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MECHANISMS IN MALIGNANCY

THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) PATHWAY IN CANCER

PART II — TARGET CANCER INDICATIONS AND TARGETING APPROACHES

MEETING COVERAGE

NEW TARGETS AND DELIVERY SYSTEMS IN CANCER DIAGNOSIS AND TREATMENT

FROM THE MARCH 2007 CONFERENCE ORGANIZED BY THE SIDNEY KIMMEL CANCER CENTER, IN SAN DIEGO, CA

Pancreatic Cancer-specific Vector	1996
Breast Cancer-specific Vector	1996
Endostatin Prodrug	1996
Viral-based Therapy for Multiple Myeloma	1997
Targeted and Shielded Adenovirus Vectors	1997
Whole Cell Vaccines for Acute Myeloid Leukemia (AML)	1997
Replication-competent Retroviral (RCR) Vectors	1998

MECHANISMS IN MALIGNANCY

THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) PATHWAY IN CANCER

PART II — TARGET CANCER INDICATIONS AND TARGETING APPROACHES

A four-part review of the role of the epidermal growth factor (EGF) receptor (EGFr) family, or ErbB receptor family, pathway in cancer began in the last issue of Future Oncology (March 28, 2007; V8, #11/12). Part I addressed ErbB receptors and ligands, downstream signaling in EGFr pathways, and clinical laboratory detection methodologies and products for diagnostic, prognostic, and theragnostic applications. This article, Part II, addresses the relationship of aberrant ErbB receptor expression and mutations within specific cancer indications and provides an overview of therapeutic approaches. Part III describes the status of commercialized agents targeting the EGFr pathway and assesses their performance in the clinic and will appear in the next issue of Future Oncology. Part IV, the last of this series, describes and analyzes the many novel drugs in development targeting this pathway.

ERBB RECEPTOR ASSOCIATION BY CANCER INDICATION

Widespread clinical use of agents targeting aberrant ErbB receptor expression and mutation in cancer began with the approval of the anti-ErbB2 (HEr2) monoclonal antibody (MAb), trastuzumab (Herceptin; Genentech), which was the first anti-EGFr family therapeutic to be approved, in this case for HEr2-positive breast cancer. FDA approval and successful commercialization of Herceptin represented a triumph of rational, targeted therapy. Selection of candidates for therapy with Herceptin requires demonstration of abundant presence of HEr2 by immunohistochemical (IHC) or fluorescent *in situ* hybridization (FISH) methodologies. Approvals of other MAb followed. Initiation of treatment with the anti-EGFr MAb, cetuximab (Erbitux; ImClone Systems), which is approved for metastatic colorectal cancer, also requires demonstration of expression of the target, in this case EGFr, by IHC or FISH for candidate selection. Panitumumab (Vectibix; Amgen) is approved for metastatic colorectal cancer that expresses EGFr. Another MAb, nimotuzumab, developed by Cuba's Centre of Molecular Immunology, is commercially available outside the USA.

Another R&D approach resulted in the development of small molecule tyrosine kinase inhibitors (TKI), which inhibit the intracellular TK domain of EGFr, activation of which initiates signal transduction in downstream pathways. FDA approved TKI include gefitinib (Iressa; AstraZeneca) and erlotinib (Tarceva; Genentech/ OSI Pharmaceuticals) for non-small cell lung cancer (nsclc), and lapatinib ditosylate (Tykerb; GlaxoSmithKline for advanced or metastatic breast cancer that overexpresses HEr2. EGFr testing for overexpression (e.g., protein by IHC or gene amplification by FISH) or activating mutations of the kinase domain (e.g., by sequencing) is performed to determine candidacy for treatment with TKI.

Approvals of Herceptin and EGFr-targeted agents stimulated research into the EGFr family of receptors and pathways and development of novel, targeted agents. However, as clinical experience accumulated, the limits of the effectiveness of EGFr family-targeted agents became clear, and questions emerged regarding the diagnostic/prognostic/theragnostic value of marker overexpression and mutations, the mechanisms of tumor sensitivity and resistance, the value of multitargeted agents and combination therapy approaches (simultaneously or sequentially administered), and others. Notably:

- Many mutations (see Exhibit 1) have been identified, which may explain the variations in response rates and resistance observed in the clinic with approved and developmental EGFr family-targeted agents.
- Only 30% to 40% of patients with HEr2-positive breast cancer (i.e., HEr2 overexpression) respond to trastuzumab treatment, which is the most efficacious of the currently available EGFr family targeted therapies. The mechanisms of sensitivity and resistance to trastuzumab are not fully understood.
- Although EGFr is overexpressed by more than 35% of all solid tumors, and increased levels of EGFr gene expression are frequently associated with increased risk of the disease and poor prognosis, the finding of EGFr overexpression, for the most part, has not translated into ability to predict response to targeted therapies. For some malignancies (e.g., lung cancer), mutations in EGFr family genes, rather than expression levels, are proving to be more useful predictors

of tumor sensitivity and the development of resistance.

- New findings reveal an increasing number of alternative, redundant EGFr pathways for activation of downstream signal transduction. The myriad issues include cooperation/interaction of EGFr family receptors to activate pathways, intranuclear events, and others. More extensive and detailed study is needed of the meaning of expression levels (gene, mRNA, protein) of ErbB receptors, ligands, and downstream effectors in the clinical setting.
- EGFr testing issues include methods of detection. Many studies now test both expression with IHC and gene amplification with FISH; and mutation studies are also performed when appropriate. Other issues include sample selection (e.g., minimizing false negative tests, the problem and meaning of variability by tumor subtype, establishing primary tumor versus metastases), duration of testing (i.e., before, during, and after clinical trials), the need to analyze test results in responding and non-responding groups of patients, and others. In the future it is anticipated that new technologies will permit simultaneous measurement of multiple markers along signal transduction pathways to better understand cancer cell signaling.
- Issues in clinical efficacy measures include the potential (controversial) development of new metrics to assess response. Survival benefit has proven to be difficult to achieve.

Exhibit 2 lists estimates of EGFr family member expression rates in human malignancies.

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

At present, EGFr is the subject of much research, more than for any of the other ErbB family receptor, because of its widespread presence in epithelial tumors, the identification of mutations that have impact on therapy in nsclc, and the availability of data from completed and ongoing clinical trials with the approved and many developmental agents targeting EGFr. Exhibit 3 lists research findings related to EGFr in specific clinical indications. The text below provides highlights of findings presented in Exhibit 3, according to specific clinical indications.

Colorectal Cancer

For colorectal cancer, a positive EGFr protein expression test result is required for initiation of treatment with cetuximab (Erbitux) or panitumumab (Vectibix). However, significant evidence suggests that EGFr expression is not predictive of response to cetuximab therapy, and much work is underway to determine the predictive roles of EGFr expression, gene amplification, and mutation in colorectal cancer. Many unanswered questions and issues remain. Among the most pressing issues addressed by studies in the exhibit are:

- variations in EGFr expression between primary and secondary lesions
- changes in expression during disease progression or in response to treatment
- intratumoral heterogeneity
- conflicting findings regarding the usefulness of EGFr in predicting response to targeted therapy
- the potential usefulness of downstream markers for prognostic and theragnostic use
- use of EGFr measurements for prediction of therapeutic response to chemotherapy and radiotherapy.

The following study exemplifies the search for EGFr family-related markers for theragnostic and prognostic use in colorectal cancer; others are listed in Exhibit 3. Although EGFr levels and EGFr mutations do not appear to correlate with response to cetuximab in colorectal cancer, gene amplification resulting in an increased number of gene copies for EGFr may be predictive. Investigators sought to identify predictive markers of response for development into molecular theragnostics to enhance the effectiveness of cetuximab therapy in colorectal cancer. Preclinical approaches using transcriptional profiles of 164 primary tumors and 21 sensitive and resistant cell lines identified a list of genes that may be predictive of response.

A phase II clinical trial of cetuximab monotherapy was conducted in patients with metastatic colorectal cancer to examine whether preclinically identified markers could be validated in a clinical setting. RNA was isolated from pretreatment core biopsies from all patients and profiled on Affymetrix gene chips. DNA isolated from the pretreatment core biopsies was used for both EGFr gene copy number and mutation analysis. An interim statistical analysis of transcriptional profiling data was performed to examine whether preclinically identified markers are differentially expressed between patients who derive clinical benefit, described as partial response (PR) or stable disease (SD), and those with progressive disease. Pathway analysis tools were used to identify biologic relationships between the predictive markers identified from this clinical study.

Several preclinically identified candidates distinguished between responders and non-responsders with high accuracy. The top candidate sensitivity markers identified from this analysis are key players in the EGFr signaling pathway. Strikingly higher expression levels in responders suggest that tumors addicted to the EGFr pathway respond to cetuximab. Approximately 20 top response prediction markers are found to be associated with the EGFr network that may be used either singly or in combination for patient selection to enhance the effectiveness of cetuximab therapy. This analysis is being extended to include a larger set of patients from this trial (Khambata Ford S, etal, AACR06, Abs. 4032).

Although tumor expression of EGFr does not serve as a prognostic marker for survival, EGFr may be used as a marker of circulating tumor cells because EGFr persistence in blood after surgery identified a subset of patients at high risk of relapse, as reported by investigators at Policlinico Umberto I (Rome, Italy). They investigated by RT-PCR assay the expression at mRNA level of EGFr, IL-6, and IL-10 in blood samples taken from 56 patients with colorectal cancer. Each gene expression was evaluated 1 day before and 20 days after primary surgery. Persistence of each gene in blood after surgery was then correlated to the relapse free time in a 3-year follow-up. In blood samples taken before surgery, EGFr, IL-6, and IL-10 were expressed in 62%, 100%, and 100% of patients, respectively. EGFr expression, but not IL-6 or IL-10, correlated with stage of disease. Among 41 patients who underwent follow-up studies, EGFr was persistently high in 67%; 94% of them relapsed. Persistence of IL-10 after surgery also identifies relapses in 89% of cases. IL-6 persistence was not found to significantly correlate to progression of disease. Persistence of both EGFr and IL-10 in blood of patients with colorectal cancer after surgery identifies those with high propensity to relapse. These findings may suggest a clinical use of preoperative EGFr/IL-10 RT-PCR assay in the prediction of tumor recurrence (Giacomelli L, etal, Clin Cancer Res, Jul 2003;9(7):2678-82). EGFr expression in blood also correlates with worse prognosis in patients with bladder cancer. However, no correlation is found between EGFr expression in the primary tumor cells and those in peripheral blood, which indicates that biologic characteristics and genetic profiles of primary tumor cells differ from cells involved in tumor progression, which may detach and enter the circulation.

Head and Neck Cancer

EGFr is overexpressed in squamous cell carcinoma of the head and neck (SCCHN), and high expression levels correlate with decreased survival. Both components of the ligand/receptor pair of transforming growth factor (TGF)-α/EGFr are overexpressed in SCCHN, suggesting a role for this autocrine growth pathway in SCCHN, and a therapeutic potential for agents that block EGFr (Grandis JR, etal, J Natl Cancer Inst, 3 Jun 1998;90(11):824-32). Response to treatment, however, has been limited in clinical trials of TKI treatment in SCCHN. A study of Japanese patients with SCCHN found no mutations of the TK domains of EGFr or HEr2, which may explain the limited response of SCCHN to TKI. More study is needed to understand the roles of EGFr gene or protein expression or signaling pathways in SCCHN. In earlier work, two antisense oligonucleotide constructs, against TGF-a mRNA and the U6 small nuclear RNA (snRNA) promoter, which is required for EGFr expression, effectively inhibited growth in *in vivo* and *in vitro* studies, respectively.

Lung Cancer

Much work has been done to investigate the role of EGFr mutations in nsclc, particularly with regard to sensitivity and resistance to treatment. EGFr mutations in patients with nsclc, particularly mutations of four exons (18-21) in the EGFr kinase region, have been associated with sensitivity and resistance to the EGFr TKI, gefitinib and erlotinib. Exons 18-21 encode part of the tyrosine kinase domain. Exon18-21 mutations are clustered around the tyrosine kinase ATP-binding pocket.

EGFr mutations are present in approximately 10% of nsclc cases from North America and Western Europe, but increases to 30-50% in individuals of East Asian descent. EGFr mutations occur in more than 50% of adenocarcinomas with bronchioloalveolar features, which are found in non-smokers with nsclc. According to some researchers, the discovery of EGFr mutations in never-smokers was one of the most important finding ever discovered in nsclc. They note that treatment of patients whose tumors bear such sensitizing mutations with small molecule TKI, gefitinib or erlotinib, results in response rates and durations never before reported, including complete responses.

EGFr mutations in nsclc include:

- Exon 18 substitutions (G719A, G719S, G719C, others): in the EGFr nucleotide-binding loop; account for 5% of EGFr mutations; associated with drug sensitivity.
- Exon 19: in-frame deletion mutations of exon 19 (residues 747-750) are the most prevalent EGFr mutations in nsclc, accounting for 45%; associated with drug sensitivity. In mouse studies, exon 19 deletions (involving the LREA motif, L858R, G719S and ins 770(NPG)-mutated EGFr proteins) have been shown to be oncogenic and increase EGFr kinase activity, which causes hyperactivation of downstream pro-survival pathways (and is oncogenic).
- Exon 19 D761Y: a secondary mutation; accounts for <1% of EGFr mutations; D761Y is reportedly associated with small molecule TKI (gefitinib and erlotinib) resistance in nsclc cells with L858R-EGFr.
- Exon 20 T790M (M=methionine) mutation: a second site mutation found in 50% of cases of acquired resistance to gefitinib and erlotinib; clinically the most relevant EGFr mutation in nsclc. The T790M mutation can render partially activated mutant EGFr fully ligand-independent (i.e., constitutively active).
- Exon 20 in-frame insertions: account for 5% of EGFr mutations; associated with drug resistance (with much less clinical significance than T790M).
- Exon 20 substitutions: account for <1% of EGFr mutations; associated with drug sensitivity.
- Exon 21 L858R substitution: in the EGFr activation loop, accounts for an estimated 40-45% of EGFr mutations; associated with drug sensitivity.

The role of EGFr mutations in lung cancer was extensively reviewed recently (Sharma S, etal, Nat Rev Cancer 2007;7(3):169-181), which was the source of some of the information above.

Prostate Cancer

EGF and EGFr levels are regulated by androgens in human prostate cancer cell lines; and levels of EGFr expression are increased in hormone-independent prostate cancer cell lines. In a mouse model of androgen responsive prostate cancer, androgen increased the activity of the EGF-network in prostate cancer by increasing ErbB1 expression, and ErbB1 activity was essential for androgen-induced proliferation and survival of prostate cancer cells. In a study of human prostatectomy specimens from patients with prostate cancer, androgen receptor and EGFr staining indices were interdependent; and low androgen receptor and increased EGFr staining were associated with increased risk with regard to relapse-free survival.

Ovarian Cancer

A study of EGFr gene status and protein expression in human primary and recurrent ovarian serous carcinoma detected EGFr amplification in 20% of primary and 22% of recurrent tumors. EGFr protein immunoexpression was observed in 28% of primary and 33% of recurrent carcinomas. In recurrent ovarian carcinoma, moderate and strong EGFr expression was associated with amplification. A larger clinical trial detected both EGFr amplification and protein overexpression in serous ovarian carcinoma. The number of EGFr copies was of greater prognostic value, and the researchers suggest that EGFr amplification is a potentially useful criterion for selecting patients with serous ovarian carcinoma for clinical trials with EGFr inhibitors.

Brain Cancer

Clinical trials to date of EGFr inhibitors in glioma have yielded inconclusive results regarding effectiveness. Results with an *in vitro* model suggest that recovery of downstream Raf/Mek/Erk kinase signaling, via recovery of MAP kinase after initial inhibition, may be the mechanism by which glioma cells overcome EGFr inhibition. This finding suggests that dual, EGFr/MAP kinase inhibition therapy may be an effective therapeutic approach for glioma.

Breast Cancer

Overexpression of EGFr occurs in about 30% of breast tumors and often correlates with poor prognosis. Findings from an *in vitro* study suggest that an amphiregulin autocrine loop and EGFr overexpression are linked, which may explain why both factors predict aggressive breast cancer. A study in breast cancer cell lines identified phosphorylation sites, including the c-Src phosphorylation site, Y845, that remained phosphorylated in resistant cells even after TKI treatment. Other tyrosine kinases may be involved in the promotion of EGFr tyrosine kinase intrinsic resistance in breast cancer. Another breast cancer study suggests that overexpressed EGFr facilitates tumor cell proliferation in part through cooperation of nuclear EGFr with E2F1 to cause activation of B-Myb gene expression.

Pancreatic Cancer

EGFr overexpression in pancreatic cancer may be associated with aggressive disease and poor prognosis. Although treatment with erlotinib, in combination with gemcitabine, has provided clinical benefit in patients with pancreatic adenocarcinoma, a link between positive therapeutic findings and EGFr overexpression or mutation has not been established. In a study of 66 patients with inoperable or metastatic pancreatic adenocarcinoma, the researchers concluded that the incidence of somatic mutations in the tyrosine kinase domains of EGFr was very low, and increased EGFr gene copy number did not influence survival.

HER2/NEU (HER2, ERBB2)

Exhibit 4 lists research findings related to HEr2 in specific clinical indications. The text below provides highlights of findings presented in Exhibit 4, according to specific clinical indications.

Breast Cancer

Clinically, 15-20% (or up to 30-40% in older reports) of invasive breast malignancies are HEr2-positive, which is associated with a worse prognosis than HEr2-negative tumors. In addition to its distinctive molecular signature, HEr2-positive breast cancer is characterized by distinctive clinical features, including high grade poorly differentiated tumor cells, rapid proliferation, lymph-node involvement, relative resistance to particular types of chemotherapy, poor prognosis, and greater risk of recurrence. HEr2-positive tumors respond to anthracycline-based adjuvant chemotherapy, but are only partially responsive to tamoxifen because, although they express estrogen or progesterone (or both), the levels of these hormones are lower than in HEr2-negative breast tumors. By some estimates, approximately 50,000 women in the USA and 250,000 worldwide are diagnosed with HEr2 positive breast cancer each year.

HEr2 amplification occurs during the *in situ* stage of tumor development. HEr2 testing can be performed on either the primary or metastatic tumor because, once HEr2 amplification occurs, the malignant phenotype is thought to be fixed. Because it does not bind ligands, the most likely role of HEr2 is that of a signal modulator, which is induced after other EGFr-family receptors bind ligand. HEr2 gene amplification causes increases of up to 100 times greater than normal expression of HEr2 on breast cancer cells. HEr2 overexpression can transform normal cells into a malignant phenotype in culture and appears to encourage HEr2 heterodimer formation with abnormal signaling properties (Burstein HJ, NEJM 2005;1652-4).

Although the clinical efficacy of the anti-HEr2 MAb, trastuzumab, is well established, the mechanisms of action of its beneficial effects are not clear. Limited laboratory and clinical data suggest downregulation of surface HEr2 expression follows trastuzumab binding to HEr2 protein,

Exhibit 1
Potential Treatment Implication(s) of Selected Related EGFr Pathway Mutations

Selected Related EGFr Pathway Mutations	Potential Treatment Implication(s)
EGFr kinase domain mutations: • point mutation G719, exon 18 • deletion of amino acids 747-750, exon 19 • in-frame insertions, exon 20 • point mutations L858 and L861, exon 21	Favorable response to anti-EGFr tyrosine kinase inhibitors (TKI); occurs more frequently in subpopulations of patients with non-small cell lung cancer (females, Japanese, and nonsmokers with bronchioalveolar adenocarcinoma)
EGFr gene amplification	Correlates with response rate to TKI; EGFr gene amplification, with coexisting EGFr mutations, strongly correlates with a high response rate to TKI
HEr2 mutations	Favorable response to anti-EGFr TKI; found in lung adenocarcinoma (like EGFr mutations)
High expression levels of ErbB3	Correlates with clinical benefit from geftinib in patients with geftinib-sensitive nsclc
K-Ras mutations	Correlates with resistance to EGFr inhibitors; found more frequently in smokers
B-Raf mutations	Found in melanoma; correlates with exquisite sensitivity to small molecule MAPK/ extracellular signal-related kinase inhibitors
Somatic mutations of PI3KCA gene (encodes p110alpha catalytic subunit of PI3K)	Found in a variable cohort of colon, brain, gastric, breast, and lung malignancies
Loss of function of PTEN (tumor suppressor phosphatase gene)	Found in many different malignancies; results in increased PI3K activity and increased importance of this pathway. [PTEN selectively dephosphorylates phosphatidylinositol 3,4,5-triphosphate.] Absence of PTEN function in tumor cells correlates withextreme sensitivity to mammalian targets of rapamycin and PI3K inhibitors
Secondary EGFr mutation (i.e., T790M)	Correlates with acquired resistance (i.e., initial responders to geftinib become resistant); in experiments, irreversible EGFr inhibitors block signaling and growth of tumor cells with T790M mutations (clinical trials are underway in patients with lung cancer refractory to erlotinib)

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

ways and regulation of cell cycle signaling, suppresses VEGF production, and potentiates chemotherapy. The lack of benefit of trastuzumab in HEr2-negative breast cancer and other tumors with moderately elevated levels of HEr2 expression suggests that critical thresholds of HEr2 expression or gene amplification are required for the effectiveness of this drug. It is notable that, although they overexpress HEr2, only 30-40% of patients with HEr2-positive breast cancer respond to trastuzumab. Resistance to trastuzumab has emerged and occurs in almost all patients. Mechanisms of trastuzumab resistance are not understood (Exhibit 3).

Ovarian Cancer

Overexpression of HEr2 in ovarian cancer is associated with aggressive disease. A wide range of HEr2 overexpression rates, from 1.8% to 76%, has been reported in the literature. In various ovarian cancer studies, HEr2 overexpression has been associated with advanced stages, poorly differentiated tumors, resistance to chemotherapy, and shortened survival. Treatment with trastuzumab as a single agent in pretreated patients with ovarian cancer and high levels of HEr2 overexpression produces a low but significant response rate. However, the clinical utility of trastuzumab is limited by the small number of patients with ovarian cancer with tumors expressing high HEr2 levels. In a study of 160 patients with epithelial ovarian cancer, IHC staining for HEr2 found that 64.4% were HEr2 negative, 24.4% were 1+, 6.9% were 2+, and 4.4% were 3+. Positive IHC staining for HEr2 was associated with poor overall survival.

Pancreatic Cancer

Pancreatic cancer is generally associated with a low frequency of HEr2 overexpression, studies of HEr2 expression in pancreatic cancer have yielded conflicting results. In one study, 29 (58%) cases of pancreatic ductal adenocarcinoma (PDAC) were HEr2-0, 11 (22%) were HEr2-1+, 6 (12%) were HEr2-2+, and 4 (8%) were HEr2-3+. Expression of HEr2 protein was significantly associated with tumor grade but not with the stage of the tumor. HEr2 expression and gene alterations were identified in two studies of PDAC. The first study found that HEr2 gene amplification occurs in a subset of PDAC but, in contrast to breast cancer, HEr2 protein overexpression does not predict HEr2 gene deregulation. This finding suggests that HEr2 protein plays a different biologic role in PDAC and that PDAC may not be responsive to anti-HEr2 therapies. The second study found, in a subset of PDAC, coamplification of HEr2 and topoisomerase IIα (TOP2A), suggesting roles for TOP2A inhibitors, trastuzumab, and the combination of the two. Another study found no overexpression of HEr2 in PDAC.

Osteosarcoma

Osteosarcoma may be associated with HEr2 dysregulation, but results of studies of HEr2 gene amplification and expression have reached conflicting conclusions. The few reported studies in osteosarcoma generally indicate low rates and intensities of HEr2 expression.

In one study, HEr2 expression was detected in a small subset of osteosarcoma, but at low levels (no 2+ or 3+ immunoreactivity), and no gene amplification was detected. In another study, HEr2 overexpression was detected in 32% cases of osteosarcoma and demonstrated prognostic value, based on event-free survival analysis. In a preclinical model, trastuzumab was not effective against osteosarcoma, unless it was combined with an anti-IGF-Ir targeting strategy.

Lung Cancer

Lung cancer does not appear to be strongly associated with HEr2 dysregulation. In a lung cancer study, Japanese researchers found very small percentages of HEr2 mutations and amplifications in patients with nsclc. Researchers from Aichi Cancer Center and Nagoya University in Japan searched for EGFr and HEr2 mutations in 349 resected primary nsclc tumors from Japanese patients to determine correlations between genetic abnormalities and clinicopathologic factors. EGFr mutations were detected in 102 (29%) of 349 tumors, and two missense mutations were identified in 8 tumors. Southern blot analysis identified 11 (5.4%) EGFr gene amplifications in 204 nsclc samples. HEr2 mutations were detected in 6 (1.7%) of 349 tumors; and HEr2 amplifications were detected in 1 (0.5%) of 204 tumors. Kras mutations were identified in 21 (6%) of 349 tumors. No tumor had two or more EGFr, HEr2, and Kras gene mutations simultaneously. EGFr and HEr2 mutations occurred more frequently in female, never or light smoking patients, and those with adenocarcinoma. Genetic alteration of EGFr or HEr2 was found in 62 (67%) of 93 patients in this subgroup. These results indicate that activation of EGFr signaling cascades caused by gene alteration is a key event in the pathogenesis of this subset of lung cancer (Toshihiko Y, etal, AACR06, Abs. 4134).

