抽出し、2容積の100%エタノールで沈殿させ、5μlのRPAサンプル用緩衝溶液(88%のホルムアルデヒド、10mMのEDTA pH8.0、1mg/mlのキシレンシアノール、1mg/mlのプロモフェノール・ブルー)に分散させた。サンプルを95℃にて10分間にわたって変性させ、TBE(89mMのトリス、89mMのホウ酸エステル、2mMのEDTA pH8.3)の中で5%ポリアクリルアミド/尿素ゲル上にて電気泳動を行なった。このゲルを真空下で乾燥させ、保護された断片をリン光画像解析によっ

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て定量した ( I P ラブ・ゲル、モレキュラー・ダイナミックス社 )。

【 0 0 9 1 】 約 4 . 8 k b の新奇な転写産物が、 p 6 8 E C D I I I a を最高レベルで発現した H E K 2 9 3 細胞の中で検出された。しかし S K B R - 3、 B T 4 7 4、 S K O V - 3 というガン細胞系のノーザンプロット解析では、新奇な転写産物は検出できなかった。そこで、より高感度のリボヌクレアーゼ保護アッセイ ( R P A ) により、新奇な転写産物の発現レベルを、完全にスプライスされた 4 . 5 k b の転写産物と比較して調べた。検出可能なレベルの p 6 8 E C D I I I a を含んでいた卵巣ガン細胞系 ( S K O V - 3 ) および乳ガン細胞系 ( S K B R - 3 と B T 4 7 4 ) からの R N A と、 H E R - 2 の c D N A が安定にトランスフェクションされた対照細胞系 1 7 - 3 - 1 からの R N A を、 E C D I I I a ( イントロン 8 ) 配列全体と、イントロン 8 に隣接した 5 ' H E R - 2 エキソン配列とにまたがる<sup>3 2</sup> P 標識アンチセンス R N A プローブとハイブリダイズさせた。 R N A ーゼで消化させ、電気泳動を行ない、放射能写真を撮影したところ、 3 7 0 個のヌクレオチドからなるバンドが、 1 7 - 3 - 1 以外の細胞系で検出された。これは、 E C D I I I a を含む H E R - 2 の m R N A によって保護されることが予想されるサイズに対応している。さらに、 8 7 個のヌクレオチドが保護された断片が、すべての細胞で検出された。これは、対照となる正常な細胞系と比べてこれらガン細胞系で 1 0 0 倍以上も過剰に発現している完全にスプライスされた H E R - 2 の情報に対して予想されるサイズである ( K r a u s 他、 E M B O J .、第 6 巻、 6 0 5 ~ 6 1 0 ページ、 1 9 8 7 年 )。保護されたそれぞれの断片の量を測定し、サイズに関して正規化して新奇な転写産物の相対量を推定し、 p 1 8 5 H E R - 2 の m R N A に対する割合として表わした。 E C D I I I a 挿入体を含む新奇な H E R - 2 の m R N A は、 S K O V - 3 細胞では完全にスプライスされた転写産物のレベルの 4 . 2 % であり、 S K B R - 3 細胞では 5 . 4 % であり、 B T 4 7 4 細胞では 0 . 8 % であった。

#### 【実施例 6】

【 0 0 9 2 】 この実施例では、 E C D I I I a 挿入体を含む新奇な転写産物がヒト胚の腎臓および肝臓で発現したことを示す。ノーザンプロット解析により、 E C D I I I a 配列を含む新奇な転写産物がヒトの正常な組織で発現したかどうかを調べた。ヒト胎児のさまざまな組織からのポリ A<sup>+</sup> m R N A をノーザンプロットとして調製し、新奇な E C D I I I a 配列に対して特異的な放射標識プローブとハイブリダイズさせた。 4 . 8 k b の m R N A が腎臓で検出され、 2 . 6 k b の転写産物が肝臓で検出された ( 図 2 )。 4 . 8 k b の転写産物は、 2 7 4 b p の挿入体を有する完全長 4 . 5 k b 転写産物に対応しているようである。 2 . 6 k b の転写産物は、以前に報告されている 2 . 3 k b の別の転写産物 ( ヤマモト他、 N a t u r e、第 3 1 9 巻、 2 3 0 ~ 2 3 4 ページ、 1 9 8 6 年 ; S c o t t 他、 M o l . C e l l . B i o l .、第 1 3 巻、 2 2 4 7 ~ 2 2 5 7 ページ、 1 9 9 3 年 ) に 2 7 4 b p の E C D I I I a 挿入体が含まれたものに対応しているのであろう。プロットを分離させて 5 ' H E R - 2 コード配列に対して特異的なプローブとハイブリダイズさせたところ、 4 . 8 k b と 4 . 5 k b の m R N A を示す広いバンドが胎児の腎臓組織で検出され、 2 . 6 k b の転写産物断片が肝臓で検出された。これは、これらの新奇な転写産物が、 H E R - 2 の E C D をコードする配列を含んでいることを示している。挿入された E C D I I I a 配列が終止コドンを含んでいるため、同じタンパク質産物がこれら m R N A のそれぞれから産生される可能性がある。

【 0 0 9 3 】 いくつかの細胞系についても E C D I I I a を含む新奇な転写産物についてノーザンプロット解析で調べた。 4 . 8 k b の新奇な転写産物が、ヒト胚の腎臓細胞系である H E K - 2 9 3 で検出された ( 図 2 )。 E C D I I I a 配列が、 H E R - 2 遺伝子が増幅されている S K B R - 3、 B T 4 7 4、 S C O V - 3 というガン細胞系の R T - P C R 解析により検出されたとはいえ、 E C D I I I a を含む新奇な転写産物はこれらの細胞をノーザンプロット解析しても検出できなかった。そこで、 E C D I I I a 配列全体と、この E C D I I I a 挿入体に隣接する 5 ' H E R - 2 エキソン配列とにまたがるアンチセンスプローブを利用して、より高感度のリボヌクレアーゼ保護アッセイ ( R P A ) を行なった。 E C D I I I a 挿入体を有する新奇な H E R - 2 の m R N A は、 S C

OV-3細胞、SKBR-3細胞、BT474細胞では、完全にスプライスされた転写産物の5%未満しか検出されなかった。これらの知見は、ECD III a配列を含む新奇な2つの転写産物が、ヒトの正常組織で組織特異的に発現したこと、4.8 kbの新奇な転写産物がHEK-293細胞系で発現したこと、遺伝子が増幅したガン細胞が、新奇な転写産物を4.5 kbのHER-2転写産物の5%未満という低いレベルで発現していることを示している。

#### 【実施例7】

【0094】この実施例では、ECD III a配列を含むタンパク質の発現を示す。新奇な配列がタンパク質産物に翻訳されるかどうかを調べるため、ECD III a配列を細菌内でポリヒスチジン・タグを有するペプチドとして発現させ、ニックル-アフィニティ・クロマトグラフィにより精製し、この精製ペプチドに対する抗血清を得た。4.8 kbの新奇なECD III aの転写産物を発現したHEK-293細胞がECD III aを含むタンパク質を発現するかどうかを、ウエスタンブロット解析で調べた。細胞抽出物と細胞外マトリックスからの68 kDaのタンパク質は、抗ECD III a抗体と反応したが(図3)、免疫確立前の血清とは反応しなかった。反応は、この抗血清を、精製ECD III aペプチドとともに予備培養することによって停止させた(図3)。いくつかの細胞抽出物で検出された大きな約125 kDaのタンパク質は、p68 HER-2の集合体であろう。新奇な転写産物のcDNAの配列(図1)からは、p185 HER-2のN末端配列中の5つの共通N結合型グリコシル化部位すべてがグリコシル化されている場合には、65~70 kDaの分泌タンパク質産物が予測されることがわかる(Stern他、Mol. Cell. Biol.、第6巻、1729~1740ページ、1986年)。他のいくつかの細胞系がp68 ECD III aを発現するかどうかを調べた。cDNAにECD III a配列を含むガン細胞系(BT474、SKOV-3、SKBR-3)も検出可能なレベルのp68 HER-2を含んでいた。

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#### 【実施例8】

【0095】この実施例では、HER-2遺伝子が増幅されているガン細胞系でp185 HER-2と比べてp68 HER-2の発現が顕著に低下していることを示す。HER-2遺伝子が増幅されているガン細胞系ではp185 HER-2のmRNAと比べてp68 HER-2のmRNAの発現レベルが非常に低いため、HER-2遺伝子が増幅されているいくつかの細胞系と増幅されていないいくつかの細胞系において、p68 HER-2タンパク質とp185 HER-2タンパク質の相対的な割合を調べた。ウエスタンブロットを調製し、p68 HER-2に対して特異的な抗血清とp185 HER-2に対して特異的な抗血清の両方について調べた。図4は、HER-2遺伝子が増幅されているガン細胞系でp185 HER-2が容易に検出できたことを示している(Kraus他、EMBO J.、第6巻、605~610ページ、1987年)。しかしp68 HER-2が対応して増加することはなかった。比較すると、p68 HER-2は、HEK-293、IOSEVAN、HBL100という非発ガン性細胞で検出された唯一のHER-2タンパク質であった。それに対してp185 HER-2は、これらの細胞中では非常に低いレベルでしか発現せず(Kraus他、EMBO J.、第6巻、605~610ページ、1987年)、過剰に露出したブロットにおいて検出された。これらのデータは、HER-2遺伝子が増幅されているガン細胞系ではp68 HER-2がp185 HER-2と比べて少ないことを示しており、p185 HER-2が過剰に発現したときにp68 HER-2が低レベルに維持される何らかのメカニズムが存在している可能性のあることを示唆している。

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#### 【実施例9】

【0096】この実施例では、p68 HER-2とECD III aペプチドがp185 HER-2に特異的に結合することを示す。p68 HER-2は分泌タンパク質であり、しかもp68 HER-2は新奇な配列に加えてp185 HER-2と一致するサブドメインIとIIを含んでいるため、p68 HER-2がp185 HER-2と相互作用する可能性を調べた。p185 HER-2とp68 HER-2のN末端に対する抗ペプチド抗体

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である抗neu(N)、またはp185HER-2に対して特異的な抗体である抗neu(C)を用い、p68HER-2を低レベルで発現し、p185HER-2を過剰発現しているガン細胞SKBR-3の免疫沈降を行なった。免疫沈降させる物質をウエスタンブロットとして調製し、プローブとしてp68HER-2に対して特異的な抗ECDIIIIaと抗neu(C)の両方を用いて調べた。抗neu(N)は、p68HER-2とp185HER-2の両方を免疫沈降させた(図5A)。さらに、p185HER-2のC末端に対して特異的な抗体はp185HER-2を免疫沈降させ、p68HER-2を共同沈降させた(図5A)。この結果は、2つのタンパク質の間に相互作用があることを示唆している。

【0097】ECD配列同士の結合相互作用は非常に弱いので(Tzahar他、EMBO J.、第16巻、4938~4950ページ、1997年; Fitzpatrick他、FEBS Letters、第431巻、102~106ページ、1998年)、結合が、新奇なプロリン・リッチなECDIIIIa領域によるものかどうかを調べた。新奇な79個のアミノ酸からなる領域をヒスチジン・タグを有するタンパク質として精製してニッケル・アガロース上に固定し、プルダウン・アッセイで使用した。対照として、ECDIIIIaとは関係のない、ヒスチジン・タグを有する2つの精製ペプチド、すなわちウィルソン病の膜タンパク質の600残基からなる断片と、CREBタンパク質のDNA結合領域を含む70残基からなる断片とを、同様にしてニッケル・アガロース樹脂上に固定した。固定されたペプチドを、HER-2をトランスフェクションした3T3細胞(17-3-1)から調製したタンパク質とともに培養した。十分に洗浄した後、結合したタンパク質を溶離させ、ウエスタンブロットとして調製し、それを、p185HER-2に対して特異的な抗体をプローブとして用いて調べた。ヒスチジン・タグを有するECDIIIIaペプチドと対照用ペプチドが同じ量だけ樹脂に結合したことが、1Mのイミダゾールで溶離させ、溶離した物質をSDSゲル中でクーマッシー染色することによって確認された。p185HER-2は、ペプチドなしの樹脂にも対照用ペプチドを有する樹脂にも保持されることがなかったのに対し、ECDIIIIaペプチドによってだけ選択的に保持された(図5B)。

【0098】プルダウン・アッセイにおいてECDIIIIa領域がp185HER-2に結合したため、このECDIIIIa領域が、p185HER-2を過剰発現している細胞に選択的に結合するかどうかを調べた。これは、HER-2をトランスフェクションした17-3-1細胞の単層培養物を用いて3T3親細胞と比較することによって調べた。細胞をさまざまな濃度のヒスチジン-ECDIIIIaペプチドとともに培養し、洗浄し、プロテアーゼ阻害剤を用いて変性用緩衝溶液中に抽出した。結合したペプチドがあるかどうかを検出するため、細胞抽出物を、ECDIIIIaに対して特異的な抗体を用いてウエスタンブロット解析により調べた。さらに、ECDIIIIaペプチドで処理した細胞を等分した複数のアリコートで、ウエスタンブロットとしてp185HER-2に対して特異的な抗体と反応させた。するとトランスフェクションされた17-3-1細胞でp185HER-2が過剰に発現した。ECDIIIIaペプチドは、nMオーダーのさまざまな濃度で完全な17-3-1細胞に好んで結合する(図5C)のに対し、同等な量の3T3親細胞にはペプチドがほとんど結合しないか、まったく結合しなかった。これは、ECDIIIIaがp185HER-2の細胞外領域と特異的な相互作用をしていることを示唆する。

#### 【実施例10】

【0099】p68ECDIIIIaとECDIIIIaペプチドがp185HER-2のチロシンリン酸化に及ぼす効果を調べた。RTKのチロシンリン酸化は、リガンド活性化とシグナル伝達があることの第1の印である。17-3-1細胞の処理を、さまざまな量の精製ECDIIIIaペプチドを用いて、またはp68HER-2を高レベルで含むHEK293細胞からのならし培地(CM)(図2A)を用いて、または対照となる、検出できるレベルのp68HER-2を含まないSKOV-3細胞からのならし培地を用いて行ない、この17-3-1細胞のチロシンリン酸化を調べた。ヒスチジン-ECDII

I a または濃縮 C M で 10 分間処理したとき ( 図 6 ) または 2 時間処理したとき、チロシンリン酸化シグナルは増加しなかった。これは、p 1 8 5 H E R - 2 が活性化されなかったことを示唆している。p 6 8 H E R - 2 を含む C M も E C D I I I a ペプチドも、p 1 8 5 H E R - 2 のチロシンリン酸化レベルが低い S K O V - 3 細胞からの p 1 8 5 H E R - 2 に対応するチロシンリン酸化シグナルを検出可能なほど変化させることはなかった。さらに、p 6 8 H E R - 2 と E C D I I I a ペプチドは、1 7 - 3 - 1 細胞抽出物から免疫沈降させた p 1 8 5 H E R - 2 の自己リン酸化活性に対して検出可能なほどの影響をインビトロで与えることはなかった。これらの結果は、p 6 8 H E R - 2 が p 1 8 5 H E R - 2 のシグナル伝達を活性化させなかったという結論を支持している。

#### 【実施例 1 1】

【0 1 0 0】この実施例では、イントロン 8 が m R N A 内に保持されている場合には、イントロン 8 の配列が、そのイントロン 8 のうちで ( p 1 8 5 H E R - 2 の細胞外領域と一緒にあって ) イン・フレーム・コード配列として機能する部分において多型であることを示す。

【0 1 0 1】ヒト H E R - 2 遺伝子のイントロン 8 は、場合によっては m R N A 内に交互に保持されて、p 1 8 5 H E R - 2 の細胞外領域の一部の C 末端の位置で新奇な 7 9 残基領域をコードしている。保持されたイントロンを有するこの転写産物の産物である “ハースタチン” は、H E R - 2 ガン遺伝子の自己阻害剤として機能する。イントロン 8 をコードしている領域は、単独では、n M のアフィニティで p 1 8 5 H E R - 2 に結合することがわかっている ( D o h e r t y 他、P r o c . N a t l . A c a d . S c i . U S A、第 9 6 巻、1 0 , 8 6 9 ~ 1 0 , 8 7 4 ページ、1 9 9 9 年 ) 。

【0 1 0 2】H E R - 2 遺伝子のイントロン 8 のヌクレオチドの多型とそこから推定されるアミノ酸配列を、別々の 1 5 人のゲノム D N A をシーケンシングすることによって同定した。図 8 と配列 I D 番号 1 は、イントロン 8 の最も一般的なヌクレオチド配列と、それに対応するアミノ酸配列をそれぞれ示している。この領域は、1 0 通りの多型 ( 配列 I D 番号 1 0 の中には文字 W ( 2 箇所 )、Y ( 3 箇所 )、R、N、M、S ( 2 箇所 ) で示し、図 8 には “ X ” で示してある ) を含んでおり、その結果として保存されないアミノ酸置換が起こっている ( 表 I の注を参照のこと )。例えば、ヌクレオチド位置 1 6 1 に多型 ( G C ) があると ( 図 8 ; 表 I )、配列 I D 番号 1 のアミノ酸残基 # 5 4 の位置、または配列 I D 番号 2 の残基 # 3 9 4 の位置でアルギニン ( R ) がプロリン ( P ) に代わるはずである。図 8 または配列 I D 番号 1 0 の位置 1 で示される N 末端のグリシン ( G ) は、“ハースタチン” 配列のアミノ酸残基 3 4 1 に対応する ( D o h e r t y 他、P r o c . N a t l . A c a d . S c i . U S A、第 9 6 巻、1 0 , 8 6 9 ~ 1 0 , 8 7 4 ページ、1 9 9 9 年 )。図 1 ( A ) に示したヌクレオチド配列は、図 8 に示した最も一般的であることがわかった配列と比べてアミノ酸残基 # 6 と # 7 3 の位置のアミノ酸が異なる多型になっている。

【0 1 0 3】この結果は、ヒトの集団において、イントロン 8 がコードされている領域にいくつかの違いが存在しており、その結果、E C D I I I a を含むタンパク質変異体相互の間で生化学的特性や生物学的特性が異なる可能性のあることを示している。同定されたいくつかの変異を表 1 にまとめておく。

表 1

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	X(4)	X(14)	X(17)	X(47)	X(54)	X(62)	X(106)	X(161)	X(191)	X(217)
変異1	T									
変異2		C								
変異3			T							
変異4				A						
変異5					A					
変異6						C,T,A				
変異7							A			
変異8								G		
変異9									T	
変異10										C
変異11			T							C

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【0104】表1。ヒトの集団（別々の15人）でイントロン8がコードされている領域に見られた配列の変異1～11をリストにしてある。このリストには、図8に示した最も一般的なDNA配列と比べて特定の“X”の位置に塩基の変化があることが示されている。それぞれのXの後ろにある括弧内の数字は、図8に示したDNA配列または配列ID番号10のヌクレオチドの位置に対応している。ここにリストとして示したDNA配列の変異は、配列ID番号1の可変アミノ酸位置（“Xaa”）に対応している。例えば、X（4）がXaa（2）に；X（14）がXaa（5）に；X（17）がXaa（6）に；X（47）がXaa（16）に；X（54）がXaa（18）に；X（62）がXaa（21）に；X（106）がXaa（36）に；X（161）がXaa（54）に；X（191）がXaa（64）に；X（217）がXaa（73）に対応している。配列ID番号2の可変アミノ酸位置に対応している変異は以下の通りである。X（4）がXaa（342）に；X（14）がXaa（345）に；X（17）がXaa（346）に；X（47）がXaa（356）に；X（54）がXaa（358）に；X（62）がXaa（361）に；X（106）がXaa（376）に；X（161）がXaa（394）に；X（191）がXaa（404）に；X（217）がXaa（413）に対応している。配列ID番号1の可変アミノ酸位置に関する（図8に示した最も一般的なDNA配列と比べた場合の）具体的なアミノ酸の変化は以下の通りである。変異1、Xaa（2）（トレオニン セリン）；変異2、Xaa（5）（ロイシン プロリン）；変異3、Xaa（6）（プロリン ロイシン）；変異4、Xaa（16）（ロイシン グルタミン）；変異5、Xaa（18）（メチオニン ロイシン）；変異6、Xaa（21）（グリシン アスパラギン酸、Alu、またはバリン）；変異7、Xaa（36）（ロイシン イソロイシン）；変異8、Xaa（54）（プロリン アルギニン）；変異9、Xaa（64）（プロリン ロイシン）；変異10、Xaa（73）（アスパラギン酸 アスパラギン）；変異11、Xaa（6）（プロリン ロイシン）とXaa（73）（アスパラギン酸 アスパラギン）。同じ置換が配列ID番号2の対応する可変アミノ酸位置にも適用される。

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【実施例12】

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【0105】この実施例では、アフリカ系アメリカ人からのDNAサンプルにおいて同定可能なHER-2のイントロン8の5つの多型（配列の変異12～16）を示す（後出の表IIIを参照のこと）。

【0106】具体的には、イントロン8がmRNA内に保持されている場合には、イントロン8の配列が、そのイントロン8のうちで（p185HER-2の細胞外領域と一緒に）イン・フレーム・コード配列として機能する部分において、4つの多型部位が同定された（すなわち配列ID番号10または図8の配列領域に含まれる配列領域内の4つの多型位置）。これら多型部位のうち2つ（変異12と15）は、実施例11に示した多型部位（それぞれ変異3と10）に対応しているのに対し、他の2つ（変異13と14）は追加の多型部位を表わしている（表II）。

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【0107】さらに、イントロン8がmRNA内に保持されている場合には、追加の多型部位（変異16）が、“非コード”配列に留まるイントロン8の領域に同定された。この“非コード”イントロン8多型部位は3'に位置し（すなわちイントロン8のうちでこの実施例と実施例11に示した他の多型部位を含む部分から見て下流の位置にある）、イントロン8がmRNA内に保持されている場合には、（p185HER-2の細胞外領域と一緒に）イン・フレーム・コード配列として機能する。

【0108】方法。HER-2遺伝子内のイントロン8のヌクレオチド配列およびそこから推定されるアミノ酸配列における多型を、215人からのゲノムDNA（血液サンプルを使用）をシーケンシングすることによって同定した。215人の内訳は、アフリカ系アメリカ人（黒人）75人、コーカサス人（白人）135人、アジア系アメリカ人（アジア人）1人、ヒスパニック4人である。上記の実施例11と同様、図8、配列ID番号1、配列ID番号10の位置1で表わされるN末端のグリシン（GまたはGly）は、配列ID番号2または配列ID番号13の“ハースタチン”のアミノ酸残基#341に対応する。

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【0109】結果。表IIは、イントロン8のコード配列中のヌクレオチドの置換および2つのアミノ酸残基の置換と、イントロン8の非コード配列中の第3のヌクレオチドの置換を表わしている。位置を表わす数字としては、“ハースタチン”タンパク質配列全体（配列ID番号2または配列ID番号13）に対応する番号を用いている。

表II

	N		
黒人	75		
白人	135		
アジア人	1		
ヒスパニック/ラテンアメリカ人	4		
合計	215		
黒人におけるハースタチンの多型分布			
		アルギニン357システイン(C108IT)	
	前立腺ガンのケース	対照	他のガン
野生型 (%)	24(96)	32(89)	13(93)
ヘテロ型 (%)	1(4)	2(6)	1(7)
突然変異型 (%)	0(0)	2(6)	0(0)
合計	25	36	14
		アルギニン371イソロイシン(G1124T)	
	前立腺ガンのケース	対照	他のガン
野生型 (%)	24(96)	36(100)	14(100)
ヘテロ型 (%)	1(4)	0(0)	0(0)
突然変異型 (%)	0(0)	0(0)	0(0)
合計	25	36	14
		C1279T(3'UTR)	
	前立腺ガンのケース	対照	他のガン
野生型 (%)	24(96)	36(100)	12(93)
ヘテロ型 (%)	2(8)	0(0)	2(7)
突然変異型 (%)	0(0)	0(0)	0(0)
合計	25	36	14

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【0110】表 I I。この表は、アフリカ系アメリカ人からの DNA の HER - 2 のイントロン 8 において（実施例 11 で同定された多型と比べて）追加された 3 つの多型の分布を示している。アミノ酸の位置は、“ハースタチン”配列のアミノ酸位置に対応している（配列 ID 番号 2 または配列 ID 番号 13）。

【0111】以下の表 I I I は、配列データから（やはり図 8 または配列 ID 番号 10 に

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示した配列領域のヌクレオチド位置に対応している)ヌクレオチド位置#17と#217が多型であることが明らかにされたことを示している。位置#17の多型(変異12)は、表Iの変異3に対応している(実施例11)。位置#217の多型(変異15)は、(少なくともタンパク質レベルで)表Iの変異10に対応している(実施例11)(配列ID番号12と配列ID番号13を参照のこと)。

【0112】さらに、配列データ(配列ID番号11を参照のこと)からは、イントロン8が、上記の実施例11に示した多型部位に加えてさらに3つの多型部位(変異13、14、16に対応)を含んでいることが明らかになった(表IIIを参照のこと)。これらのうちの2つ(変異13、14)は配列ID番号11のヌクレオチド位置#49および#92に位置している(やはり配列ID番号10(または図8)のヌクレオチド位置#49および#92に対応している)。第3番目(変異16)は、配列ID番号11のヌクレオチド位置#259に位置する[やはり配列ID番号10のヌクレオチド位置#259(または図1のAの項に示した配列の位置#264)に対応している]。したがって、変異16に対応する多型は、この実施例と実施例11に示した他の多型部位を含むイントロン8の部分(すなわち実施例10に示した部分)から19ヌクレオチドだけ3'側(下流)に位置し、イントロン8がmRNA内に保持されている場合には、(p185HER-2の細胞外領域と一緒に)イン・フレーム・コード配列として機能する。

【0113】これら多型のうちの2つによって、保存されないアミノ酸置換が起こる(表IIと表IIIおよび表IIIの注を参照のこと;配列ID番号12と配列ID番号13も参照のこと)。例えば、配列ID番号11のヌクレオチド位置#49[または配列ID番号10、または図8の位置#49](すなわち表IIの位置X(49)の多型)に対応するヌクレオチド位置に見られる多型(C T)により、配列ID番号12、配列ID番号1、配列ID番号10、または配列ID番号11のアミノ酸残基#17に対応するアミノ酸位置、あるいは配列ID番号13または配列ID番号2のアミノ酸残基#357に対応するアミノ酸位置が、アルギニン(Arg)からシステイン(Cys)に置換される。

【0114】配列ID番号11、配列ID番号12、配列ID番号13は、この実施例に記載した4つの可変アミノ酸位置と、実施例11の可変アミノ酸位置を示している。後者は、配列ID番号1、配列ID番号2、配列ID番号10にも示してある。

【0115】表IIIは、(表Iの変異3および変異10にそれぞれ対応する変異12および変異15に加え)イントロン8の“コード”配列中のヌクレオチド置換と、(実施例11の表Iのアミノ酸残基置換と比べて)追加された2つのアミノ酸残基置換(すなわち変異13と変異14)と、イントロン8の3'“非コード”領域中の第3のヌクレオチド置換を示している。それぞれのX(多型位置)の後ろにある括弧内の数字は、配列ID番号11のヌクレオチド位置を示す[または表Iにおけるように、図8または配列ID番号10に示したDNA配列中のヌクレオチド位置に対応(または関係(X(259)の場合))している]。

【0116】上記の実施例11に関しては、配列ID番号11、図8、配列ID番号1、または配列ID番号10の位置1で示したN末端のグリシン(GまたはGly)は、配列ID番号2の“ハースタチン”のアミノ酸残基#341に対応する。

表III

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	X(17)	X(49)	X(92)	X(217)	X(259)
変異12	T				
変異13		T			
変異14			T		
変異15				A	
変異16					T

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【0117】表III。(別々の215人の)ヒトの組織で見いだされた、イントロン8をコードする領域における配列の変異。配列の変異12~16をリストにしてある。それぞれのX(多型位置)の後ろにある括弧内の数字は、配列ID番号11のヌクレオチド位置[または図8または配列ID番号10に示したDNA配列中のヌクレオチド位置に対応または関係(X(259)の場合)する位置]に対応している。このリストと配列ID番号11に示したDNA配列の変異は、配列ID番号12に示した可変アミノ酸位置に対応している[配列ID番号1または配列ID番号10の可変アミノ酸位置("Xaa")にも以下のように対応している: X(17)がXaa(6)に; X(49)がXaa(17)に; X(92)がXaa(31)に; X(217)がXaa(73)に対応している]。DNA配列中の変異X(259)は、非翻訳領域に発生するため、ハースタチンのアミノ酸配列を変化させることはない。同様に、この表の変異は、配列ID番号13および配列ID番号2の可変アミノ酸位置に以下のように対応している: X(17)がXaa(346)に; X(49)がXaa(357)に; X(92)がXaa(371)に; X(217)がXaa(413)に対応している。配列ID番号11と配列ID番号12の可変アミノ酸位置に関する(図8の最も一般的なDNA配列と比べた)具体的なアミノ酸変化は以下の通りである: 変異12、Xaa(6)(プロリン ロイシン); 変異13、Xaa(17)(アルギニン システイン); 変異14、Xaa(31)(アルギニン イソロイシン); 変異15、Xaa(73)(アスパラギン酸 アスパラギン)。変異16であるX(259)は非翻訳領域にあるため、アミノ酸の変化はコードしておらず、ヌクレオチド位置259のヌクレオチド配列だけを変化させる(すなわちC T)。同じ置換が、配列ID番号13の対応する可変アミノ酸位置に適用される。

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【0118】SKOV-3 卵巣ガン細胞。ヒト卵巣ガンに由来する細胞系(SKOV-3)においてさらに2つの多型が見つかった。この2つの多型があることで、保存されないアミノ酸置換が起こる。1つの多型は、イントロン8配列のヌクレオチド#17と"ハースタチン"配列のヌクレオチド#1037における置換(CとT)である。その結果、イントロン8配列のアミノ酸残基#6と"ハースタチン"配列(すなわち配列ID番号2または配列ID番号13)のアミノ酸残基#346においてロイシンがプロリンに置換される。SKOV-3 卵巣ガン細胞系に見られる第2の多型は、イントロン8配列のヌクレオチド#217と"ハースタチン"配列のヌクレオチド#1237における置換(GとA)である。その結果、イントロン8配列のアミノ酸残基#73と"ハースタチン"配列(すなわち配列ID番号2または配列ID番号13)のアミノ酸残基#413においてアスパラギンがアスパラギン酸に置換される。

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【0119】重要なことだが、この実施例12の配列解析において同定された5つの多型部位は、アフリカ系アメリカ人(黒人)からのDNAサンプルにだけ見られた。

実施例11と12のまとめ

【0120】本発明の実施例11と12を合わせると、HER-2 遺伝子のイントロン8

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にある13個の多型位置が開示されている。実施例12はDNAサンプルのサイズが比較的大きく、同定された5つの多型部位(そのうちの3つは実施例11で同定された10個の多型部位とは異なっている)はアフリカ系アメリカ人(黒人)にだけ見られた。

