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MECHANISMS IN MALIGNANCY

THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) PATHWAY IN CANCER

PART I — LIGANDS, RECEPTORS AND DOWNSTREAM EFFECTORS

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PART I — LIGANDS, RECEPTORS AND DOWNSTREAM EFFECTORS

The epidermal growth factor receptor (EGFr) pathway plays critical roles in the regulation of cell growth, differentiation, and motility in normal as well as malignant cells. Aberrant activation of EGFr pathways is associated with oncogenesis, antiapoptosis, and other processes involved in malignancy, such as cellular proliferation, progression, resistance, invasion, migration, angiogenesis, and metastasis. At least six principal pathways of EGFr signaling support of cancer development and progression have been identified (Exhibit 1).

The EGFr pathway is the prototypical receptor system related to cancer. As long ago as the early 1980s, an anti-EGFr antibody was designed and used by John Mendelsohn, MD, and colleagues at the University of California, San Diego to shut down EGFr, which halted the spread of cancer in a xenograft mouse model (Masui H, etal, Cancer Res, Mar 1984;44(3):1002-7). This finding caused a stampede of scientists toward EGFr, and it became the receptor that "launched a thousand labs" (Vastag B, JNCI, 2005;97(9):628-30). Initiation of human clinical trials with anti-EGFr therapy followed in the 1990s amid great excitement and expectation of phenomenal response rates. Initial therapeutic approaches were based on monoclonal antibodies (MAb) designed to clamp down on the extracellular 'cup' of EGFr to block EGF and other ligands from binding and triggering the intracellular signaling cascade. The first approach was based Mendelsohn and Sato's anti-EGFr MAb, which was modified and eventually developed as cetuximab (Erbitux; ImClone). Trastuzumab (Herceptin; Genentech), an anti-ErbB (HEr2/neu) MAb was the first anti-EGFr therapeutic to be approved. It was approved for the treatment of HEr2-positive breast cancer, and its use was limited to patients with breast cancer who tested positive for HEr2 overexpression on a companion diagnostic test (HercepTest; Dako). A second therapeutic approach attacked the intracellular tyrosine kinase component of EGFr.

Along with therapeutic agents, new molecular tests for EGFr status were developed, and more are currently under development, including tests based on somatic mutations associated with various malignancies. Advanced testing approaches have been made possible by new developments in test technology and a wealth of studies and clinical trials, particularly those with pharmacogenomic components, which continue to yield findings with significance for development of new molecular tests and targeted therapeutics.

Much progress has been made since the earliest observations that patients with tumors expressing large amounts of EGFr protein show higher response rates to therapy than patients with lower amounts. Prognosis and theragnosis have come to the forefront, and the scientific race to identify mutations that predict response to anti-EGFr drugs continues to gain momentum. At present, drug resistance can be predicted more easily than drug efficacy; much more work needs to be done.

This article is Part I of a series that reviews the prominent role of the EGFr pathway in cancer. Part I outlines EGFr ligands, receptors, intracellular pathways, diagnostic/prognostic/theragnostic test methodologies, and commercial EGFr test development. Part II reviews the current knowledge base of the relationship of the EGFr pathway to clinical cancer indications. Part III describes the status of commercialized agents targeting the EGFr pathway and their performance in the clinic. Part IV describes numerous novel drugs in development targeting this pathway.

THE EPIDERMAL GROWTH FAMILY OF RECEPTORS (ERBB)

The ErbB family (Exhibit 2) comprises four wild type receptors, EGFr (erbB1/HEr1), erbB2 (HEr2, HEr2/neu, c-neu), erbB3 (HEr3), and erbB4 (HEr4). The members of this family are highly conserved across species and have both cellular and physiologic functions. The normal cellular functions of EGFr include proliferation and differentiation of many cell types. Also, EGFr plays critical roles in the normal tissue homeostasis and fetal development of a diverse spectrum of organisms. Mice lacking EGFr die soon after birth, exhibiting impairment of epithelial cell development in various organs, including the skin, lung, and gastrointestinal tract.

The four receptors in this family may be distinguished by their structures, dimerization properties, and ligand activation. Also, EGFr family members are distinguished from all other known receptor tyrosine kinases in possessing constitutive kinase activity without a phosphorylation event within their kinase domains (Stamos J, etal, J Biol Chem 2002; 277(48):46265-46272).

The extracellular, ligand binding regions of ErbB receptors consist of four domains that may assume at least two alternative conformations, extended and locked. ErbB receptors, with the exception of ErbB2, are dimerized in the presence of ligands, leading to receptor activation and internalization by endocytosis. Internalized receptors are either degraded in lysosomes or translocated to the nucleus, where they act as transcription factors or coregulators of gene transactivators. In either case, nuclear EGFr activates genes related to malignant processes. ErbB2 does not have a ligand and is not subjected to the same regulation as the other ErbB receptors. ErbB2 forms dimers with other erbB family members, which may slow internalization of the partner receptor, thereby abnormally prolonging its activation.

In the absence of ligand binding, ErbB1, ErbB3, and ErbB4 receptors adopt an autoinhibited conformation. Ligand binding induces a significant conformational change

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in the receptors, which promotes receptor dimerization and activation. Interestingly, heterodimers may form even when only one member of the pair binds its ligand. Each dimeric receptor complex initiates a distinct signaling pathway by recruiting different Src homology 2 (SH2)-containing effector proteins.

Epidermal Growth Factor (EGF) Receptor (EGFr or ErbB1)

The epidermal growth factor (EGF) receptor (EGFr or ErbB1) monomer consists of an extracellular ligand-binding domain, a helical transmembrane region, and a cytoplasmic portion (Exhibit 3). The large extracellular portion of the receptor (~100 kDa) contains two cysteine-rich domains that bind a number of ligands, including EGF, transforming growth factor α (TGFα), epiregulin, HB-EGF, epigen, amphiregulin, and betacellulin. The small (~3 kDa) central lipophilic segment anchors the receptor to the cell membrane. The intracellular domain (~60 kDa) is a protein kinase with a regulatory carboxyl (COOH)-terminal tail serving as a binding site for kinase substrates. The EGFr extracellular domain shares a 36% to 48% homology with the other members of the EGFr family, the kinase domain displays 60% to 82% homology, and the carboxyl-terminal segments are highly divergent with only 24% to 33% homology (Crovello CS, etal, J Biol Chem 1998;273:26954-26961). The carboxyl terminal region of EGFr contains endocytic motifs that regulate the internalization of EGFr.

ErbB1 is the prototypical member of the superfamily of receptors. It is widely expressed on many cell types, including those of epithelial and mesenchymal lineages, and overexpressed in a variety of human tumors. EGFr is the only homodimer in the group. ErbB2 is the preferential heterodimerization partner of EGFr, compared to the other erbB family members. EGFr is the only receptor in the group activated by EGF or TGFα.

ErbB2 (HEr2, HEr2/neu, c-neu)

ErbB2 (HEr2, HEr2/neu, c-neu) shares its structure with that of EGFr but lacks its own ligand-binding domain. HEr2 is also unique in that it appears to be fixed in an active-like conformation even in the absence of ligand, which may explain why no HEr2 ligand has been identified and why HEr2 readily dimerizes with its fellow receptors ErbB1 (HEr1), ErbB3 (HEr3), and ErbB4 (HEr4) and is the preferred binding partner for these three ErbB receptors. The fact that HEr2 does not adopt an autoinhibited conformation may also explain in part why it is unique among ErbB receptors in transforming cells when overexpressed in the absence of any ligand.

Ligand-activated ErbB1 preferentially heterodimerizes with HEr2 when both receptors are co-expressed in cells. Dimerization with ErbB2 often enhances the transforming capacity of other ErbB; in comparison with EGFr homodimers, EGFr-HEr2 heterodimers have an increased rate of recycling, stability, and signaling potency (Graus-Porta D, atal, EMBO J, 1 Apr 1997;16:1647-1655).

ErbB3 (HEr3)

ErbB3 (HEr3) also recapitulates the structure of erbB1 but lacks the carboxyl-terminal tail. This receptor is activated by members of the neuregulin (NRG) family of ligands, primarily NRG1/heregulin and NRG2. ErbB2 and ErbB3 form a high affinity heregulin coreceptor that elicits potent mitogenic and transforming signals. Activation of ErbB3/ErbB2 heterodimers can affect both proliferation and motility. Clinical studies indicate that these receptors play an important role in tumor incidence, progression, and metastasis. ErbB3-dependent signaling through ErbB3/ErbB2 heterodimers contributes to metastasis by enhancing tumor-cell invasion and intravasation *in vivo* (Yokoe S, etal, Cancer Res, 1 Mar 2007;67(5):1935-42, and Xue C, etal, Cancer Res, 1 Feb 2006;66(3):1418-26).

ErbB4 (HEr4)

ErbB-4 is a transmembrane receptor tyrosine kinase that regulates cell proliferation and differentiation. ErbB4 (HEr4) is activated by all members of the neuregulin family, as well as betacellulin, epiregulin, and HB-EGF.

After binding of heregulin or activation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA), the ErbB4 ectodomain is cleaved by a metalloprotease. Subsequent cleavage by γ-secretase releases the ErbB4 intracellular domain from the membrane facilitating its translocation to the nucleus where it activates gene expression in a more direct manner. The ErbB4 receptor undergoes proteolysis within its plasma membrane domain, a process called regulated intramembrane proteolysis (RIP), and its intracellular portion moves to the nucleus, where it may affect the transcription of target genes. This cleavage of ErbB4 may represent another mechanism for receptor tyrosine kinase-mediated signaling (Ni C-Y, etal, Science, 7 Dec 2001;294 (5549):2179-2181).

EGFR/LIGANDS

EGFr ligands are growth factors that are generally small proteins secreted by cells into the intercellular space. Ligand binding to the extracellular portion of the receptors induces dimerization. Dimers add phosphate groups to themselves (autophosphorylation), resulting in the creation of docking sites (tyrosine residues) in the cytosolic kinase domain that bind to downstream signal transduction molecules containing Src homology 2 (SH2) domains. Binding activates downstream pathways that eventually reach the nucleus, where they alter the expression of target genes.

Eight ligands, epidermal growth factor (EGF), transforming growth factor α (TGFα), epiregulin, heparin-binding EGF (HB-EGF), neuregulins 1-4, epigen, amphiregulin, and betacellulin (Exhibit 4), are the most significant of up to 30 ligands that are capable of activating the first, most upstream step in this complex, multi-layered signal-transduction network. Different activated ligand-receptor complexes vary in both the strength and type of cellular responses they induce (Yarden, Y, Eur J Cancer, Sep

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2001;37 (4):S3-8, Raab G and Klagsbrun M, Biochim Biophys Acta, 9 Dec 1997;1333(3):F179-99).

All ErbB receptor ligands are initially synthesized as transmembrane (membrane-anchored) precursors and subsequently released from the cell by proteolysis. Some growth factors are biologically active when retained on the cell surface, implying that surface localization may serve to restrict activity to the microenvironment, whereas release may lead to distal effects. In several cases, ectodomain release is critical for activation of EGFr ligands by sheddases. Membrane-anchored ligands may deliver signals that are qualitatively different from those provided by their soluble counterparts. Among sheddases of 6 primary EGFr ligands identified using mouse embryonic cells lacking candidate-releasing enzymes, ADAM10 emerged as the main sheddase of EGF and betacellulin and ADAM17 as the major convertase of epiregulin, TGFα, amphiregulin, and HB-EGF-like growth factor. Identification of EGFr ligand sheddases is a crucial step toward understanding the mechanism underlying ectodomain release and has implications for designing novel inhibitors of EGFr. Shed receptors may further serve to regulate the biological activity of these ligands by acting as agonists or antagonists, and receptor shedding may additionally render cells less responsive to their cognate ligands (Peschon JJ, etal, Science, 13 Nov 1998;282(5392): 1281-1284).

Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF) was one of the first growth factors to be discovered and is the prototype of a large family of closely related growth factors.

Transforming Growth Factor α (TGFα)

TGFα has been identified as a key modulator in the process of cell proliferation in both normal and malignant epithelial cells. The ectodomain of TGFα is released from cells by proteolysis by the protease tumor necrosis factor-α converting enzyme (TACE) to yield the soluble regulator. Extracellular TGFα binds to EGFr, activating the EGFr tyrosine kinase enzymatic activity, which triggers the intracellular signaling pathway.

Epiregulin

Epiregulin, a close relative of EGF, directly activates two members of the ErbB family, EGFr and ErbB4, as does betacellulin. There are, however, quantitative differences between these two moieties. Epiregulin stimulates higher levels of EGFr phosphorylation than betacellulin, while betacelllin stimulates higher levels of ErbB4 phosphorylation than epiregulin. ErbB2, which is not activated by epiregulin when expressed on its own, increases the sensitivity of epiregulin activation of ErbB4 (J Biol Chem, 1 May 1998;273(18):11288-11294).

The binding affinity of epiregulin for its receptors is lower than those of other EGF-family ligands. The secondary structure of the carboxyl-terminal domain of epiregulin is different from that of other EGF-family ligands because of the lack of hydrogen bonds. This structural difference may provide an explanation for the reduced binding affinity of epiregulin to the ErbB receptors (Sato K, etal, FEBS Lett, Oct 2003;553(3):232-8).

Epiregulin is regulated by 2,3,7,8-tetrachlorodibenzo-p-dioxin TCDD-activated aryl hydrocarbon receptor (Ahr). Ahr directly increases epiregulin expression, which could play an important role in TCDD-mediated tumor promotion observed in rodent models (Patel RD, Toxicol Sci, Jan 2006;89(1):75-82).

Heparin-binding EGF-like Growth Factor (HB-EGF)

Heparin-binding EGF-like growth factor (HB-EGF exerts its biologic activity through activation of EGFr and other ErbB receptors. Through the activation of signaling molecules downstream of ErbB receptors and interactions with molecules associated with HB-EGF, this factor participates in diverse biologic processes, including heart development and maintenance, skin wound healing, eyelid formation, blastocyst implantation, progression of atherosclerosis, and tumor formation. Recent studies have indicated that HB-EGF gene expression is significantly elevated in many human malignancies and its expression level in various cancer-derived cell lines is much higher than those of other EGFr ligands. HB-EGF appears to play a key role in the acquisition of malignant phenotypes, such as tumorigenicity, invasion, metastasis, and resistance to chemotherapy agents. Studies *in vitro* and *in vivo* have indicated that HB-EGF expression is essential for tumor formation of cancer-derived cell lines. Both CRM197, a specific inhibitor of HB-EGF, and an antibody against HB-EGF inhibit tumor growth in nude mice (Miyamoto S, etal, Cancer Sci, May 2006;97(5):341-7).

HB-EGF is an early response gene to chemotherapy and contributes to chemotherapy resistance. Chemotherapy-induced EGFr activation is regulated by HB-EGF. Overexpression of HB-EGF leads to apoptotic resistance to chemotherapy, whereas suppression of HB-EGF expression by siRNA results in a dramatic increase in cell death. Chemotherapy-induced HB-EGF activation represents a critical mechanism of inducible chemotherapy resistance, implying that therapeutic intervention aimed at inhibiting HB-EGF activity may be useful in cancer prevention and treatment (Wang F, etal, Oncogene, 25 Sep 2006; epub ahead of print).

Epigen

Epigen, the most recently described ligand of ErbB receptors, demonstrates intricate relationships between affinity and mitogenicity. Bioactive epigen, implicated in wound healing and cancer, is a highly mitogenic ligand of EGFr. Ectodomain shedding of epigen requires ADAM17.

The precursor of epigen is a widely expressed transmembrane glycoprotein that undergoes cleavage at two sites to release a soluble EGF-like domain. Recombinant epigen does not stimulate cells singly expressing ErbB2, but it acts as a mitogen for cells expressing ErbB1 and co-

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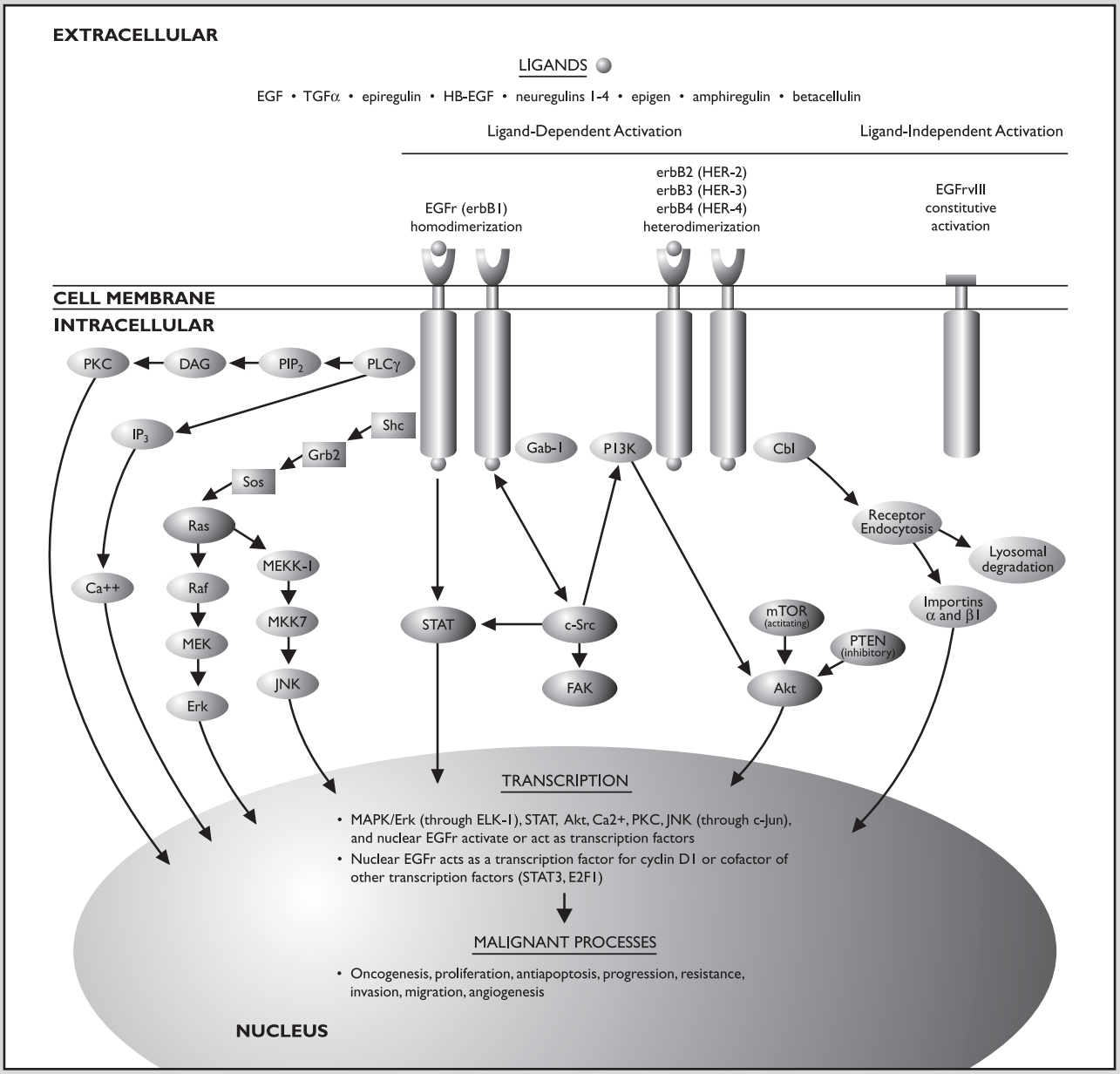
Exhibit 1
EGFr Family Receptors and Major Pathways



Legend:

- ***Akt:*** *cellular protein of v-akt oncogene*
- ***Cbl:*** *casitas-B-lineage lymphoma protein*
- ***c-Src:*** *non-receptor tyrosine kinase, cellular protein of v-Src gene*
- ***DAG:*** *1,2-diacylglycerol*
- ***E2F1:*** *member of E2F transcription factor family, cell cycle regulator*
- ***EGF:*** *epidermal growth factor*
- ***EGFr:*** *epidermal growth factor receptor*
- ***EGFrvIII:*** *epidermal growth factor receptor variant III*
- ***ELK-1:*** *serum response factor (SRF) accessory factor, activates transcription*
- ***Erk:*** *extracellular signal-regulated kinase*
- ***FAK:*** *focal adhesion kinase*
- ***Gab1:*** *Grb2-associated binder 1 adaptor protein*
- ***Grb2:*** *growth factor receptor-bound protein 2*
- ***HB-EGF:*** *heparin-binding epidermal growth factor*
- ***Importins:*** *mediate protein transport into the nucleus*
- ***IP_3:*** *inositol 1,3,5-triphosphate*
- ***JNK:*** *c-Jun NH2-terminal kinase (stress-activated protein kinase 1)*
- ***MAPK:*** *mitogen-activated protein kinase*
- ***MEK:*** *MAP/Erk kinase (a MAPK kinase)*
- ***MEKK:*** *MAPK kinase kinase*
- ***MKK:*** *MAPK kinase*
- ***mTOR:*** *mammalian target of rapamycin*
- ***PI3K:*** *phosphatidylinositol 3-kinase*
- ***PIP2:*** *phosphatidylinositol-4,5-bisphosphate)*
- ***PKC:*** *protein kinase C*
- ***PLCγ:*** *phospholipase Cγ*
- ***PTEN:*** *phosphatase and tensin homolog deleted on chromosome ten*
- ***Raf:*** *a MAPK, cellular protein of raf gene*
- ***Ras:*** *a small GTPase, cellular protein of ras gene*
- ***Shc:*** *SRC-homology–containing protein*
- ***Sos:*** *son of sevenless guanine nucleotide exchange factor*
- ***STAT:*** *signal transducers and activators of transcription*
- ***TGFα:*** *transforming growth factor α*

Source: New Medicine's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, February 2007

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expressing ErbB2 in combination with other ErbB. Soluble epigen is more mitogenic than EGF, although its binding affinity is 100-fold lower. The anomalous mitogenic power of epigen is attributed to evasion of receptor-mediated depletion of ligand molecules and inefficient receptor ubiquitylation and downregulation (Kochupurakkal BS, etal, J Biol Chem, 4 Mar 2005;280(9):8503-12).

