c-MET Expression Level in Primary Colon Cancer: A Predictor of Tumor Invasion and Lymph Node Metastases

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ABSTRACT

Purpose: Both c-MET and vascular endothelial growth factor (VEGF)-C expression are important factors in primary carcinoma progression. We hypothesized that overexpression of c-MET and/or VEGF-C mRNA in primary colorectal cancer (CRC) can predict tumor invasion and regional metastasis.

Experimental Design: The level of c-MET and VEGF-C mRNA expression was assessed using a quantitative RT-RealTime PCR assay on early stage primary CRC tumors (n = 36).

Results: The c-MET mRNA copy number ranged from 1.18 × 10^2 to 1.11 × 10^6 copies (median 5.17 × 10^4) per 250 ng of RNA from CRC specimens. c-MET mRNA copies in CRC specimens were significantly higher than that from normal colon mucosal epithelium (P = 0.0001). c-MET mRNA copies significantly correlated with the depth of invasion: T1 versus T2, P = 0.007; T1 versus T3/T4, P = 0.0001; T2 versus T3/T4, P = 0.0005; and T1/T2 versus T3/T4, P = 0.011. c-MET copy number in primary CRC of N1/N2 staged patients was significantly higher than N0 cases (P < 0.03). Expression levels of c-MET mRNA were verified with immunohistochemistry analysis of c-MET protein expression in CRC specimens and normal mucosal epithelium. The VEGF-C mRNA copies of primary CRC assessed ranged from 0 to 1.65 × 10^6 copies (median 580). Although VEGF-C mRNA copies in CRC primary tumors were significantly higher than normal colon mucosal epithelium (P = 0.0008), it did not correlate with any major clinicopathological parameters of CRC.

Conclusions: This study indicates c-MET mRNA overexpression in primary CRC may be an important prognostic marker for early stage invasion and regional disease metastasis.

INTRODUCTION

Colorectal carcinoma is the second leading cause of cancer-related deaths and the most common gastrointestinal malignancy in the United States (1). The 5-year survival rate of American Joint Committee on Cancer stage I CRC3 is 90% and decreases to 75 and 50% for stage II and III patients, respectively. Tumor invasion and regional lymph node metastasis are important factors for determining CRC prognosis. Recent data from molecular studies have elucidated some of the mechanisms and sequence of tumor progression (2, 3). There is a paucity of molecular predicative indicators for regional disease invasion and metastasis.

c-MET, a proto-oncogene, has been suggested to be associated with CRC progression (4). The c-MET protein contains a tyrosine kinase domain that initiates a range of signals to regulate various cellular functions (5, 6). HGF/SF produced by stromal fibroblasts acts in a paracrine manner on cancer cells via the c-MET receptor (4). The activation of this receptor by HGF/SF ligand can induce proliferation, motility, adhesion, and invasion in tumor cells (7–9). HGF/SF activation of the c-MET tyrosine kinase pathway has been thought of as one of the key factors influencing the events of tumor progression. Several studies have reported amplification and overexpression of c-MET in cancer cells, including CRC (10–13). Overexpression of c-MET protein has been correlated with tumor progression and prognosis in breast cancer, gastric cancer, hepatocellular, endometrial, and nasopharyngeal carcinomas (14–18). We reported previously on the utility of c-MET as an RT-PCR marker for detecting micrometastasis of CRC in SLNs (19). c-MET mRNA expression assessed by RT-PCR was detected in 40% of SLNs (19). Umeki et al. (13) have reported that overexpression of c-MET mRNA is associated with greater tumor size in primary CRC but did not assess its prognostic value. However, c-MET gene expression levels in primary CRC have not been investigated for their clinicopathological utility. c-MET expression levels have been primarily studied under the confines of qualitative assays, such as RT-PCR or IHC, for detection of...
expression as compared with respective normal epithelium. However, with RealTime technology, specific mRNA copy analysis is now quantifiable, reproducible, and less labor intensive to perform.