【0121】これら2つの実施例の13個の多型のうちの12個(すなわち実施例12の変異16を除く)は、イントロン8がmRNA内に保持されている場合に、そのイントロン8のうちで(p185HER-2の細胞外領域と一緒に)イン・フレーム・コード配列として機能する部分に存在している。

【0122】変異16に対応する多型は、イントロン8がmRNA内に保持されている場合に、そのイントロン8のうちで“非コード”配列のままになっている領域に位置する。イントロン8のこの“非コード”多型部位は、他の多型部位を含むイントロン8の部分から19ヌクレオチドだけ3'の側すなわち下流に位置し、イントロン8がmRNA内に保持されている場合には、(p185HER-2の細胞外領域と一緒に)イン・フレーム・コード配列として機能する。

【0123】HER-2のイントロン8のこれら多型は、新奇なDNA配列およびタンパク質配列と、HER-2を過剰に発現する固形ガンを治療するための新奇な薬理的組成物と、配列ID番号1、配列ID番号2、配列ID番号12、または配列ID番号13に対応するECD III a変異体と結合するモノクローナル抗体とを提供する。HER-2のイントロン8のこれら多型は、ガンの治療および予防のための予後予測方法ならびに診断方法も提供する。

#### 【図面の簡単な説明】

【図1】HER-2の細胞外領域に挿入されたヌクレオチド配列またはアミノ酸配列を示す。HER-2のECDをコードしているエキソン1~9からの配列(プライマーAとB)は、SKOV-3細胞からのcDNAライブラリーからPCRによって増幅した。約1420bpの産物が、サザンブロット解析によりHER-2に対して特異的であることがわかった。この産物をサブクローニングし、ヌクレオチド配列を決定した。Aの項には、274bpの挿入体(ボックスの外)のヌクレオチド配列と、ボックス内でヌクレオチド配列に直接隣接した5'配列および3'配列が示してある。この挿入体は、Cousens他のナンピングを用いると(Science、第230巻、1132~1139ページ、1985年)、p185HER-2のヌクレオチド残基1171と1172の間で、アミノ酸残基340の後ろに位置している。矢印で示した共通する5'スプライス部位および3'スプライス部位の拡大図が示してある。挿入された配列は、5'HER-2エキソン配列とインフレームであり、アルギニン340(R<sup>340</sup>)に続く79個のアミノ酸延長部をコードしていることが推定される。この挿入体によってコードされる新奇な79個のアミノ酸配列はプロリン・リッチ(19%)であり、アスパラギンが結合する共通グリコシル化部位を有する。終止コドンが、挿入された配列のヌクレオチド236~238の位置に見つかった。Bの項には、予測される新たな転写産物が、p185と一致するサブドメインIとIIを含む分泌タンパク質断片であり、膜貫通領域と細胞質領域を欠いていることを示してある。完全にグリコシル化された場合に予想されるサイズは65~70kDaである。このポリペプチド産物は、p68HER-2と呼ばれる。したがってこの産物は、p185HER-2で見つかった膜貫通領域と細胞質領域を欠いている分泌タンパク質断片であろう。

【図2】ECD III aを含む新たなHER-2転写産物をノーザンブロット解析で検出した結果を示す。さまざまなヒト胎児組織(クロンテック社)またはHEK-293細胞からのポリA<sup>+</sup>mRNA(2.5μg)をホルマリン・アガロース・ゲルに溶解させ、10×SSC中のブライトスター(登録商標)膜(アンピオン社)に移した。この膜を、ECD III a配列と相補的な<sup>32</sup>P標識アンチセンスRNAプローブとハイブリダイズさせ、分離させ、5'HER-2エキソン配列に対して特異的な<sup>32</sup>P標識cDNAプローブで再度調べた。膜を非常に厳しい条件下で洗浄し、リン光画像装置(モレキュラー・ダイナミックス社)で解析した。

【図3】抗ECD III aが、ヒト胚性腎臓細胞系(HEK293)の約68kDaの

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タンパク質に対して配列特異的な反応をすることを示している。細胞抽出タンパク質(20  $\mu$ g)と、HEK293細胞によって調整した20  $\mu$ lの培地に対してウエスタンブロット解析を行ない、1:10,000に希釈した抗ECD I I I a(レーン1と2)、またはヒスチジン・タグを有する50  $\mu$ g/mlの精製ECD I I I aペプチドを含む、1:10,000に希釈した抗ECD I I I a(レーン3と4)をプローブにして調べた。

【図4】HER-2遺伝子が増幅したガン細胞系ではp68 ECD I I I aの発現と比べてp185 HER-2の発現が顕著に増大していることを示す。ヒト胚性腎臓細胞系(HEK293)からの細胞抽出物(タンパク質15  $\mu$ g)、非発ガン性卵巣表面上皮細胞系(IOSEVAN)、HER-2遺伝子が増幅した卵巣ガン細胞系(SKOV-3)、非発ガン性乳房上皮細胞系(HBL100)、HER-2遺伝子が増幅した乳ガン細胞系(BT474とSKBR-3)を7.5%のアクリルアミド・ゲル中でSDS-PAGEによって分離させ、ウエスタンブロット法で解析した。ウエスタンブロット法では、プローブとしてp68 HER-2に対して特異的な抗体(抗ECD I I I a)とp185 HER-2に対して特異的な抗体(アンチneu(C))の両方を用いた。

【図5】p68 ECD I I I aがp185 HER-2に結合している様子を示す。Aの項では、非変性緩衝溶液中に抽出した2mgのSKBR-3細胞を、p68 HER-2とp185 HER-2のN末端配列に対して特異的な5  $\mu$ lのアンチneu(N)とともに、またはp185 HER-2のC末端に対して特異的な5  $\mu$ lのアンチneu(C)とともに免疫沈降させ、p68 HER-2に対して特異的な抗ECD I I I aとp185 HER-2に対して特異的なアンチneu(C)の両方をプローブとしてウエスタンブロット解析を行なった。Bの項では、100  $\mu$ gの17-3-1細胞抽出物を、ヒスチジン・タグを有する20  $\mu$ gのECD I I I aまたはヒスチジン・タグを有する20  $\mu$ gのCREB断片と結合させた充填容積が50  $\mu$ lのNiNTAアガロース樹脂(キアジェン社)とともに200  $\mu$ lの洗浄用緩衝溶液(20mMのトリス pH8.0、300mMのNaCl)中で室温にて1時間攪拌しながら培養した。なお、同じものを2つ用意した。次にこの樹脂を500  $\mu$ lの洗浄用緩衝溶液で4回洗浄し、50  $\mu$ lのSDS-サンプル緩衝溶液とともに100 で2分間培養することにより、タンパク質を溶離させた。溶離したタンパク質は、p185 HER-2のC末端に対して特異的な抗体、すなわちアンチneu(C)を用いてウエスタンブロット法で解析した。Cの項では、3T3細胞、またはHER-2をトランスフェクションした17-3-1細胞約 $10^5$ 個からなる単層を12のウエル・プレートに入れたものをPBSで2回洗浄し、次に、血清フリーで1%のBSAを含む培地0.5ml、ならびにヒスチジン・タグを有する精製した39、75、150、300nMの組み換えECD I I I aとともに4 で2時間にわたって培養した。細胞を、1%のBSAを含むPBSの中で1回洗浄し、PBSの中で2回洗浄し、変性用緩衝溶液中に抽出した。等量の複数のアリコート(タンパク質20  $\mu$ g)を、ECD I I I aに対して特異的な抗体(抗ECD I I I a)、またはp185 HER-2に対して特異的な抗体(アンチneu(C))を用いて(上の列)ウエスタンブロット法で解析した。

【図6】p68リッチにした培地もECD I I I aペプチドもp185 HER-2のチロシンリン酸化を促進できないことを示している。HER-2をトランスフェクションした約 $10^5$ 個の17-3-1細胞をPBSで2回洗浄し、血清フリーの培地中で37 にて24時間培養し、ヒスチジン・タグを有する75または150  $\mu$ lのECD I I I a、または高レベルのp68を分泌するHEK-293細胞からの50  $\times$ CM、またはp68 HER-2が検出できないSKOV-3細胞からの50  $\times$ CMを用いて10分間処理した。処理した細胞は、ホスホチロシンホスファターゼ阻害剤のバナジウム塩(2mM)を含む変性用緩衝溶液で抽出し、各サンプルからの細胞抽出タンパク質20  $\mu$ g/mlを、ホスホチロシンに対するモノクローナル抗体(シグマ社)を用いてウエスタンブロット法で解析した。62.5mMのトリスpH6.7と、2%のSDSと、100mMの2-メルカプトエタノールの中で55 にて30分間にわたって培養することによりブロットを

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ストリップし、p185HER-2に対して特異的なアンチneu(C)をプローブとしてこのプロットを再度調べた。

【図7】p68HER-2が、発ガン性細胞の基質とは独立な成長を抑制したことを示している。SCOV-3卵巣ガン細胞と、HER-2をトランスフェクションした17-3-1細胞を、0.3%の寒天を含む10%のウシ胎仔血清を加えた培地(対照条件)中に分散させ、この培地に、SCOV-3細胞によって調整した50x濃縮培地(検出可能なレベルのp68HER-2を含まない(-p68CM))、またはHEK-293細胞によって調整した50x濃縮培地(20nMのp68HER-2を含む(+p68CM))を添加した。12個のウエル・プレートに0.5%のアガロースを含む0.5mlの培地を層状に入れ、その上に5x10<sup>3</sup>個の細胞を加えたものをそれぞれの実験条件ごとに全部で3通り用意した。図示した結果は、3通り用意したウエル中の50個を超える細胞からなるコロニーの数を、培養開始後21日目のときに数えた平均値と標準偏差をプロットしたものである。同様の結果が3つの別々の実験でも観察された。

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【図8】HER-2のイントロン8のヌクレオチドと、推定されるアミノ酸配列を示す。ヒトのゲノムDNAに対し、イントロン8に隣接するプライマーを用いてPCRを行なった。PCRパラメータは、94にて1分間、62にて1分間、72にて30秒間を30サイクルの後、72にて7分間を1サイクルであった。410bpの産物をゲルで精製し、順方向と逆方向にシーケンシングした。図示した配列は、別々の約15人からのイントロン8で見つかった最も一般的な配列である。配列が変異してアミノ酸の置換が起こる可能性のある箇所はXで示してある。

20

【図1】



図1

【図2】

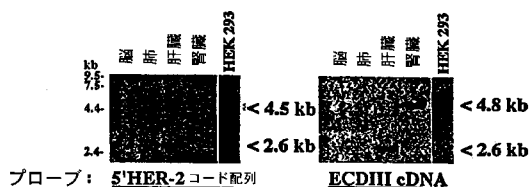


図2

【図3】

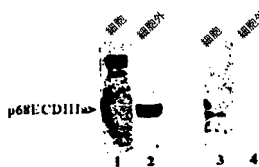


図3

【 図 4 】

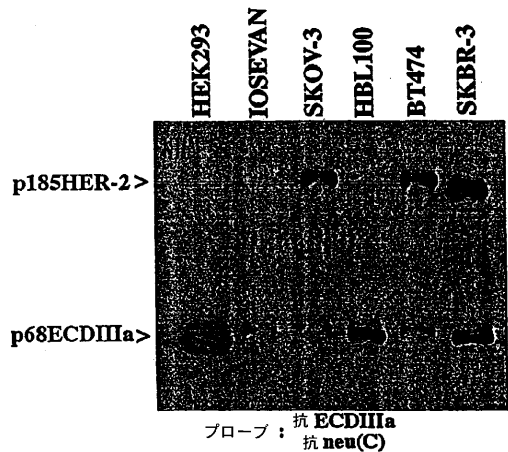


図 4

【 図 5 】

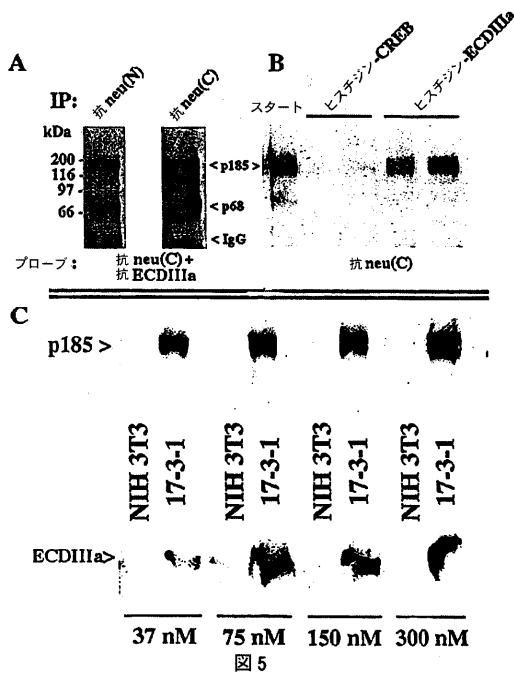


図 5

【 図 6 】

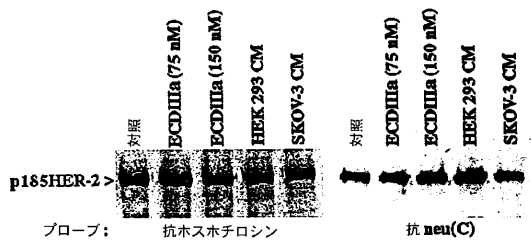


図 6

【 図 8 】

HER-2のイントロン8の多型

```

1  G T H S L P P R P A A V P V P L R M Q P G
1  GGTACCCTCACTCACTGCCCGAGGCCAGCTGCAGTTCCTGTCCCTCTGCCCATGCAGCCTGGC
   X X X X X X X X X X X X X X X X
22 P A H P V L S F L R P S N D L V S A F Y S
64 C C A G C C C A C C T G T C C T A C C T C C T C A G A C C C T C T G G G A C C T A G T C T C T G C C T T C T A C T C T
   X
43 L P L A P L S P T S V P I S P V S V G R G
127 C T A C C C T G G C C C C C T C A G C C C T A C A A G T G C C C T A T A T C C C C T G T C A G T G G G G A G G G G C
   X
64 P D P D A H V A V D L S R Y E G stop 80
190 C C G G A C C C T G A T G C T C A T G T G G C T G T T G A C C T G T C C C G G T A G A G G C T G A 240
   X X
    
```

図 8

【 図 7 】

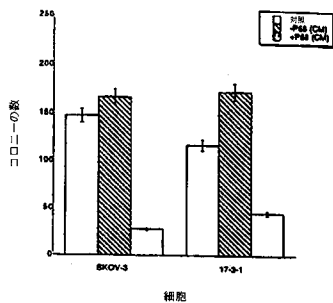


図 7

## 【国際公開パンフレット】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number  
WO 01/61356 A1

- (51) International Patent Classification: G01N 33/567, 33/574, C07K 16/00 (74) Agent: DAVISON, Barry, L., Davis Wright Tremaine, LLP, 2600 Century Square, 1501 Fourth Avenue, Seattle, WA 98101-1688 (US).
- (21) International Application Number: PCT/US01/05327 (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TI, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 16 February 2001 (16.02.2001) (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BI, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (25) Filing Language: English (30) Priority Data: 09/506,079 16 February 2000 (16.02.2000) US
- (26) Publication Language: English
- (71) Applicant (for all designated States except US): OREGON HEALTH SCIENCES UNIVERSITY [US/US]; 3181 S.W. Sam Jackson Park Rd., L335, Portland, OR 97201-3098 (US).
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- Published:  
— with international search report  
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



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(54) Title: HER-2 BINDING ANTAGONISTS

(57) Abstract: There is disclosed a pharmaceutical composition for treating solid tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO: 1 or SEQ ID NO: 12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least 10<sup>6</sup>, (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO: 2 or SEQ ID NO: 13, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Also disclosed are prognostic and diagnostic assays.

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**HER-2 BINDING ANTAGONISTS****Technical Field of the Invention**

5 The present invention provides a HER-2 binding antagonist. Specifically, intron retention has generated a novel HER-2 antagonist polypeptide that binds to the HER-2 receptor.

This work was supported by a grant from the Department of Defense (DOD) Breast Cancer Research Program. The United States Government has certain rights in this invention.

**10 Cross-reference to Related Applications**

This application is a continuation-in-part of United States Patent Application Serial No. 09/234,208, entitled HER-2 BINDING ANTAGONISTS, filed 20 January 1999.

**Background of the Invention**

15 The HER-2/neu (erbB-2) oncogene encodes a receptor-like tyrosine kinase (RTK) that has been extensively investigated because of its role in several human carcinomas (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994) and in mammalian development (Lee et al., *Nature* 378:394-398, 1995). The sequence of the HER-2 protein was determined from a cDNA that was cloned by homology to the epidermal growth factor receptor (EGFR) mRNA from placenta (Coussens et al., 20 *Science* 230:1132-1139, 1985) and from a gastric carcinoma cell line (Yamamoto et al., *Nature* 319:230-234, 1986). The HER-2 mRNA was shown to be about 4.5 kb (Coussens et al., *Science* 230:1132-1139, 1985; and Yamamoto et al., *Nature* 319:230-234, 1986) and encodes a transmembrane glycoprotein of 185 kDa in normal and malignant human tissues (p185HER-2) (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994). The function of the HER-2 gene has been examined 25 mainly by expressing the cDNA corresponding to the 4.5 kb transcript in transfected cells and from the structure and biochemical properties of the 185 kDa protein product. P185HER-2 consists of a large extracellular domain, a transmembrane segment, and an intracellular domain with tyrosine kinase activity (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994). Overexpression of p185HER-2 causes phenotypic transformation of cultured cells (DiFiore et al., *Science* 237:178-182, 1987; and Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987) and has been associated with aggressive clinical progression of breast and ovarian cancer (Slamon et al., *Science* 235:177-182, 1987; and Slamon et al., *Science* 244:707-712, 1989). p185HER-2 is 35 highly homologous to the EGFR. However, a ligand that directly binds with high affinity to p185HER-2 has not yet been identified. Moreover, the signaling activity of HER-2 may be mediated through heterodimerization with other ligand-binding members of the EGFR family (Carraway and Cantley, *Cell* 78:5-8, 1994; Earp et al., *Breast Cancer Res. Treat.* 35:115-132,

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1995; and Qian et al., *Oncogene* 10:211-219, 1995).

Divergent proteins, containing regions of the extracellular domains of HER family RTKs, are generated through proteolytic processing of full length receptors (Lin and Clinton, *Oncogene* 6:639-643, 1991; Zabrecky et al., *J. Biol. Chem.* 266:1716-1720, 1991; Pupa et al., *Oncogene* 8:2917-2923, 1993; Vecchi et al., *J. Biol. Chem.* 271:18989-18995, 1996; and  
5 Vecchi and Carpenter, *J. Cell Biol.* 139:995-1003, 1997) and through alternative RNA processing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maithe, *Oncogene* 16:3243-3252, 1998). The extracellular domain of p185HER-2 is proteolytically shed from breast carcinoma cells in culture (Petch et al.,  
10 *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maithe, *Oncogene* 16:3243-3252, 1998), and is found in the serum of some cancer patients (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) where it is may be a serum marker of metastatic breast cancer (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) and may allow escape of HER-2-rich tumors from immunological control (Baselga et al., *J. Clin.*  
15 *Oncol.* 14:737-744, 1996; and Brodowicz et al., *Int. J. Cancer* 73:875-879, 1997).

A truncated extracellular domain of HER-2 is also the product of a 2.3 kb alternative transcript generated by use of a polyadenylation signal within an intron (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). The alternative transcript was first identified in the gastric carcinoma cell line, MKN7 (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al.,  
20 *Mol. Cell. Biol.* 13:2247-2257, 1993) and the truncated receptor was located within the perinuclear cytoplasm rather than secreted from these tumor cells (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). However, no particular therapeutic, diagnostic or research utility has been ascribed to this truncated extracellular domain polypeptide. A truncated extracellular domain of the EGFR, generated by alternative splicing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990) is secreted, exhibits ligand-binding, and dimerization properties  
25 (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989), and may have a dominant negative effect on receptor function (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989; and Flickinger et al., *Mol. Cell. Biol.* 12:883-893, 1992).

Therefore, there is a need in the art to find molecules that bind to cellular HER-2 and particularly molecules that bind to different sites than humanized antibodies to HER-2 (e.g., Herceptin®). Such molecules would be useful therapeutic agents for various cancers that overexpress HER-2.

#### Summary of the Invention

The present invention provides an isolated polypeptide having from about 50 to 79  
35 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®

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(a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2).

The present invention further provides an isolated DNA sequence that codes, on expression, for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2). The present invention further provides a transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .

The present invention further provides an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present. Preferably, the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2).

The present invention further provides an isolated DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present. Preferably, the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation are present. The present invention further provides a transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present.

The present invention provides a method for treating a solid tumor characterized by overexpression of HER-2, comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a

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monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer and colon cancer. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a pharmaceutical composition for treating tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD of HER-2).

The present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation,

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immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2.

5 The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether a particular ECDIIIa variant  
10 sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database. Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

15 The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether an amount of an p68HER-2  
20 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

25 The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2  
30 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

#### 35 **Brief Description of the Drawings**

Figure 1 shows a nucleotide and amino acid sequence of the insert in the extracellular domain of HER-2. The HER-2 ECD coding sequence from exon 1-9 (primers A and B) was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ~1420 bp was

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found to be HER-2-specific by Southern blot analysis. This product was subcloned and the nucleotide sequence was determined. In panel A, the nucleotide sequence is shown for the 274 bp insert (outside the box) and for the immediately adjacent 5' and 3' sequences enclosed in the box. The insert is located between nucleotide residues 1171 and 1172 and following amino acid residue 340 in p185HER-2 using the numbering of Coussens et al. (*Science* 230:1132-1139, 1985). The consensus 5' and 3' splice sites at the arrows are shown in larger print. The inserted sequence is in-frame with 5' HER-2 exon sequence and is deduced to encode a 79 amino acid extension following Arg 340 (R<sup>340</sup>). The novel 79 novel amino acid sequence encoded by the insert is proline-rich (19%) and has a consensus asparagine linked glycosylation site, which is underlined. A stop codon was found at nucleotides 236-238 within the inserted sequence. In panel B, the predicted product of the alternative transcript is a truncated secreted protein which contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65-70 kDa. This polypeptide product is referred to as p68HER-2. Thus, the product will be a truncated secreted protein which is missing the transmembrane domain and cytoplasmic domain found in p185HER-2.

Figure 2 shows the detection of alternative HER-2 transcripts containing the ECDIIIa sequence by Northern blot analysis. PolyA+ mRNA (2.5 µg) from different human fetal tissues (Clontech) or isolated from HEK-293 cells was resolved in a formalin agarose gel and transferred to a BrightStar® membrane (Ambion) in 10xSSC. The membrane was hybridized with a <sup>32</sup>P-labeled antisense RNA probe complementary to the ECDIII sequence, stripped and reprobed with a <sup>32</sup>P-labeled cDNA probe specific for the 5' HER-2 exon sequence. The membranes were washed under high stringency conditions and analyzed by phosphorimaging (Molecular Dynamics).

Figure 3 shows a sequence-specific reactivity of anti-ECDIIIa with a protein of ~ 68 kDa in a human embryonic kidney cell line (HEK293). Cell extract protein (20 µg) and 20 µl of media conditioned by HEK-293 cells were Western blotted and probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIa diluted 1:10,000 containing 50 µg/ml purified His-tagged ECDIIIa peptide (lanes 3, 4).

Figure 4 shows the expression of p185HER-2, relative to p68ECDIIIa expression, is markedly elevated in carcinoma cell lines in which the HER-2 gene is amplified. Cell extracts (15 µg of protein) from human embryonic kidney cell line (HEK293), nontumorigenic ovarian surface epithelial cell line (IOSEVAN), ovarian carcinoma cell line with HER-2 gene amplification (SKOV-3), nontumorigenic breast epithelial cell line (HBL100), and breast carcinoma cell lines with HER-2 gene amplification (BT474 and SKBR-3), were resolved by SDS-PAGE in 7.5% acrylamide gels and analyzed as a Western blot. The Western blot was probed with both antibodies specific for p68HER-2 (anti-ECDIIIa) and for p185HER-2 (anti-neu(C)).

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Figure 5 shows that p68ECDIIIa binds to p185HER-2. In panel A: Two mg of SKBR-3 cells extracted in nondenaturing buffer were immunoprecipitated with 5 $\mu$ l anti-neu(N) specific for the N-terminal sequence of p68HER-2 and p185HER-2, or with 5  $\mu$ l anti-neu(C) specific for the C-terminus of p185HER-2 and then probed as a Western blot with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C) specific for p185HER-2. In panel B: 100  $\mu$ g of 17-3-1 cell extract were incubated in duplicate with 50  $\mu$ l packed volume of NiNTA agarose (Qiagen) coupled to 20  $\mu$ g of His-tagged ECDIIIa or to 20  $\mu$ g His-tagged CREB fragment in 200  $\mu$ l of wash buffer (20mM Tris pH 8.0, 300mM NaCl) at room temperature for 1 hr with shaking. The resin was then washed 4 times with 500  $\mu$ l of wash buffer and proteins were eluted by incubation with 50  $\mu$ l SDS-sample buffer at 100° C for 2 min. Eluted proteins were analyzed by Western blot analysis using antibodies against the C-terminus of p185HER-2, anti-neu(C). In panel C: Monolayers of  $\sim 10^5$  3T3 cells or HER-2 transfected 17-3-1 cells in 12 well plates were washed twice with PBS and then incubated with 0.5 ml of serum-free media with 1% BSA and 39, 75, 150, and 300 nM of purified recombinant His-tagged ECDIIIa for 2 hrs at 4 °C. Cells were washed 1 time in PBS containing 1% BSA and twice in PBS and then were extracted in denaturing buffer. Equal aliquots (20  $\mu$ g protein) were analyzed by western blotting with antibodies specific for ECDIIIa (anti-ECDIIIa) or, in the upper panel, with antibodies specific for p185HER-2 (anti-neu(C)).

Figure 6 shows that neither p68-rich conditioned media nor the ECDIIIa peptide stimulate tyrosine phosphorylation of p185HER-2. Monolayer cultures of  $\sim 10^5$  HER-2 transfected 17-3-1 cells were washed twice with PBS, incubated in serum-free media at 37 °C for 24 hrs, and then treated for 10 minutes with 75 or 150  $\mu$ M His-tagged ECDIIIa or with 50X CM from HEK-293 cells that secrete high levels of p68 or 50X CM from SKOV-3 cells that have no detectible p68HER-2. The treated cells were extracted with denaturing buffer containing the phosphotyrosine phosphatase inhibitor vanadate (2 mM) and 20  $\mu$ g/ml of cell extract protein from each sample were analyzed by Western blot analysis with monoclonal antibodies against phosphotyrosine (Sigma). The blot was stripped by incubation at 55° C for 30 min in 62.5 mM Tris pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol and then reprobed with anti-neu(C) specific for p185HER-2.

Figure 7 shows that p68HER-2 inhibited anchorage independent growth of tumorigenic cells. SKOV-3 ovarian carcinoma cells and HER-2 transfected 17-3-1 cells were suspended in media with 10% fetal bovine serum containing 0.3% agar (control conditions) to which was added 50X concentrated media conditioned by SKOV-3 cells (which contains no detectable p68HER-2 (-p68 CM)), or 50X concentrated media conditioned by HEK-293 cells (which contains 20 nM p68HER-2 (+p68 CM)). Five times  $10^3$  cells were plated in triplicate for each experimental condition onto a 0.5 ml layer of media containing 0.5% agarose in 12 well plates. The results shown are plotted as the mean and

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standard deviation of the number of colonies with more than 50 cells in triplicate wells counted at 21 days of incubation. Similar results were observed in three separate experiments.

Figure 8 shows the nucleotide and deduced amino acid sequence of HER-2 Intron 8.

5 Human genomic DNA was subjected to PCR using primers that flank intron 8. PCR parameters were 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 30 s, followed by 1 cycle of 72 °C for 7 min. A 410 bp product was gel purified and sequence in the forward and reverse directions. The sequence shown is the most common sequence found within intron 8 from about 15 different individuals. The positions of sequence variation that would result in amino acid substitutions are marked by Xs.

#### Detailed Description of the Invention

The present invention is based upon the initial discovery of an alternative HER-2 mRNA of 4.8 kb with a 274 bp insert identified as intron 8. The retained intron is in-frame and encodes 79 amino acids [SEQ ID NO. 1] followed by a stop codon at nucleotide 236.

15 The alternative mRNA predicts a truncated HER-2 protein that lacks the transmembrane and intracellular domains and contains 419 amino acids [SEQ ID NO. 2]; 340 residues that are identical to the N-terminus of p185HER-2 and 79 unique residues at the C-terminus [SEQ ID NO. 1]. Using specific antibodies against either the novel 79 amino acid residue C-terminal sequence [SEQ ID NO. 1] or the N-terminus of p185HER-2, a 68 kDa protein product was

20 identified [SEQ ID NO.2]. This 68 kDa protein is the product of an alternative HER-2 transcript, and is found in cell extracts and in extracellular media from several cell lines. Expression of the alternative transcript was highest in a nontransfected human embryonic kidney cell line.

The results presented here show expression of alternative HER-2 mRNA, which

25 contains an additional 274 nucleotides, probably intron 8. Consistent with this finding, an alternative transcript of ~ 4.8 kb was detected in human fetal kidney tissue and in the human embryonic kidney cell line, HEK 293. Moreover, a transcript of 2.6 kb, which is the size expected if the sequence is retained in the 2.3 kb truncated HER-2 mRNA (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993), was

30 detected in human fetal liver tissue by Northern blot analysis using a probe specific for the inserted sequence or for the HER-2 ECD coding sequence (Figure 2). The inserted sequence introduces a termination codon and predicts a novel 79 amino acid extension designated ECDIIIa at residue 340 of the p185HER-2 protein. The predicted protein therefore lacks the transmembrane and intracellular domains, but contains subdomains I and II of the

35 extracellular domain of p185HER-2. As predicted, a secreted protein that contains N-terminal sequence of p185HER-2 and the C-terminal extension provided by the inclusion of the novel sequence was detected (Figures 3 and 5). The ECDIIIa protein was found to be 68 kDa which is the approximate size expected of the protein encoded by the alternative

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transcript if the five N-linked glycosylation sites found in subdomains I and II of p185HER-2 are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

The data presented herein demonstrate that p68HER-2 specifically binds to p185HER-2. The association with p185HER-2 may be conferred by the novel proline rich ECDIIIa domain rather than the N-terminal subdomains I and II of p68HER-2. While the HER-2 ECD, generated by *in vitro* deletion mutagenesis, also contains subdomains I and II, it does not associate with the extracellular domain of p185HER-2 unless engineered to enhance their proximity (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997; and Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998). However, the unique ECDIIIa peptide binds with high affinity (nM concentrations) to p185HER-2 and to transfected 17-3-1 cells that overexpress p185HER-2 (Figure 5). Preferential binding of the ECDIIIa domain peptide to 17-3-1 cells indicates that secreted p68HER-2 interacts with the extracellular region of p185HER-2 at the cell surface. Therefore, p68HER-2 and fragments thereof appear to be a naturally occurring HER-2 binding protein, encoded by the HER-2 gene. In contrast to EGFR family ligands (Groenen et al., *Growth Factors* 11:235-257, 1994), p68HER-2 lacks an EGF homology domain and contains the first 340 amino acids of the receptor itself, p185HER.