Another lung cancer study focused on newly discovered HEr2 mutations. Researchers from Vanderbilt University (Nashville, TN), Cold Spring Harbor Laboratory in NY, and University of Texas Southwest Medical Center (Dallas, TX) examined the transforming effects of HEr2 gene mutations/insertions within exon 20 that were recently discovered in lung and other epithelial human malignancies (Stephens etal, Nature, 2005; Shigematzu etal, Cancer Res, 2005). HEr2 expression containing a YVMA insertion at G776 in human epithelial cell lines was much more potent in inducing serum-free proliferation, invasiveness, survival, and tumorigenicity *in vivo* compared to wild type (wt) HEr2. HEr2 with a YVMA insertion was autophosphorylated on tyrosine and induced transphosphorylation of EGFr and higher association with signal transducers (e.g., Shc and p85) that engage proliferative and antiapoptotic pathways compared to wt HEr2. HEr2 with a YVMA insertion also transphosphorylated kinase-dead EGFr and wt EGFr in the presence of the EGFr RTK inhibitor, gefitinib. This finding suggests that in heterodimers of EGFr and mutant HEr2, the catalytic activity of the former is dispensable. RNA interference (RNAi) of mutant HEr2 in lung cancer cells that containing a VC insertion within exon 20 of HEr2 decreased proliferation and increased apoptosis. Treatment with trastuzumab downregulated mutant receptors from the cell surface and inhibited growth of lung cancer cells with a VC insertion and HEr2 with a YVMA insertion-transfected cells. Together, these data suggest that the natural mutant of HEr2 activates cellular substrates, including EGFr, more potently than wt HEr2 and that cancer cells expressing this mutant should remain sensitive to trastuzumab, but insensitive to EGFr RTK inhibitors. This HEr2 kinase domain mutation causes constitutive phosphorylation and activation of HEr2 and EGFr (Wang SE, etal, AACR06, Abs. 1457).

Other Malignancies

Other malignancies that may also be affected by HEr2 dysregulation include gallbladder, bladder, and prostate cancer. In a small study of gallbladder cancer, 10 (25%) of 40 patients were positive for HEr2 expression, and its expression in adenoma and younger patients with gallbladder cancer suggests a role in early events of carcinogenesis. In bladder cancer, HEr2 protein overexpression has been reported to range from 2-80%; and overexpression has been correlated with invasiveness and early tumor recurrence. In one study, strong HEr2 overexpression correlated with metastatic urothelial carcinoma, suggesting a potential therapeutic target. In prostate cancer,

Exhibit 2
EGFr Family Member Expression Rates (%) in Human Malignancies[1]

Bladder	Brain	Breast	Colorectal	Head & Neck	Lung	Ovarian	Pancreatic	Prostate	Thyroid	Uterus/ cervix
Epidermal growth factor receptor (EGFr)										
31-50	40-63	14-91	25-77	90-100	40-80 (62% of nsclc)[2]	35-70	3-89	65	++	++/++
HEr2										
2-80 (45)	++ (breast metas-tases)	15-20 (30% of invasive breast cancer)	++	++	44 (nsclc) 18 (sclc)	2-76 (20-45)	42	++	2	++/22
HEr3										
--	--	+	+	+	+	53.4%	++	++	57	++/74
HEr4										
--		+	++	+	++ (nsclc)	++	+	++	0-73 (40)	++/80
Epidermal growth factor receptor variant III (EGFrvIII)										
--	40-50 (in GBM)	0-1	0-1	42	5-15 (nsclc)	++	--	+	--	--

[1]Ranges and estimates from published reports of human tumor studies.

[2]EGFr mutation in ≥30% of Asians, >50% of nonsmokers with nsclc, 10% of Westerners

Note: EGFr family expression can be detected in many tumors of epithelial cell origin. More often than mere detection of any protein expression, overexpression of protein, gene amplification, and gene mutation may be associated with poor prognosis and offer predictive information regarding treatment in terms of sensitivity/resistance.

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

HEr2 membrane expression in hormone-sensitive tumors was, surprisingly, associated with increased time-to-biochemical relapse and longer overall survival.

HEr3 (ErbB3)

ErbB3 has begun to attract attention as an activator of P13K signaling (and possibly other pathways) in HEr2 driven breast cancer and EGFr driven nsclc. Recent studies suggest possible roles for HEr3 targeted treatment of breast cancer and HEr3 testing for predicting the outcome of chemotherapy and determining prognosis in lung cancer. Exhibit 5 lists research findings related to HEr3 in specific clinical indications. The text below provides highlights of findings presented in Exhibit 5, according to specific clinical indications.

HEr2 positive breast cancer depends on tyrosine kinase activity for the promotion of carcinogenesis, metastasis, and therapeutic resistance. Of the ErbB dimers that can activate HEr2 kinase, the HEr2/HEr3 heterodimer is the most potent oncogenic complex. Research suggests that HEr2 requires HEr3 to promote cellular proliferation, although it occurs in some model systems occur without changes in HEr2 tyrosine kinase activity. Two studies suggest that HEr3 interaction with HEr2 plays roles in drug resistance in breast cancer.

The first breast cancer study listed in the exhibit found that increased HEr3 membrane expression promotes substrate resistance to EGFr family TKI. The researchers suggest that incomplete inhibition of HEr2 kinase induces oncogenic HEr3 signaling and that more potent TK or combination strategies are needed. They also suggest that transphosphorylation, rather than autophosphorylation of HEr3 is an appropriate biologic marker for measurement of EGFr TKI efficacy. The second study found that HEr3 plays a critical role in the activation of HEr2 tyrosine kinase activity and HEr2-associated tumorigenesis. HEr2/HEr3 heterodimerization was required for HEr2 tyrosine kinase activation in mammary/breast cancer cells,

and downregulation of HEr2 inhibited HEr3-associated procarcinogenic activity via inactivation of the PI3K/Akt pathway. HEr3 also contributed to HEr2-mediated tamoxifen resistance, suggesting that HEr3 may be a clinically relevant therapeutic target in HEr2 positive breast cancer.

An ovarian cancer study investigated the relationship between HEr3 expression and prognosis in 116 patients and found HEr3 expression in 53.4% and that high levels of HEr3 expression were associated with reduced median survival time, 3.31 years in patients with low levels of HEr3 expression versus 1.80 years in patients with high levels of HEr3 expression.

A lung cancer study found that HEr3 mRNA levels may serve as an ancillary marker for treatment with EGFr TKI. Another study found that EGFr-HEr3 coexpression occurs more frequently in squamous cell carcinoma than in adenocarcinoma, which may explain the different responses of these tumors to EGFr inhibitors.

HEr4 (ErbB4)

Exhibit 6 lists research findings related to HEr4 in specific clinical indications. The text below provides highlights of findings presented in Exhibit 6, according to specific clinical indications.

A breast cancer study of cells created with a HEr4 activating mutation demonstrated increased killing of breast tumor cells and potentiated proapoptotic function of HEr4 in breast (and prostate and ovarian) cancer cell lines tested. The authors suggest that active HEr4 signaling may be a mechanism for therapeutic intervention.

EGFr family overexpression has been associated with poor prognosis in ovarian cancer. Experimental evidence supports the notions that no individual EGFr member is a promising stand-alone target for ovarian cancer and that therapeutic response is influenced by cooperation among them. A study of EGFr family expression and receptor cooperation in ovarian cancer cell lines found that the entire EGFr family plays a role in ovarian cancer growth. Another study found that that EGFr family expression is useful for distinguishing between disseminated thyroid cancer (DTC) and benign thyroid lesions and that, because a high proportion of DTC expresses HEr1, HEr3, and HEr4, investigation of anticancer agents that target one or more EGFr family members warrants clinical study in patients with DTC.

EGFr is widely expressed in bladder cancer, and EGFr expression predicts disease outcome. Results of clinical trials of EGFr directed treatment in bladder cancer have been disappointing because of difficulty identifying subsets of patients who are likely to respond. A study of molecular correlates of sensitivity to EGFr-targeted therapy in bladder cancer found that expression of PDGFr and nuclear HEr4 correlated with resistance to cetuximab; membranous HEr4 was observed only in sensitive and intermediate lines.

EGFr Variant III (EGFrvIII)

Exhibit 7 lists research findings related to EGFr in specific clinical indications. The text below provides highlights of findings presented in Exhibit 7, according to specific clinical indications.

EGFrvIII (and EGFr) are glioma-associated antigens that are hyperexpressed by neoplastic glial cells, compared to normal brain cells. Glioblastoma multiforme (GBM) represents 55% of all cases of glioma, the most common primary malignant tumor of the CNS. Dysregulated EGFr occurs in about 40-50% of GBM, of which nearly half coexpress EGFrvIII (i.e., 15% to >20% of all GBM coexpress EGFrvIII). EGFrvIII has been implicated in the aggressiveness and refractory nature of GBM and may play a role in a subset of nsclc. By some estimates, up to 15% of cases of nsclc express EGFrvIII.

A mass spectrometry study of GBM, EGFrvIII, and downstream components provides rationale for the use of c-Met inhibition as a target for treatment in GBM. Another study found that EGFrvIII is an important factor for development of (dendritic cell) immunotherapy strategies against GBM.

A Japanese study genotyped the EGFrvIII status and detected EGFrvIII mutation in 8 of 252 cases of surgically treated lung cancer; all were male and smokers; and 7 of 8 had squamous cell carcinoma (SCC) versus adenocarcinoma EGFr gene copies were higher in EGFrvIII mutants than in wt EGFrvIII. A USA group found detected EGFrvIII mutations in 5% (3/56) of human SCC and none in 123 cases of human lung adenocarcinoma. In murine tumors, treatment with an irreversible EGFr inhibitor was effective, which suggests a therapeutic strategy for tumors with EGFrvIII mutation.

A study of SCCHN studied incidence of EGFrvIII expression and effects of EGFr targeting. EGFrvIII was detected in 42% (14/33) of SCCHN tumors and, in all cases, in conjunction with wt EGFr. EGFrvIII was found to contribute to increased tumor growth and resistance to agents that target wt EGFr.

TARGETING ERBB RECEPTORS FOR THE TREATMENT OF CANCER

Targeting ErbB receptors as an anticancer strategy has resulted in the regulatory approvals and commercialization of 5 drugs, to be discussed in detail in Part III of the EGFr Pathway in Cancer series. The more than 60 developmental agents addressing the ErbB pathway will be described in detail in Part IV of this series.

Intracellular Targeting

Intracellular signal transduction by EGFr family members is initiated by phosphorylation/activation of the receptor's tyrosine kinase (TK), which then activates the multiple intracellular pathways that contribute to oncogenesis and malignancy. [See Part I of this series, the EGFr Pathway in Cancer, for details of receptors, ligands, and

Exhibit 3
Epidermal Growth Factor (EGF) Receptor (EGFr) in Malignancy

Avian erythroblastic leukemia viral (v-erb-b) oncogene homolog • ErbB-1 • ErbB1 • HER-1 • HER1

Colorectal cancer

Because increasing evidence suggests that EGFr expression is not predictive of metastatic colorectal cancer response to cetuximab therapy, Institute Gustave Roussy (Villejuif, France) and Hôpital Pitié-Salpêtriére (Paris, France) assessed response to cetuximab (combined with chemotherapy) according to differential expression of the biomarkers EGFr, VEGF, pAKT, and PTEN. IHC was used for retrospective analysis of EGFr, HEr2, VEGF, pAKT, and PTEN protein expression in specimens from 48 patients with metastatic colorectal cancer treated with cetuximab in combination with chemotherapy in the third or fourth line setting. A total of 60 samples were obtained, 26 from primary lesions, 34 from hepatic metastasis, and 14 from both lesions. Results showed positive protein expression values of 20% for EGFr, 37% for VEGF, 0% for HEr2, 73% for pAKT, and 58% for PTEN. In the 14 dual biopsies, there was no difference in biomarker expression between the primary and metastatic lesions for EGFr and pAKT, while VEGF and PTEN were frequently discordant between both lesions. Preliminary results in only 12 patients showed no differences in biomarker expression according to response to cetuximab based therapy. Protein expression values from patients with PR (n=6) were 60% for EGFr, 50% for VEGF, 83% for pAKT, and 67% for PTEN. In patients with progressive disease (n=6), such values were 50% for EGFr, 50% for VEGF, 83% for pAKT, and 100% for PTEN. The researchers concluded that preliminary results suggest that protein expression of EGFr, HEr2, and pAKT primary and secondary lesions is similar, while protein expression of PTEN and VEGF differs between primary and secondary lesions. However, preliminary findings suggest that protein expression of the biomarkers tested does not correlate with response to cetuximab-based therapy in metastatic colorectal cancer (Domont J, etal, AACR07, Abs. 208).

Changes in EGFr and HEr2 gene copy numbers and protein expression during colorectal cancer (CRC) progression were investigated by researchers from UZ Gasthuisberg (Leuven, Belgium), Helios-Klinikum (Wuppertal, Germany), and Cliniques Universitaires St. Luc (Brussels, Belgium). They also investigated possible effects of intratumoral heterogeneity on these measurements. Sequential slides from 41 paired samples from either a primary colorectal cancer and one related distant metastatic lesion (n=29), or a subsequent local relapse (n=2); or two metachronous metastatic lesions (n=10). Specific copy numbers of both genes and chromosome centromeres and frequencies in tumor cells were used to evaluate EGFr and HEr2 gene status. FISH-positive status was obtained when high level trisomy or polysomy (=3 copies of the gene in =40% of cells), or an amplification pattern were observed. EGFr protein expression-positive status was defined as =1% malignant cells stained. Intratumoral heterogeneity was studied by testing EGFr status by FISH and IHC on multiple blocks originating from the same patient's tumor source. According to preliminary results, FISH analysis showed that 9 of 41 (22%) pairs were discordant for EGFr status, and 5 of 12 (41%) pairs were discordant for HEr2 status. IHC analysis of EGFr expression was discordant in 8 of 26 (30%) paired samples. When the only two pairs that showed EGFr amplification were excluded, correlation between EGFr gene status and protein expression on sequential slides could not be established. These findings suggest that EGFr and HEr2 gene status and EGFr expression might not be stable during disease progression. Furthermore, they raised the question of whether changes in EGFr and HEr2 status are caused by selective pressure, rather than as a consequence of chromosomal instability. Evaluation of multiple tumor cross sections from the same tumor source will provide a tool to interpret and validate these findings in future prospective studies (Personeni, etal, AACR07, Abs. 5030).

EGFr and HEr2 gene status was determined by FISH in primary tumor or metastatic samples from 54 patients with metastatic colorectal cancer with progressive disease, stable disease (SD), or responding disease (PR, plus SD for at least 30 weeks) after treatment with cetuximab alone or in combination with irinotecan. In 13 of 54 patients, samples originating from different tumor sources were examined to determine the potential impact of variations in EGFr gene status on response prediction. Also, Kras and Braf gene mutations were evaluated in 47 of the 54 patients. Disease control was observed in 15 (62.5%) of 24 patients with an elevated EGFr copy number in at least 40% of tumor cells or EGFr amplification in at least 10% of tumor cells. By contrast, only 10/30 (33.3%) patients with a normal or low EGFr copy number experienced disease control. Discrepancies in EGFr gene status between paired samples were observed in 2 of 13 patients. Neither HEr2 nor BRAF mutation status correlated with disease control. None of the 12 patients with PR had a Kras mutation, while 13 of 35 non-responding patients had Kras mutations. Results suggest that EGFr copy number by FISH analysis may predict cetuximab efficacy in metastatic colorectal cancer, and that the effects of intratumoral heterogeneity on response prediction must be addressed. Kras mutations predict lack of objective response to cetuximab alone or in combination with irinotecan (Personeni N, etal, ASCOGI07, Abs. 400).

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Primary tumor samples from 27 patients with metastatic colorectal cancer were evaluated for EGFr expression by IHC, EGFr gene status by FISH, Kras mutations in codons 12 and 13 by direct sequencing, and PTEN expression by IHC. Of the 27 patients, there were 11 PR, and disease stabilized in 3 and progressed in 13; SD and PD were considered as nonresponses (NR). EGFr overexpression was detected in at least 1% of cells. EGFr amplification was noted in 8 (PR=6, NR=2) patients, 16 (PR=5, NR=11) were EGFr polysomic, and 3 with NR were EGFr disomic. Kras gene mutation was detected in 10 cases (2 PR, 8 NR) and was wt in 17 (PR=9, NR=8). Normal PTEN protein expression (>50% of cells) was observed in 16 cases (PR=10, NR=6); PTEN protein expression was absent in 11 cases (PR=1, NR=10). Patients with disomic EGFr did not benefit from cetuximab therapy. Cetuximab resistance was associated with Kras mutations and loss of expression of PTEN protein. EGFr gene status by FISH represents a prerequisite for cetuximab treatment in metastatic colorectal cancer. Kras gene mutations and PTEN protein expression are predictors of cetuximab efficacy (Romagnani E, etal, ASCOGI07, Abs. 427).

Researchers from McGill University (Montreal, Quebec) investigated the predictive and prognostic value of EGFr expression in rectal cancer treated with preoperative high dose rate brachytherapy and in mismatch-repair (MMR)-proficient colorectal cancer, respectively. EGFr was analyzed by IHC on 82 rectal tumor biopsies and 1197 MMR-proficient colorectal cancer. Receiver operating characteristic (ROC) curve-derived cutoffs were used to evaluate the association of EGFr overexpression, tumor response, and clinicopathologic features, including survival. Results showed that the scoring method was reproducible in rectal cancer biopsies and colorectal cancer and that the selected cutoff scores from ROC curve analysis for each clinicopathologic feature were consistent among (3) pathologists. EGFr overexpression was associated with response to radiotherapy and worse survival time and independently associated with adverse prognosis. EGFr is a predictive marker of response to preoperative radiotherapy and an independent adverse prognostic factor colorectal cancer (Zlobec I, Br J Cancer. 2007;96(5):793-800; Epub 2007 Feb 20).

Researchers from University of Southern California (Los Angeles, CA) performed a retrospective study of molecular determinants of irinotecan (CPT-11) efficacy in 33 patients with colorectal cancer. Response rates in the 32 evaluable patients included 1 CR, 12 PR, 13 SD, and 6 progressive disease. High intratumoral mRNA levels of EGFr, ERCC1, and GSPT-P1 were each associated with response to CPT-11-based chemotherapy. Recursive partitioning analysis showed that EGFr and ERCC1 mRNA levels differentiate responders from nonresponders. In addition, the finding of high intratumoral gene expression levels of EGFr and ERCC1, in combination, was associated with progression-free survival (PFS). EGFr mRNA levels were correlated with expression levels of ERCC1, GST-P1, and VEGF. These results suggest that gene expression levels of EGFr, ERCC1, and GST-P1 may predict clinical outcome of patients with metastatic colorectal cancer treated with first line CPT-11 based chemotherapy (Vallbohmer D, etal, Int J Cancer, 2006;119(10):2435-42).

Colorectal cancer

The mutation status of EGFr and HEr2 and their downstream effectors, Kras and Braf, has not been completely established in squamous cell carcinoma of the head and neck (SCCHN). Researchers from Okayama University (Okayamashi, Japan) and Wakayama Medical University (Wakayamashi, Japan) used PCR and direct sequencing to detect mutations in EGFr (exons 19, 20, 21) and Kras (codons 12, 13, 61). They analyzed the same samples for mutations in the tyrosine kinase (TK) domain of HEr2 (exons 18, 19, 20, 21, 22, 23) and BRaf (exons 11 and 15). Because most of the Braf mutations were a substitution of V599L, mutant allele specific amplification was used to detect this mutation in exon 15. Results showed that, of 79 SCCHN samples analyzed, no mutation of EGFr, HEr2, KRas, or BRaf was detected. Although these genes are overexpressed in SCCHN, these data show that mutations are not common and may not play a significant role for the activation of the EGFr pathway in Japanese patients with SCCHN. Although SCCHN overexpress EGFr and HEr2, response to TK inhibitors (TKI) is limited in clinical trials, which may be explained by the lack of mutations in the TK domain of EGFr and HEr2. Whether these tumors depend on increased EGFr gene or protein expression or signaling pathways other than EGFr or HEr2 requires additional studies (Gunduz M, etal, AACR07, Abs. 5241).

University of Pittsburgh scientists have also constructed an EGFr antisense-expression DNA plasmid under transcriptional control of the U6 small nuclear RNA (snRNA) promoter as an *in vivo* challenge to SCCHN. When complexed with a DC-chol cationic liposome-mediated carrier and injected into subcutaneously grafted human SCCHN tumors grown in nude mice, the antisense construct demonstrated antitumor effects, including inhibition of growth, suppression of EGFr protein expression, and an enhanced level of apoptosis, documenting the potential effectiveness of an *in situ* antisense oligonucleotide EGFr expression system in the treatment of SCCHN (He Y, etal, J Natl Cancer Inst, 15 Jul 1998;90(14):1080-7).

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Prostate cancer

Researchers from Jung-Stilling-Hospital (Siegen, Germany) evaluated the potential prognostic significance of androgen and growth factor receptor staining (IHC) in prostatectomy specimens from patients with prostate cancer. This prospective study included 211 patients with locally confined prostate cancer who treated with radical prostatectomy +/- antiandrogen pretreatment (Jan-1-1990 to Aug-31-1996). A total of 68/211 demonstrate relapse or progression or died of the malignancy. Among the key findings, androgen receptor and EGFr staining indices exhibited statistical dependencies. With regard to relapse-free survival (RFS), low androgen receptor and increased EGFr staining were associated with increased risk. Low (compared to higher) androgen receptor staining index indicated a worse prognosis after data stratification according EGFr staining index. Grade 3 carcinoma had a worse prognosis than better differentiated carcinoma. Antiandrogen pretreatment had no influence on overall survival or RFS (Schafer W, etal, J Urol, 2006;176(2):532-7).

Androgen plays a critical role in controlling growth and survival of prostate cancer cells by increasing cellular proliferation and inhibiting apoptosis. Researchers used the androgen sensitive LNCaP cells and the androgen responsive prostate cancer xenograft CWR22 passed in SCID mice. In these models, the expression and activity of ErbB1-3 were analyzed, as well as the expression of the ligands activating ErbB1 from the EGF network, and the impact of ErbB1 inhibition on androgen induced proliferation and antiapoptosis. Androgen selectively increases the expression and the activity of ErbB1 mRNA and protein. Blocking the ErbB1 activity by a specific tyrosine kinase inhibitor CGP59326, abrogates the androgen-induced proliferation in LNCaP cells without affecting cell viability. These results indicate that androgen increases the activity of the EGF-network in prostate cancer by increasing ErbB1 expression, and that this activity of this tyrosine kinase receptor is essential for androgen induced proliferation and survival of prostate cancer cells (Torring N, etal, CDPOnline 2002, Abs. 1092:156).

Ovarian cancer

Researchers at Helsinki University Central Hospital, in Finland examined frequency and clinicopathological correlations of gene amplification, protein expression, and mutations of EGFr and HEr2 in 398 cases of serous carcinoma, the most common and aggressive type of ovarian cancer. EGFr amplification was detected in 12% (41/333), low-level gain in 43% (144/333), and protein overexpression in 17% (66/379). Both increased copy number and overexpression of EGFr were associated with high tumor grade, greater patient age, large residual tumor size, high proliferation index, aberrant p53, and poor patient outcome. An increased number of copies of EGFr was associated with an increased number of copies of HEr2. No EGFr mutations were identified; one insertion mutation in exon 20 of HEr2 was detected. Both EGFr amplification and protein overexpression occur in serous ovarian carcinoma, but the number of EGFr copies was of greater prognostic value. EGFr amplification is a potentially useful criterion for selecting patients in clinical trials of EGFr inhibitors in serous ovarian carcinoma (Lassus H, etal, J Mol Med, 2006;84(8):671-81).

Researchers at University Hospital of Basel, in Switzerland, studied EGFr gene status and protein expression in tumor tissues from 80 patients with primary and recurrent ovarian serous carcinoma. Classification of the patients into six groups was determined according to ascending EGFr gene copy numbers. EGFr amplification and high polysomy (FISH+) was present in 20% of the primary and 22% of the recurrent tumors. Mutational analysis revealed only one tumor with a silent EGFr mutation, which was the only carcinoma in which high level amplification was detected. EGFr protein immunoexpression was observed in 28% primary and 33% recurrent carcinoma and correlated with amplification in the primary tumors. In recurrent carcinoma, moderate and strong EGFr expression was associated with amplification. The observed molecular events may have impact on the responsiveness to EGFr targeting agents in ovarian cancer (Stadlmann S, etal, Mod Pathol 2006;19(4): 607-10).

