(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.[7]	F I		テーマコード（参考）
C12N 15/09	C12N 15/00	ZNAA	4B024
A61K 38/00	A61K 39/395	T	4B063
A61K 39/395	A61P 1/00		4B065
A61P 1/00	A61P 11/00		4C084
A61P 11/00	A61P 15/00		4C085

審査請求 未請求　予備審査請求 有　（全 159 頁）　最終頁に続く

(21)出願番号　特願2001-560693(P2001-560693)
(86)(22)出願日　平成13年2月16日(2001.2.16)
(85)翻訳文提出日　平成14年8月15日(2002.8.15)
(86)国際出願番号　PCT/US2001/005327
(87)国際公開番号　WO2001/061356
(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)

(71)出願人　502296246
オレゴン　ヘルス　サイエンセズ　ユニヴァーシティー
アメリカ合衆国　オレゴン州　９７２０１－３０９８　ポートランド　サウスウェスト　サム　ジャクソン　パーク　ロード　３１８１　エル３３５
(74)代理人　100059959
弁理士　中村　稔
(74)代理人　100067013
弁理士　大塚　文昭
(74)代理人　100082005
弁理士　熊倉　禎男
(74)代理人　100065189
弁理士　宍戸　嘉一

最終頁に続く

(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学的組成物であって、（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10^8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または配列ＩＤ番号１３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、薬理学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学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10^8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６９～７９個である、請求項１に記載の単離ポリペプチド。
【請求項３】
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、請求項１に記載の単離ポリペプチド。
【請求項４】
発現したときに、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10^8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。
【請求項５】
長さがアミノ酸約６９～７９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ＤＮＡ配列。
【請求項６】
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、請求項４に記載の単離ＤＮＡ配列。
【請求項７】
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。
【請求項８】
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。
【請求項９】
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、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】
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、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ポリペプチドをコードしている単離ＤＮＡ配列。
【請求項１２】
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、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ＤＮＡ配列。
【請求項１３】
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、ポリペプチドをコードしている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。
【請求項１４】

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10^8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。

【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。

【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学的組成物であって、（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10^8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、薬理学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、上記薬理学的組成物。

【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に記載の薬理学的組成物。

【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に記載の薬理学的組成物。

【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10^8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。

【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

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。
【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、
（ｂ）配列同定アッセイにより、この体液サンプル中にＥＣＤ　ＩＩＩａ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　ＩＩＩａ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シークエンシング、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。
【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、
（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シークエンシング、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３０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）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、この体液サンプル中にｐ６８ＨＥＲ－２のＥＣＤ ＩＩＩａ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 ＩＩＩａ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。

【請求項３４】
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、ＥＬＩＳＡ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３３に記載の診断方法。

【請求項３５】
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３３に記載の診断方法。

【請求項３６】
ｐ６８ＨＥＲ－２とｐ１８５ＨＥＲ－２のＥＣＤの量の比を測定する操作をさらに含む、請求項３５に記載の診断方法。

【請求項３７】
配列ＩＤ番号１、配列ＩＤ番号２、配列ＩＤ番号１２、配列ＩＤ番号１３のいずれかで表わされる配列のＥＣＤ ＩＩＩａ変異体に対して特異的な抗体。

【請求項３８】
ｐ６８ＨＥＲ－２のＥＣＤ ＩＩＩａ変異体３に対して特異的な抗体。

【請求項３９】
（ａ）配列ＩＤ番号１、配列ＩＤ番号２、配列ＩＤ番号１２、配列ＩＤ番号１３のいずれかで表わされる配列のＥＣＤ ＩＩＩａ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。

【請求項４０】
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３６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。
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日に出願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。
【従来の技術】
【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

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と、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Ｍａｉｈｌｅ、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Ｍａｉｈｌｅ、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、１９６６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

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。

【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。

【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学的組成物であって、（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、薬理学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学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。

【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

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。

【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ＥＣＤ　ＩＩＩａ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ＥＣＤ　ＩＩＩａ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シークエンシング、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シークエンシング、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ｐ６８ＨＥＲ－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ｐ６８ＨＥＲ－２のＥＣＤ　ＩＩＩａ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ＥＣＤ　ＩＩＩａ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。

【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ｐ１８５ＨＥＲ－２のＥＣＤの量を測定し、ｐ１８５ＨＥＲ－２と特定のｐ６８ＨＥＲ－２のＥＣＤ　ＩＩＩａ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ＩＤ番号１または配列ＩＤ番号２で表わされる配列のＥＣＤ　ＩＩＩａ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２７４ｂｐの挿入体を有する、ＨＥＲ－２の新たな４．８ｋｂのｍＲＮＡ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、７９個のアミノ酸［配列ＩＤ番号１］をコードした後、ヌクレオチド配列２３６位に終止コドンを有する。この新たなｍＲＮＡからは、膜貫通領域と細胞内領域を欠いているＨＥＲ－２タンパク質断片が予測される。このタンパク質断片は、４１９個のアミノ酸［配列ＩＤ番号２］を含む。４１９個のアミノ酸は、ｐ１８５ＨＥＲ－２のＮ末端と一致する３４０個の残基と、Ｃ末端の新奇な７９個の残基［配列ＩＤ番号１］からなる。Ｃ末端の新奇な７９個のアミノ酸残基［配列ＩＤ番号１］に対して特異的な抗体、またはｐ１８５ＨＥＲ－２のＮ末端に対して特異的な抗体を用い、６８ｋＤａのタンパク質産物を同定した［配列ＩＤ番号２］。この６８ｋＤａのタンパク質は、新奇なＨＥＲ－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、ＨＥＲ－２の新奇なｍＲＮＡ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４．８ｋｂ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ＨＥＫ２９３で検出された。さらに、２．６ｋｂ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２．６ｋｂというサイズは、挿入配列がＨＥＲ－２の２．３ｋｂ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、ｐ１８５ＨＥＲ－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、ｐ１８５ＨＥＲ－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、ｐ１８５ＨＥＲ－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６８ｋＤａであることがわかった。これは、ｐ１８５ＨＥＲ－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）。

【００２３】ここに提示したデータは、ｐ６８ＨＥＲ－２がｐ１８５ＨＥＲ－２に特異的に結合することを示している。ｐ１８５ＨＥＲ－２との会合は、ｐ６８ＨＥＲ－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ｐ１８５ＨＥＲ－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、ｐ１８５ＨＥＲ－２および、ｐ１８５ＨＥＲ－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ｐ６８ＨＥＲ－２がｐ１８５ＨＥＲ－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、ｐ６８ＨＥＲ－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、ｐ６８ＨＥＲ－２はＥＧＦ相同領域を欠いており、ｐ１８５ＨＥＲ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。

【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ｐ１８５ＨＥＲ－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ｐ１８５ＨＥＲ－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ｐ１８５ＨＥＲ－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）。

【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；Ｄｏｕｇａｌｌ他、Ｏｎｃｏｇｅｎｅ、第９巻、２１０９～２１２３ページ、１９９４年；Ｇｒｏ

ｅｎｅｎ他、Ｇｒｏｗｔｈ Ｆａｃｔｏｒｓ、第１１巻、２３５～２５７ページ、１９９４年）。ｐ６８ＨＥＲ－２もＥＣＤ ＩＩＩａ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（Ｄｏｕｇａｌｌ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 ＩＩＩａ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ギオポエチン－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（Ｍａｉｓｏｎｐｉｅｒｒｅ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（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。

薬理学的組成物

【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学的組成物であって、（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、薬理学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学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学的組成物は、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学的組成物として、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）に適した薬理学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学的製剤にする。非経口投与する薬理学的組成物は、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】経口投与する薬理学的組成物は、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。
ｐ６８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う、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う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。
ｐ６８、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10^8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、子宮頚ガン、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。

