Association Between c-Met and Lymphangiogenic Factors in Patients With Colorectal Cancer

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Purpose: Animal models show a strong relationship between lymphangiogenesis and lymph node metastasis. However, the clinical significance of lymphangiogenesis in patients with colorectal cancer (CRC) remains uncertain. This study aimed to evaluate the association between c-Met and lymphangiogenic factors and to elucidate the prognostic significance of c-Met in patients with CRC.

Methods: A total of 379 tissue samples were obtained from surgically resected specimens from patients with CRC at Soonchunhyang University Cheonan Hospital between January 2002 and December 2010. The expressions of c-Met, vascular endothelial growth factor (VEGF)-C, VEGF-D, VEGF receptor (VEGFR)-3, and podoplanin were examined using immunohistochemistry. The expression of c-Met and clinical factors were analyzed.

Results: Of the 379 tissues, 301 (79.4%) had c-Met expression. High expression of c-Met in tumor cells was significantly associated with high expression of VEGF-C (P < 0.001) and VEGFR-3 (P = 0.001). However, no statistically significant association with podoplanin (P = 0.587) or VEGF-D (P = 0.096) was found. Of the 103 evaluable patients, expression of c-Met in tumor cells was significantly associated with advanced clinical stage (P = 0.020), positive lymph node status (P = 0.038), and high expression of VEGF-C (P = 0.020). However, no statistically significant association with podoplanin (P = 0.518), VEGFR-3 (P = 0.085), VEGF-D (P = 0.203), or overall survival (P = 0.360) was found.

Conclusion: Our results provide indirect evidence for an association and possible regulatory link of c-Met with the lymphangiogenic markers, but c-Met expression in patients with CRC is not a prognostic indicator for overall survival.

Keywords: Lymphangiogenesis; c-Met; Colorectal neoplasms

INTRODUCTION

Colorectal cancer (CRC) is a major health problem and one of the leading causes of cancer-related death worldwide [1]. Patients with lymphatic invasion have a less favorable outcome, and lymph node metastasis is a very important prognostic factor in CRC [2]. Although the pattern of spread of CRC may vary, the initial step involves lymphatic invasion and metastasis to regional lymph nodes [3].

Meanwhile, several growth factors have been found to contribute to lymphangiogenesis in solid tumors; these include vascular endothelial growth factor (VEGF) C, VEGF-D, VEGF receptor-3 (VEGFR-3), podoplanin, and c-Met [4]. VEGF-C and -D have
been identified as specific lymphangiogenic factors that act via activation of VEGFR-3, which is expressed in lymphatic endothelial cells [5]. Hepatocyte growth factor (HGF) is a heparin-binding glycoprotein produced by various cells of mesenchymal origin. In vivo studies have shown that HGF plays an important role in tissue repair and promotes tumor invasiveness [6]. c-Met, as the receptor of HGF, was found to be overexpressed in various types of tumors, mediating its multiple roles, such as promoting tumor cell growth, including tumor cell invasion, and stimulating angiogenesis [7].

Recently, in experimental cancer metastasis models, a growing amount of evidence has been found that tumor lymphangiogenesis may further facilitate tumor metastases. However, the clinical significance of lymphangiogenesis in patients with CRC remains uncertain. The aim of this study was to evaluate the association between c-Met and lymphangiogenic factors and to elucidate the prognostic significance of c-Met in patients with CRC.

METHODS

Three hundred seventy-nine patients with CRC, who were diagnosed and surgically treated at Soonchunhyang University Hospital between January 2002 and December 2010, were enrolled in this study. The clinical variables, including sex, age, and tumor stage were all obtained preoperatively. Surgical specimens were evaluated for histopathologic staging. The patients were classified according to the 6th edition of the American Joint Committee on Cancer Staging System [8]. The expressions of c-Met, VEGF-C, VEGF-D, VEGFR-3, and podoplanin were examined by using immunohistochemistry (IHC). The expression of c-Met and the clinical factors was analyzed. Our study was approved by the Clinical Ethics Review Committee at Soonchunhyang University Hospital, Cheonan, Republic of Korea (approval number: 2015-08-023). Written informed clinical consent was obtained from all the patients.