The VEGF family members are known principal angiogenic factors (20). VEGF can specifically induce the proliferation and migration of endothelial cells (20). Moreover, VEGF enhances the vascular permeability of tumors that can facilitate entry of cancer cells into the blood circulation to metastasize to distant sites. A member of the VEGF family, VEGF-C is the ligand for VEGF receptor-3 described as a lymphoid endothelial receptor (20, 21). Induction of lymphangiogenesis by VEGF-C is demonstrated to enhance tumor metastasis via the lymphatic system in animal models (22, 23). VEGF-C overexpression and secretion have been found in various carcinomas. Overexpression of VEGF-C detected by IHC was correlated with lymphangiogenesis, lymph node metastasis, and a poorer prognosis in breast cancer, gastric cancer, and lung adenocarcinoma (24–26). However, the biological role of VEGF-C mRNA expression levels in primary CRC tumors still remains unknown.

We hypothesized that the enhanced levels of c-MET and/or VEGF-C in CRC primary tumor may predict tumor invasion and regional metastatic potential. Our recent development of qRT assay enabled us to compare c-MET and VEGF-C mRNA expression levels among CRC tumors accurately and quantitatively. To assess this problem, we developed a qRT assay for assessment of c-MET and VEGF-C mRNA expression as surrogates of clinicopathological status of CRC. A qRT assay predictive of early stage primary CRC invasion and regional node metastasis potential would have potential clinicopathological utility.

MATERIALS AND METHODS

Patients. Early stage CRC patients undergoing surgery were accrued from JWCI Saint Johns Health Center (Santa Monica, CA), McLaren Regional Medical Center (Flint, MI), and Century City Hospital (Los Angeles, CA). Thirty-six patients (median age, 72.5 years; range, 45–90; 17 men and 19 women), who underwent curative resection of the primary CRC between 1998 and 2000, were included in the study. All patients in the study were consented according to the guidelines set forth by JWCI and other centers human subjects Institutional Review Board committee. The SLNs, the first tumor-draining lymph nodes, assigned by JWCI and other centers according to the American Joint Committee on Cancer Tumor-Node-Metastasis classification. All CRC patients; Refs. 27–29), and reagent controls (reagent alone without RNA or cDNA) for qRT assay were included. Once the assay was established and optimized for individual markers, reproducibility patients’ specimens were run. Each assay was repeated at least twice to verify the results, and the mean copy number was used for analysis. SD between assays was not significant (<5%) for all markers studied.

The standard RealTime PCR curve was established for quantifying mRNA copy number by using nine known copy numbers of serially diluted (10^4 to 10^8 copies) plasmids containing c-MET, VEGF-C, GAPDH, or β2MG cDNA. Each cDNA was synthesized by RT-PCR and extracted from 2% agarose gels using the QIAquick gel extraction method (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The
cDNA was ligated into pCR II-TOPO cloning vector (Invitrogen, San Diego, CA), the cDNA clones were transformed into *Escherichia coli* DH5α cells, and cultures were expanded as described previously (30). Plasmids containing the target gene were purified and quantified for use in the qRT setup. To confirm that the inserted PCR product size was correct, plasmids were digested with specific restriction enzymes, and cDNA clone PCR products were then run on gel electrophoresis.