【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 ＩＩＩａ領域はｐ１８５ＨＥＲ－２との特異的結合に関与し、その結果としてｐ６８ＥＣＤＩ ＩＩＩａ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。

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。

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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａペプチドまたはｐ６８ＨＥＲ－２のｐＭ量を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

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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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変異体が存在しているかどうかや、特定の変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。

【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 ＩＩＩａ変異体ポリペプチド、ＥＣＤ ＩＩＩａ変異体ポリペプチドの断片、ＥＣＤ ＩＩＩａ変異体の機能的等価物、ＥＣＤ ＩＩＩａ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。

【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。

【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（Ｍｏｒｒｉｓｏｎ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

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 ＩＩＩａ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断片、Ｆ（ａｂ’）．ｓｕｂ．２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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ変異体に対する抗体で、ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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をシークエンシングした結果、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

＃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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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うことがある。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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ変異体配列を発現しているか

に関する情報が得られる可能性がある。
【００４９】ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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・プライマーの長さとヌクレオチド配列、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。
【００５０】さらに、特定のＥＣＤ ＩＩＩａ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 ＩＩＩａ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。
【００５２】興味の対象であるＥＣＤ ＩＩＩａ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よう。これらの方法により、所定のＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａを含むペプチドが、抗ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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。
【００５３】ＥＣＤ ＩＩＩａ変異体ポリペプチドのイムノアッセイには、ＥＣＤ ＩＩＩａ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。

固相支持体は、抗ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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（“ＥＬＩＳＡ”）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、Δ－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、α－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、β－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。

【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（ＲＩＡ）を利用してＥＣＤ ＩＩＩａ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、γ線カウンター、シンチレーション・カウンターなどの手段により、あるいは放射能写真撮影により検出することができる。

【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、１５２Ｅｕ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（ＤＴＰＡ）、エチレンジアミン四酢酸（ＥＤＴＡ）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。

【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。

【００５９】抗ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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、ＨＥＲ－２を過剰に発現しているガン患者

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 ＩＩＩａ変異体が存在しているかを明らかにし、さまざまなＥＣＤ ＩＩＩａ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。

【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 ＩＩＩａ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 ＩＩＩａ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シークエンシング、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。

【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 ＩＩＩａ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 ＩＩＩａ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。

【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 ＩＩＩａ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 ＩＩＩａ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

【００６７】理論に囚われないとすると、ｐ６８ＨＥＲ－２またはＥＣＤ ＩＩＩａ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

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、オリジーン・テクノロジーズ社（ロックヴィル、メリーランド州）から提供してもらうか、ＳＫＯＶ－３細胞から抽出したＲＮＡから調製するかした。トリリージェント（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_{260}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^+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（Ｃｏｕｓｓｅｎｓ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（バイオーラド・モデル７８５ヴァキューム・ブロッター）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、（$\alpha-^{32}P$）ｄＣＴＰ（ネン・ライフ・サイエンシーズ社）で標識したＥＣＤ ＩＩＩａ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、２．５ｍＭのＭｇＣｌ$_2$、５μｇの各プライマー、２００μＭ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・テクノロジーズ社）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、Ｃｏｕｓｓｅｎｓ他が報告しているＨＥＲ－２のｃＤＮＡ配列（Ｃｏｕｓｓｅｎｓ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’－ＴＧＡＧＣＡＣＣＡＴＧＧＡＧＣＴＧＧＣ－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（オリジーン・テクノロジーズ社）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 ＩＩＩａと表記する）新奇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’－ＡＡＣＡＣＡＧＣＧＧＴＧＴＧＡＧＡＡＧＴＧＣ－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 ＩＩＩａ配列を含むｍＲＮＡの構造を調べた。まず最初に鎖状ｃＤＮＡを、０．５μｇのオリゴ－ｄＴで処理した５μｇ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 ＩＩＩａ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 ＩＩＩａに対して特異的な配列に相補的な逆プライマー（Ｄ）（５’－ＡＴＡＣＣＧＧＧＡＣＡＧＧＴＣＡＡＣＡＧＣ－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 ＩＩＩａ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’－ＴＴＣＡＣＡＣＴＧＧＣＡＣＧＴＣＣＡＧＡＣＣ－３’［配列ＩＤ番号８］）を用いて行なった。順プライマーは、５’ＥＣＤ ＩＩＩａ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産物をサブクロー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（Ｃｏｕｓｓｅｎｓ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 ＩＩＩａ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 ＩＩＩａの特性を明らかにする実験の結果を示す。ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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Ｍａｉｈｌｅ、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 ＩＩＩａ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 ＩＩＩａ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と一致していて開始コドンを含む順プライマーと、３’ＥＣＤ ＩＩＩａ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 ＩＩＩａ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

ＥＲ－２コード配列を増幅した。２．９ｋｂ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クエンシングによって５’（１．３ｋｂ）と３’（２．９ｋｂ）の増幅産物の両方のキャラクテ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１０ｋｂの産物、３’コード配列に対しては約５ｋｂ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４．５ｋｂ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、２７４ｂｐ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４．８ｋｂとなることを示している。

【実施例４】

【００８１】この実施例では、ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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２８０ｂｐ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 ＩＩＩａ発現ベクターは、細菌株ＢＬ２１の形質転換に使用した。

【００８２】ＥＣＤ ＩＩＩａタンパク質産物を発現させるため、ｐＥＴ－ＥＣＤ ＩＩＩａ発現ベクターで形質転換したＢＬ２１細胞をＬＢ培養液の中で３０μｇ／ｍｌのカナマイシンとともに３７℃にて４時間にわたって成長させた。０．１ｍＭ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分間にわたって撹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（１０ｍＭのトリスｐＨ７．９と３００ｍＭのＮａＣｌ）で洗浄し、次に、５０ｍＭ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 ＩＩＩａタンパク質を、２５０ｍＭ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。

【００８３】要するに、コカリコ・バイオロジカルズ社（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 ＩＩＩａペプチド（以下に記載）を注射することにより抗ＥＣＤ ＩＩＩａ抗血清を生産してもらった。ｐ１８５ＨＥＲ－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）。ｐ１８５ＨＥＲ－２のカルボキシ端末の最後の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テリゼーションしたところ、精製ＥＣＤ ＩＩＩａ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。

【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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａ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℃にて撹拌しながら２時間にわたって行なった。免疫複合体は、撹拌しながら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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａ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　ＩＩＩａの発現を調べた。ｃＤＮＡにＥＣＤ　ＩＩＩａ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

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 ＩＩＩａ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 ＩＩＩａ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。

【実施例５】

【００８９】この実施例では、ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａの新奇な転写産物のサイズと発現を調べるため、ＥＣＤ ＩＩＩａ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 ＩＩＩａ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 ＩＩＩａ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’－ＧＣＡＣＧＧＡＴＣＣＡＴＡＧＣＡＧＡＣＴＧＡＧＧＡＧＧ－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・インスティチュート・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 ＩＩＩａ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、（α－^{32}Ｐ）ＣＴＰ、Ｔ７ＲＮＡポリメラーゼと、Ｔ７／ＳＰ６リボ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 ＩＩＩａ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。

【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ＲＮアーゼＡ（ベーリンガー・マンハイム社）および２μｇ／ｍｌのＲＮ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のブロモフェノール・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