Amphiregulin

Amphiregulin is a ligand of ErbB1. Like other ligands of EGFr, amphiregulin is synthesized as a precursor that is shed from the plasma membrane by metalloproteases. Amphiregulin is thought to play a non-redundant role in cancer development. Hyperactive autocrine loops involving amphiregulin production have been described in a variety of tumors.

Betacellulin

Betacellulin is a ligand for ErbB1 and ErbB4. The betacellulin precursor is a substrate for ADAM10-mediated ectodomain shedding.

Neuregulins

Neuregulins 1 (NRG1) and NRG2 are ligands for both ErbB3 and ErbB4, while NRG3 and NRG4 are ligands of ErbB4.

EGFr Downstream Signaling

Many of the features of the malignant phenotype, such as increased proliferation, angiogenesis, and evasion of apoptosis, are associated with the signaling networks that involve EGFr family members. Ligand binding and pathway activation is cell-dependent.

EGFr can be activated by either ligand-dependent or ligand-independent mechanisms. Regardless of the mechanism, activation of EGFr induces autophosphorylation of intracytoplasmic tyrosine kinase domains of the receptor. Autophosphorylation, which occurs in several tyrosine residues in the COOH-terminal tail of EGFr, induces docking sites for intracytoplasmic proteins with Src homology 2 and phosphotyrosine binding domains. The binding of these proteins to phosphotyrosine residues initiates intracellular signaling pathways related to EGFr activation. Major signaling cascades are described below. For a recent review of the EGFr pathway, see Scaltriti M and Baselga J, Clin Cancer Res, 2006;12(18):5268-72.

Ligand-dependent Activation

Ligand binding promotes the formation of dimers consisting of two of the proteins linked together, either with an identical protein, as in homodimerization, or a related protein, as in heterodimerization. Dimerization leads to phosphorylation of the intracellular tyrosine kinase domains, and the phosphorylated residues act as binding sites for other proteins and tyrosine kinase substrates.

Dimerization is mediated by a unique dimerization arm, which becomes exposed only after a dramatic domain rearrangement is promoted by growth factor binding. Ligand binding to EGFr results in receptor homodimerization; ligand binding to HER3 or HER4 results in receptor heterodimerization. ErbB2 has no ligand but forms heterodimers by constitutive exposure of its dimerization arm (Burgess AW, etal, Mol Cell, 2003;12(3):541-52). Ligand-dependent EGFr activation may occur as a result of overexpression of EGFr, which occurs frequently in malignancies, or overexpression of ligands.

Ligand-independent Activation

Ligand-independent activation can occur under particular conditions. It occurs in EGFr family members in which the extracellular domain is deleted, such as EGFr variant III (EGFrvIII), which has been associated with cancer. In cases in which the extracellular domain has been deleted, no site is available for ligand binding, and the receptor may be constitutively activated. Constitutive activation may cause constant autophosphorylation of intracytoplasmic tyrosine kinase domains (i.e., constant receptor activation). Ligand-independent activation may also occur as a result of overexpression of urokinase-type plasminogen activator receptor (via $\alpha5\beta1$ integrin) or as a result of cellular stress, such as radiation, which inhibits phosphatases that antagonize receptor tyrosine kinase activity.

Intracellular Signaling Pathways of the EGFr System

Currently, 6 major intracellular signaling pathways have been described involving members of the EGFr family. Simplified forms of these pathways are plotted in Exhibit 1.

Phospholipase Cγ (PLCγ) binds directly to activated EGFr. Binding to activated EGFr induces PLCγ-driven hydrolysis of phosphatidylinositol 1,3,5-diphosphate, which produces inositol 1,3,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 facilitates intracellular calcium release, which promotes transcription of target genes in the nucleus. DAG is a cofactor in protein kinase C (PKC) activation. Activated PKC activates the mitogen-activated protein kinases (MAPK) pathway. Through these pathways, PLCγ plays several roles in the development of cancer (Schonwasser DC, etal, Mol Cel Biol, 1998;18:790-8) and c-Jun NH(2)-terminal kinase (Kurokawa H and Arteaga CL, Clin Cancer Res, 2003 Jan;9(1 Pt 2):511S-5S). More directly, PLCγ functions as a key molecular switch in the dysregulation of cell motility and tumor migration, leading to metastasis (Wells A and Grandis JR, Clin Exp Metastasis, 2003;20(4):285-90).

Ras/Raf/mitogen-activated protein kinases (MAPK) are a group of serine/threonine kinases that play critical roles in cancer as regulators of cellular proliferation, survival, and migration. Upon EGFr dimerization and autophosphorylation, adaptor proteins bind directly, or through an adaptor molecule, to docking sites on the recep-

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tor. The activated EGFr dimer complexes with the adapter protein, Grb, coupled to the guanine nucleotide releasing factor, SOS. The Grb-SOS complex can either bind directly to phosphotyrosine sites in the receptor or indirectly through Shc. These protein interactions bring SOS in close proximity to Ras, enabling Ras-GDP recruitment and activation to become Ras-GTP (i.e., activated Ras). Ras-GTP then activates Raf-1, which, through intermediary steps, activates the MAPK and extracellular signal-related kinases-1 and -2 (Erk-1 and Erk-2, or Erk-1/2). Erk-1 and Erk-2 are then transported into the nucleus, where they phosphorylate specific transcription factors. Hyperactivation of MAPK, primarily via the Erk pathway, is a landmark of cancer. For a recent review of the four linear MAPK cascades, downstream mechanisms, the many links of MAPK to tumor biology, and the mechanisms that prolong MAPK signaling, see Katz M, etal, Biochim Biophys Acta, 2007 Jan 10; epub ahead of print.

Signal transducers and activators of transcription (STAT) comprise a family of latent cytoplasmic transcription factors that mediate cellular responsiveness to several cytokines and growth factors and convey these signals from the cytoplasm to the nucleus. After EGFr dimerization, STAT, via their Src homology 2 binding domains, interact with EGFr phosphotyrosine residues. STAT then translocate to the nucleus where they drive transcription of specifically targeted genes. Several endogenous regulators of the STAT pathway, which profoundly affect STAT signaling, are known.

STAT1, STAT3, and STAT5 are deregulated in a variety of human tumors. Increased activity of EGFr and HEr2 (and also PDGFr) is associated with persistent activation of STAT3 protein. STAT3 and STAT5 acquire oncogenic potential through constitutive phosphorylation of tyrosine, and their activity is required to sustain a transformed phenotype. STAT proteins promote oncogenesis and tumor progression (Klampfer L, Curr Cancer Drug Targets, 2006;6(2):107-21).

Src is the most prominent member of a 9-gene family of nonreceptor tyrosine kinases. Although independent in its function, Src cooperates with receptor tyrosine kinase signaling by activating many substrates, including phosphatidylinositol 3-kinase (PI3K), STAT proteins, focal adhesion kinase (FAK), which is involved in adhesion and migration, and others. Although Src was the first oncogene discovered (as the transforming protein of the Rous sarcoma virus) almost 30 years ago, the role of Src and the Src family kinases in human oncogenesis is still not completely understood because EGFr-Src interactions are complex. Src enhances EGFr activation and acts as a signal transducer; and elevated levels of Src kinase activity have been reported in various human malignancies *in vitro* and *in vivo*. However, through associations with other receptors or independent activity, Src may also contribute to resistance to EGFr therapies. Despite the various roles played by Src, it remains an attractive target for therapeutics development because it can activate STAT3, STAT5, and other downstream targets. Recent studies have shown that Src regulates cell adhesion, invasiveness, and motility in malignant cells and tumor vasculature, rather than exerting effects on cell replication (Homsi J, etal, Expert Opin Ther Targets, 2007;11(1):91-100).

Phosphatidylinositol 3-kinase (PI3K)/Akt is a serine/threonine kinase that is also known as protein kinase B (PKB). EGFr activation of the PI3K/Akt pathway is driven mostly by dimerization of HEr3. HEr3 dimerization induces activation of PI3K at phosphotyrosine binding domains. Then, through several intermediary steps, PI3K activates Akt, which then acts on transcription factors in the nucleus. Akt may also be activated or influenced by other mechanisms (e.g., inactivation of tumor suppressors). The EGFr homodimer activates Akt specifically through interaction with the docking protein Gab-1. mTOR, and other kinases also activate Akt. PTEN is a negative regulator of Akt. mTOR is a positive regulator of Akt. The PI3K/Akt pathway plays many roles in cancer. Akt is a critical enzyme in signal transduction pathways that affect transcription in the nucleus related to cellular growth, proliferation, and survival (Dillon RL, etal, Oncogene, 2007;26(9):1338-45). Other studies suggest roles for this pathway in resistance, invasion, migration, and angiogenesis. A role in diabetes is also under study.

Direct and indirect nuclear endpoints of EGFr signaling pathways are involved in transcription in the nucleus. Among others, direct pathways include the MAPK, Erk-1/2, STAT proteins, and Akt pathways, which act directly on transcription factors in the nucleus, affecting cellular proliferation and survival. Indirect pathways affecting transcription in the nucleus include Src kinase, which (among other activities) acts to enhance the STAT pathway, and PLCγ, which (among other activities) activates PKC, which then activates the MAPK and c-Jun NH2-terminal kinase pathways.

It is well established that EGFr transmits extracellular mitogenic signals (e.g., those generated by EGF and TGFα) by activating downstream signaling cascades (e.g., PLCγ, Ras, PI3K/Akt, and others), which leads to altered gene activities, which cause uncontrolled tumor proliferation, apoptosis, etc. Recent evidence suggests that the EGFr pathway also acts by a direct mode of action that is distinct from the signal transduction pathways. This direct mode of action involves cellular transport of EGFr, or direct nuclear translocation, from the cell surface to the cell nucleus. EGFr, HEr2, truncated HEr2, HEr3, and truncated HEr4 have been found to translocate to the nucleus. Although not well understood, it is known that EGFr may be internalized into the cytoplasm by endocytosis, after which it may undergo lysosomal degradation or, through a series of steps, be imported into the nucleus. In the nucleus, translocated EGFr acts as a transcription factor for target

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Exhibit 2
Transmembrane Receptors

Ligand	Description
	Ligand-Dependent Activators-Homodimers
Epidermal growth factor receptor (EGFr)/erbB1/HEr1	The epidermal growth factor receptor (EGFr) is a multisited, 170 kDa, multifunctional transmembrane glycoprotein with intrinsic tyrosine kinase activity. Upon ligand binding, the monomeric receptor undergoes dimerization resulting in kinase activation and phosphorylation of its own tyrosine residues (autophosphorylation), which is followed by activation of signal transducers. Deregulation of signaling because of EGFr gene amplification or rearrangement has been implicated in the development of a number of neoplasms. EGFr is expressed in normal tissue and overexpressed in many of the most prevalent human tumor types. Although EGFr regulates normal growth of many different cell types, it also can stimulate cancer cells to grow. In fact, many cancer cells require signals mediated by EGFr for survival. EGFr resides on the surface of tumor cells and is activated when naturally occurring proteins, such as EGF or transforming growth factor α (TGFα), bind to it, changing its shape, thus triggering internal cellular signals that stimulate tumor cell growth. When the intrinsic kinase is activated and EGFr tyrosyl-phosphorylates itself, numerous intermediary effector molecules are activated, including closely related c-erbB receptor family members. This initiates various signaling pathways, some of which attenuate receptor signaling. The integrated biologic responses to EGFr signaling are pleiotropic and include mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation. In addition to being implicated in organ morphogenesis, maintenance and repair, upregulated EGFr signaling has been associated with a wide variety of tumors with progression to invasion and metastasis. EGFr represents a potential target for therapeutic intervention in cancer (Wells A, Int J Biochem Cell Biol, Jun 1999;31(6):637-43).
	Ligand-Dependent Activators-Heterodimers
HEr2/neu (erbB2)	HEr2/neu is a 185 kDa transmembrane RTK belonging to the EGFr family. Despite similarity in sequence and predicted structure to other RTK, HEr2/neu does not have a ligand-binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGFr family members to form heterodimers, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signaling pathways, such as those involving mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K). Amplification and/or overexpression of this gene has been reported in numerous malignancies. Alternative splicing results in several additional transcript variants, some encoding different isoforms and others that have not been fully characterized to date. In human malignancies, HEr2/neu may function solely as a shared coreceptor for multiple stroma-derived growth factors. For example, it is likely that heterodimerization of HEr2 with other receptor tyrosine kinases such as ErbB3 and ErbB4, leads to trans-tyrosyl phosphorylation of the cytoplasmic regions of the multimeric receptor cluster (Pusztai L, etal, Cancer Treat Rev, Oct 1999;25(5):271-7). In addition to its oncogenic potential, HEr2/neu overexpression in tumors may render them intrinsically resistant to chemotherapy (Thor AD, etal, JNCI, 16 Sep 1998;90(18): 1346-60; Berry DA, etal, J Clin Oncol, 15 Oct 2000;18(20):3471-9).
HEr3 (erbB3)	HEr3, a member of the EGFr family of receptor tyrosine kinases, is a single membrane-bound protein with extensive homology to EGFr but lacking the C terminal catalytic domain. HEr3 has a neuregulin binding domain. Because HEr3 lacks an active kinase domain, however, it cannot convey the signal into the cell via protein phosphorylation. HEr3 does, however, form heterodimers with other EGFr family members with kinase activity.

— continued on next page

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	HEr3 acts as a proto-oncogene; amplification of the HEr3 gene and/or overexpression of HEr3 protein have been reported in many malignancies. Alternative transcriptional splice variants encoding different isoforms have been characterized. One isoform lacks the intermembrane region and is secreted outside the cell. This isoform acts to modulate the activity of the membrane-bound form. The c-erbB3 gene encodes secreted and transmembrane RTK. ErbB3 was isolated and characterized by investigators at the National Cancer Institute (NCI). Characterization of the cloned DNA fragment mapped the region of v-erbB homology to three exons with closest identity of 64% and 67% to a contiguous region within the tyrosine kinase domains of EGFr and ErbB3 proteins, respectively. cDNA cloning revealed a predicted 148-kDa transmembrane polypeptide with structural features identifying it as a member of the ErbB3 gene family, thus designated as ErbB3. It was mapped to human chromosome 12q13 and shown to be expressed as a 6.2-kilobase transcript in a variety of normal tissues of epithelial origin (Kraus MH, etal, PNAS USA, Dec 1989;86(23):9193-7).
HEr4 (erbB4)	ErbB4, a member of the EGFr family, is a transmembrane RTK that regulates cell proliferation and differentiation. It is a single-pass type I membrane protein with multiple cysteine rich domains, a trans-membrane domain, a tyrosine kinase domain, a PI3K binding site, and a PDZ domain-binding motif. The protein binds to and is activated by neuregulins and other factors and induces a variety of cellular responses, including mitogenesis and differentiation. Multiple proteolytic events allow for the release of a cytoplasmic fragment and an extracellular fragment. After binding its ligand or activating protein kinase C (PKC), the ErbB4 ectodomain is cleaved by a metalloprotease. Subsequent cleavage by γ-secretase releases the ErbB4 intracellular domain from the membrane and facilitates its translocation to the nucleus.
Ligand-independent Activators-Constitutive Activation	
EGFrvIII	EGFrvIII, an alternatively spliced form of EGFr, is the result of the deletion of exons 2 to 7, which eliminates 801 bp from the extracellular domain of the receptor. EGFrvIII is a constitutively active and ligand-independent version of EGFr and is highly oncogenic. Unlike wild-type EGFr, which is also found on normal tissues, EGFrvIII is expressed in various tumors but not on normal tissues, including those that express the wild-type receptor. This finding suggests its use as a tumor-specific therapeutic target (Zalutsky MR, Q J Nucl Med, Jun 1997;41(2):71-7, Wikstrand CJ, etal, J Neurovirol, Apr 1998;4(2):148-58). Some malignancies that frequently spread to the brain express EGFrvIII. If the original tumor expresses EGFrvIII, the cells that spread to the brain also express the protein. However, the importance of EGFrvIII as a target is debated, and there have been conflicting reports on its prevalence in specific tumors. EGFrvIII, known as the 'type III' deletion', is one of the most common mutations of the EGFr gene in human cancer and results in a receptor lacking amino acids 6 through 273 (Humphrey PA, etal, Cancer Res, 1 Apr 1988;48: 2231-2238, Ekstrand AJ, etal, PNAS USA, 1 May 1992;89:4309-4313, and Wong AJ, etal, PNAS USA, 1 April 1992;89:2965-2969). This deletion removes all of extracellular domain I and part of domain II, which abolishes ligand binding, but confers stable homodimerization to the molecule. The result is a catalytically active complex capable of transducing signals independent of ligand association. The frequent association of EGFrvIII expression with tumor growth suggests that EGFrvIII provides a strong selective advantage (Nishikawa R, etal, PNAS USA, 1 Aug 1994;91:7727-7731). MAb directed against tumor-specific EGFrvIII antigen prevent cell proliferation *in vitro*, and provide antitumor immunity to mice with EGFrvIII-expressing melanoma (Sampson JH, et al, PNAS USA, 20 Jun 2000;97:7503-7508). Substantial localization of EGFrvIII to the endoplasmic reticulum contrasts that of wild-type EGFr, which is mainly localized in the plasma membrane (Ekstrand AJ, etal, Oncogene, 6 Apr 1995;10:1455-1460). This altered intracellular pattern of expression prolongs the half-life of the protein compared with wild-type EGFr, probably by interfering with normal endosomal ubiquitination and degradation. Glycosylation of EGFrvIII is probably important in inducing the active, dimerized EGFrvIII complex. Prevention of N-linked glycosylation prevents activation (Fernandes H, etal, J Biol Chem, 9 Feb 2001;276:5375-5383).

