Abstract. c-Met is responsible for cell motility and tumour spreading. c-Met expression and signal transducers reflecting c-Met functionality were investigated in breast carcinomas, in correlation with patient outcome and tumour vasculature. Tissue microarrays of 930 breast carcinomas were constructed, categorised according to patients’ follow-up (4- to 10-year follow-up; median, 6.5 years). Standardised immunocytochemical procedures were performed using anti-c-Met, -PI3K, -FAK, -JAK, and -CD146, -FYN and an automated autostainer (Ventana). High-throughput densitometry measuring the extent of immunoprecipitates was assessed by image analysis (SAMBA). c-Met overexpression correlated with poor survival along with PI3K and FAK reflecting c-Met functionality and CD146 and FYN expression in endothelial cells. Automated quantification of immunocytochemical precipitates using image analysis was shown to provide an objective means of measuring cellular proteins that are potentially relevant for current practice in pathological diagnosis and for specific therapy combining inhibitors of both c-Met and downstream transducer pathways, and of tumour angiogenesis.

Introduction

The action of inhibitors of tyrosine kinase receptors (such as trastuzumab, imatinib, bevacizumab and gefitinib) illustrates the value of targeting this protein class for treatment of selected cancers. As a member of the receptor tyrosine kinase (RTK) family, c-Met also represents a target for cancer therapy. c-Met is a high-affinity receptor for hepatocyte growth factor (HGF) or scatter factor (SF) and is significantly overexpressed within tumour cells. c-Met and HGF are required for normal mammalian development and play an important role in epithelial mesenchymal interactions during organ morphogenesis (1,2). c-Met is expressed in most carcinomas, and elevated expression relative to normal tissue has been detected in a number of carcinomas including lung, breast, colorectal, prostate, pancreatic, head and neck, gastric, hepatocellular, ovarian and renal carcinomas and in gliomas, melanomas and some sarcomas (3-6). c-Met expression is regulated in tumours by a number of epigenetic mechanisms involving tumour-secreted growth factors, tumour hypoxia and activation of other oncogenes (7-9). In addition, c-met amplification and subsequent overexpression have been reported in gastric and colorectal cancers and gliomas (10-12). c-Met and HGF overexpression enhances the growth of neoplasms and invasion. The diverse set of cellular functions regulated by this receptor influences some obligatory steps during the metastatic process including: i) migration, secretion of proteolytic enzymes and invasive growth of tumour cells during extravasation; ii) anoikis, or survival of tumour cells in vasculature; iii) arrest in capillary beds and invasive extravasation of tumour cells to form micrometastases; and finally iv) cell growth and survival at diverse remote microenvironments supporting the growth of metastasis. In addition, the increased production of HGF by tumoural stromal cells in neoplasms at both primary and metastatic sites may affect the recruitment of endothelial cells and formation of microcapillaries during tumour neovascularisation. In support of this role, the endothelial expression of c-Met and stimulation of endothelial growth, invasion and motility by HGF have been demonstrated in vitro (9), resulting in neovascularisation (13,14). HGF has also been demonstrated to upregulate the expression of pro-angiogenic factors including VEGF, IP8 and UPA by both tumour cells and vascular smooth muscle cells, and to downregulate expression...
of antiangiogenic factors such as thrombospondin-1. In addition, it increases expression of VEGF-R2 by endothelial cells (9,13,14).