Brain cancer

EGFr amplification and overexpression frequently occur in malignant glioma and are associated with poor prognosis. Mitogen activated protein (MAP) kinase and phosphatidlylinositol-3' kinase (PI3K) pathways are among the downstream mediators activated by EGFr. To date, clinical trials using inhibitors of EGFr in glioma have yielded inconclusive results regarding their effectiveness Researchers from University of California, San Francisco hypothesize that glioma may be resistant to EGFr inhibition because of recovery of downstream signaling. This hypothesis was tested by analyzing signaling pathways in U87MG cells transduced with EGFr and treated with erlotinib. In treated cells, EGFr phosphorylation and phosphorylation of the PI3K target, Akt, remained inhibited throughout the experiment. Phosphorylation of Erk, a component of the MAP kinase pathway however, was initially inhibited but recovered almost completely, despite continuous blockade of EGFr

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signaling. This finding suggests that recovery of Raf/Mek/Erk kinase signaling may be the mechanism by which glioma cells overcome EGFr inhibition. Researchers then hypothesized that inhibition of both EGFr and MAP kinase signaling would increased efficacy. The combination of erlotinib, and U0126, an inhibitor of the MAP kinase kinase, MEK, demonstrated increased efficacy as predicted. Therefore, it appears that recovery of MAP kinase signaling contributes to the relative resistance of malignant glioma to EGFr blockade and combined blockade of EGFr and MAP kinase signaling offers a mechanistic rationale for overcoming this resistance (Nicolaides TF, AACR07, Abs. 4006).

Breast cancer

Researchers from Wayne State University (Detroit, MI) hypothesized that EGFr may provide a pathway of EGFr tyrosine kinase inhibitor (TKI) resistance through sustained tyrosine phosphorylation in the absence of intrinsic kinase activity. This hypothesis predicts that some EGFr-expressing breast tumors are resistant to EGFr TKI because of phosphorylation by other tyrosine kinases, such as c-Src and HEr2. The researchers tested 17 breast cancer cell lines for response to EGFr TKI and, as predicted, found that only 3 were sensitive to treatment at concentrations that inhibit EGFr tyrosine kinase activity, while 6 were sensitive at concentrations of up to 100 times higher. The other 8 breast cancer cell lines were completely resistant to EGFr TKI treatment. Six of the resistant cell lines, expressed EGFr at detectable levels. Kinase activity in the EGFr TKI resistant cell lines was inhibited, but tyrosine phosphorylation remained detectable in several of the cell lines. Several phosphorylation sites that remained phosphorylated in resistant cells after EGFr TKI treatment were identified, including the c-Src phosphorylation site, Y845. These findings suggest a possible role for other tyrosine kinases in the promotion of EGFr tyrosine kinase intrinsic resistance in breast cancer (Boerner, JL, Griffin KL, AACR07, Abs. 2072).

Regulation of normal cellular levels of EGFr include degradation through proteolytic pathways and recycling to the membrane for reactivation. Overexpression of EGFr protein without gene amplification occurs in the inflammatory breast cancer cell line, SUM149; and proliferation of SUM149 cells requires an amphiregulin (AR)/EGFr autocrine loop. Researchers at Karmanos Cancer Institute (Detroit, MI) studied the possible role of AR stabilization of EGFr in decreasing EGFr degradation in SUM149 cells and its contribution to EGFr overexpression. Based on their results, they propose that EGFr dynamics are altered by an amphiregulin autocrine loop that causes stabilization of EGFr and decreased EGFr degradation, which results in increased steady-state EGFr protein levels and accumulation of EGFr at the cell surface. This relationship between an AR autocrine loop and overexpression of EGFr might explain why both factors predict aggressive breast cancer (Willmarth NE and Ethier SP, AACR07, Abs. 3773).

The transcription factor, B-Myb, is overexpressed at high levels in primary breast carcinoma. Expression of B-Myb is activated by E2F1/2 in the late G1 phase of the cell cycle and sustained through the S phase. Reports of a casual correlation between EGFr and B-Myb expression in primary breast carcinoma were confirmed by a group from The University of Texas M.D. Anderson Cancer Center (Houston, TX) that studied mechanisms for coexpression of EGFr and B-Myb and reported that EGFr is important for B-Myb expression, and the underlying mechanism includes cooperative effects from EGFr and E2F1. B-Myb gene activity was significantly increased in G1 by stimulation by EGF and forced expression of EGFr. Inhibition of the downstream pathways, PI3K and ERK, did not significantly suppress EGF-induced B-Myb expression. In contrast, *in vivo* observations showed

EGF-induced association of nuclear EGFr to the B-Myb promoter, which were detected only in G1/S and abolished by treatment with an EGFr kinase inhibitor. The group further reasoned that nuclear EGFr might cooperate with E2F1 to cause activation of B-Myb because EGFr lacks a DNA-binding domain but contains transactivational activity, and E2F1 activates B-Myb expression in G1/S. In fact, EGFr was found to co-immunoprecipitate with E2F1 in an EGF-dependent manner; and EGF was found to activate *in vivo* binding of E2F1 to the B-Myb promoter. In all cases, B-Myb promoter activity was greatly increased by forced expression of both EGFr and E2F1 in EGFr-null CHO cells, compared to expression of EGFr or E2F1 alone and a control vector. Promoter mutagenesis studies confirmed this finding, showing that both E2F and EGFr target sites were required for EGFr-induced activation of B-Myb promoter. The data suggest that deregulated EGFr signaling facilitates tumor cell proliferation in part by EGFr interaction with E2F1 and subsequent activation of B-Myb gene expression (Hanada N, etal, Mol Carcinog, 2006;45(1):10-17).

Because HEr2 is constitutively phosphorylated in some breast tumors, researchers speculated that transmodulation of HEr2 may occur via EGFr signaling. To test this they examined the effect of EGFr-specific kinase inhibitors against the HEr2-overexpressing human breast tumor lines BT-474, SKBR-3, MDA-361, and MDA-453.

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Treatment of all breast cancer cell lines (except MDA-453) with 1 microM of ZD1839 almost completely eliminated HEr2 phosphorylation. In contrast, the incorporation of [gamma-(32)P]ATP *in vitro* onto HEr2 receptors isolated from BT-474 cells was unaffected by 1 microM of ZD1839. EGFr is expressed by BT-474, SKBR-3, and MDA-361 but not by MDA-453 cells, suggesting that ZD1839-mediated inhibition of the EGFr kinase explained the inhibition of HEr2 phosphorylation *in vivo*. These data imply that EGFr tyrosine kinase inhibitors will be effective against HEr2-overexpressing breast tumor cells that also express EGFr and support their use in combination with HEr2 antibodies, such as Herceptin, against mammary carcinoma with high levels of the HEr2 proto-oncogene (Moulder S, etal, Can Res 15 Dec 2001;61(24):8887-95).

Pancreatic cancer

Researchers from Sungkyunkwan University School of Medicine (Seoul, Korea) investigated the presence of EGFr mutations and increased EGFr copy numbers in pancreatic adenocarcinoma. Exons 18-21 in the tyrosine kinase domain were sequenced to identify EGFr mutation; and codons 12, 13, and 61 were sequenced to identify KRAS mutation. EGFr copy number was determined by quantitative real-time polymerase chain reaction. In 66 patients with inoperable or metastatic pancreatic adenocarcinoma, only 1 (1.5%) demonstrated an EGFr mutation (exon 20 substitution). Elevated EGFr copy numbers were detected in 26 (41%) patients. EGFR amplification did not influence survival. Point mutations of the KRAS gene were detected in 32 (49%) of 65 pancreatic adenocarcinomas examined; codon 12 (n = 31), and codon 61 (n = 1) of the. The presence of a point mutation in codon 12 was associated with an adverse effect on survival. The researchers concluded that the incidence of somatic mutations in the tyrosine kinase domains of EGFr was very low, and increased EGFr gene copy number did not influence survival (Lee J, etal, Cancer, 2007;109(8):1561-1569 [Epub ahead of print]).

In pancreatic cancer, mRNA expression has been shown to be enhanced compared with normal controls for a number of important tyrosine growth factor receptors, including EGFr (4-fold), ErbB-2 (2.5-fold), and ErbB-3 (5.2-fold), as well as for multiple ligands that bind to EGFr, including EGF, TGF-alpha, amphiregulin (AR), and heparin-binding EGF-like growth factor (HB-EGF), suggesting that coexpression of EGFr and its ligands may contribute to the aggressiveness of human pancreatic cancer (Yamanaka Y, etal, Anticancer Res, May-Jun 1993; 13(3):565-9; Korc M, Surg Oncol Clin N Am, Jan 1998;7(1):25-41; Friess H, etal, Ann Surg, Dec 1999;230(6): 767-74, discussion 774-5). In contrast, however, mRNA expression of the ErbB4 receptor is 6-fold decreased in the non-metastatic stages of pancreatic cancer when compared to tumors with lymph node involvement or distant metastases or to the normal pancreas (Graber HU, etal, Int J Cancer, 19 Feb 1999;84(1):24-7).

Lung cancer

Although non-small cell lung cancer (nsclc) with EGFr gene activating mutations is usually highly sensitive to the EGFr tyrosine kinase inhibitors (TKI), gefitinib or erlotinib, acquired resistance to TKI commonly develops after an initial striking response. Secondary mutation of threonine to methionine at codon 790 (T790M) of the EGFr gene is related to acquired resistance. In this study, exons 18-21 of the EGFr gene were sequenced in tumor samples from 14 patients with nsclc who developed acquired resistance to gefitinib after an initial response. Notably, this EGFr region corresponds with that of the ABL gene where secondary mutations have been reported in patients with chronic myelogenous leukemia (CML) and acquired resistance to imatinib. Detection of secondary Kras mutations was also undertaken. Activating mutations of the EGFr gene were identified in all 14 patients (9 exon 19 deletions, 5 L858R). Additionally, 7 of 14 patients had a T790M mutation; no other novel secondary mutations were detected. In the 7 patients with T790M mutations, T790M mutant bands were smaller than wild type bands; patients tended to be never smokers and female; and the period of treatment with gefitinib was not correlated with the presence or absence of T790M. T790M was not detected in tumors before gefitinib treatment. No patients exhibited acquired Kras gene mutation. Therefore, secondary T790M mutation of the EGFr gene accounted for half of the cases of acquired gefitinib resistance. Various types of secondary mutations were not likely to exist in the EGFr gene as a mechanism of acquired resistance, unlike cases of CML (Mitsudomi T, etal, ASCO06, Abs. 7074).

Research groups from Harvard Medical School (Boston, MA) and Massachusetts Institute of Technology (Cambridge, MA) reviewed results of their first 100 patients with lung cancer to undergo somatic EGFr mutation testing for clinical decision-making. Beginning in August 2004, mutation screening by comprehensive direct sequence analysis of exons 18 to 24 of the EGFr tyrosine kinase domain was initiated as part of clinical care, including protocol therapy. Mutations identified in the original genomic DNA sample were confirmed with 3 to 5 independent PCR procedures and compared with the patient's germ-line DNA to ensure the finding

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is somatic if not previously reported. EGFr sequence testing and analysis was found to be feasible and affected the treatment of patients with nsclc. EGFr tyrosine kinase inhibitor (gefitinib or erlotinib) therapy was more likely to be recommended for patients with EGFr mutations than those without mutations, but did not prevent treatment with TKI in selected patients. It is most likely that EGFr mutation testing (and other molecular analyses) will be clinically most useful for assessment of efficacy of TKI as first line treatment in metastatic nsclc and as adjuvant treatment in early stage resected nsclc (Lecia V, etal, Clin Cancer Res, 2006;12:4403s-4408s).

Researchers from Massachusetts General Hospital, Harvard Medical School (Charlestown, MA) evaluated the correlation of molecular markers, including mutations, with clinical outcomes in patients with advanced nsclc treated with gefitinib, chemotherapy, or chemotherapy and gefitinib in IDEAL and INTACT clinical trials. Gefitinib treatment leads to major responses in <19% of patients; symptoms improve in nearly half (IDEAL 1 & 2). The combination of gefitinib plus systemic chemotherapy produces no therapeutic gain, compared to chemotherapy alone (INTACT I & II). Somatic activating mutations of exons 18-21 in the EGFr-TK domain have been identified in patients who exhibit dramatic, rapid responses to gefitinib (Lynch etal, Paez etal, Pao, etal). In this study, tumor samples were obtained from patients enrolled in IDEAL studies (i.e., monotherapy in previously treated patients) and INTACT studies (randomized trials of chemotherapy +/- gefitinib in untreated advanced stage patients). Exons 18-21 of the EGFr TK domain were sequenced using standard protocols, and samples were analyzed for ras and p53 mutations. Correlations were made between mutational status, therapies, and outcome measures including tumor response and survival. Tumor samples were available from 119 of 416 IDEAL patients, of which 78 were fully evaluable and validated for exons 18-21. A total of 14 mutations were detected (11 deletion and 3 point mutations); 12 were in adenocarcinomas, 8 in women, 9 in smokers or former smokers, and 6 in patients with objective responses. Tumor samples were available from 672 of 2130 INTACT patients, of which 312 are evaluable to date. A total of 32 mutations have been detected (22 deletion and 10 point mutations); 25 were in adenocarcinomas, 17 in men, and 9 in never smokers. A total of 15 of 23 had objective responses to treatment; the other 9 were not evaluable. Correlations of EGFr, ras, and p53 mutation status with treatment (chemotherapy, chemotherapy plus gefitinib, or gefitinib alone) were published; see abstract below (Lynch TJ, ASCO05, Abs. 7006).

Researchers from Massachusetts General Hospital, Harvard Medical School (Charlestown, MA) examined EGFr mutations and gene amplification in nsclc in the IDEAL/INTACT gefitinib trials. Although it is known that most patients with nsclc who experience dramatic responses to gefitinib have specific EGFr activating mutations, large clinical trials were needed to determine the predictive value of these mutations. IDEAL evaluated the contribution of molecular alterations in EGFr on response and survival in patients treated with gefitinib in phase II trials; INTACT evaluated phase III trials. EGFr mutation frequency was analyzed and compared with gene amplification in lung cancer specimens from both trials. This study found that EGFr mutations correlated with previously identified clinical features of gefitinib response, including adenocarcinoma histology, absence of smoking history, female sex, and Asian ethnicity. However, no such association was identified in tumors with EGFr amplification, which suggests that EGFr mutations and EGFr gene amplification identify different biologic subsets of nsclc. Responses to gefitinib in IDEAL involved 6 of 13 tumors (46%) with an EGFr mutation, 2 of 7 (29%) with amplification, and 5 of 56 (9%) with neither mutation nor amplification. In INTACT, there was no significant difference in response to gefitinib plus chemotherapy according to EGFr genotype. This group concluded that EGFr mutations and, to a lesser extent, EGFr gene amplification appear to identify distinct nsclc subsets with increased responses to gefitinib. Combination therapy with gefitinib plus chemotherapy does not improve survival in patients with these molecular markers (Bell DW, etal, J Clin Oncol 2005;23(31):8081-92).

Although EGFr tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, induce major responses in 10-20% of unselected patients with advanced nsclc who relapse after chemotherapy, the superiority of the combination of gefitinib or erlotinib combined with chemotherapy, compared to chemotherapy alone, was not demonstrated in four large clinical trials. A group led by Giuseppe Giaccone, of Vrije Universiteit Medical Center (Amsterdam, The Netherlands), hypothesizes that the absence of patient selection by EGFr expression level may explain this negative finding, although preclinical results did not support a role for measuring and using EGFr expression levels to predict tumor sensitivity to TKI. Small restrospective studies show that tumor-associated missense mutations and small deletions in the ATP binding pocket of EGFr correlate strongly with major responses to gefitinib or erlotinib. The incidence of these EGFr mutations, however, may not correlate perfectly with treatment response. Other indicators of nsclc response to EGFr inhibitors include EGFr amplification and functional status of downstream EGFr signaling pathways, such as activated PI3K-Akt. These findings may provide the basis to establish markers for predicting nsclc response to EGFr inhibitors.

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EGFr overexpression is pronounced in virtually all cases of squamous lung carcinoma and is also found in over 65% of large cell lung cancer and adenocarcinoma. Overexpression of EGFr is one of the earliest and most consistent abnormalities in bronchial epithelium of high-risk smokers. Recent studies of the effect of inhibitors of receptor tyrosine kinases suggest that patterns of coexpression of multiple members of the EGFr family could be important in determining response. Targeted lung cancer treatment and chemoprevention trials require greater attention to molecular endpoints than has been the case in past trials (Franklin W, etal, Semin Oncol Feb 2002;29(1 Suppl 4):3-14).

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

downstream signaling pathways (Fut Onc, Mar 27, 2007;8(11/12).] Intracellular targeting of ErbB receptors is focused on inhibition of TK with small molecule tyrosine kinase inhibitors (see Exhibit 8). As small molecules, TKI have some advantages over biologicals such as the anti-EGFr MAb. Small molecule TKI are easier to manufacture, ship, and store, and they are administered orally, unlike MAb, which require injection. Clinical performance characteristics and metrics of approved TKI and MAb will be addressed in the next issue of Future Oncology.

Reversible and Irreversible Inhibitors

The three FDA-approved EGFr family TKI, gefitinib (Iressa; ErbB1), erlotinib (Tarceva; ErbB1), and lapatinib (Tykerb; ErbB1/ErbB2), are all reversible inhibitors. They bind competitively (i.e., they compete with ATP) and reversibly with the ATP binding pocket of the tyrosine kinase. In contrast, irreversible ErbB inhibitors form covalent bonds with the TK ATP binding pocket, and are in development to provide prolonged suppression of ErbB receptor-mediated signaling. Therapeutic goals of irreversible binding include increased antitumor effectiveness and overcoming the resistance that may develop with reversible inhibition. Among others, irreversible TKI compounds in development include:

- CI-1033 (canertinib; Pfizer), an irreversible pan-erbB inhibitor that binds ErbB receptors through alkylation of a cysteine residue in the ATP-binding pocket and has been tested in many phase I trials;
- HKI-272 (Wyeth), an irreversible inhibitor of ErbB1 and ErbB2 that has been tested in phase II trials of breast cancer and nsclc;
- BIBW 2992 (Boehringer Ingelheim), an irreversible inhibitor of ErbB1 and ErbB2 that has been tested in phase I trials and a phase II trial in hormone-refractory prostate cancer.

Although results of early studies with irreversible TKI have been promising, it is not yet clear what, if any, clinical advantages (or disadvantages) they will offer over reversible agents. Some researchers are skeptical that irreversible TKI will demonstrate significant advantages, citing the long half-lives and prolonged inhibition already obtained with reversible TKI and the likelihood of multiple mechanisms of resistance that irreversible inhibition may not subvert. However, recent studies suggest that resistance-conferring mutations act, at least in part, by restoring wild type ATP affinity (i.e., increasing ATP affinity of the receptor), which reduces the ability of TKI to compete with ATP for the receptor and their therapeutic effectiveness. Such a resistance mechanism might be defeated with irreversible TKI, with the following rationale: an irreversibly bound ATP binding pocket cannot bind ATP, and therefore ATP binding affinity cannot increase.

At the recent annual meeting of AACR, researchers from Dana-Farber Cancer Institute (Boston, MA) presented a study of structural and kinetic aspects of the mechanism of the T790M resistance mutation in nsclc and the inhibition of T790M by irreversible inhibitors. They note that the T790M mutant is sensitive to the irreversible inhibitors HKI-272, Cl-1033, and Cl-387,785. Like reversible inhibitors, these compounds bind in the ATP binding cleft, but they also possess an additional reactive group that binds covalently with Cys 773, which is located at the edge of the ATP binding cleft. In this study, the structures of the mutant alone and in complex with HKI-272 were determined in order to gain understanding of the effects of the T790M mutant and the mechanism by which it confers resistance. The kinetics and inhibitor-binding properties of the T790M mutant, the L858R/T790M double mutant, and the wild-type EGFr kinase were also studied.

The researchers found that gefitinib binds to the T790M and L858R/T790M mutants with low nanomolar affinity (Kd=~5 nM), which was a surprising finding because it indicates that the T790M substitution does not sterically block gefitinib binding, as previously supposed. Additional support was provided by determination of the crystal structure of HKI-272 in complex with the T790M mutant, which found that the binding mode of HKI-272 was the same as that of the wild-type kinase. Kinetic analysis showed that the T790M mutant is approximately 5 times more active that the wild-type kinase and has a similar Km for ATP. Also of interest, although the L858R and G719S single mutants demonstrate markedly impaired affinity for ATP, the L858R/T790M double mutant had a Km for ATP equivalent to that of wild-type kinase. These findings indicate that much of the resistance induced by T790M may be caused by its ability to

restore wild-type affinity for ATP, rather than by its steric effects on inhibitor binding, as previously thought. This study suggests that (reversible) TKI potency is diminished by enhanced ATP-affinity of the T790M mutant and that irreversible inhibitors overcome this effect through covalent binding (Yun C, etal, AACR07, Abs. LB-367).

Also at AACR, researchers from Case Western Reserve University (Cleveland, OH), Beth Israel Deaconess Medical Center (Boston, MA), and Yale Cancer Center (New Haven, CT) presented a model for predicting resistance mechanisms against irreversible EGFr inhibitors for guiding clinical drug development. A cell-based, *in vitro* random mutagenesis screen utilizing T790M-mutant, H1975 cells identified mutations in exons 14-22 of EGFr, including TK domain mutations that confer resistance to the irreversible TKI, CL-387,785. Mutations at 4 positions (E931G, H773L, L655H, L658P) occurred repeatedly, strongly suggesting functional relevance. The 4 variants and an EGFr-T790M-C797S mutant (predicted to affect covalent binding of CL-387,785 to EGFr) were generated and reintroduced into lung cancer cells. All 5 mutations were found to cause functional resistance to CL-387,785, demonstrating enhanced cell growth, reduced apoptosis, and persistent EGFr autophosphorylation in the presence of CL-387,785, compared to parental H1975 cells. Alternative EGFr inhibitors and a Cdk 4 inhibitor were then screened, some of which inhibited cell growth of all resistant mutants. This predictive model has applications in clinical development of irreversible EGFr inhibitors and development of alternative strategies to overcome or prevent development of resistance in EGFr-mutant nsclc (Yu Z, etal, AACR07, Abs. 2555).

Specific, Dual, and Pan-ErbB Inhibitors

The first two small molecule TKI introduced into clinical oncology practice were specific for the single EGFr family target, ErbB1. The March 2007 approval of lapatinib (Tykerb) marked the first FDA approval of a dual targeted TKI (i.e., for ErbB1 and ErbB2). Other dual targeted compounds and pan-erbB targeted agents, which demonstrate affinities for 3 or 4 ErbB receptors (ErbB1, -2, -3, -4), are in development.

As the pan-erbB inhibitor prototype, CI-1033 (canertinib dihydrochloride) has been the subject of much research (Dewji MR, J Chemother, 2004;16 Suppl 4:44-8). Bristol Myers Squibb is also developing BMS-690514, which is a pan ErbB (Erb-1, -2, -4) and VEGFr2 inhibitor. In studies presented at the AACR annual meeting, researchers from Bristol-Myers Squibb (Princeton, NJ) demonstrated that the preclinical antitumor activity of BMS-690514 is attributable to inhibition of EGFr and HEr2 signaling in tumor cells and inhibition of VEGFr2 activity in tumor endothelium. The antitumor efficacy achieved with BMS-690514 was superior to that previously observed in the same tumor models using other EGFr or HEr inhibitors (e.g., gefitinib, BMS-599626). Measurements of tumor blood flow and target gene regulation provided evidence supporting the contribution of anti-angiogenic activity of BMS-690514 to overall efficacy profile in the model tested. The preclinical safety profile was acceptable (Wong TW, etal, AACR07, Abs. 4007).

EXTRACELLULAR TARGETING

Extracellular targeting of ErbB receptors is focused on naked monoclonal antibody (MAb) therapeutics, three of which are FDA approved for clinical use, and other developmental approaches, including various types of modifications of MAb and immunotherapeutic/vaccine approaches.

Naked Monoclonal Antibodies (MAb)

MAb are produced by the fusion of a mouse myeloma cell with a specific antibody-producing mouse B cell. The result is a single cell (hybridoma), which is multiplied in the laboratory to establish a factory for the production of a single (monoclonal) antibody, which attaches to a specific antigen. Replacement of segments of mouse antibody with human segments results in the development of chimeric or humanized antibodies, depending upon the degree of humanization. The potential utility of tumor-targeted MAb may be increased by protein engineering approaches, which can be used to modify characteristics such as affinity, immunogenicity, and pharmacokinetic properties (e.g., creation of recombinant antibody fragments).