Previously described putative HER-2 ligands were found to associate indirectly with p185HER-2 only in a heterodimer with an EGFR family member (Heldin and Ostman, *Cytokine Growth Factor Rev.* 7:33-40, 1996). Although it is possible that ECDIIIa binds indirectly to p185HER-2 through a coreceptor, this seems unlikely since detergent solubilized p185HER-2 was specifically and efficiently "pulled down" by immobilized ECDIIIa peptide (Figure 5B).

For all naturally occurring or engineered ligands for mammalian EGFR family members, binding is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; Dougall et al., *Oncogene* 9:2109-2123, 1994; and Groenen et al., *Growth Factors* 11:235-257, 1994). Although they bind, neither p68HER-2 nor the ECDIIIa peptide was found to activate p185HER-2. Activation was assessed in two different cell lines that differ in the extent of p185HER-2 tyrosine phosphorylation, transfected 17-3-1 cells as well as SKOV-3 ovarian carcinoma cells. Furthermore *in vitro* self-phosphorylation activity, which is enhanced in dimeric forms of p185HER-2 (Dougall et al., *Oncogene* 9:2109-2123, 1994; and Lin et al., *J. Cell. Biochem.* 49, 290-295, 1992), was not stimulated by p68HER-2 or ECDIIIa. Similarly, the Argos protein, which is an extracellular inhibitor of the Drosophila EGF receptor and the only known antagonist of class I RTKs, did not stimulate tyrosine phosphorylation of the receptor (Schweitzer et al., *Nature* 376:699-702, 1995). Likewise, Angiopoietin-2, a natural antagonist for the Tie 2 RTK, bound the endothelial receptor but failed to activate it (Maisonpierre et al., *Science* 277:55-60, 1997).

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Without being bound by theory, since p68HER-2 occupies but does not activate, it could block dimerization of p185HER-2. By analogy, HER-2 ECD, when engineered to enhance its binding to RTKs, prevented the formation of productive dimers required for transphosphorylation and receptor activation thereby having a dominant negative effect (O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997). In contrast to the HER-2 ECD, soluble p68HER-2 exhibited strong binding to p185HER-2, yet also contains subdomain I and II of the ECD. Since subdomain I may be the low affinity, promiscuous ligand binding site required for recruitment of p185HER-2 into heteromeric complexes (Tzahar et al., *EMBO J.* 16:4938-4950, 1997), p68HER-2 could block this site and thereby obstruct recruitment of p185HER-2 into dimers. Alternatively, p68HER-2 could compete with an uncharacterized ligand for binding to p185HER-2. The tissue-specific expression of p68HER-2 in human fetal liver and kidney may function to modulate the extent to which p185HER-2 is occupied during development of these organs. Moreover, the overexpression of p185HER-2, relative to p68HER-2 in tumor cells with HER-2 gene amplification (Figure 3), could occur through a selective pressure based on overcoming the effects of a binding protein such as p68HER-2. Therefore, p68HER-2 is the first example of a naturally occurring p185HER-2 binding protein that may prevent activation of p185HER-2.

#### Pharmaceutical Composition

The present invention further provides a pharmaceutical composition for treating solid tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The inventive pharmaceutical composition, comprising either or both of the inventive polypeptides and/or monoclonal antibody, can be administered to a patient either by itself (complex or combination) or in pharmaceutical compositions where it is mixed with suitable carriers and excipients. Inventive polypeptide can be administered parenterally, such as by intravenous injection or infusion, intraperitoneal injection, subcutaneous injection, or intramuscular injection. Inventive polypeptide can be administered orally or rectally through

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appropriate formulation with carriers and excipients to form tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like. Inventive polypeptide can be administered topically, such as by skin patch, to achieve consistent systemic levels of active agent. Inventive polypeptide is formulated into topical creams, skin or mucosal patch, liquids or gels  
5 suitable to topical application to skin or mucosal membrane surfaces. Inventive polypeptide can be administered by inhaler to the respiratory tract for local or systemic treatment of cancers characterized by overexpressing HER-2.

The dosage of inventive polypeptide suitable for use with the present invention can be determined by those skilled in the art from this disclosure. Inventive polypeptide will contain  
10 an effective dosage (depending upon the route of administration and pharmacokinetics of the active agent) of inventive polypeptide and suitable pharmaceutical carriers and excipients, which are suitable for the particular route of administration of the formulation (*i.e.*, oral, parenteral, topical or by inhalation). The active inventive polypeptide is mixed into the pharmaceutical formulation by means of mixing, dissolving, granulating, dragee-making,  
15 emulsifying, encapsulating, entrapping or lyophilizing processes. The pharmaceutical formulations for parenteral administration include aqueous solutions of the inventive polypeptide in water-soluble form. Additionally, suspensions of the inventive polypeptide may be prepared as oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or  
20 triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. The suspension may optionally contain stabilizers or agents to increase the solubility of the complex or combination to allow for more concentrated solutions.

Pharmaceutical formulations for oral administration can be obtained by combining the  
25 active compound with solid excipients, such as sugars (*e.g.*, lactose, sucrose, mannitol or sorbitol), cellulose preparations (*e.g.*, starch, methyl cellulose, hydroxypropylmethyl cellulose, and sodium carboxymethyl cellulose), gelatin, gums, or polyvinylpyrrolidone. In addition, a disintegrating agent may be added, and a stabilizer may be added.  
Processes for Synthesizing p68 and 79 aa C Terminal Region

30 Polypeptide synthesis is done by a group of standard procedures for polypeptide synthesis by sequential amino acids building through peptide synthesis equipment, following manufacturer's instructions for synthesizing peptides. Preferably, shorter polypeptides, of less than 100 amino acids, are best suited for the method of synthesis through sequential amino acid building of polypeptides. In addition, heterologous polypeptides can be expressed  
35 by transformed cells using standard recombinant DNA techniques to transform either prokaryotic or eukaryotic cells, provide appropriate growth media for their expression, and then purify the inventive polypeptide either from the media or from intracellular contents depending upon the type of cell used and its expression characteristics.

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Methods for Treating Cancer with p68, 79 aa C Terminal Region, and Combinations

The present invention provides a method for treating a solid tumor characterized by overexpression of HER-2, or HER-2 variants (see Example 8) comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected

5 from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least

10 three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer, prostate cancer, gastric carcinoma, cervical cancer, esophageal carcinoma, and colon cancer.

15 Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The p68HER-2 polypeptide described herein was found to bind to HER-2 and prevent

20 signal transduction through the kinase domain. Without being bound by theory, the unique ECDIIIa domain mediates specific binding to p185HER-2 and the resulting interaction with p68ECDIIIa prevents p185HER-2 dimerization and subsequent signal transduction. Therefore, p68HER-2 functions as a HER-2 antagonist to prevent signal transduction by preventing dimerization as a necessary prerequisite for signal transduction. Thus, the

25 mechanism of p68HER-2 as a HER-2 antagonist is different from the mechanism of binding agents, such as the 79 amino acid polypeptide described herein or a monoclonal antibody that binds to the EDC of HER-2. The inventive method provides that p68HER-2 inhibits tumor cell growth in tumors that overexpress HER-2 by providing a selective pressure for such tumor cells. Similarly, the HER-2 antagonists that are binding agents also inhibit tumor cell

30 growth in tumors that overexpress HER-2 by providing selective pressure to such cells to prevent ligand binding to the ECD of HER-2 and prevent signal transduction even before potential dimerization.

Use of 79 aa C Terminal Region as a Targeting Molecule

The present invention further provides a method for targeting a therapeutic agent to

35 solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the

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isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD of HER-2). It was discovered that the 79 amino acid polypeptide [SEQ ID NO. 1] exhibited surprising high affinity binding properties to the ECD of HER-2. Moreover, the site of such binding is different and unaffected by the site of binding of a marketed humanized monoclonal antibody (Herceptin®). Therefore, the high binding affinity enables the 79 amino acid polypeptide to function as a targeting molecule to tumor cells expressing HER-2.

10 Anti-p68 Antibody as a Diagnostic/Prognostic Agent

The p68HER-2 ECDIIIa variant 3 (see TABLE 1, below) glycosylated polypeptide was expressed and used as an antigen for antibody production. Specifically, antibody specific for p68HER-2 was prepared by injecting rabbits with purified polyhistidine-tagged ECDIIIa variant 3 peptide, which is the same as the intron encoded novel C-terminus or p68HER-2, the domain that binds with high affinity to p185HER-2. The isolated polyclonal antibody detected pM quantities of ECDIIIa peptide or of p68HER-2 with high specificity (see Figures 3 and 5). Thus, an antibody specific for p68HER-2 is useful as a diagnostic agent for detecting p68HER-2 in bodily fluids and tumor tissues using diagnostic techniques, such as ELISA, immunoprecipitations, immunohistochemistry or Western analysis.

20 Antibodies that specifically recognize one or more epitopes of ECDIIIa, or epitopes of p68HER-2, or peptide fragments, and thus distinguish among ECDIIIa variants (see TABLE 1, below) are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single-chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The antibodies of the invention may be used, for example, in the detection of a particular p68HER-2 ECDIIIa variant in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients or tissue samples may be tested for the presence of particular variants, or for abnormal amounts particular variants.

30 Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of particular p69HER-2 variants. Additionally, such antibodies can be used in conjunction with the cancer treatment methods described herein.

35 For the production of antibodies, various host animals may be immunized by injection with e.g., polyhistidine-tagged ECDIIIa variant polypeptides, truncated ECDIIIa variant polypeptides, functional equivalents of the ECDIIIa variants or mutants of the ECDIIIa region. Such host animals may include but are not limited to rabbits, mice, hamsters and rats, to name but a few. Various adjuvants may be used to increase the immunological response,

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depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (*Nature* 256:495-497, 1975; and U.S. Pat. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., *Immunology Today* 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030, 1983), and the EBV-hybridoma technique (Cole et al., *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Hybridomas producing mAb may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

Additionally, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855, 1984; Neuberger et al., *Nature*, 312:604-608, 1984; Takeda et al., *Nature*, 314: 452-454, 1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region (humanized).

Alternatively, techniques described for the production of single-chain antibodies (U.S. Pat. No. 4,946,778; Bird, *Science* 242:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Ward et al., *Nature* 334:544-546, 1989) can be adapted to produce single-chain antibodies against ECDIIIa variant gene products. Single-chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., *Science*, 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

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Antibodies to particular ECDIIIa variants can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" the ECDIIIa variant, using techniques well known to those skilled in the art. (Greenspan & Bona, *FASEB J* 7 (5):437-444, 1993; and Nissinoff, *J. Immunol.* 147:2429-2438, 1991). For example antibodies which bind to an ECDIIIa variant and competitively inhibit the binding of p68HER-2 to HER-2 receptor can be used to generate anti-idiotypes that "mimic" the ECDIIIa variant and, therefore, bind and neutralize HER-2 receptor. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in cancer therapeutic regimens.

Alternatively, antibodies to particular ECDIIIa variants that can act as agonists or antagonists of the ECDIIIa variant activity can be generated. Such antibodies will bind to the ECDIIIa variant and modulate the activity of p68HER-2 vis-à-vis p185HER-2 receptor-mediated signal transduction. Such antibodies may be particularly useful for treating particular cancers and/or modulating tumor differentiation. Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis.

#### ECDIIIa region Variants as Diagnostic/Prognostic Agents

Example 11 (below) shows that the human sequence of intron 8 is both proline-rich and polymorphic. Sequencing of genomic DNA from fifteen different individuals resulted in the identification of 10 variable sequence regions within Her-2 Intron 8. See SEQ ID NO:10; Figure 8, and Table 1. Figure 8 shows the most common nucleotide and corresponding polypeptide sequences of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (see legend to TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue 341 in the "herstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999), is a polymorphic form that differs at amino acid residues #6 and

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#73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. An individual may, *inter alia*, be genetically heterozygous for two variants, homozygous for a given variant, or homozygous for a double variant. Both tumor progression and optimal treatment may vary depending upon the particular variants represented in a given individual.

This variability has both prognostic and diagnostic utility. The present invention shows that ECDIIIa-containing polypeptides can bind tightly to, and thus antagonize the HER-2 receptor. Such a specific, high-affinity interaction is dependent upon particular primary, secondary and tertiary structure of the ECDIIIa-containing polypeptide. The ECDIIIa region is proline-rich, and it is well known in the art that nonconservative substitution of proline residues, or other residues within a proline-rich sequence, in a given protein can have profound effects on its secondary and tertiary structure. Thus, the polymorphisms of the present invention are likely to embody significant structural, biochemical and biological differences relative to the most common polypeptide structure (shown in Figure 8). Structural differences among ECDIIIa variant proteins may include for example, differences in size, electronegativity, or antigenicity. Differences in biological properties among ECDIIIa variants might be seen *e.g.*, in the relative degree of cellular secretion, the nature and/or extent of modulation of the HER-2 receptor, pharmacokinetics (*e.g.*, serum half-life, elimination profile), resistance to proteolysis, N-linked glycosylation patterns, etc. These biological differences, in turn, would be expected to alter tumor progression and thus optimal treatment protocols. Thus, the knowledge that an individual contains a particular ECDIIIa variant or variants (*e.g.*, in individuals heterozygous for a given variant, or individuals with compound variants like variant 11 of Table 1), may, in itself, be prognostic of particular cancer susceptibility.

The apparent genetic heterogeneity of ECDIIIa region means that the nature of the particular ECDIIIa variation carried by an individual may have to be ascertained using sequence identity assays prior to attempting genetic diagnosis of the patient. The analysis can be carried out on any genomic DNA derived from bodily fluids of the patient, typically a blood sample from an adult or child, but alternatively may be serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, and chorionic villi samples. It is expected that standard genetic diagnostic methods, such as hybridization or amplification assays, can be used. Either DNA or RNA, may, for example, be used in hybridization or amplification assays of biological samples to detect particular ECDIIIa variant sequences. Such sequence identity assays may include, but are not limited to, Southern or Northern analyses, single-stranded conformational polymorphism analysis, *in situ* hybridization assays, and polymerase chain reaction ("PCR") analyses. Such analyses

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may reveal both quantitative and qualitative aspects of ECDIIIa variant sequence expression. Such aspects may include, for example, point mutations, and/or activation or inactivation of gene expression. Standard *in situ* hybridization techniques may be used to provide information regarding which cells within a given tissue express a particular ECDIIIa variant sequence.

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Preferably, diagnostic methods for the detection of ECDIIIa variant nucleic acid molecules involve contacting and incubating nucleic acids, derived from cell types or tissues being analyzed, with one or more labeled nucleic acid reagents, or probes, specific for particular ECDIIIa variants. More preferably, PCR, or reverse transcription PCR, can be utilized to identify nucleotide variation within the ECDIIIa domain. PCR reaction conditions should be chosen which optimize amplified product yield and specificity, and, additionally, produce amplified products of lengths that may be resolved utilizing standard gel electrophoresis techniques. Such reaction conditions are well known to those of skill in the art, and important reaction parameters include, for example, length and nucleotide sequence of oligonucleotide primers, and annealing and elongation step temperatures and reaction times. Following the PCR reaction, the PCR products can be analyzed by methods such as heteroduplex detection, cleavage of RNA-DNA hybrids using Rnase A, single-stranded conformational polymorphisms, and denaturing gradient gel electrophoresis.

Additionally, if the particular ECDIIIa sequence variant is known to add or remove a restriction site, or to have significantly altered the size of a particular restriction fragment, a protocol based upon restriction fragment length polymorphism ("RFLP") analysis may be appropriate.

ECDIIIa variants can also be analyzed at the expression level using sequence identity assays with bodily fluids derived from the patient, typically a blood sample from an adult or child, but may include serum, urine, lymph, saliva, tumor tissue, placental or umbilical cord cells, amniotic fluid, and chorionic villi samples. Well-known sequence identity assays for analyzing expression include, but are not limited to, mRNA-based methods, such as Northern blots and *in situ* hybridization (using a nucleic acid probe derived from the relevant cDNA), and quantitative PCR (as described by St-Jacques et al., *Endocrinology* 134:2645-2657, 1994).

Polypeptide-based methods (e.g., including but not limited to western blot analysis) including the use of antibodies specific for the ECDIIIa variant of interest, as discussed above, could also be used. These techniques permit quantitation of the amount of expression of a given ECDIIIa variant, at least relative to positive and negative controls. Preferably, a battery of monoclonal antibodies, specific for different ECDIIIa epitopes or variants, could be used for rapidly screening cells or tissue samples to detect those expressing particular ECDIIIa variants, or for quantifying the level of ECDIIIa variant polypeptides. Preferred diagnostic methods for the quantitative or qualitative detection of ECDIIIa variant peptide

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molecules may involve, for example, immunoassays wherein particular ECDIIIa-containing peptides are detected by their interaction with anti-ECDIIIa variant specific antibodies. This can be accomplished for example, by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or

5 fluorometric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of ECDIIIa-containing peptides. Through the use of such procedures, it is possible to determine not only the presence of particular ECDIIIa-containing polypeptides, but also their distribution in the examined tissue.

10 Immunoassays for ECDIIIa variant polypeptides preferably comprise incubating a biological sample, such as the above-named bodily fluids, which have been incubated in the presence of a detectably labeled antibody capable of identifying ECDIIIa-containing peptides, and detecting bound antibody by any of a number of techniques well known in the art. The biological sample may be brought in contact with and immobilized onto a solid phase support

15 or carrier such as nitrocellulose, or other solid support that is capable of immobilizing soluble proteins, cells, or cell particles. The support may then be washed with suitable buffers followed by treatment with the detectably labeled anti-ECDIIIa variant specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support may then be detected by

20 conventional means.

Alternatively, anti-ECDIIIa variant specific antibodies can be detectably labeled by linking the same to an enzyme for use in an enzyme immunoassay or Enzyme Linked Immunosorbent Assay ("ELISA"). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably, a chromogenic substrate, in such a manner as to

25 produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase,

30 glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

The detection can be accomplished by calorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished visually by comparison of the extent of enzymatic reaction with appropriate standards. Detection may

35 also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect ECDIIIa-containing peptides through the use of a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by

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autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, 5 allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or 10 ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, 15 isoluminol, thormatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of 20 luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The binding activity of a given lot of anti-ECDIIIa-variant specific antibody may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing 25 routine experimentation.

Accordingly, the present invention, including the unexpected discovery of a plurality of variable sequence positions within the proline-rich ECDIIIa region, along with antibodies specific for particular ECDIIIa variants, provides for valuable prognostic and diagnostic information and assays.

Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: 30 (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; and (b) 35 measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the

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amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis. Preferably, the method for determining the prognosis of tumor treatment further comprises determining which particular ECDIIIa variants are present and optimizing tumor treatment in view of particular biochemical and biological properties among ECDIIIa protein variants.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether a particular ECDIIIa variant sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database. Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

P68HER-2 as a Therapeutic Agent

Without being bound by theory, but it appears that p68HER-2 or ECDIIIa peptide inhibits the growth of tumor cells that overexpress HER-2 by binding to p185HER-2 at the

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cells surface. This hypothesis was examined by testing anchorage independent growth of cells in the presence or absence of p68HER-2 using cells that depend on p185HER-2 overexpression for their malignant growth, yet have little or no detectable p68HER-2. Anchorage independent growth of cells in soft agar was used as a predictive model for tumor cytotoxicity. This is a common and predictive procedure to examine transforming activity and reflects the tumorigenic and oncogenic potential of cells (DiFore et al., *Science* 237:178-182, 1987; Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987; and Baasner et al., *Oncogene* 13:901-911, 1996).

The effects of p68HER-2 on anchorage independent growth in soft agar was determined using SKOV-3 carcinoma cells and HER-2 transfected 17-3-1 cells, which are both tumorigenic and overexpress p185HER-2. The cells were suspended in media supplemented with fetal calf serum in the presence or absence of p68HER-2 and incubated for 21 days in a humidified incubator. Anchorage independent growth was quantitated by counting the number of colonies that contained more than 50 cells. Figure 7 shows that in the presence of p68HER-2, anchorage independent growth of both SKOV-3 cells and 17-3-1 cells was inhibited several fold. Accordingly, these data show that p68HER-2 is not just cytostatic, but cytotoxic and possibly apoptotic.

#### Example 1

This example provides the results from an experiment to investigate HER-2 mRNA diversity within the extracellular domain (ECD) coding sequence using polymerase chain reaction (PCR). A cDNA library from SKOV-3 cells (American Type Culture Collection (Rockville, MD) maintained in DMEM, supplemented with 10% fetal bovine serum and 0.05% gentamycin), an ovarian carcinoma cell line in which the HER-2 gene is amplified eight times (Tyson et al., *Am. J. Obstet. Gynecol.* 165:640-646, 1991) was examined using a forward primer specific for exon 1 (Tal et al., *Mol. Cell. Biol.* 7, 2597-2601, 1987) identical to nucleotides 142-161 and a reverse primer complementary to nucleotides 1265-1286 in exon 9 (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). Briefly, The SKOV-3 cDNA library was provided by Origene Technologies, Inc. (Rockville, MD), and was prepared from RNA extracted from SKOV-3 cells. RNA was extracted from SKOV-3 cells grown to 80% confluence on 15 cm plates with TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol, to obtain total RNA. RNA was resuspended in 10mM Tris-EDTA, pH 8.0, for reverse transcription and cDNA library construction, or in RNA hybridization buffer (80% formamide, 40mM PIPES, 4 mM NaCl, 1mM EDTA, pH 7.5) for ribonuclease protection assay (RPA). RNA concentrations were determined spectrophotometrically at OD<sub>260</sub>. Poly A<sup>+</sup> mRNA was selected from total RNA using a mRNA extraction kit (Oligotex, Qiagen).

A product of ~1420 bp, determined to be HER-2-specific by Southern blotting, was approximately 270 bp larger than the expected size of 1144 bp from the previously described

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cDNA sequence (Coussens et al., *Science* 230:1132-1139, 1985). Briefly, the Southern blotting procedure transferred nucleic acids from agarose gels under vacuum (Bio-Rad Model 785 Vacuum Blotter) in 0.4 M NaOH to Gene Screen Plus Hybridization Transfer Membrane (NEN Research Products, Boston, MA). Nucleic acids were fixed to membranes by UV crosslinking in a UV-Stratalinker (Stratagene, Inc., La Jolla, CA), and the membranes were blocked in hybridization buffer (50% formamide, 5X SSC, 1% SDS, 10 mg/ml herring sperm DNA) at 42 °C for 2 h. The membranes were hybridized at 42 °C for 16 h in hybridization buffer with 10<sup>7</sup> cpm of a 220 bp Kpn-HincII fragment from ECDIIIa cDNA labelled with ( $\alpha$ -<sup>32</sup>P)dCTP (NEN Life Sciences) using a Random Prime DNA Labelling Kit (Boehringer Mannheim).

Templates were amplified in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Emeryville, CA) using the Expand High Fidelity PCR System (Boehringer Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM MgCl<sub>2</sub>, 5  $\mu$ M of each primer, and 200  $\mu$ M dNTPs. All primers were obtained from GIBCO BRL (Life Technologies). Numbering of nucleotide and amino acid residues is according to the HER-2 cDNA sequence reported by Coussens et al. (Coussens et al., *Science* 230:1132-1139, 1985). The HER-2 extracellular domain was targeted for amplification from an SKOV-3 cDNA library (Origene Technologies, Inc.) using a forward primer (A) identical to nucleotides (nt) 142-161 of HER-2 cDNA (5'-TGAGCACCAATGGAGCTGGC-3' [SEQ ID NO 3]), which spans the initiation codon (underlined) and a reverse primer (B) (5'-TCCGGCAGAAATGCCAGGCTCC-3' [SEQ ID NO 4]), which is complementary to HER-2 exon sequence at nt 1265-1286. Cycling parameters were: 94 °C, 30 sec; 58 °C, 45 sec; 68 °C, 3 min, for 30 cycles. The region spanning the alternative sequence (denoted ECDIIIa) from genomic DNA, was amplified using a forward primer (C) (5'-AACACAGCGGTGTGAGAAGTGC-3' [SEQ ID NO 5]) identical to HER-2 exon-specific sequence at nt 1131-1152 and the reverse primer (B) [SEQ ID NO. 4] on DNA prepared as described (Bond et al., *FEBS Letters* 367:61-66, 1995) with cycling parameters: 94 °C, 30 sec; 62 °C, 30 sec; 72 °C, 60 sec, for 25 cycles.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the structure of mRNA containing the ECDIIIa sequence. First strand cDNA was reverse transcribed (Bond et al., *FEBS Letters* 367:61-66, 1995) using 5  $\mu$ g RNA primed with 0.5  $\mu$ g oligo-dT. To amplify the ECDIIIa insert and adjacent 5' HER-2 exon sequence, a forward primer (A) described above and a reverse primer (D) (5'-ATACCGGACAGGTC AACAGC-3' [SEQ ID NO 6]) which is complementary to the 3'ECDIIIa-specific sequence were used. Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 2 min, for 30 cycles.

Amplification of the ECDIIIa insert and adjacent 3' HER-2 exon-specific sequence was with a forward primer (E) (5'-TCTGGGTACCCACTCACTGC-3' [SEQ ID NO 7])

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which is identical to the 5'ECDIIIa-specific sequence and contains a *Kpn*I restriction site and a reverse primer (F) (5'-TTCACACTGGCAGTCCAGACC-3' [SEQ ID NO 8]) which is complementary to HER-2 exon sequence at nt 3898-3919 and spans the termination codon (underlined). Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 5 min, for 30 cycles.

The PCR product was subcloned and the nucleotide sequence was determined.

The results showed that the normal HER-2 coding sequence was present beginning with the 5' primer sequence and continued uninterrupted through nucleotide 1171. At this position, a 274 nucleotide insertion was found, followed by the expected coding sequence, including the 3' primer sequence. Analysis of the predicted protein product showed that the 274 nucleotide insertion encodes an extension of the known HER-2 protein, beginning at residue 340 (Cousens et al., *Science* 230:1132-1139, 1985), and introduces an in-frame stop codon 79 amino acids later (Figure 1). Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in Genbank showed no homologies.

Examination of the 5' and 3' junctions of the divergent sequence revealed consensus splice donor and acceptor sites (Sharp, and Burge, *Cell* 91:875-879, 1997) and include a pyrimidine tract and potential branchpoint adenine residues near the 3' end of the insert sequence (Figure 1). Thus, the inserted sequence is likely to be an intron.

Inspection of the predicted amino acid sequence of the novel 79 amino acids [SEQ ID NO. 1] encoded by the inserted sequence shows a consensus N-linked glycosylation site and a high proline content of 19% (Figure 1). The inserted sequence was designated ECDIIIa since it is located at the boundary between subdomains II and III in the extracellular domain of the p185HER-2 sequence (Lax et al., *Mol. Cell. Biol.* 8:1831-1834, 1988). The insert sequence is in-frame with the adjacent 5' HER-2 exon sequence for 236 nt where there is a termination codon.

#### Example 2

This example provides the results from experiments characterizing ECDIIIa as contiguous with HER-2 exons in the genome. To investigate the HER-2 gene structure in the region of the ECDIIIa sequence, a forward primer, identical to nucleotides 763-785, and a reverse primer, complementary to nucleotides 1265-1286 of the HER-2 cDNA, were used in the PCR on human genomic DNA. The amplification product was anticipated to span exon 5 (Tal et al., *Mol. Cell. Biol.* 7:2597-2601, 1987) to an exon which is immediately 3' of the ECDIIIa sequence. Intron number and sizes were estimated based on PCR product sizes, restriction digest analysis, and partial sequence analysis of amplification products.

Next, human genomic DNA was examined using HER-2 exon-specific primers that directly flank the insert to determine the sequences immediately flanking the ECDIIIa sequence. A ~430 bp product was amplified from normal human genomic DNA and from genomic DNA extracted from carcinoma cell lines SKOV-3, SKBR-3 and BT474, all of

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which have HER-2 gene amplification (Kraus et al., *EMBO J.* 6:605-610, 1987) and were found to express ECDIIIa in their cDNA. The identities of the PCR products as HER-2 were verified by Southern blot analysis using the procedure described in Example 1. Nucleotide sequence analysis showed that the PCR product from human genomic DNA contained the ECDIIIa insert, flanked immediately on both sides by known HER-2 coding sequence; no mutations or rearrangements were seen. These data show that the ECDIIIa sequence represents a wholly retained intron, likely intron 8 based on the size of products amplified following intron 4 and on the location of intron 8 in the homologous EGFR gene and HER-3 gene (Lee and Mailhe, *Oncogene* 16:3243-3252, 1998).

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**Example 3**

This example shows that ECDIIIa is the only retained intron within the coding sequence of HER-2 mRNA. To determine whether additional introns were retained in the mRNA containing the ECDIIIa insert sequence, the reverse transcriptase-polymerase chain reaction (RT-PCR) was employed. First, a forward primer identical to 5' HER-2 cDNA sequence at 142-161 which spans the initiation codon, and a reverse primer complementary to the 3' ECDIIIa sequence were employed with SKBR-3 and SKOV-3 cDNA. A product of 1.3 kb was amplified, which is the size expected if the product contained no introns other than intron 8. Amplification of the 3' HER-2 coding sequence was then performed using a forward primer identical to 5' ECDIIIa sequence and a reverse primer complementary to 3' HER-2 cDNA sequence at nucleotides 3898-3919, which spans the p185HER-2 termination codon. A product of 2.9 kb was amplified, which is the size expected from the HER-2 cDNA if no additional introns were retained.

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Further characterizations of both the 5' (1.3 kb) and 3' (2.9 kb) amplification products by restriction digest analysis and nucleotide sequencing confirmed the absence of additional retained introns. To determine the size of the products amplified when intron sequences are included, genomic DNA was used as a template for the PCR reactions, which resulted in products of approximately 10 kb for the 5' coding sequence and 5 kb for the 3' coding sequence. These results indicate that the alternative HER-2 transcript, resulting from retention of an intron of 274 bp, was expected to be about 4.8 kb in size, assuming that the 5'untranslated (5'UTR) and 3'untranslated (3'UTR) regions are identical in size to the previously described ~4.5 kb HER-2 cDNA (Coussens et al., *Science* 230:1132-1139, 1985).