At the molecular level, binding of activated HGF to the c-Met extracellular ligand-binding domain results in receptor multimerisation and phosphorylation of multiple tyrosine residues at intracellular regions, that regulate internalization, catalytic activities and docking of regulatory substrates. Activation of c-Met results in binding phosphorylation in adaptor proteins (mainly Gab 1 and to a lesser extent Gab 2), with subsequent activation of signal transducers such as PI3K, FAK, JUN (amino-terminal kinase JNK), ERK and STATs, also regulated through JAK, another signal transducer activated by the transmembrane non-tyrosine kinase cytokine IL-6 receptor (4,6,13,14). Although other RTKs signal through these pathways, c-Met differs in the following: i) the presence of the unique multisubstrate docking site at the c-terminal region of the receptor; and ii) signalling through the specific adaptor proteins Gab 1 and 2. Another particular feature of c-Met signalling is its interaction with focal adhesion complexes and non-kinase binding partners such as B4 integrin, CD44, semaphorin and ezrin (3,5,13). Downstream of Gab 1, the regulation of cell motility, cell dissociation, cell adhesion and invasion by c-Met has been shown to be dependent on both PI3K and ERK pathways. PI3K has been shown to control c-Met-dependent cell survival. FAK also acts on cell motility and invasion through the loss of tight junctions and facilitation of adhesion of tumour cells onto the extracellular matrix. JAK and STATs participate in branching morphogenesis (3,4,13).

In the present study, we used immunohistochemistry to document variation in c-Met expression in a large series (n=930) of breast carcinomas, as well as expression of the c-Met signal transducers PI3K and FAK. Our objective was to correlate expression of these proteins in tumour cells with patient outcome, in order to identify potential intratumoural targets for specific inhibitors with potential relevance for cancer therapy (3-6,13,14). We used a standardised method to identify overexpression of these proteins in tumours, and a new high-throughput method of evaluation including tissue microarray (TMA) and quantitative densitometry after automated digitisation of microscopic images of immunostaining.

In previous studies (15-17), we showed that angiogenic factors such as CD105, Tie2, VEGFR2, HIF and CD31 in frozen samples are valid objective indicators of tumour neoangiogenesis that correlate with poor survival and high metastatic risk. However, we found that TMA was not suitable for evaluation of angiogenesis due to the small diameter (0.6 mm) of cores from which paraffin sections were assessed (18). Subsequently, we more successfully examined CD146/Mel-CAM labelling of activated endothelial cells in TMA (unpublished data). Since remote metastases develop through blood vessels, we also aimed to evaluate CD146 expression as a reflection of tumour vasculature, using the same technical approach, in correlation with c-Met expression.

Various inhibitors suitable for antiangiogenic therapy have been reported (reviewed in ref. 19). However, the preliminary results of clinical studies have suggested that antiangiogenic therapy has poor activity in advanced tumours, but some controversial trials have claimed significantly better outcome when an antivascular endothelial growth factor antibody was combined with chemotherapy as front-line therapy against colorectal cancer, a finding that has renewed interest in this therapeutic strategy (19). Also, it has been shown that proinvasive effects of hypoxia activate transcription of the c-met proto-oncogene, resulting in higher levels of c-Met, thus amplifying HGF signalling. Inhibition of c-Met expression prevents hypoxia-induced invasive growth (9). Neoplastic cells escape hypoxia by invading surrounding tissues where oxygen and nutrients are available. This invasive switch that links tumour hypoxia to increased malignancy points to the therapeutic relevance of suppressing cell motility when targeting tumour vasculature in order to prevent the potential spread of cancer cells following intratumoural oxygen deprivation. Therefore, a combination of an angiogenesis inhibitor with agents blocking c-Met and the downstream regulation pathway as a multitarget therapy would have greater therapeutic efficacy (9,14,19). However, before such potential clinical tools can be developed, it is necessary to establish that at the time of diagnosis, tumour cells exhibit increased vasculature associated with c-Met dysregulation, in parallel with tumour aggressiveness and poor patient outcome.

In the present study, we have compared expression of c-Met and signal transducers in a large series of breast carcinomas from patients categorised as deceased or alive with and without metastases, in correlation with CD146 expression, as a marker of endothelial cell activation and tumour angiogenesis, in subgroups of patients classified according to clinical outcome and nodal status.