Naked MAb are defined as those without drugs or radioactive material attached and are the most commonly used MAb type. The 3 approved anti-EGFr family MAb therapeutics are naked MAb, which attach to the extracellular domains (ECD) of specific receptors (i.e., the ECD of the specific ErbB receptor is the antigen of the MAb):

- Trastuzumab (Herceptin) is a humanized IgG1 MAb that targets the HEr2 protein, which is overexpressed by a subset of breast malignancies; it is FDA approved for breast cancer with demonstrated HEr2 overexpression.
- Cetuximab (Erbitux) is a chimeric IgG2 MAb that targets the EGFr protein, which is overexpressed by many solid tumors; it is FDA approved for colorectal and head and neck cancer with demonstrated EGFr overexpression.
- Panitumumab (Vectibix) is a fully human IgG1 MAb that targets the EGFr protein; it is FDA approved for colorectal cancer with demonstrated EGFr overexpression.

Other MAb targeting EGFr family members are in development, such as zalutumumab (HuMax-EGFr; Genmab/Medarex), a fully human MAb targeting EGFr, which is in phase II trials for head and neck cancer and nsclc.

Anti-erbB MAb therapeutics bind with high specificity to the extracellular TK domains of their specific erbB targets on tumor cells. Their binding interactions are charac-

Exhibit 4
HEr2/neu (Her-2/neu) in Malignancy

ErbB-2 (ErbB2) • c-erbB-2 • p185erbB2 • TROB1 • transducer of ErbB-2 • TOB • TROB
HER-2, HER2 • NEU • NGL • TKR1 • herstatin • neuroblastoma/glioblastoma derived oncogene homolog
tyrosine kinase-type cell surface receptor

Breast cancer

High expression levels of both Notch1 and its ligand, Jagged-1, are associated with lowest overall survival rates in breast cancer, including HEr2-positive breast cancer. A study in HEr2-positive breast cancer cells, conducted by researchers from Loyola University Medical Center (Maywood, IL), demonstrated novel crosstalk between HEr2 and Notch1. A model in which HEr2 was eliminated by trastuzumab inhibition or creation of dominant negative HEr2 deletion mutant was found to activate a survival and proliferative pathway by increasing activity of the g-secretase complex, processing and nuclear accumulation of Notch1, and Notch1 signaling. A follow-up experiment in which the cells were treated with trastuzumab and the Notch1 inhibitor, GSI, resulted in synergistic inhibition of cell proliferation by HEr2-positive breast cancer cells. These findings suggest a possible mechanism of resistance to trastuzumab: HEr2 inhibition by trastuzumab increases activity of the g-secretase complex, which causes activation of Notch1 signaling, which is a potent survival and proliferative pathway (Osipo C, AACR07, Abs. 3570).

Because recombinant human erythropoietin (EPO) therapy is often used to prevent and alleviate cancer treatment-related anemia and fatigue, the possible role of EPO treatment in trastuzumab resistance was investigated. EPO is known to be cytoprotective in non-hematopoietic tissues through activation of the EPO receptor (EPOr) present in these tissues. EPOr is also found in tumor specimens obtained from some patients with cancer, particularly breast cancer. Furthermore, EPO is produced by human breast cancer cells, which suggests a potential EPO-EPOr autocrine or paracrine stimulation loop. In some established breast cancer cell lines EPOr is co-expressed with HEr2, and EPO treatment of these cells activated cell signaling, which was demonstrated by increased levels of activation-specific phosphorylation of MAPK, Akt, and STAT5. Concurrent treatment of two HEr2- and EPOr-positive breast cancer cell lines with EPO reduced the therapeutic response to trastuzumab. Hypoxic culture of another breast cancer cell line demonstrated increased secretion of EPO, which was linked to trastuzumab resistance. These findings suggest that exogenous EPO treatment and breast cancer cell-produced EPO may be clinically relevant mechanisms of cellular resistance to trastuzumab (Liang K, etal, AACR07, Abs. 344).

HEr2 amplification or protein overexpression predicts response to trastuzumab and anthracycline-based chemotherapy in women with breast cancer, provoking the question of whether it predicts response to endocrine therapy. Two oncologists from Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University (Baltimore, MD) noted that preclinical and some clinical data suggest that HEr2-positivity may also predict for relative resistance to endocrine therapy. Relevant clinical data interpretation is difficult because studies are mostly retrospective and patient populations and methods of marker detection and interpretation are heterogeneous. They conclude that, at present, HEr2 expression should not influence the decision to use endocrine therapy (Prowell TM, Armstrong DK, Semin Oncol 2006;33(6):681-7).

A large national retrospective/prospective study was conducted involving 1685 patients diagnosed in with HEr2-positive (2+, 3+ score) primary breast carcinoma, in Italy in 2000-2001. Goals were to increase understanding of the biology, prognosis, and drug-sensitivity of HEr2-positive breast cancer to help optimize treatment. A control was identified as the first consecutive patient with HEr2-negative cancer (score=0 or 1+). The four HEr2 categories (0, 1+, 2+, 3+) investigated demonstrated specific features. There was a significantly higher frequency of p53-positivity among HEr-2+ tumors, compared to the other three categories. There was also a significantly lower frequency of lymphoid infiltration and desmoplasia, accompanied by more frequent vascular invasion in HEr2-1+ tumors, compared to HEr2-0 tumors. HEr2 status was not associated with tumor size or nodal involvement. At a 4-year (median) follow-up, the relapse rate in the HEr2-positive group was 20% compared to 14% in the HEr2-negative group. Based on relapse rates, only women with HEr2-3+ tumors demonstrated a significantly poorer prognosis, in both node-positive and node-negative subgroups. When patients who underwent invasive surgery were included in the analysis, however, patients with HEr2-2+ tumors also exhibited a significantly increased relapse rate, compared to women with negative HEr2 status. The last finding is consistent with the theory that surgery releases growth factors, which stimulate tumor cells. Relapse rate analysis by type of therapy confirmed the unresponsiveness of HEr2-positive tumors to tamoxifen, but sensitivity to chemotherapy, particularly taxanes. In fact, in women treated with taxanes, HEr2-positive tumors had significantly better prognoses than HEr2-negative tumors (Ménard S, ASCO06, Abs. 10501).

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The growth factor heregulin (HRG) binds to erbB-3 or erbB-4 receptors, promoting heterodimerization with erbB-2, and induces autophosphorylation and activation of erbB-2 signaling. It is generally accepted that heregulin and erbB-2 do not interact directly. Depending on its concentration, HRG can either inhibit or stimulate cell proliferation in cell lines that overexpress erbB-2, suggesting a possible weak interaction between HRG and erbB-2 via the existence of a low-affinity binding site for erbB-2 within the EGF-like domain of HRG. Moreover a synthetic peptide, derived from sequence at the erbB-2 extracellular domain, was capable of specifically blocking HRG-induction of erbB-2 tyrosine phosphorylation. Therefore it is possible that this region constitutes a critical region of the erbB-2 receptor responsible for HRG induction of erbB-2 heterodimerization and activation (Mroczkowska JE, etal, AACR02, Abs. 3580).

EGFr and ErbB2 have been identified in mammary carcinoma growth and metastasis. Recent research suggests that type I receptor signaling may be mediated by the CD44 family of transmembrane glycoproteins that also have been implicated in mammary tumor progression. Researchers have tested whether CD44, EGFr, and ErbB2 interacted and colocalized with one another in four mammary carcinoma cell lines (MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-436) and in cytology samples obtained from patients with metastatic breast cancer. CD44 constitutively localized and coimmunoprecipitated with ErbB2 and EGFR in all four mammary carcinoma cell lines and also colocalized with ErbB2 and EGFr in all cytology samples expressing ErbB2. CD44 colocalized with EGFr in cells from only 1 of 16 ErbB2-negative cytology samples. These data indicate that CD44-EGFr-ErbB2 protein complexes occur in a high proportion of metastatic mammary carcinoma and suggest that CD44-type I receptor colocalization may be a novel prognostic marker for aggressive mammary cancers (Wobus M, etal, Appl Immunohistochem Mol Morphol March 2002;10(1):34-9).

Overexpression of c-erbB2 has been reported to be associated with a poor clinical outcome in breast cancer, however, its prognostic value remains controversial especially in patients with node-negative breast cancer, and as it pertains to estrogen receptor (Er) status. Researchers employed IHC staining for c-erbB2 on the primary breast tumors from 698 patients with a mean follow-up duration of 54 months. The c-erbB2 expression was positive in 17.2% of cases, which inversely correlated with the Er status. Both univariate and multivariate analyses indicated the c-erbB2 expression to be a significant prognostic factor for disease-free survival (DFS) and overall survival (OS), while the same effect was also seen in the patient groups with node-negative as well as node-positive breast cancer. The c-erbB2 expression is an independent significant factor for breast cancer and the prognostic significance remains in the node negative as well as node positive breast cancer, while the same effect was also found in all subgroups stratified according to the adjuvant therapies. In addition, the combination of c-erbB2 and Er made it possible to identify the subgroup with the worst clinical outcome (Tsutsui S, etal, J Surg Oncol Apr 2002;79(4):216-23).

HER2 is overexpressed in about in 20%-40% of human breast cancer. HEr2 overexpression affects roughly 20% to 25% of women with early stage breast cancer and roughly 25% to 30% of women with metastatic disease. Activation of HEr2 is pivotal in mammary tumorigenesis; overexpression and amplification of HEr2 is associated with tumor progression. Mutations are not predictive in node-negative breast cancer but appear to predict response to adjuvant chemotherapy in node-positive patients. Encoded by c-erbB-2, p185c-erbB-2 is a 185 kDa tyrosine kinase-type receptor overexpressed in 20%-30% of breast cancer cells (Suzuki T, etal, AACR97, Abs. 55:9). HEr2 overexpression in breast cancer is associated with poor prognosis; the presence of HEr2 protein in breast cancer correlates with aggressive disease and is associated with resistance to conventional-dose chemotherapy.

Ovarian cancer

Researchers from Medical University of Vienna (Vienna, Austria) evaluated the influence of HEr2 on ovarian cancer prognosis and investigated a correlation between compromised survival and CXCR4 expression and/or SDF-1 abundance. CXCR4/SDF-1 is a chemokine-chemokine receptor pair signaling pathway implicated in increased metastatic potential in breast cancer. IHC on tissue microarrays was used to detect HEr2, CXCR4, and SDF-1 in 148 ovarian tumor samples. HEr2 overexpression was found in 27.6% of ovarian cancer tissues and in 15% of ovarian borderline tumors. In patients with ovarian cancer, HEr2 overexpression correlated closely with overall survival (univariate hazard ratio (HR) 2.59; multiple corrected HR 1.92). In contrast, CXCR4 expression and SDF-1 abundance demonstrated no correlation with overall survival or with HEr2 expression. As expected, cytoplasmic CXCR4 expression and SDF-1 abundance were closely correlated. These results confirm a univariate influence of HEr2 expression on overall survival. The finding that this effect was completely independent of CXCR4 expression and SDF-1 abundance implies that there are significant differences in the HEr2 downstream pathways in ovarian cancer and breast cancer (Pils D, etal, Br J Cancer, 2007;96(3):485-91).

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Although cyclooxygenase 2 (COX2) and HEr2 overexpression have been related to poorer prognosis in epithelial ovarian cancer (EOC), data regarding the percentage of tumors with overexpression varies widely in different studies. Researchers from Vejle Hospital (Vejle, Denmark) investigated the potential prognostic value of COX2 and HEr2 expression in EOC, the possible coexpression of the two markers, and the degree of agreement between FISH and IHC in the evaluation of HEr2 status in EOC. IHC was performed for COX2 and HEr2 and FISH analysis for HEr2 gene amplification in 160 patients with EOC, FIGO stages IIB-IV. The period of follow-up was more than 10 years. IHC staining for HEr2 found: 64.4% scored as 0, 24.4% as 1+, 6.9% as 2+, and 4.4% as 3+. Positive IHC staining for HEr2 was associated with poor overall survival. Agreement between IHC and FISH was observed in all cases. Although long-term observation of patients with EOC who demonstrate HEr2-positivity predicted a grave prognosis, the low rate of HEr2 overexpression limits its potential clinical application. COX2 overexpression was found in 20.0% of the tumor samples. MST for COX2-negative tumors was 21.6 months, compared to 36 months for COX2-positive tumors. The longer survival for COX2 positive was significant by both univariate and multivariate analysis (Steffensen KD, etal, Int J Gynecol Cancer, 14 Feb 2007; epub ahead of print).

Researchers at Ludwig Maximilians University of Munich (Munich, Germany) examined the prognostic impact of KI67, p53, HEr2, topoisomerase II-alpha (Top IIa), EGFr, and nm23 expression in tumor tissues from 90 patients with ovarian cancer. Although testing for tumor biological factors for prognostic and predictive indicators is not testing in ovarian cancer, detection of hematogenous tumor spread could help to estimate the risk of metastatic disease. Bone marrow (BM) was aspirated and screened for disseminated tumor cells in the bone marrow (DTC-BM) at the time of primary diagnosis. Expression of p53, KI67, Top IIa, EGFr, HEr2, and nm23 was evaluated by IHC and classified by percentage of stained cells or immunoreactive score (IRS). Expression rates for HEr2 (2+/3+: 34.5%), KI67 (median 30%), p53 (median IRS 5), and Top IIa (median IRS 4) were relatively high. In contrast, expression rates for nm23 (median IRS 2) and EGFr (IRS 0: 61%) were low. In 21 of 90 patients (23.3%), DTC-BM could be detected. The presence of DTC-BM was inversely related to nodal status but not to the other factors examined. Tumor stage, lymph node involvement, grade, postoperative tumor residue, peritoneal seeding, and KI67 significantly correlated with OS after a median observation time of 28 months (2-105). The presence of DTC-BM and KI67 positivity, but not other tumor markers, predicted reduced distant disease-free survival. Although tumor stage and postoperative tumor residue remain the primary determinants of prognosis in patients with ovarian cancer, tumor biologic factor testing could help to stratify subgroups of patients and establish targeted therapies (Schindlbeck C, etal, Int J Gynecol Cancer, 12 Apr 2007; epub ahead of print).

HEr2 expression in epithelial ovarian cancer has been much less studied than HEr2 expression in breast cancer. Various HEr2 testing technologies (e.g., IHC, FISH, CISH, ELISA, others) report HEr2 overexpression frequencies varying from 1.8% to 76%. HEr2 overexpression has been associated with advanced stages, poorly differentiated tumors, resistance to chemotherapy, and shortened survival in some studies. Single agent trastuzumab therapy produces a low but significant response rate in pretreated patients with ovarian cancer characterized by HEr2 overexpression, but its usefulness is limited because of the low frequency of high levels of HEr2 overexpression. At present, information is insufficient for assessment and utilization of HEr2-based therapies in epithelial ovarian cancer (Serrano-Olvera A, etal, Cancer Treat Rev, 2006;32(3):180-90).

Researchers have investigated whether combined treatment with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and trastuzumab could enhance the specific killing of cells that overexpress the ErbB-2 receptor. This resulted in an enhancement of TRAIL-mediated apoptosis in all cell lines overexpressing ErbB-2 receptor compared with either reagent alone. Alternatively, there was no effect in cell lines with low levels of the Erb-B2 receptor. Trastuzumab treatment resulted in downregulation of the ErbB-2 receptor in all ErbB-2-overexpressing cell lines. Similar enhancement of TRAIL toxicity was observed when the ErbB-2 receptor was downregulated using antisense oligodeoxynucleotides (Cuello M, etal, Can Res 15 June 2001;61:4892-4900).

Six ovarian cell lines (PEO1, SKOV-3, OVCAR-5, 41M, PEO1cddp and A2780) were studied to assess the relative contribution of individual erbB family members to cellular migration. When these cell lines were stimulated with ErbB family growth factors. under identical conditions TGFα had a much greater effect on cell migration and epithelial-mesenchymal morphological transition than NRG1β, whereas growth effects were equal. Statistically significant associations were identified between the extent of migration promoted by TGFα and erbB2 expression levels. TGFα treatment resulted in phosphorylation of both EGFR and erbB2. TGFα activated both the ERK and PI3K pathways. Using specific small molecule inhibitors within a TGFα stimulated migration assay, direct block of EGFR (Iressa/ZD1839, 1μM) decreased TGFα-driven

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migration to control levels, indicating the importance of EGFR activation in migration, and hence its value as a therapeutic target. Blockade of PI3K with LY294002 and of ERK with PD98059 or UO126) had no significant effect on cell migration, suggesting these pathways did not mediate migration in the cell lines assayed, or are redundant to other cascades. However, blockade of PLC with U73122 showed a partial reversal of the TGFα stimulus, implicating this cascade as being important to migration within ovarian systems. These data indicate that the EGFR-erbB2 dimer pair is a potent drive to cell migration in ovarian cancer, which can be reversed by EGFR blockade using Iressa, and that downstream signaling via PLC is also important for migration of ovarian cancer cells (Sewell JM, etal, AACR02, Abs. 112).

Pancreatic cancer

A research group from 417VA Hospital (NIMTS; Athens, Greece) studied HEr2 expression and gene alterations in pancreatic ductal adenocarcinoma (PDAC), comparing IHC and chromogenic *in situ* hybridization (CISH) based on tissue microarrays and computerized image analysis. HEr2 gene amplification was detected in 8 (16%) of 50 histologically confirmed PDAC. Chromosome 17 aneuploidy was detected in 19 cases (38%). Correlation of IHC results obtained by conventional eye and digital microscopy significantly improved interobserver agreement, particularly in the cases of overexpression (2+, 3+). Finally, 29 (58%) of cases were characterized as HEr2-0, 11 (22%) were HEr2-1+, 6 (12%) were HEr2-2+, and 4 (8%) were HEr2-3+. Expression of HEr2 protein was significantly associated with tumor grade but not with the stage of the tumor. Chromosome 17 and gene status were not correlated with grade or stage. These findings indicate that a subset of pancreatic ductal adenocarcinomas is characterized by HEr2 gene amplification. However, protein overexpression does not predict this specific gene deregulation mechanism in pancreatic cancer, in contrast to breast cancer. This difference may reflect a different biologic role of the HEr2 protein in these tumors, which might affect the response of such targeted agents as anti-HEr2 MAb. The addition of digital image analysis to conventional eye microscopy evaluation of HEr2 expression improves the accuracy and reliability of IHC evaluation, but does not demonstrate clinical significance and prognostic value in PDAC (Tsiambas E, etal, JOP 2006;7(3):283-94).

Topoisomerase IIalpha (TOP2A) and HEr2 are coamplified in a subset of PDAC. Investigators at Johns Hopkins University examined 55 PDAC specimens for TOP2A immunolabeling and identified TOP2A protein expression in all specimens. Normal pancreatic ductal epithelium, proposed to give rise to PDAC, did not demonstrate detectable TOP2A expression. In a subset of specimens, coamplification of HEr2 was present in 8 of 9 TOP2A-amplified cases, suggesting a potential relationship between TOP2A and HEr2 in PDAC. It is, therefore, suggested that TOP2A immunolabeling be used in conjunction with a newly developed multicolor probe to screen patients with PDAC to determine the best potential therapeutic modalities, such as TOP2A inhibitors, trastuzumab, or both (Hansel DE, etal, Am J Clin Pathol, Jan 2005;123(1):28-35).

In a retrospective study on archival specimens from 33 patients with PDAC, and 25 with hepatocellular carcinoma (HCC), 2 patients with HCC and none with PDAC overexpressed HEr-2, while 2 patients with PDAC and 1 patient with HCC overexpressed CD117. Because HEr2 and CD117 are not significantly overexpressed in either cancer, there appears to be no role for the use of trastuzumab in either malignancy. Also, although it appears that there is no role for tyrosine kinase inhibitors in the treatment of HCC, larger studies are necessary to establish their role in PDAC (Potti A, etal, Anticancer Res, May-Jun 2003;23(3B):2671-4).

Osteosarcoma

University of Utah Health Sciences Center (Salt Lake City, UT) researchers used FISH and multiplex and monoplex PCR) to evaluate HEr2 gene status and determine whether HEr2 gene amplifications are present in osteosarcoma. FISH analysis (including a probe for chromosome 17) was used in 21 cases of osteosarcoma; in 11 of the cases, HEr2 gene amplification status had been previously reported. None of the osteosarcoma cases exhibited HEr2 gene amplification by FISH analysis or subsequent quantitative (multiplex) PCR. Although apparent expression of HEr2 protein was observed in several cases, immunoreactivity was localized to the cytoplasm and was not membranous in character. In an additional 35 osteosarcoma specimens subjected to monoplex PCR analysis, amplifiable DNA was recovered in 19 specimens (54%). None of these samples demonstrated amplification by monoplex PCR analysis; and membranous immunoreactivity (1+) was demonstrated in only one case. The researchers concluded that, although a small subset of osteosarcoma demonstrated weak noncircumferential membranous immunoreactivity for HEr2 protein, none demonstrated positive (2+ or 3+) immunoreactivity; and none demonstrated HEr2 gene amplification by FISH or PCR (Willmore-Payne C, etal, Arch Pathol Lab Med, 2006; 130(5): 691-8).

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The prognostic and therapeutic relevance of HEr2 expression in osteosarcoma and Ewing's sarcoma was studied by a research group at Istituti Ortopedici Rizzoli (Bologna, Italy). HEr2 protein expression was evaluated by IHC in 84 osteosarcoma and 113 Ewing's sarcoma tumor biopsies. HEr2 gene status was assessed in a panel of cell lines; and *in vitro* efficacy of trastuzumab as a single agent or in combination with insulin-like growth factor I receptor (IGF-Ir) IR3 antibody. HEr2 overexpression was detected in 32% of osteosarcoma and 16% of Ewing's sarcoma tumors and associated with increased expression of P-glycoprotein (P-gp). Based on event-free survival analysis, HEr2 and/or P-gp expression demonstrated prognostic value in osteosarcoma, but not in Ewing's sarcoma. However, no therapeutic effectiveness of trastuzumab was observed in the preclinical model in either cancer cell line, unless it was combined with an anti-IGF-Ir targeting strategy. The therapeutic potential of trastuzumab in these malignancies may be better exploited in combination therapies that include anti-IGF-Ir approaches (Scotlandi K, etal, Eur J Cancer, 2005;41(9):1349-61).

Recently, it has been demonstrated that overexpression of ErbB2 protein in osteosarcoma is associated with the presence of pulmonary metastasis and decreased survival. Alternatively, a previous study showed that the expression of ErbB2 declines in individual cases of osteosarcoma as they become metastatic. Researchers then determined the relation between ErbB2 status and outcome in a large number of selected patients with high grade osteosarcoma. This was determined immunohistochemically in biopsy specimens of osteosarcoma of the extremities from patients who were treated with surgery and chemotherapy. None of the patients had metastatic disease at presentation (Stage II), and all were followed-up for at least 5 years. The ErbB2 status was analyzed in relation to the lengths of event-free and overall survival. Of the 81 tumors examined, 51 demonstrated high levels of ErbB2 expression. The presence of increased levels of ErbB2 in osteosarcoma was significantly associated with the increased probability of event-free and overall survival. In patients with high grade osteosarcoma without metastatic disease at presentation, treated with surgery and chemotherapy, the presence of increased levels of ErbB2 in tumor cells was associated with a significantly increased probability of event-free and overall survival (Akatsuka T, etal, Cancer 1 Mar 2002;94(5):1397-404).

Lung cancer

A retrospective study, conducted by Erciyes University Medical Faculty (Kayseri, Turkey), was designed to examine the association and predictive/prognostic value of HEr2 expression and survival in patients with sclc. Of the 67 specimens tested, 12 (17.9%) exhibited HEr2 overexpression. Median overall survival of patients whose tumors were HEr2-positive was 8 +/- 0.9 months; for patients with HEr2-negative tumors, median survival was and 11 +/- 1.5 months. HEr2 overexpression, detected by IHC, had prognostic value. These findings suggest that sclc may be treatable with HEr2-targeted therapeutics (Canoz O, etal, Lung 2006;184(5):267-72).

Preclinical data suggests a role for trastuzumab the treatment of nsclc. HEr2 protein is overexpressed in 20% to 66% of resected nsclc tumors, and predicts poor patient outcome in multiple series. Experiments with nsclc cell lines show that HEr2 overexpression increases chemoresistance, invasiveness, and metastatic potential of the cells (Azzoli C, etal, Semin Oncol Feb 2002;29(1 Suppl 4):59-65).

When expression of HEr2 in archival paraffin-embedded specimens of nsclc was evaluated, testing tumor and normal tissue from the same patients, HEr2 overexpression was not detected in the normal epithelium in a majority of samples (74/81), but was detected in 22 (27%) tumor samples including adenocarcinoma, squamous cell carcinoma and large cell lung cancer (lclc), at the 2+ or 3+ level with another 26 samples at the 1+ level. A marginal reduction to 21% in the 2+ and 3+ subset resulted when expression in the few normal tissues from the corresponding tumor score was subtracted (Scheurle D, etal, ASCO00, Abs. 2012).

