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(54) Title: EGFR MUTATIONS

(57) Abstract: The present invention relates to mutations in Epidermal Growth Factor Receptor (EGFR) and methods of detecting such mutations as well as prognostic methods method for identifying a tumors that are susceptible to anticancer therapy such as chemotherapy and/or kinase inhibitor treatment. The methods involve determining the presence of a mutated (EGFR) gene or mutated (EGFR) protein in a tumor sample whereby the presence of a mutated (EGFR) gene or protein indicates the tumor is susceptible to treatment.

FIELD OF THE INVENTION

The present invention relates to cancer diagnostics and therapies and in particular to the detection of mutations that are diagnostic and/or prognostic.

BACKGROUND OF THE INVENTION

Epidermal Growth Factor Receptor (EGFR) is a member of the type 1 tyrosine kinase family of growth factor receptors, which play critical roles in cellular growth, differentiation, and survival. Activation of these receptors typically occurs via specific ligand binding, resulting in hetero- or homodimerization between receptor family members, with subsequent autophosphorylation of the tyrosine kinase domain. This activation triggers a cascade of intracellular signaling pathways involved in both cellular proliferation (the ras/raf/MAP kinase pathway) and survival (the PI3 kinase/Akt pathway). Members of this family, including EGFR and HER2, have been directly implicated in cellular transformation.

A number of human malignancies are associated with aberrant or overexpression of EGFR and/or overexpression of its specific ligands e.g. transforming growth factor α (Gullick, Br Med Bull 1991, 47:87–98; Modijtahedi and Dean, Int J Oncol 1994, 4:277–96; Salomon et al., Crit Rev Oncol Hematol 1995;19:183–232). EGFR overexpression has been associated with an adverse prognosis in a number of human cancers, including NSCLC. In some instances, overexpression of tumor EGFR has been correlated with both chemoresistance and a poor prognosis (Lei et al., Anticancer Res 1999; 19:221–8; Veale et al., Br J Cancer 1993;68:162–5). These observations suggest that agents that effectively inhibit EGFR receptor activation and subsequent downstream signaling may have clinical activity in a variety of human cancers, including NSCLC.

Tarceva™ (also known as erlotinib; OSI-774), a quinazoline, is an orally active, potent, selective inhibitor of EGFR tyrosine kinase. Erlotinib inhibits human EGFR tyrosine kinase with an IC_{50} of 2 nM (0.786 mg/mL) in an in vitro enzyme assay. This inhibition is selective for EGFR tyrosine kinase, results in cell cycle arrest at G_1, and is reversible. Oral administration of erlotinib in mice has

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demonstrated a >70% reduction in EGFR autophosphorylation in human xenografts and marked growth inhibition of HN5 and A431 xenografts in nude mice has been demonstrated. In addition to single-agent activity in *in vivo* assay systems, erlotinib has been evaluated in combination with a number of chemotherapy agents to determine possible interactions. There was an additive interaction between erlotinib and paclitaxel, cisplatin, gemcitabine, and doxorubicin.

Lung cancer represents the leading cause of cancer-related mortality for both men and women in the United States. In 2000, it was estimated that 164,000 new cases would be diagnosed and 157,000 patients would die from this disease (Greenlee et al., CA Cancer J Clin 2001, 51:15–36). Approximately 75% of these patients would have had non–small cell histologies, with the majority presenting with inoperable Stage IIIB or Stage IV disease. For those patients with more limited disease at presentation (Stages I–IIIA), relapse following standard surgical therapy, with or without adjuvant or neoadjuvant chemo- and/or radiotherapy, is common. These findings result in an overall 5-year survival in non–small cell lung cancer (NSCLC) of ~12% and serve to emphasize the unmet medical need in this disease.

The platinum compound cisplatin was the first chemotherapy agent to show clinical benefit in the management of locally advanced or metastatic NSCLC. Randomized clinical trials demonstrated improved response rates, quality of life, and survival compared with the best supportive care (Rapp et al. 1988). However, the magnitude of this improvement was modest–measured in weeks. Subsequently, a number of newer chemotherapy agents have been evaluated as single agents and in combination with the platinum salts in the first-line setting. The conclusion from these studies is that modern "doublet" chemotherapy appears to achieve response rates of 15%–20%, median time to disease progression of 3–4 months, and median survival of 7–8 months. The modest improvements in efficacy with combination therapies over the results obtained with cisplatin have established these therapies as a standard of care for patients with advanced NSCLC and an acceptable performance status (Non–Small Cell Lung

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Cancer Cooperative Group, Br Med J 1995,311:899–909; American Society of Clinical Oncology, J Clin Oncol 1997, 15:2996–3018; Breathnach et al., J Clin Oncol 2001;19:1734–42).

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

SUMMARY OF THE INVENTION

A first aspect provides a method for identifying a colorectal tumor in a human subject that is susceptible to treatment with cetuximab or panitumumab, comprising (i) determining the presence of a wild-type KRAS protein or gene in a sample of said tumor, wherein the presence of a wild-type KRAS protein or gene indicates that the tumor is susceptible to treatment with cetuximab or panitumumab; or (ii) determining the presence of a mutated KRAS protein or gene in a sample of said tumor, wherein the absence of a mutated KRAS protein or gene indicates that the tumor is susceptible to treatment with cetuximab or panitumumab.

A second aspect provides a method for determining whether a colorectal tumor in a human subject is not responsive to therapy with cetuximab or panitumumab comprising: determining the presence of a KRAS gene having a mutation in a sample of said tumor, wherein said KRAS gene mutation encodes a mutation at amino acid residue 12 or 13, and wherein the presence of the KRAS gene mutation indicates that the tumor is not responsive to treatment with cetuximab or panitumumab.

A third aspect provides a method for determining whether a colorectal tumor in a human subject is not responsive to therapy with cetuximab comprising determining the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13, wherein the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13

indicates that the tumor is not responsive to treatment with cetuximab.

A fourth aspect provides a method for determining whether a colorectal tumor in a human subject is not responsive to therapy with panitumumab comprising determining the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13, wherein the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13 indicates that the tumor is not responsive to treatment with panitumumab.

A fifth aspect provides a method for determining whether a colorectal tumor in a human subject is responsive to treatment with cetuximab, comprising determining the presence of a wild-type KRAS gene in a sample of said tumor, wherein the presence of the wild-type KRAS gene indicates that the tumor in a human subject is responsive to treatment with cetuximab.

A sixth aspect provides a method for determining whether a colorectal tumor in a human subject is responsive to treatment with panitumumab, comprising determining the presence of a wild-type KRAS gene in a sample of said tumor, wherein the presence of the wild-type KRAS gene indicates that the tumor in a human subject is responsive to treatment with panitumumab.

Disclosed herein is a method for identifying a tumor in a human subject that is susceptible to treatment comprising determining the presence of a mutated EGFR gene or mutated EGFR protein in a sample of said tumor wherein said mutation is located in exons 18-21 of EGFR whereby the presence of a mutated EGFR gene or mutated EGFR protein indicates the tumor is susceptible to treatment.

Also disclosed is a method of treating a tumor in a mammal comprising identifying the presence of an EGFR mutation in said tumor and treating said mammal with an anticancer agent.

Also disclosed is a method of identifying an EGFR mutation in a sample comprising contacting nucleic acid from said sample with a probe that is capable of specifically hybridizing to nucleic acid encoding a mutated EGFR protein, or fragment thereof incorporating a mutation, and detecting the hybridization.

Also disclosed are nucleic acid probes capable of specifically hybridizing to nucleic acid encoding a mutated EGFR protein or fragment thereof incorporating a mutation.

Also disclosed is a method of detecting a mutated EGFR gene in a sample comprising amplifying from said sample nucleic acid corresponding to the kinase domain of said EGFR gene, or a fragment thereof suspected of containing a mutation, and comparing the electrophoretic mobility of the amplified nucleic acid to the electrophoretic mobility of corresponding wild-type EGFR gene or fragment thereof.

Also disclosed is a method for identifying a tumor in a human subject that is susceptible to treatment with an EGFR inhibitor comprising (i) determining the presence of a wild-type KRAS protein or gene in a sample of said tumor whereby the presence of a wild-type KRAS protein or gene indicates that the tumor is susceptible to treatment with an EGFR inhibitor or (ii) determining the presence of a mutated KRAS protein or gene in a sample of said tumor whereby the absence of a mutated KRAS protein or gene indicates that the tumor is susceptible to treatment with an EGFR inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the amino acid sequence of wild-type EGFR1 (SEQ ID NO: 1) in which the signal sequence is residues 1-24, the extracellular domain includes residues 24-645, the transmembrane domain includes residues 646-668, and the cytoplasmic domain includes residues 669-1210. The tyrosine kinase domain region is residues 718-964, and the threonine phosphorylation site is residue 678.

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Figure 2a through 2d is the cDNA sequence (SEQ ID NO: 2) of wild-type EGFR in which exon 18 corresponds to nucleotides 2308-2430; exon 19 corresponds to nucleotides 2431-2529; exon 20 corresponds to nucleotides 2530-2715 and exon 21 corresponds to 2716-2871.

Figure 3 is a graphical representation of extracellular (top) and intracellular (bottom) regions of EGFR.

Figure 4 is a Kaplan-Meier curve showing time to progression of patients having NSCLC tumors expressing wild-type EGFR (solid line) and mutant EGFR (dashed line).

Figure 5 is a Kaplan-Meier curve showing survival of patients having NSCLC tumors expressing wild-type EGFR (solid line) and mutant EGFR (dashed line).

Figure 6 is an autoradiograph illustrating inhibition of autophosphorylation of wild-type EGFR, and mutant EGFR (L858R and del746-752) with varying concentrations of erlotinib in transiently transfected COS7 cells.

Figure 7 is a graph showing inhibition of autophosphorylation of wild-type EGFR and mutant EGFR (L858R and del746-752) with varying concentrations of erlotinib in transiently transfected COS7 cells.

Figure 8 illustrates mutations in exons 18 and 19 of EGFR gene and protein sequences. Amino acid and nucleotide changes, and insertions are in bold, underlined font while deletions are shown as dashes (-).

Figure 9 illustrates mutations in exons 20 and 21 of EGFR gene and protein sequences. Amino acid and nucleotide changes, and insertions are in bold, underlined font while deletions are shown as dashes (-).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is a discovery of the present invention that mutational events associated with tumorigenesis occur in Epidermal Growth Factor Receptor (EGFR). Although it was previously known that aberrant EGFR activity was associated with various cancers, it was unknown that mutations in the EGFR kinase domain region (KDR) existed that caused aberrant signaling activity associated with some cancers. Surprisingly patients suffering from tumors having EGFR KDR mutations have a better prognosis than those with wild-type EGFR. The KDR mutations of the EGFR gene can involve rearrangements such as insertions and deletions as well as point mutations.