Materials and methods

Breast tumour samples and patients' data. Breast carcinoma samples were obtained from 930 patients who had undergone initial surgery in the Hôpital de la Conception (PB). Tissue fragments were selected from our archival tumour paraffin blocks. All tumour samples used for TMA construction were fixed in buffered formalin, paraffin-embedded at controlled temperature and stored at 18-22°C. Patient follow-up ranged from 4-10 years (median, 6.5 years). All patients were informed at the time of operation that tissue blocks stored in the laboratory archives might be used for delayed diagnostic and research purposes, and gave their agreement.

Methods

Tissue microarray (TMA) construction. TMA were constructed as previously described (20-23). Briefly, for each tumour, two representative tumour areas were delineated by circling within tissue sections (SG, CC, JD, SCM) appropriate areas with a permanent black pen on hematoxylin and eosin-stained paraffin sections in order to guide the technicians' punches of cores from the primary paraffin block. Cores were sampled using an ALPHELYS arraying device (78370 Plaisir, France). Core cylinders of 0.6-mm diameter punched from the donor block were then deposited in the recipient paraffin block. TMA sections (4-μm thick) were cut 24 h before immunohistochemical processing.

A total of six TMA blocks were constructed enclosing altogether 1860 cores, corresponding to 2 cores per individual tumour and 930 patients.
Comparison of c-Met expression in different cores punched from the same tumour. Evaluation of c-Met immunoexpression in 50 breast carcinoma cases selected for TMA showed similar immunostaining in multiple cores sampled from different areas (but of similar cellularity) of the donor blocks (Spearman’s correlation coefficient r=0.784, p<0.01). This result indicates that a limited number of cores (two in the present study) was sufficient when areas in the donor blocks were properly delineated and representative of the tumour.

Comparison of semiquantitative and quantitative analysis using image analysis. Semiquantitative evaluation was first used to classify the immunoexpression as negative or positive. The positive cores were further categorised as: weak (1+), intermediate (2+), or intense (3+) staining, based on the intensity and extent of immunoreaction. This evaluation significantly correlated with the densitometric quantification of immunoreactivity assessed by SAMBA image analysis, when both methods were applied on 120 cores and compared for one antibody (Spearman’s correlation coefficient r=0.728, p<0.01).

Image analysis procedure. The TMA analysis with the SAMBA 2050 automated device (SAMBA Technologie, Meylan/Grenoble, France) (26-28) was performed according to the following protocol. First, an image of the entire slide was built up using a low-power magnification (x2, pixel dimension 3.7 μm). This image was made of a mosaic of images acquired along a rectangular grid with contiguous fields. Second, the area of the slide containing the TMA cores was automatically delineated and scanned at higher magnification (x10, pixel dimension 7.4 μm). Third, after autofocusing, the images were acquired with an overlap greater than the largest mechanical positioning error. Using the image contents, a matching algorithm determined precisely the relative position of each image with respect to its neighbours. Calculated overlap was removed from images to produce a new set of higher magnification (x10) images, thus covering precisely the cores of interest. A specially developed tool referred to as TMA crop then allowed superimposition of the TMA grid onto the reduced image and precise alignment of each node of the grid with the core location within the image. The final step was performed automatically using the core image contents to ensure pixel precision of the match. From the images acquired with x20 magnification, a new set of images was next computed, one for each core. After colour analysis of the core images, the SAMBA ‘immuno’ software was applied as previously reported (15-17,25-28) in usual full tissue sections. Several parameters per core were computed: the area of counterstaining, the ratio of the positive area versus counterstained areas and a quick score (percentage of positive area x mean optical density). Optical density (OD) was evaluated on a scale of grey levels (arbitrary units) ranging from 0 (100% transmission, OD = 0) to 255 (1% transmission, OD = 2). The computation of each parameter
obtained provided numerical values consisting of continuous variables for statistical tests.

For the present study, the immunostained 60 TMA and 18,600 immunostained core sections resulting from the 6 different TMA and 10 antibodies were run according to this procedure.