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**Example 4**

This example illustrates the expression of a protein containing an ECDIIIa sequence. To assess whether the alternative sequence is translated into a protein product, the ECDIIIa sequence was expressed as a polyhistidine-tagged peptide in bacteria, purified the peptide by nickel-affinity chromatography, and raised antisera against the purified peptide. Briefly, the bacterial expression vector was prepared by amplifying the ECDIIIa sequence from the SKOV-3 cDNA library using primer E and a reverse primer complementary to the 3' end of

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the ECDIIIa insert sequence. The reverse primer contained a *Bam*H1 restriction site sequence, and was identical to that used for template construction in the RPA (described in examples 1 and 2). The PCR amplification product of ~280 bp was digested with *Kpn*I and *Bam*H1, gel purified (Qiaex II, Qiagen, Chatsworth, CA), and cloned into the pET30a vector, which encodes a six histidine tag at the amino-terminus of the expressed protein (Novagen, Madison, WI). The resulting expression vector, pET-ECDIIIa, was used for transformation of bacterial strain BL21.

To express the ECDIIIa protein product, BL21 cells transformed with the pET-ECDIIIa expression vector were grown in LB broth with 30 µg/ml Kanamycin for 4 h at 37 °C. Expression was induced with 0.1 mM IPTG for 3 h and the harvested cells were lysed by sonication, and then centrifuged at 39,000 x g for 20 min. The supernatant was absorbed onto Ni-NTA agarose (Qiagen), by shaking for 60 min at room temperature. The resin was washed with ten volumes of wash buffer (10 mM Tris pH 7.9 and 300 mM NaCl), followed by ten volumes of wash buffer with 50 mM imidazole. The his-tagged ECDIIIa protein was eluted in wash buffer with 250 mM imidazole. The his-tagged protein, which was estimated to be approximately 90% pure by Coomassie Blue staining of gels, was used to generate and characterize antibodies.

Briefly, anti-ECDIIIa antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA) by injection of two rabbits with purified polyhistidine-tagged ECDIIIa peptide (described below). Polyclonal anti-*neu* (N) was produced against a peptide identical to amino acid residues 151-165 of p185HER-2 (Lin and Clinton, *Oncogene* 6:639-643, 1991). Polyclonal anti-*neu* (C) was made against a peptide identical to the last 15 residues of the carboxy-terminus of p185HER-2 (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). Antisera from two immunized rabbits were characterized and found to contain antibodies of high titer that reacted with the purified ECDIIIa peptide.

A Western blot analysis examined whether SKBR-3 cells, which expressed the alternative sequence in its cDNA, produced a protein that reacts with anti-ECDIIIa antibody. A 68 kDa protein from the cell extract and from the extracellular media reacted with anti-ECDIIIa antibody from two different rabbits diluted at least 20,000 fold, but not with preimmune sera. Inspection of the cDNA sequence of the alternative transcript (Figure 1) predicted a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence were glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

If the 68 kDa ECDIIIa protein [SEQ ID NO. 2] is the translation product of the alternative HER-2 mRNA, then its N-terminal residues should be identical to the N-terminal 340 residues of p185HER-2. Therefore, cell extract from SKBR-3 cells was immunoprecipitated with anti-peptide antibody against an N-terminal sequence of HER-2, anti-*neu* (N) (Lin and Clinton, *Oncogene* 6:639-643, 1991) or with anti-ECDIIIa, and the

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immune complexes were examined by Western blot analysis with both antibodies. Briefly, three to 5  $\mu$ l of antisera were added to 2 mg of protein from cell lysates prepared in M-RIPA buffer (1% Nonidet P-40, 50 mM Tris pH 7.4, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 1% aprotinin), which had been centrifuged to remove nuclei.

- 5 Immunoprecipitation was for 2 h with shaking at 4 °C as described (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). The immune complexes were bound to Protein G Sepharose (Pharmacia) by incubation for 1 h at 4 °C with shaking, collected by centrifugation, and washed four times with M-RIPA. The proteins were released from the immune complex by  
10 incubation at 95° C for 2 min in SDS-PAGE sample buffer and resolved by SDS-PAGE in 7.5% gels (Mini-Protean II electrophoresis cell, Bio-Rad).

- Western blotting was conducted following SDS-PAGE. Proteins were electroblotted onto nitrocellulose (Trans-blot, BioRad) using a semi-dry transfer unit (Bio-Rad) at 15 V for 20 min per gel (0.75 mm thick) equilibrated with 25 mM Tris pH 8.3, 192 mM glycine, 50 mM NaCl, and 20% methanol. The membranes were blocked with 5% nonfat dry milk at 25  
15 °C for one hour. The blots were then incubated with primary antibody, washed twice for 15 min, and four times for 5 min with TBS-Tween (Tris-buffered saline containing 0.05% Tween), and then incubated for 40 min with goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase (Bio-Rad), diluted 1:10,000 in TBS-Tween. After incubation with secondary antibody, the membranes were washed as described above and reacted with  
20 chemiluminescent reagent (Pierce) and then were exposed to Kodak X-OMAT BLU film.

- As expected, p68HER-2 was detected when anti-ECDIIIa was used for immunoprecipitation and for Western blot analysis. When anti-ECDIIIa was used for immunoprecipitation and anti-neu (N) was the probe in the Western blot, a 68kDa protein was detected, indicating that p68ECDIIIa contained the N-terminal sequence of p185HER-2.  
25 Further, anti-neu (N) precipitated p68HER-2, which was detected by probing with anti-ECDIIIa antibody. These results demonstrate that p68HER-2 contains both ECDIIIa and the N-terminal sequence of HER-2.

- Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3) also had  
30 p68HER-2. Of several cell lines examined, HEK293 cells, derived from normal human embryonic kidney cells, expressed the highest levels of p68ECDIIIa in the cell extract and in the extracellular media, at about 5 to 10-fold higher amounts than SKBR-3 cells. In comparison to the carcinoma cell lines examined (SKBR-3, SKOV-3, and BT474) which overexpress p185HER-2, the HEK293 cells contained about 20 fold lower amounts of  
35 p185HER-2. Therefore, the relative proportion of p68HER-2 to p185HER-2 was at least 100 fold greater in HEK293 cells than in the three carcinoma cell lines studied. Reactivity with p68HER-2 as well as with a protein of ~120 kDa, particularly apparent in the HEK293 extracts, was blocked by preincubation of the antisera with purified ECDIIIa peptide

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demonstrating sequence-specific reactivity. The larger protein may be a dimer of p68HER-2. Therefore, p68HER-2 was expressed and secreted from several carcinoma cell lines and is at 5-10 fold elevated levels in HEK293.

#### Example 5

5 This example illustrates expression of an alternative HER-2 transcript containing the ECDIIIa intron sequence. Results of the RT-PCR analysis indicated that the ECDIIIa sequence was inserted into an otherwise normal-sized HER-2 mRNA. These data suggest an alternative transcript of ~4.8 kb. To examine the size and expression of the ECDIIIa alternative transcript, Northern blot analysis was conducted using an ECDIIIa-specific probe. Briefly, a template for antisense RNA probe synthesis was constructed from SKOV-3 cDNA  
10 by PCR amplification of a 389 bp sequence spanning the entire ECDIIIa insert sequence and containing adjacent 5'HER-2 exon sequence. The PCR was done using the forward primer C [SEQ ID NO. 5] that is identical to HER-2 cDNA sequence at nt 1131-1152 and a reverse primer (5'-GCACGGATCCATAGCAGACTGAG GAGG-3' [SEQ ID NO. 9]) which  
15 contains a 3' *Bam*H1 restriction endonuclease site and is complementary to the sequence spanning the 3' splice site of the ECDIIIa sequence. The PCR product was then digested with *Bam*H1, liberating a 375 bp fragment, which was cloned into pBluescript SK (Stratagene). The plasmid was sequenced by the Vollum Institute Core Sequencing Facility (Portland, OR) with m13 forward and reverse primers. An antisense RNA probe  
20 complimentary to the entire ECDIIIa sequence and to 87 nt of HER-2 exon sequence 5' to the insert was transcribed from 1 µg of linearized template using ( $\alpha$ -<sup>32</sup>P) CTP, T7 RNA polymerase, and the T7/SP6 Riboprobe Synthesis System (Promega, Madison, WI). This probe was expected to protect a 370 nt fragment when hybridized with mRNA containing ECDIIIa and adjacent HER-2 exon sequence, and to protect an 87 nt fragment when  
25 hybridized with fully spliced HER-2 mRNA.

To prepare the RNA hybrids, 30 µg of RNA were hybridized with approximately 50,000 cpm of antisense RNA probe at 48 °C for 16 h. RNA hybrids were digested for 30 min at 37 °C with 40 µg/ml RNaseA (Boehringer Mannheim) and 2 µg/ml RNase T1 (Life Technologies) in a solution of 250 mM NaCl, 5 mM EDTA, and 10 mM Tris pH 7.5.  
30 Proteinase K (100 µg) (Life Technologies) in 20 µl 10% SDS was added to stop the digestion. Samples were extracted with acid phenol (pH 4.5; Life Technologies) and chloroform, precipitated with two volumes of 100% ethanol, and suspended in 5 µl of RPA sample buffer (88% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue). Samples were denatured at 95° C for 10 min and electrophoresed on a  
35 5% polyacrylamide/urea gel in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA pH 8.3). Gels were dried under vacuum and subjected to phosphorimager analysis for quantitation of the protected fragments (IP Lab Gel, Molecular Dynamics).

An alternative transcript of approximately 4.8 kb was detected in HEK293 cells which

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expressed the highest levels of p68ECDIIIa. However an alternative transcript could not be detected by Northern analysis of the SKBR-3, BT474, or SKOV-3 carcinoma cell lines.

Therefore, the more sensitive ribonuclease protection assay (RPA) was employed to examine the expression levels of the alternative transcript relative to the fully spliced 4.5 kb transcript.

- 5 RNA from ovarian (SKOV-3) and breast (SKBR-3 and BT474) carcinoma cell lines, which contained detectable levels of p68ECDIIIa, and a control cell line, 17-3-1, stably transfected with HER-2 cDNA, were hybridized with an antisense <sup>32</sup>P-labeled RNA probe which spanned the entire ECDIIIa (intron 8) sequence and 5' HER-2 exon sequence flanking intron 8.
- 10 Following RNase digestion, electrophoresis, and autoradiography, a band of 370 nucleotides was detected in each cell line except for 17-3-1, which corresponds to the expected size protected by an ECDIIIa-containing HER-2 mRNA. In addition, an 87 nucleotide protected fragment was detected in all cells and is the size expected for the fully-spliced HER-2 message which is overexpressed by more than 100 fold in these carcinoma cell lines compared to normal control cell lines (Kraus et al., *EMBO J.* 6:605-610, 1987). The amounts of each protected fragment were quantitated and normalized for size to estimate the relative abundance of the alternative transcript, expressed as a percentage of the p185HER-2 mRNA.
- 15 The alternative HER-2 mRNA with the ECDIIIa insert was at 4.2% the level of the fully spliced transcript in SKOV-3; 5.4% in SKBR-3, and 0.8% in BT474 cells.

#### Example 6

- 20 This example shows that alternative transcripts containing the ECDIIIa insert were expressed in human embryonic kidney and liver. A Northern blot was conducted to examine whether an alternative transcript, which contains the ECDIIIa sequence, was expressed in normal human tissue. PolyA<sup>+</sup> mRNA from a variety of human fetal tissues prepared as a Northern blot was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8 kb mRNA was detected in kidney and a 2.6 kb transcript was detected in liver (Figure 2). The 4.8 kb transcript likely corresponded to the full length 4.5 kb transcript with the 274bp insert and the 2.6 kb transcript may have corresponded to a previously described 2.3 kb alternative transcript (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993) with the 274bp ECDIIIa insert. When the blot was stripped and hybridized with a probe specific for the 5' HER-2 coding sequence, a broad band representing the 4.8 and 4.5 kb mRNAs was detected in fetal kidney tissues and the truncated 2.6 kb transcript was detected in liver showing that these alternative transcripts contain sequences that encode the HER-2 ECD. Because the inserted ECDIIIa sequence contained a termination codon, the same protein product may be produced from each of these
- 35 mRNAs.

Several cell lines were also investigated for the ECDIIIa-containing alternative transcript by Northern blot analysis. The 4.8 kb alternative transcript was detected in the human embryonic kidney cell line, HEK-293 (Figure 2). Although the ECDIIIa sequence

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was detected by RT-PCR analysis of SKBR-3, BT474, and SKOV-3 carcinoma cell lines, which all contain HER-2 gene amplification, an ECDIIIa-containing alternative transcript could not be detected by Northern analysis of these cells. Therefore, the more sensitive ribonuclease protection assay (RPA) was employed using an antisense probe which spanned the entire ECDIIIa sequence and 5' HER-2 exon sequence flanking the ECDIIIa sequence. The alternative HER-2 mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcript in SKOV-3, SKBR-3, and BT474 cells. These findings show that two alternative transcripts containing the ECDIIIa sequence were expressed in a tissue-specific manner in normal human tissues, that the 4.8 kb alternative transcript was expressed in the HEK-293 cell line, and that the carcinoma cells with gene amplification express reduced amounts of the alternative transcript at less than 5% of the 4.5 kb HER-2 transcript.

**Example 7**

This example illustrates expression of a protein containing the ECDIIIa sequence. To assess whether the alternative sequence was translated into a protein product, the ECDIIIa sequence, as a polyhistidine-tagged peptide in bacteria, was expressed and purified by nickel-affinity chromatography, and raised antisera against the purified peptide. The HEK-293 cells, which expressed the 4.8 kb ECDIIIa alternative transcript, were examined for expression of an ECDIIIa-containing protein by Western analysis. A 68 kDa protein from the cell extract and from the extracellular media reacted with the anti-ECDIIIa antibody (Figure 3) but not with preimmune sera and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Figure 3). The larger protein of ~125 kDa detected in some cell extracts may be an aggregate of p68HER-2. The cDNA sequence of the alternative transcript (Figure 1) predicts a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986). Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3, SKBR-3) also had detectable levels of p68HER-2.

**Example 8**

This example illustrates the expression of p68HER-2 relative to p185HER-2 was markedly reduced in carcinoma cell lines in which the HER-2 gene is amplified. Because the p68HER-2 mRNA was expressed at very low levels relative to the p185HER-2 mRNA in carcinoma cell lines with HER-2 gene amplification, the relative proportions of p68HER-2 and p185HER-2 proteins in several cell lines were examined with and without HER-2 gene amplification. Western blots were prepared and probed with both antisera specific for p68HER-2 and for p185HER-2. Figure 4 shows that p185HER-2 was readily detected in the carcinoma cells lines that have their HER-2 gene amplified about 8 times (Kraus et al., *EMBO J.* 6:605-610, 1987). However, there was not a corresponding elevation in p68HER-2. In comparison, p68HER-2 was the only HER-2 protein detected in the HEK-293, IOSEVAN,

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and HBL100 nontumorigenic cells, although p185HER-2 was expressed at very low levels in these cells (Kraus et al., *EMBO J.* 6:605-610, 1987) and was detected in overexposed blots. These data show that p68HER-2 was low in proportion to p185HER-2 in carcinoma cells with HER-2 gene amplification and suggests that a mechanism may exist to maintain low levels of p68HER-2 when p185HER-2 is overexpressed.

#### Example 9

This example illustrates that p68HER-2 and the ECDIIIa peptide specifically bind to p185HER-2. Because p68HER-2 is secreted and contains subdomains I and II identical to p185HER-2, in addition to a novel sequence, the possibility that p68HER-2 may interact with p185HER-2 was investigated. Antipeptide antibody against the N-terminus of p185HER-2 and p68HER-2, anti-neu (N), or antibody specific for p185HER-2, anti-neu(C), were used for immunoprecipitations of SKBR-3 carcinoma cells, which express low levels of p68HER-2 and overexpress p185HER-2. The immunoprecipitated material was prepared as a Western blot and probed with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C). Anti-neu (N) immunoprecipitated both p68HER-2 and p185HER-2 (Figure 5A). In addition, antibodies specific for the C-terminus of p185HER-2 immunoprecipitated p185HER-2 and coprecipitated p68HER-2 (Figure 5A), suggesting an interaction between the two proteins.

Since binding interactions between ECD sequences are very weak (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998), the possibility that binding may be conferred by the novel proline rich ECDIIIa domain was examined. The unique 79 amino acid domain, purified as a His-tagged protein, was immobilized on nickel agarose and used in a pull-down assay. For controls, two purified His-tagged peptides unrelated to ECDIIIa, a 600 residue fragment of the Wilson's disease membrane protein, and a 70 residue fragment containing the DNA binding domain of the CREB protein, were likewise immobilized on nickel agarose resin. The immobilized peptides were incubated with protein extracts prepared from HER-2 transfected 3T3 cells (17-3-1). Following extensive washes, the bound proteins were eluted and prepared as a Western blot which was probed with an antibody specific for p185HER-2. Equal amounts of His-tagged ECDIIIa peptide and control peptide were bound to the resin as confirmed by elution with 1M imidazole and Coomassie staining of the eluted material in SDS-gels. While no p185HER-2 was retained by resin without peptide or with control peptide, p185HER-2 was selectively retained by the ECDIIIa peptide (Figure 5B).

Since the ECDIIIa domain bound to p185HER-2 in a pulldown assay, the question of whether the ECDIIIa domain preferentially binds to cells that overexpress p185HER-2 was examined. This was investigated using monolayer cultures of 17-3-1 cells transfected with HER-2 compared to the parental 3T3 cells. The cells were incubated with different concentrations of the His-ECDIIIa peptide, washed, and extracted in denaturing buffer with protease inhibitors. To detect any bound peptide, the cell extracts were examined by Western

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blot analysis using antibodies specific for ECDIIIa. In addition, equal aliquots of the ECDIIIa peptide treated cells were reacted as a Western blot with antibodies specific for p185HER-2, demonstrating the overexpression of p185HER-2 in the transfected 17-3-1 cells. The ECDIIIa peptide preferentially bound to intact 17-3-1 cells at nM concentrations (Figure 5C) whereas little or no peptide was found to bind to equivalent amounts of parental 3T3 cells suggesting a specific interaction with the extracellular domain of p185HER-2.

#### Example 10

Effect of p68ECDIIIa and the ECDIIIa peptide on tyrosine phosphorylation of p185HER-2 was examined. Tyrosine phosphorylation of RTKs is the initial indication of ligand activation and signal transduction. Tyrosine phosphorylation in 17-3-1 cells treated with different amounts of the purified ECDIIIa peptide, with conditioned media (CM) from HEK293 cells that contained high levels of p68HER-2 (Figure 2A), or with control, conditioned media from SKOV-3 cells that had no detectable p68HER-2 were examined. There was no increase in the tyrosine phosphorylation signal at 10 minutes (Figure 6) or 2 hrs of treatment with His-ECDIIIa or with concentrated CM suggesting that p185HER-2 was not activated. Neither p68HER-2-containing CM nor the ECDIIIa peptide detectably altered the phosphotyrosine signal corresponding to p185HER-2 from SKOV-3 cells in which p185HER-2 tyrosine phosphorylation levels were low. Additionally, p68HER-2 and the ECDIIIa peptide had no discernable effect on *in vitro* self-phosphorylation activity of p185HER-2 immunoprecipitated from 17-3-1 cell extracts. These results support the conclusion that p68HER-2 did not activate p185HER-2 signal transduction.

#### Example 11

This example illustrates that the sequence of intron 8 is polymorphic within that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

Intron 8 of the human HER-2 gene is alternatively retained in mRNA, and encodes a novel 79-residue domain at the C-terminus of a part of the extracellular domain of p185HER-2. The product, "herstatin," of the alternative transcript with the retained intron functions as an autoinhibitor of the HER-2 oncogene. The intron 8 encoded domain, alone, was shown to bind with nM affinity to p185HER-2. (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999).

Polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA from 15 different individuals. Figure 8 and SEQ ID NO:1 show the most common nucleotide and corresponding amino acid sequences, respectively, of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (*see* legend to

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TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "berstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) is a polymorphic form that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. Some identified variants are summarized in Table 1:

TABLE 1

	X(4)	X(14)	X(17)	X(47)	X(54)	X(62)	X(106)	X(161)	X(191)	X(217)
Variant 1	T									
Variant 2		C								
Variant 3			T							
Variant 4				A						
Variant 5					A					
Variant 6						C, T, A				
Variant 7							A			
Variant 8								G		
Variant 9									T	
Variant 10										C
Variant 11			T							C

Table 1. Sequence variants in the intron-8 encoded domain found in the human population (based on 15 different individuals). Sequence variants 1-11 are listed, showing the base changes at particular "X" positions relative to that found in the most common DNA sequence shown in Figure 8. The numbers in parenthesis after each X correspond to the nucleotide position in the DNA sequence shown in Figure 8 or SEQ ID NO:10. The DNA sequence variants listed here correspond to the variable amino acid positions ("Xaa") of SEQ ID NO:1 as follows: X(4) to Xaa(2); X(14) to Xaa(5); X(17) to Xaa(6); X(47) to Xaa(16); X(54) to Xaa(18); X(62) to Xaa(21); X(106) to Xaa(36); X(161) to Xaa(54); X(191) to Xaa(64); X(217) to Xaa(73); and to the variable amino acid positions of SEQ ID NO:2 as follows: X(4) to Xaa(342); X(14) to Xaa(345); X(17) to Xaa(346); X(47) to Xaa(356); X(54) to Xaa(358); X(62) to Xaa(361); X(106) to Xaa(376); X(161) to Xaa(394); X(191) to Xaa(404); X(217) to

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Xaa(413). The specific amino acid changes (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:1 are: Variant 1, Xaa(2)(Thr→Ser); Variant 2, Xaa(5) (Leu→Pro); Variant 3, Xaa(6) (Pro→Leu); Variant 4, Xaa(16) (Leu→Gln); Variant 5, Xaa(18) (Met→Leu); Variant 6, Xaa(21) (Gly→Asp, Alu or Val); Variant 7, Xaa(36) (Leu→Ile); Variant 8, Xaa(54) (Pro→Arg); Variant 9, Xaa(64) (Pro→Leu); Variant 10, Xaa(73) (Asp→Asn), and Variant 11, Xaa(6) (Pro→Leu) and Xaa(73) (Asp→Asn). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:2.

10

**EXAMPLE 12**

This example shows (*see* Table III, below) five polymorphic HER-2 intron 8 polymorphisms (sequence variants 12-16) identifiable in DNA samples from African Americans.

Specifically, four polymorphic sites were identified within that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA (*i.e.*, four polymorphic sites within the sequence region encompassed by SEQ ID NO:10, or within that encompassed by the sequence region of Figure 8). Two of these polymorphic sites (variants 12 and 15) correspond in position to those (variants 3 and 10, respectively) disclosed above in Example 11, whereas the other two (variants 13 and 14) represent additional polymorphic sites (Table II).

Furthermore, (*see* Table II and Table III, below) an additional polymorphic site (variant 16) was identified in a region of intron 8 that remains as "non-coding" sequence when intron 8 is alternatively retained in mRNA. This "non-coding" intron 8 polymorphic site is located 3', or downstream from that portion of intron 8 that contains the other polymorphic sites shown in this Example and Example 11, and that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

*Methods.* Polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA (using blood samples) from 215 individuals corresponding to 75 African Americans (Black), 135 Caucasians (White), one Asian American (Asian) and 4 Hispanics. As for Example 11, above, the N-terminal Glycine (G or Gly) designated as position 1 in Figure 8 or SEQ ID NO:1 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence of SEQ ID NO:2 or SEQ ID NO:13.

*Results.* Table II designates the nucleotide substitutions and the two amino acid residue substitutions in the coding sequence of intron 8 and a third nucleotide substitution in a non coding sequence of intron 8 using numbering corresponding to the entire "herstatin" protein sequence (SEQ ID NO:2 or SEQ ID NO:13):

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Table II

	N		
Black	75		
White	135		
Asian	1		
Hispanic/Latino	4		
Total	215		
Herstatin Polymorphism Distributions Among Blacks			
Arg357Cys (C1081T)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	32 (89)	13 (93)
het (%)	1 (4)	2 (6)	1 (7)
mut (%)	0 (0)	2 (6)	0 (0)
total	25	36	14
Arg371Ile (G1124T)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	36 (100)	14 (100)
het (%)	1 (4)	0 (0)	0 (0)
mut (%)	0 (0)	0 (0)	0 (0)
total	25	36	14
C1279T (3'UTR)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	36 (100)	12 (93)
het (%)	2 (8)	0 (0)	2 (7)
mut (%)	0 (0)	0 (0)	0 (0)
total	25	36	14

Table II. This table shows the distribution of three additional (relative to those identified in Example 11) polymorphic regions in HER-2 intron 8 of the DNA from African American individuals. Amino acids position designations correspond to amino acid positions in the "Herstatin" sequence (SEQ ID NO:2 or SEQ ID NO:13).

Table III, below, illustrates that the sequence data revealed polymorphisms at nucleotide positions #17 and #217 (also corresponding to nucleotide positions of the sequence region shown in Figure 8 or SEQ ID NO:10). The polymorphism at position #17 (variant 12) corresponds to variant 3 of Table I (Example 11). The polymorphism at position #217 (variant 15) corresponds (at least at the protein level) to variant 10 of Table I (Example 11) (see SEQ ID NO:12 and SEQ ID NO:13).

Additionally, the sequence data (see SEQ ID NO:11) revealed (see Table III) that intron 8 contains three polymorphic sites (corresponding to variants 13, 14 and 16) in addition to those disclosed in Example 11, above. Two of these (variants 13 and 14) are located at nucleotide positions #49 and #92 of SEQ ID NO:11 (also corresponding to

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nucleotide positions #49 and #92 of SEQ ID NO:10 (or Figure 8). The third (variant 16) is located at a nucleotide position #259 of SEQ ID NO:11 [also corresponding to nucleotide position #259 relative to the sequence region of SEQ ID NO:10 (or to position #264 of the sequence shown in Figure 1, panel A)]. Thus, the polymorphism corresponding to variant 16 is located 19 nucleotide positions 3' (downstream) from that portion of intron 8 that contains the other polymorphic sites shown in this Example and Example 11 (*i.e.*, that portion represented by SEQ ID NO:10), and that serves as in-frame (with the extracellular domain of p185HER-2) "coding" sequence when intron 8 is alternatively retained in mRNA.

Two of these polymorphisms result in nonconservative amino acid substitutions (*see* Table II and Table III, and legend of Table III; *also see* SEQ ID NO:12 and SEQ ID NO:13). For example, the polymorphism (C→T) found at the nucleotide position corresponding to nucleotide #49 of SEQ ID NO:11 [or to position # 49 of SEQ ID NO:10 or Figure 8] (*i.e.*, the polymorphism at position X(49) of Table 2) would result in a substitution of Arginine (Arg) for Cysteine (Cys) at the amino acid position corresponding to amino acid residue #17 of SEQ ID NO:12, SEQ ID NO:1, SEQ ID NO:10 or SEQ ID NO:11) or to amino acid residue # 357 of SEQ ID NO:13 or SEQ ID NO:2.

SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:13 show the four variant amino acid positions described in this example, along with those of Example 11 that are also shown in SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:10.

Table III designates (in addition to variants 12 and 15, which correspond to variants 3 and 10, respectively of Table I) the nucleotide substitutions and the corresponding two additional (relative to those of Table I of Example 11) amino acid residue substitutions (*i.e.*, variants 13 and 14) in the "coding" sequence of intron 8, along with the third nucleotide substitution in the 3' "non-coding" region of intron 8. The numbers in parenthesis after each X (polymorphic position) refer to nucleotide positions of SEQ ID NO:11 [or, as in Table I, correspond to (or are relative to, in the case of X(259)) the nucleotide positions in the DNA sequences shown in Figure 8 or SEQ ID NO:10].

As for Example 11, above, the N-terminal Glycine (G or Gly) designated as position 1 in SEQ ID NO:11, Figure 8, SEQ ID NO:1 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence of SEQ ID NO:2.

TABLE III

	X(17)	X(49)	X(92)	X(217)	X(259)
Variant 12	T				
Variant 13		T			
Variant 14			T		
Variant 15				A	
Variant 16					T

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Table III. Sequence variants in the intron-8 encoded domain found in human tissues (based on 215 different individuals). Sequence variants 12-16 are listed. The numbers in parenthesis after each X (polymorphic position) refer to nucleotide positions of SEQ ID NO:11 [or to positions that correspond to, or are relative to (in the case of X(259)) the nucleotide positions in the DNA sequences shown in Figure 8 or SEQ ID NO:10]. The DNA sequence variants listed here and in SEQ ID NO:11 correspond to variable amino acid positions shown in SEQ ID NO:12 [and also correspond to variable amino acid positions ("Xaa") of SEQ ID NO:1 or SEQ ID NO:10 as follows: X(17) to Xaa(6); X(49) to Xaa(17); X(92) to Xaa(31); X(217) to Xaa(73)]. The DNA sequence variant X(259) occurs in an untranslated region, and therefore does not alter the amino acid sequence of herstatin. Likewise, the variants of this table correspond to variable amino acid positions of SEQ ID NO:13 and SEQ ID NO:2 as follows: X(17) to Xaa(346); X(49) to Xaa(357); X(92) to Xaa(371); X(217) to Xaa(413). The specific amino acid changes (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:11 and SEQ ID NO:12 are: Variant 12, Xaa(6)(Pro→Leu); Variant 13, Xaa(17) (Arg→Cys); Variant 14, Xaa(31) (Arg→Ile); Variant 15, Xaa(73) (Asp→Asn). Variant 16, X(259) is in an untranslated region and does not code for an amino acid alteration, but instead alters only the nucleotide sequence at nucleotide position 259 (*i.e.*, C→T). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:13.

*SKOV3 ovarian carcinoma cells.* Two additional polymorphisms were found in a cell line derived from human ovarian cancer (SKOV3). These two polymorphisms result in nonconservative amino acid substitutions. One polymorphism is a substitution (C-T) at nucleotide #17 in the intron 8 sequence and nucleotide # 1037 in the "herstatin" sequence resulting in a substitution of leucine for proline at amino acid residue #6 in the intron 8 sequence and at amino acid residue # 346 in the "herstatin" sequence (*i.e.*, of SEQ ID NO:2 or SEQ ID NO:13). The second polymorphism found in the SKOV3 ovarian carcinoma cells line is a substitution (G-A) at nucleotide #217 in the intron 8 sequence and nucleotide #1237 in the "herstatin" sequence resulting in a substitution of Asparagine for Aspartic Acid at amino acid residue # 73 in the intron 8 sequence and amino acid #413 in the "herstatin" sequence (*i.e.*, of SEQ ID NO:2 or SEQ ID NO:13).

Significantly, the five polymorphic sites identified in the sequence analysis of this Example 12 were found only in DNA samples from African Americans (Black).

#### Summary of Examples 11 and 12

Together, Examples 11 and 12 of the present invention disclose 13 polymorphic positions in intron 8 of the Her-2 gene. Example 12, involved a relatively large DNA sample

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size, and indicated that the five polymorphic sites identified (three of which are distinct from the ten polymorphic sites identified in Example 11) are unique to African Americans (Black).