**IHC.** Expression of c-MET in CRC cells was assessed by IHC. Specimens were fixed in 10% formalin and paraffin embedded by conventional techniques. Freshly cut 5-μm sections were deparaffinized in xylene, and the slides were bathed in 0.01M sodium citrate and heated in a microwave oven for 12 min. The sections were incubated with polyclonal rabbit anti-human c-MET antibody (Zymed Laboratories, South San Francisco, CA) at a dilution of 1:200 and kept at 4°C overnight. Pathologically verified metastatic malignant melanoma specimens were used as positive control (31). Negative control slides were treated with nonimmunized rabbit immunoglobulin fraction only under equivalent conditions. For the secondary developing reagents, a labeled streptavidin-biotin kit (DAKO, Carpinteria, CA) was used. Slides were developed with diaminobenzaminidine and counterstained with hematoxylin. The specimens were evaluated independently by two of the authors (H. T. and M. T.) in a blind fashion without previous knowledge of the qRT results on c-MET mRNA expression. The IHC results for c-MET were arbitrarily classified into four scores dependent on the intensity of immunoreactivity: (a) −, negative immunostaining; (b) ±, very weak immunostaining; (c) +, medium positive immunostaining; and (d) ++, strongly positive immunostaining.

**Statistical Analysis.** To investigate the association between the c-MET and VEGF-C mRNA expression and clinicopathological parameters, ANOVA and Student’s *t* test with log transformations were used. Spearman correlation coefficient analysis was used to assess the relationship among each marker mRNA expressions. All *P* values, which were two sided at a value of 0.05, were considered statistically significant.

**RESULTS**

**c-MET mRNA Expression.** Serially diluted c-MET cDNA was used to construct a standard curve. The qRT amplification of the serially diluted c-MET cDNA plasmid showed a logarithmic signal increase (Fig. 1). The standard curve was generated by using the Ct of the plasmid controls, at which the fluorescence signal of the reporter dye rose above the baseline. The Ct of each sample was plotted on the standard curve, and the mRNA copy number was calculated. Initially, we assessed c-MET mRNA levels in CRC lines to establish and optimize the assay. The c-MET mRNA copy level in three established CRC cell lines (HT29, SW480, and JWCISP) were 4.51 × 10^6, 2.98 × 10^6, and 1.78 × 10^6 (mean 3.09 × 10^6 ± 1.37 × 10^6), respectively. As controls, normal donor peripheral blood lymphocytes were assessed. The mean c-MET mRNA copies were insignificant in normal lymphocytes (8.5 ± 3.3/250 ng of total RNA).

The c-MET mRNA expression was detectable by qRT in 36 of 36 (100%) CRC specimens. The c-MET mRNA copies ranged from 118 to 1.11 × 10^6 (median 51,700 copies) per 250 ng of total RNA from CRC specimens (Table 1). The c-MET mRNA copies in CRC specimens were significantly higher than that from eight specimens of normal colon mucosal epithelium.
We then evaluated the significance of c-MET mRNA copy numbers as a surrogate of primary CRC tumor invasion, T level. The median c-MET mRNA copy numbers per 250 ng of RNA in primary CRC for individual pathological T levels were as follows: (a) T1 was 5,275 copies; (b) T2 was 6.145 × 10^4 copies; and (c) T3/T4 was 1.415 × 10^5 copies. The c-MET mRNA copy numbers significantly correlated with the depth of tumor invasion (T level): (a) T1 versus T2, P = 0.007; (b) T1 versus T3/T4, P = 0.0001; (c) T1 versus T2 versus T3/T4, P = 0.0005; and (d) T1/T2 versus T3/T4, P = 0.011 (Tables 1 and 2).

We next assessed primary tumor c-MET mRNA copies to determine whether they could predict lymph node pathology status (N level). The median c-MET mRNA copy numbers per 250 ng of RNA for pathological N levels were as follows: (a) N0 was 3.53 × 10^2 copies; and (b) N1/N2 was 7.08 × 10^4 copies. c-MET mRNA copy numbers in N1/N2 cases were significantly higher than N0 (P = 0.026; Tables 1 and 2). For patients without lymph node metastasis, the c-MET mRNA copy number was still significantly different among the three T groups (overall T groups analysis by ANOVA; P = 0.024): (a) T1/N0 was 5,275 copies (median); (b) T1/N1 was 7.815 × 10^4 copies; and (c) T1/N2 was 1.30 × 10^5 copies. Significant differences were also found between T1/N0 and T1/N1 (P = 0.034) and between T1/N0 and T1/T2/N0 (P = 0.012). The c-MET mRNA expression did not correlate with the tumor site and differentiation.

