Expression of tyrosine kinases in human malignancies as potential targets for kinase-specific inhibitors

Christian F Singer^{*}, Gernot Hudelist^{1*}, Wolfgang Lamm, Ruth Mueller, Klaus Czerwenka² and Ernst Kubista

Department of Senology and Ludwig Boltzmann-Institute of Clinical Experimental Oncology, University of Vienna, Austria

¹Department of Obstetrics and Gynecology, Villach Hospital, Villach, Austria

²Department of Gynecopathology, Vienna, University of Vienna, Austria

(Requests for offprints should be addressed to C F Singer, Department of Obstetrics and Gynecology, Division of Senology, Vienna Medical University, Waehringer Guertel 18-20, A-1090 Vienna, Austria; Email: christian.singer@meduniwien.ac.at)

^{*}(C F Singer and G Hudelist contributed equally to this work)

Abstract

Tyrosine kinase (TK) inhibition has been identified as a promising strategy in the treatment of human malignancies and several synthetic inhibitors have been developed. While the selective blockage of specific TKs is highly effective *in vitro*, clinical results have been less impressive. It has been suggested that the simultaneous inhibition of multiple TKs might lead to more favorable therapeutic results *in vivo*. We have therefore performed a systematic analysis of intra-tumoral TK expression in order to identify potential targets for a simultaneous kinase inhibition. To this end, we have analyzed the protein expression of membrane-associated epidermal growth factor receptor (EGF-R), Her-2/neu, platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor receptor (IGF-R), c-Kit and of cytoplasmatic c-Abl in 500 human tumors of epithelial, stromal and mesenchymal origin by immunohistochemistry, and found a distinct pattern of kinase expression: EGF-R, PDGF-R and c-Abl were expressed in the majority of malignant tumors, whereas c-Kit, Her-2/neu and IGF-R protein expression was considerably less frequent. Overall, the EGF-R protein expression was correlated with PDGF-R, c-Kit and c-Abl immunoreactivity ($P = 0.003$, $P = 0.001$ and $P < 0.001$, respectively). c-Abl was co-expressed with IGF-R and PDGF-R ($P = 0.003$ and $P < 0.001$, respectively). Kinase co-expression was also seen in tumor subgroups and was particularly significant in breast cancer where IGF-R protein was expressed together with PDGF-R and c-Abl ($P = 0.003$ and $P = 0.004$, respectively), and in colon cancer where PDGF-R was correlated with EGF-R ($P < 0.001$). With the exception of Her-2/neu expression and age, intra-tumoral TK expression was not associated with parameters such as grading or histological subtypes. Taken together, we have found a specific pattern of kinase co-expression and have identified several potential targets for a tumor-specific multimodal TK inhibition.

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Introduction

Tyrosine kinase (TK) expression analysis is emerging as an important prognostic tool in the prediction of the clinical course in several human malignancies: the amplification/over-expression of Her-2/neu in invasive breast cancer has been associated with shortened disease-free survival and overall survival, and thus represents an adverse prognostic parameter (Lohrisch & Piccart 2001).

Expression of the epidermal growth factor receptor (EGF-R) in prostate cancer is correlated with disease relapse and progression to androgen-independence, and in ovarian malignomas platelet-derived growth factor receptor (PDGF-R) protein expression is a negative prognostic parameter in terms of overall survival (Henriksen *et al.* 1993, Di Lorenzo *et al.* 2002).

TKs are also increasingly gaining attention as potential therapeutic targets since a number of synthetic

TK inhibitors (TKIs) have been developed over recent years and several large clinical trials are currently investigating their use in human solid tumors: gefitinib (ZD1839, Iressa) is a synthetic EGF-R TKI which has been shown to be effective in the treatment of advanced refractory non-small-cell lung cancer with nearly 50% of patients deriving substantial palliative benefit from gefitinib therapy in one study (Hainsworth *et al.* 2003). Phase II and III trials of the EGF-R kinase inhibitor erlotinib (OSI-774, Tarceva) are currently underway in patients with advanced non-small-cell lung cancer, ovarian cancer, and head and neck squamous cell cancer (Herbst 2003).