Twelve of the thirteen polymorphisms (*i.e.*, except for variant 16 of Example 12) of these two Examples are present in that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

The polymorphism corresponding to variant 16 is located in a region of intron 8 that remains as "non-coding" sequence when intron 8 is alternatively retained in mRNA. This "non-coding" intron 8 polymorphic site is located 19 nucleotide positions 3', or downstream from that portion of intron 8 that contains the other polymorphic sites, and that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

These HER-2 intron 8 polymorphisms provide for novel DNA and protein sequences, novel pharmaceutical compositions for treating solid tumors that overexpress HER-2, and monoclonal antibodies that bind to ECDIIIa variants corresponding to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13. These HER-2 intron 8 polymorphisms also provide for prognostic and diagnostic assays for the treatment and prevention of cancer.

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We claim:

1. An isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
- 5 2. The isolated polypeptide of claim 1, wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
3. The isolated polypeptide of claim 1, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the
- 10 ECD or HER-2).
4. An isolated DNA sequence that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
- 15 5. The isolated DNA sequence that codes on expression for a polypeptide of claim 4 wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
6. The isolated DNA sequence of claim 4, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
7. A transfected cell comprising an expression vector having a DNA sequence
- 20 that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
8. An isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79
- 25 amino acids are present, and wherein at least three N-linked glycosylation sites are present.
9. The isolated and glycosylated polypeptide of claim 8, wherein the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present.
10. The isolated and glycosylated polypeptide of claim 8, wherein the isolated
- 30 polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
11. An isolated DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-
- 35 linked glycosylation sites are present.
12. The isolated DNA sequence that codes on expression for a polypeptide of claim 11, wherein the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present.

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13. A transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present.
- 5 14. A method for treating a solid tumor characterized by overexpression of HER-2, comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 10 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone.
- 15 15. The method of claim 14, wherein the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer and colon cancer.
16. The method of claim 14, wherein the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID 20 NO:12.
17. The method of claim 16, wherein the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, and the monoclonal antibody that binds to the ECD of HER-2.
18. A pharmaceutical composition for treating solid tumors that overexpress HER- 25 2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C 30 terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier.
19. The pharmaceutical composition for treating solid tumors that overexpress 35 HER-2 of claim 18, wherein the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12.
20. The pharmaceutical composition for treating solid tumors that overexpress HER-2 of claim 19, wherein the agent is a combination of the isolated polypeptide having

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from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12 and the monoclonal antibody that binds to the ECD of HER-2.

21. A method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
22. The method for targeting a therapeutic agent to solid tumor tissue of claim 21, wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
23. The method for targeting a therapeutic agent to solid tumor tissue of claim 21, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
24. A method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from a patient, wherein the bodily fluid is selected from the group consisting blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis.
25. The method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2 of claim 24, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid.
26. The method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2 of claim 25, further comprising determining a ratio between the amount of p68HER-2 and p185HER-2, whereby the higher the p68HER-2 to p185HER-2 ratio, the better the prognosis of the patient.
27. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:
- (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof;
- (b) determining whether an ECDIIIa variant protein or DNA sequence, or a HER-2 intron 8 variant DNA sequence is present in the bodily fluid sample using a sequence identity assay; and
- (c) correlating the presence of the ECDIIIa variant protein or DNA sequence, or the HER-2 intron 8 variant DNA sequence to cancer treatment and diagnosis using an historical database.

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28. The diagnostic assay of claim 27, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.
29. The diagnostic assay of claim 27, further comprising measuring the amount of  
5 p185HER-2 ECD in the bodily fluid.
30. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:  
(a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations  
10 thereof;  
(b) determining whether a HER-2 intron 8 variant DNA sequence is present in the bodily fluid sample using a sequence identity assay; and  
(c) correlating the presence of the HER-2 intron 8 variant DNA sequence to cancer treatment and diagnosis using an historical database.
31. The diagnostic assay of claim 30, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, hybridization assays, and combinations thereof.
32. The diagnostic assay of claim 30, further comprising measuring the amount of  
15 p185HER-2 ECD in the bodily fluid.
33. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:  
(a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations  
20 thereof;  
(b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and  
(c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer  
25 treatment and diagnosis using an historical database.
34. The diagnostic assay of claim 33, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, hybridization assays, and combinations thereof.
35. The diagnostic assay of claim 33, further comprising measuring the amount of  
35 p185HER-2 ECD in the bodily fluid.
36. The diagnostic assay of claim 35, further comprising determining a ratio between the amount of p68HER-2 and p185HER-2 ECD.
37. An antibody specific for an ECDIIIa variant of the sequence in SEQ ID NO:1,

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SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13.

38. An antibody specific for p68HER-2 ECDIIIa variant 3.

39. A diagnostic kit comprising:

5 (a) a monoclonal antibody or antigen-binding fragment thereof that specifically binds to an ECDIIIa variant of the sequence in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13; and

(b) a detectable label, whereby the binding of the antibody in step (a) can be detected.

40. The diagnostic kit of claim 36, wherein the label is selected from the group consisting of enzymes, radiolabels, chromophores, chemiluminescent tags, and fluorescers.



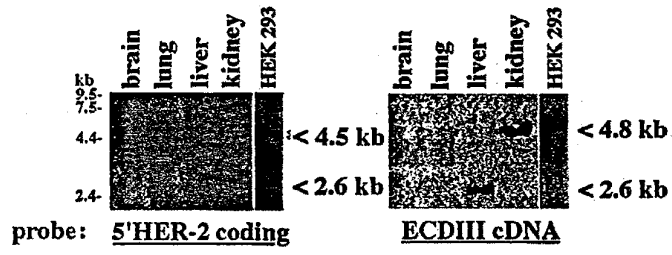


Figure 2

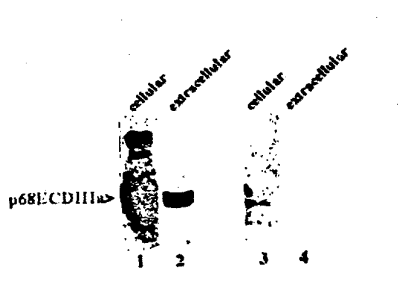


Figure 3

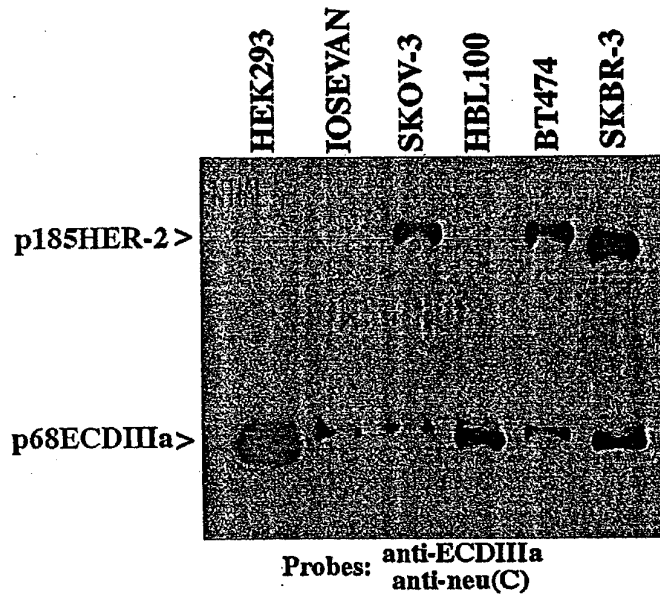


Figure 4

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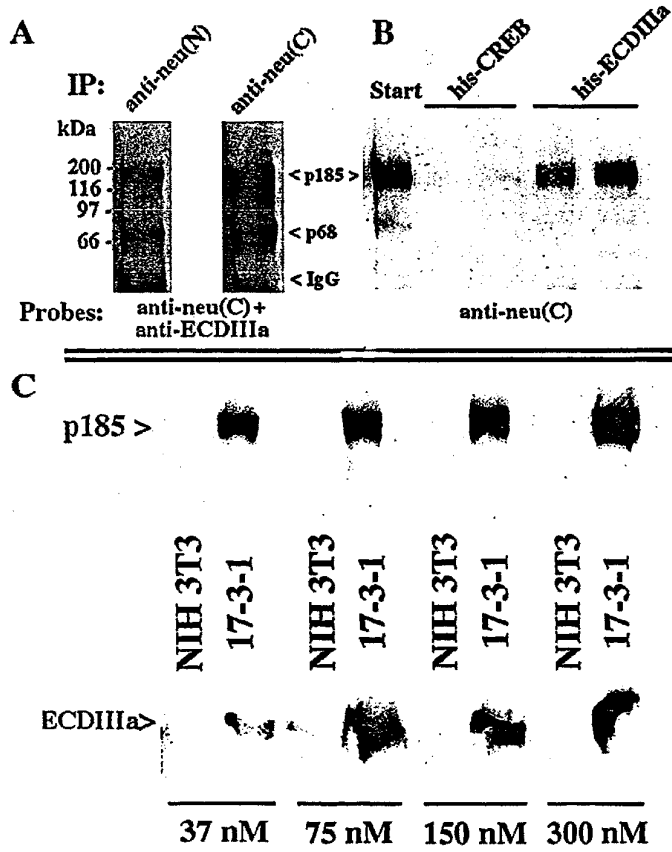


Figure 5

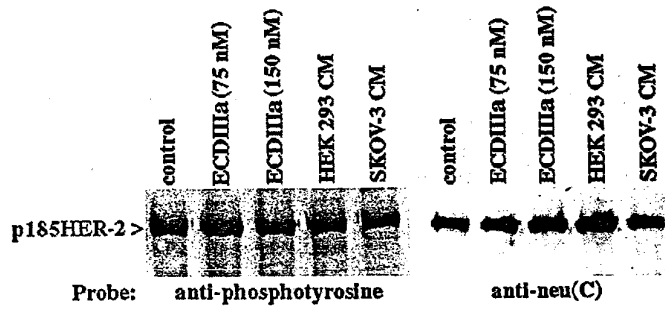


Figure 6

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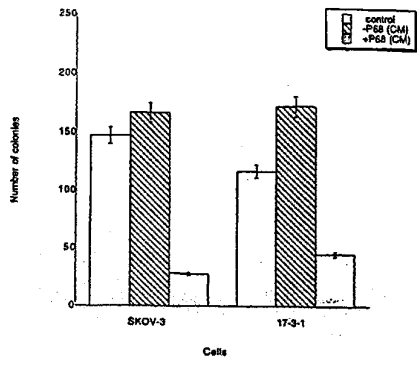


Figure 7

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**HER-2 Intron 8 Polymorphisms**

```

1   G T H S L P P R P A A V P V P L R M Q P G
1   GGTACCCACTCACTGCCCGAGGCCAGCTGCAGTTCCTGTCCCTCTGCGCATGCAGCCTGGC
    X       X X               X       X       X

22  P A H P V L S F L R P S W D L V S A F Y S
64  CCAGCCCACCTGTCTATCCTTCCTCAGACCCTCTGGGACCTAGTCTCTGCCTTCTACTCT
    X

43  L P L A P L S P T S V P I S P V S V G R G
127 CTACCCCTGGCCCCCTCAGCCCTACAAGTGTCCCTATATCCCTGTGTCAGTGTGGGAGGGGC
    X

64  P D P D A H V A V D L S R Y E G stop 80
190 CCGGACCCTGATGTCATGTGGCTGTGACCTGTCCCGGTATGAAGGCTGA 240
    X       X

```

Figure 8

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANTS: Clinton, Gail M., Henner, William D. and Evans,  
5 Adam

(ii) TITLE OF INVENTION: HER-2 BINDING ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 13  
10

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(F) ZIP: 98101  
15

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: PC compatible  
(C) OPERATING SYSTEM: Windows95  
(D) SOFTWARE: Word  
20

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: to be assigned  
(B) FILING DATE: 16 February 2001  
(C) CLASSIFICATION:  
25

(viii) ATTORNEY/AGENT INFORMATION:

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30

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35

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 79  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
40

(ii) MOLECULE TYPE: HER-2 ECD antagonist

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
45

Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro Xaa  
5 10 15  
Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu Arg Pro  
20 25 30  
Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu Ala Pro Leu  
50





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- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 (ii) MOLECULE TYPE: oligonucleotide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
- 10 ATACCGGGAC AGGTCAACAG C 21
- (2) INFORMATION FOR SEQ ID NO:7:  
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 (A) LENGTH: 20  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 (ii) MOLECULE TYPE: oligonucleotide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
- 20 TCTGGGTACC CACTCACTGC 20
- (2) INFORMATION FOR SEQ ID NO:8:  
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 (A) LENGTH: 22  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 (ii) MOLECULE TYPE: oligonucleotide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
- 30 TTCACACTGG CACGTCCAGA CC 22
- (2) INFORMATION FOR SEQ ID NO:9:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 (ii) MOLECULE TYPE: oligonucleotide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
- 40 GCACGGATCC ATAGCAGACT GAGGAGG 27
- (2) INFORMATION FOR SEQ ID NO:10:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 240 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
- 50

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GGT WCC CAC TCA CYG CYC CCG AGG CCA GCT GCA GTT CCT GTC CCT 45  
 Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro  
 5 10 15

5 CWG CGC ATR CAG CCT GNC CCA GCC CAC CCT GTC CTA TCC TTC CTC 90  
 Xaa Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu  
 20 25 30

10 AGA CCC TCT TGG GAC MTA GTC TCT GCC TTC TAC TCT CTA CCC CTG 135  
 Arg Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu  
 35 40 45

GCC CCC CTC AGC CCT ACA AGT GTC CST ATA TCC CCT GTC AGT GTG 180  
 Ala Pro Leu Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val  
 15 50 55 60

GGG AGG GGC CYG GAC CCT GAT GCT CAT GTG GCT GTT SAC CTG TCC 225  
 Gly Arg Gly Xaa Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser  
 20 65 70 75

CGG TAT GAA GGC TGA 240  
 Arg Tyr Glu Gly

25 (2) INFORMATION FOR SEQ ID NO:11:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 274 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 30 (ii) MOLECULE TYPE: DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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 Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro  
 35 5 10 15

CWG YGC ATR CAG CCT GNC CCA GCC CAC CCT GTC CTA TCC TTC CTC 90  
 Xaa Xaa Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu  
 40 20 25 30

AKA CCC TCT TGG GAC MTA GTC TCT GCC TTC TAC TCT CTA CCC CTG 135  
 Xaa Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu  
 45 35 40 45

GCC CCC CTC AGC CCT ACA AGT GTC CST ATA TCC CCT GTC AGT GTG 180  
 Ala Pro Leu Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val  
 50 55 60

50 GGG AGG GGC CYG GAC CCT GAT GCT CAT GTG GCT GTT VAC CTG TCC 225  
 Gly Arg Gly Xaa Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser  
 65 70 75

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CGG TAT GAA GGC TGAGACGGCC CCTTCCCCCA CYCACCCCCA CCTCTC 274  
 Arg Tyr Glu Gly

5 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 79

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

10

(ii) MOLECULE TYPE: HER-2 ECD antagonist

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro Xaa  
 5 10 15  
 Xaa Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu Xaa Pro  
 20 25 30  
 Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu Ala Pro Leu  
 35 40 45  
 20 Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val Gly Arg Gly Xaa  
 50 55 60  
 Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser Arg Tyr Glu Gly  
 65 70 75

25 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 419

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

30

(ii) MOLECULE TYPE: polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35 Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu  
 5 10 15  
 Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Cys Lys  
 20 25 30  
 Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His  
 35 40 45  
 40 Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr  
 50 55 60  
 Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val  
 65 70 75 80  
 45 Gln Gly Tyr Val Leu Cys Ala His Asn Gln Val Arg Gln Val Pro Leu  
 85 90 95  
 Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr  
 100 105 110  
 Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Agn Agn Thr Thr Pro  
 115 120 125  
 50 Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser  
 130 135 140  
 Leu Thr Glu Cys Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln



## 【国際公開パンフレット(コレクトバージョン)】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number  
WO 01/061356 A1

- (51) International Patent Classification: G01N 33/567, 33/574, C07K 16/00
- (21) International Application Number: PCT/US01/05327
- (22) International Filing Date: 16 February 2001 (16.02.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
09/506,079 16 February 2000 (16.02.2000) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KR, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GI, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IL, IT, LU, MC, NL, PT, SI, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
with international search report
- (48) Date of publication of this corrected version:  
31 October 2002
- (15) Information about Correction:  
see PCT Gazette No. 44/2002 of 31 October 2002, Section II
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

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(54) Title: HIER-2 BINDING ANTAGONISTS

(57) Abstract: There is disclosed a pharmaceutical composition for treating solid tumors that overexpress HIER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO: 1 or SEQ ID NO: 12, wherein the polypeptide binds to the extracellular domain ECD of HIER-2 at an affinity of at least 10<sup>6</sup>, (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO: 2 or SEQ ID NO: 13, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HIER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Also disclosed are prognostic and diagnostic assays.

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**HER-2 BINDING ANTAGONISTS****Technical Field of the Invention**

5 The present invention provides a HER-2 binding antagonist. Specifically, intron retention has generated a novel HER-2 antagonist polypeptide that binds to the HER-2 receptor.

This work was supported by a grant from the Department of Defense (DOD) Breast Cancer Research Program. The United States Government has certain rights in this invention.

**10 Cross-reference to Related Applications**

This application is a continuation-in-part of United States Patent Application Serial No. 09/234,208, entitled HER-2 BINDING ANTAGONISTS, filed 20 January 1999.

**Background of the Invention**

15 The HER-2/neu (erbB-2) oncogene encodes a receptor-like tyrosine kinase (RTK) that has been extensively investigated because of its role in several human carcinomas (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994) and in mammalian development (Lee et al., *Nature* 378:394-398, 1995). The sequence of the HER-2 protein was determined from a cDNA that was cloned by homology to the epidermal growth factor receptor (EGFR) mRNA from placenta (Coussens et al.,  
20 *Science* 230:1132-1139, 1985) and from a gastric carcinoma cell line (Yamamoto et al., *Nature* 319:230-234, 1986). The HER-2 mRNA was shown to be about 4.5 kb (Coussens et al., *Science* 230:1132-1139, 1985; and Yamamoto et al., *Nature* 319:230-234, 1986) and encodes a transmembrane glycoprotein of 185 kDa in normal and malignant human tissues (p185HER-2) (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994). The function of the HER-2 gene has been examined  
25 mainly by expressing the cDNA corresponding to the 4.5 kb transcript in transfected cells and from the structure and biochemical properties of the 185 kDa protein product. P185HER-2 consists of a large extracellular domain, a transmembrane segment, and an intracellular domain with tyrosine kinase activity (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; and Dougall et al., *Oncogene* 9:2109-2123, 1994). Overexpression of p185HER-2 causes phenotypic transformation of cultured cells (DiFiore et al., *Science* 237:178-182, 1987; and Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987) and has been associated with aggressive clinical progression of breast and ovarian cancer (Slamon et al., *Science* 235:177-182, 1987; and Slamon et al., *Science* 244:707-712, 1989). p185HER-2 is  
35 highly homologous to the EGFR. However, a ligand that directly binds with high affinity to p185HER-2 has not yet been identified. Moreover, the signaling activity of HER-2 may be mediated through heterodimerization with other ligand-binding members of the EGFR family (Carraway and Cantley, *Cell* 78:5-8, 1994; Earp et al., *Breast Cancer Res. Treat.* 35:115-132,

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1995; and Qian et al., *Oncogene* 10:211-219, 1995).

Divergent proteins, containing regions of the extracellular domains of HER family RTKs, are generated through proteolytic processing of full length receptors (Lin and Clinton, *Oncogene* 6:639-643, 1991; Zabrecky et al., *J. Biol. Chem.* 266:1716-1720, 1991; Pupa et al., *Oncogene* 8:2917-2923, 1993; Vecchi et al., *J. Biol. Chem.* 271:18989-18995, 1996; and Vecchi and Carpenter, *J. Cell Biol.* 139:995-1003, 1997) and through alternative RNA processing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maihle, *Oncogene* 16:3243-3252, 1998). The extracellular domain of p185HER-2 is proteolytically shed from breast carcinoma cells in culture (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990; Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993; and Lee and Maihle, *Oncogene* 16:3243-3252, 1998), and is found in the serum of some cancer patients (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) where it is may be a serum marker of metastatic breast cancer (Leitzel et al., *J. Clin. Oncol.* 10:1436-1443, 1992) and may allow escape of HER-2-rich tumors from immunological control (Baselga et al., *J. Clin. Oncol.* 14:737-744, 1996; and Brodowicz et al., *Int. J. Cancer* 73:875-879, 1997).

A truncated extracellular domain of HER-2 is also the product of a 2.3 kb alternative transcript generated by use of a polyadenylation signal within an intron (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). The alternative transcript was first identified in the gastric carcinoma cell line, MKN7 (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993) and the truncated receptor was located within the perinuclear cytoplasm rather than secreted from these tumor cells (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). However, no particular therapeutic, diagnostic or research utility has been ascribed to this truncated extracellular domain polypeptide. A truncated extracellular domain of the EGFR, generated by alternative splicing (Petch et al., *Mol. Cell. Biol.* 10:2973-2982, 1990) is secreted, exhibits ligand-binding, and dimerization properties (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989), and may have a dominant negative effect on receptor function (Basu et al., *Mol. Cell. Biol.* 9:671-677, 1989; and Flickinger et al., *Mol. Cell. Biol.* 12:883-893, 1992).

Therefore, there is a need in the art to find molecules that bind to cellular HER-2 and particularly molecules that bind to different sites than humanized antibodies to HER-2 (e.g., Herceptin®). Such molecules would be useful therapeutic agents for various cancers that overexpress HER-2.

#### Summary of the Invention

The present invention provides an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®

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(a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2).

The present invention further provides an isolated DNA sequence that codes, on expression, for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^3$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2). The present invention further provides a transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^6$ .

The present invention further provides an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present. Preferably, the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2).

The present invention further provides an isolated DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present. Preferably, the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation are present. The present invention further provides a transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present.

The present invention provides a method for treating a solid tumor characterized by overexpression of HER-2, comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^3$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C-terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a

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monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer and colon cancer. Preferably, the agent is the isolated

5 polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a pharmaceutical composition for treating  
10 tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79  
15 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably,  
20 the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The present invention further provides a method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-  
25 2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of  
30 Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD of HER-2).

The present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group  
35 consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation,

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immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2.

5 The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether a particular ECDIIIa variant  
10 sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database. Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

15 The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; (b) determining whether an amount of an p68HER-2  
20 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

25 The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2  
30 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

### 35 **Brief Description of the Drawings**

Figure 1 shows a nucleotide and amino acid sequence of the insert in the extracellular domain of HER-2. The HER-2 ECD coding sequence from exon 1-9 (primers A and B) was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ~1420 bp was

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found to be HER-2-specific by Southern blot analysis. This product was subcloned and the nucleotide sequence was determined. In panel A, the nucleotide sequence is shown for the 274 bp insert (outside the box) and for the immediately adjacent 5' and 3' sequences enclosed in the box. The insert is located between nucleotide residues 1171 and 1172 and following amino acid residue 340 in p185HER-2 using the numbering of Coussens et al. (*Science* 230:1132-1139, 1985). The consensus 5' and 3' splice sites at the arrows are shown in larger print. The inserted sequence is in-frame with 5' HER-2 exon sequence and is deduced to encode a 79 amino acid extension following Arg 340 (R<sup>340</sup>). The novel 79 novel amino acid sequence encoded by the insert is proline-rich (19%) and has a consensus asparagine linked glycosylation site, which is underlined. A stop codon was found at nucleotides 236-238 within the inserted sequence. In panel B, the predicted product of the alternative transcript is a truncated secreted protein which contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65-70 kDa. This polypeptide product is referred to as p68HER-2. Thus, the product will be a truncated secreted protein which is missing the transmembrane domain and cytoplasmic domain found in p185HER-2.

Figure 2 shows the detection of alternative HER-2 transcripts containing the ECDIIIa sequence by Northern blot analysis. PolyA<sup>+</sup> mRNA (2.5 µg) from different human fetal tissues (Clontech) or isolated from HEK-293 cells was resolved in a formalin agarose gel and transferred to a BrightStar® membrane (Ambion) in 10xSSC. The membrane was hybridized with a <sup>32</sup>P-labeled antisense RNA probe complementary to the ECDIII sequence, stripped and reprobed with a <sup>32</sup>P-labeled cDNA probe specific for the 5' HER-2 exon sequence. The membranes were washed under high stringency conditions and analyzed by phosphorimaging (Molecular Dynamics).

Figure 3 shows a sequence-specific reactivity of anti-ECDIIIa with a protein of ~ 68 kDa in a human embryonic kidney cell line (HEK293). Cell extract protein (20 µg) and 20 µl of media conditioned by HEK-293 cells were Western blotted and probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIIa diluted 1:10,000 containing 50 µg/ml purified His-tagged ECDIIIa peptide (lanes 3, 4).

Figure 4 shows the expression of p185HER-2, relative to p68ECDIIIa expression, is markedly elevated in carcinoma cell lines in which the HER-2 gene is amplified. Cell extracts (15 µg of protein) from human embryonic kidney cell line (HEK293), nontumorigenic ovarian surface epithelial cell line (IOSEVAN), ovarian carcinoma cell line with HER-2 gene amplification (SKOV-3), nontumorigenic breast epithelial cell line (HBL100), and breast carcinoma cell lines with HER-2 gene amplification (BT474 and SKBR-3), were resolved by SDS-PAGE in 7.5% acrylamide gels and analyzed as a Western blot. The Western blot was probed with both antibodies specific for p68HER-2 (anti-ECDIIIa) and for p185HER-2 (anti-neu(C)).

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Figure 5 shows that p68ECDIIIa binds to p185HER-2. In panel A: Two mg of SKBR-3 cells extracted in nondenaturing buffer were immunoprecipitated with 5 µl anti-neu(N) specific for the N-terminal sequence of p68HER-2 and p185HER-2, or with 5 µl anti-neu(C) specific for the C-terminus of p185HER-2 and then probed as a Western blot with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C) specific for p185HER-2. In panel B: 100 µg of 17-3-1 cell extract were incubated in duplicate with 50 µl packed volume of NiNTA agarose (Qiagen) coupled to 20 µg of His-tagged ECDIIIa or to 20 µg His-tagged CREB fragment in 200 µl of wash buffer (20mM Tris pH 8.0, 300mM NaCl) at room temperature for 1 hr with shaking. The resin was then washed 4 times with 500 µl of wash buffer and proteins were eluted by incubation with 50 µl SDS-sample buffer at 100° C for 2 min. Eluted proteins were analyzed by Western blot analysis using antibodies against the C-terminus of p185HER-2, anti-neu(C). In panel C: Monolayers of ~10<sup>5</sup> 3T3 cells or HER-2 transfected 17-3-1 cells in 12 well plates were washed twice with PBS and then incubated with 0.5 ml of serum-free media with 1% BSA and 39, 75, 150, and 300 nM of purified recombinant His-tagged ECDIIIa for 2 hrs at 4 °C. Cells were washed 1 time in PBS containing 1% BSA and twice in PBS and then were extracted in denaturing buffer. Equal aliquots (20 µg protein) were analyzed by western blotting with antibodies specific for ECDIIIa (anti-ECDIIIa) or, in the upper panel, with antibodies specific for p185HER-2 (anti-neu(C)).

Figure 6 shows that neither p68-rich conditioned media nor the ECDIIIa peptide stimulate tyrosine phosphorylation of p185HER-2. Monolayer cultures of ~10<sup>5</sup> HER-2 transfected 17-3-1 cells were washed twice with PBS, incubated in serum-free media at 37 °C for 24 hrs, and then treated for 10 minutes with 75 or 150 µM His-tagged ECDIIIa or with 50X CM from HEK-293 cells that secrete high levels of p68 or 50X CM from SKOV-3 cells that have no detectable p68HER-2. The treated cells were extracted with denaturing buffer containing the phosphotyrosine phosphatase inhibitor vanadate (2 mM) and 20 µg/ml of cell extract protein from each sample were analyzed by Western blot analysis with monoclonal antibodies against phosphotyrosine (Sigma). The blot was stripped by incubation at 55° C for 30 min in 62.5 mM Tris pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol and then reprobed with anti-neu(C) specific for p185HER-2.

Figure 7 shows that p68HER-2 inhibited anchorage independent growth of tumorigenic cells. SKOV-3 ovarian carcinoma cells and HER-2 transfected 17-3-1 cells were suspended in media with 10% fetal bovine serum containing 0.3% agar (control conditions) to which was added 50X concentrated media conditioned by SKOV-3 cells (which contains no detectable p68HER-2 (-p68 CM)), or 50X concentrated media conditioned by HEK-293 cells (which contains 20 nM p68HER-2 (+p68 CM)). Five times 10<sup>3</sup> cells were plated in triplicate for each experimental condition onto a 0.5 ml layer of media containing 0.5% agarose in 12 well plates. The results shown are plotted as the mean and

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standard deviation of the number of colonies with more than 50 cells in triplicate wells counted at 21 days of incubation. Similar results were observed in three separate experiments.

Figure 8 shows the nucleotide and deduced amino acid sequence of HER-2 Intron 8.

- 5 Human genomic DNA was subjected to PCR using primers that flank intron 8. PCR parameters were 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 30 s, followed by 1 cycle of 72 °C for 7 min. A 410 bp product was gel purified and sequence in the forward and reverse directions. The sequence shown is the most common sequence found within intron 8 from about 15 different individuals. The positions of sequence variation that would result in amino acid substitutions are marked by Xs.

#### Detailed Description of the Invention

The present invention is based upon the initial discovery of an alternative HER-2 mRNA of 4.8 kb with a 274 bp insert identified as intron 8. The retained intron is in-frame and encodes 79 amino acids [SEQ ID NO. 1] followed by a stop codon at nucleotide 236.

- 15 The alternative mRNA predicts a truncated HER-2 protein that lacks the transmembrane and intracellular domains and contains 419 amino acids [SEQ ID NO. 2]; 340 residues that are identical to the N-terminus of p185HER-2 and 79 unique residues at the C-terminus [SEQ ID NO. 1]. Using specific antibodies against either the novel 79 amino acid residue C-terminal sequence [SEQ ID NO. 1] or the N-terminus of p185HER-2, a 68 kDa protein product was identified [SEQ ID NO.2]. This 68 kDa protein is the product of an alternative HER-2 transcript, and is found in cell extracts and in extracellular media from several cell lines. Expression of the alternative transcript was highest in a nontransfected human embryonic kidney cell line.