We assessed the expression of two internal reference genes, GAPDH and β2MG, using a qRT assay. All CRC specimens were positive for GAPDH and β2MG mRNA verifying the integrity of the RNA in the tissue specimens. The GAPDH mRNA copy number ranged from 1.64 × 10^4 to 5.79 × 10^6 (median 6.76 × 10^5 copies), and the β2MG mRNA copy number ranged from 1.24 × 10^3 to 2.98 × 10^6 copies (median 4.79 × 10^5 copies) per 250 ng of total RNA from primary CRC. Spearman correlation coefficient analysis indicated a positive relationship between GAPDH and β2MG mRNA expressions (correlation coefficient 0.789, P < 0.0001). However, the copy number of these two reference genes mRNA was not always synchronous, and the GAPDH:β2MG mRNA ratio of primary CRC varied from 0.4 to 890 (mean ratio 31.01). In particular, β2MG mRNA copy numbers in T3/T4 cases (mean 1.78 × 10^5 copies) had a tendency to be lower than that in T1/T2 cases (mean 4.44 × 10^5 copies; P = 0.16).

**IHC Analysis of c-MET Protein Expression.** IHC was performed on respective primary CRC in which reverse transcriptase was performed to determine concordance of the results. Analysis of an individual marker mRNA expression by RT-PCR does not always correlate with the respective marker protein expression. There are many factors, such as translation efficacy, post-translational modification, and protein half-life. IHC results for c-MET protein in CRC specimens, and normal mucosal epithelium was evaluated (Table 3; Fig. 2). A negative or very weak immunostaining for c-MET protein was observed in normal epithelial cells of the normal colon mucosal sections. In contrast, primary CRC cells showed a variety of immunore-
activity in the cytoplasm. CRC tumors with low c-MET mRNA copy number had a tendency to display weak immunostaining, whereas CRC tumors with high c-MET mRNA copies demonstrated strong immunostaining. The studies overall showed a very good correlation between the two assays. This indicated that analysis of quantitative levels of c-MET mRNA is informative and representative of c-MET protein expression.

VEGF-C mRNA Expression. A qRT assay for VEGF-C mRNA expression was established and optimized using CRC cell lines before development of an assay for CRC tumor specimens. The VEGF-C mRNA expression was detectable by qRT in 32 of 36 (89%) CRC specimens and in two of the three CRC cell lines. The VEGF-C mRNA copy number ranged from 0 to 2.75 × 10^3 (median, 580 mRNA copies) per 250 ng of total RNA from CRC specimens was significantly higher than that from normal colon mucosal epithelium (normal colon mucosal epithelium versus CRC tumors, P = 0.0008; normal colon mucosal epithelium versus T1 tumor, P = 0.002; Table 2). The VEGF-C mRNA expression did not correlate with any major clinicopathological parameters, differentiation states, T level, or N level of primary CRC. Spearman correlation coefficient did not reveal significant correlation between c-MET and VEGF-C mRNA copy levels (correlation coefficient 0.11, P = 0.52).

**DISCUSSION**

High levels of c-MET expression (IHC) have been correlated with metastatic spread of tumors and disease outcome in multiple tumors (4). However, the analysis of primary CRC has been controversial as to the role of c-MET expression in relation to early stage invasion and metastasis. In this study, we approached this problem by developing and using the qRT assay on early stage primary CRC. The qRT assay is a rapid and reproducible quantitative approach with high sensitivity and specificity. Moreover, the assay does not require tedious methodologies to determine the quantity of PCR products, as RT-PCR methodologies reported previously. To evaluate c-MET expression roles in early stages of primary CRC growth, a rigorous nonsubjective quantitative assay was developed. The overall study demonstrated that qRT can be used in place of IHC as a predictive factor to evaluate primary CRC potential outcome.