Source: New Medicine's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, February 2007

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genes (e.g., cyclin D1 gene) or as a cofactor of STAT3 and E2F1 transcription factors (Lo HW, etal, Breast Cancer Res Treat, 2006;95(3):211-8).

ABERRANT RECEPTOR/LIGAND STATUS

Abnormal EGFr signaling, caused by ligand overproduction and/or receptor dysfunction, is often associated with adverse prognosis and can be useful for selection of targeted therapeutics. Abnormal signaling may result from increased gene copy number (amplification), overexpression, ligand overproduction (autocrine stimulation), or activating mutations. The presence of amplification and ligand overexpression is associated with a poorer prognosis than increased expression of either the receptor or activating ligand alone.

Gene Amplification

Malignant cells may have hundreds of copies of the normal ErbB receptor genes, referred to as double-minutes because they come in pairs. The normal cell expresses between 40,000 and 100,000 receptors on its surface, while in tumor cells this number may exceed 2 million. EGFr gene amplification, however, is uncommon.

Autocrine Stimulation

Autocrine stimulation describes elevated production and expression of both the receptor and its activating ligands by tumor cells. Concomitant overexpression of EGFr and its ligands is present in many tumors. Overproduction of EGF or TGFα transforms cells in culture and stimulate expression of EGFr.

Mutations

The roles of EGFr signaling abnormalities and mutations in cancer indications will be reviewed in part II of this article.

Less than 3 years ago, it was discovered that activating mutations in the tyrosine kinase domain of the EGFr gene occurred in a subset of non-small cell lung cancer (nsclc) tumors, particularly adenocarcinoma, and predict, although not precisely, the response to small molecule tyrosine kinase inhibitors (TKI). EGFr mutations are limited to the first four exons (exons 18–21) of the tyrosine kinase (TK) domain. Two predominant somatic mutations account for 85% of all mutations. The Exon 21 L858R point mutation accounts for 39%, and exon 19 deletions (729-761), particularly deletion 15 (E746-A750), account for 46%. A third mutation type, exon 20 (762-823) insertions, accounts for another 9%. Taken together, these 3 mutations account for 94% of EGFr mutations (Gazdar AF, Minna JD, PLoS Med 2005;2(11): e377).

Before the recent discovery of EGFr kinase domain somatic mutations in nsclc, deletions of the EGFr extracellular domain (i.e., EGFrvIII) were thought to be the most frequent EGFr mutations in the different tumor types. A truncated extracellular domain has an activating effect on EGFr; and cells expressing truncated receptors demonstrate a proliferative advantage. The deletion mutant, EGFrvIII (delta 801EGFr, del2-7 EGFr), is the most common truncated receptor. It was initially characterized at the genomic level in glioblastoma and is present in approximately 5% of human lung squamous cell carcinoma (SCC) but not in adenocarcinoma (Hongbin J, etal, PNAS USA 2006;103(20):7817-7822).

Common somatic mutations of the EGFr gene include:

- EGFrvIII (in-frame deletion of exons 2–7 (801 bp)
- Exon 21 L858R point mutation
- Exon 19 deletion 15 (E746 - A750)
- Other exon 19 deletions (18)
- T790M
- L858R
- L861Q
- G719S, G719A, G719C
- S768I
- Exon 20 (3 insertions)
- Mutant base at position 2369

DIAGNOSIS/PROGNOSIS/THERAGNOSIS/ PHARMACOGENOMICS

New molecular technologies in the fields of genomics and proteomics and their applications in molecular pathology have significant clinical potential in the diagnosis, prognosis, and treatment (e.g., theragnosis) of cancer. An overview of recent advances and their implications was presented at a session held at the September 2006 meeting of the AACR.

The EGFr family receptors, ligands, and signaling pathways comprise the preeminent and most well defined 'cancer gene' system. Genomic changes in the EGFr system drive transformation of normal cells into malignant derivatives and progression into invasive cancer. Better understanding of the tumorigenic roles of EGFr system members at the molecular level has led to the development of targeted drugs and molecular diagnostics.

As such, new molecular technologies have relevance to, and are in various stages of development for, applications related to the EGFr pathway. Although research in identifying the molecular origins of malignancy in all its manifestations has been ongoing for decades, the primary motivation for the rapid transformation of research modalities into commercialized products was to develop a test to select patients to be treated with trastuzumab (Herceptin; Genentech). The link between patient selection and drug treatment options is becoming the norm. More and more drugs are being used for indications where the status of a molecular marker appears to be a critical determinant of an individual patient's response to a drug. It is a win-win situation; the right patient gets the right drug, ineligible patients are not treated with expensive, often toxic agents, and a considerable outlay of funds and services is avoided when treatment reponses cannot

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rationally be predicted. Testing methodologies will be increasingly linked to treatment guidelines for specific agents and used.

New diagnostic technologies are also expanding classification of tumors based on individual genetic, proteomic, and functional profiles. Still, many challenges remain, particularly for the improved treatment of individual patients based on genetic and proteomic aspects of their individual tumors. Applications of these new technologies are expected to have an increasing impact on the discovery and development of targeted therapies and development of diagnostic and predictive tumor determinants that will guide their use.

The field of molecular oncology is having an impact on every aspect of cancer drug development, from basic R&D, lead selection, and optimization to preclinical evaluation to clinical trial design, patient selection, endpoint determination (e.g., surrogate markers), to disease monitoring.

Pharmacogenetic profiling of individual tumors is needed because of the substantial inter-individual variation in anticancer drug targets, disposition, efficacy, and toxicity. Variations in specific genes that are in the target cellular pathway or influence absorption, metabolism, or disposition of drugs, as well as the genetic make-up of the tumor may determine whether a particular therapeutic would be efficacious. Pharmacogenetic profiles help understand the functional consequences of chemoprevention, chemotherapy, or radiotherapy response and toxicity, as they relate to an individual patient. The goal, promise, and potential of pharmacogenetics is to enable clinicians to select therapeutics that are most effective and least toxic for individual patients.

The evolution of higher throughput and low cost molecular technologies is allowing comprehensive evaluation of changes in tumor DNA (genomic analysis), RNA (transcriptional profiling), and proteins (proteomic analysis) and the determination of the consequences of those changes. An individual patient's personal genetic make-up (SNP analysis) can be used to identify molecular markers of susceptibility to toxic effects of particular drugs.

Along these same lines, the use of biomarkers also characterize individual patient risk factors, the biological nature or tumors, and early and late responses to therapy that are essential for more efficient and effective development of cancer therapeutics.

Pharmacogenetic profiling approaches for selecting patients who are most likely to respond to a particular drug's mechanism of action are expected to allow developers to claim indications of efficacy based on smaller clinical trials, which may speed the drug development process. Also, the increased use of biomarkers is likely to more often become a regulatory requirement. Although diagnostics have been historically developed and approved in much shorter timeframes, it is anticipated that, moving forward, regulatory approvals of diagnostics and therapeutics will be much more tightly linked. This linkage will raise new clinical development and regulatory challenges.

Maximum tolerated dose (MTD), the current holy grail of clinical endpoints, may not be the optimal metric to determine dosing of targeted (and often cytostatic) drugs. Genomic assays, tissue biomarkers, and other technologies are under study for use in optimizing cancer therapy. These approaches identify appropriate patients for specific targeted therapy; analyze individual genetic alterations that may affect drug metabolism, transport and pharmacokinetics; measure early drug pharmacodynamics; and relate these parameters to tumor response.

Increased knowledge of the crucial genes and proteins expressed in tumors, mechanisms of activation, and the signaling pathways related to cancer, is leading to the emergence of genomic signatures that are associated with important clinical features.

More reliable assays and models that improve the ability to predict clinical outcome on an individualized basis are needed. A great diversity of methodologies is under investigation for reliability in predicting and optimizing therapeutic responsiveness of human tumors. Genomics approaches are providing insights into the genetic determinants that characterize tumors and tumor subtypes.

Current Clinical Laboratory Detection Methodologies

The principal technologies commonly employed in the clinical laboratory for HEr2 and EGFr testing are immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), and fluorescent *in situ* hybridization (FISH). Polymerase chain reaction (PCR), a DNA amplification technique, is also used in many test systems to increase sensitivity.

Immunohistochemistry (IHC) is a method for detection and visualization of proteins within a cell or on the cell surface, in a tissue section. Monoclonal or polyclonal antibodies are used to target specific cells or tissue antigens, followed by immunoenzymatic staining for visualization by traditional light microscopy. Antibodies may be conjugated to an enzyme that catalyzes a color-producing reaction (chromogenic stains such as horseradish peroxidase, DAB, AEC, Fast Red) that is visible by light microscopy. Alternatively, immunofluorescence may be employed, in which antibodies are conjugated to a fluorophore (e.g., rhodamine) for visualization by light microscopy or specialized microscopic methods (e.g., confocal laser scanning microscopy can visualize interactions between multiple proteins). IHC is widely used to identify molecular markers useful in cancer diagnosis and drug development, including HEr2 and EGFr.

Enzyme-linked immunosorbent assay (ELISA) is another method for detection of an antigen within a tissue sample. Two antibodies are employed. One is specific to the antigen; the other, which is coupled to an enzyme, reacts

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to the antigen-antibody complexes. This second antibody is linked to a chromogenic or fluorogenic substrate as in IHC. ELISA is used to determine the presence of antigens (or antibodies) in a sample and for determining serum antibody concentrations.

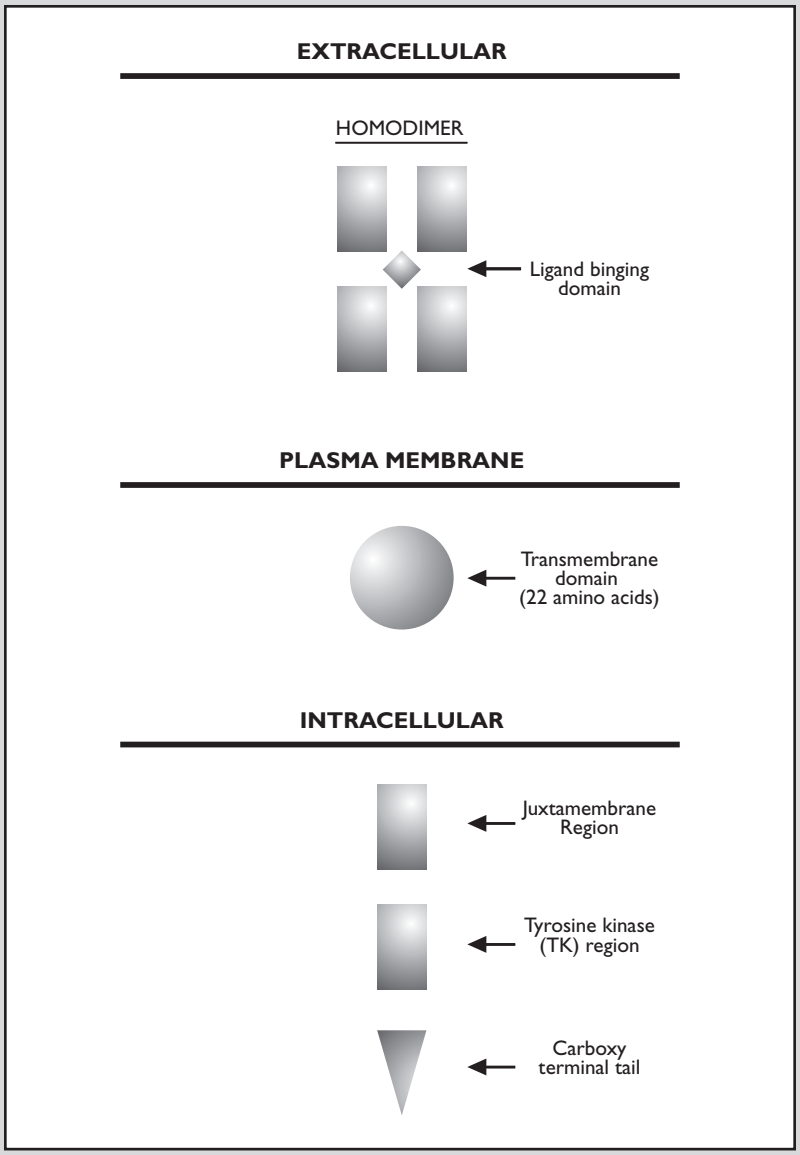
Fluorescent in situ hybridization (FISH) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH probes bind only to sequences with a high degree of similarity and may be designed to detect the absence or presence of very short sequences or with much less resolution to detect gross chromosomal characteristics. Quantification in FISH studies is achieved by counting fluorescent dots or making color comparisons. An alternative to FISH, known as chromogenic *in situ* hybridization (CISH) has been developed by Invitrogen and is addressed below.

Polymerase chain reaction (PCR) is an *in vitro* technique for enzymatic replication of DNA outside of a living organism (e.g., E. coli, yeast). PCR permits exponential amplification of DNA, much like that which occurs in living organisms, and is used with many detection methodologies to increase sensitivity.

High-resolution melting analysis (HRMA) is a recently introduced closed-tube fluorescence-based method for rapid mutation screening and detection. HRMA distinguishes specific mutations from the wild type sequence with little labor and time and at low cost. PCR and the melting analysis are performed in the same capillary tube within a few hours, at a cost of only about $1 per sample. In a recent study, HRMA was shown to be a practical and precise method for detection of DEL and L858R mutations, EGFr mutations that predict a better response and longer survival in patients with nsclc treated with gefitinib (Takano T, etal, ASCO06, Abs. 7075). HRMA is expected to become a practical method for detection of major EGFr mutations in cytologic materials from patients with nsclc, even when archived cytologic samples are used.

Quantitative mass spectrometry is another new, promising detection methodology. In one study it was used to characterize the dynamics of tyrosine phosphorylation in human mammary epithelial cells with varying HEr2 expression levels after treatment with EGF or heregulin. A computational linear mapping technique, partial least squares regression (PLSR), was used to detail and characterize signaling mechanisms responsible for HEr2-mediated effects (via changes in tyrosine phosphorylation) on migration and proliferation. Endocytosis and activation of PI3K-mediated pathways together representedparticularly strong loci for the integration of the multiple pathways mediating HEr2 control of mammary epithelial cell proliferation and migration. The PLSR modeling approach reveals critical signaling processes regulating HEr2-mediated cell behavior (Kumar N, etal, PLoS Comput Biol 2007;3(1): e4; published online 2007 January 5).

Exhibit 3
Structure of the Epidermal Growth Factor Receptor (EGFr)



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RT-PCR for detection of EGFrvIII is a sensitive, one-step RT-PCR method for detection of EGFrvIII mRNA from total tumor RNA, which was developed to help resolve discrepancies in the presence of EGFrvIII in various tumors. The competitive effect of the wild type (wt) EGFr sequence was overcome by designing primers with dideoxy-C termini from sequences in exons 2 and 7 to inhibit amplification of wt EGFr. The high G-C content and RNA secondary structure of EGFrvIII were overcome by adding DMSO to a final concentration of 10%, which enhanced amplification. Slight degradation of total RNA further increased amplification efficiency. Using this method, 56% (13/23) of primary breast carcinomas, 52% (25/48) of ovarian tumors, and 56% (9/16) of colon malignancies were found positive for EGrRvIII, which is comparable to published IHC results (Trotter MG, Wong AJ, AACR05, Abs. 194).

Newer detection methodologies are in limited use or under evaluation for detection of EGFr mutations, particularly to address the detection challenge posed by small tumor samples contaminated with normal cells. Studies of some of the newer techniques report impressive results for EGFr mutation detection. However, they often require intensive labor or sophisticated instruments, which has limited or prevented their adoption in clinical practice.

About 90% of sequence variants in humans are differences in single bases of DNA, called single nucleotide polymorphisms (SNP). When two individuals are compared, their genomic DNA differs at ~1/1000 nucleotides. Neutral polymorphisms may be responsible for subtle differences between individuals, such as hair and eye color, or may be silent identifiers of variability and relatedness. Other polymorphisms cause genetic diseases such as hemophilia and are referred to as mutations. Polymorphisms within an individual may cause cancer or other diseases, are responsible for the variability of immune responses, and have been implicated in the aging process. Identification of polymorphisms can therefore be useful in diagnosis of genetic disease, detection of tumors, study of immune response and aging, identification of microbial strains, and developing relatedness trees between humans or other organisms.

Several methods exist to detect single nucleotide DNA polymorphisms, the two most common being DNA sequencing and restriction fragment-length polymorphism (RFLP) analysis. Sequencing of cloned genomic DNA or polymerase chain reaction PCR-amplified products is the most direct polymorphism detection method, but can be labor intensive and expensive. RFLP analysis of restricted genomic DNA by Southern blotting, or of digested PCR products by agarose gel electrophoresis requires that the polymorphism create or eliminate a restriction site. Both sequencing and RFLP analysis are ineffective when the polymorphism exists as a small percentage of the total DNA population. Allele-specific PCR-based methods of polymorphism identification are able to detect small levels of point mutations, but the approach is limited by the fact that most primer-template mismatches have no significant effect on the amplification process.

HEr2 Testing: IHC versus FISH

The clinical benefit of HEr2 testing is well established. In clinical data from trials in the mid- and late-1990s HEr2 emerged as a prognostic factor and predictor of disease-free and overall survival in breast cancer. Furthermore, data from the initial efficacy trials with trastuzumab (Herceptin; Genentech), a chimeric human-mouse MAb that binds an exposed portion of the HEr2/neu receptor on the cell surface, suggested that the beneficial treatment effects of the drug were largely limited to patients with tumors expressing high levels of HEr2 protein. HercepTest (Dako), an IHC test for HEr2 protein, was developed by Genentech and Dako as a companion diagnostic for Herceptin, specifically to identify patients with HEr2+ tumors for selection for Herceptin therapy. Since the approval of the original BLA for Herceptin in September 1998, FDA has required HEr2 testing for the selection of patients for treatment with the drug. The indication reads, "Herceptin should only be used in patients whose tumors have HEr2 protein overexpression."