PDGF-R and the TKs c-Kit and Bcr-Abl are targets for the highly specific TKI imatinib (Glivec), and treatment of c-Kit-expressing gastrointestinal stromal tumors and of chronic myeloid leukemia with constitutively activated c-Abl results in impressive clinical responses (Joensuu *et al.* 2002, Goldman & Melo 2003).

Unfortunately, although most TKIs are highly effective *in vitro*, their potential use *in vivo* is limited to those tumors which express target TKs and in which these kinases also have a role in tumorigenesis. And even then, inhibition of a single TK does not appear to be sufficient to produce a clinically meaningful response. Indeed, until now — with the notable exception of imatinib — most inhibitors have failed to meet the high expectations originally placed on them (Dancey & Freidlin 2003). The reasons for the disappointing clinical results are largely unknown, but the recruitment of alternative stimulatory pathways and the simultaneous activation of other membrane-bound TKs have been suggested (Normanno *et al.* 2003). This hypothesis has led to the evaluation of dual inhibitors of EGF-R and Her-2 (PKI 166, GW 572016, EKB 569) which have been or are being tested in phase I trials (Torrance *et al.* 2000, Mellinghoff *et al.* 2002, Bonmi 2003).

In order to identify those human malignancies which most likely profit from such a simultaneous pharmacological TK inhibition, a thorough knowledge of the expression of intra-tumoral TKs is essential. Until now, however, no such comprehensive survey exists. We have therefore used tissue arrays to analyze the protein expression of the six TKs which are believed to play the most significant roles in the progression of a number of human malignancies, and which are potential clinical targets for kinase inhibition (Blume-Jensen & Hunter 2001). We selected EGF-R, Her-2/neu, PDGF-R, insulin-like growth factor 1 receptor (IGF-1R), c-Abl and c-Kit for their pivotal involvement in a number of solid tumors and for the fact that each of these kinases can be targeted by newly developed, clinically available TKIs (George 2001, Scheijen & Griffin 2002, Janmaat & Giaccone 2003,

Camirand & Pollak 2004). Furthermore, in each case, pre-clinical studies have already confirmed that these targets can be selectively neutralized by highly specific TKIs. We then investigated potential TK co-expression patterns in order to identify tumor entities that might be particularly vulnerable to combinations of specific TK inhibitors.

Materials and methods

Tissue arrays

Fourth Generation Tissue Arrays (TARP-4) were purchased from the National Cancer Institute Tissue Array Program. Each array contained 500 tissue samples from biopsies obtained from malignant brain tumors (25 samples), malignant breast tumors (75 samples), malignant colon tumors (75 samples), lung cancer (75 samples), lymphomas (50 samples), malignant melanomas (25 samples), ovarian cancer (50 samples), prostate cancer (75 samples) and corresponding normal tissues (50 samples). Table 1 shows the mean age, sex and histological classification of the eight tumor subgroups. A detailed listing of these parameters can be viewed for each individual biopsy at http://ccr.cancer.gov/tech_initiatives/tarp.

Immunohistochemistry

Tissue arrays were de-paraffinized with three washes of xylene for 5 min each, followed by two washes in 100% ethanol for 10 min each. The slides were then incubated in two washes of 95% ethanol for 10 min and washed in twice in dH₂O for 5 min. For antigen unmasking, sections were heated in 1 mM EDTA for 10 min. After three washes in dH₂O for 5 min each, sections were incubated in peroxidase blocking agent (0.3–1% hydrogen peroxide) for 5 min to quench endogenous peroxidase activity and rinsed in PBS for 2 min. The sections were then blocked with serum (dependent on the primary antibody with goat, horse or donkey serum; all from ImmunoCruz Staining System, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 4 min. After a consecutive PBS wash, slides were incubated with IGF-1R (Ab-1 clone 24-31, mouse monoclonal, 1:100; NeoMarkers, Fremont, CA, USA), EGF-R (clone 31G7, mouse monoclonal, 1:200; Zymed Laboratories, San Francisco, CA, USA), Her-2/neu (Dako A/S, Glostrup, Denmark), PDGF-R- α (C-20, rabbit polyclonal antibody, 1:200; Santa Cruz Biotechnology), c-Abl (no. 2862, rabbit polyclonal antibody, 1:50; Cell Signaling Technology, Beverly, MA, USA) and c-Kit (no. A4502, rabbit polyclonal, 1:100; Dako Cytomation Denmark A/S, Glostrup, Denmark) antibodies for 2–8 h at room temperature. Negative controls were performed on all tissue arrays by replacing primary

Table 1 Patient and tumor characteristics.