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Samples from approximately 250 patients who participated a randomized, double-blinded phase III clinical trial referred to as Tribute were sequenced for mutations occurring in exons 18-21 of EGFR. Tribute studied 1,079 patients at approximately 150 centers in the United States having histological confirmed NSCLC who had not received prior chemotherapy comparing erlotinib + chemotherapy (carboplatin/paclitaxel) with chemotherapy alone. Patients received paclitaxel (200 mg/m^2 3 hour i.v. infusion) followed by carboplatin (AUC = 6 mg/ml x minute infused over 15-30 minutes using Calvert formula) with or without erlotinib (100 mg/day p.o. escalated to 150mg/day for tolerant patients). Tumor samples, formalin-fixed paraffin-embedded blocks or unstained slides, from approximately 250 patients collected from the Tribute trial were enriched for tumor cells by laser capture mircrodissection followed by DNA extraction. Exons 18-21 were amplified by nested PCR and bi-directional sequences were obtained from each PCR product using fluorescent dye-terminator chemistry. Mutations discovered from the sequencing are shown in table 1:

Table 1

protein mutation	nucleic acid mutation	exon
G719A	2402G>C	18
G719C	2401G>T	18
G719S	2401G>A	18
E746-R748 del	2482-2490 del GGAATTAAGA (SEQ ID NO: 32)	19
E746-A750 del	2481-2495 del GGAATTAAGAGAAGC (SEQ ID NO: 33)	19
E746-R748 del E749Q A750P	2482-2490 del GAATTAAGA 2491G>C 2494G>C	19
L747-E749 del A750P	2485-2493 del TTAAGAGAA 494G>C	19
L747S R748-P753 del	2486-2503 del TAAGAGAAGCAACATCTC (SEQ ID NO: 34)	19
L747-S752 del E746V	2485-2502 del TTAAGAGAAGCAACATCT 2483A>T (SEQ ID NO: 35)	19
L747-T751 del ins S	2486-2494del TAAGAGAAGCAA (SEQ ID NO: 36)	19
	2499-2522 del ATCTCCGAAAGCCAACAAGGAAAT	19

S752-I759 del	(SEQ ID NO: 37)	
M766-A767 AI ins	2544-2545 ins GCCATA	20
S768-V769 SVA ins	2554-2555 ins CCAGCGTGG (2556C>T silent)	20
L858R	2819T>G	21
G719C S768I	2401G>T 2549G>T {2607G>A SNP silent}	18 20
G719C V765M S768I	2401G>T 2539G>A 2549G>T	18 20 20
A755V	2510C>T	19
L747S	2486T>C	19
E746K	2482G>A	19
P772-H773 V ins	2561-2562 ins GGT	20
L858P	2819T>C	21
L861Q	2576T>A	21
P772-H773 NS ins H773Y	2562-2563 ins AACTCC 2563C>T	20
T790M L858R	2615C>T 2819T>G	20 21
S784F L858R		21 21

ins = insertion del = deletion

Nucleotide numbering for mutations is based on reference sequence shown in figures 2a-2d.

Clinical outcome of patients having tumors with EGFR mutations and wild-type EGFR were analyzed according to response (complete + partial) benefit (response + stable disease) and progressive disease. Lesions were evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) criteria whereby "complete response" (CR) is defined as the disappearance of all target lesions; "partial response" (PR) is defined as at least a 30% decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum longest diameter; "progressive disease" (PD) is defined as at least a 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum longest diameter recorded since the treatment started or the appearance of one or more new lesions; and "stable disease" (SD) is defined as neither sufficient shrinkage to qualify for partial response nor sufficient increase to

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qualify for progressive disease, taking as reference the smallest sum longest diameter since the treatment started.

Results of the analysis are summarized in table 2.

Table 2

	Mutant EGFR n=24	Wild-Type EGFR n=181
Response / Benefit Rate		
response (CR + PR)	11 46%	46 25%
benefit (CR + PR + SD)	18 75%	105 58%
SD	7 29%	59 33%
PD	6 25%	76 42%
Survival (days)		
median	435	309
range	133-687	9-643

CR=complete response; PR=partial response; SD=stable disease; PD=progressing disease

Analysis of clinical outcome revealed that patients with tumors expressing a mutation in exons 18-21 of EGFR have better prognosis than those with tumors expressing wild-type EGFR. Mutant EGFR patients exhibited greater response rate, benefit rate and survival when treated with chemotherapy or chemotherapy plus erlotinib. These results are useful for predicting outcome such that patients who's tumors have EGFR mutations in any or all of exons 18 through 21 have more favorable prognosis than patients who's tumors do not have such mutations.

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Accordingly, the present invention provides a method for determining the prognosis of a patient having a tumor comprising determining in a sample of said tumor the presence or absence of one or more EGFR mutations in exons 18-21 (or the amino acid sequence corresponding to exons 18-21) whereby the presence of said one or more EGFR mutation indicates better prognosis compared to the absence of said one or more EGFR mutation. By "prognosis" is meant response and/or benefit and/or survival. By "EGFR mutations" means an amino acid or nucleic acid sequence that differs from wild-type EGFR protein or nucleic acid respectively found on one allele (heterozygous) or both alleles (homozygous) and may be somatic or germ line. In a particular embodiment said mutation is found in the kinase domain region (KDR) of EGFR. In another particular embodiment the mutation is an amino acid substitution, deletion or insertion as shown in table 1. In an embodiment the amino acid mutation is one or more of the following: G719A, E746K, L747S, E749Q, A750P, A755V, S768I, L858P, E746-R748 del, R748-P753 del, M766-A767 AI ins, and S768-V769 SVA ins.. In another particular embodiment, the mutation is a nucleic acid point mutation, deletion or insertion as shown in table 1. In an embodiment, the nucleic acid mutation is one or more the following: 2402G>C; 2482G>A; 2486T>C; 2491G>C; 2494G>C; 2510C>T; 2549G>T; 2819T>C; 2482-2490 del; 2486-2503 del; 2544-2545 ins GCCATA; and 2554-2555 ins CCAGCGTGG.

EGFR exons 18-21 from an H1975 tumor cell line that exhibited resistance to treatment with erlotinib was sequenced and found to incorporate a mutation T790M in combination with an L858R mutation. Accordingly the present invention further provides a method for determining the prognosis of a patient having a tumor comprising determining in a sample of said tumor the presence or absence of the T790M EGFR mutation whereby the presence of said T790M EGFR mutation indicates poorer prognosis compared to the absence of said T790M EGFR mutation. Further, there is provided a method of identifying patients having a tumor that is less responsive to therapy of an EGFR inhibitor such as erlotinib or gefitinib, whether in combination with chemotherapy or not, comprising determining the presence or absence of a T790M EGFR mutation in the patient's tumor whereby the presence of said mutation indicates the patient will respond less to said therapy compared to a patient having a tumor that does not have said T790M EGFR mutation. Further, there is provided a method of identifying a tumor that is resistant to treatment with an EGFR inhibitor, such as a kinase domain binding inhibitor (for example erlotinib or gefitinib), whether in combination with chemotherapy or not, comprising determining the presence or absence of a T790M EGFR mutation in a sample of the tumor whereby the presence of said mutation indicates the tumor is resistant to said treatment. It is understood that determination of the mutation is at the protein level or nucleic acid level (genomic DNA or mRNA) and are accomplished using techniques such as those described herein. In a particular embodiment, said

EGFR inhibitor competes with ATP at the EGFR kinase domain. In a particular embodiment the EGFR inhibitor is erlotinib.

Also disclosed is a method of treating a patient having a tumor incorporating a T790M mutant EGFR protein or gene (or treating a tumor incorporating a T790M mutant EGFR protein or gene) comprising co-administering to said patient (or contacting said tumor with) a first compound that binds to and/or inhibits signaling of said T790M mutant EGFR in combination with a second compound that binds to and/or inhibits signaling of wild-type EGFR or EGFR incorporating an activating mutation. In a particular embodiment said activating mutation is one or more of those described in Table 1 (other than T790M). In a particular embodiment said first and second compounds are administered sequentially or concommitantly. In a particular embodiment said second compound is erlotinib.

Also disclosed is a method of screening for compounds that inhibit signaling of a mutant EGFR protein that incorporates a T790M mutation, comprising contacting said mutant EGFR with a test compound in the presence of a phosphorylation substrate and ATP and detecting a change in the amount of phosphorylation of said substrate whereby a reduction of phosphorylation of said substrate compared to a control, or compared to phosphorylation of the substrate in the absence of the test compound, indicates said test compound is an inhibitor of mutant EGFR signaling. In an embodiment, said method is performed *in vitro* in the presence of a ligand for said mutant EGFR such as EGF or TGF-alpha.

In a particular embodiment the inhibitory activity of a test compound can be determined *in vitro* by the amount of inhibition of the phosphorylation of an exogenous substrate (e.g. Lys_3 -Gastrin or polyGluTyr (4:1) random copolymer (I. Posner et al., J. Biol. Chem, 267 (29), 20638-47 (1992)) on tyrosine by epidermal growth factor receptor kinase by a test compound relative to a control. Purified, soluble human T790M mutant EGFR (96 ng) is preincubated in a microfuge tube with EGF (2 μg/ml) in phosphorylation buffer+vanadate (PBV: 50 mM HEPES, pH 7.4; 125 mM NaCl; 24 mM $MgCl_2$; 100 μM sodium

orthovanadate), in a total volume of 10 µl, for 20-30 minutes at room temperature. The test compound, dissolved in dimethylsulfoxide (DMSO), is diluted in PBV, and 10 µl is mixed with the mutant EGFR/EGF mix, and incubated for 10-30 minutes at 30°C. The phosphorylation reaction is initiated by addition of 20 µl ^{33}P-ATP/substrate mix (120 µM Lys_3 -Gastrin (sequence in single letter code for amino acids, KKKGPWLEEEEEAYGWLDF - SEQ ID NO: 38), 50 mM Hepes pH 7.4, 40 µM ATP, 2µCi γ-[^{33}P]-ATP) to the mutant EGFR/EGF mix and incubated for 20 minutes at room temperature. The reaction is stopped by addition of 10 µl stop solution (0.5M EDTA, pH 8; 2mM ATP) and 6 µl 2N HCl. The tubes are centrifuged at 14,000 RPM, 4°C., for 10 minutes. 35 µl of supernatant from each tube is pipetted onto a 2.5 cm circle of Whatman P81 paper, bulk washed four times in 5% acetic acid, 1 liter per wash, and then air dried. This results in the binding of substrate to the paper with loss of free ATP on washing. The [^{33}P] incorporated is measured by liquid scintillation counting. Incorporation in the absence of substrate (e.g., lys_3 -gastrin) is subtracted from all values as a background and percent inhibition is calculated relative to controls without test compound present. Such assays, carried out with a range of doses of test compounds, allow the determination of an approximate IC_{50} value for the *in vitro* inhibition of T790M mutant EGFR kinase activity.

Also disclosed is a method for identifying a tumor in a human subject that is susceptible to treatment comprising determining the presence of a mutated EGFR gene or mutated EGFR protein in a sample of said tumor wherein said mutation is located in exons 18-21 of EGFR whereby the presence of a mutated EGFR gene or mutated EGFR protein indicates that the tumor is susceptible to treatment with an anticancer agent. In a particular embodiment the anticancer agent is a chemotherapeutic agent which may be a cytotoxic or cytostatic. Tumors include neuroblastoma, intestine carcinoma such as rectum carcinoma, colon carcinoma, familiary adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tong carcinoma, salivary gland carcinoma, gastric

carcinoma, adenocarcinoma, medullary thyroidea carcinoma, papillary thyroidea carcinoma, renal carcinoma, kidney parenchym carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeloid leukemia (AML), chronic mycloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyo sarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma. Particular tumors include those of the brain, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, breast, lung, vulval, thyroid, colorectal, oesophageal, hepatic carcinomas, sarcomas, glioblastomas, head and neck, leukemias and lymphoid malignancies.