Variability between different test results raised questions regarding the optimal method for selection of patients who might benefit from Herceptin. HercepTest results differed from the clinical trial assay, which were also IHC tests used to determine HEr2 overexpression. Among the questions was whether to treat patients with 2+ or 3+ HEr2 expression or only those with a 3+ level of expression. At that time, Genentech made a postmarketing commitment "to assess the clinical outcome of patients selected for treatment on the basis of the Dako HercepTest and other HEr2 diagnostics in the context of Herceptin clinical trials."

During the next several years a few refinements in IHC testing were made, and FISH testing for HEr2 was introduced. At a December 2001 clinical review meeting of the FDA Oncologic Drugs Advisory Committee (ODAC) Meeting, Genentech presented an sBLA requesting a labeling supplement for Herceptin to include FISH testing as a method to select patients for treatment. At that time the FDA did not consider HEr2 assessment to be straightforward for several reasons. There was significant variability in results between different laboratories. There was considerable off label use of IHC tests (with unapproved antibodies) and FISH tests. Direct comparative statements of equivalence or superiority between FISH and IHC could not be performed; and there were other problems. FDA seemed to agree that, in a preselected population, FISH is a useful method for selection of patients who are known to be IHC 2+ or 3+. In January 2002, the Vysis PathVysion HER2 DNA Probe was approved as a method to select patients for therapy with Herceptin.

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FDA Approved Products

Diagnostic tests related to HEr2 and EGFr are considered to be medical devices by FDA. In order to obtain FDA approval, manufacturers must submit either a premarket approval (PMA) application or a 510(k) submission. The latter requires demonstration of substantial equivalence to another legally marketed device (i.e., the new product is at least as safe and effective as the predicate). FDA approved HEr2 and EGFr tests are based on IHC detection of HEr2 or EGFr protein overexpression, ELISA detection of HEr2 overexpression, and HEr2 gene amplification determined by FISH. Exhibit 6 lists FDA approved HEr2 and EGFr tests, intended applications, and approval dates.

Abbott Molecular (Des Plaines, IL) markets the FDA approved PathVysion HER2 DNA Probe Kit, which detects HEr2 gene amplification status by FISH. The December 1998 approval describes intended use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in patients with Stage II, node-positive breast cancer. It is also indicated as an aid in predicting disease-free and overall survival in patients with Stage II, node-positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy. Since the introduction of Herceptin, this kit has been used to determine the appropriateness of initiating Herceptin therapy. In January 2002, the PathVysion HER2 DNA Probe was approved as a method to select patients for therapy with Herceptin. Vysis, which developed this and other PathVysion DNA probe products, is now a wholly owned subsidiary of Abbott Laboratories.

Applied Imaging (San Jose, CA) developed the Ariol technology for quantitative image analysis automated processing of *in vitro* diagnostic tests. The Ariol Automated FISH Enumeration System was approved in April 2005 as an accessory to the PathVysion kit. The approval allows the company's automated scanning microscope and image analysis system to detect amplification of the HEr2/neu gene via FISH in human breast cancer biopsy samples. This FDA indication complements and completes the breast cancer panel on the Ariol system. Applications for the HEr2/neu IHC, estrogen receptor, and progesterone receptor assays were previously approved. The breast panel indication is for *in vitro* evaluation as an aid to the pathologist in the detection, classification and counting of cells of interest based on particular color, intensity, size, pattern, and shape. Assay results, as an adjunct to clinical and pathologic information, are used as prognostic factors in patients with Stage II node-positive breast cancercer and to predict disease-free and overall survival with adjuvant CAF chemotherapy. The assay is also indicated as an aid in assessing the eligibility of patients with metastatic breast cancer for treatment with Herceptin.

BioGenex (San Ramon, CA) received FDA approval in December 2004 for the Insite HEr2/neu Kit for detection of HEr2 protein overexpression by IHC. The test was designed to determine eligibility of patients with breast cancer for Herceptin therapy. The kit was available for manual use and in an automated form compatible with the BioGenex i6000 Automated Staining System and the OptiMax Plus Consolidated Staining System. In October 2004 BioGenex entered into an agreement with Abbott to develop solutions using an automated system for Abbott's FISH-based tests. BioGenex granted Abbott distribution rights for marketing the new system worldwide in the FISH diagnostics field.

FDA requested a recall of the BioGenex InSite test, which began in early 2006, because "Products produce granular staining in immunostained tissue sections, which may lead to misdiagnosis from false positive result." Biogenex voluntarily ceased the sale of and recalled the InSite kits and antibodies. The company also voluntarily requested the withdrawal of the associated PMA (P040030 and P040030/S001). In July 2006 the company received a warning letter from the FDA regarding manufacturing practices and distribution of products that do not conform to specifications. According to FDA, the identified deficiencies are system failures and transcend the InSite HER-2/neu kit and antibody devices.

Dako (Glostrup, Denmark and Carpinteria, CA) markets three FDA-approved tests. HercepTest detects HEr2 protein overexpression by semi-quantitative IHC and is indicated as an aid in the assessment of patients for whom Herceptin treatment is under consideration. The HercepTest was developed in collaboration with Genentech and approved in September 1998.

Dako's HER2 FISH pharmDx Kit is a direct FISH assay designed to quantitatively determine HEr2 gene amplification in breast cancer tissue specimens. Gene amplification is determined from the ratio between the number of signals from the hybridization of the HEr2 gene probe and the number from the hybridization of the reference chromosome 17 probe. It was FDA approved in May 2005 for determination of appropriateness of Herceptin treatment in patients with breast cancer.

EGFR pharmDx Kit detects EGFr protein overexpression. It is FDA indicated as an aid in identifying patients with colorectal cancer who are eligible for treatment with Erbitux (cetuximab) or Vectibix (panitumumab). One kit is designed for manual use, a second kit is designed for use on the Dako Autostainer. FDA approval was granted in February 2004.

In March 2007, BioImagene (Cupertino, CA), a provider of digital pathology solutions, received FDA clearance for the use of its Pathiam Imaging Software for HER2/neu for *in vitro* diagnostic use. BioImagene's Pathiam is intended for use as an accessory to the Dako HercepTest to aid a pathologist in semi-quantitative measurement of HEr2/neu in breast cancer tissue. When used with the Dako HercepTest, it is an aid in the assessment of patients with breast cancer being considered for Herceptin treatment. The imaging software detects and classifies cells

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of interest by analyzing digitized images of microscope slides based on recognition of cellular objects of a color, size and shape.

Genzyme (Cambridge, MA) develops diagnostic tests and provides reference laboratory services on a nationwide basis through its Genzyme Genetics division. The company provides HEr2 testing services using products from other companies, including HercepTest and PathVysion.

Genzyme is developing a portfolio of genetic tests related to lung cancer. In 2004, the company acquired selected cancer testing assets of Impath to expand its cancer diagnosis capabilities. In a May 2005 agreement with the Massachusetts General Hospital and Dana-Farber Cancer Institute, Genzyme obtained exclusive, worldwide diagnostic rights to EGFr mutations found in some patients with nsclc, which were discovered by the two research groups. The presence of particular EGFr mutations has been shown to correlate with the clinical response to targeted lung cancer therapies, including the TKI, erlotinib (Tarceva; OSI Pharmaceuticals), and gefitinib. Genzyme has used the EGFr markers to develop three tests to help identify patients with nsclc who are most likely to respond to TKI. Research is underway to investigate the occurrence of the same EGFr mutations in other tumor types to determine whether mutation testing may play a role in selecting therapeutic options in patients with other malignancies.

In September 2005, the FDA approved and Genzyme launched its EGFR Mutation Assay to detect the presence of EGFr mutations in patients with nsclc, to help identify those likely to respond to targeted therapies. This assay combines PCR and gene sequencing testing technologies for the detection of somatic mutations in nsclc tumor tissue. Cells from tumor-rich areas are microdissected, followed by DNA extraction, PCR amplification, and bi-directional sequencing of exons 18 through 21 in the tyrosine kinase domain of the EGFr gene. Analysis is performed in the company's CLIA certified high complexity molecular testing laboratory. EGFr mutation analyses are reviewed by a team of 17 surgical pathologists and PhD geneticists.

Genzyme launched its EGFr by FISH test, for detection of EGFr gene amplification, in February 2006. The test was introduced as a complement to the company's EGFr Mutation Assay.

In December 2006, Genzyme introduced its third genetic test for mutations related to therapeutic response to TKI therapies. The KRAS Mutation Analysis test helps identify patients with nsclc who may not respond to TKI therapies. The test incorporates allele-specific primer extensions for detecting mutations in codons 12 and 13 of the Kras gene. Between 15 and 30% of patients with nsclc test positive for specific Kras mutations, which indicate resistance to TKI. Genzyme Genetics is currently the only national commercial laboratory in the USA to offer this test.

Siemens (Munich, Germany and New York, NY) markets an FDA approved HEr-2/neu ELISA test. Reagents for this test are directed to the p105 extracellular domain (ECD) of the HEr2/neu oncoprotein and do not distinguish phosphorylation states of the full length molecule. This ELISA test is intended for use with human serum and/or plasma samples; use with plasma samples is intended for research purposes only. The intended use of the HER-2/neu ELISA for clinical purposes is for measurement of HEr2/neu protein in serum samples when monitoring patients with metastatic breast cancer.

These tests were developed by Bayer Diagnostics. In 2002, Dako and Bayer (Diagnostics division) had agreed on 5-year, global distribution agreement for oncology-related serum assays in the plate ELISA format. The tests are designed to aid in the confirmation, monitoring, staging, screening, or prognosis of human cancer. These assays, developed and manufactured by Bayer Diagnostics' Oncogene Science Group, include proprietary serum tests for the shed extracellular domain of the HEr2/neu oncoprotein (HER-2/neu ECD), EGFr, urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), and an assay that detects the complexes between uPA and PAI-1. The serum HEr2/neu and EGFr diagnostic tests target oncogenes that are present in many types of cancer including breast, ovarian, prostate, and gastrointestinal malignancies. Separate tests for measuring the total and complexed forms of prostate-specific antigen (PSA) to aid in the detection of prostate cancer were also included in the agreement. In January 2007, the acquisition of Bayer's Diagnostic Division by Siemens was finalized. The new business is now known as Siemens Medical Solutions Diagnostics and is headquartered in the USA.

Ventana Medical Systems (Tucson, AZ) markets two FDA approved HEr2 tests. The Oncor Inform HER-2/neu Gene Detection System, approved in December 1997, detects HEr2 gene amplification by FISH "as an aid to stratify breast cancer patients according to risk for recurrence or disease-related death. It is indicated for use as an adjunct to existing clinical and pathologic information currently used as prognostic indicators in the risk stratification of breast cancer in patients with a primary, invasive, localized breast carcinoma who are lymph node-negative."

HER-2 (c-erbB-2) Pathway, an IHC mouse MAb test based on clone CB11, is used for the detection of HEr2 oncoprotein. HER-2 Pathway was approved by FDA in November 2000 to select patients with breast cancer for Herceptin treatment. This system was changed in January 2007 to the Pathway Anti-Her-2/neu (4b5) Primary Antibody. The January 2007 supplementary approval included a change in the primary antibody from the mouse MAb cb11 to the rabbit MAb 4b5. Other modifications included changes in the detection format from basic DAB detection kit to the Ventana Iview DAB detection kit, the optional Ultraview universal DAB detection kit for biotin free detection format, and the Ventana Image Analysis System (VIAS).

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Exhibit 4
Extracellular Ligands of the ErbB Receptor Pathway

Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF) is a mitogenic polypeptide with a molecular weight of 6 kDa. EGF exerts a profound effect on differentiation of specific cells *in vivo* and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin. The EGF precursor is believed to exist as a membrane-bound molecule that is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells to divide.

EGF plays an important role in cell proliferation, survival, migration, and differentiation, and stimulates cells to divide by activating members of the EGFr family. The highly conserved EGF-EGFr signaling module plays an important role in the morphogenesis of a diverse spectrum of organisms, ranging from nematodes to humans, and has also been identified in the development and growth of many types of human tumor cells.

Transforming Growth Facor (TGFα)

Transforming growth factors (TGF) are biologically active polypeptides that reversibly confer the transformed phenotype on cultured cells. TGFα is a single chain mitogenic peptide composed of 50 amino acid residues cross linked by three disulphide bonds. TGFα shares about 40% sequence homology with EGF and competes with EGF for binding to EGFr, stimulating EGFr phosphorylation, and producing a mitogenic response. Expression of TGFα occurs transiently in some fetal and adjacent maternal tissues during embryonal development and in a number of adult tissues. However, *in vitro* evidence suggests that in normal cells, TGFα is downregulated in nondividing or quiescent states. TGFα is initially synthesized as a high molecular weight, glycosylated, membrane-associated precursor of approximately 160 amino acids. The low-molecular weight peptide and the precursor are biologically active in a number of systems and can function as transforming proteins when overexpressed.

Abnormal TGFα expression has been consistently associated with tumors of epithelial origin. *In vitro*, TGFα is associated with increased levels of oncogene expression. TGFα binds to and activates EGFr. Upon activation, EGFr tyrosyl-phosphorylates itself and numerous intermediary effector molecules, including closely related c-erbB receptor family members, initiating numerous signaling pathways. TGFα and EGFr are coexpressed at elevated levels in a number of human tumors and tumor cell lines, suggesting that TGFα functions as an autocrine cellular growth factor (Roberts AB and Sporn MB, Cancer Surv 1985;4(4):683-705; Keski-Oja J, etal, J Cell Biochem, Feb 1987;33(2):95-107; Prog Growth Factor Res 1989;1(1):23-32; Yeh J and Yeh YC, Biomed Pharmacother 1989;43(9):651-9; Di Marco E, etal, Nat Immun Cell Growth Regul 1990;9(3):209-21; Salomon DS, etal, Cancer Cells, Dec 1990;2(12):389-97; Groenen LC, etal, Growth Factors 1994;11(4):235-57; Brattain MG, etal, Curr Opin Oncol, Jan 1994;6(1):77-81; Wells A, Int J Biochem Cell Biol, Jun 1999;31(6):637-43; Waksal HW, Cancer Metastasis Rev 1999;18(4):427-36).

Epiregulin

Epiregulin, a member of the EGF family, may function as a ligand of EGFr and most of the other members of the ErbB family. Epiregulin is a growth regulating peptide purified from conditioned medium of the mouse fibroblast-derived tumor cell line NIH3T3 (clone T7). It has a length of 46 amino acids and exhibits 24% to 50% amino acid sequence identity with sequences of other growth factors related to EGF. The human epiregulin gene encodes a putative transmembrane precursor of 163 amino acids. Recombinant expression in mammalian cells shows that the protein is secreted as a soluble form of approximately 5 kDa, which is biologically active on the basis of stimulation of DNA synthesis. Epiregulin inhibits growth of several types of epithelial tumor cells and stimulates growth of fibroblasts and various other cell types. Human epiregulin is expressed mainly on peripheral blood macrophages and in the placenta in normal tissues, and it is highly expressed in some epithelial tumor cell lines.

Heparin-binding EGF-like Growth Factor (HB-EGF)

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family. It was initially identified in the conditioned medium of human macrophages. The soluble mature form of HB-EGF is proteolytically released from a larger membrane-anchored precursor. HB-EGF is a potent mitogen and chemotactic factor processed for fibroblasts and smooth muscle cells (SMC), but not endothelial cells. HB-EGF activates two EGFr subtypes, HEr1 and HEr4, and binds to cell surface heparan sulfate rich proteoglycan (HSPG).

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HB-EGF gene expression is highly regulated by cytokines, growth factors, and transcription factors. It plays a part in a variety of processes, such as blastocyst implantation and wound healing, and in pathologic processes, such as tumor growth, SMC hyperplasia, and atherosclerosis. The transmembrane form of HB-EGF is a juxtacrine growth and adhesion factor and is uniquely the receptor for diphtheria toxin (Raab G and Klagsbrun M, Biochim Biophys Acta, 9 Dec 1997;1333(3):F179-99).

HB-EGF gene expression is significantly elevated in many human malignancies, and its expression level in a number of cancer-derived cell lines is much higher than those of other EGFr ligands. HB-EGF appears to play a key role in the acquisition of malignant phenotypes, such as tumorigenicity, invasion, metastasis, and resistance to chemotherapy. Studies *in vitro* and *in vivo* indicate that HB-EGF expression is essential for tumor formation of cancer-derived cell lines (Miyamoto S, etal, Cancer Sci, May 2006;97(5):341-7).

Neuregulins (NRG)

Neuregulins (NRG) comprise a family of heparin sulfate proteoglycans secreted into the neuromuscular junction by innervating motor and sensory neurons. These cell-cell signaling proteins are ligands for receptor tyrosine kinases of the ErbB family. Neuregulins are a diverse family of proteins that arise by alternative splicing from a single gene. They play an important role in controlling the growth and differentiation of glial, epithelial, and muscle cells. The neuregulin family of genes has four members, NRG1, NRG2, NRG3, and NRG4.

In mice, NRG treatment results in a transient increase of several members of the early growth response (Egr) family of transcription factors. Egr1, Egr2, and Egr3 are induced within the first hour of NRG treatment, with Egr1 and Egr3 RNA levels showing the most significant increases of 9- and 16-fold, respectively. There is also a corresponding increase in protein levels for both of these transcription factors (Jacobson C, etal, PNAS USA, 17 Aug 2004;101(33):12218-23).

Neuregulin 1 (NRG1) is a growth factor that functions as a ligand for the tyrosine kinases ErbB3 and ErbB4, stimulating cell proliferation, differentiation, and apoptosis. NRG1 was originally identified as a 44 kDa glycoprotein that interacts with the ErbB RTK to increase its phosphorylation on tyrosine residues. NRG1 plays essential roles in the nervous system, heart, and breast.

An extraordinary variety of different isoforms are produced from the NRG1 gene by alternative splicing. These isoforms include heregulins (HRG), glial growth factors (GGF), and sensory and motor neuron-derived factor (SMDF). They are tissue-specifically expressed and differ significantly in their structures. The HRG isoforms all contain immunoglobulin (Ig) and EGF-like domains. GGF and GGF2 isoforms contain a kringle-like sequence plus Ig and EGF-like domains. The SMDF isoform shares only the EGF-like domain with other isoforms. Receptors for all NRG1 isoforms are the ErbB family of tyrosine kinase transmembrane receptors. Through interaction with ErbB receptors, NRG1 isoforms induce growth and differentialtion of epithelial, neuronal, glial, and other types of cells.

The many NRG1 isoforms that differ in their N-terminal region or in their EGF-like domain differ in their *in vivo* functions. These differences in function may arise because of differences in expression pattern or may reflect differences in intrinsic biological characteristics. While differences in expression pattern certainly contribute to the observed differences in *in vivo* functions, there are also significant differences in intrinsic characteristics that may tailor isoforms for specific signaling requirements (Falls DL, etal, Exp Cell Res, 10 Mar 2003;284(1):14-30).

Neuregulin 2 (NRG2) is a novel member of the neuregulin family. Through interaction with its primary receptors, ErbB3 and ErbB4, NRG2 induces growth and differentiation of epithelial, neuronal, glial, and other types of cells. The NRG2 gene consists of 12 exons, and the genomic structure is similar to that of NRG1. NRG1 and NRG2 mediate distinct biologic processes by acting at different sites in tissues and eliciting different biologic responses in cells. The NRG2 gene is located close to the region for the demyelinating Charcot-Marie-Tooth disease locus, but is not responsible for this disease. Alternative transcripts encoding distinct isoforms have been described.