Recent studies using a qRT assay have regarded the Ct as a value of the mRNA quantity without cDNA copy number standardization. However, the value of Ct is subtly changeable and not reproducible depending on the conditions of a thermal cycler and reagents. This can be misleading in interpreting data and comparing assay results. The standardization of the assay using known cDNA copy numbers serially diluted produced a more accurate account of copy numbers versus a relative value. In other studies using a qRT assay, the correction of the targeted mRNA quantity by an internal reference gene has been performed, and the ratio between targeted mRNA and an internal reference housekeeping gene mRNA was generally used (32). However, our results showed that mRNA expression of the two commonly used internal reference genes, GAPDH and β2MG, in CRC tumors did not always show similar trends. In addition, because of the heterogeneity in tumor tissues, the expression of the internal reference housekeeping genes varies across tissues. The results obtained with the adjustments may be skewed by the selection of the internal reference gene. For these reasons, we used an absolute value of the mRNA copy number per defined amount of RNA without any correction by an internal reference gene to analyze the correlation with clinicopathological parameters (33, 34). Few studies have documented on the variability of known housekeeping gene expression relative to the pathological state in the specimens. The study demonstrates the variability of expression of specific housekeeping genes. Loss of β2MG mRNA expression has been reported to be found in primary CRC tumors (35–37).

Expression of c-MET mRNA and protein in CRC has been reported with frequencies between 30 and 91% in Northern blot and RT-PCR assays, respectively, and between 57 and 100% by Western blot and IHC, respectively (10–13, 38, 39). Our studies demonstrated a significantly higher expression of c-MET mRNA and protein in CRC tumor compared with the expression in corresponding normal colon mucosa. The HGF/SF ligands have been reported to induce various morphogenetic responses in the development of CRC (4). Tumorigenic activity of c-MET depends on deregulation of HGF/c-MET signaling pathway. Constitutive activation of tyrosine kinase receptors can be obtained by c-MET overexpression and activation by ligand (4). Not only does constitutive activation of c-MET induce cellular transformation but it can also enhance tumor cell migration and invasion. Moreover, HGF/SF triggers destabilization of adherent junctions by transcriptional down-regulation of cadherins (4). HGF/SF also enhances the transcriptional levels of matrix metalloproteinase. Collectively, these events facilitate tumor cell invasion and metastasis ability. In addition, HGF/SF increases the adherence of c-MET-positive cancer cells to the extracellular matrix via integrins (40).

In CRC cell lines, highly metastatic cell lines showed increased c-MET mRNA expression (41). Cell lines are not representative of in vivo tissues. However, our results, which demonstrated that the overexpression of c-MET significantly increases with CRC primary tumor depth of invasion and lymph

### Table 3 Immunohistochemical analysis for c-MET mRNA expression

<table>
<thead>
<tr>
<th>Specimen</th>
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<th>TN factor</th>
<th>mRNA copies</th>
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<td>Normal colon epithelium</td>
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<td>Primary CRC</td>
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a Tumor site: A, ascending colon; C, cecum; T, transverse colon; S, sigmoid colon; R, rectum.
b Immunostaining scale: –, no immunostaining; ±, very weak immunostaining; +, medium positive immunostaining; ++, strongly positive immunostaining.
node metastasis, were compatible with other investigations in vitro. Our results indicated a significant increase of c-MET mRNA copy number between T1 and T2, suggesting that c-MET overexpression plays an important role in aggressive proliferation and development of regional metastatic potential in early stage of CRC development. Recent investigations also have demonstrated that c-MET protein expression correlated with tumor size and stage classification in patients with bladder carcinoma (42). These studies suggest the clinicopathologic utility of c-MET in early stages of carcinoma progression and as a potential target for preventative treatment of early stage disease. There was no correlation between c-MET copy number and histological differentiation, although it is known that, in general, poorly differentiated tumors are associated with in-