Gallbladder cancer

A preliminary study of p53 and HEr2 expression in gallbladder cancer in Indian patients was conducted by researchers at Banaras Hindu University (Varanasi, India). Among 40 patients with gallbladder cancer, 10 (25%) of were positive for HEr2 expression, and 8 (20%) were positive for p53 expression. HEr2 positivity decreased with increasing grade of gallbladder cancer; p53 positivity increased with increasing grade. Only one patient with gallbladder cancer co-expressed HEr2 and p53, which suggests that they may play independent roles in the carcinogenesis of gallbladder cancer, HEr2 overexpression in adenoma and the younger age group indicates that it may play a role in an early event in carcinogenesis (Chaube A, etal, BMC Cancer, 2006;6:126).

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Bladder cancer

Researchers from Cleveland Clinic (Cleveland, OH) studied HEr2 protein expression in metastatic and nonmetastatic urothelial carcinoma. Overexpression of HEr2 protein has been detected in 27-80% of urothelial carcinoma in previous studies and correlated with early tumor recurrence and invasiveness. Downregulation of HEr2 protein has also been described in distant metastases. The study included 9 nonmetastatic primary tumors, 44 metastatic primary tumors, and 43 metastases (41 paired). IHC staining was scored according to standard protocol as 0-4. Although normal urothelium demonstrated overall negativity for HEr2, 5/11 (45%) cases demonstrated weak (2+) positivity in the umbrella cell layer. Among 9 nonmetastatic primary tumors, 6/9 (67%) were negative for HEr2 protein expression, but 2 cases were weakly positive (2+), and 1/9 (11%) was strongly positive (3+). In the metastatic primary group, 26/44 (59%) were negative, 14/44 (32%) weakly positive, and 4/44 (9%) were strongly positive. In metastatic lesions, a higher percentage of lesions, 9/43 (21%) were strongly positive (3+), 25/43 (59%) were negative, and 9/43 (21%) were weakly positive (2+). The group concluded that HEr2 protein expression is stronger in metastatic lesions, but the difference in HEr2 expression in primary metastatic and nonmetastatic tumors was not significant. These findings may indicate a potential therapeutic target in metastatic urothelial carcinoma (Hansel DE, etal, AACR07, Abs. 467).

HEr2 protein overexpression has been reported to range from 2% to 74% in bladder cancer, and HEr2 gene amplification to range from 4% to 59%. Detection of HEr2 expression by IHC and gene amplification by FISH and CISH was studied in noninvasive and invasive transitional cell carcinoma (TCC) of the bladder at Paracelsus University (Salzburg, Austria). HEr2 protein overexpression and HEr2 gene amplification were evaluated in archival tissues from 87 patients with noninvasive papillary (n=25) and invasive (n=62) TCC. HEr2 protein overexpression (3+ and 2+) by standard IHC was detected in 37 (58%) of the invasive and 19 (76%) the noninvasive TCC. HEr2 gene amplification assays were then performed on positive cases and was detected in 81% of 3+ overexpressing cases and 43% of 2+ overexpressing invasive cases, and in 21% of noninvasive papillary bladder tumors. CISH and FISH were 100% concordant. Validation of HEr2 gene detection in bladder cancer may allow for the use of trastuzumab therapy. This study indicated that CISH could provide an accurate and practical alternative to FISH for the clinical diagnosis of HEr2 oncogene amplification in bladder cancer (Hauser-Kronberger C, etal, J Urol. 2006;175 (3 Pt 1):875-80).

Cox-2 and HEr2 coexpression in invasive bladder cancer was studied by a group of researchers from University of Munster in Germany. Tumor tissue from 153 consecutive patients who had undergone radical cystectomy for bladder cancer was analyzed for HEr2 gene amplification by FISH, and for HEr2 and Cox-2 protein expression by IHC. Cox-2 and HEr2 coexpression was detected in 44/132 (33%) of TCC. There was no significant association between Cox-2 and HEr2 gene amplification status. Neither marker was associated with primary tumor stage, lymph-node status, or histologic grading. Coexistence of HEr2 gene amplification and Cox-2 expression, however, correlated with distant metastases. Of 5 Cox-2-positive samples, 2 (40%) demonstrated HEr2 gene amplification (borderline statistical significance; p=0.046). There was no relationship between Cox-2 or HEr2 expression or amplification, alone or in combination, and overall or disease-free survival. Analysis of coexpression of Cox-2 and HEr2 status did not provide prognostic information in patients with bladder cancer. Nevertheless, combined treatment with HEr2 and Cox-2 inhibitors may be beneficial for the patient subgroup of HEr2- and Cox-2-expressing tumors (Eltze E, etal, Int J Oncol. 2005;26(6):1525-31).

Approximately 50% of bladder tumors exhibit strong IHC staining for ErbB1 and approximately 45% express ErbB2, both associated with poor prognosis. Activation of these receptors leads to stimulation of downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) and the anti-apoptotic kinase AKT pathways. They are also implicated in activation of STAT1, a signal transducer and transcription activator. Ultimately, activation of these pathways results in increased cell survival and proliferation. Thus, inhibition of ErbB1 and ErbB2 function may be methods of improving outcome for patients with bladder cancer (McHugh LA, etal, AACR04, Abs. 4398).

Researchers have studied the frequency and the role of c-erbB-2 gene amplification, relative increase in c-erbB-2 gene copy number, and gain of chromosome 17 in bladder cancer. Cancer specimens were examined using FISH. Dual labeling hybridization with a directly labeled centromere probe for chromosome 17 together with a probe for the c-erbB-2 locus was performed. c-erbB-2 gene amplification was found in 3.4% of the specimens. Relative increases in c-erbB-2 gene copy numbers was found in 41.4%

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of specimens and was significantly associated with tumor grade. Gain of chromosome 17 was identified in 65.5% of specimens and was significantly associated with tumor grade and tumor stage. Results suggest that c-erbB-2 gene amplification, relative increase in c-erbB-2 gene copy number, and gain of chromosome 17 may play important roles in the development and progression of bladder cancer. Additionally, the use of c-erbB-2 amplification, relative increase in c-erbB-2 gene copy number, and gain of chromosome 17 using FISH, together with tumor grade and stage, may provide a more useful clinical indicator in bladder cancer (Ohta J, etal, Clin Can Res Aug 2001;7:2463-67).

Prostate cancer

Researchers from Glasgow Royal Infirmary (Glasgow, UK) performed IHC analysis of protein expression of EGFr family members, including EGFrvIII, and phosphorylated Akt (pAkt) in matched hormone-sensitive and hormone-refractory prostate tumors. A high level of HEr2 membrane expression in hormone-sensitive tumors was, surprisingly, associated with increased time-to-biochemical relapse and longer overall survival. EGFrvIII membrane expression was associated with shorter time to biochemical relapse. Furthermore, EGFrvIII nuclear expression was associated with decreased time to death from biochemical relapse and decreased overall survival. HEr4 membrane expression in hormone-sensitive tumors was associated with longer time to biochemical relapse. Increased expression of pAkt was associated with reduced survival. HEr2 was an independent positive predictor of time-to-relapse in hormone-sensitive prostate tumors. A high level of HEr2 in hormone-refractory tumors, in contrast, was associated with increased time to death from biochemical relapse. These findings suggest multiple roles for EGFr family members in prostate cancer. Determination of expression levels alone is not sufficient for determining the biological responses they may elicit (Edwards J, etal, Clin Cancer Res, 2006;12(1):123-30).

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

ter ized as competitive (inhibition) with high functional affinities (that are considered irreversible in cases of bivalent binding). For a review of the structural basis for attachment to and inhibition of EGFr by cetuximab (Li S, Cancer Cell 2005;7:301-311).

Anti-EGFr naked MAb exert their therapeutic actions by mechanisms that include interference with ligand binding (e.g., cetuximab, panitumumab), downregulation of receptors (e.g., trastuzumab downregulates HEr2 receptors), other mechanisms of inhibition of homo- and heterodimer formation, mediation of antibody-dependent cellular cytotoxicity (i.e., Fc domain interactions with Fc receptors on monocytes, natural killer cells, and activated granulocytes provoke immune destruction of tumor cells), complement activation, and others.

Bispecific MAb

A bispecific monoclonal antibody (bsMAb) is a MAb that binds two different types of antigen. bsMAb are produced by the fusion of two different hybridomas in the laboratory. Ertumaxomab (Rexonum; Fresenius Biotech), an intact bispecific MAb targeting HEr2 and CD3, is in phase II development for metastatic breast cancer.

Immunoconjugates

An immunoconjugate consists of a MAb (or other immune system targeting compound), chemically conjugated to a chemotherapy drug, toxin, or radioactive material. The MAb serves as a targeting mechanism to deliver the drug, toxic compound, or radioactive material with specificity to cancer cells, thereby minimizing toxic effects on normal tissues. Immunoconjugates targeted to EGFr family members and EGFr ligand-targeted agents in development include, among others:

- Herceptin-DM1 (Genentech/ ImmunoGen) consists of Herceptin and a prodrug of the toxin, DMI. After binding to cell-surface HEr2 and internalization, active DM1 is released intracellularly. Phase I trials are underway in metastatic breast cancer expressing HEr2.
- SAI-EGFR-ECD (Micromet) targets the extracellular domain of the EGFr and is in preclinical development for advanced, solid tumors.
- Zemab (TopoTarget) is a recombinant MAb targeting ErbB2 linked to a toxin in phase I development for solid tumors.
- TP-38 (Cervene; PE-38; Ivax) is a recombinant chimeric protein consisting of a genetically engineered form of the cytotoxic Pseudomonas exotoxin (PE38) fused to transforming growth factor (TGF)-α. The TGF-α segment targets this compound to tumor cells that overexpress ErbB receptors. A phase II trial in newly diagnosed Grade 4 glioblastoma multiforme (GBM), which overexpresses EGFrvIII, is underway.

IMMUNOTHERAPIES/VACCINES USING ERBB AS ANTIGENS

Immunotherapies and vaccines for cancer indications may be defined as treatment approaches that induce host immune system rejection of cancerous tissues, by either

antigen-specific or non-specific strategies. Non-specific strategies aim to augment the immune response to the tumor, mostly through injection of immune stimulating substances, such as bacterial extracts, cytokines, or non-specific gene therapy agents. Antigen-specific approaches include both the use of tumor cells themselves as sources of antigens and the use of tumor-associated antigens incorporated into vaccine vectors for activation of the immune system. In the case of MAb-based therapeutics, the definition of cancer immunotherapies/vaccine above distinguishes between the passive MAb approaches described and active immunotherapy/vaccine approaches, in which MAb are used to provoke active, specific immune system responses or, hypothetically, activate non-specific immune system responses.

Therapeutic vaccination against tumor-associated antigens is viewed as a very attractive treatment option because of mechanisms of action that suggest this approach will demonstrate high specificity for tumor tissue, high degree of efficacy, (the possibility of) long lasting, cell-mediated immunity against the treated malignancy, and attractive safety profiles. It is a fundamental of cancer vaccine development that strategies used for prophylactic vaccinations against infectious diseases are not necessarily useful for therapeutic cancer vaccination. Vaccination against tumor-associated antigens is often challenging because patients with cancer are usually immunosuppressed, and most cancer-associated antigens are self antigens. Investigative immunostimulation strategies aim to both increase patients' immune system responses and overcome immune tolerance to self antigens. They include new approaches for stimulating antigen presentation, T-cell reactivity, and innate immune activities. Clinical trials are underway that combine various immunotherapy approaches, including blocking immune regulation, in some cases in combination with chemotherapy (e.g., specific T-cell stimulation plus chemotherapy). Two recent publications from researchers at Transgene, SA, review overall discovery, development, challenges, and outlook for therapeutic cancer vaccines (Paul S, etal, IDrugs, 2007 May;10(5):324-8; Acres B, etal, Curr Opin Drug Discov Devel, 2007 Mar;10(2):185-92).

Exhibit 9 provides a sampling of developmental cancer immunotherapies/vaccine approaches based on ErbB receptor targets. This incomplete list of therapeutics and indications is provided to demonstrate the variety of developmental approaches under investigation. New drugs in development that target erbB receptors, including immunotherapies/vaccines, will be extensively reviewed in Part IV of the EGFr Pathway in Cancer series.

MECHANISMS OF RESISTANCE AND TOXICITY

Despite early optimistic predictions regarding their clinical performance, targeted anticancer agents, like cytotoxic therapies, demonstrate limited effectiveness, in part because of resistance mechanisms, and they may cause serious side effects.

RESISTANCE

The effectiveness of targeted therapeutics, which are often used in combination regimens with cytotoxics or multimodality regimens with radiotherapeutics, is limited by resistance. Resistance may result in low initial response rates in patients who would be expected to respond to targeted therapies (e.g., those with EGFr or HEr2 receptor overexpression). Acquired resistance may emerge during initial treatment, causing a declining or lack of response in patients who initially responded to therapy. The underlying mechanisms of resistance remain poorly understood; and currently employed clinical methods for predicting treatment responses to targeted drugs remain imprecise. Recent and ongoing research, however, has increased knowledge of mechanisms of drug resistance, especially with regard to currently approved ErbB receptor-targeted TKI and MAb therapeutics. The identification of ErbB receptor mutations in lung cancer, which may arise before (primary resistance) or after (acquired resistance) treatment, represented a critical advance in our understanding of resistance to TKI. Basic research and R&D related to resistance to TKI and MAb targeting ErbB receptors now focuses on:

- identification of new mutations and elucidation of how mutations cause resistance;
- identification and elucidation of other mechanisms of resistance, not associated with mutations (e.g., activation of alternative ErbB receptors, alterations in EGFr internalization and degradation, novel escape mechanisms, others);
- development of new model systems of resistance; and
- R&D of new therapeutic strategies to prevent the development of resistance and/or overcome it (e.g., irreversible TKI).

Much work has focused on resistance to the small molecule EGFr TKI, gefitinib and erlotinib. Less is known about mechanisms of resistance to lapatinib, which was FDA approved in March 2007. A different resistance profile is anticipated for lapatinib because of its dual inhibition of ErbB1and ErbB2, which may delay the onset of tumor resistance.

Clinically, the most important EGFr mutation in nsclc is the second site mutation T790M (on exon 20). T790M can cause constitutive activation of a partially activated mutant EGFr. Clinically, an estimated 50% of patients who experienced initial dramatic responses to resistance to gefitinib and erlotinib and then acquire TKI resistance have secondary T790M mutations. The mechanism of resistance of T790M is widely thought to be steric blocking of TKI binding to the ATP binding pocket of the receptor by the large methionine residue substitution, although other mechanisms have been proposed, such as enhanced ATP-affinity of the T790M mutant (above see: Yun C, etal, AACR07, Abs. LB-367). The L858R and G719S nsclc mutants also demonstrate markedly impaired affinity for

ATP. The D761Y (exon 19) mutation is reportedly associated with TKI resistance in nsclc cells with L858R-EGFr.

Numerous other mutations have been described for which clinical significance is not yet fully known. Researchers from Vanderbilt University School of Medicine (Nashville, TN) described HEr2 kinase domain mutations in lung cancer that cause constitutive phosphorylation and activation of HEr2 and EGFr and resistance to EGFr TKI. Their findings suggest that cancer cells expressing this mutation remain sensitive to HEr2-targeted therapies but insensitive to EGFr TKI. Expression of a HEr2 mutant containing a G776(YVMA) insertion in exon 20 was more potent than wild type HEr2 in associating with and activating signal transducers, phosphorylating EGFr, and inducing survival, invasiveness, and tumorigenicity. HEr2(YVMA) transphosphorylated kinase-dead EGFr(K721R) and wild type EGFr in the presence of EGFr TKI. Knockdown of mutant HEr2 in H1781 lung cancer cells increased apoptosis and restored sensitivity to EGFr TKI. The HEr2 inhibitors lapatinib, trastuzumab, and CI-1033 inhibited growth of H1781 cells and cells expressing exogenous HEr2(YVMA). These findings suggest that HEr2(YVMA) activates cellular substrates more potently than wild type HEr2 and that HEr2(YVMA) mutants are insensitive to EGFr TKI but remain sensitive to HEr2-targeted therapies (Wang SE, etal, Cancer Cell, 2006 Jul;10(1):25-38).

Activating EGFr mutations are rare in colorectal cancer and do not appear to confer sensitivity to gefitinib and chemotherapy in the way that activating EGFr mutations identify nsclc sensitivity to gefitinib. Resistance to the combination of chemotherapy with gefitinib in colorectal cancer is predicted by p21 expression, especially in combination with p53 mutation. Overexpression of phosphorylated AKT1 may also be useful as a predictor of resistance to gefitinib therapy (Ogino S, etal, Clin Cancer Res, 2005;11(18):6650-6). In a study of 30 patients with colorectal cancer conducted at Universite Paris-Descartes, Institut National de la Sante et de la Recherche Medicale (Paris, France), Kras mutations predicted resistance to cetuximab therapy and were associated with a worse prognosis (Lievre A, etal, Cancer Res, 2006;66(8):3992-5).

Acquired resistance to trastuzumab is common in patients with breast cancer who have shown an initial response to the drug. Although the basis of this resistance is not understood, studies suggest several possible mechanisms, some of which are also implicated in resistance to EGFr TKI in breast cancer. A research group from Cancer Research UK (London), College de France (Paris), and Weatherall Institute of Molecular Medicine (Oxford, UK) showed that activation of alternative ErbB receptors mediates resistance to EGFr TKI and trastuzumab in breast cancer. They used the Forster Resonance Energy Transfer (FRET) technique to study the responses of ErbB receptors in breast cancer cell lines to treatment with EGFr TKI or trastuzumab. They found that the TKI induced activation of alternative ErbB receptors and mediated resistance to these drugs. Furthermore, trastuzumab induced activation of all ErbB pathways in SKBR3, despite its ability to downregulate HEr2 in the long term. These findings showed, for the first time, that EGFr TKI and trastuzumab treatment in breast cancer cells induce activation of alternative ErbB pathways, providing possible mechanisms of resistance to targeted therapies (Kong A, etal, AACR07, Abs. 2337).

Researchers at University of Wisconsin (Madison, WI) investigated mechanisms of acquired resistance to EGFr inhibition in cetuximab-resistant (Cet-R) clones from the nsclc (H226) cell lines, which were developed with long-term exposure to cetuximab. The Cet-R cells exhibited increases in total and active forms of EGFr and increased release of the EGFr ligands TGF-α and neuregulin. The potential roles of constitutive EGFr expression and activation in acquired resistance were assessed by investigating differences between Cet-R and parental cells with regard to patterns of ligand-stimulated EGFr internalization. In parental H226 cells, 45 minutes of EGF stimulation resulted in a reduced level of surface EGFr and evidence of receptor internalization; whereas no similar reduction of surface EGFr or evidence of receptor internalization was observed in Cet-R cells after EGF stimulation. Differences in ligand-stimulated EGr degradation were evaluated by investigating Cbl-mediated ubiquitinylation of EGFr, which is required for lysosomal sorting of the receptor. After EGF stimulation, EGFr is ubiquitinized and recruits Cbl protein in parental but not in Cet-R cells. An increased association of EGFr with hsp90, a negative regulator of Cbl-mediated downregulation of EGFr was observed in Cet-R cells. These findings indicate that EGFr is not readily internalized and degraded following EGF stimulation in Cet-R cells, suggesting that alterations in EGFr trafficking and degradation may play important roles in acquired resistance to cetuximab (Huang S, etal, AACR07, Abs. 2348).

Researchers at Roswell Park Cancer Institute, of the State University of New York at Buffalo, recently proposed a novel mechanism of resistance to EGFr antagonism in solid tumors, particularly colorectal cancer. This group created a model of constitutive EGFr signaling in colon cancer cells by transfection with human TGF-α cDNA (a ligand for EGFR) under repressible control by tetracycline. Constitutive TGF-α expression causes constitutive EGFr activation that allows for cancer cell survival in response to environmental stress. The reversal of constitutive EGFr activation caused the loss of downstream mitogen-activated protein kinase and Akt activation and reduced the size of the xenograft size (through decreased proliferation and increased apoptosis). Dissection of an activation pathway with the irreversible TKI, CI-1033, showed that ErbB2 can activate Akt, but not Erk, when EGFr is antagonized. This pathway may describe a novel escape mechanism and explain the disappointing results observed with anti-EGFr therapies in clinical trials (Rajput A, etal, Cancer Res, 2007;67(2):665-73).

Exhibit 5
ErbB3 in Malignancy

HEr3 • erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) tyrosine kinase-type cell surface receptor

Gastric cancer

There have been few recent reports of HEr3 association with gastric cancer.

Investigators at National Cancer Center Research Institute (Tokyo, Japan) report that the 6.2-kb c-erbB3 transcript was expressed in all gastric cancer cell lines examined, and that 1.4-kb c-erbB3 transcript was expressed as highly as 6.2-kb transcript in MKN45 cells. erbB3-S cDNA, corresponding to 1.4-kb c-erbB3 transcript, was cloned. Sequence analysis of erbB3-S cDNA showed that this 1.4-kb c-erbB3 mRNA encoded a secreted receptor. Analysis of partial genomic structure of c-erbB3 gene revealed that the exon specific to secreted receptor was identical with the 5' portion of the intron in c-erbB3 gene. Therefore, c-erbB3 gene encodes secreted as well as transmembrane receptor tyrosine kinase because of alternative splicing (Katoh M, etal, Biochem Biophys Res Commun, 14 May 1993;192(3):1189-97).

Breast cancer

Elevated ErbB3 mRNA levels have been observed in some human mammary tumor cell lines.

Although tyrosine kinase inhibitors (TKI) against the EGFr family exhibit effective *in vivo* inhibition of EGFr and HEr2, their activities against HEr2-driven breast cancer are limited, for reasons that are not understood. The phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway is critically important and driven mostly by transphosphorylation of the kinase-inactive HEr3. Researchers from University of California, San Francisco demonstrated that HEr3, and consequently PI3K/Akt signaling, evades inhibition by currently employed EGFr family TKI in *in vitro* and *in vivo* tumor models. This evasion is caused by a compensatory shift in HEr3 phosphorylation-dephosphorylation equilibrium, which is driven by increased membrane expression of HEr3 driving the phosphorylation reaction and reduced HEr3 phosphatase activity, which impedes the dephosphorylation reaction. Akt-negative feedback signaling mediates these compensatory changes. HEr3 is not a target of TKI, but HEr3 substrate resistance undermines TKI efficacy. Experiments using small interfering RNA knockdown abrogate HEr3 resistance and restore potent pro-apoptotic activity to EGFr TKI. This finding reaffirms oncogene addiction in HEr2-driven tumors and the therapeutic potential of HEr2 as a target. One therapeutic implication of the buffering of HEr3 signaling against incomplete inhibition of HEr2 kinase is that, in order to effectively silence oncogenic HEr3 signaling, more potent TKI or combination strategies are required. Transphosphorylation of HEr3, rather than autophosphorylation, is the appropriate biologic marker for assessment of EGFr TKI efficacy (Sergina NV, etal, Nature, 2007;445(7126):437-41).

The roles of erbB3 in erbB2-associated kinase activity in tamoxifen resistance were investigated by a group from University of Colorado at Denver and Health Sciences Center (Aurora, CO). In a study of tumor-derived cell lines from wild type rat c-neu transgenic mice and human breast cancer, downregulation of HEr3 by specific siRNA reduced HEr2 tyrosine phosphorylation, decreased PI3K/Akt signaling, and inhibited mammary/breast cancer cell proliferation and colony formation. Specific HEr3 siRNA sensitized HEr2 transfected MCF-7 cells (MCF-7/HEr2) to tamoxifen-associated inhibition of cell growth and colony formation and enhanced tamoxifen-induced apoptosis. In contrast, control siRNA transfected MCF-7/HEr2 cells were tamoxifen-resistant. The researchers concluded that HEr2/HEr3 heterodimerization is required for HEr2 tyrosine kinase activation in mammary/breast cancer cells and that downregulation of HEr2 inhibits HEr3-associated procarcinogenic activity via inactivation of the PI3K/Akt pathway. HEr3 also contributed to HEr2-mediated tamoxifen resistance, suggesting that HEr3 may be a clinically relevant therapeutic target in HEr2 positive breast cancer (Liu B, etal, Int J Cancer, 2007;120(9):1874-82).

Ovarian cancer

After dimerization with other members of the EGFr family several signal transduction cascades may be activated, including phosphoinosite 3'-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (ERK1/2). Investigators from University of Mainz, in Germany, studied a possible association between expression of HEr3 expression and prognosis in patients with ovarian cancer. They examined tumor tissue obtained from 116 consecutive patients diagnosed with primary epithelial ovarian cancer between 1986 and 1995 for HEr3 expression. HEr3 expression was observed in 53.4% of the patients and associated with decreased

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histologic grade and type, residual disease, and age. HEr3 expression (hazard ratio=1.71), FIGO stage (HR=4.78), residual tumor (HR= 2.69), and age (HR=2.06) correlated with prognosis. HEr3 expression clearly influenced survival time. Median survival time was 3.31 years for patients with low HEr3 expression, compared to 1.80 years for patients with overexpression of HEr3. Although validation of this study is required, HEr3 may represent a new prognostic factor in primary epithelial ovarian cancer, and investigation of therapeutic strategies for blocking HEr3 may be warranted (Tanner B, etal, J Clin Oncol, 2006;24(26):4317-23).