Particular chemotherapeutic agents include, but are not limited to (i) antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuiridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, such as bleomycin, (iii) DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; (iv) intercalating agents such as adriamycin (doxorubicin) or mitoxantrone; (v) protein synthesis inhibitors, such as L asparaginase, cycloheximide, puromycin or diphtheria toxin; (vi) topoisomerase I poisons, such as camptothecin or topotecan; (vii) topoisomerase II poisons, such as etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; (ix) kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents such as thioplatin, PS-341, phenylbutyrate,

ET-18- OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinie acid and derivatives thereof; (xi) hormones such as glucocorticoids or fenretinide; (xii) hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists. In an embodiment, the chemotherapeutic compound is one or more of gemcitabine, cisplatin, doxorubicin, daunarubicin, paclitexel, taxotere and mitomycin C. In a particular embodiment the chemotherapeutic compound is one or more of gemcitabine, cisplatin and paclitaxel. In another embodiment the treatment is an inhibitor of EGFR. In an embodiment the EGFR inhibitor is an antibody such as Erbitutux™ (cetuximab, Imclone Systems Inc.) and ABX-EGF (panitumumab, Abgenix, Inc.). In another embodiment the EGFR inhibitor is a small molecule that competes with ATP such as Tarceva™ (erlotinib, OSI Pharmaceuticals), Iressa™ (gefitinib, Astra-Zeneca), tyrphostins described by Dvir, et al., J Cell Biol., 113:857-865 (1991); tricyclic pyrimidine compounds disclosed in U.S. Patent 5,679,683; compound 6- (2,6-dichlorophenyl)-2-(4-(2-diethylaininoethoxy)phenylamino)-8-methyl-8H- pyrido(2,3- d)pyrimidin-7-one (known as PD166285) disclosed in Panek, et al., Journal of Pharmacology and Experimental Therapeutics 283, 1433-1444 (1997).

Also disclosed is a method of identifying an EGFR mutation in a sample comprising contacting nucleic acid from said sample with a nucleic acid probe that is capable of specifically hybridizing to nucleic acid encoding a mutated EGFR protein, or fragment thereof incorporating a mutation, and detecting said hybridization. In a particular embodiment said probe is detectably labeled such as with a radioisotope (^3H, ^{32}P, ^{33}P etc), a fluorescent agent (rhodamine, fluorescene etc.) or a chromogenic agent. In a particular embodiment the probe is an antisense oligomer, for example PNA, morpholino-phosphoramidates, LNA or 2'-alkoxyalkoxy. The probe may be from about 8 nucleotides to about 100 nucleotides, or about 10 to about 75, or about 15 to about 50, or about 20 to about 30. In another aspect said probes of the invention are provided in a kit for identifying EGFR mutations in a sample, said kit comprising an

oligonucleotide that specifically hybridizes to or adjacent to a site of mutation in the EGFR gene. The kit may further comprise instructions for treating patients having tumors that contain EGFR mutations with an EGFR inhibitor based on the result of a hybridization test using the kit.

Also disclosed is a method of detecting a mutated EGFR gene in a sample comprising amplifying from said sample nucleic acid corresponding to the kinase domain of said EGFR gene, or exons 18-21, or a fragment thereof suspected of containing a mutation, and comparing the electrophoretic mobility of the amplified nucleic acid to the electrophoretic mobility of corresponding wild-type EGFR gene or fragment thereof. A difference in the mobility indicates the presence of a mutation in the amplified nucleic acid sequence. Electrophoretic mobility may be determined on polyacrylamide gel.

Alternatively, amplified EGFR gene or fragment nucleic acid may be analyzed for detection of mutations using Enzymatic Mutation Detection (EMD) (Del Tito et al, Clinical Chemistry 44:731-739, 1998). EMD uses the bacteriophage resolvase T_4 endonuclease VII, which scans along double-stranded DNA until it detects and cleaves structural distortions caused by base pair mismatches resulting from point mutations, insertions and deletions. Detection of two short fragments formed by resolvase cleavage, for example by gel eletrophoresis, indicates the presence of a mutation. Benefits of the EMD method are a single protocol to identify point mutations, deletions, and insertions assayed directly from PCR reactions eliminating the need for sample purification, shortening the hybridization time, and increasing the signal-to-noise ratio. Mixed samples containing up to a 20-fold excess of normal DNA and fragments up to 4 kb in size can been assayed. However, EMD scanning does not identify particular base changes that occur in mutation positive samples requiring additional sequencing procedures to identify the mutation if necessary. CEL I enzyme can be used similarly to resolvase T_4 endonuclease VII as demonstrated in US5869245.

Another simple kit for detecting the EGFR mutations of the invention is a reverse hybridization test strip similar to Haemochromatosis StripAssay™ (Viennalabs http://www.bamburghmarrsh.com/pdf/4220.pdt) for detection of multiple mutations in HFE, TFR2 and FPN1 genes causing Haemochromatosis. Such an assay is based on sequence specific hybridisation following amplification by PCR. For single mutation assays, a microplate-based detection system may be applied, whereas for multi-mutation assays, teststrips may be used as "macro-arrays". Kits may include ready-to use reagents for sample prep, amplification and mutation detection. Multiplex

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amplification protocols provide convenience and allow testing of samples with very limited volumes. Using the straightforward StripAssay format, testing for twenty and more mutations may be completed in less than five hours without costly equipment. DNA is isolated from a sample and the EGFR gene (or exons 18-21 or KDR or segments thereof) is amplified in vitro (e.g. PCR) and biotin-labelled, preferably in a single ("multiplex") amplification reaction. The PCR products are the selectively hybridized to oligonucleotide probes (wild-type and mutant specific) immobilized on a solid support such as a test strip in which the probes are immobilized as parallel lines or bands. Bound biotinylated amplicons are detected using streptavidin-alkaline phosphatase and color substrates. Such an assay can detect all or any subset of the mutations in table 1. With respect to a particular mutant probe band one of three signalling patterns are possible: (i) a band only for wild-type probe which indicates normal EGFR (ii) bands for both wild-type and a mutant probe which indicates heterozygous genotype and (iii) band only for the mutant probe which indicates homozygous mutant EGFR genotype. Accordingly there is further provides a method of detecting EGFR mutations of the invention comprising isolating nucleic acid from a sample, amplifying the EGFR gene, or fragment thereof (e.g. the KDR or exons 18-21 or smaller) such that the amplified nucleic acid comprises a ligand, contacting the amplified EGFR gene or fragment with a probe which comprises a detectable binding partner to the ligand and the probe is capable of specifically hydribizing to an EGFR mutation, and then detecting the hybridization of said probe to said amplified EGFR gene or fragment. In a particular embodiment the ligand is biotin and the binding partner is comprises avidin or streptavidin. In a particular embodiment the binding partner is steptavidin-alkaline which is detectable with color substrates. In a particular embodiment the probes are immobilized for example on a test strip wherein probes complementary to different mutations are separated from one another. Alternatively, the amplified nucleic acid is labelled with a radioisotope in which case the probe need not comprise a ligand.

The tumor samples were also analyzed for mutations in KRAS (as referred to as p21a). Particular mutations detected in exon 1 are: G12C; G12A; G12D; G12R; G12S; G12V; G13C; G13D which correlated with poor prognosis to chemotherapy as well as chemotherapy with erlotinib therapy. Accordingly, the invention further provides a method of identifying patients not responsive to therapy of an EGFR inhibitor such as erlotinib or erlotinib in combination with chemotherapy comprising determining the presence or absence of a KRAS mutation whereby the presence of said mutation indicates a patient will not respond to said therapy. Alternatively, there is provided a method for identifying a tumor in a human subject that is susceptible to treatment with an EGFR inhibitor comprising (i) determining the presence of a wild-type KRAS protein or gene in a sample of said tumor whereby the presence of a wild-type KRAS protein or gene indicates that the tumor is susceptible to treatment with an EGFR inhibitor or (ii) determining the presence of a mutated KRAS protein or gene in

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a sample of said tumor whereby the absence of a mutated KRAS protein or gene indicates that the tumor is susceptible to treatment with an EGFR inhibitor. In a particular embodiment the mutation is in exon 1 of K-Ras. In another embodiment the K-Ras mutation is at least one of G12C; G12A; G12D; G12R; G12S; G12V; G13C; G13D. Alternatively, individuals who have tumors which harbor mutant K-Ras may be treated with EGFR inhibitors when in concomitantly with a K-Ras inhibitor. Methods for determining the presence of K-Ras mutations are analogous to those used to identify EGFR mutations described in detail herein.

According to the diagnostic and prognostic method of the present invention, alteration of the wild-type EGFR gene is detected. Alterations of a wild-type gene according to the present invention encompasses all forms of mutations such as insertions, inversions, deletions, and/or point mutations. Somatic mutations are those which occur only in certain tissues, e.g., in the tumor tissue, and are not inherited in the germ line. Germ line mutations can be found in any of a body's tissues. If only a single allele is somatically mutated, an early neoplastic state is indicated. However, if both alleles are mutated then a late neoplastic state is indicated. The finding of EGFR mutations is therefore a diagnostic and prognostic indicator as described herein.

The EGFR mutations found in tumor tissues may result in increased signaling activity relative to wild-type EGFR leading to a cancerous state. In order to detect the alteration of the wild-type EGFR gene a sample or biopsy of the tumor is obtained by methods well known in the art and appropriate for the particular type and location of the tumor. For instance, samples of lung cancer lesions may be obtained by resection, bronchoscopy, fine needle aspiration, bronchial brushings, or from sputum, pleural fluid or blood. Means for enriching a tissue preparation for tumor cells are known in the art. For example, the tissue may be isolated from paraffin or cryostat sections. Cancer cells may also be separated from normal cells by flow cytometry or laser capture microdissection. These as well as other techniques for separating tumor from normal cells are well known in the art. If the tumor tissue is highly contaminated with normal cells, detection of mutations is more difficult.

Detection of point mutations may be accomplished by molecular cloning of the EGFR allele (or alleles) and sequencing that allele(s) using techniques well known in the art. Alternatively, the polymerase chain reaction (PCR) can be used to amplify gene sequences directly from a genomic DNA preparation from the tumor tissue. The DNA sequence of the amplified sequences can then be determined and mutations identified therefrom. The polymerase chain reaction is well known in the art and described in Saiki et al., Science 239:487, 1988; U.S. 4,683,203; and U.S. 4,683,195.