て定量した（ＩＰラブ・ゲル、モレキュラー・ダイナミックス社）。
【００９１】約４．８ｋｂの新奇な転写産物が、ｐ６８ＥＣＤ ＩＩＩａを最高レベルで発現したＨＥＫ２９３細胞の中で検出された。しかしＳＫＢＲ－３、ＢＴ４７４、ＳＫＯＶ－３というガン細胞系のノーザンブロット解析では、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（ＲＰＡ）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４．５ｋｂの転写産物と比較して調べた。検出可能なレベルのｐ６８ＥＣＤ ＩＩＩａを含んでいた卵巣ガン細胞系（ＳＫＯＶ－３）および乳ガン細胞系（ＳＫＢＲ－３とＢＴ４７４）からのＲＮＡと、ＨＥＲ－２のｃＤＮＡが安定にトランスフェクションされた対照細胞系１７－３－１からのＲＮＡを、ＥＣＤ ＩＩＩａ（イントロン８）配列全体と、イントロン８に隣接した５’ＨＥＲ－２エキソン配列とにまたがる^{32}Ｐ標識アンチセンスＲＮＡプローブとハイブリダイズさせた。ＲＮアーゼで消化させ、電気泳動を行ない、放射能写真を撮影したところ、３７０個のヌクレオチドからなるバンドが、１７－３－１以外の細胞系で検出された。これは、ＥＣＤ ＩＩＩａを含むＨＥＲ－２のｍＲＮＡ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、８７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１００倍以上も過剰に発現している完全にスプライスされたＨＥＲ－２の情報に対して予想されるサイズである（Ｋｒａｕｓ他、ＥＭＢＯ Ｊ．、第６巻、６０５～６１０ページ、１９８７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、ｐ１８５ＨＥＲ－２のｍＲＮＡに対する割合として表わした。ＥＣＤ ＩＩＩａ挿入体を含む新奇なＨＥＲ－２のｍＲＮＡは、ＳＫＯＶ－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、ＳＫＢＲ－３細胞では５．４％であり、ＢＴ４７４細胞では０．８％であった。
【実施例６】
【００９２】この実施例では、ＥＣＤ ＩＩＩａ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。ノーザンブロット解析により、ＥＣＤ ＩＩＩａ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$^{+}$ｍＲＮＡをノーザンブロットとして調製し、新奇なＥＣＤ ＩＩＩａ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。４．８ｋｂのｍＲＮＡが腎臓で検出され、２．６ｋｂの転写産物が肝臓で検出された（図２）。４．８ｋｂの転写産物は、２７４ｂｐの挿入体を有する完全長４．５ｋｂ転写産物に対応しているようである。２．６ｋｂの転写産物は、以前に報告されている２．３ｋｂの別の転写産物（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２７４ｂｐのＥＣＤ ＩＩＩａ挿入体が含まれたものに対応しているのであろう。ブロットを分離させて５’ＨＥＲ－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、４．８ｋｂと４．５ｋｂのｍＲＮＡを示す広いバンドが胎児の腎臓組織で検出され、２．６ｋｂ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、ＨＥＲ－２のＥＣＤ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ＥＣＤ ＩＩＩａ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ｍＲＮＡのそれぞれから産生される可能性がある。
【００９３】いくつかの細胞系についてもＥＣＤ ＩＩＩａを含む新奇な転写産物についてノーザンブロット解析で調べた。４．８ｋｂの新奇な転写産物が、ヒト胚の腎臓細胞系であるＨＥＫ－２９３で検出された（図２）。ＥＣＤ ＩＩＩａ配列が、ＨＥＲ－２遺伝子が増幅されているＳＫＢＲ－３、ＢＴ４７４、ＳＣＯＶ－３というガン細胞系のＲＴ－ＰＣＲ解析により検出されたとはいえ、ＥＣＤ ＩＩＩａを含む新奇な転写産物はこれらの細胞をノーザンブロット解析しても検出できなかった。そこで、ＥＣＤ ＩＩＩａ配列全体と、このＥＣＤ ＩＩＩａ挿入体に隣接する５’ＨＥＲ－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（ＲＰＡ）を行なった。ＥＣＤ ＩＩＩａ挿入体を有する新奇なＨＥＲ－２のｍＲＮＡは、ＳＣ

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 ＩＩＩａ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、４．８ｋｂ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４．５ｋｂ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 ＩＩＩａ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 ＩＩＩａ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。４．８ｋｂの新奇なＥＣＤ ＩＩＩａの転写産物を発現したＨＥＫ－２９３細胞がＥＣＤ ＩＩＩａ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６８ｋＤａのタンパク質は、抗ＥＣＤ ＩＩＩａ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 ＩＩＩａ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１２５ｋＤａのタンパク質は、ｐ６８ＨＥＲ－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、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ｐ６８ＥＣＤ ＩＩＩａを発現するかどうかを調べた。ｃＤＮＡにＥＣＤ ＩＩＩａ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ｐ６８ＨＥＲ－２を含んでいた。

【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ｐ１８５ＨＥＲ－２と比べてｐ６８ＨＥＲ－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ｐ１８５ＨＥＲ－２のｍＲＮＡと比べてｐ６８ＨＥＲ－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、ｐ６８ＨＥＲ－２タンパク質とｐ１８５ＨＥＲ－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、ｐ６８ＨＥＲ－２に対して特異的な抗血清とｐ１８５ＨＥＲ－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遺伝子が約８倍に増幅されているガン細胞系でｐ１８５ＨＥＲ－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ｐ６８ＨＥＲ－２が対応して増加することはなかった。比較すると、ｐ６８ＨＥＲ－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ｐ１８５ＨＥＲ－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ｐ６８ＨＥＲ－２がｐ１８５ＨＥＲ－２と比べて少ないことを示しており、ｐ１８５ＨＥＲ－２が過剰に発現したときにｐ６８ＨＥＲ－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。

【実施例９】

【００９６】この実施例では、ｐ６８ＨＥＲ－２とＥＣＤ ＩＩＩａペプチドがｐ１８５ＨＥＲ－２に特異的に結合することを示す。ｐ６８ＨＥＲ－２は分泌タンパク質であり、しかもｐ６８ＨＥＲ－２は新奇な配列に加えてｐ１８５ＨＥＲ－２と一致するサブドメインＩとＩＩを含んでいるため、ｐ６８ＨＥＲ－２がｐ１８５ＨＥＲ－２と相互作用する可能性を調べた。ｐ１８５ＨＥＲ－２とｐ６８ＨＥＲ－２のＮ末端に対する抗ペプチド抗体

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ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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、ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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、ＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ領域がｐ１８５ＨＥＲ－２に結合したため、このＥＣＤ ＩＩＩａ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－ＥＣＤ ＩＩＩａ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、ＥＣＤ ＩＩＩａ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、ＥＣＤ ＩＩＩａペプチドで処理した細胞を等分した複数のアリコートを、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。ＥＣＤ ＩＩＩａ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、ＥＣＤ ＩＩＩａ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】ｐ６８ＥＣＤ ＩＩＩａとＥＣＤ ＩＩＩａ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ＥＣＤ ＩＩＩａ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－ＥＣＤ ＩＩ

ＩａまたはCM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、ｐ１８５ＨＥＲ－２が活性化されなかったことを示唆している。ｐ６８ＨＥＲ－２を含むＣＭもＥＣＤ ＩＩＩａペプチドも、ｐ１８５ＨＥＲ－２のチロシンリン酸化レベルが低いＳＫＯＶ－３細胞からのｐ１８５ＨＥＲ－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、ｐ６８ＨＥＲ－２とＥＣＤ ＩＩＩａペプチドは、１７－３－１細胞抽出物から免疫沈降させたｐ１８５ＨＥＲ－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、ｐ６８ＨＥＲ－２がｐ１８５ＨＥＲ－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１１】