Neuregulin 3 (NRG3), a novel protein structurally related to NRG1, is predicted to contain an extracellular domain with an EGF motif, a transmembrane domain, and a large cytoplasmic domain. The EGF-like domain of NRG3 binds to the extracellular domain of ErbB4 *in vitro*. In addition, NRG3 binds to ErbB4 expressed on cells and stimulates tyrosine phosphorylation of this receptor, sugggesting that NRG3 is a novel, neural-enriched ligand for ErbB4 (Zhang D, etal, PNAS USA, 2 Sep 1997;94(18):9562-7).

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Epigen (EPGN, EPG)

Epigen is a protein of 152 amino acids that contains features characteristic of the EGF superfamily, including one EGF-like domain, with a 24% to 37% identity to EGF superfamily members including EGF, TGFα, and epiregulin.

Epigen was identified by researchers at Genesis Research and Development (Auckland, New Zealand), using high throughput sequencing of a mouse keratinocyte library. Two hydrophobic regions, corresponding to a putative signal sequence and transmembrane domain, flank a core of amino acids encompassing 6 cysteine residues and 2 putative N-linked glycosylation sites. Epigen is present in testis, heart, and liver. Recombinant Epigen was synthesized in E. coli and refolded, and its biologic activity was compared with that of EGF and TGFα, in several assays. In epithelial cells, Epigen stimulated phosphorylation of c-erbB1 and mitogen-activated protein kinases (MAPK), and also activated a reporter gene containing enhancer sequences present in the c-fos promoter. Epigen also stimulated the proliferation of HaCaT cells, which was blocked by an antibody to the extracellular domain of c-erbB1. Thus, epigen is the newest member of the EGF superfamily and, with its ability to promote the growth of epithelial cells, may constitute a novel molecular target for wound healing (Strachan L, etal, J Biol Chem, 2001 May 25;276(21):18265-71).

Recombinant epigen cannot stimulate cells singly expressing ErbB2, but it acts as a mitogen for cells expressing ErbB1 and co-expressing ErbB2 in combination with the other ErbB. Interestingly, soluble epigen is more mitogenic than EGF, although its binding affinity is 100-fold lower. These findings attribute the anomalous mitogenic power of epigen to evasion of receptor-mediated depletion of ligand molecules, as well as to inefficient receptor ubiquitylation and downregulation. Epigen may represent the last EGF-like growth factor and define a category of low affinity ligands, whose bioactivity differs from the more extensively studied high affinity ligands (Kochupurakkal BS, etal, J Biol Chem, 4 Mar 2005;280(9):8503-12).

Amphiregulin

Amphiregulin, a member of the EGF family, is an extremely hydrophilic glycoprotein 84 amino acids in length. A truncated form of 78 amino acids has also been described. Amphiregulin contains 6 cysteine residues engaged in the formation of disulfide bonds (positions 46/59; 54/70; 72/81), which are essential for its biologic activity. The carboxyl-terminal from residues 46 to 84 exhibits striking homology to EGF family of proteins.

Amphiregulin is synthesized as a membrane-bound precursor of 252 amino acids, which is then processed into the mature molecule by proteolytic events. Amphiregulin contains putative nuclear targeting sequences involved in amphiregulin-mediated growth responses.

Amphiregulin functions as an autocrine growth factor and also as a mitogen for astrocytes, Schwann cells, and fibroblasts. It interacts with the EGF/TGFα receptor to promote the growth of normal epithelial cells and inhibits growth of certain aggressive carcinoma cell lines. Amphiregulin is associated with a psoriasis-like skin phenotype.

Betacellulin (BTC)

Betacellulin, a member of the mammalian EGF family of growth factors, is synthesized primarily as a transmembrane precursor, which is then processed to mature molecule by proteolytic events. Several alternatively spliced transcript variants of this gene have been described, but the full length nature of some of these variants has not been determined. Betacellulin directly binds to both EGFr and HEr4 and induces growth of certain epithelial cell types.

The solution structure of the EGF-like domain of betacellulin that interacts with both ErbB1 and ErbB4 was determined using 2-dimensional nuclear magnetic resonance spectroscopy. The overall structure of betacellulin is stabilized by 3 disulfide bonds, a hydrophobic core, and 23 hydrogen bonds. It appears that betacellulin is comprised of five β strands and one short 3(10) helical turn. The secondary structural elements of betacellulin are basically similar to those of the other EGF family proteins, except for several significant structural variations that may affect the specific receptor-recognition properties of ErbB ligands (Miura K, etal, Biochem Biophys Res Commun, 28 Jun 2002;294(5):1040-6).

Source: New Medicine's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, February 2007

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It is indicated "as an aid in the assessment of breast cancer patients being considered for Herceptin treatment."

Ventana also markets the Confirm anti-Epidermal Growth Factor Receptor, which is an IHC test directed against the extracellular domain of human EGFr protein of 170 kD. The antibody also recognizes the type III variant of 145kD. This test is intended for laboratory use, via light microscopy, for the qualitative detection of EGFr in formalin fixed, paraffin embedded tissue on a Ventana automated slide stainer.

Other Products

Many diagnostic tests used in clinical practice in the USA are not approved by FDA. Only FDA approved products may be labeled "for use as an *in vitro* diagnostic;" and third party payers reimburse only for diagnostic kits that are FDA-cleared for their intended use. However, sophisticated, non-FDA approved products and services, sometimes known as "home brew" diagnostics, may be used in clinical practice and reimbursed by third party payers according to CPT codes for established procedures, if testing is adequately supported by the medical literature. Reference laboratories, for example, typically seek reimbursement by billing for all accepted procedures performed during the laboratory testing process (e.g., billing for DNA extraction, DNA amplification, etc.).

Abbott Molecular is currently developing a molecular diagnostic test for bladder cancer based on the detection of EGFr amplification by FISH technology. The test will be used to determine early recurrence of bladder cancer and is expected to be on the market within a couple of years.

AdnaGen (Langenhagen, Germany) uses a proprietary technique, based on tumor type-specific antibody-linked magnetic particles, to isolate tumor cells from peripheral blood of patients with cancer, after which RT-PCR is applied for molecular diagnosis. The presence in peripheral blood of circulating tumor cells (CTC) indicates active growth and disease progression (relapse or metastasis). Shedded tumor cells survive for only a short period in the circulation. In one study, AdnaGen's technology demonstrated specificity of >97% and an analytical sensitivity (or limit of detection) of one tumor cell in 5 mL blood. Molecular profiling of isolated cells allows detection of HEr2, EGFr, and other tumor markers. The CTC isolation step is useful for prognosis and theragnosis. Determination of molecular target expression levels in CTC show that they may differ significantly from those of the primary tumor, reflecting the nature of the metastasis. The AdnaGen technology can eliminate reliance solely on histopathology of the primary tumor to target therapy by allowing specific targeting of the metastases (Albert WH, etal, First AACR Int Conf Mol Diag Cancer, Sep 2006, Abs. A46).

Beckman Coulter (Fullerton, CA) markets the ProteomeLab PF 2D system, which provides an integrated solution that resolves the thousands of proteins present in cell lysates. The system provides a combination of chromatofocusing followed by non-porous reverse phase chromatography. The result is ultra high resolution of proteins and delivery of information that is relatable to 2-dimensional gel data. Detailed protein maps can then be constructed in the second dimension for comparison using the ProteomeLab Software Suite supplied with the system. Liquid fractions can be collected and stored for future analysis or the eluent can be connected directly to ESI-mass spectrometry. Much less time and attention than traditional labor-intensive techniques is required. The entire two dimensional process can be performed in less than 20 hours.The ProteomeLab PF 2D system is being used to study EGFr mutations in human glioma xenografts. High levels of EGFr are common in glioma, usually in combination with wild-type and/or mutant EGFr gene amplification that has been associated with reduced survival. The EGFrvIII mutation, which is caused by the deletion of exons 2–7 in the EGFr mRNA, results in an in-frame deletion of 801 bp of sequence coding for a portion of the extracellular domain. EGFrvIII is expressed primarily in Grade IV glioblastoma multiforme (GBM)/astrocytoma. EGFrvIII confers increased tumorigenicity to glioma cells *in vivo*, enhances cell tumorigenicity and growth rates, and reduces apoptosis. EGFrvIII is constitutively active; it is phosphorylated in a ligand-independent fashion in the absence of significant internalization and downregulation. Understanding the aberrant signaling of this potent glioma oncogene is important. Victoria Ioffe and colleagues of Henry Ford Hospital (Detroit, MI) and Mayo Clinic (Rochester, MN) are using the ProteomeLab PF 2D system to identify molecular differences between glioma cells bearing amplified EGFr and amplified EGFrvIII in human glioma xenografts. This protemics system allows easier detection of changes in signaling pathways (e.g., phosphorylation levels) than is achievable with genomics technologies.

DxS (Manchester, UK) offers wide ranging EGFr mutation testing capability using technology that combines ARMS (allele specific PCR)4 with the Scorpions signaling system5 to develop sensitive, quantitative and robust real-time PCR tests for EGFr mutations in tumors. ARMS technology was licensed from AstraZeneca.

The company's EGFr Mutation Test Kit tests for the two predominant somatic mutations in the EGFr tyrosine kinase domain, the Exon 21 L858R point mutation and the Exon 19 deletion 15 (E746 - A750). It is intended for research purposes only.

The DsX Extended EGFR Mutation Test Kit tests for the 29 most common somatic mutations in the EGFr gene, including 19 deletions in exon 19, T790M, L858R, L861Q, G719X (detects the presence of G719S, G719A or G719C but does not distinguish between them), S768I, and 3 inser-

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tions in exon 20 (detects the presence of any of 3 insertions but does not distinguish between them).

The T790M EGFR Mutation Test Kit tests for a secondary mutation in the EGFr kinase domain, T790M, which appears to confer drug resistance in patients with cancer. The cytosine to thymidine change is predicted to change threonine to methionine in the catalytic site of the tyrosine kinase domain. Such tumors demonstrate sensitivity to a class of irreversible inhibitors of T790M-mutant EGFr. The assay allows approximate determination of the percentage of mutant T790M in a sample. This kit is sold as a research tool to enable researchers to understand the association between T790M mutations and progression of lung cancer and is also useful for drug company studies to determine the T790M status of patients in clinical trials.

Epigenomics (Berlin, Germany and Seattle, WA) is a molecular diagnostic company with a focus on the development of novel products for cancer. The company's tests detect and interpret DNA methylation patterns. Epigenomics' product pipeline contains a validated biomarker panel for the early detection of colorectal cancer in blood plasma, and further proprietary DNA methylation biomarkers at various stages of development for prostate, breast and lung cancer detection in body fluids. The company offers these programs as partnering opportunities for development, marketing, and sales of cancer molecular diagnostics. In cooperation with industry partners, the company develops diagnostic screening tests for the early detection of cancer, based on easily obtainable body fluid samples (e.g., blood and urine), aimed at finding cancer at an early stage before symptoms occur. Epigenomics also develops tissue-based prognostic tests for prostate and breast cancer in strategic partnerships with Qiagen (Amsterdam, The Netherlands) and Affymetrix (Santa Clara, CA).

In May 2006, AstraZeneca and Epigenomics announced that Epigenomics' Clinical Solutions division will test the potential of novel DNA methylation cancer markers discovered during the companies' earlier collaboration to predict response to EGFr inhibitors, such as AstraZeneca's Iressa (gefitinib), in a panel of preclinical cancer models. Epigenomics will receive R&D fees and may participate in the development of any potential theragnostic products resulting from the collaboration.

In December 2006, Roche Diagnostics terminated its 4-year collaboration with Epigenomics to develop prostate, breast, and colorectal cancer screening diagnostic tests based on Epigenomics' DNA methylation technologies. Roche determined that the colorectal cancer screening data presented by Epigenomics to date did not meet its criteria for development as *in vitro* diagnostic tests.

In January 2007, Epigenomics entered into a R&D collaboration with Centocor to identify and analyze potential biomarkers for use in Centocor's oncology program. Epigenomics will use its proprietary Differential Methylation Hybridization (DMH) microarray platform to perform genome-wide DNA methylation profiling study on samples provided by Centocor. The goal is to identify candidate DNA methylation biomarkers that may support drug development and could be used for selecting patients that have a higher chance of benefiting from a drug candidate under development at Centocor.

Genomic Health (Redwood City, CA) markets Oncotype DX, a high-throughput reverse-transcriptase-PCR (RT-PCR) assay of 21 prospectively selected genes in paraffin-embedded tumor tissue. Tests are conducted by Genomic Health Laboratory, which is a licensed, reference laboratory with a CLIA certificate of accreditation and College of American Pathologists accreditation. Oncotype DX was used to determine HEr2 status in patients with node-negative, tamoxifen-treated breast cancer who were enrolled in the National Surgical Adjuvant Breast and Bowel Project clinical trial B-14. It has also been used in quantitative gene expression studies, including EGFr, to identify correlates of response to EGFr kinase inhibitors. Specifically, the Oncotype DX high-throughput RT-PCR assay was found to be feasible and possibly useful for studying the correlation between quantitative gene expression in tumor and response to gefitinib monotherapy in patients with nsclc. Selected genes included those implicated in HEr-kinase signaling, including STAT5A, STAT5B, amphiregulin, epiregulin, MMP2, TIMP2, PDGFr, and a 'growth cluster' of Ki-67, survivin, Chk1, and topoisomerase II. The study was conducted by RB Natale and colleagues at Cedars-Sinai Medical Center (Los Angeles, CA), AstraZeneca (Wilmington, DE), and Genomic Health.

Idaho Technology (Salt Lake City, UT) offers Hi-Res Melting as a post-PCR technique for homogeneous mutation scanning and genotyping. The system detects single base changes in the sample by simply melting the PCR product. The PCR plate or tube is transferred to the company's LightScanner instrument, using LCGreen Plus+ Dye. Hi-Res Melting reduces the amount of DNA sequencing by quickly differentiating and ruling out specimens that do not have sequence variants. The use of unlabeled probe technology Hi-Res Melting can determine common or known sequence variants, identifying samples with unknown variants that need to be sequenced. Mutations in PCR products are detected by changes in the shape of the melting curve compared to a reference sample.

Invitrogen (Carlsbad, CA) is developing the chromogenic *in situ* hybridization (CISH) technology for the development of analyte-specific reagents, as an alternative to FISH. CISH technology was acquired by Invitrogen with its February 2005 acquistion of Zymed (South San Francisco, CA). According to the company, CISH is a flexible molecular technology that allows for detection of a broad array of gene amplifications and deletions and morphological interpretation using a standard bright field light microscope. Simultaneous detection of gene amplification and morphology under bright field microscope is achieve by using Zymed's Subtraction Probe Technology (SPT).

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Exhibit 5
Members of Intracellular Pathways Modulated by Activation of ErbB Receptors

Akt (Akt1, Akt2, Akt3)

Akt, a cytosolic protein, comprises a subfamily of serine/threonine kinases containing Src homology 2-like (SH2-like) domains. Akt plays roles in multiple cellular processes by mediating extracellular signals (e.g., mitogenic growth factor, insulin, and stress) and intracellular signals (e.g., altered tyrosine receptor kinases, Ras, and Src). Activated Akt plays a major role in promoting cell survival by inactivating components of the apoptotic machinery. More than 30 Akt substrates have been identified. Phosphorylation of Akt mediates its effects on cell survival, growth, differentiation, angiogenesis, migration, and metabolism. Furthermore, the PI3K/PTEN/Akt pathway is frequently altered in many human malignancies and overexpression of Akt induces malignant transformation and chemoresistance (Kim D, etal, Front Biosci, 1 Jan 2005;10:975-87).

Akt is activated by growth factors leading to the activation of phosphoinositol-3-kinase (PI3K). Production of a phospholipid, phosphatidylinositol (3,4,5) trisphosphate by PI3K is enhanced in a wide range of tumor types. This lipid and Akt trigger a cascade of responses, from cell growth and proliferation to survival and motility that drive tumor progression (Vivanco I and Sawyers CL, Nat Rev Cancer, Jul 2002;2(7):489-501).

Upon activation, PI3K phosphorylates the inositol ring at the D3 position, which in turn serves to anchor Akt to the plasma membrane where it is phosphorylated and fully activated by PDK1 and PDK2. PTEN and SHIP, which decrease the pool of available phospholipids, are negative regulators of Akt. Activated Ras, at least in certain circumstances, can upregulate PI3K and, hence, is also a potential activator of Akt. Overall, positive regulators of Akt are commonly upregulated in human malignancies while PTEN is frequently lost or inactivated by mutations. Furthermore, heterozygous deletion of PTEN in mice elicits a wide range of spontaneous tumors, attributed mainly to activation of Akt. Taken together these observations suggest that Akt activation plays a potent role in carcinogenesis. Several targets of Akt have been implicated in mediating cell survival directly or indirectly. It appears that activation of Akt promotes cell survival through maintaining mitochondrial integrity in a mechanism distinct from that of Bcl-2 or Bcl-xL. This mechanism is dependent on the availability of glucose and, at least in part, on mitochondrial associated hexokinases. Based on genetic evidence, provided through the use of Akt knockout mice, Akt is required for both cell survival and oncogenic transformation. It is possible that activation of Akt could be oncogenic by preventing normal apoptosis of cells, thereby enabling accumulation of more oncogenic mutations in these cells. However, activation of Akt can also abrogate cell-cycle checkpoints and overcome G2/M cell-cycle arrest, mediated by DNA mismatch repair. Thus, cells in which Akt is activated can accumulate mutations because the G2 cell-cycle point is abrogated, survive, and continue to divide because of the antiapoptotic activity of Akt. It is proposed that this dual mechanism of Akt may explain its frequent activation in human malignancies (Hay N, etal, AACR02).

The 3 isoforms of Akt, Akt1, Akt2, and Akt3, have distinct functions in the cell. Akt1 and Akt2 are activated by platelet-derived growth factor (PDGF). This activation that occurs through PI3K, is rapid and specific, and it is abrogated by mutations in the pleckstrin homology domain of Akt1. In the developing nervous system, Akt is a critical mediator of growth factor-induced neuronal survival. Survival factors can suppress apoptosis in a transcription-independent manner by activating Akt1, which then phosphorylates and inactivates components of the apoptotic machinery. Multiple alternatively spliced transcript variants have been identified for this gene. Akt2 is a putative oncogene, amplified and overexpressed in various malignancies.

c-SRC

The viral src (v-src) gene, originally identified by homology to the Rous sarcoma virus oncogene protein pp60(v-Src), was the first retroviral oncogene to be identified, and its cellular counterpart was the first proto-oncogene to be discovered in the vertebrate genome. Src has been important as an entry point into the molecular genetics of cancer (Martin GS, Nat Rev Mol Cell Biol, Jun 2001;2(6):467-75). The 3-dimensional structure of c-Src was delineated by investigators at Children's Hospital (Boston, MA) (Xu W, etal, Nature, 13 Feb 1997;385(6617):595-602; comment:582-3, 585).

