Advances in Brief

Overexpression and Amplification of the Met/HGF Receptor Gene during the Progression of Colorectal Cancer


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Abstract

The c-met oncogene encodes the receptor for hepatocyte growth factor/scatter factor, a potent mitogen for epithelial cells that also promotes cell motility and invasiveness. We have studied the changes of c-met gene expression that occur during the progression of colorectal tumors. Sixteen adenomas, 123 primitive carcinomas, and 25 liver metastases were examined. In several instances it was possible to compare same-patient samples of normal colon mucosa against primary tumor and primary carcinoma against synchronous metastasis. The expression of the c-met gene was increased from 5- to 50-fold in about 50% of tumors, at any stage of progression, and in 70% of liver metastases. Overexpression was associated with amplification of the c-met gene in only 10% of carcinomas, but in 8 of 9 metastases examined. These data suggest that overexpression of the c-met oncogene contributes a selective growth advantage to neoplastic colorectal cells at any stage of tumor progression. Moreover, amplification appears to give a further selective advantage for the acquisition of metastatic potential.

Introduction

A variety of genetic alterations have been associated with colorectal cancer, including deletion of tumor suppressor genes and activation of dominant oncogenes (for a review, see Ref. 1). In general, genetic alterations could occur at any stage of neoplastic transformation, resulting in heterogeneity of the tumor population. The so-called tumor progression is due to the selection of the most malignant clones. Colorectal neoplasia provides an excellent opportunity to study this progression. Most carcinomas appear to arise from adenomas and to carry several types of genetic alterations accumulated during the transition from the benign to the malignant phenotype (2, 3). No specific genetic change has been related to the progression of carcinomas toward the metastatic phenotype, which therefore appears to require the accumulation of alterations other than those so far described.

The c-met oncogene (for a review, see Ref. 4) encodes a transmembrane tyrosine kinase identified as the receptor for a polypeptide known as HGF or SF (for a review, see Ref. 5). The receptor is a 190-kDa heterodimer of two disulfide-linked subunits: an extracellular 50-kDa α chain and a membrane-spanning 145-kDa β chain (6) showing tyrosine kinase activity (7). Both chains are derived from a 170-kDa precursor that is glycosylated and cleaved to give the mature heterodimer (8). The c-met-encoded receptor is the prototype of a new class of growth factor receptors that includes the proteins encoded by the c-ras (9) and c-sea (10) genes.

met was originally identified in a transfection assay as an oncogene rearranged following treatment of a cell line with a chemical carcinogen (11). The c-met protooncogene was also found to be constitutively activated in a human gastric carcinoma cell line (12). We have also previously shown that the c-met gene, expressed in several normal human epithelial tissues (13, 14), is often overexpressed in carcinomas (13–15). The ligand of the receptor encoded by the c-met gene, HGF/SF, is a unique growth factor that shows both mitogenic and motogenic activities, and is able to induce epithelial cells to invade collagen matrices in vitro (16). In addition, the expression of a functional met/HGF receptor has been shown to be sufficient to transfer an invasive phenotype to transfected cells (17). These data suggest that the met/HGF receptor and its ligand may be involved in the pathogenesis and/or in the progression of cancer of epithelial origin.

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Materials and Methods

Tissue Samples. The tissues were obtained from the Pathology Department after surgery of patients. Normal and
neoplastic tissues were immediately frozen in liquid nitrogen. For molecular analyses, tissues were pulverized in the presence of liquid nitrogen. In the case of liver metastasis analysis, given that only minute specimens were available, the 25 samples were divided as follows: 16 (of which 2 were synchronous metastases) were used only for Northern blot analysis, 6 (all synchronous metastases) were used for both Western and Southern blot analyses, and 3 were used only for Southern blot analysis. Metastasis samples used for Western blot analysis were from patients not subjected to chemotherapy and radiotherapy.

**Antibodies.** mAbs DQ13, used for Western blot analysis, were raised against a peptide corresponding to 19 C-terminal amino acids (Ser1372 to Ser1390) of the c-met human sequence (EMBL Data-Bank reference no. X54559). mAbs DO-24, used for immunohistochemistry, were directed against the extracellular domain of the met protein. Both antibodies were kindly provided by Dr. M. Prat (14).