Fig. 2 Representative immunohistochemical staining for c-MET protein in paraffin-embedded specimens of primary CRC. A, negative staining with focally very weak immunoreactivity (c-MET mRNA 1.34 × 10^3 copies). B, medium positive immunostaining (c-MET mRNA 8.17 × 10^4 copies). C, strongly positive immunostaining (c-MET mRNA 2.44 × 10^5 copies). D, normal colon epithelium. E, negative control. Photograph magnification, ×200.
increased nodal involvement in CRC. The study findings may be attributable to several factors, such as sample size assessed, variable classification of individual cell differentiation states within the tumor, heterogeneity of tumor cells within the tumor, and/or adjacent tissue influence.

One of the major questions that comes up in assessing mRNA copy numbers of a gene is the relative association with respective protein expression in a specimen. The mRNA copy number of a gene does not always correlate with the expression of protein product because of the variant post-transcription events, such as mRNA modification, translation efficacy, half-life of the mRNA, translation modification, and protein turnover in the cell. Our results demonstrated that c-MET mRNA copy number detected by a qRT assay in general correlated well with c-MET protein expression. The studies indicated that a qRT assay can be used as a surrogate for IHC analysis. The qRT assay for c-MET showed concordance with both normal mucosal epithelial cells and primary CRC.

VEGF-C mRNA expression was significantly increased in primary CRC compared with normal mucosal epithelium. However, to our surprise, there was no correlation between VEGF-C mRNA expression and tumor depth or lymph node metastasis. VEGF-C protein expression has been reported to correlate with lymphatic vessel invasion and lymph node metastasis in several types of carcinomas (24–26). The overall association of elevated VEGF-C expression with CRC tumors remains controversial. Akagi et al. (43) reported that the expression of VEGF-C correlated with lymph node metastasis using IHC analysis of CRC. However, other investigations demonstrated that lymph node metastasis did not correlate with VEGF-C but with VEGF-A mRNA expression (44). Most of the studies assessing VEGF-C expression have used IHC for VEGF-C protein analysis in tumor cells in vivo. Additional clarification of the regulatory functions of VEGF-C in CRC is required. Moreover, induction of lymphangiogenesis and lymph node metastasis involve multiple factors, not only the VEGF-C family of genes but also other tumor-related genes (45, 46). One could hypothesize as a tumor reached a larger size the development of both lymphatic and blood vessels would increase. The development of lymphatic vessels may occur at specific stages as a tumor develops and not be directly proportional to tumor growth. The development of blood and lymphatic vessels involves multiple factors produced by tumor cells. VEGF-C expression in tumors may be one of several factors that promote tumor metastasis but not a predominant factor in facilitating metastasis as many have thought.

HGF stimulation has shown to enhance VEGF-A mRNA expression in renal tubular cell (47). However, the association of HGF/c-MET pathway with VEGF-C mRNA expression has not yet been defined in any carcinomas. In this study, we could not find any association between c-MET and VEGF-C mRNA expression. This study demonstrates c-MET overexpression, which suggests the biological behavior of primary CRC is independent from VEGF-C mRNA expression.

In summary, we have demonstrated c-MET and VEGF-C mRNA expression of individual CRC tumors using a qRT assay. The study demonstrated c-MET mRNA overexpression as a potential prognostic marker associated with T and N factors. VEGF-C mRNA expression was significantly increased in CRC tumors compared with normal mucosal epithelium; however, VEGF-C mRNA expression did not correlate with any clinicopathological parameters in CRC. To our knowledge, this is the first study to determine c-MET mRNA expression level of primary CRC is predictive of T and N levels. The molecular classification for c-MET expression of primary CRC may be a useful prognostic indicator for invasion and regional lymph node metastasis. Additional studies on biological behavior of the CRC tumors expressing c-MET may allow the development of new targeted therapy inhibiting the c-MET signal pathway.
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