Ovarian cancer

Researchers from Catalan Institute of Oncology, Hospital Germans Trias i Pujol (Badalona, Spain) and the University of Messina in Italy addressed pathways and markers that correlate with the outcome of chemotherapy in lung cancer. mRNA transcripts that are involved in DNA repair pathways (e.g., ERCC1 and BRCA1) cause selective resistance to cisplatin or taxanes Thioredoxin causes broad spectrum of resistance to chemotherapy agents. DNA repair gene polymorphisms and checkpoint gene methylation in circulating serum DNA have great potential as predictive markers of survival in particular cisplatin-based regimens. Epithelial-mesenchymal transitions and HEr3 mRNA levels have potential as ancillary markers for treatment with EGFr RTK inhibitors (Rosell R, etal, Curr Opin Pharmacol, 2006;6(4):323-31; erratum in Curr Opin Pharmacol, 2007;7(1):119).

A study to identify immunohistochemical (IHC) biomarkers for improved prognostication in patients with non-small cell lung cancer (nsclc) used tissue samples from 609 patients with nsclc, including 243 adenocarcinoma (ACA), 272 squamous cell carcinoma (SCC), 35 large cell carcinoma, 32 non-small cell carcinoma NOS, and 6 others (giant cell carcinoma); 21 patients with other histologies were excluded. Survival data for 535 cases was available. A total of 11 biomarkers were tested, including EGFr, HEr2, HEr3, p53, p63, Bcl1, Bcl-2, TTF1, CEA, Ch, and SNP. Bcl-2 was the only biomarker that predicted better overall survival (OS). Bcl-2 and p63 predicted disease-specific survival (DSS) in all nsclc. p63 was significant for DSS in SCC but not in ACA. Bcl2 was not significant for DSS in either subgroup. Expression of EGFr was associated with improved DSS in SCC but not in ACA. Co-expression of EGFr-HEr3 occurred more frequently in SCC than in ACA. Correlation between outcome and any combination or clustering of biomarkers was observed. The researchers concluded that p63 and Bcl-2 are predictive of DSS in nsclc. Expression of EGFr predicts DSS in SCC. Histopathologic subclassification of nsclc is important for retention of the relevance of some biomarkers (e.g., EGFr). p63, Bcl-2, and EGFr may be used as prognostic markers in patients with nsclc. EGFr-HEr3 coexpression occurs more frequently in SCC than in ACA, which may help explain the differential responses of SCC and ACA to EGFr inhibitors (Renouf D, etal, ASCO06, Abs. 7211).

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

TOXICITY

Potential side effects of most currently marketed ErbB-pathway targeting drugs include mostly relatively minor and several major (life-threatening) toxicities.

The most common side effect of anti-EGFr treatment with TKI or MAb is an acneiform skin rash, the development of which appears to be associated with a positive clinical outcome of treatment. Anecdotal observations of this association emerged soon after clinical trials with these agents were initiated. Data from several clinical trials with EGFr-targeted agents show positive correlations or positive trends between the development of rash and response to treatment and/or survival. Such findings suggest that rash might be a surrogate marker of efficacy and useful as a tool to predict response. Large, prospective studies of the correlation between grade of rash and response and survival in clinical trials are needed. Studies of the etiology of the rash are also needed to understand the relationship of the rash to response (Pérez-Soler R, etal, The Oncologist, 2005;10(5)345-356).

A serious cardiovascular side effect has been linked to treatment with the MAb Herceptin. In August 2005, Genentech and the FDA issued an advisory to physicians regarding a significant increase in cardiotoxicity among patients treated with Herceptin. According to a preliminary analysis of safety data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-31, the 3-year cumulative incidence of congestive heart failure and cardiac death was 4.1% among women treated with Herceptin and chemotherapy, compared to 0.8% for women randomized to chemotherapy alone.

Gefinitib treatment for lung cancer has been associated with severe albeit infrequent cases (about 1%) of interstitial lung disease (ILD), described as interstitial pneumonia, pneumonitis, and alveolitis; approximately 1/3 of the ILD cases were fatal. Although the reported incidence of ILD in the 23,000 patient USA expanded access program

was about 0.3%, Japanese postmarketing experience estimated the rate of ILD at about 2%. As a consequence, in December 2002, use of Iressa in Japan was restricted to medical institutions capable of treating patients who experience sudden serious side effects.

MEETING COVERAGE

NEW TARGETS AND DELIVERY SYSTEMS IN CANCER DIAGNOSIS AND TREATMENT

FROM THE MARCH 2007 CONFERENCE ORGANIZED BY THE SIDNEY KIMMEL CANCER CENTER, IN SAN DIEGO, CA

This report summarizes selected presentations from the "New Targets and Delivery Systems in Cancer Diagnosis and Treatment" conference organized by the Sidney Kimmel Cancer Center and held in San Diego, CA, in March, 2007.

ADOPTIVE IMMUNOTHERAPY (AIT)

Adoptive immunotherapy (AIT) describes a process in which previously sensitized immunologic agents (cells or sera) are transferred to non-immune recipients (adoptive transfer). AIT is a common immunotherapy strategy in cancer.

Adoptive Transfer of IL-2-activated Natural Killer (NK) Cells

Adoptive transfer of IL-2-activated NK cells, administered via a proper route, migrate to and heavily infiltrate metastases from murine tumors of different origin, according to a presentation by Marianne Hokland, MD, from Aarhus University in Denmark.

NK cells, a subset of lymphocytes, are large granular lymphocytes (LGL) able to kill virally infected cells and certain types of tumor cells. NK cells are considered to be the major component of antitumor immunity responsible for rapid elimination from the circulation of malignant cells that express low levels of major histocompatibility complex (MHC) class I molecules on their surfaces, sparing most normal cells. NK cells express receptors that bind to various ligands on target cells and, thus, regulate the cytolytic activities of these cells or bind to the Fc portions of antibodies causing antibody-dependent cellular cytotoxicity (ADCC). The cytolytic, secretory, and proliferative functions of NK cells are upregulated by a variety of biologic agents, including interleukin-2 (IL-2), IL-12, or interferon γ (IFN-γ). In addition, adoptively transferred, IL-2-activated NK cells appear to selectively enter and destroy established tumors/micrometastases (Basse PH, etal, Mol Biotechnol, Jun 2002;21(2):161-70; basse@imap.pitt.edu).

NK cells play an important role in the lungs in terms of tumor cell clearance and resistance of this organ to metastasis. This correlation between NK activity and clearance of tumor cells is found only in the lungs. NK cells, activated *ex vivo* with IL-2, localize to lung tumors following intravenous (IV) adoptive transfer, significantly reducing the growth of the tumors they infiltrate. Therefore, NK cells are very potent tumor killer cells capable of eliminating not only circulating tumor cells but also well established micrometastases (Yang Q, etal, Immunol Res 2006;36(1-3):13-25). IL-2-activated natural killer (A-NK) and phytohemagglutinin and IL-2 activated killer T (T-LAK) cells localize and accumulate in murine lung tumor metastases following adoptive transfer. Morphologic appearance, content of extracellular matrix (ECM), and vascular density of lung metastases predicts permissiveness to infiltration by adoptively transferred NK and CTL (Yang Q, etal, Cancer Immunol Immunother, Jun 2006;55(6):699-707).

Dr. Hokland and colleagues at the University of Pittsburgh demonstrated close contact between adherent lymphokine-activated killer (A-LAK) cells and various tumor cells. Spleen cells isolated from mice were stimulated and expanded *ex vivo* with IL-2 to generate LAK cells. These were labeled with fluorochromes for identification and reinjected into mice xenografted with B16 melanoma, MCA 102 sarcoma, or Lewis lung carcinoma. Approximately 5- to 10-fold higher numbers of A-LAK cells were found in malignant foci compared to the surrounding normal tissue. An A-LAK cell/tumor cell ratio higher than 1:1 was observed in most lung metastases. However, about 5% of the lung metastases were not infiltrated even though neighboring metastases were highly infiltrated. The existence of lung metastases that are resistant to infiltration by transferred effector cells at time of treatment may reduce the efficacy of cell-based immunotherapy (Yang Q, etal, Int J Cancer, 1 Jul 2003;105(4):512-9), which may necessitate tumor biopsy analysis of the distribution of extracellular matrix (ECM) and vasculature for selection of patients most likely to benefit from cellular adoptive immunotherapy (Yang Q, etal, Cancer Immunol Immunother, Jun. 2006;55(6):699-707). In addition, although substantial infiltration of lung metastases was observed when A-LAK cells were administered IV, significant infiltration of liver metastases was seen only upon intraportal injection of A-LAK cells, suggesting that the traffic of IV injected A-LAK cells was impaired through the lung capillaries. According to these findings, A-LAK cells, when administered via a proper route, migrate to and heavily infiltrate metastases from murine tumors of different origin. Close contact between A-LAK cells and tumor cells is believed to be responsible for the events leading to tumor cell lysis.

Based on these findings, in order to optimize adoptive immunotherapy, it is important to identify a subtype of LAK cells that demonstrates maximal tumor infiltration and significant cytotoxicity. For this purpose, both short and long term cultured murine adherent natural killer (A-NK) cells and mitogen-stimulated, lymphokine-activated T-

killer (T-LAK) cells were studied with respect to their abilities to proliferate, cytotoxic attributes, IL-2 requirements, and abilities to infiltrate B16 pulmonary metastases following adoptive transfer. In short term (5 days) cultures, A-NK and T-LAK cells both accumulated substantially in tumor tissues. Loose tumors were infiltrated by A-NK or T-LAK cells, whereas compact tumors or normal tissues were not penetrated. A-NK cells gradually lost their ability to accumulate in tumor tissues during *in vitro* culture, whereas T-LAK cells cultured for as long as 20 days were able to infiltrate metastases as efficiently as their short term culture counterparts. A-NK cells proliferated only up to day 15, whereas T-LAK cells continued their growth beyond this time period. In addition, T-LAK cells required lower amounts of IL-2 than A-NK cells to maximally penetrate tumors. Based on these findings, T-LAK cells are attractive candidates for adoptive immunotherapy

NOVEL DRUG DELIVERY FOR TUMOR TARGETING AND IMAGING

Although targeted biologic therapies hold tremendous potential for treatment of cancer, their use has been limited by constraints on delivery and effective tumor targeting. Development of delivery vehicles capable of locating and entering tumors before delivering a therapeutic payload is expected to enable the design of more effective and less toxic treatment strategies.

Biologic Agents as Delivery Vehicles

One possible strategy to improve the effectiveness of the various viral-based and immune-cell therapies proposed in the treatment of cancer is to combine these biologic agents to take advantage of naturally occurring immune cell-pathogen relationships. This approach may enable systemic delivery of targeted therapeutics to tumors, namely oncolytic viruses, attenuated bacteria, and eukaryotic cells such as cellular immunotherapeutics and progenitor/stem cells, to treat disseminated disease and micrometastases (Thorne SH, Expert Opin Biol Ther, Jan 2007;7(1):41-51; sthorne@stanford.edu). Also, immune cells may be used as carrier vehicles to deliver viral therapies to tumors (Thorne SH, and Contag CH, Cell Mol Life Sci, 2 Apr 2007; epub ahead of print).

Cytokine-induced killer (CIK) cells, described in a presentation by Christopher Contag, PhD, from Stanford University School of Medicine (Stanford, CA), are *ex vivo* activated and expanded CD8+ natural killer T cells with antitumor activity. According to Dr. Contag, although T-cell adoptive transfer may be effective, isolation of specific cells can be laborious, and these cells may have a limited effect as monotherapy. This therapeutic approach should be combined with chemotherapy, radiation, or monoclonal antibody (MAb)-based therapy. The factors determining efficacy of such treatments include the proper selection of modalities used in combination, appropriate treatment timing, and carefully selected patient populations.

Although CIK cells alone exhibit antitumor activity, Dr. Contag's group attempted to identify a subpopulation that may be more cytotoxic by designing the first study to explore cell killing of primary ovarian carcinoma cells by retargeting autologous CIK cells with bispecific antibodies. Primary tumor cells and autologous CIK cells were obtained from women with epithelial ovarian cancer. Bispecific antibodies against cancer antigen-125 (BSAbxCA125) and Her2 (BSAbxHer2) were developed using chemical heteroconjugation. Expansion of CIK cells resulted in a significant increase of CD3+CD8+ and CD3+CD56+ T cells. When cell killing of primary ovarian carcinoma cells was investigated with and without bispecific antibodies, enhancement by bispecific antibodies increased the mean percentage lysis of fresh ovarian cancer cells exposed to autologous CIK cells from 21.7% +/- 0.3% to 89.4% +/- 2.1% at an effector-to-target (E:T) ratio of 100:1 ($p<0.001$). Anti-NKG2D antibodies attenuated the CIK activity by 56.8% on primary cells ($p< 0.001$). NKG2D is a transmembrane protein receptor present on NK cells; engagement of this molecule can trigger cytolytic activity. Four hours after CIK cell injection in SCID mice, it was possible to visualize CD8+NKG2D+ CIK cells infiltrating HEr2-expressing cancer cells by fluorescence microscopy. Tumor burden was significantly reduced in mice that underwent adoptive transfer of CIK cells redirected with BSAbxCA125 and BSAbxHer2; and survival improved compared to those treated with CIK cells alone. Therefore, bispecific antibodies significantly enhanced the cytotoxicity of CIK cells in primary ovarian cancer cells and in a mouse model. The mechanism of cytolysis seems to be mediated in part by the NKG2D receptor (Chan JK, Clin Cancer Res, 15 Mar 2006;12(6):1859-67).

The cytolytic ability of the CIK cells was also investigated in combination with an oncolytic viral therapy. CIK cells, preinfected with modified vaccinia virus, were introduced in both immunodeficient and immunocompetent mouse models. According to whole body imaging studies, these cells retained their ability to traffic to and infiltrate the tumor effectively before releasing the virus. Importantly, the virus remained hidden within the CIK cells until the time of interaction of the cells with tumor cells. This dual, synergistic approach effectively targeted delivery of a cell-killing therapy to tumors in animal models (Thorne SH, etal, Science, 24 March 2006;311(5768):1780-84).

In another set of studies, an oncolytic virus approach was used for angiogenesis inhibition. An antiangiogenic gene therapy vector, Ad Flk1-Fc, which expresses a soluble vascular endothelial growth factor receptor (VEGFr) capable of inhibiting tumor angiogenesis and growth, was used in combination with an oncolytic virus, dl922/947. Replication and subsequent cytotoxicity of dl922/947 are restricted to tumor cells with a loss of the G1-S cell-cycle checkpoint. This approach resulted in a significantly greater antitumor effect compared to that obtained with

Exhibit 6
ErbB4 in Malignancy

Her4 • Her-4 • ErbB-4

Breast cancer

In normal breast, HEr4 regulates epithelial differentiation and functions as a nuclear chaperone for signal transducer and activator of transcription (STAT) 5A. This mechanism stimulates milk-gene expression. A research group from Tulane Cancer Center (New Orleans, LA) suggests that these HEr4 activities suppress breast tumor growth. A HEr4 allele harboring an activating transmembrane mutation (HEr4-CA) was created by substituting isoleucine 658 for glutamic acid. This base substitution resulted in the formation of the valine-glutamic acid-glycine activation domain that was first identified in oncogenic HEr2. Receptor tyrosine phosphorylation was increased by a factor of five as a result of ectopic expression of HEr4-CA in HEK293T cells. HEr4-CA exhibited higher levels of nuclear translocation than wt HEr4 leading to HEr4-induced STAT5A simulation of the ß-casein promoter. Activated HEr4 induces cell killing of breast tumor cells Notably, HEr4-CA potentiated the proapoptotic function of HEr4 in each breast, prostate, and ovarian cancer cell line tested, while untransformed cell lines were resistant to both HEr4 and HEr4-CA-mediated apoptosis. Active HEr4 signaling is a potential mechanism for therapeutic intervention in human cancer (Vidal GA, etal, Oncogene, 2007;26(3):462-6).

To delineate the biological function of ErbB4 receptors in breast cancer, researchers used a hammerhead ribozyme strategy to downregulate ErbB4 receptors in various breast cancer cell lines. Downregulation of ErbB-4 in estrogen receptor-positive (Er+) human breast cancer cell lines (MCF-7 and T47D), which express relatively high levels of ErbB4, significantly inhibited colony formation. Little effects were observed in estrogen receptor-negative (Er-) MDA-MB-453 cells, which express low levels of endogenous ErbB4 and high levels of ErbB2 and ErbB3. Downregulation of ErbB4 in T47D and MCF-7 cells significantly inhibited tumor formation in athymic nude mice. In addition, NRG-stimulated phosphorylation of ErbB4 and NRG-induced colony formation was significantly reduced in ribozyme-transfected T47D cells. These data provide the first evidence that elevation of ErbB4 expression plays a role in the proliferation of some ER+ human breast cancer cell lines (T47D and MCF-7) that express high levels of ErbB4 (Tang C, etal, Can Res 15 Oct 1999;59:5315-22).

Medulloblastoma

There are few recent reports of studies of HEr4 in medulloblastoma.

When levels of expression of the EGFr family and 14 of their ligands was evaluated in a series of 32 medulloblastoma samples, the most striking result was the high levels of expression of an erbB4 transcript that in many cases was greater that 250 times that found in normal cerebellum. ErbB4 transcript levels greater than 10 times that of cerebellum were seen in 88% of the series and only one case showed no evidence of expression. ErbB2 transcript levels exceeding 10 times that of cerebellum were seen in 31% of cases, with lower levels of expression in all but 7 tumors (22%). ErbB1 and erbB3 transcripts only rarely reached levels greater that 10 times that of cerebellum (one case each) with 41% showing no evidence of transcript for erbB1 and almost 70% showing no evidence of transcript for erbB3. Only one case showed no evidence of expression of any of the 4 receptors. Analysis of tumor subgroup showed a relatively good correlation between transcript levels and protein expression. One or more ligands for erbB4 (epiregulin, betacellulin, NRG, or NRG2 including splice variants) were expressed at varying levels in all of the cases with erbB4 expression with no obvious common pattern emerging. The single case showing no detectable expression of any of the 4 receptors also showed ligand expression. Gene amplification was assessed in all cases with high expression of any of the transcripts and none was found, thus other mechanisms must be responsible for these high expression levels. The findings indicate the complex receptor-ligand interactions that may be occurring in medulloblastomas and suggest that various forms of autocrine, paracrine, and juxtacrine stimulation of the EGFr family may contribute to the malignant phenotype in these tumors. Whether the findings correlate to any aspect of the clinical behavior of the tumors remains to be elucidated (Liu L, etal, AACR02, Abs 249).

Ovarian cancer

HEr1 was overexpressed in 3/9 ovarian cancer cell lines, HEr2 in 1/9, HEr3 in 2/9, and HEr4 in 4/9. Blocking EGFr with cetuximab, gefitinib, or the combination of both resulted in minimal or no growth inhibition in all 9 cell lines. In 3 cell lines selected for EGFr and HEr2 expression, combination treatment with cetuximab and trastuzumab did not inhibit growth. Combination treatment increased resistance in 1 of the 3 cell lines, which overexpressed EGFr, HEr2, and HEr4. In this study, ovarian cancer demonstrated

— *continued on next page*

variable EGFr family expression. No correlation between EGFr and HEr2 overexpression and response to their targeted inhibition was observed. These findings support the concept the entire EGFr family plays a role in ovarian cancer growth and suggests that an shift in the equilibrium of EGFr heterodimerization may play an important role the maintenance of cell signaling (Bull SL, etal, ASCO06, Abs. 13120).

Studies using immunohistochemical expression identified ErbB4 in 93% of ovarian tumors using the HFR-1 antibody and in 89% of ovarian tumors using the H4.77.16 antibody. Tumors of serous histology were more likely to express a higher level of ErbB4 than endometrioid tumors, and for stage III serous tumors, long term survival was associated with moderate to high coexpression of ErbB4 and ErbB2. In ovarian cancer cell lines, high ErbB4 expression was associated with cisplatin resistance. Using RT-PCR, the presence of multiple isoforms of ErbB4 mRNA was identified in both ovarian primary tumors and cell lines. The use of an anti-ErbB4 blocking antibody suggested that it was not the mediator of the growth stimulatory effects of neuregulin in ovarian cancer cells and indeed could potentially antagonize this effect (Gilmour L, Can Res 1 Mar 2001;61(5):2169-76).

Thyroid cancer

A study conducted at Steven Jones University of British Columbia (Vancouver, BC) evaluated diagnostic and prognostic utility of HEr1, HEr2, HEr3, and HEr4 in differentiated thyroid cancer (DTC). Percentages of cases in which markers were expressed were HEr1 (76%), HEr2 (2%), HEr3 (57%), and HEr4 (73%). Expression of HEr1 and HEr3 demonstrated increased expression in DTC, compared to benign thyroid lesions (76.3% versus 59.6%). HEr4 expression was decreased in DTC, compared to benign thyroid lesions (72.7% versus 85.9%). HEr2 expression did not differ in benign and DTC lesions. Using all 4 markers as potential predictors of benign versus malignant status demonstrated accuracy, sensitivity, and specificity of 67.2%, 60.4%, and 74.0%, respectively. This group concluded that EGFr family expression is useful for distinguishing between DTC and benign thyroid lesions and that, because a high proportion of DTC expressed HEr1, HEr3, and HEr4, investigation of anticancer agents that target one or more EGFr family members warrants clinical study in patients with DTC (Wiseman SM, etal, AACR07, Abs. 382).

Bladder cancer

Molecular correlates of sensitivity to EGFR-targeted therapy in bladder cancer were reviewed by a group from University of Texas M. D. Anderson Cancer Center; 12 bladder cancer cell lines containing only wild type EGFr were stratified as sensitive, intermediate, or resistant to the anti-human EGFr antagonist MAb, cetuximab. EGFr ligand expression (TGF-alpha, PDGF isotypes, IGF-1), growth factor receptors (EGFr, HEr2, HEr3, HEr4, PDGFralpha and PDGFrβ, IGFr, and FGFr3), and markers of epithelial and mesenchymal differentiation were evaluated. Tumorigenicity was assessed in an orthotopic nude mouse model, and invasive potential was determined. All cell lines expressed EGFr and EGFr ligands in amounts that were not predictive of response. Expression of PDGFr and nuclear HEr4 correlated with resistance to C225; and membranous HEr4 was observed only in sensitive and intermediate lines. Resistance to C225 was also correlated with loss of E-cadherin expression. FGFr3 mutations were found only in intermediate and resistant cell lines. Cell lines that were resistant to C225 were the most invasive, while those that were sensitive were least invasive. All cell lines were highly tumorigenic, except for 3 of the 4 most sensitive cell lines, which were non tumorigenic in the orthotopic model. In this study, expression of specific growth factors and loss of epithelial markers correlated with resistance to EGFr-targeted therapy and invasive phenotype. This information will be used in clinical trials for prospective evaluation of patient response to EGFr-targeted therapy. The researchers will examine molecular mechanisms of the relation ships described and potential targeting of other growth factor receptors, particularly PDGFr-β and FGFr3 (Black PC, etal, AACR07, Abs. 5417).

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

the respective monotherapies. Replication and repackaging of the replication-deficient Ad Flk1-Fc component were associated with enhanced soluble VEGFr expression. In addition, co-administration of these viral therapies *in vivo* produced significantly enhanced antitumor effects in colon HCT 116 and prostate PC-3 xenografts in mice. The increased therapeutic benefit correlated with replication of Ad Flk1-Fc viral genomes, increased intratumoral levels of Flk1-Fc protein, and decreased microvessel density, consistent with enhanced antiangiogenic activity (Thorne SH, etal, Mol Ther. May 2006;13(5):938-46).

In another project, Dr. Contag's group evaluated the role of the myc oncogene in hepatocellular carcinoma, which is generally refractory to current treatments. Inact-

ivation of the myc oncogene causes tumor cells to differentiate into hepatocytes and biliary cells forming bile duct structures. Rapid loss of expression of the tumor marker alpha fetoprotein (AFP) was observed using bioluminescence imaging in these cells, whereas an increase was noted in the expression of liver cell markers, cytokeratin 8 and carcinoembryonic antigen (CEA), and, in some cells, the liver stem cell marker, cytokeratin 19. This state is referred to as 'tumor dormancy'. Inactivation of the myc oncogene also induced sustained regression of invasive liver tumors. Using array comparative genomic hybridization technology, investigators confirmed that cells that differentiated into hepatocytes, and those in which tumor characteristics were restored, were clonally derived from the tumor cells. This was evident because they retained identical molecular signatures. Therefore, oncogene inactivation unmasked the pluripotent capacity of tumors, leading to their differentiation into normal cellular lineages and tissue structures. However, although these cells existed in a state of tumor dormancy, they retained their latent, tumorigenic potential and and could become malignant after reversal of myc inactivation (Shachaf CM, etal, Nature, 28 Oct 2004;431(7012):1112-7).