**Western Blotting.** Pulverized tissues were solubilized in boiling Laemmli buffer (19) containing the reducing agent β-mercaptoethanol. Under these conditions, the 50-kDa α and the 145-kDa β chain which constitute the 190-kDa receptor are distinct. Equal amounts of proteins (200 μg) were loaded into each lane. Proteins were separated by PAGE and transferred to nitrocellulose sheets. Western blot analysis was carried out as described by Towbin et al. (20). Blots were probed with the anti-met protein mAbs and then with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, revealed by enhanced chemiluminescence (Amersham Corp.). The relative expression of the met protein was quantified by laser densitometric scanning of X-ray films.

**Isolation of RNA and Northern Blot Hybridization.** Total cellular RNA was isolated from snap-frozen fresh human tissues by guanidine isothiocyanate extraction and cesium chloride density gradient ultracentrifugation. After denaturation, RNA samples (15 μg) were separated by electrophoresis on 1% denaturing agarose gels and transferred to nitrocellulose. Hybridization was carried out at 42°C in the presence of 50% formamide using the entire met cDNA as a probe. The cDNA probe encompassed the entire c-met coding sequence (EMBL Data-Bank reference no. X54559). The relative expression of the c-met transcripts was quantified by densitometric analysis of X-ray films, normalized for the accumulation of the mRNA encoding the human elongation factor 1 (21).

**Southern Blotting.** DNA was extracted and Southern blot analysis was performed as described in Sambrook et al. (22). Ten μg of DNA were digested either with the EcoRI or the Hind III restriction enzymes for 12 h at 37°C and loaded into each lane. Hybridization was carried out at 65°C using the cDNAs encompassing the entire coding sequences of either the met or HGF genes as probes. Probes were generated using the Mega Prime method (Amersham Corp.) in the presence of [α-32P]CTP. Nitrocellulose sheets were washed twice in 2X SSC-0.1% SDS at room temperature for 15 min, and once at 65°C for 20 min. Filters were rehybridized with a 32P-labeled β-globin probe to estimate the amount of DNA loaded. The intensity of labeled bands was estimated by laser densitometric scanning of X-ray films.

### Results

**Colorectal Adenomas.** Sixteen samples of colorectal adenomas were obtained at surgery or via endoscopy and snap frozen. Samples of unaffected adjacent tissue were also taken (10 cases). The expression of the c-met gene has been evaluated either at RNA or at protein level using Northern and Western blot analysis, respectively (Table 1). In 13 of 16 adenomas the

### Table 1  Overexpression and amplification of the c-met gene in colorectal tumors

<table>
<thead>
<tr>
<th>Samples</th>
<th>c-met Overexpression</th>
<th>c-met Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At RNA level</td>
<td>Relative score</td>
</tr>
<tr>
<td></td>
<td>subtotal</td>
<td>(+) (+) (+) (+)</td>
</tr>
<tr>
<td>Adenomas (total)</td>
<td>13/16</td>
<td>9/10</td>
</tr>
<tr>
<td>Moderately dysplastic</td>
<td>11/14</td>
<td>9/10</td>
</tr>
<tr>
<td>Severely dysplastic</td>
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<td>0/0</td>
</tr>
<tr>
<td>Carcinomas (total)</td>
<td>76/123</td>
<td>38/56</td>
</tr>
<tr>
<td>Dukes' classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1/2</td>
<td>0/0</td>
</tr>
<tr>
<td>B1</td>
<td>5/9</td>
<td>2/5</td>
</tr>
<tr>
<td>B2</td>
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<tr>
<td>C1</td>
<td>5/8</td>
<td>3/3</td>
</tr>
<tr>
<td>C2</td>
<td>23/42</td>
<td>8/14</td>
</tr>
<tr>
<td>D</td>
<td>12/20</td>
<td>10/14</td>
</tr>
<tr>
<td>Metastases (total)</td>
<td>15/22</td>
<td>12/16</td>
</tr>
</tbody>
</table>

* c-met gene overexpression was evaluated at RNA level with Northern blot analysis and at protein level with Western blot analysis; the score relative to normal tissues of the same patient or to average value of normal tissues was as follows: (+), 2-5-fold; (++), 5-20-fold; (+++), more than 20-fold increase. Metastases were compared to samples of normal liver, that shows the highest level of expression.