Src is a prototype of a nonreceptor type tyrosine kinase. The product of the human src gene, pp60c-src, is overexpressed and highly activated in a wide variety of human malignancies. There appears to be a significant relationship between Src activation and cancer progression, and Src may influence the development of the metastatic phenotype. Therefore, Src may play a role as a critical component of the signal transduction

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pathways that control cancer cell development and growth. At the cellular level, Src plays an important role in proliferation, adhesion, and motility. Also, the role of Src in bone metabolism, first observed in Src-deficient mice, has since been confirmed using low-molecular-weight Src inhibitors in animal models of osteoporosis. Src is involved in cell survival and intracellular trafficking in various specialized cell types, suggesting that Src inhibitors might have therapeutic value in the suppression of tumor growth, tumor angiogenesis, and bone resorption (Susva M, etal, Trends Pharmacol Sci, Dec 2000;21(12):489-95).

In cells, endogenous Src is switched from an inactive to an active state by a variety of mechanisms that simultaneously relieve constraints on the kinase and protein-interacting Src homology (SH) domains. As a result, Src moves to the cell periphery, often to sites of cell adhesion, where myristylation mediates attachment to the inner surface of the plasma membrane. From these peripheral sites, Src's catalytic activity initiates intracellular signal transduction pathways that influence cell growth and adhesion, contributing to control of cell migration. Deregulation of Src in cancer cells may, therefore, enhance tumor growth and/or stimulate relatively nonmotile cells to become migratory or invasive. Src may also influence the life or death decisions that cells make during many biological processes and, therefore, Src modulation in cancer cells can alter cell responses that are often perturbed in cancer (Frame MC, Biochim Biophys Acta, 21 Jun 2002;1602(2):114-30).

Overexpression and/or hyperactivation of c-Src kinase is a common feature of many malignancies. Also, c-Src kinase has been implicated in a number of processes important for malignant growth, including growth-factor-driven cell proliferation, vascular endothelial growth factor (VEGF)-dependent angiogenesis, and metastasis. c-Src is a target with potential broad applications in neoplastic disease.

Cyclin D1 (CDND1)

Cyclin D1 belongs to the cyclin D subfamily, a member of the highly conserved cyclin family. Cyclins are the regulatory subunits of kinases that control progress through the cell cycle. They are characterized by dramatic periodicity in protein abundance throughout the cell cycle. They function as regulators of cyclin dependent kinases (cdk). Different cyclins exhibit distinct expression and degradation patterns that contribute to the temporal coordination of each mitotic event.

Cyclin D1 is involved in regulation of both the normal cell cycle and the cell-cycle in neoplasia. Overexpression of cyclin D1 protein releases a cell from its normal controls and causes transformation to a malignant phenotype (Donnellan R and Chetty R, Mol Pathol, Feb 1998;51(1):1-7). Cyclin D1 forms a complex with and functions as a regulatory subunit of cdk4 or cdk6 required for cell cycle G1/S transition. Cyclin D1 and cdk4 phosphorylate tumor suppressor retinoblastoma protein (Rb), which is the mechanism of transition to S phase (DNA synthesis). Rb regulates expression of CCND1 positively. Mutations, amplification, and overexpression of CCND1, which alter cell-cycle progression, are observed frequently in and may contribute to tumorigenesis of a variety of tumors.

Cyclins, particularly cyclin D1, are targets for extracellular signaling and frequently deregulated during oncogenesis. Receptor tyrosine kinases (RTK) and adhesion molecules act through various effector pathways to modulate cyclin D1 abundance at multiple levels including transcription, translation and protein stability. The importance of this pathway during oncogenesis is illustrated by the dependence of oncogenes such as Ras and ErbB2 on cyclin D1 (Musgrove EA, Growth Factors, 2006 Mar;24(1):13-9).

The cyclin D1 protooncogene exercises powerful control over the mechanisms that regulate the mitotic cell cycle, and excessive cyclin D1 expression and/or activity is common in human malignancies. Although somatic mutations of the cyclin D1 locus are rarely observed, mounting evidence demonstrates that a specific polymorphism of cyclin D1 (G/A870) and a protein product of a potentially related alternate splicing event (cyclin D1b) may influence cancer risk and outcome (Knudsen KE, etal, Oncogene, 2006 Mar 13;25(11):1620-8).

E2F transcription factor 1 (E2F1)

E2F1 belongs to the E2F family of transcription factors that play a critical role in the transcription of certain genes required for cell-cycle progression and tumor suppressor protein activity. E2F1 is regulated during the cell cycle at the mRNA level by changes in transcription of the E2F1 gene and at the protein level by complex formation with proteins such as Rb, and cyclin A (Mudryj M, etal, Cell, 28 Jun 1991;65(7):1243-53), and the differentiation-regulated transcription factor protein 1 (DP1).

E2F1 binds preferentially to Rb in a cell-cycle-dependent manner. It controls genes regulating S phase entry and DNA synthesis. The role of E2F1 in cell-cycle progression is largely facilitated by this factor's ability to induce genes involved in cell-cycle regulation and DNA replication. However, in response to cell damage, the

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E2F1 pathway also plays an important role in mediating apoptosis and DNA repair. E2F1 mediates both cell proliferation and p53-dependent/independent apoptosis *in vitro* and *in vivo*, both as a potent inducer and as a mediator. E2F1 can directly bind to and activate the promoter of DIABLO, a mitochondrial proapoptotic gene, through the E2F1-binding sites BS2 and BS3, promoting p53-independent apoptosis.

E2F1 behaves paradoxically as an oncogene and as a tumor suppressor gene. E2F1 can override a Rb-induced G1/S block and behave as an oncogene in certain cells. To address the function of E2F1 and RB/E2F1 complexes *in vivo*, investigators at the Massachusetts General Hospital Cancer Center, (Charlestown, MA) produced transgenic mice homozygous for a nonfunctional E2F1 allele. These mice are viable and fertile, yet experience testicular atrophy and exocrine gland dysplasia. Surprisingly, mice lacking E2F1 develop a broad and unusual spectrum of tumors. Although overexpression of E2F1 in tissue culture cells can stimulate cell proliferation and be oncogenic, loss of E2F1 in mice results in tumorigenesis, demonstrating that E2F1 also functions as a tumor suppressor (Yamasaki L, etal, Cell, 17 May 1996;85(4):537-48). Also, mice lacking E2F1 exhibit a defect in T-lymphocyte development leading to an excess of mature T cells because of a maturation stage-specific defect in thymocyte apoptosis. As these mice age they exhibit a second phenotype marked by aberrant cell proliferation. These findings suggest that while certain members of the E2F family may positively regulate cell-cycle progression, E2F1 functions to regulate apoptosis and to suppress cell proliferation (Field SJ, etal, Cell, 17 May 1996;85(4):549-61).

Although E2F1 binds DNA as a homodimer increasing promoter activity, optimal DNA-binding and transcriptional activity occurs in the heterodimeric form composed of two partners, such as E2F1 and DP1. It is likely that binding of growth-suppressing proteins, such as Rb, inhibits the transactivation potential of E2F1, either by blocking the interaction of E2F1 with a separate component of the transcription complex, or by bringing a repressor domain to the transcription complex. Phosphorylation or sequestration of Rb by viral oncoproteins can free E2F. Interaction of free E2F induces a bend in the DNA that may also play a role in transactivation, perhaps by bringing proteins separated by distance on the promoter DNA into contact. Because E2F targets genes encoding proteins critical for cell growth, deregulation of E2F activity can have severe consequences, such as apoptosis or uncontrolled proliferation (Slansky JE and Farnham PJ, Curr Top Microbiol Immunol 1996;208:1-30).

The DNA damage-responsive ataxia-telangiectasia mutated (ATM) kinase is required for E2F1 to induce both p53 phosphorylation and apoptosis. E2F1 physically associates with ATM in response to several DNA damaging agents. Also, E2F1 localizes to discrete foci in response to DNA damage in an Nijmegen breakage syndrome protein (NBS1)-dependent manner. E2F1 associates with key DNA damage-responsive proteins and may play a specific role in the DNA damage response. E2F1 induces ATM-dependent p53 phosphorylation that is independent of transcription and DNA damage. These findings further suggest that E2F1-induced apoptosis may be both independent of its transcriptional capacity, and an extension of a physiologic role in response to DNA damage (Powers JT Jr, etal, AACR06, Abs. 5623).

Investigators at Oregon Health & Science University (Portland, OR) show that E2F1 stimulates base excision repair in part through transcriptional activation of XRCC1, which encodes a protein involved in DNA base excision repair (BER) (Chen D, etal, AACR06, Abs. 5624).

Increased levels of free E2F1 have been associated with increases in thymidine kinase, dihydrofolate reductase (DHFR), thymidylate synthase (TS), and ribonucleotide reductase in tumor cells exhibiting loss of Rb function. This has been shown to result in cellular resistance to methotrexate, which targets DHFR, and 5-fluorodeoxyuridine, which targets TS (Li W, etal, PNAS USA, 24 Oct 1995;92(22):10436-40, Hochhauser D, JNCI, 18 Sep 1996;88(18):1269-75, Bertino JR, etal, AACR99, Abs. 771).

Extracellular signal-regulated kinases (ERK1/2) or mitogen activated protein kinase 1 (MAPK1)

ERK1/2 or MAPK1 is a member of the mitogen-activated protein (MAP) kinase family. ERK-1 and ERK-2 are two alternatively spliced transcript variants encoding the same protein, but differing in the untranslated regions (UTR); they are sometimes referred to as ERK1/2. MAP kinases act as an integration point for many biochemical signals. In response to a variety of extracellular signals, they are involved in a wide variety of cellular processes, including progression, proliferation, differentiation, transcription regulation, embryogenesis, and development. They are also involved in short term changes required for homeostasis and acute hormonal responses. Activation of MAPK1 occurs as a result of a variety of mitogenic stimuli as well as differentiation signals and requires phosphorylation by upstream kinases. Mitogens such as polypeptide growth factors

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PDGF, CSF-1, IGF-1, etc., as well as insulin, potently activate the ERK family of MAPK. Most of these agents exhibit very weak (or no) induction of the other MAPK kinases, SAPK, and p38, in most cell types. EGF can also induce this pathway, and Ras plays a role in ERK activation. Upon activation, MAPK1 translocates to the nucleus of the stimulated cell, where it phosphorylates nuclear targets. Downstream substrates include Elk1, phospholipase A2 and another protein kinase, p90Rsk1. ERK are inactivated by dephosphorylation by specific protein phosphatases such as MKP1 (CL100) and PAC1.

Several key growth factors, cytokines, and proto-oncogenes transduce their growth and differentiation-promoting signals through the MAPK/ERK cascade. Overexpression or constitutive activation of this pathway plays an important role in the pathogenesis and progression of breast cancer and other malignancies, making the components of this signaling cascade potentially important as therapeutic targets (Allen LF, etal, Semin Oncol, Oct 2003;30(5 Suppl 16):105-16).

ERK1/2 are the most important members of MAPK family in terms of seven trans-membrane-domain receptor (7TMr)-mediated regulation of mitogenic processes. Regulation of the ERK1/2 signaling cascade by 7TMr is highly complex and cell type-specific. Recent advances in the knowledge of this effector pathway have revealed that its regulation is at least partly independent of traditional G protein-mediated actions arising from the stimulation of 7TMr. ERK1/2 regulation represents a wealth of potential targets available for the development of new strategies for the treatment of proliferative and other ERK-related disorders (Werry TD, etal, Curr Pharm Des, 2006;12(14):1683-702).

ERK1/2 can be activated transiently or persistently by MEK1/2 and upstream MAP3K in conjunction with regulation and involvement of scaffolding proteins and phosphatases. Activation of ERK1/2 generally promotes cell survival; but under certain conditions, ERK1/2 can have proapoptotic functions (Lu Z, Xu S, IUBMB Life, 2006 Nov;58(11):621-31).

Focal adhesion kinase (FAK), PTK2

FAK is a 119.2-kDa cytoplasmic, nonreceptor protein tyrosine kinase concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents. It is a member of the FAK subfamily of protein tyrosine kinases but lacks significant sequence similarity to kinases from other subfamilies. FAK is a major phosphotyrosine-containing protein that mediates cell-extracellular matrix signaling events (Sheta EA, etal, JNCI, 5 Jul 2000;92(13):1065-73).

Activation of FAK may be an important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix. At least 4 transcript variants encoding 4 different isoforms have been found for this gene.

FAK mediates a direct interaction with the C terminal SH2 domain of phospholipase C (PCLG1) and is a primary mediator of integrin signaling after integrin mediated cell adhesion and FAK phosphorylation. FAK plays an important role in the response of migrating cells to mechanical input. FAK is the kinase signal transducer for integrins which, when constitutively active, inhibits anoikis allowing anchorage-independent cell growth.

FAK is activated upon integrin-ligand binding and by other signaling pathways and is a key regulator in cell adhesion, motility, and invasion, in part by mediating cell contact induction of VEGF transcription (Renshaw MW, etal, J Cell Biol, 1 Nov 1999;147(3):611-8; Maung K, etal, Oncogene, 18 Nov 1999;18(48):6824-8; Sheta EA, etal, *ibid*).

FAK, implicated in the regulation of cell migration, is overexpressed in various highly metastatic tumors. Overexpression of the PTK2 and elevation of FAK protein has been observed in many cancer cell lines. FAK is only weakly expressed, if at all, in normal and benign tissues as well as in noninvasive, yet hypercellular, neoplastic tissues, but is overexpressed at moderate or strong levels as epithelial and mesenchymal cells become invasive, with its upregulation apparently occurring at an early stage of tumorigenesis (Weiner TM, etal, Lancet, 23 Oct 1993;342(8878):1024-5; Owens LV, etal, Cancer Res, 1 Jul 1995;55(13):2752-5; Owens LV, etal, Ann Surg Oncol, Jan 1996;3(1):100-5; Agochiya M, etal, Oncogene, 7 Oct 1999;18(41):5646-53; Judson PL, etal, Cancer, 15 Oct 1999;86(8):1551-6; Cance WG, etal, Clin Cancer Res, Jun 2000;6(6):2417-23).

In addition to such cellular functions as spreading, proliferation, migration, and invasion, FAK signaling plays a critical role in the production of matrix metalloproteinases (MMP) such as MMP-2 and MMP-9, subsequently activating tumor invasion. Moreover, TNFα activates FAK signaling and enhances tumor invasion. Therefore, FAK signaling provides a linkage between inflammation and cancer (Mon NN, etal, Ann NY Acad Sci, Nov 2006;1086:199-212).

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MUC4/sialomucin complex (SMC)

The major constituents of mucus, the viscous secretion that covers epithelial surfaces such as those in the trachea, colon, and cervix, are highly glycosylated proteins called mucins. Sialomucin complex (SMC)/MUC4, encoded on a single gene, is a large, heterodimeric glycoprotein complex that plays important roles in the protection of the epithelial cells and has been implicated in intestinal epithelial cell differentiation and in the integrity of the renewal of the epithelium. The MUC4 gene is expressed in numerous normal tissues including stomach, ovary, salivary gland, uterus, prostate, thymus, thyroid, breast, esophagus, small intestine, testis, and placenta, and is also found in secreted fluid, such as tears, milk, and saliva. Secreted isoforms of MUC4 may also exist. At least two dozen transcript variants of this gene have been found, although for many of them the full-length transcript has not been determined or they are found only in tumor tissues. This gene contains a region in the coding sequence which has a variable number (>100) of 48 nucleotide (nt) tandem repeats, although only two repeats are included in these sequences.

MUC4 is composed of two subunits, a heavily glycosylated mucin extracellular domain ASGP-1, stably associated with the N-glycosylated transmembrane subunit ASGP-2. The ASGP-2 sequence contains two EGF-like domains within the extracellular region, suggesting a potential ligand role. Characterization of an ASGP-2/ErbB2-containing signal transduction particle in the constitutively activated ascites cells provides support for this ligand hypothesis. Co-infection of insect cells with ASGP-2 and members of the EGFr family demonstrated that ASGP-2 binds selectively via EGF-1 to ErbB2, upregulating the receptor's kinase activity and receptor autophosphorylation, and modulating the effects of the soluble ligand NDF/HRG on the ErbB2/ErbB3 heterodimer (Carraway CC, etal, Scientific World J, 1 Jan 2001;1(1 Suppl 3):5149).

The transmembrane subunit ASGP-2 has two EGF-like domains, one of which acts as an intramembrane ligand for RTK ErbB2, which has been strongly implicated in cancer progression.

In a study, performed on a series of 100 non-small cell lung cancer (nsclc) samples to investigate expression of human MUC4 in neoplastic and corresponding non-neoplastic tissues and the relationship of MUC4 expression in neoplastic tissues to ErbB2 expression, apoptosis, proliferation, differentiation, and tumor stage, MUC4 expression was detected in all of the non-neoplastic bronchial tissues and 85% of nsclc cells. However, MUC4 expression was higher in neoplastic than in non-neoplastic tissues. MUC4 expression was inversely correlated with apoptotic index and correlated with ErbB2 expression, but not with cell proliferation or tumor stage. These results indirectly suggest that MUC4, in association with ErbB2, might be involved in the repression of apoptosis and differentiation rather than proliferation in nsclc cells (Karg A, etal, Pathol Res Pract 2006; 202(8):577-830).

Muc4 physically interacts with the ErbB2 and augments receptor tyrosine phosphorylation in response to the neuregulin-1b (NRG1b). Investigators at the University of California, Davis Cancer Center demonstrated that Muc4 expression in A375 human melanoma cells, as well as MCF7 and T47D human breast cancer cells, enhances NRG1beta signaling through the phosphatidylinositol 3-kinase pathway. In examining the mechanism underlying Muc4-potentiated ErbB2 signaling, Muc4 expression augmented NRG1b binding to A375 cells without altering the total quantity of receptors expressed by the cells. Muc4 induces the relocalization of ErbB2 and ErbB3 from intracellular compartments to the plasma membrane. Moreover, Muc4 interferes with the accumulation of surface receptors within internal compartments following NRG1b treatment by suppressing the efficiency of receptor internalization. Therefore, it appears that transmembrane mucins may modulate RTK signaling by influencing receptor localization and trafficking and thus contribute to tumor growth and progression (Funes M, etal, J Biol Chem, 14 Jul 2006;281(28):19310-9).

Phospholipase C, γ1 (PLCγ1, PLCG1)

PLCγ1 is a large protein with a complex sub-domain structure that integrates a wide variety of signaling pathways. PLCγ1 is commonly activated by tyrosine kinase receptor engagement. The by-products of PLCγ1 activation act as second messengers and propagate signal transduction from surface receptors to the nucleus where gene transcription events control subsequent cell behavior.

PLCγ1 catalyzes the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate. This reaction uses calcium as a cofactor and plays an important role in the intracellular transduction of receptor-mediated tyrosine kinase activators. For example, when activated by SRC, PLCγ1 causes the Ras guanine nucleotide exchange factor RasGRP1 to translocate to the Golgi, where it activates Ras. Also, PLCγ1 has been shown to be a major substrate for heparin-binding growth factor 1 (acidic fibroblast

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growth factor)-activated tyrosine kinase. Two transcript variants encoding different isoforms have been found for this gene.

The tumor suppressor gene, PTEN, plays a role in regulating activity of PLCγ1 and phospholipase D (PLD). The lipid phosphatase activity of PTEN decreases phosphatidylinositol-(3,4,5)-P3, which antagonizes the proliferative PI3K/Akt pathway (Alvarez-Breckenridge CA, etal, AACR05, Abs. 5473).

Activation of the PLCγ1 pathway has been implicated in the regulation of growth factor-mediated chemotaxis in a variety of cell types. PLCγ1 plays a key role in regulating cytoskeletal dynamics required for morphologic changes and motility in tumor and endothelial cells contributing to metastasis (Peak JC, etal, AACR06, Abs. 1766).