【０１００】この実施例では、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なって）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。

【０１０１】ヒトＨＥＲ－２遺伝子のイントロン８は、場合によってはｍＲＮＡ内に交互に保持されて、ｐ１８５ＨＥＲ－２の細胞外領域の一部のＣ末端の位置で新奇な７９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、ＨＥＲ－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、ｎＭのアフィニティでｐ１８５ＨＥＲ－２に結合することがわかっている（Ｄｏｈｅｒｔｙ他、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ、第９６巻、１０，８６９～１０，８７４ページ、１９９９年）。

【０１０２】ＨＥＲ－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１５人のゲノムＤＮＡをシークエンシングすることによって同定した。図８と配列ＩＤ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、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、ヌクレオチド位置１６１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＃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。

【０１０３】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、ＥＣＤ ＩＩＩａ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

	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

【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。

【実施例１２】

【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なって）イン・フレーム・コード配列として機能する部分において、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の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）。

【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なって）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）をシークエ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。

【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

【０１１０】表ＩＩ。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ＩＩＩは、配列データから（やはり図８または配列ＩＤ番号１０に

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なって）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

	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

【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。

【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。

【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

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なって）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なって）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学的組成物と、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 ＩＩＩａ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、Ｃｏｕｓｓｅｎｓ他のナンバリングを用いると（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^{340}）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 ＩＩＩａ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^{+}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（アンビオン社）に移した。この膜を、ＥＣＤ ＩＩＩ配列と相補的な^{32}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^{32}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 ＩＩＩａが、ヒト胚性腎臓細胞系（ＨＥＫ２９３）の約６８ｋＤａの

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（２０μｇ）と、ＨＥＫ２９３細胞によって調整した２０μｌ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 ＩＩＩａ（レーン１と２）、またはヒスチジン・タグを有する５０μｇ／ｍｌの精製ＥＣＤ ＩＩＩａペプチドを含む、１：１０，０００に希釈した抗ＥＣＤ ＩＩａ（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ｐ６８ＥＣＤ ＩＩＩａの発現と比べてｐ１８５ＨＥＲ－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１５μｇ）、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ｐ６８ＨＥＲ－２に対して特異的な抗体（抗ＥＣＤ ＩＩＩａ）とｐ１８５ＨＥＲ－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】ｐ６８ＥＣＤ ＩＩＩａがｐ１８５ＨＥＲ－２に結合している様子を示す。Ａの項では、非変性性緩衝溶液中に抽出した２ｍｇのＳＫＢＲ－３細胞を、ｐ６８ＨＥＲ－２とｐ１８５ＨＥＲ－２のＮ末端配列に対して特異的な５μｌのアンチｎｅｕ（Ｎ）とともに、またはｐ１８５ＨＥＲ－２のＣ末端に対して特異的な５μｌのアンチｎｅｕ（Ｃ）とともに免疫沈降させ、ｐ６８ＨＥＲ－２に対して特異的な抗ＥＣＤ ＩＩＩａとｐ１８５ＨＥＲ－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、１００μｇの１７－３－１細胞抽出物を、ヒスチジン・タグを有する２０μｇのＥＣＤ ＩＩＩａまたはヒスチジン・タグを有する２０μｇのＣＲＥＢ断片と結合させた充填容積が５０μｌのＮｉＮＴＡアガロース樹脂（キアジェン社）とともに２００μｌ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時間撹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５００μｌの洗浄用緩衝溶液で４回洗浄し、５０μｌ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、ｐ１８５ＨＥＲ－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 ＩＩＩａ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２０μｇ）を、ＥＣＤ ＩＩＩａに対して特異的な抗体（抗ＥＣＤ ＩＩＩａ）、またはｐ１８５ＨＥＲ－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 ＩＩＩａペプチドもｐ１８５ＨＥＲ－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１５０μｌのＥＣＤ ＩＩＩａ、または高レベルのｐ６８を分泌するＨＥＫ－２９３細胞からの５０×ＣＭ、またはｐ６８ＨＥＲ－２が検出できないＳＫＯＶ－３細胞からの５０×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２０μｇ／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

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ブ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５０×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５０×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5×10^{3}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。

【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クエンシングした。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。

【図１】

図１

【図２】

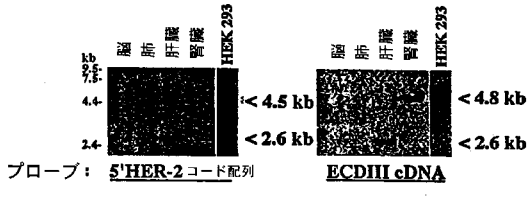

図２

【図３】

図３

【図４】

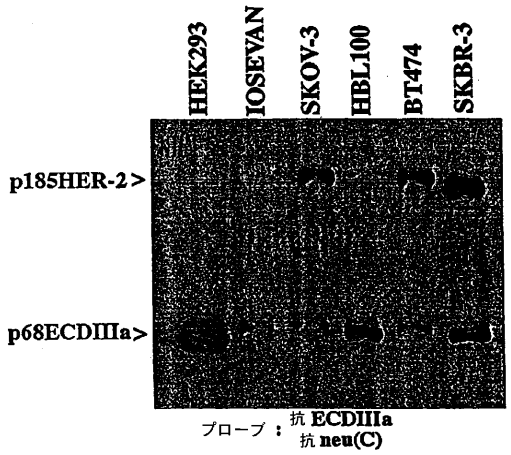

図4

【図５】

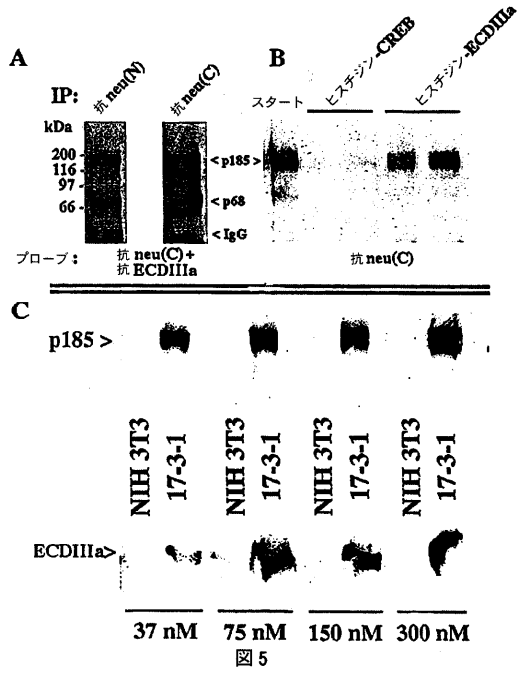

図5

【図６】

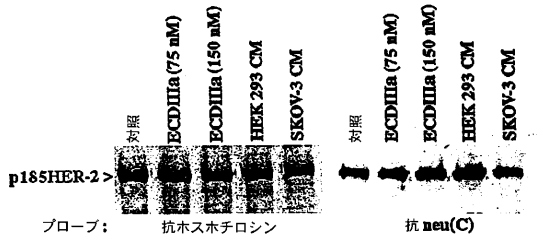

図6

【図８】

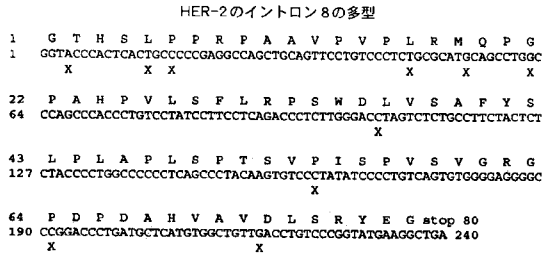

図8

【図７】

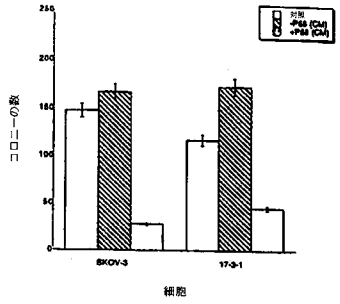

図7

【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[7]: 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

(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).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CLINTON, Gail** [US/US]; 3840 S.W. Beaverton Avenue, Portland, OR 97201 (US). **HENNER, William, D.** [US/US]; 1050 Coralan Avenue, Apt. 210, Burlingame, CA 94010 (US). **EVANS, Adam** [US/US]; 7414 S.E. 18th Avenue, Portland, OR 97202 (US).