* nd, not determined.

* subclassified according to O'Brien et al. (57).

* Dukes' classifications are as modified by Astler and Coller (25): A, tumor restricted to the mucosa; B1, tumor penetrating into but not through the muscularis propria; B2, tumor penetrating through the bowel wall; C1, as A or B1, but with lymph node invasion; C2, as B2, but with lymph node invasion; D, any type of tumor with distant metastases.
expression was increased relative to normal tissues; the increase varied from 2- to 20-fold that of normal tissues. Adenomas showed overexpression irrespective to the degree of dysplasia. All of the adenomas were classified as tubulovillous. In Western blot analysis, the c-met receptor p145 β chain and the p170 precursor of the met/HGF receptor were labeled by mAbs raised against a synthetic peptide corresponding to the C-terminal tail of the human receptor. Bound antibodies were labeled with horse-radish peroxidase-conjugated rabbit anti-mouse immunoglobulins and revealed with enhanced chemiluminescence (Amersham Corp.).

Overexpression of the c-met gene in samples of colorectal adenomas might be the consequence of a very high expression in a few cells or the result of an increased number of receptor in all of the cells. To explore, immunohistochemical analysis of frozen sections was performed with mAbs against an extracellular epitope of the receptor. The staining was found to be homogeneously distributed among adenoma cells (not shown).

**Colorectal Carcinomas.** One hundred twenty-three samples of primitive colorectal carcinomas were obtained at surgery and snap frozen. In 99 cases, samples of normal adjacent mucosa were also available. Overexpression of the c-met gene was evaluated with either Western blot analysis (67 cases) or Northern blot analysis (56 cases). Table 1 summarizes data on expression ranging from 2 to 50 times that of adjacent normal tissues. These data agree with those previously reported by Prat et al. (14), in which immunohistochemical analysis showed that the receptor was expressed in normal colorectal tissues, but was homogenously increased in carcinoma cells.

Examples of the comparison between carcinomas and normal mucosa are seen in Figs. 2 and 3. Again, the c-met-encoded receptor detected in carcinomas with Western blot analysis had features like that of normal tissues. In Northern blot analysis the c-met transcript of 7.1 kilobases was the most abundant in all cases.

The primary colorectal carcinomas were divided into groups according to their local invasiveness and their tendency to spread to distant organs using Dukes' classification modified by Astler and Coller (Ref. 25 and Table 1). Statistical analysis was used to compare Dukes' B1, B2, and C2 tumor stage and to compare tumor grading to the level of c-met gene expression using Pearson's and Mantel-Haenszel $\chi^2$ tests and Pearson's and Spearman's coefficient of correlation. Overexpressing tumors were found to be distributed in all Dukes' classifications and to show varying degrees of differentiation.

Overexpression of the c-met gene observed in the colorectal carcinomas may be due to c-met gene rearrangement or amplification. To examine these hypotheses, Southern blot analysis was performed using a cDNA encompassing the entire c-met coding sequence as a probe. Of the 76 carcinomas over-expressing the c-met gene, 32 were analyzed and compared to the normal tissues taken from the same patients. The restriction patterns detected after EcoRI (Fig. 4) and HindIII digestion (not shown) of normal tissues and tumors were comparable. They differed only by the absence of a band in some of the tumor samples (Fig. 4). As the samples also contained inflammatory and stromal cells, the difference might be attributed to a different level of methylation of the c-met gene in different cells. This hypothesis is currently under investigation. No differences were observed by comparing the restriction pattern of tumor samples to that obtained after digestion of DNA from a cell line where the c-met cDNA was cloned and sequenced (12).

Of the 32 cases examined by Southern blot analysis, the c-met gene was amplified in 3. Fig. 4 shows one of these cases (case P69). Using laser densitometric scanning of X-ray films, the labeling intensity of the restriction fragments was quantified: in these three cases, the amount of c-met-specific DNA detected in the tumor was twice as much as in the normal tissue (not shown).