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Specific primer pairs which can be used for PCR amplification of EGFR exons 18-21 include:

<5pEGFR.ex18.out> CAAATGAGCTGGCAAGTGCCGTGTC (SEQ ID NO: 39)
<3pEGFR.ex18.out> GAGTTTCCCAAACACTCAGTGAAAC (SEQ ID NO: 40)

<5pEGFR.ex19.out> GCAATATCAGCCTTAGGTGCGGCTC (SEQ ID NO: 41)
<3pEGFR.ex19.out> CATAGAAAGTGAACATTTAGGATGTG (SEQ ID NO: 42)

<5pEGFR.ex20.out> CCATGAGTACGTATTTTGAAACTC (SEQ ID NO: 43)
<3pEGFR.ex20.out> CATATCCCCATGGCAAACTCTTGC (SEQ ID NO: 44)

<5pEGFR.ex21.out> CTAACGTTCGCCAGCCATAAGTCC (SEQ ID NO: 45)
<3pEGFR.ex21.out> GCTGCGAGCTCACCCAGAATGTCTGG (SEQ ID NO: 46)

<5pEGFR.ex18.in.m13f> TGTAAAACGACGGCCAGTCAAGTGCCGTGTCCTGGCACCCAAGC (SEQ ID NO: 47)
<3pEGFR.ex18.in.m13r> CAGGAAACAGCTATGACCCCAAACACTCAGTGAAACAAAGAG (SEQ ID NO: 48)

<5pEGFR.ex19.in.m13f> TGTAAAACGACGGCCAGTCCTTAGGTGCGGCTCCACAGC (SEQ ID NO: 49)
<3pEGFR.ex19.in.m13r> CAGGAAACAGCTATGACCCATTTAGGATGTGGAGATGAGC (SEQ ID NO: 50)
<5pEGFR.ex20.in.m13f> TGTAAAACGACGGCCAGTGAAACTCAAGATCGCATTCATGC (SEQ ID NO: 51)
<3pEGFR.ex20.in.m13r> CAGGAAACAGCTATGACCGCAAACTCTTGCTATCCCAGGAG (SEQ ID NO: 52)
<5pEGFR.ex21.in.m13f> TGTAAAACGACGGCCAGTCAGCCATAAGTCCTCGACGTGG (SEQ ID NO: 53)
<3pEGFR.ex21.in.m13r> CAGGAAACAGCTATGACCCATCCTCCCCTGCATGTGTTAAAC (SEQ ID NO: 54)

Specific primer pairs which can be used for PCR amplification of K-Ras exon 1 include:

<5pKRAS-out> TACTGGTGGAGTATTTGATAGTG (SEQ ID NO: 55)
<3pKRAS-out> CTGTATCAAAGAATGGTCCTG (SEQ ID NO: 56)

<5pKRAS-in.m13f> TGTAAAACGACGGCCAGTTAGTGTATTAACCTTATGTG (SEQ ID NO: 57)
<3pKRAS-in.m13r> CAGGAAACAGCTATGACCACCTCTATTGTTGGATCATATTCG (SEQ ID NO: 58)

The ligase chain reaction, which is known in the art, can also be used to amplify EGFR sequences. See Wu et al., Genomics, Vol. 4, pp. 560-569 (1989). In addition, a technique known as allele specific PCR can be used. (See Ruano and Kidd, Nucleic Acids Research, Vol. 17, p. 8392, 1989.) According to this technique, primers are used which hybridize at their 3'ends to a particular EGFR mutation. If the particular EGFR mutation is not present, an amplification product is not observed. Amplification Refractory Mutation System (ARMS) can also be used as disclosed in European Patent Application Publication No. 0332435 and in Newton et al., Nucleic Acids Research, Vol. 17, p.7, 1989. Insertions

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and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism, (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. Single stranded conformation polymorphism (SSCP) analysis can also be used to detect base change variants of an allele. (Orita et al., Proc. Natl. Acad. Sci. USA Vol. 86, pp. 2766-2770, 1989, and Genomics, Vol. 5, pp. 874-879, 1989.) Other techniques for detecting insertions and deletions as are known in the art can be used.

Alteration of wild-type genes can also be detected on the basis of the alteration of a wild-type expression product of the gene. Such expression products include both the EGFR mRNA as well as the EGFR protein product. Point mutations may be detected by amplifying and sequencing the mRNA or via molecular cloning of cDNA made from the mRNA. The sequence of the cloned cDNA can be determined using DNA sequencing techniques which are well known in the art. The cDNA can also be sequenced via the polymerase chain reaction (PCR).

Mismatches, according to the present invention are hybridized nucleic acid duplexes which are not 100% complementary. The lack of total complementarity may be due to deletions, insertions, inversions, substitutions or frameshift mutations. Mismatch detection can be used to detect point mutations in the gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumor samples. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter et al., Proc. Natl. Acad. Sci. USA, Vol. 82, p. 7575, 1985 and Meyers et al., Science, Vol. 230, p. 1242, 1985. In the practice a the present invention the method involves the use of a labeled riboprobe which is complementary to the human wild-type EGFR gene coding sequence (or exons 18-21 or KDR thereof). The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the EGFR mRNA or gene but can be exons 18 through 21 or the EGFR KDR or segments thereof. If the riboprobe comprises only a segment of the EGFR mRNA or gene it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

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In a similar manner, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., Proc. Natl. Acad. Sci. USA, Vol. 85, 4397, 1988; and Shenk et al., Proc. Natl. Acad. Sci. USA, Vol. 72, p. 989, 1975. Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., Cariello, Human Genetics, Vol. 42, p. 726, 1988. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR before hybridization. Changes in DNA of the EGFR gene can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the EGFR gene which have been amplified by use of polymerase chain reaction may also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the EGFR gene sequence harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the EGFR gene sequence. By use of a battery of such allele-specific probes, PCR amplification products can be screened to identify the presence of a previously identified mutation in the EGFR gene. Hybridization of allele-specific probes with amplified EGFR sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

Alteration of wild-type EGFR genes can also be detected by screening for alteration of wild-type EGFR protein. For example, monoclonal antibodies immunoreactive with EGFR can be used to screen a tissue. Lack of cognate antigen would indicate an EGFR mutation. Antibodies specific for products of mutant alleles could also be used to detect mutant EGFR gene product. Antibodies may be identified from phage display libraries. Such immunological assays can be done in any convenient format known in the art. These include Western blots, immunohistochemical assays and ELISA assays. Any means for detecting an altered EGFR protein can be used to detect alteration of wild-type EGFR genes.

Mutant EGFR genes or gene products can be detected from tumor or from other body samples such as urine, sputum or serum. The same techniques discussed above for detection of mutant EGFR genes or gene products in tumor samples can be applied to other body samples. Cancer cells are sloughed off from tumors and appear in such body samples. By screening such body samples, a simple early diagnosis can be achieved for many types of cancers. In addition, the progress of chemotherapy or radiotherapy can be monitored more easily by testing such body samples for mutant EGFR genes or gene products.

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The methods of diagnosis of the present invention are applicable to any tumor in which EGFR has a role in tumorigenesis for example lung, breast, colon, glioma, bladder, liver, stomach and prostate. The diagnostic method of the present invention is useful for clinicians so that they can decide upon an appropriate course of treatment. For example, a tumor displaying alteration of both EGFR alleles might suggest a more aggressive therapeutic regimen than a tumor displaying alteration of only one EGFR allele.

The primer pairs of the present invention are useful for determination of the nucleotide sequence of a particular EGFR allele using the polymerase chain reaction. The pairs of single stranded DNA primers can be annealed to sequences within or surrounding the EGFR gene on in order to prime amplifying DNA synthesis of the EGFR gene itself. A set of these primers allows synthesis of all of the nucleotides of the EGFR exons 18 through 21. Allele specific primers can also be used. Such primers anneal only to particular EGFR mutant alleles, and thus will only amplify a product in the presence of the mutant allele as a template. In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme site sequences appended to their ends. Thus, all nucleotides of the primers are derived from EGFR exons 18-21 or sequences adjacent thereto except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art. The primers themselves can be synthesized using techniques which are well known in the art. Generally, the primers can be made using oligonucleotide synthesizing machines which are commercially available. Design of particular primers is well within the skill of the art.

The nucleic acid probes provided by the present invention are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in the RNase protection method for detecting point mutations already discussed above. The probes can be used to detect PCR amplification products. They may also be used to detect mismatches with the EGFR gene or mRNA using other techniques. Mismatches can be detected using either enzymes (e.g., S1 nuclease), chemicals (e.g., hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids. These techniques are known in the art. See Novack et al., Proc. Natl. Acad. Sci. USA, Vol. 83, p. 586, 1986. Generally, the probes are complementary to EGFR exon 18-21 sequences, although generally probes to the kinase domain and segments thereof are also contemplated. An entire battery of nucleic acid probes may be used to compose a kit for detecting alteration of wild-type EGFR genes. The kit allows for hybridization to the entire exon 18-21 sequence of the EGFR gene. The probes may overlap with each other or be contiguous.

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If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the EGFR gene. The riboprobe thus is an antisense probe in that it does not code for the EGFR protein because it is complementary to the sense strand. The riboprobe generally will be labeled with a radioactive, colorimetric, or fluorometric material, which can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches.

Predisposition to cancers can be ascertained by testing any tissue of a human for mutations of the EGFR gene. For example, a person who has inherited a germ line EGFR mutation would be prone to develop cancers. This can be determined by testing DNA from any tissue of the body. For example, blood can be drawn and DNA extracted from the cells of the blood. In addition, prenatal diagnosis can be accomplished by testing fetal cells, placental cells, or amniotic fluid for mutations of the EGFR gene. Alteration of a wild- type EGFR allele, whether for example, by point mutation or by deletion, can be detected by any of the means discussed above.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

EXAMPLES:

Example 1 Slide Preparation - Deparaffinization and Staining

Submersed sections in the following solutions:

1. Fresh xylenes (to depariffinize the sections) - 5 min
2. Fresh xylenes - 5 min
3. 100% ethanol - 15 sec

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4. 95% ethanol - 15 sec
5. 70% ethanol-15 sec
6. Deionized water - 15 sec
7. Mayer's Hematoxylin - 30 sec
8. Deionized water - rinse (x 2) - 15 sec
9. 70% ethanol - 15 sec
10. Eosin Y - 5 sec
11. 95% ethanol- 15 sec
12. 95% ethanol - 15 sec
13. 100% ethanol - 15 sec

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14. 100% ethanol - 15 sec
15. Xylenes (to ensure dehydration of the section) - 60 sec
16. Air-dried for approximately 2 minutes or gently used air gun to completely remove xylenes.
17. The tissue was then ready for LCM.

Example 2 Laser Capture Microdissection and DNA Extraction

Materials:

PixCell II LCM System
CapSure HS or CapSure Macro LCM caps
ExtractSure device (HS only)
Razor blades (factory sterile)
0.5 ml tubes
0.2 ml tubes
PicoPure DNA extraction Kit
65°C incubator

Procedure:

1. Placed CapSure cap over area of tissue to be collected
2. Lased over desired area
2. Lifted cap off tissue.
3. Dispensed 20 ul of PicoPure digest buffer with Proteinase K into 0.5ml tube.
4. Placed cap with dissected material into tube to form a tight seal.
5. Inverted tube such that digest buffer covered cap.
6. Incubated at 65°C for 24 hours.
7. Spun tube with cap to collect digested material in the bottom of the tube.
8. Transferred digest to 0.2 ml strip tube.
9. Inactivated Proteinase K at 95°C for 10 minutes in a thermocycler with a heated lid.
10. Used 1-2 ul of sample in a 50 ul PCR reaction. No clean-up was necessary.