Statistical analysis. Statistical analyses were performed using NCSS 2005 (25-28) and Statistica 2006 statistical softwares. The concordance between the immunoeexpression of markers: i) in frozen and fixed tissues; ii) in full section and TMA sections; and iii) by semiquantitative and automated analysis, were examined using Spearman's ρ rank correlation. Comparison of mean parameters for each marker was assessed by non-parametric tests. Mean values were computed from

Figure 1. Steps of SAMBA software processing before densitometry on TMA. (A) Reference grid, (B and C) precrop of digitized TMA and superimposed grid, (D) cropped final image before image analysis (densitometry).

Figure 2. High magnification of one core (0.6 mm in diameter) from an individual breast cancer tumour included in TMA: immunostaining with anti-c-Met, Ventana Benchmark autostainer and kit.

Figure 3. High magnification of one core (0.6 mm in diameter) from an individual breast cancer tumour included in TMA: immunostaining with anti-PDK, Ventana Benchmark autostainer and kit.
measurements in the 2 cores from each tumour, before correlation. Contingency table analysis was used to analyse the relationship between protein expression in TMA of tumour sets. All p-values were two-sided. For each antibody, univariate analysis was conducted using Mann-Whitney and/or Kolgomorov, and $\chi^2$ tests. Only one parameter of immunostaining (percentage of positive surface) was retained in order to limit the amount of data and to facilitate the statistical analysis in the present initial part of our study design.

Procedures for the quantification and cropping stage are shown in Fig. 1 and the distribution of immunolabelling within sections of tumour cores with the various antibodies are shown in Figs. 2-7.

Results

c-Met overexpression in tumours with unfavorable outcome (tumour spreading). Immunostaining with anti-c-Met produced higher levels of cytoplasmic labelling (Fig. 2) in tumours from deceased or metastatic patients (Table II), in terms of both ratio of positive to negative cases ($p=0.01$) and greater c-Met-positive surface ($p<0.0001$), particularly in node-negative patients ($p<0.0001$) (Table II). Similarly, PI3K expression (Fig. 3) was greater in tumours of poor prognosis ($p<0.0001$), in terms of positive/negative cases and extent of positive staining (Table II). Results were similar for FAK expression (Fig. 4) ($p=0.0157$ and $p<0.0001$ for positive/negative cases and for extent of staining, respectively) (Table II). However,
Table II. Quantitative distribution of c-Met, PI3K, FAK and JAK immunostaining in TMA, evaluated by SAMBA device.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Deceased/metastasis</th>
<th>Alive/no metastasis</th>
<th>Deceased/metastasis</th>
<th>Alive/no metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients (n=916)</td>
<td>270/283</td>
<td>13/283</td>
<td>p&lt;0.01</td>
<td>50/633</td>
</tr>
<tr>
<td>Node-negative patients (n=477)</td>
<td>73/75</td>
<td>2/75</td>
<td>p&lt;0.00001</td>
<td>2/402</td>
</tr>
<tr>
<td>Node-positive patients (n=439)</td>
<td>p=0.1614</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients (n=928)</td>
<td>245/283</td>
<td>39/283</td>
<td>p&lt;0.00001</td>
<td>163/645</td>
</tr>
<tr>
<td>Node-negative patients (n=477)</td>
<td>75/75</td>
<td>0/75</td>
<td>p&lt;0.00001</td>
<td>0/402</td>
</tr>
<tr>
<td>Node-positive patients (n=451)</td>
<td>p&gt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients (n=925)</td>
<td>217/287</td>
<td>70/287</td>
<td>p=0.0157</td>
<td>183/638</td>
</tr>
<tr>
<td>Node-negative patients (n=477)</td>
<td>p&gt;0.05</td>
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</tr>
<tr>
<td>Node-positive patients (n=446)</td>
<td>p&gt;0.05</td>
<td></td>
<td></td>
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<tr>
<td>JAK</td>
<td></td>
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</tr>
<tr>
<td>All patients (n=918)</td>
<td>140/283</td>
<td>143/283</td>
<td>p&lt;0.01</td>
<td>494/635</td>
</tr>
<tr>
<td>Node-negative patients (n=477)</td>
<td>70/75</td>
<td>5/75</td>
<td>p&lt;0.0001</td>
<td>141/402</td>
</tr>
<tr>
<td>Node-positive patients (n=441)</td>
<td>p&gt;0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Variations in numbers of patients among the 930 included in the series, result from core section losses in TMA during technical procedure.
FAK expression retained prognostic significance in node-negative patients only when the extent of surface stained was considered.