Tumor type	Age $\pm$ S.D.	Sex (n)	Histology (n)
Malignant brain tumor	n.a.	n.a.	Glioblastoma multiforme: 25
Breast cancer	63.2 ($\pm$ 13.7)	Female: 75 Male: 0	Invasive ductal: 63 Invasive lobular: 9 DCIS: 1 Unknown: 2
Colon cancer	67.5 ($\pm$ 9.98)	Female: 30 Male: 28 Unknown: 17	Adenocarcinoma: 75
Lung cancer	63.7 ($\pm$ 10.1)	Female: 24 Male: 34 Unknown: 17	Squamous: 36 Mucinous: 24 Bronchoalveolar: 4 Unknown: 11
Lymphoma	47.1 ($\pm$ 25.7)	Female: 15 Male: 29 Unknown: 6	Hodgkin lymphoma: 5 Other: 45
Melanoma	51.5 ($\pm$ 21.3)	Female: 8 Male: 9 Unknown: 8	Primary tumor: 20 Metastatic: 5
Ovarian cancer	62.5 ($\pm$ 11.7)	Female: 50 Male: 0	Serous papillary: 34 Endometrioid: 5
Prostate cancer	n.a.	Female: 0 Male: 75	Mucinous or clear cell: 11 Adenocarcinoma: 75

n.a., not available; DCIS, ducted carcinoma *in situ*.

antibodies with diluted isotype immunoglobulins (Immunocruz Staining System). The slides were then incubated with a mouse, rabbit or goat biotinylated secondary antibody provided in the detection kit, and horseradish peroxidase agent for 60 min, and incubated with the substrate chromogen 3,3'-diaminobenzidine for 5 min. Her-2/neu protein expression was determined by using the HercepTest according to the manufacturer's instructions (Dako Cytomation A/S). All sections were counterstained with hematoxylin for 10 s.

Immunostaining quantification and statistical analysis

Protein expression levels were assessed by two experienced pathologists (W L and K C) using the HercepTest in the case of Her-2/neu. A semi-quantitative scoring system (immunoreactive score) was used to allow for a reproducible evaluation of PDGF-R, EGF-R, IGF-1R, c-Abl and c-Kit in immunohistochemically stained tissue sections (Remmele & Stegner 1987). The immunoreactive score (staining intensity: negative, weak, moderate and strong membranous staining was multiplied by the percentage of positive cells) ranged from 0 to 12; 0–2 points were considered to be negative; weak staining (3–5 points) was defined as 1+, moderate staining (6–8 points) as 2+ and strong staining (9–12 points) as 3+. The SAS

8.2 TS2M0 Win software package was used to perform statistical analyses and $P < 0.05$ from a two-tailed test was considered statistically significant.

Results

TK protein expression in malignant tumors

Table 2 shows the expression of EGF-R, Her2/neu, PDGF-R, IGF-R, c-Abl and c-Kit in each of the eight tumor entities analyzed. EGF-R was detectable in the cell membrane of tumor cells in all types of malignancies with the most consistent protein expression in brain tumors (64% of cases), prostate cancer (52%) and lung cancer (49%). EGF-R staining was absent from all of the five Hodgkin lymphomas analyzed, but was present in eight of the 45 non-Hodgkin lymphomas investigated (18%). In these cases, EGF-R staining was mostly detected in the tumor cells of CD 19 (+), CD20 (+), CD 5 (–) and CD 10 (–) lymphomas, although information about the CD expression pattern was not available in all EGF-R-positive cases. Positive staining in lymphoma cells appeared either as a linear to finely granular pattern in the cell membrane and adjacent cytoplasm, or as a coarsely granular cytoplasmatic staining. A representative picture of an EGF-R-positive tumor (breast cancer) shown in Fig. 1A. c-Abl protein was equally found in all tumor types with expression being predominant in