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Example 3 PCR amplification

PCR Primers:

Primer pairs were designed for each exon to be sequenced (*EGFR* exons 18, 19, 20 and 21). Primer sequences used were as follows:

<5pEGFR.ex18.out> CAAATGAGCTGGCAAGTGCCGTGTC (SEQ ID NO: 39)
<3pEGFR.ex18.out> GAGTTTCCCAAACACTCAGTGAAAC (SEQ ID NO: 40)

<5pEGFR.ex19.out> GCAATATCAGCCTTAGGTGCGGCTC (SEQ ID NO: 41)
<3pEGFR.ex19.out> CATAGAAAGTGAACATTTAGGATGTG (SEQ ID NO: 42)

<5pEGFR.ex20.out> CCATGAGTACGTATTTTGAAACTC (SEQ ID NO: 43)
<3pEGFR.ex20.out> CATATCCCCATGGCAAACTCTTGC (SEQ ID NO: 44)

<5pEGFR.ex21.out> CTAACGTTCGCCAGCCATAAGTCC (SEQ ID NO: 45)
<3pEGFR.ex21.out> GCTGCGAGCTCACCCAGAATGTCTGG (SEQ ID NO: 46)

<5pEGFR.ex18.in.m13f> TGTAAAACGACGGCCAGTCAAGTGCCGTGTCCTGGCACCCAAGC
(SEQ ID NO: 47)
<3pEGFR.ex18.in.m13r> CAGGAAACAGCTATGACCCCAAACACTCAGTGAAACAAAGAG
(SEQ ID NO: 48)

<5pEGFR.ex19.in.m13f> TGTAAAACGACGGCCAGTCCTTAGGTGCGGCTCCACAGC
(SEQ ID NO: 49)
<3pEGFR.ex19.in.m13r> CAGGAAACAGCTATGACCCATTTAGGATGTGGAGATGAGC
(SEQ ID NO: 50)

<5pEGFR.ex20.in.m13f> TGTAAAACGACGGCCAGTGAAACTCAAGATCGCATTCATGC
(SEQ ID NO: 51)
<3pEGFR.ex20.in.m13r> CAGGAAACAGCTATGACCGCAAACTCTTGCTATCCCAGGAG
(SEQ ID NO: 52)

<5pEGFR.ex21.in.m13f> TGTAAAACGACGGCCAGTCAGCCATAAGTCCTCGACGTGG
(SEQ ID NO: 53)
<3pEGFR.ex21.in.m13r> CAGGAAACAGCTATGACCCATCCTCCCCTGCATGTGTTAAAC
(SEQ ID NO: 54)

K-Ras oligos for PCR

<5pKRAS-out> TACTGGTGGAGTATTTGATAGTG (SEQ ID NO: 55)
<3pKRAS-out> CTGTATCAAAGAATGGTCCTG (SEQ ID NO: 56)

<5pKRAS-in.m13f> TGTAAAACGACGGCCAGTTAGTGTATTAACCTTATGTG
(SEQ ID NO: 57)
<3pKRAS-in>.m13r CAGGAAACAGCTATGACCACCTCTATTGTTGGATCATATTCG
(SEQ ID NO: 58)

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Nested amplification of the primary PCR product was performed using intron-specific primer pairs located within the primary PCR product. These nested primers pairs were tagged with M13f and M13rev sequences.

First round of PCR:

PCR Reaction:

DNA	0.5 to 30ng
Primers	250nM/ each outer primers
dNTPs	0.2mM each (Roche cat#1581295)
$MgCl_2$	1.5mM (15mM 10 X buffer)
Enzyme	1.5 U/ RX Expand High fidelity Taq (Roche cat#1759078)
	50ul reaction volume

Thermocycler conditions:

95°C - 3minutes
94°C - 30seconds repeat 35 times
58°C - 30seconds
72°C - 1minute
72°C - 8minutes
4°C - forever

Second round of PCR:

PCR Reaction:

DNA	1ul from first round PCR reaction
Primers	250nM/ each inner primers
dNTPs	0.2mM each (Roche cat#1581295)
$MgCl_2$	1.5mM (15mM 10 X buffer)
Enzyme	1.5 U/ RX Expand High fidelity Taq (Roche cat#1759078)
	50ul reaction volume

Thermocycler conditions:

95°C - 3minutes
94°C - 30seconds repeat 30 times
58°C - 30seconds
72°C - 1minute
72°C - 8 minutes
4°C - forever

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Isolation of PCR Products:

PCR reaction products were run on E-Gel 2% agarose gels (Invitrogen, cat# G6018-02) for quality control. PCR products were purified directly using the Qiaquick 96 PCR purification kit (Qiagen, cat#28181) or gel purified as was necessary. For gel purification, the PCR product was excised from the E-gel and the DNA purified using Qiaquick 96 PCR purification kit with a gel extraction protocol.(Qiagen, cat#28181).

Example 4 Sequencing

Nested sequencing primers or standard M13f and M13rev sequencing primers for tagged PCR products were used to sequence the purified PCR products. Sequences were as follows:

<m13f> TGTAAAACGACGGCCAGT (SEQ ID NO: 59)
<m13r> CAGGAAACAGCTATGACC (SEQ ID NO: 60)

Purified PCR products were diltuted and cycle-sequenced using the BigDye Terminator Kit (ABI, Foster City, CA) according to manufacturer's instructions.

Reaction Mix:

5 ul DNA (25-100ng PCR product)
6 ul water
1 ul primer diluted to .25 OD/100ul with water (m13f or m13r or sequence specific primer)
2 ul BigDye v3.1
6 ul Dilution Buffer (equivalent of ABI 5x Dilution Buffer)

Cycle Sequencing:

Conditions:
96°C - 2.5 minutes - initial denaturation
96°C - 10 seconds
50°C - 5 seconds
60°C - 4 minutes
repeated for 25 to 50 total cycles

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Reaction Cleanup:

Removed unincorporated nucleotides using:

8% sephadex

500 ul in Edge BioSystem 96-well block

spin @ 750g for 2 minutes

Analysis:

Reaction products were electrophoresed on ABI3700 or ABI3730 sequencing instruments. Electropherograms were analyzed for mutations using commercially available analysis programs, such as Sequencher (Gene Codes, Corp), and with custom tools.

Example 5 Dose Response

Human epidermal growth factor receptor (EGFR) wild-type and mutant constructs used in this study were epitope-tagged at the N-terminus with the herpes simplex virus signal sequence of gD, replacing the endogenous EGFR signal sequence (Schaefer et al. 1999 J. Biol. Chem. 274, 859-866). Cos7 cells were seeded in 12 well dishes in normal growth medium 24 hours prior to transfection. Cells were transfected with 0.25ug per well with expression plasmid DNAs (pRK5.gD.EGFR wild-type, pRK5.gD.EGFR. L858R, or pRK5.gD.EGFR.del(E746-S752)) using LipofectAMINE 2000 following manufacturer's recommended protocol (Invitrogen). Twenty-four hours post-transfection, cells were serum starved for six hours in serum free DMEM. One hour prior to stimulation, transfected cells were preincubated with the indicated concentrations of erlotinib. Transfected cells were stimulated with 1 nM TGFα for 10 minutes. Cells were lysed directly in the wells using reducing Laemmli buffer. Receptor autophosphorylation, an index of EGFR receptor activation by growth factor stimulation, was detected by Western blotting using an HRP-conjugated anti-phosphotyrosine antibody (Oncogene Sciences, AB-4). Transfection efficiency was evaluated using an antibody specific for the gD epitope tag (5B6). Level of receptor activation was evaluated from the autoradiograms using NIH Image software. These data were then used to generate a graph from which an IC50 was calculated using a 4 parameter fit function. As illustrated by the results below, erlotinib has a greater affinity to EGFR containing mutations compared to wild-type EGFR.

EGFR construct	inhibition (IC50)
WT EGFR-gD	50 nM
L858R EGFR-gD	20 nM
del(746-752) EGFR-gD	5 nM

2005250484 13 Jul 2011

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for identifying a colorectal tumor in a human subject that is susceptible to treatment with cetuximab or panitumumab, comprising (i) determining the presence of a wild-type KRAS protein or gene in a sample of said tumor, wherein the presence of a wild-type KRAS protein or gene indicates that the tumor is susceptible to treatment with cetuximab or panitumumab; or (ii) determining the presence of a mutated KRAS protein or gene in a sample of said tumor, wherein the absence of a mutated KRAS protein or gene indicates that the tumor is susceptible to treatment with cetuximab or panitumumab.

2. The method of claim 1 wherein said KRAS protein mutation is at least one of G12C; G12A; G12D; G12R; G12S; G12V; G13C; or G13D.

3. A method for determining whether a colorectal tumor in a human subject is not responsive to therapy with cetuximab or panitumumab comprising: determining the presence of a KRAS gene having a mutation in a sample of said tumor, wherein said KRAS gene mutation encodes a mutation at amino acid residue 12 or 13, and wherein the presence of the KRAS gene mutation indicates that the tumor is not responsive to treatment with cetuximab or panitumumab.

4. The method of claim 3, wherein said mutation at amino acid residue 12 or 13 is at least one of the following protein mutations: G12A; G12D; G12R; G12S; G12V; G13C; or G13D.

5. The method of claim 1, wherein determination of the presence of a wild-type KRAS gene in the tumor sample comprises contacting nucleic acid from said tumor sample with a nucleic acid probe that is capable of hybridizing to nucleic acid comprising a KRAS gene or fragment thereof, and detecting said hybridization.

6. The method of claim 1 or claim 3, wherein determination of the presence of a mutated KRAS gene in the tumor sample comprises contacting nucleic acid from said tumor sample with a nucleic acid probe that is capable of hybridizing to nucleic acid comprising a KRAS gene or fragment thereof, and detecting said hybridization.

7. The method of claim 5 or claim 6, wherein the nucleic acid probe hybridizes to a mutated KRAS gene or fragment thereof incorporating a mutation.

8. A method for determining whether a colorectal tumor in a human subject is not responsive to therapy with cetuximab comprising determining the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13, wherein the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13 indicates that the tumor is not responsive to treatment with cetuximab.

9. A method for determining whether a colorectal tumor in a human subject is not responsive to therapy with panitumumab comprising determining the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13, wherein the presence of a KRAS gene in a sample of said tumor that encodes a mutation at amino acid residue 12 or 13 indicates that the tumor is not responsive to treatment with panitumumab.

10. A method for determining whether a colorectal tumor in a human subject is responsive to treatment with cetuximab, comprising determining the presence of a wild-type KRAS gene in a sample of said tumor, wherein the presence of the wild-type KRAS gene indicates that the tumor in a human subject is responsive to treatment with cetuximab.

11. A method for determining whether a colorectal tumor in a human subject is responsive to treatment with panitumumab, comprising determining the presence of a wild-type KRAS gene in a sample of said tumor, wherein the presence of the wild-type KRAS gene indicates that the tumor in a human subject is responsive to treatment with panitumumab.