(74) Agent: **DAVISON, Barry, L.**; Davis Wright Tremaine, LLP, 2600 Century Square, 1501 Fourth Avenue, Seattle, WA 98101-1688 (US).

(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, 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 (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, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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.

WO 01/61356 A1

(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 10^8, (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.

HER-2 BINDING ANTAGONISTS

Technical Field of the Invention

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.

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

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., *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 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 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,

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, 1966; 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®

(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

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 or 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,

immunohistocytochemistry, 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 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, immunohistocytochemistry, 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.

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

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^{340}). 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 ^{32}P-labeled antisense RNA probe complimentary to the ECDIII sequence, stripped and reprobed with a ^{32}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)).

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 5µl 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 ~10^5 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 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 ~10^5 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 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 detectible 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 cancinoma 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

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. 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 an 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. 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

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).

Without being bound by theory, since p68HER-2 occupies but does not activate, it could block dimerization of p185HR-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 though 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

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), gelaten, gums, or polyvinylpyrrolidone. In addition, a desintegrating 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.

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 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. 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. 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 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 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 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 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 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.

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.

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 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.

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.

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,

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.

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, immunohistocytochemistry, 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

#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

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.

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 eptitopes 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

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.

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.

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, fluorimetirc 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.

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

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.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}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).

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, theromatic 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.

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.

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 proivides 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, immunohistocytochemistry, 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. 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, immunohistocytochemistry, 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

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_{260}. Poly A^+ 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

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 10^7 cpm of a 220 bp Kpn-HincII fragment from ECDIIIa cDNA labelled with (α-^{32}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 (Boerhinger Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM $MgCl_2$, 5 µM of each primer, and 200 µM 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 µg RNA primed with 0.5 µg 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])

which is identical to the 5'ECDIIIa-specific sequence and contains a *Kpn*1 restriction site and a reverse primer (F) (5'-TTCACACTGGCACGTCCAGACC-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

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 Maihle, *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

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 1and 2). The PCR amplification product of ~280 bp was digested with *Kpn*1 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

immune complexes were examined by Western blot analysis with both antibodies. Briefly, three to 5 µl 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. 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 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 °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 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. 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 to10-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 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

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

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 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 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 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 (α-^{32}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 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 (Boerhinger 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. 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 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

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 ^{32}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$^+$ 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

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,

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

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

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) toXaa(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

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.

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, respectivley) 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):

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

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, respectivley 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

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) toXaa(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

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.

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.

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 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.

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 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 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 are present.

10. The isolated and glycosylated polypeptide of claim 8, wherein the isolated 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-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.

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.

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 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. 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 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-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 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 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

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, immunohistocytochemistry, 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.

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, immunohistocytochemistry, 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,

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:

(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, chromohphores, chemiluminescent tags, and fluorescers.

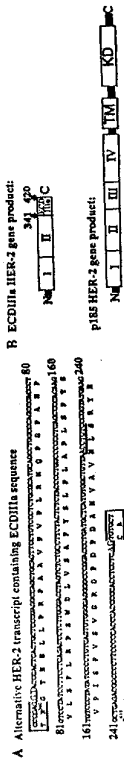
A Alternative HER-2 transcript containing ECDIIIa sequence
B ECDIIIa HER-2 gene product:
p185 HER-2 gene product:
TM
KD

Figure 1

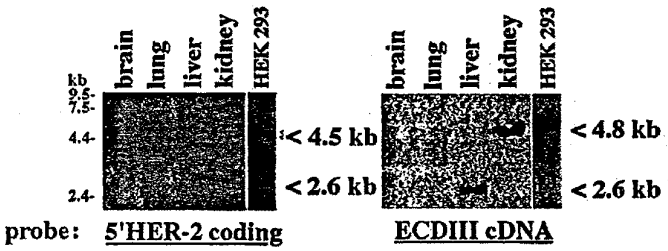
brain
lung
liver
kidney
HEK 293
kb
7.5-
4.4-
2.4-
< 4.5 kb
< 2.6 kb
brain
lung
liver
kidney
HEK 293
< 4.8 kb
< 2.6 kb
probe: 5'HER-2 coding
ECDIII cDNA

Figure 2

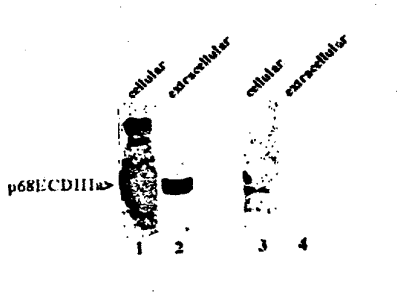
cellular
extracellular
cellular
extracellular
p68ECDIII
1
2
3
4

Figure 3

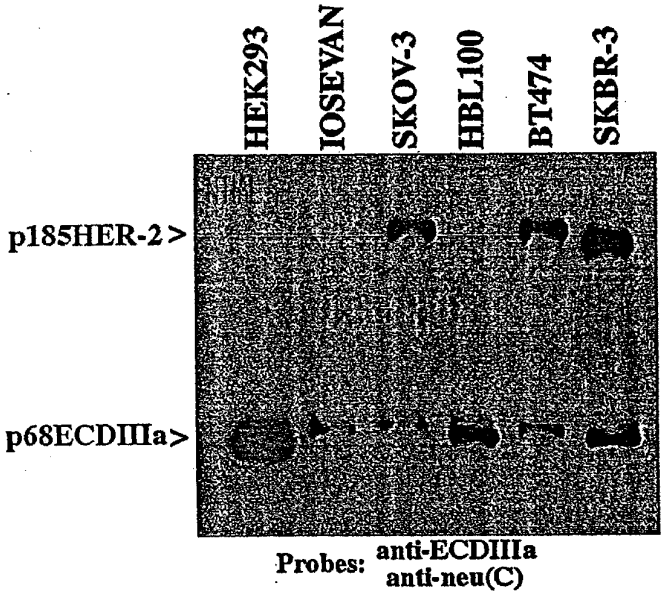
HEK293
IOSEVAN
SKOV-3
HBL100
BT474
SKBR-3
p185HER-2>
p68ECDIIIa>
Probes: anti-ECDIIIa
anti-neu(C)