Protein kinase C (PKC) family

PKC was first identified in 1977 as a protein kinase that phosphorylated histone and protamine. PKC comprises a family of serine and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol. PKC family enzymes differ in structure, cofactor requirements and function. They are ubiquitously expressed, closely related phospholipid-dependent enzymes that regulate cell growth and differentiation and are believed to play important roles in tumorigenesis (Lockwood AH, etal, J Cell Biochem, Apr 1987;33(4):237-55; Blobe GC, etal, Cancer Metastasis Rev, Dec 1994;13(3-4):411-31; Kiley SC, etal, J Mammary Gland Biol Neoplasia, Apr 1996;1(2):177-87; Uchida N, etal, Oncol Rep, Jul-Aug 2000;7(4):793-6). PKC family members phosphorylate a wide variety of protein targets and are known to be involved in diverse cellular signaling pathways. PKC family members also serve as major receptors for phorbol esters, a class of tumor promoters.

The PKC family comprises at least 12 members divided into three subgroups, differing in the enzymes' cofactor requirements, including conventional or classical PKC (cPKC) isoforms (a, b), which are dependent upon Ca+2 calcium and diacylglycerol (DAG) for activation; novel PKC (nPKC) isoforms, which require only DAG; and atypical PKC (aPKC) isoforms, which require neither calcium nor DAG. The existence of a fourth subfamily of PKC is also being defined. All PKC possess a phospholipid-binding domain for membrane interaction. The PKC-related kinases (PRK) have also been classified as members of the PKC superfamily. Each isoenzyme has a specific expression profile and is believed to play a distinct role in cells. These isoforms vary in tissue expression and cellular compartmentalization allowing for specific interactions with substrates.

Generally, a PKC molecule consists of a catalytic and a regulatory domain, composed of a number of conserved regions, interspersed with the variable domains regions of lower homology. Activation of cPKC involves translocation from the cytosol to binding domains at cell membranes. Specific anchoring proteins, such as receptors for activated C-kinase (RACK) and adducins, immobilized at particular intracellular sites, localize the kinase to its site of action. After an increase in intracellular calcium concentration, cPKC interacts with the cell membrane in an inactive, but conformationally distinct form. DAG facilitates penetration of these isoenzymes into the cell membrane (tumor-promoting phorbol esters are used experimentally as synthetic DAG-analogs). When attached, the affinity of PKC for calcium is increased such that activation of the enzyme is achieved (depending on its phosphorylation status).

PKC family enzymes' two regulatory domains, C1 and C2, are the targets of different second messengers. The recently deduced 3D structures of the C2 domains show that they can additionally act as PtdIns(4,5)P(2)-binding or phosphotyrosine-binding modules depending on the isoenzyme. The ability to play different roles in the cell-wide web of signals underscore the notion that PKC is a multifunctional family of enzymes which, after 30 years of investigation, is only now beginning to be understood (Corbalan-Garcia S, and Gomez-Fernandez JC, Biochim Biophys Acta, Jul 2006;1761(7):633-54).

PKC isoforms are thought to reside in the cytoplasm in inactive conformations and translocate to the plasma membrane or cytoplasmic organelles upon cell activation by different stimuli. However, a sizable body of evidence collected over the last 20 years indicates that PKC is capable of translocating to the nucleus. Furthermore, PKC isoforms are resident within the nucleus. Many nuclear proteins that are PKC substrates have been identified and nuclear PKC-binding proteins have been characterized that may be critical for fine-tuning PKC function in this cell microenvironment. Several lines of evidence suggest that nuclear PKC isozymes are involved in the regulation of biologic processes as important as cell proliferation and differentiation, gene expression, neoplastic transformation, and apoptosis (Martelli AM, etal, Biochim Biophys Acta, May-Jun 2006;1761(5-6):542-51).

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PKC is involved in mediating intracellular responses to external signals, such as growth factors, hormones, and neurotransmitters, and is involved in cell proliferation and oncogenesis (Cho-Chung YS, Pharmacol Ther, May-Jun 1999;82(2-3):437-49). PKC family members play crucial roles in signaling pathways that regulate diverse cellular functions, including proliferation, differentiation, and apoptosis (Jiffar T, etal, AACR-NCI-EORTC05, Abs. C46).

In vitro, PKC stimulates the activities of urokinase plasminogen activator (uPA), matrix metalloproteinases (MMP), and cell-adhesion molecules, all of which are known to increase invasiveness in human cancer cell lines (Stoll BA, Eur J Cancer Prev, Apr 2000;9(2):73-9).

PKC subtypes perform distinct functions in radiation-induced apoptosis. Moreover, some participants in PKC-related signaling cascades have been identified in radiation-induced apoptosis. Interestingly, PKC-related signaling cascades have been found to be regulated in part by ATM, a protein related to cell-cycle checkpoints and cell radiosensitivity that also regulates radiation-induced apoptosis (Nakajima T, Med Sci Monit, Oct 2006; 12(10):RA220-4).

PKC are known to modulate multidrug resistance, providing a rational for the combination of PKC modulators with classical cytotoxic drugs. Inhibition and/or activation of specific PKC is thought to control tumor growth by interacting directly with cancer cells and indirectly by blocking tumor angiogenesis (Serova M, etal, Semin Oncol, Aug 2006;33(4):466-78).

Raf-1

Raf-1, a serine/threonine protein kinase, is part of the Ras/Raf/MEK/ERK pathway, known as the mitogen-activated protein kinase (MAPK) signal transduction cascade that promotes tumorigenesis. Raf-1 is a MAP kinase (MAP3K) that functions downstream of the Ras family of membrane associated GTPases. Raf-1 binds directly to Ras-GTP and may be thought of as the effector kinase of Ras. Raf-1 is a crucial integrator in the mitogenic cytoplasmic kinase MAPK pathway. Once activated, Raf-1 can phosphorylate to activate the dual specificity protein kinases, MEK1 and MEK2, which in turn phosphorylate to activate ERK1/2 and thus affect gene expression involved in the cell division cycle, apoptosis, cell differentiation, and cell migration.

Raf kinase isoforms, such as wild-type Raf-1 or the b-raf V600E oncogene, are overactivated in a variety of solid tumors.

Ras Family

Ras and Ras-subfamily proteins are small guanine nucleotide-binding proteins that are implicated in signal transduction and regulation of growth stimulatory signaling pathways. Small monomeric G-proteins in the Ras family play a pivotal role in some RTK pathways. Ras proteins transduce biological information from the cell surface to cytoplasmic components within cells; the signal is transduced to the cell nucleus through second messengers, and it ultimately induces cell division (Scharovsky OG, etal, J Biomed Sci, Jul-Aug 2000;7(4):292-8).

The Ras and Ras-subfamily comprises four major isoforms, Nras, Hras, Ka-ras (K-rasA), and Kb-ras (K-rasB), that code for protein products of approximately 21 kDa, known collectively as p21ras. Interaction between Ras and its effectors requires an intact Ras domain and involves preferential recognition of active GTP-bound Ras (Ras-GTP). Ras maturation into its biologically active form requires post translational modifications to the carboxyl (COOH)-terminal CAAX (cysteine, aliphatic acid and any amino acid) motif, which culminate with the anchoring of p21ras to the plasma membrane, where it relays growth regulatory signals from RTK to various pathways of cell signal transduction. The most critical modification is farnesylation, the addition of a 15-carbon farnesyl isoprenoid moiety in a reaction catalyzed by the enzyme protein farnesytransferase (FTase). The isoforms differ mainly in the post translationally modified C-terminus. Oncogenic mutations in Ras prevent conversion of the active Ras-GTP to the basal inactive Ras-GDP by GAP, thereby locking Ras in an active state, relaying uncontrolled proliferative signals (Reuter CW, etal, Blood, 1 Sep 2000;96(5):1655-69; Rowinsky EK, etal, J Clin Oncol, Nov 1999;17(11):3631-52). Each major isoform of ras differs mainly in the post translationally modified C-terminus of Ras. The distribution of the four isoforms differs between different types of cancer, suggesting that the extreme C-terminus of Ras may be of fundamental importance in malignant progression.

Ras proteins play a direct causal role in human cancer and in other diseases. Mutant H-Ras, N-Ras, and K-Ras occur in varying frequencies in different tumor types but their role has not been clarified. Other members of the Ras superfamily may also contribute to cancer. Mutations also occur in downstream pathways, notably B-Raf, PTEN, and PI3K. These pathways interact at multiple points, including cyclin D1, and act synergistically. In some cases mutations in Ras and effectors are mutually exclusive, while in other cases, they coexist (Rodriguez-Viciana P, etal, Cold Spring Harb Symp Quant Biol, 2005;70:461-7).

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Oncogenic variants of the c-ras genes are found in transformed DNA preparations obtained from various primary tumors and tumor cell lines, and each ras proto-oncogene can give rise to a transforming oncogene by a single base mutation. Such an oncogene can be associated with the appearance of a spontaneous tumor in the organism (Yamamoto T, etal, J Biochem (Tokyo), Nov 1999;126(5):799-803; Scharovsky OG, etal, *ibid*).

Ras is often mutated or overexpressed in human tumors, where it becomes constitutively active and deregulates cell growth. Ras mutations are present in 15% of all malignancies and, unlike p53, a limited number of specific DNA base substitutions have been described. Furthermore, because ras mutations are essential for the malignant phenotype, they cannot, theoretically, be downregulated by the tumor to prevent immune detection. Several peptides derived from mutations commonly found in the ras gene bind to the class I molecule, HLA A2.1.

Ras family GTPases (RFG), when in their active GTP-bound state, interact with a wide array of downstream effectors to regulate many biologic functions in different cell types. How signal specificity among the closely related family members is achieved is still poorly understood. There is both promiscuity and specificity in the ability of RFG to interact with and regulate the various effector families, as well as isoforms within those families. RFG seem to have individual blueprints of effector interactions, and specificity should be considered in the context of the full spectrum of effectors they regulate. A remarkably diverse number of proteins with domains homologous to the Ras-binding domain (RBD) of known Ras effectors have emerged from the sequencing of the genome, with the potential to interact with Ras and/or other RFG. In addition, other proteins without known RBD types are known to behave as RFG effectors, suggesting even more complexity in the number of effector interactions. Determining which of these many candidates are 'true' effectors and characterizing their specificity is a critical step to understanding the specific signaling properties and biologic functions of the various RFG (Rodriguez-Viciana P, McCormick F, Methods Enzymol, 2005;407:187-94).

In its simplest manifestation, Ras functions as a relay switch that is positioned downstream of growth factor RTK such as EGFr and PDGFr, and upstream of a cytoplasmic cascade of kinases that includes the mitogen-activated protein kinases (MAPK); activated MAPK in turn regulate the activities of nuclear transcription factors. Although this rather elegant linear signaling pathway was initially thought to explain the growth-promoting actions of mutated Ras proteins in tumorigenesis and enhanced proliferation of tumors, it is now known that it is just one component of a complex array of signaling pathways (Campbell SL, etal, Oncogene, 17 Sep 1998;17(11 Reviews):1395-413).

For instance, substantial evidence has established that the Raf-1 serine/threonine kinase is a critical effector of Ras function, but there is also evidence that Ras function can be mediated through interaction with multiple effectors to trigger Raf-independent signaling pathways. In addition to p120-GAP and neurofibromin 1-GAP (NF1-GAP), other candidate effectors include activators of the Ras-related Ral proteins (RalGDS and RGL) as well as PI3K. These functionally diverse effectors share a consensus Ras binding sequence, establishing a structural basis for the ability of diverse effector proteins to interact with GTP-bound Ras (Clark GJ, etal, PNAS USA, 20 Feb 1996;93(4):1577-81).

Oncogenic forms of ras are locked in their active state and transduce signals essential for transformation, angiogenesis, invasion, and metastasis through downstream pathways involving the RAF/MEK/ERK cascade of cytoplasmic kinases, the small GTP-binding proteins RAC and RHO, and PI3K, among others. Sequence and expression analysis of more than 1,200 subtracted cDNA fragments revealed transcriptional stimulation or repression of 104 EST, 45 novel sequences, and 244 known genes in Hras-transformed cells were compared with normal cells. Furthermore, common and distinct targets were identified in cells transformed by mutant Hras, Kras and Nras, as well as 61 putative target genes controlled by the RAF/MEK/ERK pathway in reverted cells treated with the MEK-specific inhibitor PD 98059 (Zuber J, etal, Nat Genet, Feb 2000;24(2):144-52).

Ras acts with other proteins to induce neoplasia, and strong Ras signaling may, by itself, suppress proliferation of normal cells. Oncogenic Ras transiently decreases cyclin-dependent kinase 4 (CDK4) expression in primary epidermal cells, in association with cell-cycle arrest in G1 phase. CDK4 coexpression circumvents Ras growth suppression, and induces invasive human neoplasia resembling squamous cell carcinoma. Tumorigenesis is dependent on CDK4 kinase function. Although cyclin D1 is required, it is not sufficient for this process. In facilitating escape from G1 growth restraints, Ras and CDK4 alter the composition of cyclin D and cyclin E complexes and promote resistance to growth inhibition by INK4 cyclin-dependent kinase inhibitors. These data identify a new role for oncogenic Ras in CDK4 regulation and highlight the functional importance of CDK4 suppression in preventing uncontrolled growth (Lazarov M, etal, Nature Medicine, October 2002;8(10): 1105-1114).

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Signal transducers and activators of transcription (STAT)

STAT are latent cytoplasmic transcription factors that are involved in normal cytokine and growth factor signaling. In response to cytokines and growth and other activation factors, STAT family members are phosphorylated by RTK, such as JAK kinases (Janus kinases 1, 2, and 3), and form homodimers or heterodimers through their SH2 domains. These dimers translocate to the cell nucleus where they act as transcription activators of cytokine-responsive genes.

In the cell nucleus, STAT bind directly to promoter elements upstream of regulated genes. STAT proteins interact with other transcription factors and regulators either through their C-terminal transactivation domain or their less well characterized N-terminal domain. STAT proteins provide a means to integrate signaling between the membrane and cytosol of the cell and nuclear events (Silva CM, etal, AACR04, Abs. 198).

Originally, two proteins, STAT1 and STAT2, were discovered to be involved in interferon-α (IFNα) and IFNγ signal transduction. Since then, several additional STAT proteins, STAT3, 4, 5a, 5b, and 6, have been identified. For maximum activation of these proteins, phosphorylation at specific tyrosine and serine residues may be required in STAT1a, 3, 4, and 5. Specific functions of the various members of the STAT family are poorly understood.

Signal transducer and activator of transcription 3 (STAT3)

STAT3, a member of the STAT protein family, is the main mediator of interleukin 6 (IL-6)-type cytokine signaling and plays a critical role in hematopoiesis. Four isoforms, the full length STAT3, truncated β and γ, and δ, are differentially expressed during granulocytic differentiation. The importance of STAT3 is underscored by the fact that deletion of the STAT3 gene in knockout mice is lethal at the early embryonic stage. STAT3 mediates the expression of a variety of genes in response to cell stimuli and plays a key role in many cellular processes such as cell growth and apoptosis.

STAT3 is located in the cytoplasm and translocated to the nucleus after tyrosine phosphorylation. STAT3 is activated through phosphorylation in response to various cytokines and growth factors including IFNα but not IFNβ, EGF, growth hormone, G-CSF, IL5, IL6, HGF, LIF, and BMP2. Crosstalk from pathways other than JAK kinases also leads to phosphorylation and activation of STAT3 as indicated by roles for mTOR and MAP kinase pathways in STAT3 activation and signaling. STAT3 is constitutively activated by various oncoproteins, such as Src. Constitutive activation of STAT3 signaling occurs with high frequency in diverse human malignancies.

Downstream target genes of STAT3 contribute to malignant progression. Genes shown to be regulated by STAT3 include cyclin D1, p21WAF1, Bcl-x, Mcl-1, and c-Myc, which are important in cell cycle control and apoptosis. STAT3, like STAT5, induces progression through the cell cycle, prevents apoptosis, and upregulates oncogenes (e.g., c-myc and bcl-X). The transcription factors associated with STAT3 are c-Jun and cyclic AMP-responsive enhancer binding protein (CREB). The small GTPase, Rac1, also binds to STAT3 and regulates its activity. PIAS3 protein is a specific inhibitor of STAT3.

CISH results are 100% concordant with FISH results, but CISH has the advantage of longer signal stability. Invitrogen has developed 30 CISH probes, known as SpoT-Light CISH, and intends to market several of them following regulatory approval. In 2006 the company submitted its first PMA, for the SpotLight HER-2/neu probe, which identifies patients with breast cancer with tumors overexpressing HEr2/neu for use as a companion diagnostic for Herceptin therapy. Outside the USA, the SPoT-Light HER2 CISH Kit is recommended as an *in vitro* diagnostic for determination of patients with HEr2 amplification in the Herceptin product insert.

Invitrogen also markets a HER-2 ELISA kit and the SPoT-Light EGFR Probe (Zymed), and also offers reference laboratory services.

Oncotech (Tustin, CA) offers molecular oncology services to physicians and pharmaceutical companies. In October 2005, the company launched its EGFr analysis by FISH technology test used to identify patients with nsclc cells with large numbers of EGFr gene copies who are most likely to respond to new targeted therapies such as Iressa or Tarceva. The company also offers EGFr (170 kD) cell surface receptor expression analysis by IHC, HEr2 (185 kD) expression by IHC for metastatic breast cancer for prognosis and suitability for Herceptin therapy, and HEr2 image analysis for automated quantification of IHC staining for HEr2 on breast cancer specimens.

Orion Genomics (Saint Louis, MO) is developing diagnostic products based on detection of errors in DNA methylation. The company's pipeline of methylation-based

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molecular diagnostic tests indicate changes in DNA methylation (so-called second code), which are related to the presence and progression of cancer and determination of appropriate treatment options.

The company's MethylScope technology identifies DNA methylation patterns in normal and tumor tissue, enables comparison of those patterns, and allows development of profiles specific to each. A MethylScope microarray can quantitatively detect the methylation status of every human gene. DNA from a tumor sample is labeled with different colors to distinguish between methylated and unmethylated fragments, which are hybridized to the array and scanned to generate a methylation score for every gene on the array. By comparing methylation profiles of two or more samples, Orion can discover novel DNA methylation-based biomarkers associated with cancer. The company has identified a suite of promising breast cancer biomarkers that demonstrate high sensitivity and specificity. MethylScope technology the subject of patent applications by Cold Spring Harbor Laboratory for Quantitative Biology and Orion.

MethylScreen identifies tumor profiles in clinical samples and can serve as the basis for the development of cancer screening and therapy response tests. It detects trace amounts of specific methylated biomarkers in a background of normal DNA, as would be required in a serum test for cancer. It also quantifies the relative methylation density within each detected biomarker. The technology is based on treating DNA with methylation-specific enzymes and quantification using real time quantitative PCR. For each biomarker locus, the assay measures both unmethylated and methylated copies. This information is used to ascertain the presence of different classes of methylated biomarkers related to disease, stage of disease, or response to treatment. MethylScreen assays may be performed on automated instruments available in most reference testing laboratories.