12. The method of claim 8 or claim 9, wherein said mutation at amino acid residue 12 or 13 is a G12A, G12C, G12D, G12R, G12S, G12V, G13C, or G13D mutation.

13. The method of claim 8 or claim 9, wherein determination of the presence of a KRAS gene that encodes a mutation at amino acid

residue 12 or 13 said tumor sample comprises contacting nucleic acid from said tumor sample with a nucleic acid probe that is capable of hybridizing to nucleic acid comprising a KRAS gene or fragment thereof, and detecting said hybridization.

14. The method of claim 10 or claim 11, wherein determination of the presence of a wild-type KRAS gene in said tumor sample comprises contacting nucleic acid from said tumor sample with a nucleic acid probe that is capable of hybridizing to nucleic acid comprising a KRAS gene or fragment thereof, and detecting said hybridization.

15. The method of claim 8 or claim 9, wherein determination of the presence of a KRAS gene that encodes a mutation at amino acid residue 12 or 13 in said tumor sample comprises sequencing the KRAS gene or a fragment thereof.

16. The method of claim 10 or claim 11, wherein determination of the presence of a wild-type KRAS gene in said tumor sample comprises sequencing the KRAS gene or a fragment thereof.

17. A method according to any one of claims 1, 3, or 8 to 11, substantially as hereinbefore described with reference to any one of the examples or figures.

```
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  51 LQRMFNNCEV VLGNLEITYV QRNYDLSFLK TIQEVAGYVL IALNTVERIP
 101 LENLQIIRGN MYYENSYALA VLSNYDANKT GLKELPMRNL QEILHGAVRF
 151 SNNPALCNVE SIQWRDIVSS DFLSNMSMDF QNHLGSCQKC DPSCPNGSCW
 201 GAGEENCQKL TKIICAQQCS GRCRGKSPSD CCHNQCAAGC TGPRESDCLV
 251 CRKFRDEATC KDTCPPLMLY NPTTYQMDVN PEGKYSFGAT CVKKCPRNYV
 301 VTDHGSCVRA CGADSYEMEE DGVRKCKKCE GPCRKVCNGI GIGEFKDSLS
 351 INATNIKHFK NCTSISGDLH ILPVAFRGDS FTHTPPLDPQ ELDILKTVKE
 401 ITGFLLIQAW PENRTDLHAF ENLEIIRGRT KQHGQFSLAV VSLNITSLGL
 451 RSLKEISDGD VIISGNKNLC YANTINWKKL FGTSGQKTKI ISNRGENSCK
 501 ATGQVCHALC SPEGCWGPEP RDCVSCRNVS RGRECVDKCN LLEGEPREFV
 551 ENSECIQCHP ECLPQAMNIT CTGRGPDNCI QCAHYIDGPH CVKTCPAGVM
 601 GENNTLVWKY ADAGHVCHLC HPNCTYGCTG PGLEGCPTNG PKIPSIATGM
 651 VGALLLLLVV ALGIGLFMRR RHIVRKRTLR RLLQERELVE PLTPSGEAPN
 701 QALLRILKET EFKKIKVLGS GAFGTVYKGL WIPEGEKVKI PVAIKELREA
 751 TSPKANKEIL DEAYVMASVD NPHVCRLLGI CLTSTVQLIT QLMPFGCLLD
 801 YVREHKDNIG SQYLLNWCVQ IAKGMNYLED RRLVHRDLAA RNVLVKTPQH
 851 VKITDFGLAK LLGAEEKEYH AEGGKVPIKW MALESILHRI YTHQSDVWSY
 901 GVTVWELMTF GSKPYDGIPA SEISSILEKG ERLPQPPICT IDVYMIMVKC
 951 WMIDADSRPK FRELIIEFSK MARDPQRYLV IQGDERMHLP SPTDSNFYRA
1001 LMDEEDMDDV VDADEYLIPQ QGFFSSPSTS RTPLLSSLSA TSNNSTVACI
1051 DRNGLQSCPI KEDSFLQRYS SDPTGALTED SIDDTFLPVP EYINQSVPKR
1101 PAGSVQNPVY HNQPLNPAPS RDPHYQDPHS TAVGNPEYLN TVQPTCVNST
1151 FDSPAHWAQK GSHQISLDNP DYQQDFFPKE AKPNGIFKGS TAENAEYLRV
1201 APQSSEFIGA
```

FIGURE 1

CCCCGGCGCAGCGCGGCCGCAGCAGCCTCCGCCCCCCGCACGGTGTGAGCGCCCGACGCG 60

GCCGAGGCGGCCGGAGTCCCGAGCTAGCCCCGGCGGCCGCCGCCGCCCAGACCGGACGAC 120

AGGCCACCTCGTCGGCGTCCGCCCGAGTCCCCGCCTCGCCGCCAACGCCACAACCACCGC 180

GCACGGCCCCCTGACTCCGTCCAGTATTGATCGGGAGAGCCGGAGCGAGCTCTTCGGGGA 240

GCAGCGATGCGACCCTCCGGGACGGCCGGGGCAGCGCTCCTGGCGCTGCTGGCTGCGCTC 300

TGCCCGGCGAGTCGGGCTCTGGAGGAAAAGAAAGTTTGCCAAGGCACGAGTAACAAGCTC 360

ACGCAGTTGGGCACTTTTGAAGATCATTTTCTCAGCCTCCAGAGGATGTTCAATAACTGT 420

GAGGTGGTCCTTGGGAATTTGGAAATTACCTATGTGCAGAGGAATTATGATCTTTCCTTC 480

TTAAAGACCATCCAGGAGGTGGCTGGTTATGTCCTCATTGCCCTCAACACAGTGGAGCGA 540

ATTCCTTTGGAAAACCTGCAGATCATCAGAGGAAATATGTACTACGAAAATTCCTATGCC 600

TTAGCAGTCTTATCTAACTATGATGCAAATAAAACCGGACTGAAGGAGCTGCCCATGAGA 660

AATTTACAGGAAATCCTGCATGGCGCCGTGCGGTTCAGCAACAACCCTGCCCTGTGCAAC 720

GTGGAGAGCATCCAGTGGCGGGACATAGTCAGCAGTGACTTTCTCAGCAACATGTCGATG 780

GACTTCCAGAACCACCTGGGCAGCTGCCAAAAGTGTGATCCAAGCTGTCCCAATGGGAGC 840

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TGCTCCGGGCGCTGCCGTGGCAAGTCCCCCAGTGACTGCTGCCACAACCAGTGTGCTGCA 960

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GGAATAGGTATTGGTGAATTTAAAGACTCACTCTCCATAAATGCTACGAATATTAAACAC 1320

TTCAAAAACTGCACCTCCATCAGTGGCGATCTCCACATCCTGCCGGTGGCATTTAGGGGT 1380

Figure 2a

GACTCCTTCACACATACTCCTCCTCTGGATCCACAGGAACTGGATATTCTGAAAACCGTA 1440

AAGGAAATCACAGGGTTTTTGCTGATTCAGGCTTGGCCTGAAAACAGGACGGACCTCCAT 1500

GCCTTTGAGAACCTAGAAATCATACGCGGCAGGACCAAGCAACATGGTCAGTTTTCTCTT 1560

GCAGTCGTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCTCAAGGAGATAAGTGAT 1620

GGAGATGTGATAATTTCAGGAAACAAAAATTTGTGCTATGCAAATACAATAAACTGGAAA 1680

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GAGCCCAGGGACTGCGTCTCTTGCCGGAATGTCAGCCGAGGCAGGGAATGCGTGGACAAG 1860

TGCAACCTTCTGGAGGGTGAGCCAAGGGAGTTTGTGGAGAACTCTGAGTGCATACAGTGC 1920

CACCCAGAGTGCCTGCCTCAGGCCATGAACATCACCTGCACAGGACGGGGACCAGACAAC 1980

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GGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTC 2640

CTGGACTATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGT 2700

Figure 2b

GTGCAGATCGCAAAGGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTG 2760

GCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTG 2820

GCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTGCCTATC 2880

AAGTGGATGGCATTGGAATCAATTTTACACAGAATCTATACCCACCAGAGTGATGTCTGG 2940

AGCTACGGGGTGACCGTTTGGGAGTTGATGACCTTTGGATCCAAGCCATATGACGGAATC 3000

CCTGCCAGCGAGATCTCCTCCATCCTGGAGAAAGGAGAACGCCTCCCTCAGCCACCCATA 3060

TGTACCATCGATGTCTACATGATCATGGTCAAGTGCTGGATGATAGACGCAGATAGTCGC 3120

CCAAAGTTCCGTGAGTTGATCATCGAATTCTCCAAAATGGCCCGAGACCCCCAGCGCTAC 3180

CTTGTCATTCAGGGGGATGAAAGAATGCATTTGCCAAGTCCTACAGACTCCAACTTCTAC 3240

CGTGCCCTGATGGATGAAGAAGACATGGACGACGTGGTGGATGCCGACGAGTACCTCATC 3300

CCACAGCAGGGCTTCTTCAGCAGCCCCTCCACGTCACGGACTCCCCTCCTGAGCTCTCTG 3360

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CCCATCAAGGAAGACAGCTTCTTGCAGCGATACAGCTCAGACCCCACAGGCGCCTTGACT 3480

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AAAAGGCCCGCTGGCTCTGTGCAGAATCCTGTCTATCACAATCAGCCTCTGAACCCCGCG 3600

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Figure 2c

CTTTCAAAGAGGTATATTTGAAAAAAAAAAAAAGTATATGTGAGGATTTTTATTGATTGG 4200

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GAAGAAGCTTGCTGGTAGCACTTGCTACCCTGAGTTCATCCAGGCCCAACTGTGAGCAAG 4320

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Figure 2d

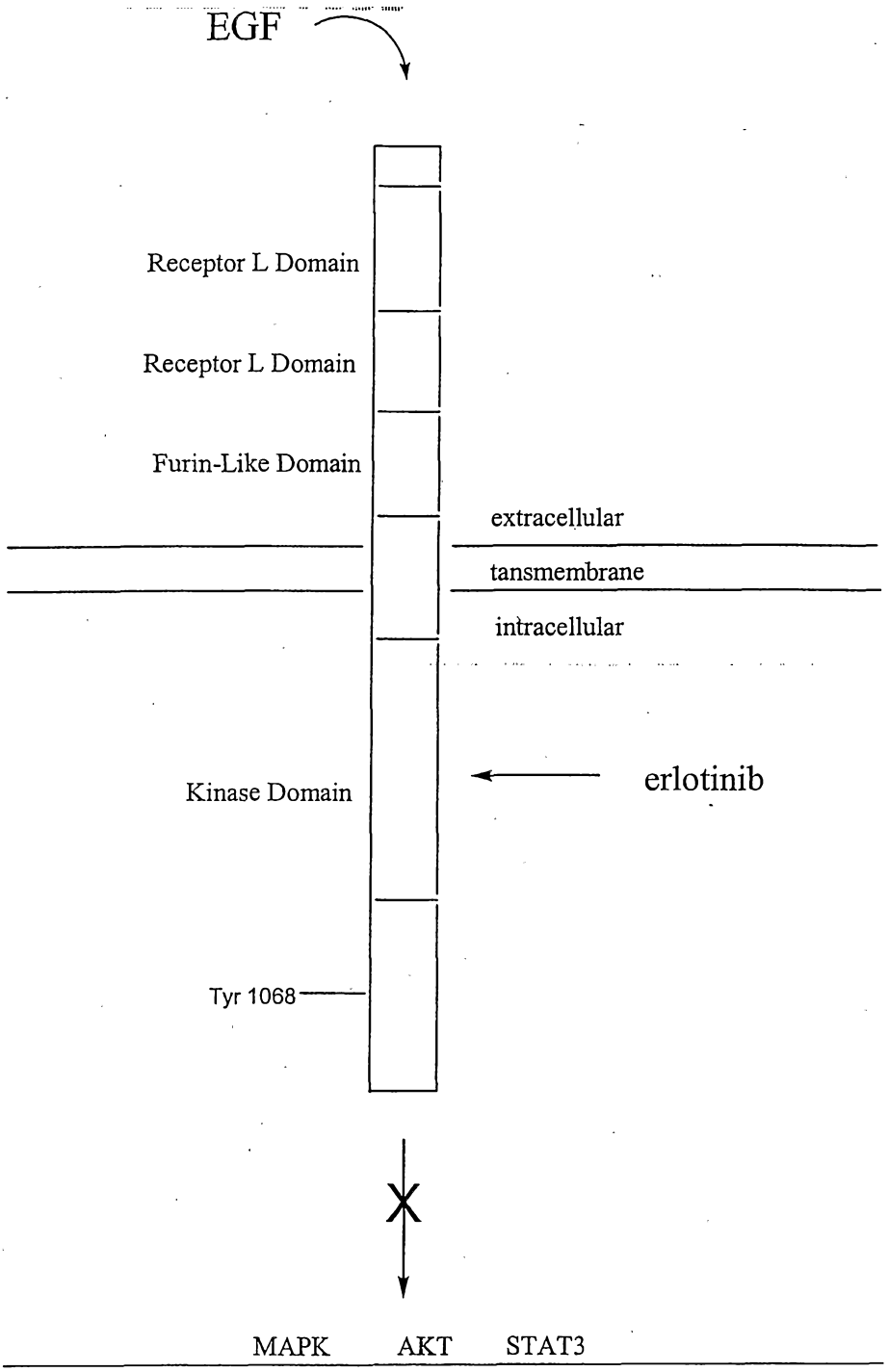
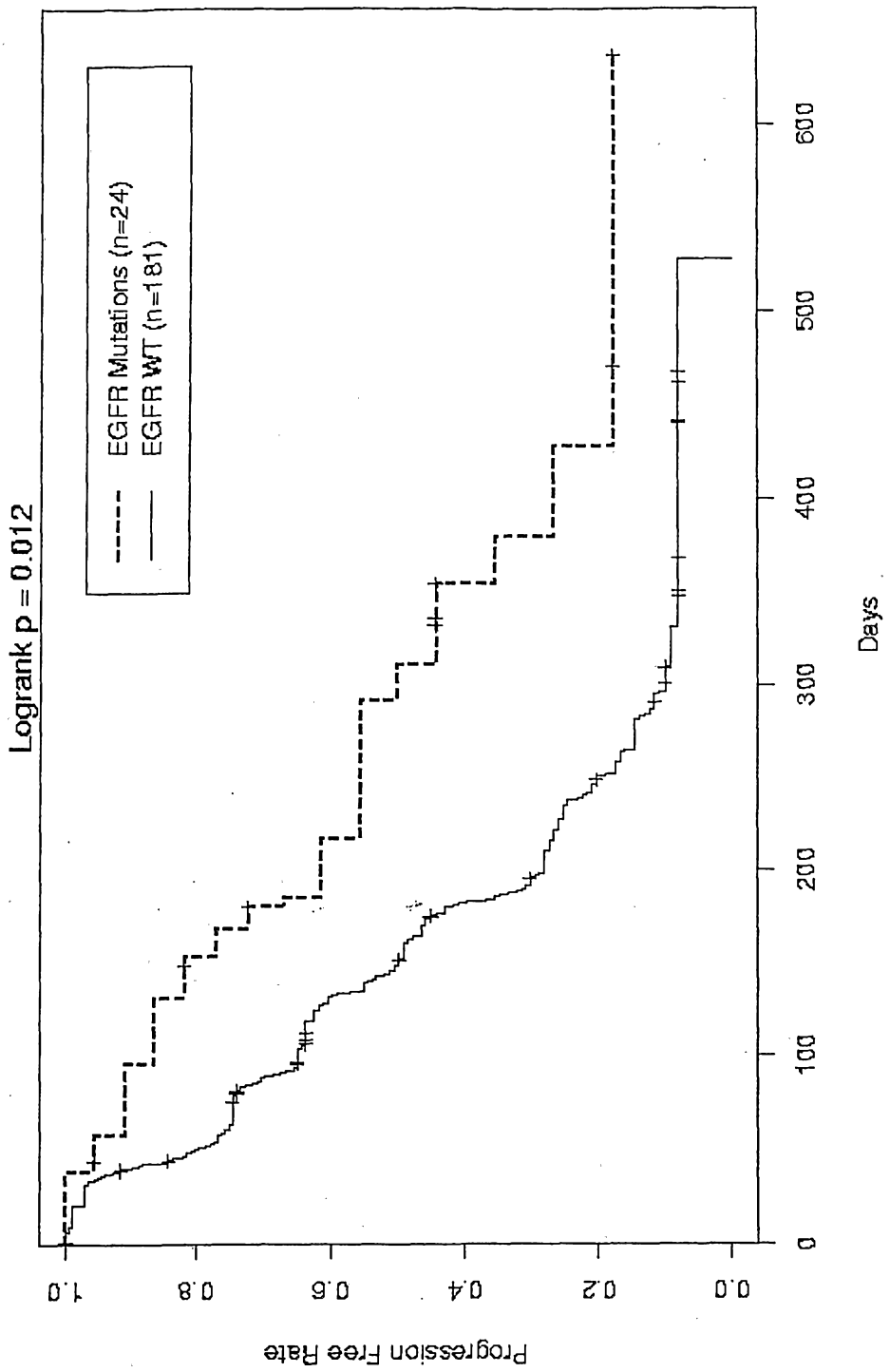

EGF
Receptor L Domain
Receptor L Domain
Furin-Like Domain
extracellular
tansmembrane
intracellular
Kinase Domain
erlotinib
Tyr 1068
MAPK
AKT
STAT3

Figure 3


Logrank p = 0.012
EGFR Mutations (n=24)
EGFR WT (n=181)
Progression Free Rate
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0.2
0.4
0.6
0.8
1.0
0
100
200
300
400
500
600
Days

Figure 4

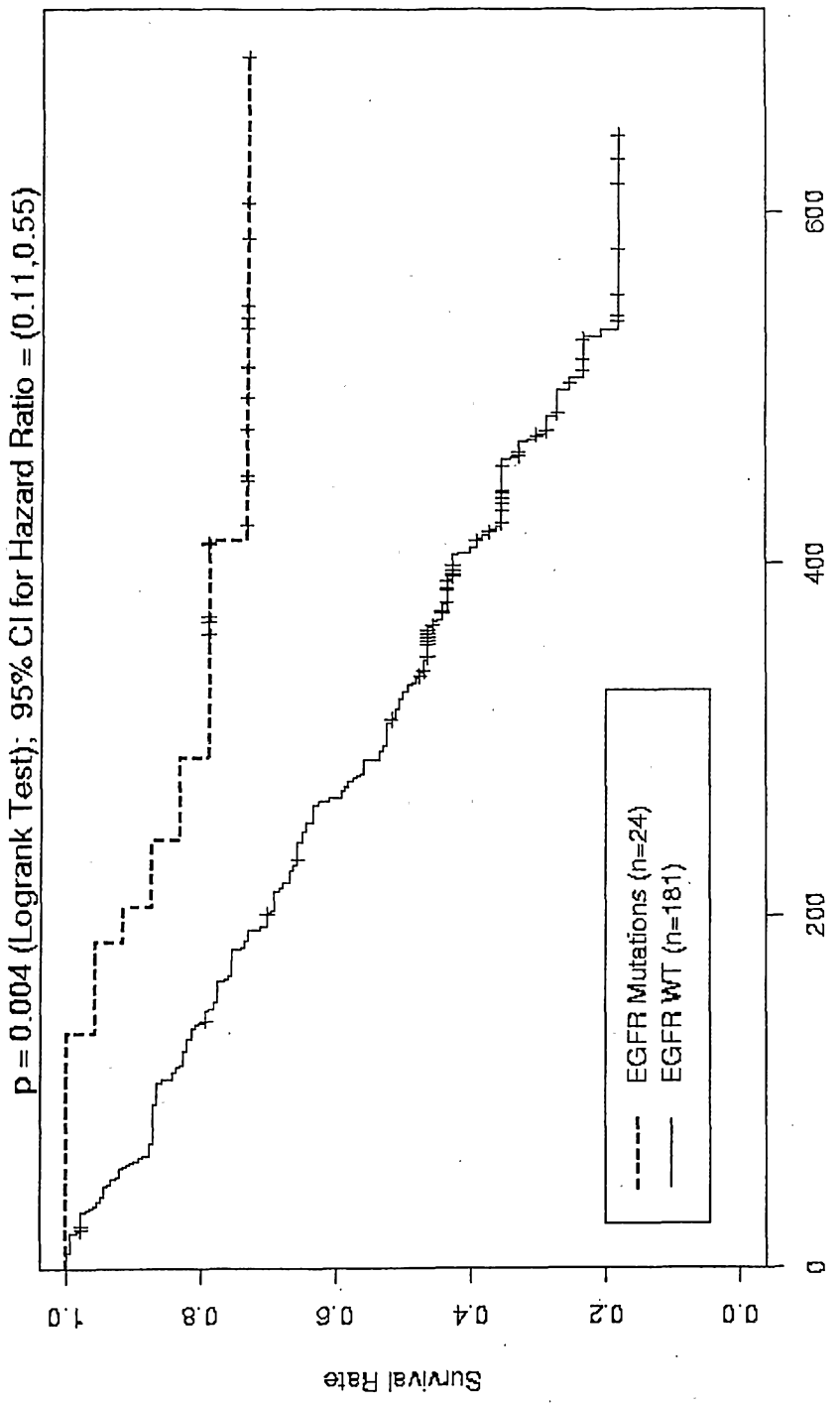
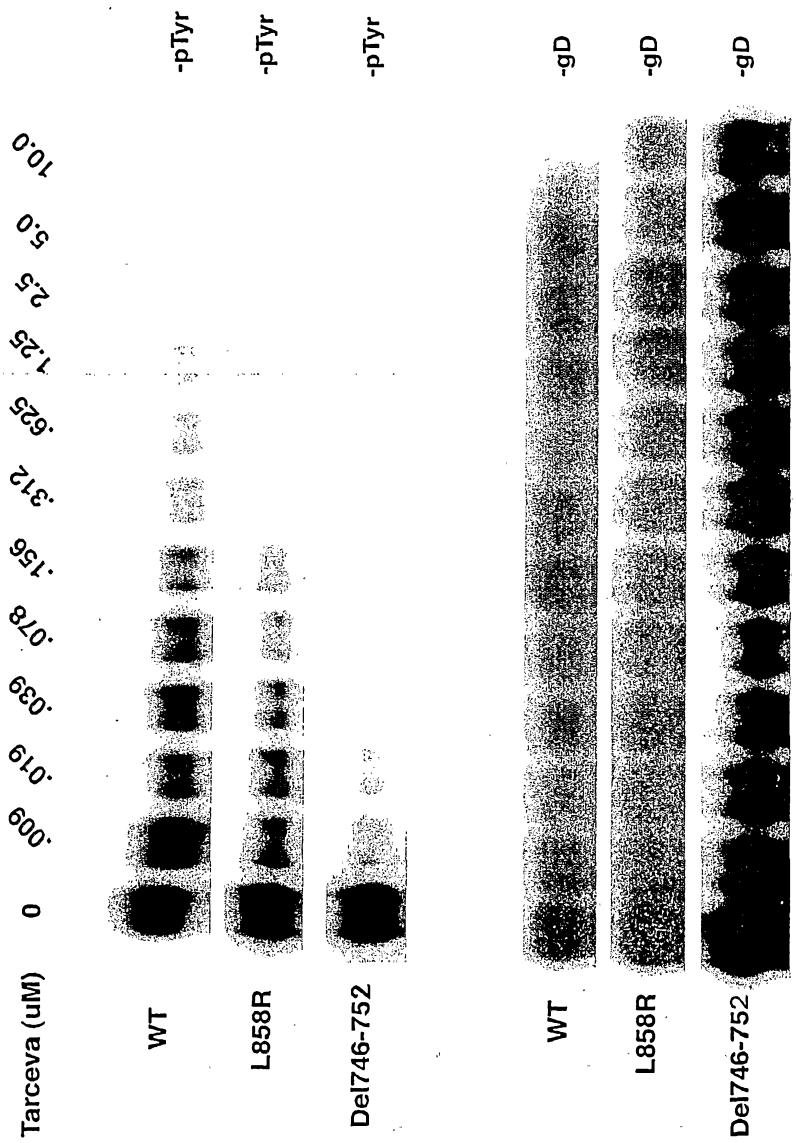