JAK (Fig. 5) was overexpressed in tumours with unfavorable outcome (Table II), suggesting that activation of c-Met signal transducers may also result from simultaneous activation of non-tyrosine kinase receptors.

Together, these data show that these molecules are overexpressed in malignant breast tumours with unfavorable outcome and suggest that they can be regarded as potential candidate targets for specific therapy.

**CD146 overexpression in tumours with unfavorable outcome (tumour vasculature).** CD146 was observed in tumours as a thin and well-delineated labelling of tumour endothelial cells of small vessels (Fig. 6) greater in metastatic or deceased patients, in terms of both the ratio of positive to negative cases and extent of immunostained surfaces, similarly to FYN expression (Fig. 7), reflecting CD146 activation (Table III).

**Discussion**

**Poor prognosis and c-Met overexpression.** In breast carcinomas, c-Met expression has been shown in several studies to correlate with tumour invasiveness, metastasis and shorter overall survival (29-32). Our results confirm these data in a significantly larger series (n=930) using objective quantification through standardised image analysis and immunohistochemical procedures in TMA. The overexpression of c-Met, despite lack of mutations or amplification (3-6,13) supports the hypothesis that specific therapy must target the protein itself and that immunohistochemical tests can be regarded as suitable for identifying protein targets in individual tumours. Antibodies against c-Met, small molecules such as PHA66752 and kerin that target c-Met, or the NK4 that blocks HGF binding to c-Met have been reported to act as specific c-Met inhibitors (33-35), while other tyrosine kinase inhibitors such as Iressa, Tarceva, Herceptin and Genifinib do not inhibit c-Met activity (6,13).

No TMA study of signal transducers in human breast carcinomas has been previously reported, although some immunohistochemical studies using large sections have been reported (33,34,36,37) The simultaneous expression of downstream transducers supports the hypothesis of overexpressed c-Met functionality in breast cancer, resulting in cell motility, dissociation, invasion and adhesion to extracellular matrix via pathways involving PI3K, FAK and ERK/PAK-P21, and associated branching morphogenesis through
angiogenesis and actin skeleton rearrangement. Experimental c-Met and CD146 cellular signals which both also act upon in 38). In our study, increased FAK immunostaining in breast that are present in focal adhesion plaques (reviewed in ref. several intracellular proteins including FAK (and paxillin) endothelial-endothelial cell adhesion through tight junctions, finding that CD146 antibody reacted only with endothelial malignant tumours do not (38,39). This is consistent with our CAM) along with endothelial cells, trophoblastic cells, expresses CD146 (Melanoma Cell Adhesion Molecule; Mel- progression marker in melanomas. Melanocytic tumour cells or size of the vessels, although it was initially reported as a three dimensional endothelial capillary tubes in vitro (3-6) and neovascularisation in tumour graft (13,14). HGF has also been demonstrated to regulate the expression of pro-angiogenic factors including VEGF and angiogenic response by endothelial cells such as VEGF-R2.