- The results presented here show expression of alternative HER-2 mRNA, which contains an additional 274 nucleotides, probably intron 8. Consistent with this finding, an alternative transcript of ~ 4.8 kb was detected in human fetal kidney tissue and in the human embryonic kidney cell line, HEK 293. Moreover, a transcript of 2.6 kb, which is the size expected if the sequence is retained in the 2.3 kb truncated HER-2 mRNA (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993), was detected in human fetal liver tissue by Northern blot analysis using a probe specific for the inserted sequence or for the HER-2 ECD coding sequence (Figure 2). The inserted sequence introduces a termination codon and predicts a novel 79 amino acid extension designated ECDIIIa at residue 340 of the p185HER-2 protein. The predicted protein therefore lacks the transmembrane and intracellular domains, but contains subdomains I and II of the extracellular domain of p185HER-2. As predicted, a secreted protein that contains N-terminal sequence of p185HER-2 and the C-terminal extension provided by the inclusion of the novel sequence was detected (Figures 3 and 5). The ECDIIIa protein was found to be 68 kDa which is the approximate size expected of the protein encoded by the alternative

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transcript if the five N-linked glycosylation sites found in subdomains I and II of p185HER-2 are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

The data presented herein demonstrate that p68HER-2 specifically binds to p185HER-2. The association with p185HER-2 may be conferred by the novel proline rich ECDIIIa domain rather than the N-terminal subdomains I and II of p68HER-2. While the HER-2 ECD, generated by *in vitro* deletion mutagenesis, also contains subdomains I and II, it does not associate with the extracellular domain of p185HER-2 unless engineered to enhance their proximity (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997; and Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998). However, the unique ECDIIIa peptide binds with high affinity (nM concentrations) to p185HER-2 and to transfected 17-3-1 cells that overexpress p185HER-2 (Figure 5). Preferential binding of the ECDIIIa domain peptide to 17-3-1 cells indicates that secreted p68HER-2 interacts with the extracellular region of p185HER-2 at the cell surface. Therefore, p68HER-2 and fragments thereof appear to be a naturally occurring HER-2 binding protein, encoded by the HER-2 gene. In contrast to EGFR family ligands (Groenen et al., *Growth Factors* 11:235-257, 1994), p68HER-2 lacks an EGF homology domain and contains the first 340 amino acids of the receptor itself, p185HER.

Previously described putative HER-2 ligands were found to associate indirectly with p185HER-2 only in a heterodimer with an EGFR family member (Heldin and Ostman, *Cytokine Growth Factor Rev.* 7:33-40, 1996). Although it is possible that ECDIIIa binds indirectly to p185HER-2 through a coreceptor, this seems unlikely since detergent solubilized p185HER-2 was specifically and efficiently "pulled down" by immobilized ECDIIIa peptide (Figure 5B).

For all naturally occurring or engineered ligands for mammalian EGFR family members, binding is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (Hynes and Stern, *Biochim. et Biophys. Acta* 1198:165-184, 1994; Dougall et al., *Oncogene* 9:2109-2123, 1994; and Groenen et al., *Growth Factors* 11:235-257, 1994). Although they bind, neither p68HER-2 nor the ECDIIIa peptide was found to activate p185HER-2. Activation was assessed in two different cell lines that differ in the extent of p185HER-2 tyrosine phosphorylation, transfected 17-3-1 cells as well as SKOV-3 ovarian carcinoma cells. Furthermore *in vitro* self-phosphorylation activity, which is enhanced in dimeric forms of p185HER-2 (Dougall et al., *Oncogene* 9:2109-2123, 1994; and Lin et al., *J. Cell. Biochem.* 49, 290-295, 1992), was not stimulated by p68HER-2 or ECDIIIa. Similarly, the Argos protein, which is an extracellular inhibitor of the Drosophila EGF receptor and the only known antagonist of class I RTKs, did not simulate tyrosine phosphorylation of the receptor (Schweitzer et al., *Nature* 376:699-702, 1995). Likewise, Angiopoietin-2, a natural antagonist for the Tie 2 RTK, bound the endothelial receptor but failed to activate it (Maisonpierre et al., *Science* 277:55-60, 1997).

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Without being bound by theory, since p68HER-2 occupies but does not activate, it could block dimerization of p185HER-2. By analogy, HER-2 ECD, when engineered to enhance its binding to RTKs, prevented the formation of productive dimers required for transphosphorylation and receptor activation thereby having a dominant negative effect (O'Rourke et al., *Proc. Natl. Acad. Sci. USA* 94:3250-3255, 1997). In contrast to the HER-2 ECD, soluble p68HER-2 exhibited strong binding to p185HER-2, yet also contains subdomain I and II of the ECD. Since subdomain I may be the low affinity, promiscuous ligand binding site required for recruitment of p185HER-2 into heteromeric complexes (Tzahar et al., *EMBO J.* 16:4938-4950, 1997), p68HER-2 could block this site and thereby obstruct recruitment of p185HER-2 into dimers. Alternatively, p68HER-2 could compete with an uncharacterized ligand for binding to p185HER-2. The tissue-specific expression of p68HER-2 in human fetal liver and kidney may function to modulate the extent to which p185HER-2 is occupied during development of these organs. Moreover, the overexpression of p185HER-2, relative to p68HER-2 in tumor cells with HER-2 gene amplification (Figure 3), could occur through a selective pressure based on overcoming the effects of a binding protein such as p68HER-2. Therefore, p68HER-2 is the first example of a naturally occurring p185HER-2 binding protein that may prevent activation of p185HER-2.

#### Pharmaceutical Composition

The present invention further provides a pharmaceutical composition for treating solid tumors that overexpress HER-2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier. Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The inventive pharmaceutical composition, comprising either or both of the inventive polypeptides and/or monoclonal antibody, can be administered to a patient either by itself (complex or combination) or in pharmaceutical compositions where it is mixed with suitable carriers and excipients. Inventive polypeptide can be administered parenterally, such as by intravenous injection or infusion, intraperitoneal injection, subcutaneous injection, or intramuscular injection. Inventive polypeptide can be administered orally or rectally through

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appropriate formulation with carriers and excipients to form tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like. Inventive polypeptide can be administered topically, such as by skin patch, to achieve consistent systemic levels of active agent. Inventive polypeptide is formulated into topical creams, skin or mucosal patch, liquids or gels suitable to topical application to skin or mucosal membrane surfaces. Inventive polypeptide can be administered by inhaler to the respiratory tract for local or systemic treatment of cancers characterized by overexpressing HER-2.

The dosage of inventive polypeptide suitable for use with the present invention can be determined by those skilled in the art from this disclosure. Inventive polypeptide will contain an effective dosage (depending upon the route of administration and pharmacokinetics of the active agent) of inventive polypeptide and suitable pharmaceutical carriers and excipients, which are suitable for the particular route of administration of the formulation (*i.e.*, oral, parenteral, topical or by inhalation). The active inventive polypeptide is mixed into the pharmaceutical formulation by means of mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping or lyophilizing processes. The pharmaceutical formulations for parenteral administration include aqueous solutions of the inventive polypeptide in water-soluble form. Additionally, suspensions of the inventive polypeptide may be prepared as oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. The suspension may optionally contain stabilizers or agents to increase the solubility of the complex or combination to allow for more concentrated solutions.

Pharmaceutical formulations for oral administration can be obtained by combining the active compound with solid excipients, such as sugars (*e.g.*, lactose, sucrose, mannitol or sorbitol), cellulose preparations (*e.g.*, starch, methyl cellulose, hydroxypropylmethyl cellulose, and sodium carboxymethyl cellulose), gelatin, gums, or polyvinylpyrrolidone. In addition, a disintegrating agent may be added, and a stabilizer may be added.

Processes for Synthesizing p68 and 79 aa C Terminal Region

Polypeptide synthesis is done by a group of standard procedures for polypeptide synthesis by sequential amino acids building through peptide synthesis equipment, following manufacturer's instructions for synthesizing peptides. Preferably, shorter polypeptides, of less than 100 amino acids, are best suited for the method of synthesis through sequential amino acid building of polypeptides. In addition, heterologous polypeptides can be expressed by transformed cells using standard recombinant DNA techniques to transform either prokaryotic or eukaryotic cells, provide appropriate growth media for their expression, and then purify the inventive polypeptide either from the media or from intracellular contents depending upon the type of cell used and its expression characteristics.

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Methods for Treating Cancer with p68, 79 aa C-Terminal Region, and Combinations

The present invention provides a method for treating a solid tumor characterized by overexpression of HER-2, or HER-2 variants (*see* Example 8) comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected

5 from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO. 2, wherein the C terminal 79 amino acids are present, and wherein at least

10 three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone. Preferably, the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer, prostate cancer, gastric carcinoma, cervical cancer, esophageal carcinoma, and colon cancer.

15 Preferably, the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1. Most preferably, the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1 and the monoclonal antibody that binds to the ECD of HER-2.

The p68HER-2 polypeptide described herein was found to bind to HER-2 and prevent

20 signal transduction through the kinase domain. Without being bound by theory, the unique ECDIIIa domain mediates specific binding to p185HER-2 and the resulting interaction with p68ECDIIIa prevents p185HER-2 dimerization and subsequent signal transduction. Therefore, p68HER-2 functions as a HER-2 antagonist to prevent signal transduction by preventing dimerization as a necessary prerequisite for signal transduction. Thus, the

25 mechanism of p68HER-2 as a HER-2 antagonist is different from the mechanism of binding agents, such as the 79 amino acid polypeptide described herein or a monoclonal antibody that binds to the EDC of HER-2. The inventive method provides that p68HER-2 inhibits tumor cell growth in tumors that overexpress HER-2 by providing a selective pressure for such tumor cells. Similarly, the HER-2 antagonists that are binding agents also inhibit tumor cell

30 growth in tumors that overexpress HER-2 by providing selective pressure to such cells to prevent ligand binding to the ECD of HER-2 and prevent signal transduction even before potential dimerization.

Use of 79 aa C-Terminal Region as a Targeting Molecule

The present invention further provides a method for targeting a therapeutic agent to

35 solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO. 1, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ . Preferably, the

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isolated polypeptide is from about 69 to 79 amino acids in length. Preferably, the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin® (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the ECD or HER-2). It was discovered that the 79 amino acid polypeptide [SEQ ID NO. 1] exhibited surprising high affinity binding properties to the ECD of HER-2. Moreover, the site of such binding is different and unaffected by the site of binding of a marketed humanized monoclonal antibody (Herceptin®). Therefore, the high binding affinity enables the 79 amino acid polypeptide to function as a targeting molecule to tumor cells expressing HER-2.

10 Anti-p68 Antibody as a Diagnostic/Prognostic Agent

The p68HER-2 ECDIIIa variant 3 (see TABLE 1, below) glycosylated polypeptide was expressed and used as an antigen for antibody production. Specifically, antibody specific for p68HER-2 was prepared by injecting rabbits with purified polyhistidine-tagged ECDIIIa variant 3 peptide, which is the same as the intron encoded novel C-terminus or p68HER-2, the domain that binds with high affinity to p185HER-2. The isolated polyclonal antibody detected pM quantities of ECDIIIa peptide or of p68HER-2 with high specificity (see Figures 3 and 5). Thus, an antibody specific for p68HER-2 is useful as a diagnostic agent for detecting p68HER-2 in bodily fluids and tumor tissues using diagnostic techniques, such as ELISA, immunoprecipitations, immunohistochemistry or Western analysis.

20 Antibodies that specifically recognize one or more epitopes of ECDIIIa, or epitopes of p68HER-2, or peptide fragments, and thus distinguish among ECDIIIa variants (see TABLE 1, below) are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single-chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The antibodies of the invention may be used, for example, in the detection of a particular p68HER-2 ECDIIIa variant in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients or tissue samples may be tested for the presence of particular variants, or for abnormal amounts particular variants.

30 Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of particular p68HER-2 variants. Additionally, such antibodies can be used in conjunction with the cancer treatment methods described herein.

For the production of antibodies, various host animals may be immunized by injection with e.g., polyhistidine-tagged ECDIIIa variant polypeptides, truncated ECDIIIa variant polypeptides, functional equivalents of the ECDIIIa variants or mutants of the ECDIIIa region. Such host animals may include but are not limited to rabbits, mice, hamsters and rats, to name but a few. Various adjuvants may be used to increase the immunological response,

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depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

5 Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These  
10 include, but are not limited to, the hybridoma technique of Kohler and Milstein, (*Nature* 256:495-497, 1975; and U.S. Pat. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., *Immunology Today* 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030, 1983), and the EBV-hybridoma technique (Cole et al., *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985). Such antibodies may be of any  
15 immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Hybridomas producing mAb may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

Additionally, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855, 1984; Neuberger et al., *Nature*,  
20 312:604-608, 1984; Takeda et al., *Nature*, 314: 452-454, 1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin  
25 constant region (humanized).

Alternatively, techniques described for the production of single-chain antibodies (U.S. Pat. No. 4,946,778; Bird, *Science* 242:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Ward et al., *Nature* 334:544-546, 1989) can be adapted to  
30 produce single-chain antibodies against ECDIIIa variant gene products. Single-chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments.  
35 Alternatively, Fab expression libraries may be constructed (Huse et al., *Science*, 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

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Antibodies to particular ECDIIIa variants can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" the ECDIIIa variant, using techniques well known to those skilled in the art. (Greenspan & Bona, *FASEB J* 7 (5):437-444, 1993; and Nissinoff, *J. Immunol.* 147:2429-2438, 1991). For example antibodies which bind to an ECDIIIa variant and competitively inhibit the binding of p68HER-2 to HER-2 receptor can be used to generate anti-idiotypes that "mimic" the ECDIIIa variant and, therefore, bind and neutralize HER-2 receptor. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in cancer therapeutic regimens.

Alternatively, antibodies to particular ECDIIIa variants that can act as agonists or antagonists of the ECDIIIa variant activity can be generated. Such antibodies will bind to the ECDIIIa variant and modulate the activity of p68HER-2 vis-à-vis p185HER-2 receptor-mediated signal transduction. Such antibodies may be particularly useful for treating particular cancers and/or modulating tumor differentiation. Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis.

#### ECDIIIa region Variants as Diagnostic/Prognostic Agents

Example 11 (below) shows that the human sequence of intron 8 is both proline-rich and polymorphic. Sequencing of genomic DNA from fifteen different individuals resulted in the identification of 10 variable sequence regions within Her-2 Intron 8. See SEQ ID NO:10; Figure 8, and Table 1. Figure 8 shows the most common nucleotide and corresponding polypeptide sequences of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (see legend to TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue 341 in the "herstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999), is a polymorphic form that differs at amino acid residues #6 and

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#73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. An individual may, *inter alia*, be genetically  
5 heterozygous for two variants, homozygous for a given variant, or homozygous for a double variant. Both tumor progression and optimal treatment may vary depending upon the particular variants represented in a given individual.

This variability has both prognostic and diagnostic utility. The present invention shows that ECDIIIa-containing polypeptides can bind tightly to, and thus antagonize the  
10 HER-2 receptor. Such a specific, high-affinity interaction is dependent upon particular primary, secondary and tertiary structure of the ECDIIIa-containing polypeptide. The ECDIIIa region is proline-rich, and it is well known in the art that nonconservative substitution of proline residues, or other residues within a proline-rich sequence, in a given protein can have profound effects on its secondary and tertiary structure. Thus, the  
15 polymorphisms of the present invention are likely to embody significant structural, biochemical and biological differences relative to the most common polypeptide structure (shown in Figure 8). Structural differences among ECDIIIa variant proteins may include for example, differences in size, electronegativity, or antigenicity. Differences in biological properties among ECDIIIa variants might be seen *e.g.*, in the relative degree of cellular  
20 secretion, the nature and/or extent of modulation of the HER-2 receptor, pharmacokinetics (*e.g.*, serum half-life, elimination profile), resistance to proteolysis, N-linked glycosylation patterns, etc. These biological differences, in turn, would be expected to alter tumor progression and thus optimal treatment protocols. Thus, the knowledge that an individual contains a particular ECDIIIa variant or variants (*e.g.*, in individuals heterozygous for a given  
25 variant, or individuals with compound variants like variant 11 of Table 1), may, in itself, be prognostic of particular cancer susceptibility.

The apparent genetic heterogeneity of ECDIIIa region means that the nature of the particular ECDIIIa variation carried by an individual may have to be ascertained using  
30 sequence identity assays prior to attempting genetic diagnosis of the patient. The analysis can be carried out on any genomic DNA derived from bodily fluids of the patient, typically a blood sample from an adult or child, but alternatively may be serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, and chorionic villi samples. It is expected that standard genetic diagnostic methods, such as hybridization or  
35 amplification assays, can be used. Either DNA or RNA, may, for example, be used in hybridization or amplification assays of biological samples to detect particular ECDIIIa variant sequences. Such sequence identity assays may include, but are not limited to, Southern or Northern analyses, single-stranded conformational polymorphism analysis, *in situ* hybridization assays, and polymerase chain reaction ("PCR") analyses. Such analyses

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may reveal both quantitative and qualitative aspects of ECDIIIa variant sequence expression. Such aspects may include, for example, point mutations, and/or activation or inactivation of gene expression. Standard *in situ* hybridization techniques may be used to provide information regarding which cells within a given tissue express a particular ECDIIIa variant sequence.

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Preferably, diagnostic methods for the detection of ECDIIIa variant nucleic acid molecules involve contacting and incubating nucleic acids, derived from cell types or tissues being analyzed, with one or more labeled nucleic acid reagents, or probes, specific for particular ECDIIIa variants. More preferably, PCR, or reverse transcription PCR, can be utilized to identify nucleotide variation within the ECDIIIa domain. PCR reaction conditions should be chosen which optimize amplified product yield and specificity, and, additionally, produce amplified products of lengths that may be resolved utilizing standard gel electrophoresis techniques. Such reaction conditions are well known to those of skill in the art, and important reaction parameters include, for example, length and nucleotide sequence of oligonucleotide primers, and annealing and elongation step temperatures and reaction times. Following the PCR reaction, the PCR products can be analyzed by methods such as heteroduplex detection, cleavage of RNA-DNA hybrids using Rnase A, single-stranded conformational polymorphisms, and denaturing gradient gel electrophoresis.

Additionally, if the particular ECDIIIa sequence variant is known to add or remove a restriction site, or to have significantly altered the size of a particular restriction fragment, a protocol based upon restriction fragment length polymorphism ("RFLP") analysis may be appropriate.

ECDIIIa variants can also be analyzed at the expression level using sequence identity assays with bodily fluids derived from the patient, typically a blood sample from an adult or child, but may include serum, urine, lymph, saliva, tumor tissue, placental or umbilical cord cells, amniotic fluid, and chorionic villi samples. Well-known sequence identity assays for analyzing expression include, but are not limited to, mRNA-based methods, such as Northern blots and *in situ* hybridization (using a nucleic acid probe derived from the relevant cDNA), and quantitative PCR (as described by St-Jacques et al., *Endocrinology* 134:2645-2657, 1994).

Polypeptide-based methods (*e.g.*, including but not limited to western blot analysis) including the use of antibodies specific for the ECDIIIa variant of interest, as discussed above, could also be used. These techniques permit quantitation of the amount of expression of a given ECDIIIa variant, at least relative to positive and negative controls. Preferably, a battery of monoclonal antibodies, specific for different ECDIIIa epitopes or variants, could be used for rapidly screening cells or tissue samples to detect those expressing particular ECDIIIa variants, or for quantifying the level of ECDIIIa variant polypeptides. Preferred diagnostic methods for the quantitative or qualitative detection of ECDIIIa variant peptide

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molecules may involve, for example, immunoassays wherein particular ECDIIIa-containing peptides are detected by their interaction with anti-ECDIIIa variant specific antibodies. This can be accomplished for example, by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or fluorometric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of ECDIIIa-containing peptides. Through the use of such procedures, it is possible to determine not only the presence of particular ECDIIIa-containing polypeptides, but also their distribution in the examined tissue.

10       Immunoassays for ECDIIIa variant polypeptides preferably comprise incubating a biological sample, such as the above-named bodily fluids, which have been incubated in the presence of a detectably labeled antibody capable of identifying ECDIIIa-containing peptides, and detecting bound antibody by any of a number of techniques well known in the art. The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support that is capable of immobilizing soluble proteins, cells, or cell particles. The support may then be washed with suitable buffers followed by treatment with the detectably labeled anti-ECDIIIa variant specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support may then be detected by conventional means.

15       Alternatively, anti-ECDIIIa variant specific antibodies can be detectably labeled by linking the same to an enzyme for use in an enzyme immunoassay or Enzyme Linked Immunosorbent Assay ("ELISA"). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably, a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

20       The detection can be accomplished by calorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished visually by comparison of the extent of enzymatic reaction with appropriate standards. Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect ECDIIIa-containing peptides through the use of a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by

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autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

5 The antibody can also be detectably labeled using fluorescence emitting metals such as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

10 The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, therramic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

15 The binding activity of a given lot of anti-ECDIIIa-variant specific antibody may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

25 Accordingly, the present invention, including the unexpected discovery of a plurality of variable sequence positions within the proline-rich ECDIIIa region, along with antibodies specific for particular ECDIIIa variants, provides for valuable prognostic and diagnostic information and assays.

30 Accordingly, the present invention further provides a method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue, and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis. Preferably, the method for determining the prognosis of tumor treatment further comprises measuring the

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amount of p185HER-2 ECD in the bodily fluid, and determining a ratio between the amount of p68HER-2 and p185HER-2. The higher the ratio of p68HER-2:p185HER-2, the better the treatment prognosis. Preferably, the method for determining the prognosis of tumor treatment further comprises determining which particular ECDIIIa variants are present and optimizing tumor treatment in view of particular biochemical and biological properties among ECDIIIa protein variants.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether a particular ECDIIIa variant sequence is present in the bodily fluid sample with a sequence identity assay; and (c) correlating the presence of the ECDIIIa variant sequence to cancer treatment and diagnosis using an historical database. Preferably, the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.

The present invention further provides an assay for cancer treatment, prognosis or diagnosis in a patient comprising: (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; (b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample.

The present invention further provides for the above-mentioned cancer treatment, prognostic or diagnostic assays further comprising measuring the amount of p185HER-2 ECD in the bodily fluid sample, and determining a ratio between the amount of p185HER-2 ECD and a particular p68HER-2 ECDIIIa variant.

The present invention further provides for antibodies specific for ECDIIIa variants of the sequence in SEQ ID NO:1 or SEQ ID NO:2, below.

P68HER-2 as a Therapeutic Agent

Without being bound by theory, but it appears that p68HER-2 or ECDIIIa peptide inhibits the growth of tumor cells that overexpress HER-2 by binding to p185HER-2 at the

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cells surface. This hypothesis was examined by testing anchorage independent growth of cells in the presence or absence of p68HER-2 using cells that depend on p185HER-2 overexpression for their malignant growth, yet have little or no detectable p68HER-2. Anchorage independent growth of cells in soft agar was used as a predictive model for tumor cytotoxicity. This is a common and predictive procedure to examine transforming activity and reflects the tumorigenic and oncogenic potential of cells (DiFore et al., *Science* 237:178-182, 1987; Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987; and Baasner et al., *Oncogene* 13:901-911, 1996).

The effects of p68HER-2 on anchorage independent growth in soft agar was determined using SKOV-3 carcinoma cells and HER-2 transfected 17-3-1 cells, which are both tumorigenic and overexpress p185HER-2. The cells were suspended in media supplemented with fetal calf serum in the presence or absence of p68HER-2 and incubated for 21 days in a humidified incubator. Anchorage independent growth was quantitated by counting the number of colonies that contained more than 50 cells. Figure 7 shows that in the presence of p68HER-2, anchorage independent growth of both SKOV-3 cells and 17-3-1 cells was inhibited several fold. Accordingly, these data show that p68HER-2 is not just cytostatic, but cytotoxic and possibly apoptotic.

#### Example 1

This example provides the results from an experiment to investigate HER-2 mRNA diversity within the extracellular domain (ECD) coding sequence using polymerase chain reaction (PCR). A cDNA library from SKOV-3 cells (American Type Culture Collection (Rockville, MD) maintained in DMEM, supplemented with 10% fetal bovine serum and 0.05% gentamycin), an ovarian carcinoma cell line in which the HER-2 gene is amplified eight times (Tyson et al., *Am. J. Obstet. Gynecol.* 165:640-646, 1991) was examined using a forward primer specific for exon 1 (Tal et al., *Mol. Cell. Biol.* 7, 2597-2601, 1987) identical to nucleotides 142-161 and a reverse primer complementary to nucleotides 1265-1286 in exon 9 (Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993). Briefly, The SKOV-3 cDNA library was provided by Origene Technologies, Inc. (Rockville, MD), and was prepared from RNA extracted from SKOV-3 cells. RNA was extracted from SKOV-3 cells grown to 80% confluence on 15 cm plates with TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol, to obtain total RNA. RNA was resuspended in 10mM Tris-EDTA, pH 8.0, for reverse transcription and cDNA library construction, or in RNA hybridization buffer (80% formamide, 40mM PIPES, 4 mM NaCl, 1mM EDTA, pH 7.5) for ribonuclease protection assay (RPA). RNA concentrations were determined spectrophotometrically at OD<sub>260</sub>. Poly A<sup>+</sup> mRNA was selected from total RNA using a mRNA extraction kit (Oligotex, Qiagen).

A product of ~1420 bp, determined to be HER-2-specific by Southern blotting, was approximately 270 bp larger than the expected size of 1144 bp from the previously described

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cDNA sequence (Coussens et al., *Science* 230:1132-1139, 1985). Briefly, the Southern blotting procedure transferred nucleic acids from agarose gels under vacuum (Bio-Rad Model 785 Vacuum Blotter) in 0.4 M NaOH to Gene Screen Plus Hybridization Transfer Membrane (NEN Research Products, Boston, MA). Nucleic acids were fixed to membranes by UV crosslinking in a UV-Stratalinker (Stratagene, Inc., La Jolla, CA), and the membranes were blocked in hybridization buffer (50% formamide, 5X SSC, 1% SDS, 10 mg/ml herring sperm DNA) at 42 °C for 2 h. The membranes were hybridized at 42 °C for 16 h in hybridization buffer with 10<sup>7</sup> cpm of a 220 bp Kpn-HincII fragment from ECDIIIa cDNA labelled with ( $\alpha$ -<sup>32</sup>P)dCTP (NEN Life Sciences) using a Random Prime DNA Labelling Kit (Boehringer Mannheim).

Templates were amplified in a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Cetus, Emeryville, CA) using the Expand High Fidelity PCR System (Boehringer Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM MgCl<sub>2</sub>, 5  $\mu$ M of each primer, and 200  $\mu$ M dNTPs. All primers were obtained from GIBCO BRL (Life Technologies). Numbering of nucleotide and amino acid residues is according to the HER-2 cDNA sequence reported by Coussens et al. (Coussens et al., *Science* 230:1132-1139, 1985). The HER-2 extracellular domain was targeted for amplification from an SKOV-3 cDNA library (Origene Technologies, Inc.) using a forward primer (A) identical to nucleotides (nt) 142-161 of HER-2 cDNA (5'-TGAGCACCATGGAGCTGGC-3' [SEQ ID NO 3]), which spans the initiation codon (underlined) and a reverse primer (B) (5'-TCCGGCAGAAATGCCAGGCTCC-3' [SEQ ID NO 4]), which is complementary to HER-2 exon sequence at nt 1265-1286. Cycling parameters were: 94 °C, 30 sec; 58 °C, 45 sec; 68 °C, 3 min, for 30 cycles. The region spanning the alternative sequence (denoted ECDIIIa) from genomic DNA, was amplified using a forward primer (C) (5'-AACACAGCGGTGTGAGAAGTGC-3' [SEQ ID NO 5]) identical to HER-2 exon-specific sequence at nt 1131-1152 and the reverse primer (B) [SEQ ID NO. 4] on DNA prepared as described (Bond et al., *FEBS Letters* 367:61-66, 1995) with cycling parameters: 94 °C, 30 sec; 62 °C, 30 sec; 72 °C, 60 sec, for 25 cycles.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to investigate the structure of mRNA containing the ECDIIIa sequence. First strand cDNA was reverse transcribed (Bond et al., *FEBS Letters* 367:61-66, 1995) using 5  $\mu$ g RNA primed with 0.5  $\mu$ g oligo-dT. To amplify the ECDIIIa insert and adjacent 5' HER-2 exon sequence, a forward primer (A) described above and a reverse primer (D) (5'-ATACCGGGACAGGTCAACAGC-3' [SEQ ID NO 6]) which is complementary to the 3'ECDIIIa-specific sequence were used. Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 2 min, for 30 cycles.

Amplification of the ECDIIIa insert and adjacent 3' HER-2 exon-specific sequence was with a forward primer (E) (5'-TCTGGGTACCCACTCACTGC-3' [SEQ ID NO 7])

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which is identical to the 5'ECDIIIa-specific sequence and contains a *Kpn*I restriction site and a reverse primer (F) (5'-TTCACTGGCAGTCCAGACC-3' [SEQ ID NO 8]) which is complementary to HER-2 exon sequence at nt 3898-3919 and spans the termination codon (underlined). Cycling parameters were: 94 °C, 30 sec; 60 °C, 40 sec; 68 °C, 5 min, for 30 cycles.

The PCR product was subcloned and the nucleotide sequence was determined.

The results showed that the normal HER-2 coding sequence was present beginning with the 5' primer sequence and continued uninterrupted through nucleotide 1171. At this position, a 274 nucleotide insertion was found, followed by the expected coding sequence, including the 3' primer sequence. Analysis of the predicted protein product showed that the 274 nucleotide insertion encodes an extension of the known HER-2 protein, beginning at residue 340 (Coussens et al., *Science* 230:1132-1139, 1985), and introduces an in-frame stop codon 79 amino acids later (Figure 1). Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in Genbank showed no homologies. Examination of the 5' and 3' junctions of the divergent sequence revealed consensus splice donor and acceptor sites (Sharp, and Burge, *Cell* 91:875-879, 1997) and include a pyrimidine tract and potential branchpoint adenine residues near the 3' end of the insert sequence (Figure 1). Thus, the inserted sequence is likely to be an intron.

Inspection of the predicted amino acid sequence of the novel 79 amino acids [SEQ ID NO. 1] encoded by the inserted sequence shows a consensus N-linked glycosylation site and a high proline content of 19% (Figure 1). The inserted sequence was designated ECDIIIa since it is located at the boundary between subdomains II and III in the extracellular domain of the p185HER-2 sequence (Lax et al., *Mol. Cell. Biol.* 8:1831-1834, 1988). The insert sequence is in-frame with the adjacent 5' HER-2 exon sequence for 236 nt where there is a termination codon.

#### Example 2

This example provides the results from experiments characterizing ECDIIIa as contiguous with HER-2 exons in the genome. To investigate the HER-2 gene structure in the region of the ECDIIIa sequence, a forward primer, identical to nucleotides 763-785, and a reverse primer, complementary to nucleotides 1265-1286 of the HER-2 cDNA, were used in the PCR on human genomic DNA. The amplification product was anticipated to span exon 5 (Tal et al., *Mol. Cell. Biol.* 7:2597-2601, 1987) to an exon which is immediately 3' of the ECDIIIa sequence. Intron number and sizes were estimated based on PCR product sizes, restriction digest analysis, and partial sequence analysis of amplification products.