**Liver Metastases.** Twenty-five samples of liver metastases were obtained at surgery. Seventeen of these samples were obtained from liver metastases of patients having previously had colorectal carcinomas (metachronous metastases) removed. In this instance, samples of the primary tumors were not available. The other eight samples were from patients affected by colorectal carcinomas with liver metastases at diagnosis (synchronous metastases).

By comparing the metastases to the normal colonic tissues, c-met gene overexpression was found in 70% of the metastases available for either Northern or Western blot analysis (Fig. 2 and Table 1). Moreover, by comparing synchronous metastases to primary carcinomas, six of eight cases showed a level of ex-
expression in the metastasis significantly higher than that of the primary tumor.

Southern blot analysis was performed on three metachronous and six synchronous liver metastases. Patients who had received radiotherapy or chemotherapy were excluded. Eight of nine showed amplification of the c-met gene (Table 1); Fig. 4 shows a representative experiment. In a synchronous metastasis c-met gene amplification was already detectable in the primary carcinoma (Fig. 4, case P69).

The degree of amplification in individual cases was determined by dilution analysis of DNA followed by laser densitometric scanning (Fig. 5). DNA prepared from normal tissues was used for titration. The number of additional copies of the c-met gene ranged from 2 to 3. This value may have been underestimated because of the dilution of tumor cell DNA with DNA from normal tissues and stromal or inflammatory cells. As reported in the case of erbB-2 amplification in breast cancer (26), the extent of amplification detected by Southern blot analysis may have been also underestimated as a consequence of cell to cell variation in gene copy number.

An increased number of c-met gene copies could be ascribed to specific gene amplification or to chromosome 7 duplication. The c-met gene has been mapped to chromosome 7 and trisomy 7 has been reported as a numeric abnormality detectable in 40% of colorectal carcinomas analyzed after short-term culture (27). To further examine these two hypotheses, the filters hybridized with the c-met probe were subsequently labeled with a HGF probe, as the gene encoding HGF also maps
Fig. 4. Southern blot analysis of samples of colorectal carcinomas (ca) and liver metastases (m) compared to samples of normal colon mucosa (n) of the same patient. Numbers on the top of the lanes, different cases. Ten μg of DNAs were digested with the EcoRI restriction enzyme for 12 h at 37°C. A cDNA encompassing the entire met coding sequence, labeled with 32P, was used as a probe.

Fig. 5. Estimate of c-met gene copy number in a synchronous metastasis (case P37). Normal and metastasis DNAs were digested with EcoRI. 10 μg of the former and serial dilutions of the latter were loaded into agarose gels and blotted to filter. Amount loaded is indicated on the top. The filter was first hybridized with the 32P-labeled c-met probe (A) and then rehybridized with a 32P-labeled β-globin probe (B) to evaluate if comparable amounts of DNA were loaded into each lane. Right, densitometric scanning of the bands indicated by the asterisks. The absorbance is expressed in arbitrary units. Each peak represents the absorbance of the examined band, plotted separately for each of the lanes shown on the top.

to the long arm of chromosome 7 (28). Amplification of the HGF gene was not detected (data not shown), showing that the increased number of c-met gene copies in liver metastases is due to specific gene amplification.

Discussion

Colorectal carcinogenesis is a complex multistep process involving deletion of tumor suppressor genes and activation of dominant oncogenes. The genetic changes so far described include loss of potential tumor suppressor genes located on chromosome 5, identified as APC (29–32); on chromosome 18, described as DCC (3); on chromosome 17, where the p53 gene is located (33); and on chromosome 8 at a locus as yet unidentified (34). Activation of dominant oncogenes includes the most common point mutations of ras (35), amplification of cyclin genes (36), amplification of c-myc (37), overexpression and amplification of erbB-2 (38), and activation of cytoplasmic tyrosine kinases, such as pp60v-src (39) and pp61v-src (40). Recently, a new colon cancer gene, that appears to destabilize the genome, has been identified on chromosome 2 (41, 42). Each of these changes occur with varying frequencies in colorectal tumors. However, none of them has been clearly associated with the ability of tumor cells to metastasize.