Figure 4

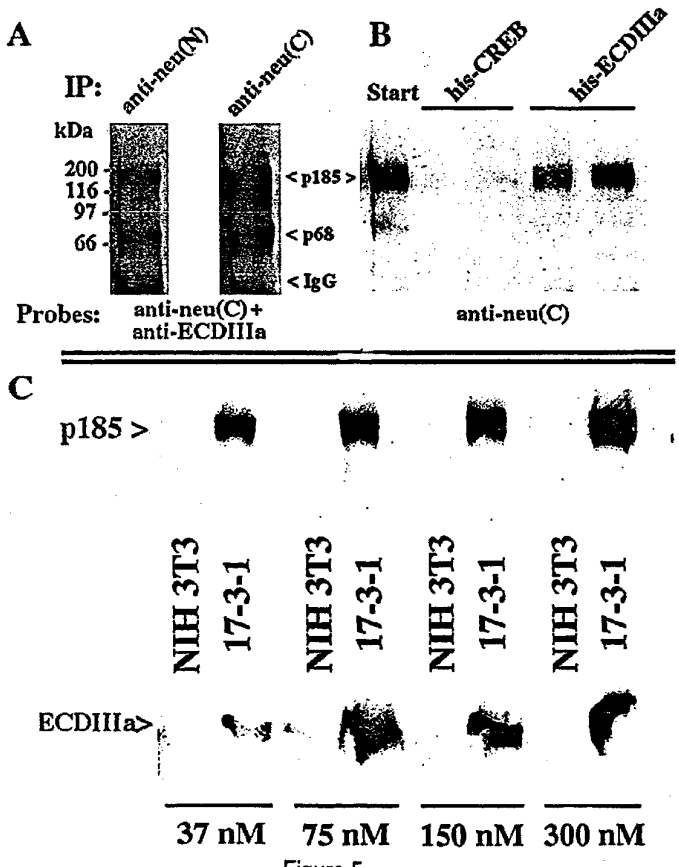
A
IP:
anti-neu(N)
anti-neu(C)
kDa
200
116
97
66
< p185 >
< p68
< IgG
Probes:
anti-neu(C) + anti-ECDIIIa
B
Start
his-CREB
his-ECDIIIa
anti-neu(C)
C
p185 >
NIH 3T3
17-3-1
NIH 3T3
17-3-1
NIH 3T3
17-3-1
NIH 3T3
17-3-1
ECDIIIa>
37 nM
75 nM
150 nM
300 nM

Figure 5

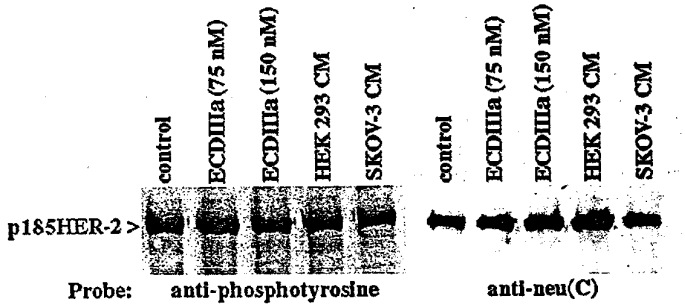
control
ECDIIIa (75 nM)
ECDIIIa (150 nM)
HEK 293 CM
SKOV-3 CM
control
ECDIIIa (75 nM)
ECDIIIa (150 nM)
HEK 293 CM
SKOV-3 CM
p185HER-2 >
Probe:
anti-phosphotyrosine
anti-neu(C)

Figure 6

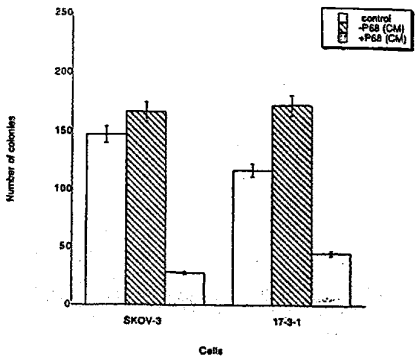
250
200
150
100
50
0
Number of colonies
control
+P68 (CM)
SKOV-3
17-3-1
Cells

Figure 7

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   GGTACCCACTCACTGCCCCCGAGGCCAGCTGCAGTTCCTGTCCCTCTGCGCATGCAGCCTGGC
      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  CCAGCCCACCCTGTCCTATCCTTCCTCAGACCCTCTTGGGACCTAGTCTCTGCCTTCTACTCT
                                          X

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

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

Figure 8

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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

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

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIS WRIGHT TREMAINE
(B) STREET: 1501 Fourth Avenue, 2600 Century Square
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: U.S.A.
(F) ZIP: 98101

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

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

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Davison, Barry L.
(B) REGISTRATION NUMBER: 47,309
(C) REFERENCE/DOCKET NUMBER: 49321-19

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206 628 7621
(B) TELEFAX: 206 628 7699

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(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
```

```
        35                  40                  45
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
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: polypeptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
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
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
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
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
145                 150                 155                 160
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175
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
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
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
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
Pro Val Pro Xaa Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser
355 360 365
Phe Leu Arg Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro
370 375 380
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
Tyr Glu Gly

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: oligonucleotide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAGCACCAT GGAGCTGGC 19

(2) INFORMATION FOR SEQ ID NO:4:

(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:4:

TCCGGCAGAA ATGCCAGGCT CC 22

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

AACACAGCGG TGTGAGAAGT GC 22

(2) INFORMATION FOR SEQ ID NO:6:

(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:

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:

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:

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:

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:

```
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

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
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
    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

CGG TAT GAA GGC TGA                                         240
Arg Tyr Glu Gly
```

(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

(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
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
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

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

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
```

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

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(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
1 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
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

(2) INFORMATION FOR SEQ ID NO:13:

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

Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
1 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
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
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
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