Next, human genomic DNA was examined using HER-2 exon-specific primers that directly flank the insert to determine the sequences immediately flanking the ECDIIIa sequence. A ~430 bp product was amplified from normal human genomic DNA and from genomic DNA extracted from carcinoma cell lines SKOV-3, SKBR-3 and BT474, all of

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which have HER-2 gene amplification (Kraus et al., *EMBO J.* 6:605-610, 1987) and were found to express ECDIIIa in their cDNA. The identities of the PCR products as HER-2 were verified by Southern blot analysis using the procedure described in Example 1. Nucleotide sequence analysis showed that the PCR product from human genomic DNA contained the ECDIIIa insert, flanked immediately on both sides by known HER-2 coding sequence; no mutations or rearrangements were seen. These data show that the ECDIIIa sequence represents a wholly retained intron, likely intron 8 based on the size of products amplified following intron 4 and on the location of intron 8 in the homologous EGFR gene and HER-3 gene (Lee and Mailhe, *Oncogene* 16:3243-3252, 1998).

**Example 3**

This example shows that ECDIIIa is the only retained intron within the coding sequence of HER-2 mRNA. To determine whether additional introns were retained in the mRNA containing the ECDIIIa insert sequence, the reverse transcriptase-polymerase chain reaction (RT-PCR) was employed. First, a forward primer identical to 5' HER-2 cDNA sequence at 142-161 which spans the initiation codon, and a reverse primer complementary to the 3' ECDIIIa sequence were employed with SKBR-3 and SKOV-3 cDNA. A product of 1.3 kb was amplified, which is the size expected if the product contained no introns other than intron 8. Amplification of the 3' HER-2 coding sequence was then performed using a forward primer identical to 5' ECDIIIa sequence and a reverse primer complementary to 3' HER-2 cDNA sequence at nucleotides 3898-3919, which spans the p185HER-2 termination codon. A product of 2.9 kb was amplified, which is the size expected from the HER-2 cDNA if no additional introns were retained.

Further characterizations of both the 5'(1.3 kb) and 3'(2.9 kb) amplification products by restriction digest analysis and nucleotide sequencing confirmed the absence of additional retained introns. To determine the size of the products amplified when intron sequences are included, genomic DNA was used as a template for the PCR reactions, which resulted in products of approximately 10 kb for the 5' coding sequence and 5 kb for the 3' coding sequence. These results indicate that the alternative HER-2 transcript, resulting from retention of an intron of 274 bp, was expected to be about 4.8 kb in size, assuming that the 5'untranslated (5'UTR) and 3'untranslated (3'UTR) regions are identical in size to the previously described ~4.5 kb HER-2 cDNA (Coussens et al., *Science* 230:1132-1139, 1985).

**Example 4**

This example illustrates the expression of a protein containing an ECDIIIa sequence. To assess whether the alternative sequence is translated into a protein product, the ECDIIIa sequence was expressed as a polyhistidine-tagged peptide in bacteria, purified the peptide by nickel-affinity chromatography, and raised antisera against the purified peptide. Briefly, the bacterial expression vector was prepared by amplifying the ECDIIIa sequence from the SKOV-3 cDNA library using primer E and a reverse primer complementary to the 3' end of

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the ECDIIIa insert sequence. The reverse primer contained a *Bam*HI restriction site sequence, and was identical to that used for template construction in the RPA (described in examples 1 and 2). The PCR amplification product of ~280 bp was digested with *Kpn*I and *Bam*HI, gel purified (Qiaex II, Qiagen, Chatsworth, CA), and cloned into the pET30a vector, which encodes a six histidine tag at the amino-terminus of the expressed protein (Novagen, Madison, WI). The resulting expression vector, pET-ECDIIIa, was used for transformation of bacterial strain BL21.

To express the ECDIIIa protein product, BL21 cells transformed with the pET-ECDIIIa expression vector were grown in LB broth with 30 µg/ml Kanamycin for 4 h at 37 °C. Expression was induced with 0.1 mM IPTG for 3 h and the harvested cells were lysed by sonication, and then centrifuged at 39,000 x g for 20 min. The supernatant was absorbed onto Ni-NTA agarose (Qiagen), by shaking for 60 min at room temperature. The resin was washed with ten volumes of wash buffer (10 mM Tris pH 7.9 and 300 mM NaCl), followed by ten volumes of wash buffer with 50 mM imidazole. The his-tagged ECDIIIa protein was eluted in wash buffer with 250 mM imidazole. The his-tagged protein, which was estimated to be approximately 90% pure by Coomassie Blue staining of gels, was used to generate and characterize antibodies.

Briefly, anti-ECDIIIa antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA) by injection of two rabbits with purified polyhistidine-tagged ECDIIIa peptide (described below). Polyclonal anti-*neu* (N) was produced against a peptide identical to amino acid residues 151-165 of p185HER-2 (Lin and Clinton, *Oncogene* 6:639-643, 1991). Polyclonal anti-*neu* (C) was made against a peptide identical to the last 15 residues of the carboxy-terminus of p185HER-2 (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). Antisera from two immunized rabbits were characterized and found to contain antibodies of high titer that reacted with the purified ECDIIIa peptide.

A Western blot analysis examined whether SKBR-3 cells, which expressed the alternative sequence in its cDNA, produced a protein that reacts with anti-ECDIIIa antibody. A 68 kDa protein from the cell extract and from the extracellular media reacted with anti-ECDIIIa antibody from two different rabbits diluted at least 20,000 fold, but not with preimmune sera. Inspection of the cDNA sequence of the alternative transcript (Figure 1) predicted a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence were glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986).

If the 68 kDa ECDIIIa protein [SEQ ID NO. 2] is the translation product of the alternative HER-2 mRNA, then its N-terminal residues should be identical to the N-terminal 340 residues of p185HER-2. Therefore, cell extract from SKBR-3 cells was immunoprecipitated with anti-peptide antibody against an N-terminal sequence of HER-2, anti-*neu* (N) (Lin and Clinton, *Oncogene* 6:639-643, 1991) or with anti-ECDIIIa, and the

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immune complexes were examined by Western blot analysis with both antibodies. Briefly, three to 5  $\mu$ l of antisera were added to 2 mg of protein from cell lysates prepared in M-RIPA buffer (1% Nonidet P-40, 50 mM Tris pH 7.4, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 1% aprotinin), which had been centrifuged to remove nuclei.

- 5 Immunoprecipitation was for 2 h with shaking at 4 °C as described (Lin et al., *Mol. Cell. Endocrin.* 69:111-119, 1990). The immune complexes were bound to Protein G Sepharose (Pharmacia) by incubation for 1 h at 4 °C with shaking, collected by centrifugation, and washed four times with M-RIPA. The proteins were released from the immune complex by incubation at 95° C for 2 min in SDS-PAGE sample buffer and resolved by SDS-PAGE in  
10 7.5% gels (Mini-Protean II electrophoresis cell, Bio-Rad).

- Western blotting was conducted following SDS-PAGE. Proteins were electroblotted onto nitrocellulose (Trans-blot, BioRad) using a semi-dry transfer unit (Bio-Rad) at 15 V for 20 min per gel (0.75 mm thick) equilibrated with 25 mM Tris pH 8.3, 192 mM glycine, 50 mM NaCl, and 20% methanol. The membranes were blocked with 5% nonfat dry milk at 25  
15 °C for one hour. The blots were then incubated with primary antibody, washed twice for 15 min, and four times for 5 min with TBS-Tween (Tris-buffered saline containing 0.05% Tween), and then incubated for 40 min with goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase (Bio-Rad), diluted 1:10,000 in TBS-Tween. After incubation with secondary antibody, the membranes were washed as described above and reacted with  
20 chemiluminescent reagent (Pierce) and then were exposed to Kodak X-OMAT BLU film.

- As expected, p68HER-2 was detected when anti-ECDIIIa was used for immunoprecipitation and for Western blot analysis. When anti-ECDIIIa was used for immunoprecipitation and anti-neu (N) was the probe in the Western blot, a 68kDa protein was detected, indicating that p68ECDIIIa contained the N-terminal sequence of p185HER-2.  
25 Further, anti-neu (N) precipitated p68HER-2, which was detected by probing with anti-ECDIIIa antibody. These results demonstrate that p68HER-2 contains both ECDIIIa and the N-terminal sequence of HER-2.

- Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3) also had p68HER-2. Of several cell lines examined, HEK293 cells, derived from normal human embryonic kidney cells, expressed the highest levels of p68ECDIIIa in the cell extract and in the extracellular media, at about 5 to 10-fold higher amounts than SKBR-3 cells. In comparison to the carcinoma cell lines examined (SKBR-3, SKOV-3, and BT474) which overexpress p185HER-2, the HEK293 cells contained about 20 fold lower amounts of  
35 p185HER-2. Therefore, the relative proportion of p68HER-2 to p185HER-2 was at least 100 fold greater in HEK293 cells than in the three carcinoma cell lines studied. Reactivity with p68HER-2 as well as with a protein of ~120 kDa, particularly apparent in the HEK293 extracts, was blocked by preincubation of the antisera with purified ECDIIIa peptide

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demonstrating sequence-specific reactivity. The larger protein may be a dimer of p68HER-2. Therefore, p68HER-2 was expressed and secreted from several carcinoma cell lines and is at 5-10 fold elevated levels in HEK293.

#### Example 5

5 This example illustrates expression of an alternative HER-2 transcript containing the ECDIIIa intron sequence. Results of the RT-PCR analysis indicated that the ECDIIIa sequence was inserted into an otherwise normal-sized HER-2 mRNA. These data suggest an alternative transcript of ~4.8 kb. To examine the size and expression of the ECDIIIa alternative transcript, Northern blot analysis was conducted using an ECDIIIa-specific probe. Briefly, a template for antisense RNA probe synthesis was constructed from SKOV-3 cDNA  
10 by PCR amplification of a 389 bp sequence spanning the entire ECDIIIa insert sequence and containing adjacent 5'HER-2 exon sequence. The PCR was done using the forward primer C [SEQ ID NO. 5] that is identical to HER-2 cDNA sequence at nt 1131-1152 and a reverse primer (5'-GCACGGATCCATAGCAGACTGAG GAGG-3' [SEQ ID NO. 9]) which  
15 contains a 3' *Bam*H1 restriction endonuclease site and is complementary to the sequence spanning the 3' splice site of the ECDIIIa sequence. The PCR product was then digested with *Bam*H1, liberating a 375 bp fragment, which was cloned into pBluescript SK (Stratagene). The plasmid was sequenced by the Vollum Institute Core Sequencing Facility (Portland, OR) with m13 forward and reverse primers. An antisense RNA probe  
20 complimentary to the entire ECDIIIa sequence and to 87 nt of HER-2 exon sequence 5' to the insert was transcribed from 1 µg of linearized template using ( $\alpha$ -<sup>32</sup>P) CTP, T7 RNA polymerase, and the T7/SP6 Riboprobe Synthesis System (Promega, Madison, WI). This probe was expected to protect a 370 nt fragment when hybridized with mRNA containing ECDIIIa and adjacent HER-2 exon sequence, and to protect an 87 nt fragment when  
25 hybridized with fully spliced HER-2 mRNA.

To prepare the RNA hybrids, 30 µg of RNA were hybridized with approximately 50,000 cpm of antisense RNA probe at 48 °C for 16 h. RNA hybrids were digested for 30 min at 37 °C with 40 µg/ml RNaseA (Boehringer Mannheim) and 2 µg/ml RNase T1 (Life Technologies) in a solution of 250 mM NaCl, 5 mM EDTA, and 10 mM Tris pH 7.5.  
30 Proteinase K (100 µg) (Life Technologies) in 20 µl 10% SDS was added to stop the digestion. Samples were extracted with acid phenol (pH 4.5; Life Technologies) and chloroform, precipitated with two volumes of 100% ethanol, and suspended in 5 µl of RPA sample buffer (88% formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue). Samples were denatured at 95 °C for 10 min and electrophoresed on a  
35 5% polyacrylamide/urea gel in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA pH 8.3). Gels were dried under vacuum and subjected to phosphorimager analysis for quantitation of the protected fragments (IP Lab Gel, Molecular Dynamics).

An alternative transcript of approximately 4.8 kb was detected in HEK293 cells which

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expressed the highest levels of p68ECDIIIa. However an alternative transcript could not be detected by Northern analysis of the SKBR-3, BT474, or SKOV-3 carcinoma cell lines. Therefore, the more sensitive ribonuclease protection assay (RPA) was employed to examine the expression levels of the alternative transcript relative to the fully spliced 4.5 kb transcript. RNA from ovarian (SKOV-3) and breast (SKBR-3 and BT474) carcinoma cell lines, which contained detectable levels of p68ECDIIIa, and a control cell line, 17-3-1, stably transfected with HER-2 cDNA, were hybridized with an antisense <sup>32</sup>P-labeled RNA probe which spanned the entire ECDIIIa (intron 8) sequence and 5' HER-2 exon sequence flanking intron 8. Following RNase digestion, electrophoresis, and autoradiography, a band of 370 nucleotides was detected in each cell line except for 17-3-1, which corresponds to the expected size protected by an ECDIIIa-containing HER-2 mRNA. In addition, an 87 nucleotide protected fragment was detected in all cells and is the size expected for the fully-spliced HER-2 message which is overexpressed by more than 100 fold in these carcinoma cell lines compared to normal control cell lines (Kraus et al., *EMBO J.* 6:605-610, 1987). The amounts of each protected fragment were quantitated and normalized for size to estimate the relative abundance of the alternative transcript, expressed as a percentage of the p185HER-2 mRNA. The alternative HER-2 mRNA with the ECDIIIa insert was at 4.2% the level of the fully spliced transcript in SKOV-3; 5.4% in SKBR-3, and 0.8% in BT474 cells.

#### Example 6

This example shows that alternative transcripts containing the ECDIIIa insert were expressed in human embryonic kidney and liver. A Northern blot was conducted to examine whether an alternative transcript, which contains the ECDIIIa sequence, was expressed in normal human tissue. PolyA<sup>+</sup> mRNA from a variety of human fetal tissues prepared as a Northern blot was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8 kb mRNA was detected in kidney and a 2.6 kb transcript was detected in liver (Figure 2). The 4.8 kb transcript likely corresponded to the full length 4.5 kb transcript with the 274bp insert and the 2.6 kb transcript may have corresponded to a previously described 2.3 kb alternative transcript (Yamamoto et al., *Nature* 319:230-234, 1986; and Scott et al., *Mol. Cell. Biol.* 13:2247-2257, 1993) with the 274bp ECDIIIa insert. When the blot was stripped and hybridized with a probe specific for the 5' HER-2 coding sequence, a broad band representing the 4.8 and 4.5 kb mRNAs was detected in fetal kidney tissues and the truncated 2.6 kb transcript was detected in liver showing that these alternative transcripts contain sequences that encode the HER-2 ECD. Because the inserted ECDIIIa sequence contained a termination codon, the same protein product may be produced from each of these mRNAs.

Several cell lines were also investigated for the ECDIIIa-containing alternative transcript by Northern blot analysis. The 4.8 kb alternative transcript was detected in the human embryonic kidney cell line, HEK-293 (Figure 2). Although the ECDIIIa sequence

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was detected by RT-PCR analysis of SKBR-3, BT474, and SKOV-3 carcinoma cell lines, which all contain HER-2 gene amplification, an ECDIIIa-containing alternative transcript could not be detected by Northern analysis of these cells. Therefore, the more sensitive ribonuclease protection assay (RPA) was employed using an antisense probe which spanned the entire ECDIIIa sequence and 5' HER-2 exon sequence flanking the ECDIIIa sequence. The alternative HER-2 mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcript in SKOV-3, SKBR-3, and BT474 cells. These findings show that two alternative transcripts containing the ECDIIIa sequence were expressed in a tissue-specific manner in normal human tissues, that the 4.8 kb alternative transcript was expressed in the HEK-293 cell line, and that the carcinoma cells with gene amplification express reduced amounts of the alternative transcript at less than 5% of the 4.5 kb HER-2 transcript.

#### Example 7

This example illustrates expression of a protein containing the ECDIIIa sequence. To assess whether the alternative sequence was translated into a protein product, the ECDIIIa sequence, as a polyhistidine-tagged peptide in bacteria, was expressed and purified by nickel-affinity chromatography, and raised antisera against the purified peptide. The HEK-293 cells, which expressed the 4.8 kb ECDIIIa alternative transcript, were examined for expression of an ECDIIIa-containing protein by Western analysis. A 68 kDa protein from the cell extract and from the extracellular media reacted with the anti-ECDIIIa antibody (Figure 3) but not with preimmune sera and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Figure 3). The larger protein of ~125 kDa detected in some cell extracts may be an aggregate of p68HER-2. The cDNA sequence of the alternative transcript (Figure 1) predicts a secreted protein product of 65-70 kDa if all 5 consensus N-linked glycosylation sites in the N-terminal p185HER-2 sequence are glycosylated (Stern et al., *Mol. Cell. Biol.* 6:1729-1740, 1986). Several other cell lines were examined for expression of p68ECDIIIa. The carcinoma cell lines which contained ECDIIIa sequence in their cDNA (BT474, SKOV-3, SKBR-3) also had detectable levels of p68HER-2.

#### Example 8

This example illustrates the expression of p68HER-2 relative to p185HER-2 was markedly reduced in carcinoma cell lines in which the HER-2 gene is amplified. Because the p68HER-2 mRNA was expressed at very low levels relative to the p185HER-2 mRNA in carcinoma cell lines with HER-2 gene amplification, the relative proportions of p68HER-2 and p185HER-2 proteins in several cell lines were examined with and without HER-2 gene amplification. Western blots were prepared and probed with both antisera specific for p68HER-2 and for p185HER-2. Figure 4 shows that p185HER-2 was readily detected in the carcinoma cells lines that have their HER-2 gene amplified about 8 times (Kraus et al., *EMBO J.* 6:605-610, 1987). However, there was not a corresponding elevation in p68HER-2. In comparison, p68HER-2 was the only HER-2 protein detected in the HEK-293, IOSEVAN,

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and HBL100 nontumorigenic cells, although p185HER-2 was expressed at very low levels in these cells (Kraus et al., *EMBO J.* 6:605-610, 1987) and was detected in overexposed blots. These data show that p68HER-2 was low in proportion to p185HER-2 in carcinoma cells with HER-2 gene amplification and suggests that a mechanism may exist to maintain low levels of p68HER-2 when p185HER-2 is overexpressed.

#### Example 9

This example illustrates that p68HER-2 and the ECDIIIa peptide specifically bind to p185HER-2. Because p68HER-2 is secreted and contains subdomains I and II identical to p185HER-2, in addition to a novel sequence, the possibility that p68HER-2 may interact with p185HER-2 was investigated. Antipeptide antibody against the N-terminus of p185HER-2 and p68HER-2, anti-neu (N), or antibody specific for p185HER-2, anti-neu(C), were used for immunoprecipitations of SKBR-3 carcinoma cells, which express low levels of p68HER-2 and overexpress p185HER-2. The immunoprecipitated material was prepared as a Western blot and probed with both anti-ECDIIIa specific for p68HER-2 and with anti-neu(C). Anti-neu (N) immunoprecipitated both p68HER-2 and p185HER-2 (Figure 5A). In addition, antibodies specific for the C-terminus of p185HER-2 immunoprecipitated p185HER-2 and coprecipitated p68HER-2 (Figure 5A), suggesting an interaction between the two proteins.

Since binding interactions between ECD sequences are very weak (Tzahar et al., *EMBO J.* 16:4938-4950, 1997; Fitzpatrick et al., *FEBS Letters* 431:102-106, 1998), the possibility that binding may be conferred by the novel proline rich ECDIIIa domain was examined. The unique 79 amino acid domain, purified as a His-tagged protein, was immobilized on nickel agarose and used in a pull-down assay. For controls, two purified His-tagged peptides unrelated to ECDIIIa, a 600 residue fragment of the Wilson's disease membrane protein, and a 70 residue fragment containing the DNA binding domain of the CREB protein, were likewise immobilized on nickel agarose resin. The immobilized peptides were incubated with protein extracts prepared from HER-2 transfected 3T3 cells (17-3-1). Following extensive washes, the bound proteins were eluted and prepared as a Western blot which was probed with an antibody specific for p185HER-2. Equal amounts of His-tagged ECDIIIa peptide and control peptide were bound to the resin as confirmed by elution with 1M imidazole and Coomassie staining of the eluted material in SDS-gels. While no p185HER-2 was retained by resin without peptide or with control peptide, p185HER-2 was selectively retained by the ECDIIIa peptide (Figure 5B).

Since the ECDIIIa domain bound to p185HER-2 in a pulldown assay, the question of whether the ECDIIIa domain preferentially binds to cells that overexpress p185HER-2 was examined. This was investigated using monolayer cultures of 17-3-1 cells transfected with HER-2 compared to the parental 3T3 cells. The cells were incubated with different concentrations of the His-ECDIIIa peptide, washed, and extracted in denaturing buffer with protease inhibitors. To detect any bound peptide, the cell extracts were examined by Western

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blot analysis using antibodies specific for ECDIIIa. In addition, equal aliquots of the ECDIIIa peptide treated cells were reacted as a Western blot with antibodies specific for p185HER-2, demonstrating the overexpression of p185HER-2 in the transfected 17-3-1 cells. The ECDIIIa peptide preferentially bound to intact 17-3-1 cells at nM concentrations (Figure 5C) whereas little or no peptide was found to bind to equivalent amounts of parental 3T3 cells suggesting a specific interaction with the extracellular domain of p185HER-2.

#### Example 10

Effect of p68ECDIIIa and the ECDIIIa peptide on tyrosine phosphorylation of p185HER-2 was examined. Tyrosine phosphorylation of RTKs is the initial indication of ligand activation and signal transduction. Tyrosine phosphorylation in 17-3-1 cells treated with different amounts of the purified ECDIIIa peptide, with conditioned media (CM) from HEK293 cells that contained high levels of p68HER-2 (Figure 2A), or with control, conditioned media from SKOV-3 cells that had no detectable p68HER-2 were examined. There was no increase in the tyrosine phosphorylation signal at 10 minutes (Figure 6) or 2 hrs of treatment with His-ECDIIIa or with concentrated CM suggesting that p185HER-2 was not activated. Neither p68HER-2-containing CM nor the ECDIIIa peptide detectably altered the phosphotyrosine signal corresponding to p185HER-2 from SKOV-3 cells in which p185HER-2 tyrosine phosphorylation levels were low. Additionally, p68HER-2 and the ECDIIIa peptide had no discernable effect on *in vitro* self-phosphorylation activity of p185HER-2 immunoprecipitated from 17-3-1 cell extracts. These results support the conclusion that p68HER-2 did not activate p185HER-2 signal transduction.

#### Example 11

This example illustrates that the sequence of intron 8 is polymorphic within that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

Intron 8 of the human HER-2 gene is alternatively retained in mRNA, and encodes a novel 79-residue domain at the C-terminus of a part of the extracellular domain of p185HER-2. The product, "herstatin," of the alternative transcript with the retained intron functions as an autoinhibitor of the HER-2 oncogene. The intron 8 encoded domain, alone, was shown to bind with nM affinity to p185HER-2. (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999).

Polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA from 15 different individuals. Figure 8 and SEQ ID NO:1 show the most common nucleotide and corresponding amino acid sequences, respectively, of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (*see legend to*

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TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) is a polymorphic form that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. Some identified variants are summarized in Table 1:

TABLE 1

	X(4)	X(14)	X(17)	X(47)	X(54)	X(62)	X(106)	X(161)	X(191)	X(217)
Variant 1	T									
Variant 2		C								
Variant 3			T							
Variant 4				A						
Variant 5					A					
Variant 6						C, T, A				
Variant 7							A			
Variant 8								G		
Variant 9									T	
Variant 10										C
Variant 11			T							C

Table 1. Sequence variants in the intron-8 encoded domain found in the human population (based on 15 different individuals). Sequence variants 1-11 are listed, showing the base changes at particular "X" positions relative to that found in the most common DNA sequence shown in Figure 8. The numbers in parenthesis after each X correspond to the nucleotide position in the DNA sequence shown in Figure 8 or SEQ ID NO:10. The DNA sequence variants listed here correspond to the variable amino acid positions ("Xaa") of SEQ ID NO:1 as follows: X(4) to Xaa(2); X(14) to Xaa(5); X(17) to Xaa(6); X(47) to Xaa(16); X(54) to Xaa(18); X(62) to Xaa(21); X(106) to Xaa(36); X(161) to Xaa(54); X(191) to Xaa(64); X(217) to Xaa(73); and to the variable amino acid positions of SEQ ID NO:2 as follows: X(4) to Xaa(342); X(14) to Xaa(345); X(17) to Xaa(346); X(47) to Xaa(356); X(54) to Xaa(358); X(62) to Xaa(361); X(106) to Xaa(376); X(161) to Xaa(394); X(191) to Xaa(404); X(217) to

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Xaa(413). The specific amino acid changes (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:1 are: Variant 1, Xaa(2)(Thr→Ser); Variant 2, Xaa(5) (Leu→Pro); Variant 3, Xaa(6) (Pro→Leu); Variant 4, Xaa(16) (Leu→Gln); Variant 5, Xaa(18) (Met→Leu); Variant 6, Xaa(21) (Gly→Asp, Alu or Val); Variant 7, Xaa(36) (Leu→Ile); Variant 8, Xaa(54) (Pro→Arg); Variant 9, Xaa(64) (Pro→Leu); Variant 10, Xaa(73) (Asp→Asn), and Variant 11, Xaa(6) (Pro→Leu) and Xaa(73) (Asp→Asn). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:2.

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**EXAMPLE 12**

This example shows (*see* Table III, below) five polymorphic HER-2 intron 8 polymorphisms (sequence variants 12-16) identifiable in DNA samples from African Americans.

Specifically, four polymorphic sites were identified within that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA (*i.e.*, four polymorphic sites within the sequence region encompassed by SEQ ID NO:10, or within that encompassed by the sequence region of Figure 8). Two of these polymorphic sites (variants 12 and 15) correspond in position to those (variants 3 and 10, respectively) disclosed above in Example 11, whereas the other two (variants 13 and 14) represent additional polymorphic sites (Table II).

Furthermore, (*see* Table II and Table III, below) an additional polymorphic site (variant 16) was identified in a region of intron 8 that remains as "non-coding" sequence when intron 8 is alternatively retained in mRNA. This "non-coding" intron 8 polymorphic site is located 3', or downstream from that portion of intron 8 that contains the other polymorphic sites shown in this Example and Example 11, and that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

*Methods.* Polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA (using blood samples) from 215 individuals corresponding to 75 African Americans (Black), 135 Caucasians (White), one Asian American (Asian) and 4 Hispanics. As for Example 11, above, the N-terminal Glycine (G or Gly) designated as position 1 in Figure 8 or SEQ ID NO:1 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence of SEQ ID NO:2 or SEQ ID NO:13.

*Results.* Table II designates the nucleotide substitutions and the two amino acid residue substitutions in the coding sequence of intron 8 and a third nucleotide substitution in a non coding sequence of intron 8 using numbering corresponding to the entire "herstatin" protein sequence (SEQ ID NO:2 or SEQ ID NO:13):

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Table II

	N		
Black	75		
White	135		
Asian	1		
Hispanic/Latino	4		
Total	215		
Herstatin Polymorphism Distributions Among Blacks			
Arg357Cys (C1081T)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	32 (89)	13 (93)
het (%)	1 (4)	2 (6)	1 (7)
mut (%)	0 (0)	2 (6)	0 (0)
total	25	36	14
Arg371Ile (G1124T)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	36 (100)	14 (100)
het (%)	1 (4)	0 (0)	0 (0)
mut (%)	0 (0)	0 (0)	0 (0)
total	25	36	14
C1279T (3'UTR)			
	Prostate Cases	Controls	Other Cancers
wt (%)	24 (96)	36 (100)	12 (93)
het (%)	2 (8)	0 (0)	2 (7)
mut (%)	0 (0)	0 (0)	0 (0)
total	25	36	14

Table II. This table shows the distribution of three additional (relative to those identified in Example 11) polymorphic regions in HER-2 intron 8 of the DNA from African American individuals. Amino acids position designations correspond to amino acid positions in the "Herstatin" sequence (SEQ ID NO:2 or SEQ ID NO:13).

Table III, below, illustrates that the sequence data revealed polymorphisms at nucleotide positions #17 and #217 (also corresponding to nucleotide positions of the sequence region shown in Figure 8 or SEQ ID NO:10). The polymorphism at position #17 (variant 12) corresponds to variant 3 of Table I (Example 11). The polymorphism at position #217 (variant 15) corresponds (at least at the protein level) to variant 10 of Table I (Example 11) (see SEQ ID NO:12 and SEQ ID NO:13).

Additionally, the sequence data (see SEQ ID NO:11) revealed (see Table III) that intron 8 contains three polymorphic sites (corresponding to variants 13, 14 and 16) in addition to those disclosed in Example 11, above. Two of these (variants 13 and 14) are located at nucleotide positions #49 and #92 of SEQ ID NO:11 (also corresponding to

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nucleotide positions #49 and #92 of SEQ ID NO:10 (or Figure 8). The third (variant 16) is located at a nucleotide position #259 of SEQ ID NO:11 [also corresponding to nucleotide position #259 *relative* to the sequence region of SEQ ID NO:10 (or to position #264 of the sequence shown in Figure 1, panel A)]. Thus, the polymorphism corresponding to variant 16 is located 19 nucleotide positions 3' (downstream) from that portion of intron 8 that contains the other polymorphic sites shown in this Example and Example 11 (*i.e.*, that portion represented by SEQ ID NO:10), and that serves as in-frame (with the extracellular domain of p185HER-2) "coding" sequence when intron 8 is alternatively retained in mRNA.

Two of these polymorphisms result in nonconservative amino acid substitutions (*see* Table II and Table III, and legend of Table III; *also see* SEQ ID NO:12 and SEQ ID NO:13). For example, the polymorphism (C→T) found at the nucleotide position corresponding to nucleotide #49 of SEQ ID NO:11 [or to position #49 of SEQ ID NO:10 or Figure 8] (*i.e.*, the polymorphism at position X(49) of Table 2) would result in a substitution of Arginine (Arg) for Cysteine (Cys) at the amino acid position corresponding to amino acid residue #17 of SEQ ID NO:12, SEQ ID NO:1, SEQ ID NO:10 or SEQ ID NO:11) or to amino acid residue #357 of SEQ ID NO:13 or SEQ ID NO:2.

SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:13 show the four variant amino acid positions described in this example, along with those of Example 11 that are also shown in SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:10.

Table III designates (in addition to variants 12 and 15, which correspond to variants 3 and 10, respectively of Table I) the nucleotide substitutions and the corresponding two additional (relative to those of Table I of Example 11) amino acid residue substitutions (*i.e.*, variants 13 and 14) in the "coding" sequence of intron 8, along with the third nucleotide substitution in the 3' "non-coding" region of intron 8. The numbers in parenthesis after each X (polymorphic position) refer to nucleotide positions of SEQ ID NO:11 [or, as in Table I, correspond to (or are relative to, in the case of X(259)) the nucleotide positions in the DNA sequences shown in Figure 8 or SEQ ID NO:10].