Metastasization is, in turn, a complex multistep process involving a broad range of host-tumor cell interactions. Tumor cells have long been thought to acquire the metastatic phenotype...
through genetic and epigenetic changes arising during progression in the primary tumor. Among the alterations listed above, pp60c-src activity was found to be significantly increased in liver metastasis of colorectal carcinomas (39). This suggests that the tyrosine kinases signaling pathway might be relevant to the acquisition of the metastatic phenotype. Activation of the src family kinases is part of a cascade of signaling events triggered by growth factor receptors such as the platelet-derived growth factor receptor (43), the colony-stimulating factor 1 receptor (44), and the HGF receptor encoded by the c-met gene (45). As far as colorectal carcinomas are concerned, activation of a tyrosine kinase receptor gene by structural alteration seems so far to be an extremely rare event: only one single case of TRK gene rearrangement has been reported (46). In addition, data on overexpression of tyrosine kinase receptors, such as that for epidermal growth factor and the one encoded by erbB-2, are contradictory: increased levels of the protein (38, 47), but no increased levels of specific mRNAs (18), have been demonstrated. In this article, we show that the c-met gene that encodes a tyrosine kinase receptor is overexpressed at both protein and RNA levels with a high frequency in colorectal carcinomas.

Studies of human tumor cell lines and of c-met-transfected cells showed that the c-met gene may be activated by structural alterations such as gene fusion with unrelated sequences (48) or truncation of the extracellular and transmembrane domains (49). Activation may also be achieved via a defective posttranslational processing of the precursor protein (50). Overexpression of the c-met gene also activates its oncogenic potential. NIH3T3 cells become transformed and tumorigenic if the met gene is overexpressed following transfection with mouse met cDNA (51) on the establishment of an autocrine circuit. Overexpression associated with gene amplification of the mouse c-met gene has been observed in a high proportion of spontaneously transformed NIH3T3 mouse cell lines (52). Overexpression of the human c-met gene has been found in a human gastric carcinoma cell line (6); in this cell line overexpression of the c-met-encoded receptor leads to the constitutive activation of the tyrosine kinase signaling pathway.

Overexpression of the c-met oncogene in colorectal tumors has been previously reported by us and confirmed by others (13, 18). We now extend the study to a larger series, which include adenomas, carcinomas at different stages, and liver metastases. Overexpression of the met gene was detectable in more than 50% of lesions at each stage, including adenomas with different degrees of dysplasia. This suggests that c-met overexpression may contribute to the loss of growth control at the early stage. The lack of correlation between c-met overexpression and grading of the tumor, as well as between overexpression and staging, suggests once again that a single genetic or epigenetic alteration cannot be associated to a category of tumors classified according to classical histological criteria. The sum of molecular, histological, and clinical information might be combined in a “profile” of a single tumor that would be more helpful in predicting its outcome and in planning therapeutic strategies.

The study of several colorectal carcinomas showed that c-met gene overexpression is associated with gene amplification only in a few cases. This agrees with previous reports showing c-met amplification in a small fraction of colorectal carcinomas (53) and esophageal carcinomas (54). Similarly, we have described c-met gene overexpression without amplification in thyroid carcinomas derived from the follicular epithelium (15). However, c-met overexpression has been associated with gene amplification in gastric cancer (24, 53).

In colorectal carcinoma progression, c-met gene amplification seems to be a late event, occurring frequently only in metastases. This is intriguing, since a role of the HGF/SF and its receptor in the metastatic behavior of tumor cells has been proposed. In vitro HGF/SF is able to stimulate epithelial cells to invade collagen matrices (16). In addition, the expression of a functional c-met-encoded receptor is sufficient to transfer an invasive phenotype to transfected cells in the presence of HGF (17).

The analysis of synchronous metastases shows that c-met gene amplification reflects tumor progression and metastatic clone selection. These data suggest that, when “fixed” by gene amplification, c-met gene overexpression is an inheritable genetic alteration contributing to the metastatic phenotype of tumor clones. It has been shown that HGF is produced by nonparenchymal liver cells and might regulate adjacent hepatocyte proliferation during liver development (55, 56). This suggests that the growth factor might also support, via a paracrine interaction, homing and cloning of metastatic cells with an abnormally high number of met/HGF receptors in the liver.

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