```
145                 150                 155                 160
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175
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
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
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
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
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
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
Tyr Glu Gly
```

【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[7]: 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

(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).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CLINTON, Gail** [US/US]; 3840 S.W. Beaverton Avenue, Portland, OR 97201 (US). **HENNER, William, D.** [US/US]; 1050 Coralan Avenue, Apt. 210, Burlingame, CA 94010 (US). **EVANS, Adam** [US/US]; 7414 S.E. 18th Avenue, Portland, OR 97202 (US).

(74) Agent: **DAVISON, Barry, L.**; Davis Wright Tremaine, LLP, 2600 Century Square, 1501 Fourth Avenue, Seattle, WA 98101-1688 (US).

(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, 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 (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, 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.

WO 01/061356 A1

(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 10^8, (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.

HER-2 BINDING ANTAGONISTS

Technical Field of the Invention

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.

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

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., *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 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 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,

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, 1966; 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®

(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

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 or 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,

immunohistocytochemistry, 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 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, immunohistocytochemistry, 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.

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

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^{340}). 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 ^{32}P-labeled antisense RNA probe complimentary to the ECDIII sequence, stripped and reprobed with a ^{32}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)).

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 5μl 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 ~10^5 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 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 ~10^5 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 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 detectible 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 cancinoma 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

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. 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 an 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. 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

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).

Without being bound by theory, since p68HER-2 occupies but does not activate, it could block dimerization of p185HR-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 though 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

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), gelaten, gums, or polyvinylpyrrolidone. In addition, a desintegrating 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.

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 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. 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. 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 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 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 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 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 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.

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.

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 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.

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.

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,

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.

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, immunohistocytochemistry, 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

#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

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.

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 eptitopes 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

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.

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.

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, fluorimetirc 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.

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

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.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}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).

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, theromatic 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.

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.

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 proivides 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, immunohistocytochemistry, 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. 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, immunohistocytochemistry, 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

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_{260}. Poly A^+ 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

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 10^7 cpm of a 220 bp Kpn-HincII fragment from ECDIIIa cDNA labelled with (α-^{32}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 (Boerhinger Mannheim) with 1X High Fidelity PCR buffer containing 2.5 mM $MgCl_2$, 5 μM of each primer, and 200 μM 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 μg RNA primed with 0.5 μg 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])

which is identical to the 5'ECDIIIa-specific sequence and contains a *Kpn*1 restriction site and a reverse primer (F) (5'-TTCACACTGGCACGTCCAGACC-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

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 Maihle, *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

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 1and 2). The PCR amplification product of ~280 bp was digested with *Kpn*1 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

immune complexes were examined by Western blot analysis with both antibodies. Briefly, three to 5 μl 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. 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 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 °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 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. 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 to10-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 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

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

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 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 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 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 (α-^{32}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 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 (Boerhinger 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. 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 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

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 ^{32}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$^+$ 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

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,

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

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

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) toXaa(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

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.

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, respectivley) 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):

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

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, respectivley 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

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) toXaa(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

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.

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.

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 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.

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 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 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 are present.

10. The isolated and glycosylated polypeptide of claim 8, wherein the isolated 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-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.

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.

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 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. 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 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-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 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 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

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, immunohistocytochemistry, 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.

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, immunohistocytochemistry, 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,

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:

(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, chromohphores, chemiluminescent tags, and fluorescers.

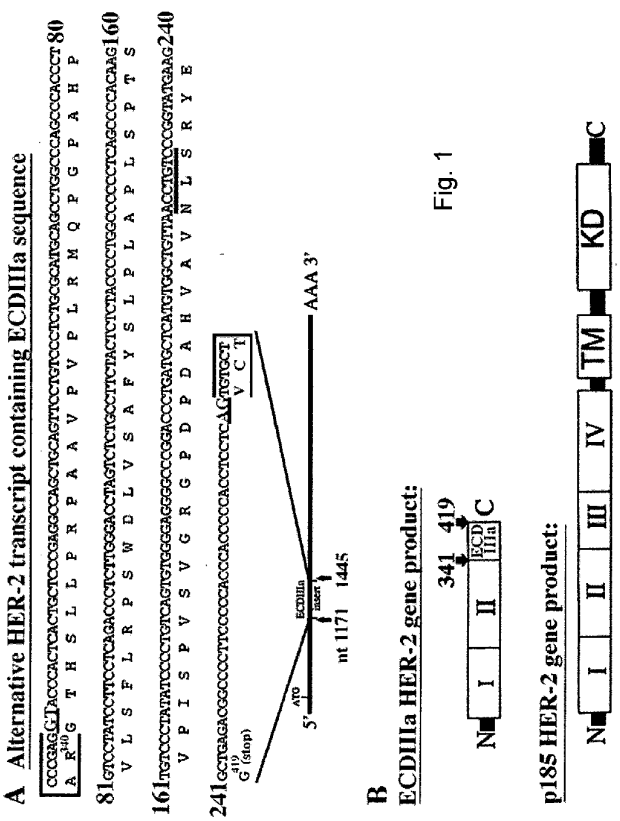
A Alternative HER-2 transcript containing ECDIIIa sequence
B
ECDIIIa HER-2 gene product:
341 419
N I II ECD IIIa C
p185 HER-2 gene product:
N I II III IV TM KD C

Fig. 1

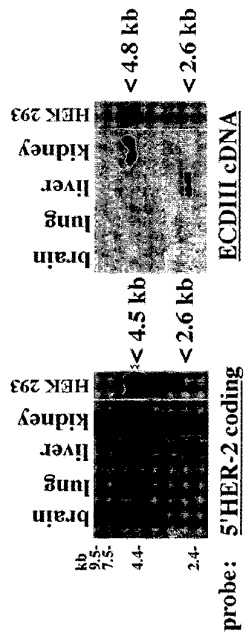
probe: 5'HER-2 coding
kb
9.5-
7.5-
4.4-
2.4-
brain
lung
liver
kidney
HEK 293
< 4.5 kb
< 2.6 kb
ECDIII cDNA
brain
lung
liver
kidney
HEK 293
< 4.8 kb
< 2.6 kb

Fig. 2

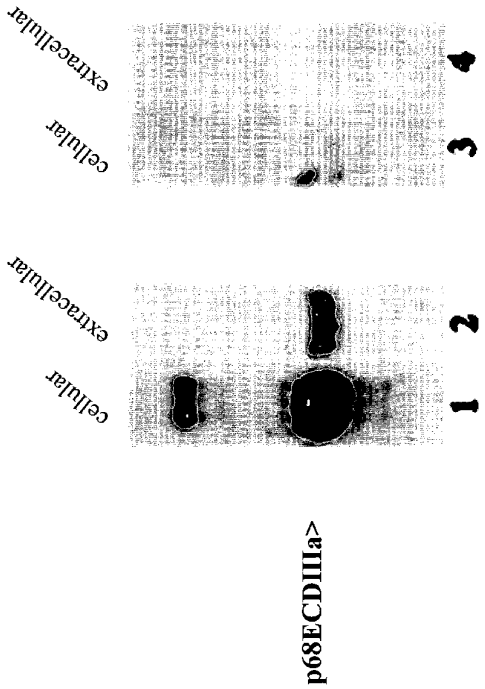
extracellular
cellular
extracellular
cellular
1
2
3
4
p68ECDIIIa>

Fig. 3

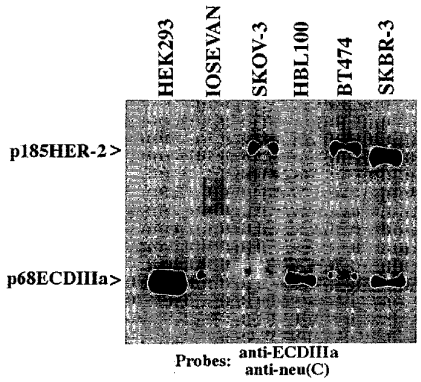
HEK293
IOSEVAN
SKOV-3
HBL100
BT474
SKBR-3
p185HER-2>
p68ECDIIIa>
Probes: anti-ECDIIIa
anti-neu(C)

Fig. 4

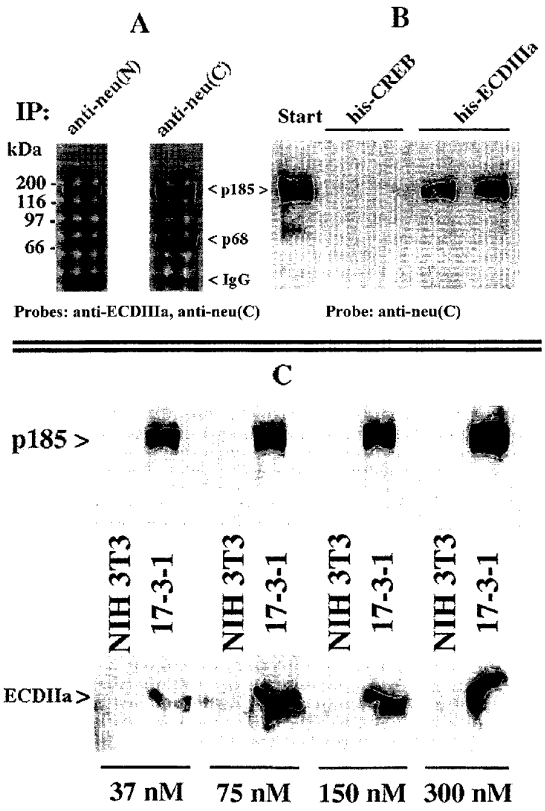
A
B
IP:
anti-neu(N)
anti-neu(C)
Start
his-CREB
his-ECDIIIa
kDa
200
116
97
66
< p185 >
< p68
< IgG
Probes: anti-ECDIIIa, anti-neu(C)
Probe: anti-neu(C)
C
p185 >
NIH 3T3
17-3-1
NIH 3T3
17-3-1
NIH 3T3
17-3-1
NIH 3T3
17-3-1
ECDIIa >
37 nM
75 nM
150 nM
300 nM

Fig. 5

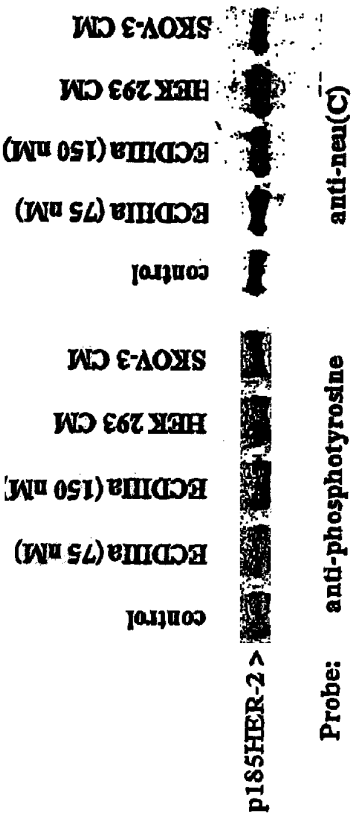
p185HER-2 >
Probe:
anti-phosphotyrosine
control
EGDIIIa (75 nM)
EGDIIIa (150 nM)
HEK 293 CM
SKOV-3 CM
anti-neu(C)
control
EGDIIIa (75 nM)
EGDIIIa (150 nM)
HEK 293 CM
SKOV-3 CM

Fig. 6

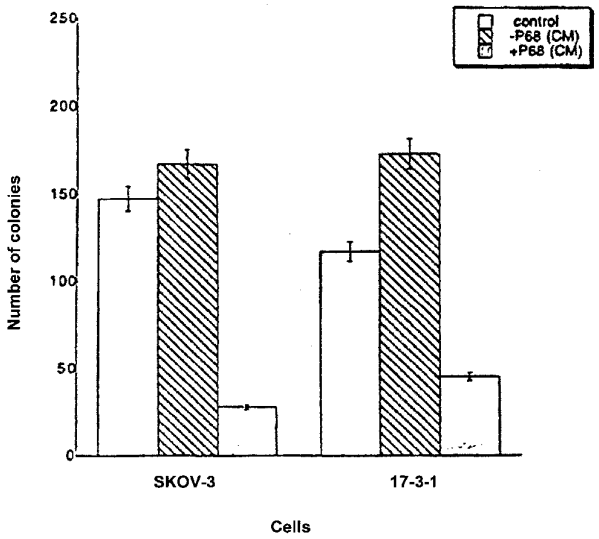
250
200
150
100
50
0
Number of colonies
control
-P68 (CM)
+P68 (CM)
SKOV-3
17-3-1
Cells

Fig. 7

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   GGTACCCACTCACTGCCCCCGAGGCCAGCTGCAGTTCCTGTCCCTCTGCGCATGCAGCCTGGC
      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  CCAGCCCACCCTGTCCTATCCTTCCTCAGACCCTCTTGGGACCTAGTCTCTGCCTTCTACTCT
                          X
43  L P L A P L S P T S V P I S P V S V G R G
127 CTACCCCTGGCCCCCCTCAGCCCTACAAGTGTCCCTATATCCCCTGTCAGTGTGGGGAGGGGC
                        X
64  P D P D A H V A V D L S R Y E G stop 80
190 CCGGACCCTGATGCTCATGTGGCTGTTGACCTGTCCCGGTATGAAGGCTGA 240
      X
                  X
```