Table 2 TK receptor protein expression in human malignancies

Tumor type	Cases	EGF-R	Her-2/neu	PDGF-R	IGF-1R	c-ABL	c-Kit
Malignant brain tumor	25	16/25 (64%)	0/24 (0%)	18/22 (82%)	0/24 (0%)	13/22 (59%)	0/25 (0%)
Breast cancer	75	14/69 (20%)	11/68 (16%)	15/64 (23%)	10/68 (15%)	41/71 (58%)	0/69 (0%)
Colon cancer	75	27/69 (39%)	3/68 (4%)	56/75 (75%)	2/68 (3%)	47/72 (65%)	4/72 (6%)
Lung cancer	75	33/67 (49%)	0/63 (0%)	55/65 (85%)	4/61 (7%)	48/69 (70%)	4/67 (6%)
Lymphomas	50	8/50 (16%)	0/48 (0%)	32/46 (70%)	0/50 (0%)	31/46 (67%)	1/49 (2%)
Melanomas	25	7/24 (29%)	2/23 (9%)	21/23 (91%)	23/25 (92%)	18/24 (75%)	3/24 (13%)
Ovarian cancer	50	7/49 (14%)	4/46 (9%)	29/39 (74%)	1/49 (4%)	20/49 (41%)	0/47 (0%)
Prostate cancer	75	34/65 (52%)	3/67 (5%)	63/65 (97%)	2/67 (3%)	44/68 (65%)	10/68 (15%)

melanomas (75%), lung cancer (70%), lymphomas (70%), colon cancer (65%) and malignant brain tumors (59%). Its expression was detected in the cytoplasm in the majority of tumor cells. A c-Abl-positive malignant breast tumor is shown in Fig. 1E.

With the exception of breast cancer (23%), PDGF-R was also immunolocalized in the majority of tumors, regardless of their identity. In the case of prostate cancer and melanomas PDGF-R was even immunodetectable in almost all cases (97 and 91%, respectively). PDGF-R-

Figure 1 Protein expression of EGF-R, Her-2/neu, PDGF-R, IGF-1R, c-Abl and c-Kit in human malignancies by immunohistochemistry. Representative array tissues are shown: (A) EGF-R protein expression (shown in breast cancer tissue); (B) Her-2/neu protein expression (shown in breast cancer); (C) PDGF-R protein expression (shown in a breast cancer sample); (D) IGF-1R protein expression (shown in breast cancer tissue); (E) c-Abl protein expression (shown in breast cancer); (F) c-Kit protein expression (shown in a colon cancer sample). All tissues are shown in low-power magnification (100 $\times$, left part of each picture) and in high-power magnification ($\times$ 300, right part of each picture). (A), (C) and (E) scale bar represents 0.2 mm; (B), (D) and (F) scale bar represents 0.07 mm.

Table 3 TK receptor protein expression in normal human tissues

Tissue	Cases	EGF-R	Her-2/neu	PDGF-R	IGF-1R	c-ABL	c-Kit
Brain	3	0/3 (0%)	0/3 (0%)	2/3 (67%)	0/3 (0%)	1/3 (33%)	0/3 (0%)
Breast	5	0/5 (0%)	0/5 (0%)	n.a.	0/5 (0%)	n.a.	0/5 (0%)
GI tract	10	2/10 (20%)	0/9 (0%)	2/10 (20%)	0/10 (0%)	1/8 (13%)	0/10 (0%)
Respiratory tract	5	0/5 (0%)	0/5 (0%)	2/5 (40%)	0/5 (0%)	3/5 (60%)	0/5 (0%)
Lymph nodes	2	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Skin	2	0/2 (0%)	0/2 (0%)	0/1 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Reproductive tract	4	1/4 (25%)	0/4 (0%)	0/3 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
Glandular tissues	5	2/5 (40%)	0/5 (0%)	3/5 (50%)	0/5 (0%)	2/5 (40%)	0/5 (0%)
Spleen	2	2/2 (100%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	2/2 (100%)	0/2 (0%)
Urinary tract	6	3/6 (50%)	0/6 (0%)	2/5 (40%)	0/5 (0%)	1/5 (20%)	0/6 (0%)