As for Example 11, above, the N-terminal Glycine (G or Gly) designated as position 1 in SEQ ID NO:11, Figure 8, SEQ ID NO:1 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence of SEQ ID NO:2.

TABLE III

	X(17)	X(49)	X(92)	X(217)	X(259)
Variant 12	T				
Variant 13		T			
Variant 14			T		
Variant 15				A	
Variant 16					T

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Table III. Sequence variants in the intron-8 encoded domain found in human tissues (based on 215 different individuals). Sequence variants 12-16 are listed. The numbers in parenthesis after each X (polymorphic position) refer to nucleotide positions of SEQ ID NO:11 [or to positions that correspond to, or are relative to (in the case of X(259)) the nucleotide positions in the DNA sequences shown in Figure 8 or SEQ ID NO:10]. The DNA sequence variants listed here and in SEQ ID NO:11 correspond to variable amino acid positions shown in SEQ ID NO:12 [and also correspond to variable amino acid positions ("Xaa") of SEQ ID NO:1 or SEQ ID NO:10 as follows: X(17) to Xaa(6); X(49) to Xaa(17); X(92) to Xaa(31); X(217) to Xaa(73)]. The DNA sequence variant X(259) occurs in an untranslated region, and therefore does not alter the amino acid sequence of herstatin. Likewise, the variants of this table correspond to variable amino acid positions of SEQ ID NO:13 and SEQ ID NO:2 as follows: X(17) to Xaa(346); X(49) to Xaa(357); X(92) to Xaa(371); X(217) to Xaa(413). The specific amino acid changes (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:11 and SEQ ID NO:12 are: Variant 12, Xaa(6)(Pro→Leu); Variant 13, Xaa(17) (Arg→Cys); Variant 14, Xaa(31) (Arg→Ile); Variant 15, Xaa(73) (Asp→Asn). Variant 16, X(259) is in an untranslated region and does not code for an amino acid alteration, but instead alters only the nucleotide sequence at nucleotide position 259 (*i.e.*, C→T). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:13.

*SKOV3 ovarian carcinoma cells.* Two additional polymorphisms were found in a cell line derived from human ovarian cancer (SKOV3). These two polymorphisms result in nonconservative amino acid substitutions. One polymorphism is a substitution (C-T) at nucleotide #17 in the intron 8 sequence and nucleotide # 1037 in the "herstatin" sequence resulting in a substitution of leucine for proline at amino acid residue #6 in the intron 8 sequence and at amino acid residue # 346 in the "herstatin" sequence (*i.e.*, of SEQ ID NO:2 or SEQ ID NO:13). The second polymorphism found in the SKOV3 ovarian carcinoma cells line is a substitution (G-A) at nucleotide #217 in the intron 8 sequence and nucleotide #1237 in the "herstatin" sequence resulting in a substitution of Asparagine for Aspartic Acid at amino acid residue # 73 in the intron 8 sequence and amino acid #413 in the "herstatin" sequence (*i.e.*, of SEQ ID NO:2 or SEQ ID NO:13).

Significantly, the five polymorphic sites identified in the sequence analysis of this Example 12 were found only in DNA samples from African Americans (Black).

#### Summary of Examples 11 and 12

Together, Examples 11 and 12 of the present invention disclose 13 polymorphic positions in intron 8 of the Her-2 gene. Example 12, involved a relatively large DNA sample

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size, and indicated that the five polymorphic sites identified (three of which are distinct from the ten polymorphic sites identified in Example 11) are unique to African Americans (Black).

Twelve of the thirteen polymorphisms (*i.e.*, except for variant 16 of Example 12) of these two Examples are present in that portion of intron 8 that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

The polymorphism corresponding to variant 16 is located in a region of intron 8 that remains as "non-coding" sequence when intron 8 is alternatively retained in mRNA. This "non-coding" intron 8 polymorphic site is located 19 nucleotide positions 3', or downstream from that portion of intron 8 that contains the other polymorphic sites, and that serves as in-frame (with the extracellular domain of p185HER-2) coding sequence when intron 8 is alternatively retained in mRNA.

These HER-2 intron 8 polymorphisms provide for novel DNA and protein sequences, novel pharmaceutical compositions for treating solid tumors that overexpress HER-2, and monoclonal antibodies that bind to ECDIIIa variants corresponding to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13. These HER-2 intron 8 polymorphisms also provide for prognostic and diagnostic assays for the treatment and prevention of cancer.

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We claim:

1. An isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
- 5 2. The isolated polypeptide of claim 1, wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
3. The isolated polypeptide of claim 1, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin (a marketed humanized monoclonal antibody that is used for the treatment of cancer and that binds to the
- 10 ECD of HER-2).
4. An isolated DNA sequence that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
- 15 5. The isolated DNA sequence that codes on expression for a polypeptide of claim 4 wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
6. The isolated DNA sequence of claim 4, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
7. A transfected cell comprising an expression vector having a DNA sequence
- 20 that codes on expression for a polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
8. An isolated and glycosylated polypeptide having from about 300 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79
- 25 amino acids are present, and wherein at least three N-linked glycosylation sites are present.
9. The isolated and glycosylated polypeptide of claim 8, wherein the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present.
10. The isolated and glycosylated polypeptide of claim 8, wherein the isolated
- 30 polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
11. An isolated DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-
- 35 linked glycosylation sites are present.
12. The isolated DNA sequence that codes on expression for a polypeptide of claim 11, wherein the isolated polypeptide is from about 350 to 419 amino acids in length and four N-linked glycosylation sites are present.

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13. A transfected cell comprising an expression vector having a DNA sequence that codes on expression for a polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present.
- 5 14. A method for treating a solid tumor characterized by overexpression of HER-2, comprising administering an agent that binds to the extracellular domain (ECD) of HER-2, wherein the agent is selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 10 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone.
- 15 15. The method of claim 14, wherein the solid tumor that overexpresses HER-2 is selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer and colon cancer.
16. The method of claim 14, wherein the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID 20 NO:12.
17. The method of claim 16, wherein the agent is a combination of the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, and the monoclonal antibody that binds to the ECD of HER-2.
18. A pharmaceutical composition for treating solid tumors that overexpress HER- 25 2, comprising an agent selected from the group consisting of (a) an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ , (b) an isolated and glycosylated polypeptide having from about 80 to 419 amino acids taken from the sequence of SEQ ID NO:2 or SEQ ID NO:13, wherein the C 30 terminal 79 amino acids are present, and wherein at least three N-linked glycosylation sites are present, (c) a monoclonal antibody that binds to the ECD of HER-2, and (d) combinations thereof, with the proviso that the agent cannot be the monoclonal antibody alone, and pharmaceutically acceptable carrier.
19. The pharmaceutical composition for treating solid tumors that overexpress 35 HER-2 of claim 18, wherein the agent is the isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12.
20. The pharmaceutical composition for treating solid tumors that overexpress HER-2 of claim 19, wherein the agent is a combination of the isolated polypeptide having

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from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12 and the monoclonal antibody that binds to the ECD of HER-2.

21. A method for targeting a therapeutic agent to solid tumor tissue, wherein the solid tumor tissue is characterized by overexpression of HER-2, comprising attaching the therapeutic agent to an isolated polypeptide having from about 50 to 79 amino acids taken from the sequence of SEQ ID NO:1 or SEQ ID NO:12, wherein the polypeptide binds to the extracellular domain ECD of HER-2 at an affinity of at least  $10^8$ .
22. The method for targeting a therapeutic agent to solid tumor tissue of claim 21, wherein the isolated polypeptide is from about 69 to 79 amino acids in length.
23. The method for targeting a therapeutic agent to solid tumor tissue of claim 21, wherein the isolated polypeptide binds to a site on the ECD of HER-2 that is different from the site of binding of Herceptin®.
24. A method for determining the prognosis of tumor treatment in a patient for a tumor that overexpresses HER-2, comprising: (a) obtaining a bodily fluid sample from a patient, wherein the bodily fluid is selected from the group consisting blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof; and (b) measuring the amount of p68HER-2 expressed using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis.
25. The method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2 of claim 24, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid.
26. The method for determining the prognosis of tumor treatment for a tumor that overexpresses HER-2 of claim 25, further comprising determining a ratio between the amount of p68HER-2 and p185HER-2, whereby the higher the p68HER-2 to p185HER-2 ratio, the better the prognosis of the patient.
27. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:
- (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof;
- (b) determining whether an ECDIIIa variant protein or DNA sequence, or a HER-2 intron 8 variant DNA sequence is present in the bodily fluid sample using a sequence identity assay; and
- (c) correlating the presence of the ECDIIIa variant protein or DNA sequence, or the HER-2 intron 8 variant DNA sequence to cancer treatment and diagnosis using an historical database.

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28. The diagnostic assay of claim 27, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, immunoassays, hybridization assays, and combinations thereof.
29. The diagnostic assay of claim 27, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid.
30. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:
- (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof;
  - (b) determining whether a HER-2 intron 8 variant DNA sequence is present in the bodily fluid sample using a sequence identity assay; and
  - (c) correlating the presence of the HER-2 intron 8 variant DNA sequence to cancer treatment and diagnosis using an historical database.
31. The diagnostic assay of claim 30, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, hybridization assays, and combinations thereof.
32. The diagnostic assay of claim 30, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid.
33. An assay for cancer treatment, prognosis or diagnosis in a patient comprising:
- (a) obtaining a bodily fluid sample from the patient, wherein the bodily fluid is selected from the group consisting of blood, serum, urine, lymph, saliva, tumor tissue, placental tissue, umbilical cord tissue, amniotic fluid, chorionic villi tissue and combinations thereof;
  - (b) determining whether an amount of an p68HER-2 ECDIIIa variant is present in the bodily fluid sample using an anti-p68HER-2 antibody-based assay, wherein the assay is selected from the group consisting of ELISA, immunoprecipitation, immunohistochemistry, and Western analysis; and
  - (c) correlating the presence or amount of the p68HER-2 ECDIIIa variant to cancer treatment and diagnosis using an historical database.
34. The diagnostic assay of claim 33, wherein the sequence identity assay is selected from the group consisting of DNA sequencing, PCR assays, ELISA immunologic assays, hybridization assays, and combinations thereof.
35. The diagnostic assay of claim 33, further comprising measuring the amount of p185HER-2 ECD in the bodily fluid.
36. The diagnostic assay of claim 35, further comprising determining a ratio between the amount of p68HER-2 and p185HER-2 ECD.
37. An antibody specific for an ECDIIIa variant of the sequence in SEQ ID NO:1,

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SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13.

38. An antibody specific for p68HER-2 ECDIIIa variant 3.

39. A diagnostic kit comprising:

5 (a) a monoclonal antibody or antigen-binding fragment thereof that specifically binds to an ECDIIIa variant of the sequence in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:12 or SEQ ID NO:13; and

(b) a detectable label, whereby the binding of the antibody in step (a) can be detected.

40. The diagnostic kit of claim 36, wherein the label is selected from the group consisting of enzymes, radiolabels, chromophores, chemiluminescent tags, and fluorescers.



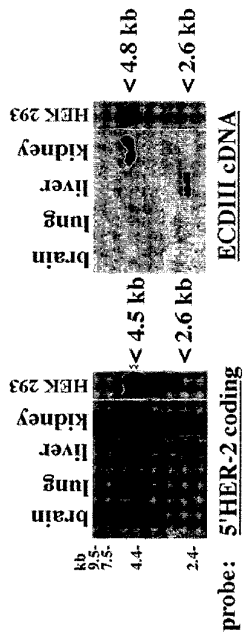


Fig. 2

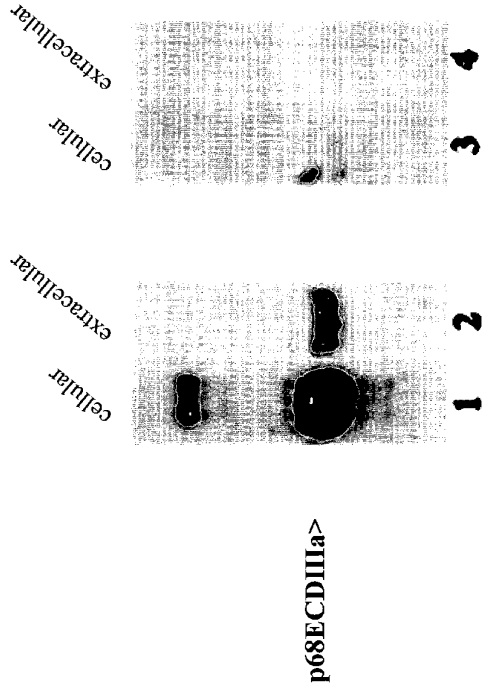


Fig. 3

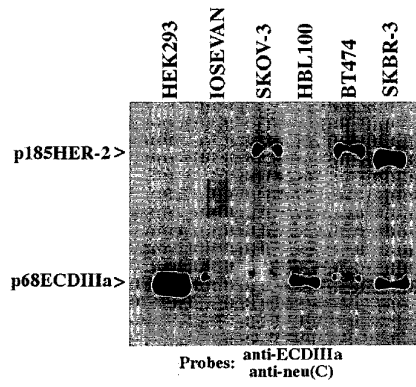


Fig. 4

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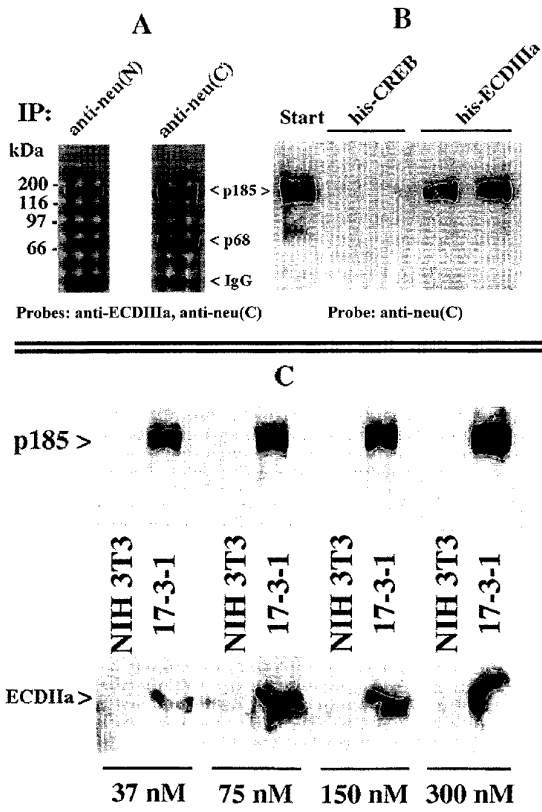


Fig. 5

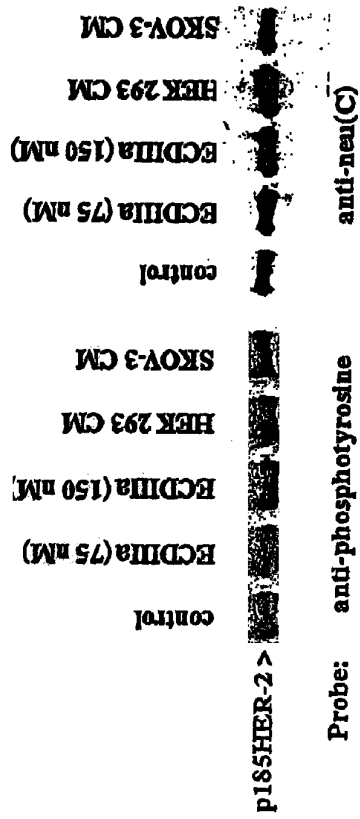


Fig. 6

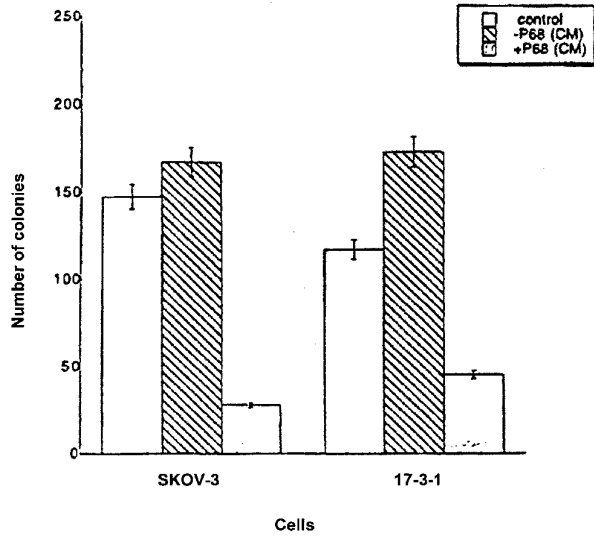


Fig. 7



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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANTS: Clinton, Gail M., Henner, William D. and Evans, Adam
- (ii) TITLE OF INVENTION: HER-2 BINDING ANTAGONISTS
- 10 (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: DAVIS WRIGHT TREMAINE
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  - (C) CITY: Seattle
  - 15 (D) STATE: Washington
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 98101
- 20 (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: PC compatible
  - (C) OPERATING SYSTEM: Windows95
  - (D) SOFTWARE: Word
- 25 (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: to be assigned
  - (B) FILING DATE: 16 February 2001
  - (C) CLASSIFICATION:
- 30 (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Davison, Barry L.
  - (B) REGISTRATION NUMBER: 47,309
  - (C) REFERENCE/DOCKET NUMBER: 49321-19
- 35 (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 206 628 7621
  - (B) TELEFAX: 206 628 7699

(2) INFORMATION FOR SEQ ID NO:1:

- 40 (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 79
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS: single
    - 45 (D) TOPOLOGY: unknown
  - (ii) MOLECULE TYPE: HER-2 ECD antagonist
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro Xaa  
 5 10 15  
 Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu Arg Pro  
 20 25 30  
 Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu Ala Pro Leu





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(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
(ii) MOLECULE TYPE: oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 ATACCGGGAC AGGTCAACAG C 21

(2) INFORMATION FOR SEQ ID NO:7:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
(ii) MOLECULE TYPE: oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20 TCTGGGTACC CACTCACTGC 20

(2) INFORMATION FOR SEQ ID NO:8:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
(ii) MOLECULE TYPE: oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

30 TTCACACTGG CACGTCCAGA CC 22

(2) INFORMATION FOR SEQ ID NO:9:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  
(ii) MOLECULE TYPE: oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

40 GCACGGATCC ATAGCAGACT GAGGAGG 27

(2) INFORMATION FOR SEQ ID NO:10:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 240 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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GGT WCC CAC TCA CYG CYC CCG AGG CCA GCT GCA GTT CCT GTC CCT 45  
 Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro  
 5 10 15

5 CWG CGC ATR CAG CCT GNC CCA GCC CAC CCT GTC CTA TCC TTC CTC 90  
 Xaa Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu  
 20 25 30

AGA CCC TCT TGG GAC MTA GTC TCT GCC TTC TAC TCT CTA CCC CTG 135  
 10 Arg Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu  
 35 40 45

GCC CCC CTC AGC CCT ACA AGT GTC CST ATA TCC CCT GTC AGT GTG 180  
 15 Ala Pro Leu Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val  
 50 55 60

GGG AGG GGC CYG GAC CCT GAT GCT CAT GTG GCT GTT SAC CTG TCC 225  
 Gly Arg Gly Xaa Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser  
 65 70 75

20 CGG TAT GAA GGC TGA 240  
 Arg Tyr Glu Gly

25 (2) INFORMATION FOR SEQ ID NO:11:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 274 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 30 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGT WCC CAC TCA CYG CYC CCG AGG CCA GCT GCA GTT CCT GTC CCT 45  
 35 Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro  
 5 10 15

CWG YGC ATR CAG CCT GNC CCA GCC CAC CCT GTC CTA TCC TTC CTC 90  
 40 Xaa Xaa Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu  
 20 25 30

AKA CCC TCT TGG GAC MTA GTC TCT GCC TTC TAC TCT CTA CCC CTG 135  
 Xaa Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu  
 35 40 45

45 GCC CCC CTC AGC CCT ACA AGT GTC CST ATA TCC CCT GTC AGT GTG 180  
 Ala Pro Leu Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val  
 50 55 60

50 GGG AGG GGC CYG GAC CCT GAT GCT CAT GTG GCT GTT VAC CTG TCC 225  
 Gly Arg Gly Xaa Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser  
 65 70 75

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CGG TAT GAA GGC TGAGACGGCC CCTTCCCCCA CYCACCCCCA CCTCCTC 274  
 Arg Tyr Glu Gly

5 (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 79  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 10 (ii) MOLECULE TYPE: HER-2 ECD antagonist  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val Pro Val Pro Xaa  
 15 Xaa Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser Phe Leu Xaa Pro  
 Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro Leu Ala Pro Leu  
 20 Ser Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val Gly Arg Gly Xaa  
 Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser Arg Tyr Glu Gly  
 65 70 75

25 (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 419  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown  
 30 (ii) MOLECULE TYPE: polypeptide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35 Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu  
 Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Cys Lys  
 Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His  
 40 Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr  
 Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val  
 Gln Gly Tyr Val Leu Cys Ala His Asn Gln Val Arg Gln Val Pro Leu  
 Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr  
 Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Agn Agn Thr Thr Pro  
 50 Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser  
 Leu Thr Glu Cys Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln

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145                                    150                                    155                                    160  
 Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn  
    165                                    170                                    175  
 5 Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys  
    180                                    185                                    190  
 His Pro Cys Ser Pro Cys Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser  
    195                                    200                                    205  
 Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys  
    210                                    215                                    220  
 10 Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys  
    225                                    230                                    235                                    240  
 Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu  
    245                                    250                                    255  
 His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val  
    260                                    265                                    270  
 15 Thr Tyr Asn Thr Asp Thr Phe Glu Ser Cys Pro Asn Pro Glu Gly Arg  
    275                                    280                                    285  
 Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Lys Leu  
    290                                    295                                    300  
 20 Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln  
    305                                    310                                    315                                    320  
 Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys  
    325                                    330                                    335  
 Pro Cys Ala Arg Gly Xaa His Ser Xaa Xaa Pro Arg Pro Ala Ala Val  
    340                                    345                                    350  
 25 Pro Val Pro Xaa Xaa Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser  
    355                                    360                                    365  
 Phe Leu Xaa Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro  
    370                                    375                                    380  
 30 Leu Ala Pro Leu Asp Pro Thr Ser Val Xaa Ile Ser Pro Val Ser Val  
    385                                    390                                    395                                    400  
 Gly Arg Gly Xaa Asp Pro Asp Ala His Val Ala Val Xaa Leu Ser Arg  
    405                                    410                                    415  
 35 Tyr Glu Gly

## 【 国際調査報告 】

INTERNATIONAL SEARCH REPORT		International application No. PCT/US01/05327
<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : G01N 33/567, 33/574; C07K 16/00 US CL : 435/7.1, 7.23; 530/387.1, 387.7 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.1, 7.23; 530/387.1, 387.7 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN, EAST (medline, embase, biosis, caplus, cancerlit, scisearch, toxline, USPATENT, PGPUB, EPO, JPO, Derwent)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOHERTY et al. The HER-2/neu receptor tyrosine kinase gene encodes a secreted autoinhibitor. Proc. Natl. Acad. Sci. USA. September 1999, Vol. 96, pages 10869-10874, especially page 10869.	24-36, 38, 40
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *B* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family		
Date of the actual completion of the international search 02 JULY 2001		Date of mailing of the international search report 01 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Jennifer E. Hunt</i> JENNIFER E. HUNT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) \*

INTERNATIONAL SEARCH REPORT	International application No. PCT/US01/05327
<b>Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)</b>	
This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:	
1. <input type="checkbox"/> Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:	
2. <input checked="" type="checkbox"/> Claims Nos.: 1-26, 37, and 39 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  The claims recite sequences, however the sequence listing is not in compliance and thus the sequences cannot be searched.	
3. <input type="checkbox"/> Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).	
<b>Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)</b>	
This International Searching Authority found multiple inventions in this international application, as follows:  Please See Extra Sheet.	
1. <input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.	
2. <input checked="" type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.	
3. <input type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:	
4. <input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:	
Remark on Protest	<input type="checkbox"/> The additional search fees were accompanied by the applicant's protest. <input type="checkbox"/> No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No. PCT/US01/05327
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**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 24-36 and 40 drawn to a method of determining prognosis and the corresponding kit.  
Group IV, claim(s)38, drawn to an antibody.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The application contains additional categories of invention.

The method of Group I is distinct from the product of Group II, and the categories are not related as a combination of category of invention.

## フロントページの続き

(51) Int.Cl. <sup>7</sup>	F I	テーマコード(参考)
A 6 1 P 15/00	A 6 1 P 35/00	4 H 0 4 5
A 6 1 P 35/00	C 0 7 K 14/47	
C 0 7 K 14/47	C 0 7 K 16/18	
C 0 7 K 16/18	C 1 2 N 1/15	
C 1 2 N 1/15	C 1 2 N 1/19	
C 1 2 N 1/19	C 1 2 N 1/21	
C 1 2 N 1/21	C 1 2 Q 1/68	A
C 1 2 N 5/10	G 0 1 N 33/53	D
C 1 2 Q 1/68	G 0 1 N 33/53	M
G 0 1 N 33/53	G 0 1 N 33/566	
G 0 1 N 33/566	C 1 2 N 5/00	A
	A 6 1 K 37/02	

(81) 指定国 AP(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), EA(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), EP(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OA(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG), AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW

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F ターム(参考) 4B024 AA01 AA12 AA20 BA10 BA36 BA63 BA80 CA04 CA07 CA09

CA12 CA20 DA06 EA04 GA11 HA03 HA11 HA13 HA14 HA20

4B063 QA01 QA13 QA17 QA19 QQ03 QQ27 QQ43 QQ53 QQ79 QQ91

QR08 QR32 QR35 QR40 QR42 QR56 QR62 QS16 QS25 QS34

QS36 QS39 QX02 QX10

4B065 AA26X AA58X AA72X AA87X AA93Y AB01 AC14 BA02 BB01 BC01

BC03 BD01 BD14 BD15 CA24 CA29 CA44 CA46

4C084	AA02	BA20	BA44	CA59	CA62	DA27	NA14	ZA592	ZA662	ZA812
	ZB262									
4C085	AA14	BB33	BB34	BB35	BB36	BB37	BB41	BB43	EE01	
4H045	AA10	AA11	AA20	AA30	BA10	BA19	BA20	BA21	BA41	BA53
	CA40	DA50	DA55	DA75	DA86	DA89	EA20	EA50	FA71	FA74
	GA01	GA15								

专利名称(译)	与HER-2结合的拮抗剂		
公开(公告)号	<a href="#">JP2004501605A</a>	公开(公告)日	2004-01-22
申请号	JP2001560693	申请日	2001-02-16
[标]申请(专利权)人(译)	俄勒冈健康科学说盐湖城大学		
申请(专利权)人(译)	俄勒冈健康Saiensezu盐湖城大学		
[标]发明人	クリントンゲイル ヘンナーウィリアムディー エヴァンスアダム		
发明人	クリントン ゲイル ヘンナー ウィリアム ディー エヴァンス アダム		
IPC分类号	G01N33/53 A61K38/00 A61K39/395 A61P1/00 A61P11/00 A61P15/00 A61P35/00 C07K14/47 C07K16/18 C07K16/32 C12N1/15 C12N1/19 C12N1/21 C12N5/10 C12N15/09 C12Q1/68 G01N33/566		
CPC分类号	A61P1/00 A61P11/00 A61P15/00 C07K16/32 Y10T436/143333		
FI分类号	C12N15/00.ZNA.A A61K39/395.T A61P1/00 A61P11/00 A61P15/00 A61P35/00 C07K14/47 C07K16/18 C12N1/15 C12N1/19 C12N1/21 C12Q1/68.A G01N33/53.D G01N33/53.M G01N33/566 C12N5/00.A A61K37/02		
F-TERM分类号	4B024/AA01 4B024/AA12 4B024/AA20 4B024/BA10 4B024/BA36 4B024/BA63 4B024/BA80 4B024/CA04 4B024/CA07 4B024/CA09 4B024/CA12 4B024/CA20 4B024/DA06 4B024/EA04 4B024/GA11 4B024/HA03 4B024/HA11 4B024/HA13 4B024/HA14 4B024/HA20 4B063/QA01 4B063/QA13 4B063/QA17 4B063/QA19 4B063/QQ03 4B063/QQ27 4B063/QQ43 4B063/QQ53 4B063/QQ79 4B063/QQ91 4B063/QR08 4B063/QR32 4B063/QR35 4B063/QR40 4B063/QR42 4B063/QR56 4B063/QR62 4B063/QS16 4B063/QS25 4B063/QS34 4B063/QS36 4B063/QS39 4B063/QX02 4B063/QX10 4B065/AA26X 4B065/AA58X 4B065/AA72X 4B065/AA87X 4B065/AA93Y 4B065/AB01 4B065/AC14 4B065/BA02 4B065/BB01 4B065/BC01 4B065/BC03 4B065/BD01 4B065/BD14 4B065/BD15 4B065/CA24 4B065/CA29 4B065/CA44 4B065/CA46 4C084/AA02 4C084/BA20 4C084/BA44 4C084/CA59 4C084/CA62 4C084/DA27 4C084/NA14 4C084/ZA592 4C084/ZA662 4C084/ZA812 4C084/ZB262 4C085/AA14 4C085/BB33 4C085/BB34 4C085/BB35 4C085/BB36 4C085/BB37 4C085/BB41 4C085/BB43 4C085/EE01 4H045/AA10 4H045/AA11 4H045/AA20 4H045/AA30 4H045/BA10 4H045/BA19 4H045/BA20 4H045/BA21 4H045/BA41 4H045/BA53 4H045/CA40 4H045/DA50 4H045/DA55 4H045/DA75 4H045/DA86 4H045/DA89 4H045/EA20 4H045/EA50 4H045/FA71 4H045/FA74 4H045/GA01 4H045/GA15		
代理人(译)	中村稔 竹内英人 小川伸男 西岛隆义		
优先权	09/506079 2000-02-16 US		
其他公开文献	JP2004501605A5		
外部链接	<a href="#">Espacenet</a>		

#### 摘要(译)

一种用于治疗过度表达HER-2的癌症的药物组合物，其包含：(a)选自SEQ ID NO：1或SEQ ID NO：12的约50-79个氨基酸。从SEQ ID NO：2或SEQ ID NO：13的序列中以至少108，(b)约300-419的亲合力与HER-2的细胞外区域(ECD)结合的分离的多肽。与(c)HER-2的ECD结合，该分离的含氨基酸的糖基化多肽存在C端79个氨基酸，并且存在至少3个N-连接的糖基化位

点。单克隆抗体，(d)选自由以下组成的组的药物和药理上可接受的碱，但该药不能单独由单克隆抗体组成的药理学公开了组合物。还公开了预后和诊断方法。