Fig. 8

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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

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

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIS WRIGHT TREMAINE
(B) STREET: 1501 Fourth Avenue, 2600 Century Square
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: U.S.A.
(F) ZIP: 98101

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

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

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Davison, Barry L.
(B) REGISTRATION NUMBER: 47,309
(C) REFERENCE/DOCKET NUMBER: 49321-19

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206 628 7621
(B) TELEFAX: 206 628 7699

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(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
```

```
        35                  40                  45
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
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: polypeptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
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
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
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
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
145                 150                 155                 160
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175
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
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
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
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
Pro Val Pro Xaa Arg Xaa Gln Pro Xaa Pro Ala His Pro Val Leu Ser
355 360 365
Phe Leu Arg Pro Ser Trp Asp Xaa Val Ser Ala Phe Tyr Ser Leu Pro
370 375 380
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
Tyr Glu Gly

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: oligonucleotide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAGCACCAT GGAGCTGGC 19

(2) INFORMATION FOR SEQ ID NO:4:

(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:4:

TCCGGCAGAA ATGCCAGGCT CC 22

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

AACACAGCGG TGTGAGAAGT GC 22

(2) INFORMATION FOR SEQ ID NO:6:

(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:

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:

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:

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:

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:

```
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

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
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
    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

CGG TAT GAA GGC TGA                                         240
Arg Tyr Glu Gly
```

(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

(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
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
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

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

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
```

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

(2) INFORMATION FOR SEQ ID NO:12:

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

(2) INFORMATION FOR SEQ ID NO:13:

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

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
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
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
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

```
145                 150                 155                 160
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175
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
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
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
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
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
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
Tyr Glu Gly
```

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/05327

A. CLASSIFICATION OF SUBJECT MATTER
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. FIELDS SEARCHED
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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

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 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

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ 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 comliance and thus the sequences cannot be searched.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ 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. ☐ 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

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998) *

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/05327

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 catergories 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.[7]	FI		テーマコード（参考）
Ａ６１Ｐ 15/00	Ａ６１Ｐ 35/00		４Ｈ０４５
Ａ６１Ｐ 35/00	Ｃ０７Ｋ 14/47		
Ｃ０７Ｋ 14/47	Ｃ０７Ｋ 16/18		
Ｃ０７Ｋ 16/18	Ｃ１２Ｎ 1/15		
Ｃ１２Ｎ 1/15	Ｃ１２Ｎ 1/19		
Ｃ１２Ｎ 1/19	Ｃ１２Ｎ 1/21		
Ｃ１２Ｎ 1/21	Ｃ１２Ｑ 1/68	Ａ	
Ｃ１２Ｎ 5/10	Ｇ０１Ｎ 33/53	Ｄ	
Ｃ１２Ｑ 1/68	Ｇ０１Ｎ 33/53	Ｍ	
Ｇ０１Ｎ 33/53	Ｇ０１Ｎ 33/566		
Ｇ０１Ｎ 33/566	Ｃ１２Ｎ 5/00	Ａ	
	Ａ６１Ｋ 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

(74)代理人 100096194
弁理士 竹内 英人
(74)代理人 100074228
弁理士 今城 俊夫
(74)代理人 100084009
弁理士 小川 信夫
(74)代理人 100082821
弁理士 村社 厚夫
(74)代理人 100086771
弁理士 西島 孝喜
(74)代理人 100084663
弁理士 箱田 篤
(72)発明者 クリントン ゲイル
アメリカ合衆国 オレゴン州 ９７２０１ ポートランド サウスウェスト ビーヴァートン アヴェニュー ３８４０
(72)発明者 ヘンナー ウィリアム ディー
アメリカ合衆国 カリフォルニア州 ９４０１０ バーリンゲイム コララン アヴェニュー １０５０ アパートメント ２１０
(72)発明者 エヴァンス アダム
アメリカ合衆国 オレゴン州 ９７２０２ ポートランド サウスイースト エイティーンス アヴェニュー ７４１４

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)	<无法获取翻译>		
公开(公告)号	JP2004501605A5	公开(公告)日	2006-06-22
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	C12N15/09 A61K39/395 A61P1/00 A61P11/00 A61P15/00 A61P35/00 C07K14/47 C07K16/18 C12N1/15 C12N1/19 C12N1/21 C12Q1/68 G01N33/53 G01N33/566 C12N5/10 A61K38/00		
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	JP2004501605A		

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的亲和力与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。