GI tract, gastrointestinal tract.

positive membrane staining is shown in a breast cancer in Fig. 1C. We observed a very different pattern of expression for IGF-R, which was either absent in the case of malignant brain tumors and lymphomas or only present in less than 5% of samples (colon cancer, ovarian cancer and prostate cancer). Melanomas, however, represent a notable exception with 92% of tumors being IGF-R-immunopositive. An IGF-R-positive malignant breast tumor is shown in Fig. 1D.

c-Kit protein was absent in all of the malignant brain tumor, breast cancer and ovarian cancer samples and was only present in few tumor tissues in the case of colon cancer, lung cancer, lymphomas, melanomas and prostate cancers. A weakly positive c-Kit-positive colon carcinoma is shown in Fig. 1F. A similar pattern was observed for Her-2/neu: protein expression was immunolocalized in 16% of malignant breast tumors, 4% of colon cancer cases, 9% of melanomas and ovarian cancers, and 5% of prostate cancers and was absent in all other tumor entities. A representative picture of Her-2/neu protein expression in a malignant breast tumor sample is shown in Fig. 1B.

TK co-expression pattern in different human malignancies

In order to identify potential interactions between different TKs, we performed a correlation analysis in each of the tumor subgroups. Since immunohistochemical receptor staining was performed on consecutive serial tissue arrays, we were able to evaluate a TK protein

expression pattern for each individual tumor biopsy (Table 3). The immunohistochemical TK expression was graded on an ordinal scale as described in the Materials and Methods section.

Overall, EGF-R protein expression was correlated with PDGF-R, c-Kit and c-Abl immunoreactivity ($P = 0.003$, $P = 0.001$ and $P < 0.001$, respectively), and c-Abl was co-expressed with IGF-R and PDGF-R ($P = 0.003$ and $P < 0.001$, respectively). Using Kendall's tau test, we then analyzed possible TK co-expression pattern within each of the different tumor entities and found that in breast cancer tissue IGF-R was significantly correlated with the expression of c-Abl ($P = 0.004$, Kendall's tau) and PDGF-R ($P = 0.003$, Kendall's tau). There was a trend towards a statistically significant co-expression of EGF-R and c-Abl protein ($P = 0.052$, Kendall's tau), but no such association was observed between EGF-R and PDGF-R, IGF-R, or Her-2/neu protein expression in malignant breast tumors. Similarly, no correlation was detected between c-Abl and PDGF-R or Her-2/neu protein expression, and between Her-2/neu and IGF-R immunostaining.

In colon carcinoma, EGF-R and c-Abl protein expression were highly correlated ($P < 0.0001$, Kendall's tau), and there was also a trend towards a co-expression of c-Abl and PDGF-R ($P = 0.055$, Kendall's tau). All other TKs did not exhibit a significant pattern of co-expression when being analyzed by immunohistochemistry. In lung cancer, EGF-R and IGF-R ($P = 0.049$, Kendall's tau) were correlated, as well as c-Abl and PDGF-R ($P = 0.025$,

Kendall's tau). Similarly, membrane-associated c-Abl and IGF-R proteins were also found to be associated with each other ($P = 0.036$, Kendall's tau).

In lymphomas, only c-Abl and PDGF-R ($P = 0.02$) were significantly co-expressed and we were unable to detect an association in the protein expression of any of the other TKs investigated. While we detected at least a trend towards a statistically significant co-expression of EGF-R and c-Abl ($P = 0.056$), no such observation was made for all other TKs.

Interestingly, a distinct pattern of TK distribution was observed in melanomas, where most TKs were correlated: EGF-R was co-expressed with c-Abl ($P = 0.025$) and c-Kit ($P = 0.031$), and the immunohistochemical detection of PDGF-R protein was associated with the detection of c-Abl ($P = 0.027$) and c-Kit ($P = 0.042$) protein. Quite similarly, we also noticed a co-expression of EGF-R and c-Abl ($P = 0.032$) and of c-Abl and PDGF-R ($P = 0.023$) in prostate cancers.