Angiogenesis, like vascular homeostasis, is linked to the functional state of interendothelial junctions which are modulated by growth and activation of endothelial cells. Molecules located in adherens junctions (e.g., VE-cadherin) or occluding and tight junctions (e.g., CD146/Mel-CAM, CD105/endoglin, CD34/PE-CAM) are crucial to angiogenesis. CD146 is a transmembrane protein belonging to the immunoglobulin superfamily, the ligand of which remains unknown. It is a structural component of interendothelial junctions, present on endothelial cells regardless of the site or size of the vessels, although it was initially reported as a progression marker in melanomas. Melanocytic tumour cells express CD146 (Melanoma Cell Adhesion Molecule; Mel-CAM) along with endothelial cells, trophoblastic cells, activated and tumouric T lymphocytes, but other human malignant tumours do not (38,39). This is consistent with our finding that CD146 antibody reacted only with endothelial cells but not with carcinomatous cells.

While the extracellular domain of CD146 is involved in endothelial-endothelial cell adhesion through tight junctions, the intracellular domain promotes the recruitment of the SCR family kinase FYN as well as tyrosine phosphorylation of several intracellular proteins including FAK (and paxillin) that are present in focal adhesion plaques (reviewed in ref. in 38). In our study, increased FAK immunostaining in breast carcinomas of poor outcome probably results from increased c-Met and CD146 cellular signals which both also act upon angiogenesis and actin skeleton rearrangement. Experimental studies have shown that anti-CD146 monoclonal antibodies induce inhibition of proliferation and migration of endothelial cells and of angiogenesis reflected by a reduction in blood vessel density, associated with tumour growth inhibition (38,39). In particular, treatment with AA98 monoclonal anti-CD146 of human xenographed tumour models using subcutaneously injected malignant cells (leiomyosarcoma, pancreatic and hepatocellular carcinomas) induced significant tumour reduction with no metastasis. This treatment produced non-cytotoxic effects in animals and its efficacy increased when AA98 was combined with other anticancer agents (39).

However, despite the fact that CD146 could constitute a target for cancer therapy as well as being an excellent marker of tumour vasculature through its expression in activated endothelial cells, no study has yet shown anti-CD146 therapy to be efficient in patients with carcinomas. Our data suggest that inhibition of CD146 and c-Met in tumours overexpressing both markers should have a synergistic effect in potentially reducing angiogenesis and cell spreading, but clinical studies are required to demonstrate the efficacy of this strategy for human therapy.

Hypoxia promotes tumour growth by transcriptional activity of the c-met proto-oncogene through a complementary pathway that is independent of the angiogenic response and mediated by upregulated VEGF through the HIF and CA IX pathway (40). This observation raised the possibility that antiangiogenic therapy, by reducing vascularisation in a primary tumour, promotes the spread of cancer cells towards a more highly oxygenated environment or distant tissue, thus favoring metastasis. It also provides support for the proposal (19,40) that a combination of selective angiogenic inhibitors with c-Met or HGF inhibitors should significantly reduce tumour progression. Also, in experimental studies, non-invasive assessment of tumour angiogenesis is possible using magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET), providing dynamic images of microvasculature that can be used in assessing inhibition of angiogenesis. Some initial study of angiogenesis inhibitors has demonstrated changes in vascular permeability and in volume fraction of metabolism after therapy which may be predictive of clinical efficiency of this therapy, in correlation with tumour regression (19). This procedure may be of value for patient management.

In conclusion, we used TMA from a series of 930 patients with breast carcinoma and developed a standardised high-throughput method for quantifying immunoprecipitates on core sections, different from that previously described (41,42) and poorly documented in the literature as a suitable tool to evaluate proteomic immunohistochemical expression of tumour cells in major human tumours. Our results show that overexpression of c-Met, as well as of PI3K and FAK, signal transducers reflecting c-Met functionality and acting on tumour cell spreading, correlates with poor survival. CD146 labelling of activated endothelial cells, that are implicated in neoangiogenesis and the subsequent metastatic process through blood vessels, also show overexpression in breast cancers with poor outcome. These observations suggest that standardised immunodetection procedures applied to individual carcinomas at the time of diagnosis, could not only yield significant prognostic data but also provide a basis for a potential specific therapy strategy combining inhibitors of c-Met and angiogenesis with cytotoxic chemotherapy and/or radiotherapy.
Acknowledgements

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References