P = 0.004 (Logrank Test); 95% CI for Hazard Ratio = (0.11,0.55)
Survival Rate
0.0
0.2
0.4
0.6
0.8
1.0
0
200
400
600
Days
EGFR Mutations (n=24)
EGFR WT (n=181)

Figure 5


Tarceva (uM)
0
.009
.019
.039
.078
.156
.312
.625
1.25
2.5
5.0
10.0
WT
L858R
Del746-752
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-pTyr
-pTyr
WT
L858R
Del746-752
-gD
-gD
-gD

Figure 6

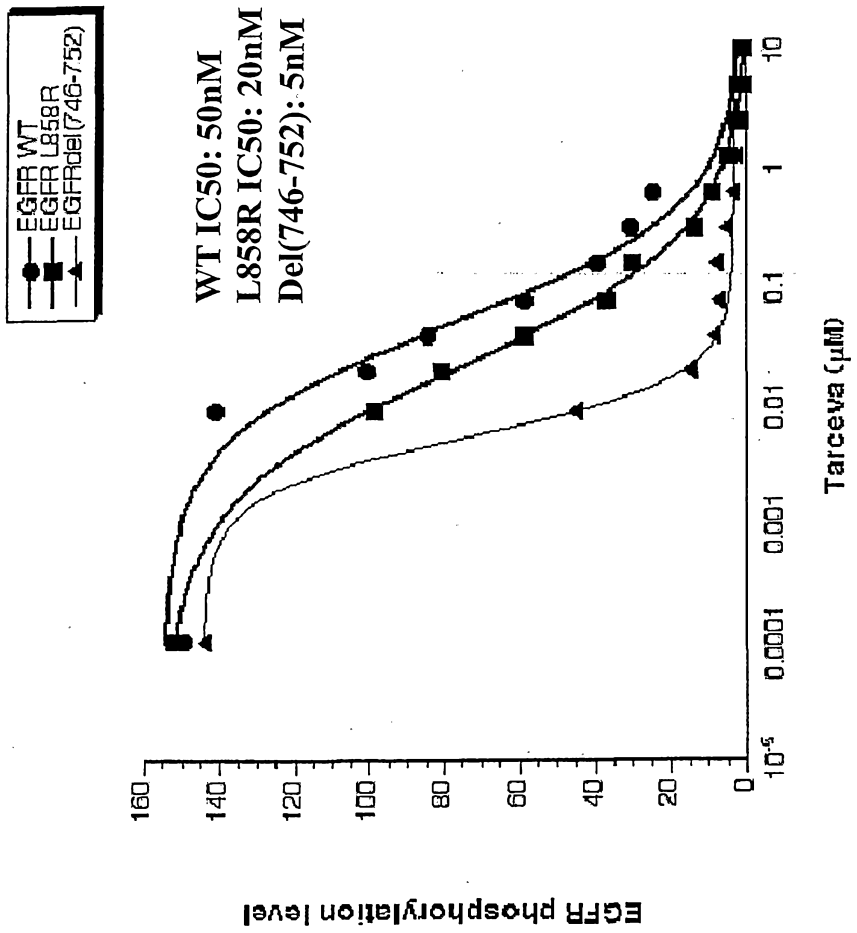
EGFR WT
EGFR L858R
EGFRdel(746-752)
WT IC50: 50nM
L858R IC50: 20nM
Del(746-752): 5nM
EGFR phosphorylation level
160
140
120
100
80
60
40
20
0
10⁻⁵
0.0001
0.001
0.01
0.1
1
10
Tarceva (μM)

Figure 7

EXON 18

EGFR protein	716	K V L G S G (SEQ ID NO: 3)
EGFR gene	2392	AAAGTGCTGGGCTCCGGT (SEQ ID NO: 4)
M1(G719A)		AAAGTGCTGTGCTCCGGT {KVLASG}
		(SEQ ID NO: 5) (SEQ ID NO: 6)
M2(G719C)		AAAGTGCTGGCCTCCGGT {KVLCSG}
		(SEQ ID NO: 7) (SEQ ID NO: 8)

EXON 19

EGFR protein	743	A I K E L R E A T S P K A N K E I L D
		(SEQ ID NO: 9)
EGFR gene	2473	GCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 10)
M3(E746-A750del)		GCTATCAA----------AACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 11)
M4(E748-R748del E749Q A750P)		GCTATCAAG---------CAACCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 12)
M5(L747S R748-P753del)		GCTATCAAGGAAT----------CGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 13)
M6(S752-I759del)		GCTATCAAGGAATTAAGAGAAGCAAC----------CCTCGAT
		(SEQ ID NO: 14)
M11(A755V)		GCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGTCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 15)
M12(L747S)		GCTATCAAGGAATCAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 16)
M13(E746K)		GCTATCAAGAAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
		(SEQ ID NO: 17)

Figure 8

EXON 20

EGFR protein	766	M A S V D N P (SEQ ID NO: 18)
EGFR gene	2542	ATGGCC---------AGCGTGGACAACCCC (SEQ ID NO: 19)
M7(M766-A767 AI ins)		ATGGCCATA---GCCAGCGTGGACAACCCC {MAIASVDNP}
		(SEQ ID NO: 20) (SEQ ID NO: 21)
M8(S768-V769 SVA ins)		ATGGCCAGCGTGGCCAGCGTGGATAACCCC {MASVASVDNP}
		(SEQ ID NO: 22) (SEQ ID NO: 23)
M9(S768I)		ATGGCC---------ATCGTGGACAACCCC {MAIVDNP}
		(SEQ ID NO: 24) (SEQ ID NO: 25)

EXON 21

EGFR protein	856	F G L A K L (SEQ ID NO: 26)
EGFR gene	2812	TTTGGGCTGGCCAAACTG (SEQ ID NO: 27)
M10(L858R)		TTTGGGCGGGCCAAACTG {FGRAKL}
		(SEQ ID NO: 28) (SEQ ID NO: 29)
M14(L858P)		TTTGGGCCGGCCAAACTG {FGPAKL}
		(SEQ ID NO: 30) (SEQ ID NO: 31)

Figure 9

20052504841 16 Jan 2009

<110> GENENTECH, INC.
SOMASEKAR SESHAGIRI

<120> EGFR Mutations

<130> 39766-0153 PCT

<140> Unknown
<141> 2005-06-02

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<160> 60

<210> 1
<211> 1210
<212> PRT
<213> Homo sapiens

<400> 1

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  1               5                  10                  15

Ala Ala Leu Cys Pro Ala Ser Arg Ala Leu Glu Glu Lys Lys Val
             20                  25                  30

Cys Gln Gly Thr Ser Asn Lys Leu Thr Gln Leu Gly Thr Phe Glu
         35                  40                  45

Asp His Phe Leu Ser Leu Gln Arg Met Phe Asn Asn Cys Glu Val
     50                  55                  60

Val Leu Gly Asn Leu Glu Ile Thr Tyr Val Gln Arg Asn Tyr Asp
 65                  70                  75

Leu Ser Phe Leu Lys Thr Ile Gln Glu Val Ala Gly Tyr Val Leu
             80                  85                  90

Ile Ala Leu Asn Thr Val Glu Arg Ile Pro Leu Glu Asn Leu Gln
         95                 100                 105

Ile Ile Arg Gly Asn Met Tyr Tyr Glu Asn Ser Tyr Ala Leu Ala
    110                 115                 120

Val Leu Ser Asn Tyr Asp Ala Asn Lys Thr Gly Leu Lys Glu Leu
    125                 130                 135

Pro Met Arg Asn Leu Gln Glu Ile Leu His Gly Ala Val Arg Phe
    140                 145                 150

Ser Asn Asn Pro Ala Leu Cys Asn Val Glu Ser Ile Gln Trp Arg
    155                 160                 165

Asp Ile Val Ser Ser Asp Phe Leu Ser Asn Met Ser Met Asp Phe
    170                 175                 180

Gln Asn His Leu Gly Ser Cys Gln Lys Cys Asp Pro Ser Cys Pro
    185                 190                 195
```

2005250484 16 Jan 2009

Asn Gly Ser Cys Trp Gly Ala Gly Glu Glu Asn Cys Gln Lys Leu
200 205 210

Thr Lys Ile Ile Cys Ala Gln Gln Cys Ser Gly Arg Cys Arg Gly
215 220 225

Lys Ser Pro Ser Asp Cys Cys His Asn Gln Cys Ala Ala Gly Cys
230 235 240

Thr Gly Pro Arg Glu Ser Asp Cys Leu Val Cys Arg Lys Phe Arg
245 250 255

Asp Glu Ala Thr Cys Lys Asp Thr Cys Pro Pro Leu Met Leu Tyr
260 265 270

Asn Pro Thr Thr Tyr Gln Met Asp Val Asn Pro Glu Gly Lys Tyr
275 280 285

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Pro Gly Leu Glu Gly Cys Pro Thr Asn Gly Pro Lys Ile Pro Ser
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