When TK expressions were compared with other histopathological parameters such as patient age, tumor size and grading, we only found a positive correlation between Her-2/neu and age ($P = 0.017$, Mann-Whitney test exact P value) in melanomas and a trend towards IGF-R expression and age in ovarian carcinomas ($P = 0.051$). None of the other parameters appeared to predispose for a specific TK expression pattern.

TK protein expression in normal human tissues

The TK protein expression pattern in an array of 50 normal human tissues is shown in Table 3. None of the TKs investigated was detected in any of the samples obtained from lymph nodes, skin or normal breast tissue (although for technical reasons PDGF-R and c-Abl could not be assessed in breast tissue). Furthermore, out of the four samples derived from the reproductive tract, only one ovarian tissue biopsy stained weakly positive for PDGF-R, while all other TKs remained undetectable (Table 3 and results not shown). Interestingly, PDGF-R and c-Abl were selectively expressed in brain tissues and in tissues obtained from the respiratory tract while all other TKs were absent. In the remaining tissues (gastrointestinal tract, glandular tissues, spleen and urinary tract) variable amounts of EGF-R, PDGF-R and c-Abl proteins were expressed, while Her-2/neu, IGF-1R and c-Kit remained undetectable in all cases.

Discussion

Although we now have an array of potent and highly specific TKIs at our disposal, many compounds have not been able to keep their promise in the treatment of human

malignancies. Disappointing clinical trials involving the use of TKIs in several tumor entities have demonstrated that, with the notable exception of imatinib in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors, and of erlotinib in glioblastomas, the targeting of a single TK does not appear to be sufficient to produce a clinically meaningful response. In principle, several reasons can be held responsible for the apparent lack of efficacy *in vivo*: (1) the expression of a membrane-associated TK does not necessarily imply that the protein is indeed a key player in pathological processes. It is thus unlikely that neutralization of a non-relevant TK will lead to a selective anti-proliferative response in tumor cells. (2) Most trials involving novel TKIs are set in advanced disease, when tumors are already highly de-differentiated and the development of additional mutations and clonal selection have lead to tumor cells that no longer depend on external signals by TK-specific ligands. It has therefore been suggested to evaluate TKIs in the early (i.e. in the neo-adjuvant) setting rather than in the metastatic phase. Finally, (3) malignant tumors can recruit alternative pathways which, upon blockage of a specific TK, can substitute and circumvent TKI-mediated anti-proliferative effects. In order to avoid these intra-tumoral escape mechanisms, less specific inhibitors such as the pan-Her-2 TKI CI1033 have been developed (Grunwald & Hidalgo 2003). Another approach might involve the combinatory use of highly specific TKIs which could be custom-tailored to the TK expression of a malignant tumor (Sridhar *et al.* 2003).

We have therefore analyzed the co-expression pattern of six TKs, which are believed to have important pathophysiological roles in a number of human malignancies and which can also be effectively neutralized by synthetic TKIs *in vitro*.

We found that the majority of malignant brain tumors express EGF-R, PDGF-R and c-Abl, which would render them potential targets for TK inhibitors such as imatinib, erlotinib or SU-101, which is currently evaluated in phase II and III studies in recurrent glioblastomas and oligoastrocytomas (Vlassenko *et al.* 2000). Indeed, imatinib has already been evaluated in human glioma cells and has shown to enhance their radio-sensitivity *in vivo*, thus indicating a key involvement of PDGF-R and possibly also of c-Abl in malignant brain tumors (Russell *et al.* 2003). Recent phase I and phase II studies in patients with gliomas have also shown very promising results with erlotinib, a highly specific EGF-R inhibitor (Prados *et al.* 2003).