Nanotechnology

Shuming Nie, PhD, from Emory University and Georgia Tech (Atlanta, GA), described a nanotechnology-based class of molecules consisting of highly luminescent semiconductor quantum dots (zinc sulfide-capped cadmium selenide) covalently coupled to biomolecules, which are useful in ultrasensitive biologics detection. This class of luminescent labels is 20 times brighter, 100 times more stable against photobleaching, and one-third as wide in spectral line-width, compared to organic dyes such as rhodamine. Because these conjugates are water soluble and biocompatible, they are suitable for studies using live cells.

Encapsulation of luminescent QD with an ABC triblock copolymer linked to an antibody against prostate-specific membrane antigen (PSMA) may be employed to target prostate cancer cells. This probe may also be delivered to tumor sites by passive targeting via both enhanced permeation and retention. An advantage of this approach is that multicolor fluorescence imaging of as few as 10-100 cancer cells may be achieved under *in vivo* conditions. Thus an imaging modality using QD probes may be adopted for real time visualization of cancer cell metastasis in live animals (Gao etal, Methods Mol Biol, 2007;374:135-46).

In another approach, hyperbranched copolymer ligands such as polyethylene glycol (PEG)-grafted polyethylenimine (PEI-g-PEG) encapsulate and solubilize luminescent QD through direct ligand-exchange reactions. The positive charges and a 'proton sponge effect' associated with multivalent amine groups confer this class of ligand-exchanged QD the ability to penetrate cell membranes and also disrupt endosomal organelles in living cells. The PEG component reduces the cytotoxicity of PEI and improves its overall nanoparticle stability and biocompatibility. This new class of cell-penetrating QD are smaller in size and considerably more stable in acidic environments than QD encapsulated with amphiphilic polymers. PEI-g-PEG QD are rapidly internalized by endocytosis. Initial storage in vesicles is followed by slow endosomal escape and release into the cytoplasm. These QD represent an important advance with respect to design and development of nanoparticle agents for intracellular imaging and therapeutic applications (Duan H and Nie S, J Am Chem Soc, 21 Mar 2007;129(11):3333-8.)

INTEGRATING MOLECUAR TARGETS AND BIOMARKERS

Correlation of Apoptosis, p53 and Mitochondria

Douglas Green, PhD, from St. Jude's Children's Hospital (Memphis, TN) addressed the correlation between apoptosis, mitochondria, and p53. The p53 tumor-suppressor protein prevents tumor development through various mechanisms. A p53-inducible gene, TP53-induced glycolysis and apoptosis regulator (TIGAR) is believed to modulate the apoptotic response to p53, allowing survival under conditions of mild or transient stress that may be reversed or repaired. In *in vitro* studies, expression of TIGAR lowered fructose-2, 6-bisphosphate levels in cells leading to inhibition of glycolysis and an overall decrease in levels of intracellular reactive oxygen species (ROS). TIGAR expression may protect cells from apoptosis or the accumulation of genomic damage associated with ROS (Bensaad K, etal, Cell, 14 July 2006;126(1):107-20).

Mitochondria play a major role in apoptosis. A subset of the Bcl-2 family of proapoptotic proteins induces disruptions in the outer mitochondrial membrane, releasing such death-promoting proteins as cytochrome C, caspase-activating molecules, and caspase-independent death effectors, which cause metabolic failure in the mitochondria. This event is known as mitochondrial outer membrane permeabilization (MOMP), and it governs various pathways leading to cell death. It is believed that changes in the cytosol determine whether or not apoptosis will occur (Chipuk JE, etal, Cell Death Differ, Aug 2006;13(8):1396-402). Therapeutic induction of MOMP may be key to the restoration of apoptosis in tumor cells, while suppression of excessive MOMP may avert pathologic cell death (Green DR and Kroemer G, Science, 30 Jul 2004;305(5684):626-629).

The proapoptotic proteins Bax and Bak, which belong to the Bcl-2 family, are required for MOMP while antiapoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL, Mcl-1, among others, prevent MOMP. Different proapoptotic Bcl-2 homology 3-only (BH3-only) proteins regulate the functions of the antiapoptotic Bcl-2 members and may activate Bax and Bak (Green DR, Cancer Cell, May 2006;9(5):351-65). Mitochondrial lipids, which regulate bioenergetic metabolite flux, and putative components of the permeability transition pore, also regulate MOMP.

Exhibit 7
Epidermal Growth Factor Receptor Variant III (EGFrvIII) in Malignancy

Delta2-7 EGFr • de2-7 EGFr • DEGFr • deltaEGFr

Brain cancer

Although EGFrvIII, has been implicated in progression of GBM, the downstream components involved in tumor progression have not been adequately assessed. Researchers from Massachusetts Institute of Technology (MIT; Cambridge, MA) and Ludwig Institute for Cancer Research (La Jolla, CA) developed a GBM model system based on the U87MG glioblastoma cell line, which was retrovirally transfected to express 3 different levels of EGFrvIII. Global tyrosine phosphorylation events in the 3 cell lines were quantitatively measured by a mass spectrometric strategy previously developed by these researchers. Critical signaling proteins, which are differentially tyrosine phosphorylated as EGFrvIII levels increase, were identified. PI3 kinase and the cellular migration/invasion machinery were among the critical pathways identified. Pathways that are normally activated by wt EGFr (e.g., MAP kinase cascades) but are not responsive to EGFrvIII were also identified. Phosphoproteomic data analysis revealed a cluster of phosphorylation sites that are highly responsive to EGFrvIII expression levels, including tyrosine phosphorylation sites in the activation loop of the c-Met receptor, PLC-g, and pyruvate kinase 3. These findings may represent alternative pathways for the development of therapeutic candidates to overcome the known resistance of EGFrvIII-positive tumors to EGFr kinase inhibitors. Initial target validation was performed with the c-Met receptor. The ligand independence of c-Met activation was demonstrated by analysis of c-Met phosphorylation in the presence of an anti-HGF antibody; and treatment with an EGFrvIII tyrphostin inhibitor showed that c-Met activation is caused by direct crosstalk with the EGFrvIII receptor. Treatment of EGFrvIII expressing cells with a c-Met specific inhibitor resulted in a dose-dependent decrease in cell growth and increased apoptosis. Finally, siRNA-mediated knockdown of the c-Met receptor resulted in decreased cell proliferation. This data suggests provides rationale for use of the c-Met receptor as an alternative target for treatment of EGFrvIII positive tumors. Phosphoproteomic analysis by mass spectrometry is established as a means of drug target discovery (Huang PH, etal, AACR07, Abs. 2529).

The mRNA and protein expression of 9 tumor antigens in human GBM were investigated by researchers at Hopital Pontchaillou (Rennes, France) for possible use in dendritic cell (DC)-based immunotherapy. ALK, EGFrvIII, GALT3, gp100, IL-13Ralpha2, MAGE-A3, NA17-A, TRP-2, and tyrosinase expression were studied in 47 tumor samples from patients with GBM. Results were compared with, as these latter two antigens. Tumor antigens showing a 5-fold increase in mRNA expression over non-neoplastic brain expression or GBM samples with very low levels of expression near the limits of detection for EGFrvIII and MAGE-A3 were considered as positive (EGFrvIII and MAGE-A3 were not detected in non-neoplastic brain). An additional requirement for IHC analysis was demonstration of mRNA overexpression in a s ignificant number of cases. Percentages of positive cases were EGFrvIII (64%), gp100 (38%), IL-13Ralpha2 (32%), and TRP-2 (21%). No overexpression for ALK, GALT3 and tyrosinase was observed 3 of 47 were positive for MAGE-3, and 1 was positive for NA17-A. More than 25% of tumor cells exhibited strong protein expression in gp100 (13%), TRP-2 (34%), EGFrvIII (85%), and IL-13Ralpha2 (96%). Protein expression of at least 3 antigens was observed in 38% of cases. EGFrvIII, IL-13Ralpha2, and, to a lesser degree, gp100 and TRP-2, are important factors for development of immunotherapy strategies against GBM (Saikali S, etal, J Neurooncol, 2007;81(2):139-48).

Preliminary surveys have indicated that oligodendroglial tumors produce EGFr and its variant, EGFrvIII; however, in an examination of 50 oligodendroglial tumors, including 25 well differentiated oligodendrogliomas (WDO) and 12 GBM exhibiting high proportions of oligodendroglia-like cells, the production of wt EGFr, but not EGFrvIII, was observed, with antigen production increasing with tumor grade (McLendon RE, etal, J Histochem Cytochem, Aug 2000;48(8):1103-10).

In an assessment of the qualitative distribution and quantitative expression at both the population and cellular levels of EGFrvIII in 21 biopsy samples of cases of human glioma by indirect analytical and quantitative flow cytometry and by IHC assay of frozen and formalin-fixed tissue, 50% of glioma cases tested (1 of 2 anaplastic astrocytomas, 7 of 12 GBM, and 2 of 6 oligodendrogliomas) expressed EGFrvIII, as determined by a minimum of 2 separate assays. Minimum estimates of the proportion of positive tumor cells in these populations ranged from 37-86%; in 4/5 cases in which EGFrvIII density/cell was quantified, values of 2.7-6.8 x 10^5 were obtained with EGFrvIII-specific MAb L8A4, representing levels consistent with successful *in vivo* immunotargeting. Confocal microscopic analysis confirmed that the subcellular

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localization of EGFrvIII was identical to that described for EGFr, i.e., predominant cell membrane expression, with some perinuclear distribution suggestive of localization to the Golgi region. Neither EGFr nor EGFrvIII was found within the nucleus (Wikstrand CJ, etal, Cancer Res, 15 Sep 1997;57(18):4130-40).

Breast cancer

A research group from University of Michigan Medical Center (Ann Arbor, MI) investigated the expression of EGFrvIII and wt EGFr in cell lines and primary breast cancer. Except for EGFrvIII transfected cells, only wt EGFr was detected. No samples were positive for EGFrvIII expression except for control transfectants and GBM. Wt EGFr, in contrast, was expressed at various levels in most samples tested. The researchers concluded that EGFrvIII expression is extremely rare in breast cancer and does not contribute to the malignant phenotype (Rae JM, etal, Breast Cancer Res Treat, 2004;87(1):87-95).

To delineate the biologic significance of EGFrvIII in human breast cancer, the MCF-7 human breast cancer cell line was transduced with the cDNA for EGFrvIII. Expression of EGFrvIII in MCF-7 cells produced a constitutively activated EGFrvIII receptor, and also elevated ErbB-2 phosphorylation, presumably through heterodimerization and crosstalk. The MCF-7/EGFrvIII transfectants exhibited an approximately 3-fold increase in colony formation in 1% serum with no significant effect observed at higher percentages of serum; a similar result was seen in anchorage-dependent assays. EGFrvIII expression also significantly enhanced the tumorigenicity of MCF-7 cells in athymic nude mice. Collectively, these results suggest that EGFrvIII could play a pivotal role in human breast cancer progression (Tang CK, etal, Cancer Res, 1 Jun 2000;60(11):3081-7).

Prostate cancer

Various studies have demonstrated an unexplained depletion of EGFr protein expression in prostate cancer. Recently, this phenomenon was attributed to the presence of the EGFrvIII variant that is highly expressed in malignant prostatic neoplasms. In a retrospective study, normal, benign hyperplastic, and malignant prostatic tissues were examined at the mRNA and protein levels for the presence of this mutant receptor. Results demonstrated that while EGFrvIII was not present in normal prostatic glands, the level of expression of this variant protein increased progressively with the gradual transformation of the tissues to the malignant phenotype. The selective association of high EGFrvIII levels with the cancer phenotype underlines the role that this mutant receptor may maintain in the initiation and progression of malignant prostatic growth (Olapade-Olaopa EO, etal, Br J Cancer, Jan 2000;82(1):186-94).

Lung cancer

A group from Nagoya City University Medical School (Nagoya, Japan) genotyped the EGFrvIII mutation status of 252 surgically treated lung cancer cases. Presence or absence of EGFrvIII mutation, EGFr copy number, and EGFr were assessed. EGFrvIII mutation was detected in 8 of 252 patients with lung cancer, all of whom were male and smokers. A total of 7 of the 8 had squamous cell carcinoma, which is a significant correlation with pathologic subtype (squamous cell carcinoma versus adenocarcinoma). Among EGFr mutations detected at the kinase domain, 60 existed exclusively with EGFrvIII mutations. The number of EGFr gene copies was significantly higher in EGFrvIII mutants (4.7) than in non-EGFrvIII mutants (2.3). These findings suggest that EGFrvIII gene mutation may be a mechanism of increased EGFR copy number and play a role in development of treatments for lung cancer (Sasaki H, etal, Oncol Rep, 2007;17(2):319-23).

Researchers at Dana-Farber Cancer Institute (Boston, MA) detected EGFrvIII mutations in 5% (3/56) of human lung squamous cell carcinoma (SCC) examined and none (0/123) in human lung adenocarcinoma. Analysis of the role of EGFrvIII mutation in lung tumorigenesis and its response to tyrosine kinase inhibition indicate that tissue-specific expression of EGFrvIII in murine lung led to the development of nsclc. The most important finding was that of the dependence of lung tumors on EGFrvIII expression for maintenance. Treatment of EGFrvIII-driven murine tumors with the irreversible EGFr inhibitor, HKI-272, dramatically reduced the size of these in 1 week. Ba/F3 cells transformed with the EGFrvIII mutant were relatively resistant to gefitinib and erlotinib, but were sensitive to HKI-272 treatment *in vitro*. These findings suggest a therapeutic strategy for tumors with EGFrvIII mutation (Ji H, etal, Proc Natl Acad Sci U S A. 2006;103(20):7817-22).

Ovarian cancer

Few reports in the literature address a role of EGFrvIII expression in ovarian cancer.

According to researchers from University of New Mexico Health Sciences Center (Albuquerque, NM), EGFr overexpression occurs frequently in ovarian cancer and is associated with poor prognosis; and EGFrvIII has been detected at a high frequency in human ovarian tumors. In an epithelial ovarian cancer

— continued on next page

cell line (OVCA 433) transfected with EGFrvIII, cells displayed a dissociated, motile phenotype and fibroblastic morphology. Adherens and desmosomal junctions were disrupted in cells expressing EGFrvIII; levels of cellular plakoglobin and beta-catenin were decreased; and E-cadherin protein and mRNA were nearly absent. E-cadherin loss was associated with decreased expression of other ovarian epithelial markers, including keratins 7, 8, and 18 and mucins 1 and 4. EGFrvIII expressing cells, in contrast, demonstrated elevations in the mesenchymal markers, N-cadherin and vimentin. Transition to a mesenchymal phenotype is characterized by a switch from E-cadherin to N-cadherin, coupled with increased expression of vimentin and loss of the epithelial keratins and mucins. This transition to characteristics of well differentiated epithelial ovarian carcinoma was an outcome of EGFrvIII expression. These findings suggest that EGFrvIII expression may regulate phenotypic plasticity in ovarian cancer and thereby contribute to more aggressive disease (Zeineldin R, etal, Mol Carcinog, 2006;45(11):851-60).

Head and neck cancer

Researchers from University of Pittsburgh, (Pittsburgh, PA) studied incidence of EGFrvIII expression in SCCHN and biologic consequences of EGFrvIII on tumor growth in response to EGFr targeting. A total of 33 SCCHN tumors were evaluated for EGFrvIII expression. EGFrvIII expression was detected in 42% of SCCHN tumors and, in all cases, in conjunction with wt EGFr. A SCCHN cell line was transfected with an EGFrvIII expression construct. *In vitro* and *in vivo* growth rates, chemotherapy-induced apoptosis, and consequences of EGFr inhibition with cetuximab were assessed in EGFrvIII-expressing cells and vector-transfected controls. Compared to controls, proliferation *in vitro* and tumor volumes *in vivo* were increased in SCCHN cells expressing EGFrvIII. Furthermore, EGFrvIII-transfected SCCHN cells demonstrated decreased apoptosis after treatment with cisplatin and decreased growth inhibition after treatment with C225, compared with vector-transfected control cells. The researchers concluded that EGFrvIII is expressed in SCCHN and contributes to increased growth and resistance to treatments that target wt EGFr. Efficacy of EGFr targeting strategies may be increased by strategies that block EGFrvIII (Sok JC, etal, Clin Cancer Res 2006;12(17):5064-73).

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

Mitochondria that have released cytochrome c are removed from the cell by autophagy, a mechanism during which a portion of the cytoplasm is delivered to lysosomes to be degraded. Upon induction of autophagy, microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is conjugated to phosphatidylethanolamine and targeted to autophagic membranes. As a result, LC3 has been used as a marker of autophagy. However, LC3 protein may also aggregate independently of autophagy when it is transiently overexpressed by transfection. It is also formed in autophagy-deficient hepatocytes, neurons, or senescent fibroblasts. Because of these other mechanisms of LC3 accumulation, punctate dots containing LC3 do not always represent autophagic structures, and LC3 localization data must be monitored and interpreted with caution (Kuma A, etal, Autophagy, 12 Jul 2007;3(4); epub ahead of print).

TARGETING THE TUMOR MICROENVIRONMENT

Vaccines

A novel DNA vaccine targeting fibroblast activation protein (FAP) was described by Ralph Reisfeld, PhD, of the Scripps Research Institute (La Jolla, CA), as an anticancer approach exploiting the properties of tumor stroma. For instance, tumor-associated fibroblast cells within the stromal compartment, which are the primary source of collagen type I, contribute to decreased chemotherapeutic drug uptake in tumors. This is an important determinant for chemotherapeutic outcome. Because tumor-associated fibroblasts are also genetically more stable compared to tumor cells, they are better targets for cancer immunotherapy. In general, targeting tumor-associated fibroblasts enhances intratumoral drug uptake.

Dr. Reisfeld's group constructed a novel, oral DNA vaccine targeting fibroblast activation protein (FAP), which is specifically overexpressed by fibroblasts in the tumor stroma. This vaccine was tested in murine models xenografted with multidrug-resistant (MDR) murine colon and breast carcinomas. Successful suppression of primary tumor cell growth and metastasis were observed and associated with CD8+ T cell-mediated killing of tumor-associated fibroblasts. In addition, decreased collagen type I expression and up to 70% greater uptake of chemotherapeutic drugs were detected in tumor tissue of FAP-vaccinated mice. Overall, a 3-fold prolongation in life span and significant suppression of tumor growth was noted in the vaccinated mice. Also, approximately 50% of the vaccinated animals completely rejected subsequent challenge with tumor cells (Loeffler M, etal, J Clin Invest, Jul 2006;116(7):1955-62).

Legumain-based vaccines have also been developed by Dr. Reisfeld's group against tumor-associated macrophages (TAM) that play an important role in tumor

progression and metastasis. Legumain, a member of the asparaginyl endopeptidase family functioning as a stress protein, is overexpressed by TAM and provides an ideal target molecule for this purpose. Functionally, legumain is a lysosomal cysteine protease involved in antigen presentation within class II MHC positive cells and pro-protein processing.

A legumain-based DNA vaccine induced a robust CD8+ T cell response against TAM, dramatically reducing their density in tumor tissues. In addition, a remarkable decrease was seen in the levels of proangiogenic factors released by TAM, such as transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)-9, and VEGF, resulting in suppression of tumor angiogenesis, growth, and metastasis.

In murine models of metastatic breast, colon, and non-small cell lung cancer, 75% of vaccinated mice survived lethal tumor cell challenges, and 62% were completely free of metastases. Thus, decreasing the number of TAM in the tumor stroma suppressed tumor growth and metastasis via inhibition of angiogenesis. This DNA-based vaccine against TAM could serve as a foundation for development of new and improved anticancer strategies (Luo Y, et al, J Clin Invest, Aug 2006;116(8):2132-2141).

Prodrugs

Dr. Reisfeld's team also developed LEG-3, a novel legumain-activated, cell-impermeable doxorubicin prodrug designed for activation exclusively within the tumor microenvironment. Because legumain is highly expressed by neoplastic, stromal, and endothelial cells in solid tumors, it is the molecule of choice for this approach. In addition, legumain is present extracellularly in the tumor microenvironment, associated with matrix and cell surfaces, and functions locally in the reduced pH of the tumor microenvironment, making it an excellent candidate for the development of a prodrug. The cell-impermeable prodrug activation approach targeting the tumor microenvironment is believed to significantly reduce drug toxicity to normal tissues. There is a profound increase in the level of doxorubicin in nuclei of cells in tumors, but little in other tissues. Moreover, the activated prodrug does not selectively delete target-producing tumor cells, which minimizes emergence of drug-resistant tumor populations. Doxorubicin prodrug LEG-3 arrested the growth of a variety of tumors and prolonged survival, without such adverse effects as myelosuppression or cardiac toxicity. The doxorubicin prodrug LEG-3 approach also holds promise for proteases other than legumain; and compounds other than doxorubicin may be employed for the development of additional targeted cancer therapeutics (Wu W, et al, Cancer Res, 15 Jan 2006;66(2):970-80).

Peritumoral Acinar Tissue Imaging

Juri Gelovani, MD, PhD, from the University of Texas, M. D. Anderson Cancer Center (Houston, TX) described peritumoral acinar tissue imaging, which may provide an early marker of pancreatic cancer, a disease that poses significant challenges with respect to diagnosis and therapy. Challenges include lack of symptoms early in the disease, 5-year survival rate of less than 5%,lack of novel biomarkers for screening, and lack of diagnostic imaging methods sensitive enough for detection of early stage disease.

Inflammation, fibrosis, acinar cell loss, and small duct-like metaplasia of acinar cells are present in the non-neoplastic pancreatic parenchyma adjacent to infiltrating pancreatic ductal adenocarcinoma (PDAC). Similar morphologic changes are also observed in the setting of chronic pancreatitis. In addition, peritumoral acini have alterations in gene expression even in the absence of morphologic changes. Fresh-frozen pancreatic acinar tissue samples were microdissected from nine patients, three each with pancreatic cancer, chronic pancreatitis, or normal pancreata, using laser capture microdissection. RNA, extracted from each microdissection, was subjected to two rounds of linear amplification and hybridized to oligonucleotide microarrays. Quantitative RT-PCR and/or immunohistochemistry (IHC) were used to investigate gene expression patterns of pancreatic acinar tissue adjacent to infiltrating PDAC versus gene expression patterns of acinar tissue affected by chronic pancreatitis or those of normal pancreatic acini. Among 20 genes that were overexpressed in peritumoral acinar tissue compared to normal acinar tissue and acini affected by chronic pancreatitis, were the genes that encode pancreatitis-associated protein (HIP/PAP), and cartilage glycoprotein-39 (HC gp-39 or TKL-40). HIP/PAP is a gene overexpressed in acini adjacent to infiltrating pancreatic cancer. In patients with pancreatic cancer and those with chronic pancreatitis, serum HC gp-39 protein levels were significantly higher than in controls without pancreatic disease, while there was no significant difference in the levels of serum HC gp-39 in patients with pancreatic cancer and those with chronic pancreatitis (Fukushima N and Koopmann J, Mod Pathol, Jun 2005;18(6):779-87).

IMAGING OF TUMORS

Proteomic Mapping and Imaging

Jan Schnitzer, MD, of the Sidney Kimmel Cancer Center and his group are differentially screening phage libraries to select organ-targeting antibodies. The source of these molecules is luminal endothelial cell plasma membranes isolated directly from tissue and highly enriched in natively expressed proteins exposed to the bloodstream. The rationale behind the use of endothelia is that the luminal endothelial cell surface exhibits molecular diversity arising from local variations in genetic expression and tissue microenvironment. Phage-displayed antibodies were used to generate fusion proteins to prevent liver uptake of IV-injected phage and assist in rapid targeting of selected organs *in vivo*. Phage display may be used for mapping cognate antigens on vascular endothelia natively in tissue and for vascular targeting of specific tissues *in vivo* for the

development of molecular therapeutics (Valadon P, et al, PNAS USA,10 Jan 2006;103(2):407-12).

The presence of two key endothelial cell surface proteins, aminopeptidase-P and annexin A1, in lungs and solid tumors, respectively, was confirmed by the use of subcellular fractionation, subtractive proteomics, bioinformatics, expression profiling, and gamma-scintigraphic imaging with antibodies. Because radioimmunotherapy to annexin A1 destroys tumors, this molecule can serve as a useful marker for testing the efficacy of tissue-specific treatment of cancer (Oh P, etal, Nature, 10 Jun 2004; 429(6992); 629-35). Quantitative proteomics was used to show that aminopeptidase P is concentrated in caveolae of lung endothelium. Caveolae are discrete microdomains on the cell surface that bud from plasma membranes by a fission process, which requires cytosol and is driven by GTP hydrolysis (Schnitzer JE, etal, Science, 1996;274:239-242).

Using electron microscopy it was shown that an antibody against aminopeptidase P targets nanoparticles to caveolae. Via a process mediated by caveolin-1, the antibody is actively transported from the blood across the endothelium into lung tissue within seconds, even against a concentration gradient. Thus, the targeted caveolae operate effectively as pumps. Whole body gamma-scintigraphic imaging shows rapid, specific delivery into lung, which is significantly greater than that achieved by standard vascular targeting. This approach, which combines organellar proteomics with multiple imaging techniques, may be applied for identification of target molecules, which could be harnessed for developing delivery systems for imaging agents, drugs, gene-therapy vectors, and nanomedicine applications (Oh P, etal, Nat Biotechnol, Mar 2007;25(3):327-37).