Transgenomic (Omaha, NE) markets the WAVE Systems and associated consumables. This system couples heteroduplex analysis (HA) and chromatography in an automated system. First, PCR products amplified from a potentially mutated test gene and its normal counterpart are denatured and reannealed to form homo- and heteroduplexes. The mixture is then separated by coupling ion-pair reverse phase liquid chromatography on a DNASep column with temperature-modulated heteroduplex analysis (TMHA). TMHA partially denatures heteroduplexes but leaves homoduplexes completely annealed. Eluted heteroduplex DNA is suitable for direct sequencing without further manipulation. Elution profiles are monitored by UV or fluorescence detectors and plotted as chromatographic peaks. A sensitive fluorescence detector is added on WAVE-HS systems to monitor picogram quantities of fluorescently tagged PCR products. WAVE systems are specifically designed for use in genetic variation detection and single- and double-strand DNA/RNA analysis and purification.

The company's SURVEYOR Mutation Detection Kits and the SURVEYOR Check-It Kit provide reagents and protocols for the detection of mutations in DNA. Transgenomic Labs provides molecular-based testing for hematology, oncology and certain inherited diseases for physicians and third-party laboratories and genomic biomarker analysis services for pharmaceutical and biopharmaceutical companies.

Veridex (Warren, NJ), a subsidiary of Johnson & Johnson, is developing two CellSearch products for identification of tumor cells, one for the enumeration and characterization of circulating tumor cells (CTC) and the other for further molecular characterization of CTC using gene expression profiling.

The CellSearch Circulating Tumor Cell Kit identifies and counts CTC in a blood sample. This kit was approved by the FDA in January 2004 as a diagnostic tool to predict progression-free and overall survival in patients with metastatic breast cancer. In December 2006, the indication was expanded to include use as an aid in the monitoring of metastatic breast cancer.

The CellSearch Epithelial Cell Kit, labeled for research use only, has the same components as the CellSearch Circulating Tumor Cell Kit, but also provides further molecular characterization of CTC. Phenotyping reagents used with this kit are fluorescein conjugated MAb directed to key molecules expressed by epithelial cells. These reagents are used in conjunction with the CellSearch Epithelial Cell Kit, processed with the CellTracks AutoPrep System, and analyzed on the CellTracks Analyzer II. Available phenotyping reagents include HEr2/neu, EGFr, and MUC-1.

Veridex also is developing GeneSearch genetic tests for use in cancer diagnosis and prognosis, particular for breast, colon, and prostate cancer. The GeneSearch BLN (breast lymph node) Assay is CE marked to the *In Vitro* Diagnostic Device Directive in the European Union and became commercially available there in November 2006.

Market Opporunities

HEr2 testing is the paradigm of a theragnostic approach to individualized treatment. The goals of HEr2 testing include identification of both patients who would be expected to benefit from treatment with Herceptin, and those who would be not expected to derive benefit (sparing those in the latter group ineffective treatment, treatment toxicities, and unnecessary expense).

Initially, HEr2 testing was used only in women with metastatic breast cancer (i.e., advanced disease). In order to initiate treatment with Herceptin, a test result demonstrating sufficiently elevated gene amplification/expression of HEr2 was required. Three levels of expression were defined, 1+, 2+ and 3+; 3+ was considered optimal for patient selection, while 2+ was deemed sufficient for initiation of Herceptin therapy.

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Exhibit 6
FDA Approved HER2 and EGFR Tests

Company	Product	Detects ❑ Methodology	Clinical Application ❑ FDA Approval Date
Abbott Molecular 1300 E. Touhy Ave. Des Plaines, IL 60018-3315 Tel: 224-361-7000 Fax: 224-361-7008 www.abbottmolecular.com	PathVysion HER2 DNA Probe Kit (PathVysion Kit)	HER2 gene amplification ❑ FISH	Breast cancer ❑ FDA approved Dec 1998
Applied Imaging 120 Baytech Drive San Jose, CA 95134-2302 Tel: 408-719-6400 Fax: 408-719-6401 www.aicorp.com	Ariol Automated FISH Enumeration System	HER2 gene amplification ❑ FISH	Breast cancer ❑ FDA approved Apr 2005, as an accessory to the PathVysion® HER-2/neu DNA Probe kit
BioGenex 4600 Norris Canyon Rd. San Ramon, CA 94583 Tel: 925-275-0550 Fax: 925-275-0580 www.biogenex.com	Insite Her2/neu Kit	HER2 protein overexpression ❑ IHC	Breast cancer ❑ FDA approved Jan 2005, but not available since FDA recall in early 2006
Dako Produktionsvej 42 DK-2600 Glostrup Denmark Tel: 45 44 85 95 00 Fax: 45 44 85 95 95 www.dako.com	HercepTest	HER2 protein overexpression ❑ Semi-quantitative IHC	Breast cancer ❑ FDA approved Sep 1998
6392 Via Real Carpinteria, CA 93013 Tel: 805-566-6655 Fax: 805-566-6688 www.dakousa.com	HER2 FISH pharmDx Kit	HER2 gene amplification ❑ FISH	Breast cancer ❑ FDA approved May 2005
	EGFR pharmDx Kit	EGFR protein overexpression	Colorectal cancer ❑ FDA approved Feb 2004
Genzyme 500 Kendall Street Cambridge, MA 02142 Tel: 617-252-7500 Fax: 617-252-7600 www.genzyme.com www.genzymegenetics.com	EGFR Mutation Analysis	PCR amplification and bi-directional gene sequencing of exons 18 through 21 of the EGFr tyrosine kinase domain. For mutation positive tumors, germline mutation analysis is performed on a separate DNA sample (peripheral blood or mouthwash)	Non-small cell lung cancer (nsclc) ❑ FDA approved Sep 2005
Siemens Medical Solutions Diagnostics 511 Benedict Avenue Tarrytown, NY 10591 Tel: 914-631-8000 Fax: 914-524-2132 www.diagnostics.siemens.com	HER-2/neu ELISA	HER2 oncoprotein (p105 extracellular domain) ❑ ELISA	Breast cancer (metastatic) ❑ FDA approved Dec 2000 (Bayer Diagnostics; test developed in collaboration with Oncogene Science, which was acquired by Bayer in Dec 1999)

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Ventana Medical Systems 1910 Innovation Park Dr. Tucson, AZ 85755 Tel: 520-887-2155 Fax: 520-229-4207 www.ventanamed.com	(Oncor) Inform HER-2/neu Gene Detection System	HER2 gene amplification ❑ FISH	Breast cancer ❑ FDA approved Dec 1997
	Pathway Anti-Her-2/neu (4b5) Primary Antibody ❑ IHC	HER2 oncoprotein ❑ IHC	Breast cancer ❑ initial FDA approval Nov 2000 as HER-2 (c-erbB-2) Pathway®; Supplementary approval in Jan 2007 as Pathway Anti-Her-2/neu (4b5) Primary Antibody)

Initially, most HEr2 tests were performed by immunochemistry (IHC), a relatively inexpensive test. Later, a new methodology, FISH, became available and began gaining ground as a more accurate approach. However, the cost FISH testing was more than an order of magnitude greater than that of IHC, both in terms of reagent costs and laboratory charges. USA facilities quickly embraced FISH, but other countries with more frugal health care budgets, such as the UK and Canada, at first balked at the high cost. The cost of reagents/consumables, alone, for FISH testing in the USA is more than $140, compared to about $10 for IHC. In the UK, consumables associated with FISH cost about £73, compared to £21 for IHC. In the USA, FISH laboratory reimbursement rates range from $315 to $350.

Initially performed only in women with advanced disease, the market opportunity for HEr2 testing significantly expanded in November 2006, when Herceptin was approved for the adjuvant treatment of early stage, node-positive, HEr2+ breast cancer, in combination with standard chemotherapy. Historically, this diagnostic test/drug relationship is one in which drug development and targeting was enabled by the development of a meaningful test for patient selection, and FDA approval was absolutely dependent upon the availability of that test to screen out and prevent ineffective treatment in the large percentage of women who would be unlikely to respond. Then, years after the initial approvals of the drug and test, the market opportunity for the test increased when the applications (FDA indications) of the drug expanded.

The cost of HEr2 testing remains a small fraction of the cost of Herceptin therapy, which is estimated at over $40,000 per patient. Nevertheless, test developers do not face the same high cost and long development cycle challenges associated with drug development. Furthermore, as shown in the example above, testing markets continually expand as new indications are approved for targeted drugs. It is notable that HEr2 is expressed in many other types of tumor cells, and the market for HEr2 diagnostics is likely to expand further as new (and existing) therapies target these indications.

The current market for HEr2 testing is represented by patients diagnosed with any stage of breast cancer, in order to identify those with early but potentially aggressive disease who may be candidates for adjuvant treatment with Herceptin. According to the American Cancer Society (ACS), an estimated 180,500 women, and 2,030 men will be diagnosed with breast cancer, and approximately 40,460 women will die of the disease in the USA in 2007. The numbers are higher in Europe, estimated at 270,000 new cases, and 88,000 deaths. Adoption of HEr2 testing in these two regions will generate an annual volume of approximately 450,000 tests, resulting in a reagents market of up to $63.0 million, depending on the testing methodology used and local prices. In addition to the European and USA markets, another 550,000 women are diagnosed annually with breast cancer in the rest of the world. In addition, overexpression of HEr2 may also be a prognostic factor in other malignancies, including lung and bladder cancer, among others.

In recent years, tests for other EGFr aberrations (e.g., receptor overexpression and mutations) have become available, and FDA has approved two for use in the clinic. The Dako EGFR pharmDx Kit, an IHC-based system that detects EGFr protein overexpression was approved in February 2004 for use in colorectal cancer. It is indicated as an aid in identifying patients with colorectal cancer who are eligible for treatment with Erbitux (cetuximab). The Genzyme EGFR Mutation Assay was approved by FDA and launched in September 2005 for the detection of EGFr mutations in patients with nsclc. Other tests are marketed or in development for EGFr amplification (e.g., by Abbott Molecular, Beckman Coulter, Genzyme, Oncotech) and EGFr mutations (e.g., by DxS, Genzyme).

Although EGFr testing is expected to gain greater acceptance and achieve some level of routine clinical use during the next five years, the level of clinical use achieved will depend largely on clinical trial data supporting correlations of specific EGFr aberrations with the initiation, maintenance, or progression of particular malignancies. That is, novel tests for detection of biomarkers may be devel

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Exhibit 7
Non-FDA Approved and Developmental HEr2 and EGFr Tests

Company	Product	Detects ❑ Methodology	Commercial Status ❑ Application
Abbott Molecular	EGFR test	EGFR amplification ❑ FISH	Developmental; launch anticipated in 2008-9 ❑ bladder cancer (early recurrence)
AdnaGen Ostpassage 7 D30853 Langenhagen Germany Tel: 49-0-511-725950-50 Fax: 49-0-511-725950-40 http://www.adnagen.com	AdnaTests, Breast Cancer Select, Breast Cancer Detect	Circulating tumor cells and HER2, EGFR Isolation via antibody-linked magnetic particles, then RT-PCR	Marketed ❑ breast cancer, other
Beckman Coulter 4300 N. Harbor Boulevard P.O. Box 3100 Fullerton, CA 92834-3100 Tel: 800-742-2345 Fax: 800-643-4366 www.beckmancoulter.com	ProteomeLab PF 2D Analysis	EGFR amplification ❑ proteomics (chromato-focusing followed by non-porous reverse phase chromatography)	Marketed ❑ glioma/astrocy-toma studies
Clarient 31 Columbia Aliso Viejo, CA 92656 Tel: 949-425-5700 Fax: 949-425-5701 www.clarientinc.com	Automated Cellular Imaging System	HER2, EGFR ❑ quantitative IHC	Marketed ❑ breast and colorectal cancer
DxS 48 Grafton Street, Manchester, M13 9XX UK tel: +44 (0) 161 606 7201 fax: +44 (0) 161 606 7313 www.dxsgenotyping.com	EGFR Mutation Test Kit	EGFR Exon 21 L858R point mutation and Exon 19 deletion 15 (E746 - A750)	Marketed
	Extended EGFR Mutation Test Kit	29 most common somatic mutations in the EGFr gene: • 19 deletions in exon 19 • T790M • L858R • L861Q • G719X (G719S, G719A, G719C) • S768I • 3 exon 20 insertions	Marketed
	T790M EGFR Mutation Test Kit	Mutant base at position 2369 of the EGFr gene	Marketed ❑ intended for research of the association between T790M mutations andprogression of lung cancer; also useful for deter-mination of the T790M status of patients in clinical trials.
Epigenomics Kleine Präsidentenstr. 1 10178 Berlin Germany Tel: 49 30 24345-0 Fax: +49 30 24345-555 www.epigenomics.com	DNA methylation test	EGFR, HER2 ❑ DNA methylation	Developmental

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Genomic Health 301 Penobscot Drive Redwood City, CA 94063 Tel: 650-556-9300 Fax: 650-556-1132 www.genomichealth.com	Oncotype DX system	EGFR, HER2 ❑ RT-PCR	Marketed ❑ breast cancer, non-small cell lung cancer (nsclc)
Genzyme	EGFR Amplification	EGFR Amplification ❑ FISH	Marketed ❑ nsclc
	KRAS Mutation Analysis	Mutations in codons 12 and 13 of the KRAS gene	Marketed ❑ nsclc
Idaho Technology 390 Wakara Way Salt Lake City, UT 84108 Tel: 801-736-6354 Fax: 801-588-0507 www.idahotech.com	Hi-Res Melting	HER2, EGFr single-base change in sample by melting the PCR product	Breast cancer, other
Invitrogen 1600 Faraday Avenue PO Box 6482 Carlsbad, CA 92008 Tel: 760-603-7200 Fax: 760-602-6500 www.invitrogen.com	HER-2 CISH	HER2 chromogenic *in situ* hybridization (CISH)	PMA submitted to FDA; marketed abroad breast cancer (as a companion diagnostic for Herceptin therapy)
Oncotech 15501 Red Hill Avenue Tustin, CA 92780 Tel: (800) 576-6326 Fax: (714) 566-0421 http://oncotech.com	EGFR by FISH	EGFR gene amplification ❑ FISH	Marketed ❑ nsclc
	EGFr (170kD) cell surface receptor overexpression	EGFr (170kD) overexpression ❑ IHC	Marketed ❑ nsclc
	HER-2 overexpression	HER2 (185kD) overexpression ❑ IHC	Marketed ❑ breast cancer
	HER-2 image analysis	HER2 ❑ Automated quantification of IHC staining	Marketed ❑ breast cancer
Orion Genomics Center for Emerging Technology 4041 Forest Park Ave Saint Louis, MO 63108 Tel: 314-615-6977 Fax: 314-615-6975 www.oriongenomics.com	MethylScope	EGFR, HER2, KRAS ❑ DNA methylation	Developmental
	MethylScreen	EGFR, HER2, KRAS ❑ real-time quantitative PCR and DNA methylation	Developmental
Siemens Medical Solutions Diagnostics	EGFR ELISA	EGFR oncoprotein (p110 extracellular domain) ❑ ELISA	Marketed for research use only
Transgenomic 12325 Emmet Street Omaha, NE 68164 Tel: 888-233-9283 Fax: 402-452-5453 www.transgenomic.com	WAVE Systems and SURVEYOR Mutation Detection Kits	HER2, EGFR gene amplification ❑ PCR, heteroduplex analysis + chromatography (automated)	Marketed
	SURVEYOR Mutation Detection Kits	HER2, EGFR gene amplification ❑ PCR, SURVEYOR Nuclease	Marketed

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Ventana Medical Systems	Confirm anti-EGFR	EGFR protein (extracellular domain of 170 kD and type III variant of 145 kD) ❑ IHC	Marketed for research use only
Veridex (Johnson & Johnson) 33 Technology Drive P.O. Box 4920 Warren, NJ 07059 Tel: 585-453-3240 Fax: 585-453-3344 www.veridex.com	CellSearch Epithelial Cell Kit	HER2, EGFR ❑ fluorescein conjugated MAbs againts epithelial cell markers	Marketed for research use only
	GeneSearch	Genetic tests	Marketed in EU, where GeneSearch BLN (breast lymph node) is approved ❑ breast, colon, and prostate cancer

oped and useful in clinical trials, but wider clinical use will not follow for tests that do not demonstrate clinical utility.

The use of EGFr testing during the development and after FDA approval of cetuximab (Erbitux; ImClone Systems) provides an interesting example of the complexities. ImClone used EGFr testing to select for patients with EGFr overexpression during the clinical trials of Erbitux because EGFr is the drug's target. However, difficulties with the IHC test and, more importantly, the problem of the high prevalence of EGFr in many malignancies, made EGFr testing more of a hindrance than a benefit. ImClone has requested that FDA remove the requirement for EGFr testing for new indications, and remove EGFr testing from the drug label.

At present, detection of EGFr overexpression offers less clinical utility than HEr2 testing in patients with breast cancer for determination of Herceptin therapy; and both EGFr and HEr2 testing offer less clinical utility than Philadelphia chromosome (BCR-ABL gene) testing for determining patients with chronic myelogenous leukemia who may respond to treatment with imatinib (Gleevec; Novartis). Still, tests of EGFr overexpression may prove valuable for determining patients with who are more likely to respond to EGFr-based therapies. Improved tests and testing methodologies, more precise application of EGFr test data to individual patients' diagnoses, progression, and therapeutic responses, and more clinical trial data are needed. Much work is underway to define the clinical role of EGFr overexpression in malignancies, including clinical trials in bladder cancer, glioma/astrocytoma, nsclc, pancreatic cancer, and many other solid tumors.

EGFr mutation testing may provide information that is more specific and useful for determining therapy. For example, the Genzyme EGFR Mutation Assay identifies the mutation in up to 20% of patients with nsclc; and approximately 85% of patients with nsclc and somatic mutations in the tyrosine kinase domain of EGFr have been reported to respond to treatment with tyrosine kinase inhibitors. Many clinical trials are underway to define the role of EGFr mutations and EGFr mutation testing in various malignancies.

Some developers of molecular diagnostic tests are responding to the current dynamics by seeking partnerships with drug companies and increasing proactive marketing activities. Tests that enable an initial drug approval or additional indications (e.g., EGFr mutation tests may enable second or third line treatments to achieve first line indications) have revenue potentials that are large enough to justify significant R&D investment (e.g., PMA submissions for regulatory approval of claims).

According to NEW MEDICINE's *Oncology KnowledgeBASE (nm|OK), more than 46 novel agents are in current development that target the EGFr pathway; 2/3 of these compounds have already entered clinical trials. The nm|OK database provides detailed, current information about developmental agents that target the many points of potential intervention along the EGFr pathway. Pharmacologic strategies include direct inhibition, novel drug delivery approaches, and vaccine strategies. Targets include the various mechanisms of EGFr activation, such as gene amplification, transcriptional abnormalities, gene mutations, and overproduction of the EGF family ligands, and many downstream targets. The first effective anti-EGFr agents included monoclonal antibodies (MAb), which bind to the extracellular domain of the receptor, and small molecule drugs, which bind to and inhibit intracellular tyrosine kinases. Other approaches include synthetic nucleic sequences (including siRNA), which prevent transcription and receptor/ligand production; gene transfer to repress transcription of receptor tyrosine kinase proteins; EGFr/ligand-directed vaccines, which stimulate production of antibodies to enhance host immune response; and drug delivery approaches (e.g., immunoconjugates), which target the extracellular domain of EGFr to haul cytotoxics, toxins or radioisotopes to cancer cells.*

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