The fact that the inhibition of a single TK leads to a clinically relevant anti-tumoral effect indicates a critical involvement of this kinase in malignant cells. This is possibly the reason for the great success of imatinib-based

therapies in gastrointestinal stromal tumors and chronic myeloid leukemia, and could also explain the encouraging responses to erlotinib in tumors such as non-small-cell lung cancer, where EGF-R expression has already been identified previously as a negative prognostic parameter (Meert *et al.* 2002, Miller *et al.* 2003, Tiseo *et al.* 2004). Glioblastoma multiforme might be a specifically promising tumor entity for EGF-R TK inhibition, since this subgroup of malignant brain tumors is characterized by a high rate of EGF-R gene amplification and activating mutations. The presence of the receptor variant EGF-RvIII is thus associated with considerably shortened survival, but at the same time renders these tumors specifically interesting targets for EGF-R TK inhibition (Buckner *et al.* 2004).

It is nevertheless interesting that within a tumor entity, two TKIs that target the same kinase (erlotinib and gefitinib) can still show considerable differences in tumor response. Although we can only speculate, reasons such as patient selection, metabolization or drug dosing might also greatly influence whether a specific TK is successful *in vivo* (Adis 2003).

In the vast majority of samples from prostate cancer and in lung cancer, we also observed c-Abl and PDGF-R co-expression. In these cases the use of imatinib would again be a suitable therapeutic approach, especially in the light of a recent publication in which imatinib has been shown to exert an additional benefit on the anti-proliferative effect of paclitaxel on PDGF-R-expressing prostate tumors in a mouse model (Vehara *et al.* 2003).

Although the TKs that were expressed most consistently in colon tumors were c-Abl and PDGF-R, the most commonly co-expressed proteins were c-Abl and EGF-R. Consequently, a combination of c-Abl-inhibitory imatinib and EGF-R-blocking gefitinib would appear to be the most effective therapy. This hypothesis is supported by work of Attoub *et al.* (2002) who found imatinib to be highly effective in the inhibition of colon cancer cell growth *in vitro*, and by observations of Tortora *et al.* (2003) who was able to elicit a dramatic cooperative anti-tumoral effect in nude mice bearing human colon cancer xenografts, when adding gefitinib to a combination of a cyclooxygenase-2 inhibitor and a proteinase K inhibitor. The latter publication also provides the first demonstration that the three novel agents blocking multiple signaling pathways, in the absence of cytotoxic drugs, may have a potent anti-tumoral and anti-angiogenic activity after oral administration. Unfortunately, until now, no clinical data are available that would confirm these observations.

In contrast to the other malignancies investigated, malignant breast tumors were found to be considerably more heterogeneous in their TK expression pattern and, although variable amounts of c-Abl were expressed in more than 50% of tumors, the protein expression of other TKs

was considerably less frequent in the samples analyzed. Since in breast cancer the c-Abl expression is still significantly correlated with IGF-1R and PDGF-R, a combination of imatinib and an IGF-1R-inhibitory antibody might in principle exert a synergetic effect (Maloney *et al.* 2003). Nevertheless, the fact that in our samples IGF-1R is only expressed in 15% of cases suggests that breast tumors are less likely to be targets for successful TK inhibition.

Our observation of EGF-R, PDGF-R and c-Abl protein expression in a number of normal tissues is neither surprising nor contradictory: several authors have described these TKs in various organs and during different stages of development previously (Maxwell *et al.* 1998, Nagao *et al.* 2002, Schmandt *et al.* 2003, Yano *et al.* 2003). These findings simply indicate that TKs also have a physiological, tissue-specific role, and that an abrogation of TK-mediated signal transduction can potentially also be associated with undesired side effects. Therefore, a potential benefit, especially in the case of less-specific synthetic TKIs, will have to be weighed carefully against an unwanted disruption of physiological signal-transduction mechanisms in healthy tissues (Ebnoether *et al.* 2002, Esmali *et al.* 2002).

Taken together, we have demonstrated the presence of TKs in several human malignancies, and have identified potential targets for a simultaneous TK inhibition. We hypothesize that, with the exception of a few selective tumor entities in which the neutralization of one single receptor is already enough to provide for impressive treatment results, the majority of human malignancies are multi-factorial and therefore the key to successful anti-neoplastic treatment is the use of a custom-tailored array of several receptor-specific antibodies and kinase inhibitors rather than the sole application of a single TKI.

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