Imaging of Signal Transduction Pathways

Juri Gelovani, MD, PhD, from the University of Texas, M. D. Anderson Cancer Center, described novel imaging methods and their usefulness in tracking signal transduction pathways, including (11C)-acetate, a tracer for detection of prostate cancer, which is used in combination with positron emission tomography (PET). Patients with prostate cancer were administered (11)C-acetate, with or without (18)F-FDG PET. Uptake of (11)C-acetate resulted in entry of the compound into catabolic or anabolic metabolic pathways mediated by acetyl-coenzyme A, demonstrating that (11)C-acetate can be used as a probe of tissue metabolism. Standardized uptake values (SUV) for each tumor were investigated for tracer activity at 10-20 minutes after (11)C-acetate and 40-60 minutes after (18)F-FDG administration. Significantly higher uptake of (11C)-acetate was seen, compared to that of (18)F-FDG, in patients with adenocarcinoma of the prostate. In addition, accumulation of (11)C-acetate was detected in all of the patients with primary prostate tumors and those with tumor metastases to lymph nodes or bone. Thus, (11)C-acetate is taken up significantly in prostate cancer, more sensitive in detection of prostate cancer than (18)F-FDG, and may also measure radiopharmaceutical uptake pathways that are different from those measured by (18)F-FDG, aiding in tumor identification (Oyama N, etal, J Nucl Med, Feb 2002;43(2):181-6).

Another approach to noninvasive imaging is based on poly(L-glutamic acid) (L-PG), a biodegradable drug carrier. In order to study the kinetics of its degradation *in vivo*, L-PG was coupled with a near-infrared fluorescence (NIRF) dye, NIR813. L-PG-NIR813 was injected IV in orthotopic human U87/TGL glioma in nude mice, and levels were was assessed using NIRF optical imaging. Upon exposure to cathepsin B, the fluorescence intensity of L-PG-NIR813 increased 10-fold. In addition, L-PG-NIR813 was degraded by another cysteine protease, cathepsin L, but not by MMP-2, cathepsin E, cathepsin D, or plasmin. Fluorescence activation was blocked in the presence of an inhibitor of cathepsin B. Degradation of L-PG-NIR813 was visualized primarily in the liver, where it peaked at 4 hours post-injection. The activation of L-PG-NIR813 within tumors was isomer-specific; D-PG-NIR813 was not activated. Thus, L-PG-NIR813 may be used to monitor the *in vivo* degradation of L-PG-based polymeric drugs, and this agent may prove useful in noninvasive imaging of protease activity (Melancon MP, et al, Pharm Res, 22 Mar 2007; epub ahead of print).

Dr. Gelovani and his group are also currently investigating magnetic resonance imaging of radiation- or chemotherapy-induced necrosis, using gadolinium-chelated polyglutamic acids. The selective accumulation of the paramagnetic magnetic resonance (MR) contrast agents, gadolinium p-aminobenzyl-diethylenetriaminepentaacetic acid-poly(glutamic acid) (L-PG-DTPA-Gd and D-PG-DTPA-Gd), was evaluated in necrotic tissue. The solid tumor models used were human Colo-205 xenograft and syngeneic murine OCA-1 ovarian tumors. Poly(L-glutamic acid)-paclitaxel conjugate (PG-TXL) was used to induce necrotic response. The property of high molecular weight, rather than *in vivo* biodegradation, of the MR agents is necessary for their specific localization to necrotic tissue. Because necrosis is common in tumors and surrounding normal tissues after radiation therapy or chemotherapy, accurate measurement of necrosis may serve as an indicator of treatment efficacy or toxic adverse effects. These gadolinium compounds may serve as noninvasive tools to achieve this purpose (Jackson EF, etal, Int J Radiat Oncol Biol Phys, 20 Mar 2007; epub ahead of print).

Dr. Gelovani's group has also developed another methodology, for serial monitoring (every 24 hours) of *in vivo* early bone marrow (BM) cell engraftment/expansion. A combined bioluminescence (BLI) and positron emission tomography (PET) imaging of a reporter gene into mouse bone marrow cells was used in a standard mouse model of bone BM transplantation. Significant cell engraftment/expansion was noted by greatly increased bioluminescence about 1 week post-transplant. In addition, PET and computed tomography (CT), employed, respectively, for

Exhibit 8
Selected EGFr Family Small Molecule Tyrosine Kinase Inhibitors (TKI)

Compound	Selectivity/Reversibility	Status
Gefitinib (Iressa)	ErbB1/reversible	Approved (nsclc)
Erlotinib (Tarceva)	ErbB1/reversible	Approved (colorectal cancer)
Lapatinib (Tykerb)	ErbB1and ErbB2/reversible	Approved (breast cancer)
CI-1033 (canertinib)	Pan-erbB/irreversible	Phase I /II (solid tumors)
HKI-272	ErbB1 and ErbB2/irreversible	Phase II (breast cancer, nsclc)
BIBW 2992	ErbB1 and ErbB2/irreversible	Phase II (prostate cancer)
BMS-690514	Pan-ErbB (Erb-1, -2. -4) and VEGFr2 inhibitor/reversible	Phase I (solid tumors)

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

whole body imaging and for localization of cells in the skeleton, revealed numerous BM cell engraftment sites (Mayer-Kuckuk P, etal, Cell Transplant 2006;15(1):75-82).

Imaging of Tumors/Microenvironment

Bonnie Sloane, PhD, from Wayne State University (Detroit, MI) shared her findings on protease expression in orthotopic lung cancer models and normal lung.

A customized Affymetrix (Santa Clara, CA) protease microarray (Hu/Mu ProtIn chip) was used to distinguish human and mouse genes in order to analyze the expression of proteases and protease inhibitors in lung cancer. According to the results, murine MMP-12, MMP-13, and cathepsin K, were upregulated in tumor tissue, compared to normal mouse lung. Comparable results with respect to these enzymes were obtained with human lung adenocarcinoma specimens. These data were validated by IHC, which detected MMP-12 expression in the stroma of human lung tumor samples. In animal studies, murine Lewis lung carcinoma cells were injected into the tail vein of syngeneic wt and MMP-12-null mice. Although both groups of mice developed the same number of lung tumors, there was a 2-fold increase in the number of tumors that reached >2 mm in diameter in MMP-12-null mice, compared with wt controls. Increase in tumor size was accompanied by an increase in blood vessels in the tumors. MMP-12 appears to protect against tumor growth (Acuff HB, etal, Cancer Res, 15 Aug 2006;66(16):7968-75).

Caveolin-1 has been shown to affect the expression and localization of cathepsin B, pro-urokinase plasminogen activator (pro-uPA), and their receptors. This regulation is believed to be important for cell-surface proteolytic events that contribute to invasiveness of colon cancer cells (Cavallo-Medved DJ, etal, Cell Sci, 1 Apr 2005;118(Part 7):1493-503). On the other hand, cystatin M is a potent endogenous inhibitor of lysosomal cysteine proteases, which significantly suppresses *in vitro* cell proliferation, migration, and Matrigel invasion of human breast carcinoma MDA-MB-435S cells. Whole animal studies, using SCID mice implanted with breast cancer cells expressing cystatin M, show delayed tumor growth and decreased number of metastases in lungs and liver (Zhang J, etal, Cancer Res, 1 Oct 2004;64(19):6957-64, and Shridhar R, etal, Oncogene, 18 Mar 2004;23(12):2206-15).

The roles of MMP-9, MMP-7, and MMP-2 were analyzed in experimental metastasis assays in wt mice and mice in which the genes for the 3 types of metalloproteinases were knocked out. An 81% reduction in the number of Lewis lung carcinoma tumors was noted in MMP-9 null mice, compared to a 42% increase in the number of tumors in MMP-7 null mice. There was no difference in the number of tumors in MMP-2 null mice, compared to wt controls. Likewise, in an orthotopic model of lung cancer, lung tumors were established in 50% fewer MMP-9 null mice, compared to control mice, although the size of the tumors was not different. Bioluminescence imaging of luciferase-expressing human lung cancer-derived A549 cells detected fewer tumor cells in the lungs of MMP-9 null mice 19 hours post-injection, compared to controls; however no difference in subsequent growth rates was observed. The number of tumor cells undergoing apoptosis was also much higher in MMP-9 null mice, compared to controls. Overall, MMP-9 appears to be important in survival of tumors at an early stage, but have no effect on subsequent growth (Acuff HB, etal, Cancer Res, 1 Jan 2006;66(1):259-66).

Monitoring of Cyclooxygenase-2 (Cox-2) to Assess Tumor Progression

Cyclooxygenase is a key enzyme in prostanoid biosynthesis. Mammalian species have two cyclooxygenases, constitutively expressed cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2), which is induced in response to

many distinct stimuli. The nonsteroidal anti-inflammatory drugs (NSAID), one of the largest classes of pharmaceutical agents, exert most of their biologic effects by inhibiting cyclooxygenase production of prostaglandins.

High levels of Cox-2 expression are detectable in skin cancer induced by 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA). Cox-2 null mice, however, are totally resistant to DMBA/TPA-induced carcinogenesis. These findings suggest that Cox-2 levels are good predictors of tumor progression.

Harvey Herschman, PhD, from Jonsson Comprehensive Cancer Center, at the University of California, Los Angeles (UCLA), described a number of Cox-2-based approaches for tumor imaging and anticancer strategies.

Dr. Herschman's group constructed sCAR-EGF, a recombinant, bispecific molecule containing the soluble portion of coxsackie and adenovirus receptor (sCAR) fused to EGF. The sCAR moiety binds to the virus and blocks CAR-dependent adenovirus infection, whereas the EGF moiety binds to cellular EGFr. Thus, systemic injection of sCAR-EGF-containing adenovirus, referred to as 'transductional retargeting', reduces expression of the reporter gene in the normal liver but facilitates expression of the reporter gene in tumor xenografts expressing high levels of EGFr (Liang Q, etal, Mol Imaging Biol, Nov-Dec 2004;6(6):395-404).

In order to monitor and quantify Cox-2 expression noninvasively *in vivo*, Dr. Herschman's group generated a 'knock-in' mouse in which the firefly luciferase reporter enzyme is expressed at the start site of translation of the endogenous Cox-2 gene, so that monitoring of luciferase activity is a measure of Cox-2 activity. The correlation of luciferase and Cox-2 expression was confirmed using heterozygous Cox-2luc/+ mouse embryonic fibroblasts isolated from the knock-in mouse.

Ex vivo imaging demonstrated simultaneous induction of Cox-2 and luciferase in multiple organs. Cox-2luc/+ knock-in mice are a valuable tool for the analysis of Cox-2 expression (Ishikawa TO, etal, Mol Imaging Biol, May-Jun 2006;8(3):171-87).

Dr. Herschman's group also constructed a Cox-2 conditional overexpression transgenic mouse (Cox-2 COE). This transgene contains a very strong and ubiquitous CAG promoter, which drives Cox-2 and humanized Renilla luciferase (hRL)-coding regions, linked by an internal ribosomal entry site. Expression of Cox-2/hRL expression may be monitored multiple times noninvasively *in vivo* by imaging hRL activity. Hepatic Cox-2 expression and hRL signal were elevated when a nonreplicating adenovirus carrying Cre recombinase (Ad-CMV-Cre) was injected IV. In addition, in *in vitro* assays, Cox-2 COE embryonic fibroblasts expressed both Cox-2 and hRL following Ad-CMV-Cre infection. This system may be used to evaluate Cox-2 overexpression in pathologic conditions, including cancer (Kamei K, etal, Genesis, Apr 2006;44(4):177-82).

Conditional knockout mice in which critical exons of the Cox-2 gene are flanked with loxP sites [Cox-2(flox/flox)] have also been generated to investigate the cell type-specific role of Cox-2 in many disease models (Ishikawa TO and Herschman HR, Mar 2006;44(3):143-9).

VECTORS

Pancreatic Cancer-specific Vector

Mien-Chie Hung, PhD, from the University of Texas, M. D. Anderson Cancer Center described new targets and delivery systems in cancer diagnosis and treatment, with emphasis on the development of a pancreatic cancer-specific vector. Dr. Hung and his team have developed the VISA expression vector (Vp16-gal4-wpre Integrated Systemic Amplifier), which confers prolonged luciferase expression, compared to that of a CMV-luciferase construct. C-VISA-BikDD selectively kills pancreatic cancer cells, but not normal cells or other types of tumor cells.

In order to be specific and effective, pancreatic gene therapy must employ a pancreatic cancer-specific promoter. Promoter of cholecystokinin type A receptor (CCKAR) is such a promoter and drives gene expression in a more tissue-specific manner than the CMV promoter.

When Bcl-2 interacting killer (Bik), a pro-apoptotic protein mutant, was coupled to the CCKAR promoter, suppression of pancreatic cancer progression was observed in a nude mouse xenograft model (Li Z, etal, Cancer Lett, 8 May 2006;236(1):58-63).

Breast Cancer-specific Vector

Similarly, Dr. Hung's group demonstrated that a topoisomerase IIα promoter containing an inverted CCAAT box (ICB) is selectively activated in breast cancer cells. This promoter, together with an enhancer sequence from the CMV immediate early gene promoter, is known as CT90. The composite promoter shows similar or higher activity than that of the CMV promoter in breast cancer cells, but is much less active in normal cell lines and normal organs than the CMV promoter. This targeted CT90-driven construct expressing BikDD, selectively kills breast cancer cells *in vitro* and suppresses mammary tumor development in an animal models, but is not detectable in the normal organs of animals that were treated with CT90- BikDD (Day CP, et al, Cancer Gene Ther, Jul 2006;13(7):706-19).

Endostatin Prodrug

Dr. Huang's team also generated a construct containing endostatin in combination with a prodrug-converting enzyme. Endostatin is an angiogenesis inhibitor that selectively targets neovascular endothelial cells and suppresses tumor growth. In this construct endostatin was fused with cytosine deaminase, which converts the prodrug, 5-flucytosine, into a cytotoxic 5-fluorouracil. In animal studies, endostatin-cytosine deaminase fusion protein treatment provided greater tumor growth suppression and increased mean survival time in mice, compared to endostatin alone, cytosine deaminase alone, or endostatin plus

cytosine deaminase in combination. The endostatin-cytosine deaminase protein significantly inhibited the growth of endothelial cells and also preferentially induced tumor cell apoptosis. This approach may be used to develop other targeted therapies for cancer (Ou-Yang F, etal, Cancer Res, 1 Jan 2006;66(1);378-84).

Viral-based Therapy for Multiple Myeloma

Stephen Russell MD, PhD, from Mayo Clinic (Rochester, MN) described his group's work on a viral-based therapy for multiple myeloma based on oncolytic measles virus (MV). Other viral vectors for the treatment of multiple myeloma, under investigation by Dr. Russell's group, include vesicular stomatitis virus and Coxsackieviruses.

MV is known to target CD46 molecules, which are much more abundant on the surface of multiple myeloma cells, compared to hematopoietic cells of various lineages. However, MV induces IFN synthesis in human myeloma and ovarian cancer cells, and generation of viral progeny is inhibited by IFN. Dr. Russell and his group have generated a chimeric virus that includes a gene responsible for antagonizing IFN induction and response (Haralambieva I, etal, Mol Ther, Mar 2007;15(3):588-97, and Ong HT, etal, Exp Hematol, Jun 2006;34(6);713-20). In addition, because neutralizing antiviral antibodies may interfere with systemic virotherapy, activated T cells are employed as carriers to deliver oncolytic measles viruses to multiple myeloma xenografts in the presence of such antibodies (Ong HT, etal, Gene Ther, Jun 2007;14(4):324-33).

Targeted and Shielded Adenovirus Vectors

Imre Kovesdi, PhD, the CEO of VectorLogics (VLI; Birmingham, AL) discussed targeted and shielded adenovirus vectors. VLI has developed technology to modify the outer coat proteins of adenovirus vectors to increase transduction of tumor cells. VLI's lead product, AdDelta24-RGD, is a transduction-enhanced, conditionally replicative adenovector (CRAd) that can effectively destroy solid tumors. CRAd overcomes the lack of Coxsackievirus and adenovirus receptors (CAR) on cancer cells to deliver its payload much more effectively than the unmodified first generation adenovectors used in previous clinical trials. VLI's transduction enhanced vectors may be used to treat such malignancies as ovarian, pancreatic, colorectal, and prostate cancer, which have not been effectively treatable with first generation adenovectors. First generation adenovectors proved safe for use in humans, but are neither tissue nor tumor-specific. Although CRAd demonstrates enhanced infectivity, it is sensitive to the host's immune response. This vulnerability is overcome by genetic modification of virion capsid protein (pIX), which hields it from neutralizing antibodies. Preliminary data using an Ad vector expressing HSV-TK, fused to the pIX protein, indicates that a shield against neutralizing antibodies is achievable. It was shown that the incorporation of HSV-TK in the virion capsids functionally converts gancyclovir. The utility of various proteins as shielding molecules is currently under investigation. AdDelta24S-RGD, an infectivity enhanced and shielded Ad vector, provides the next step in the development of clinically and commercially feasible CRAd that can be dosed multiple times for maximum effectiveness (Hedley SJ, etal, Cancer Immunol Immunother, Nov 2006;55(11):1412-9). A luciferase gene linked to pIX-HSV-TK complex has been used for *in vivo* imaging of tumors in whole animals, providing a noninvasive imaging approach.

Whole Cell Vaccines for Acute Myeloid Leukemia (AML)

Farzin Farzaneh, PhD, from the Rayne Institute, King's College (London, UK) described two major obstacles to immunotherapy of cancer, editing of the properties of tumor by the immune system and editing of the properties of the immune system by the tumor. In short, tumor and immune system influence and alter each other. The tumor may already be resistant to standard treatment by the time a patient reaches the clinic. This may explain why the most potent antigens do not provide the best vaccination targets and residual tumor burden, corresponding to treatment-resistant tumor cells, may persist despite therapy.

Acute myeloid leukemia (AML) is an aggressive and difficult to treat disease. Although 50% of patients with AML and suitable donors who meet hematopoietic stem cell-transplant (HSCT) fitness requirements survive for 5 years, older patients and those without suitable donors have a much worse prognosis. Patients in the latter category are likely to benefit most from immunotherapy (Cheuk AT, etal, Cancer Immunol Immunother, Jan 2006;55(1):68-75). The graft versus leukemia (GvL) effect observed following HSCT demonstrates the potential of the immune system to target and eradicate AML cells.

AML blasts express both human leukocyte antigen (HLA) class-I and class-II molecules. They express AML-associated antigens, such as GP250, WTI, and PRAME, among others. Autologous tumor cell vaccines in clinical trials for patients with AML face the challenges of poor gene transfer and inefficient expression of multiple transgenes encoded by single vectors. Dr. Farzaneh described the development of a self-inactivating lentiviral vector encoding B7.1 and IL-2 as a single fusion protein, which is postsynthetically cleaved to generate biologically active membrane-anchored B7.1 and secreted IL-2. Using this construct, efficient transduction of both established and primary AML blasts was achieved, resulting in expression of the transgenes in the majority of the cells following a single round of infection. Because syngeneic tumor cells genetically modified to express B7.1 (CD80) induce rejection of previously established murine solid tumors, and transduction with IL-2 can further increase survival, this approach holds promise as a strategy for generation of whole cell vaccines for AML (Chan L, etal, Mol Ther, Jan 2005;11(1):120-31).

Exhibit 9
Selected ErbB Receptor-Based Immunotherapies/Vaccine Approaches

Therapeutic ❑ Company	Approach	Status ❑ Cancer Indication
Lovaxin B ❑ Advaxis	Recombinant, live, attenuated Listeria monocytogenes vector linked to protein LLO-PEST and expressing antigen HEr2	Preclinical ❑ breast cancer
AE37 ❑ Antigen Express	Invariant chain (Ii)-key/HEr2 antigenic epitope hybrid peptide vaccine; stimulates T-helper cells	Phase I ❑ breast and prostate cancer
PX 103.2, HER-2 AutoVac MVA-BN ❑ Bavarian Nordic	MVA-BN vector combined with HEr2 DNA AutoVac vaccine	IND approved ❑ breast and prostate cancer
CDX-110 ❑ Celldex Therapeutics	Peptide vaccine targeting EGFrvIII	Phase II ❑ glioblastoma multiforme)
Lapuleucel-T (Neuvenge; APC8024) ❑ Dendreon	Autologous antigen-presenting cells loaded with BA7072, an antigen construct consisting of recombinant sequences from extracellular and intracellular domains of HEr2 (HER500) fused to human GM-CSF	Phase I ❑ solid tumors
JX-963 ❑ Jennerex Biotherapeutics	Vaccinia virus-based; 2 viral genes deleted to restrict replication to cancer cells with large nucleotide pools and cells with activated EGFr-Ras pathway; plus a gene for GM-CSF	Preclinical ❑ solid tumors
MDX-214 ❑ Medarex	Fusion protein consisting of CD89, a trigger molecule found on immune effector cells, ligated to EGF; directs CD89-positive effector cells to tumor cells overexpressing EGFr	Phase I/II ❑ solid tumors
Her2(1-683)PyVLP ❑ Responsif	Polyomavirus VP1/VP2Her2 virus-like particles	Preclinical ❑ solid tumors
6D12 ❑ U Cincinnati, U Pittsburgh	Anti-idiotype antibody raised against parental antibody for trastuzumab	Preclinical ❑ HEr2-positive cancer

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), Targets in Oncology Module, May 2007

In 2006, Dr. Farzaneh's group initiated a phase I clinical trial to investigate the use of lentiviral vectors to genetically modify AML cells to express B7.1 (CD80) and IL-2. The goal of this trial, in which treatment is administered in combination with allogeneic HSCT, is to stimulate immune eradication of residual cells in patients with poor prognosis AML (Chan L, etal, Cancer Immunol Immunother, Aug 2006;55(8):1017-24).

Replication-competent Retroviral (RCR) Vectors

Noriyuki Kasahara, MD, PhD, from UCLA, described vector-based approaches for the treatment of glioblastoma multiforme (GBM). According to Dr. Kasahara, one of the challenges in using oncolytic viruses, such as adenovirus, influenza virus, vaccinia virus, parvovirus, poliovirus, among others, is the lack of understanding of the basis for selectivity and viral persistence, which result in tumor recurrence.

The newer generation of replication-competent retroviral (RCR) vectors enables highly efficient, selective, and persistent gene transfer to tumor cells, possibly offering significant advantages as oncolytic agents. In a variety of preclinical models, RCR vectors have demonstrated efficient and persistent gene delivery. Virus was shown to replicate throughout an entire tumor mass after inoculation; and initial multiplicities of infection as low as 0.001 were observed. RCR vectors replicate within primary glioma, without spreading to adjacent normal cells in the brain (Dalba C, et al, Curr Gene Ther, Dec 2005;5(6);655-67, and Tai CK, etal, Mol Ther, Nov 2005;12(5):842-51).

Dr. Kasahara, and his team employed RCR in RG2, an immunocompetent intracranial tumor model generated in Fischer 344 rats. Immune response to the RCR vector was established by quantification of CD4, CD8, and CD11/b levels in tumors. RCR was coupled with the cytosine deaminase (CD) suicide gene in converting prodrug 5-flu-

orocytosine (5-FC) to 5-fluorouracil (5-FU). RCR-CD converted 5-FC to 5-FU effectively *in vitro*, which was demonstrated by 19F-NMR spectroscopy studies. RCR-CD transduction was seen only in tumor but not in normal brain cells or systemic organs, and there was no evidence of inflammation. Overall, infection of RG2 brain tumors with RCR-CD and subsequent treatment with 5-FC significantly prolonged survival compared with controls that were injected with buffer alone (Wang W, etal, Neurosurg Focus, 15 Apr 2006;20(4):E25). Delivery of RCR-CD to mice with CT26 colon cancer also resulted in tumor growth inhibition, and tumor regression (Hiraoka K, etal, Clin Cancer Res, 1 Dec 2006;12(23):7108-16).

Currently, Dr. Kasahara and his team are experimenting with tumor-specific T-cell delivery of replicating infectious nanovector for intratumoral transduction and immunotherapy (TTRINITI).

Editor's Note:

In the next issue of Future Oncology, Part III of this four-part review of the role of the epidermal growth factor (EGF) receptor (EGFr) family, or ErbB receptor family, pathway in cancer reviews the status of commercialized agents targeting the EGFr pathway and assesses their performance in the clinic. Part III presents detailed information on these drugs in approved and developmental indications, their domestic and foreign markets, and costs associated with treatment regimens employing these agents. Part IV, the last of this series, describes in detail the many novel drugs in development targeting the EGFr family pathway. Much of the information presented in this series is derived from NEW MEDICINE's Oncology KnowledgeBASE (nm/OK) residing at www.nmok.net. This strategically organized information is updated daily from numerous sources.

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