For construction of the tissue microarrays (TMAs), areas representative of cancer were marked on slides stained with hematoxylin and eosin (H&E), and TMAs were constructed. TMAs were created from formalin-fixed (10% neutral buffered formalin), paraffin-embedded tissues by using a 2-mm diameter punch (UNITMA, Unitech Science, Seoul, Korea). TMA blocks were assembled by getting duplicate cores from one patient block and re-embedding the 2 cores in an arrayed recipient block (UNITMA, Unitech Science, Seoul, Korea). TMA blocks were assembled from formalin-fixed (10% neutral buffered formalin), paraffin-embedded tissues by using a 2-mm diameter punch (UNITMA, Unitech Science, Seoul, Korea). TMA blocks were assembled by getting duplicate cores from one patient block and re-embedding the 2 cores in an arrayed recipient block (UNITMA, Unitech Science, Seoul, Korea). A TMA block contains 60 cores from 30 samples.

In the preparation for IHC staining, the TMAs were sectioned at 4-micron intervals, deparaffinized three times in xylene for 30 minutes, dehydrated with graded alcohol (100% ethyl alcohol for 5 minutes, 95% ethyl alcohol for 3 minutes, and 75% ethyl alcohol for 3 minutes), and then heated in antigen-retrieval solution (sodium citrate, pH 6.0) in a microwave for 20 minutes. Sections were incubated in H2O2 for 10 minutes at room temperature. Next, the sections were incubated with 150 mL of the primary antibody of c-Met (1:50, AbFrontier, Seoul, Korea, #LF-PA20708), VEGF-C (1:200, R&D systems, Minneapolis, MN, USA, #AF752), VEGF-D (1:100, Abcam plc, Cambridge, UK, #ab103685), VEGFR-3 (1:50, Abcam plc, #ab72240), and podoplanin (1:200, ReliaTech GmbH, Wolfenbüttel, Germany, #101-M41), at 4°C overnight. The sections were then washed in phosphate buffered saline (PBS) buffer three times for 3 minutes, treated with 150 mL of secondary antibody for 1 hour at room temperature, and stained with DAB solution (Dako, Carpinteria, CA, USA). The sections were then washed in PBS buffer for 10 minutes. Finally, the sections were counterstained with hematoxylin for 3 minutes at room temperature, washed in distilled water 3 times for 3 minutes, and mounted on coverslips.

For the IHC analysis, the c-Met, VEGF-C, VEGF-D, VEGFR-3, and podoplanin stained tissue cores were examined by 2 independent pathologists, and a consensus score was determined for each specimen. A positive reaction for both antibodies was scored into 4 grades according to the intensity of the staining: 0, 1+, 2+, and 3+. The percentages of positive cells were also scored into four categories: 0 (0%), 1 (1%–33%), 2 (34%–66%), and 3 (67%–100%). The final score, calculated as the product of the intensity and the percentage score, was classified as follows: 0 for negative, 1–3 for weak, 4–6 for moderate, and 7–9 for strong. Then, we re-categorized negative & weak positive into low c-Met expression and moderate & strong positive into high c-Met expression.

For the statistical analysis, the correlations between c-Met and lymphangiogenic factors was evaluated by using the chi-square or Fisher exact test. The Kaplan-Meier method was used to generate overall survival curves, and differences between cohorts were tested using log-rank statistics. All P-values quoted were 2-sided, and P < 0.05 was considered statistically significant. All the analyses were performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The median age of the 379 patients with CRC was 55 years (range, 24–88 years). By sex, 219 (57.8%) were male, and 160 (42.2%) were female patients. On the c-Met expression profiles, c-Met immunohistostaining positivity was observed in the cytoplasm and the cell membrane as brown staining (Fig. 1). Cases with high c-Met expression outnumbered cases showing low c-Met expression (191 cases (50.4%) of high expression and 188 cases (49.6%) of low expression). The percentages of positive cells were also scored into four categories: 0 (0%), 1 (1%–33%), 2 (34%–66%), and 3 (67%–100%). The final score, calculated as the product of the intensity and the percentage score, was classified as follows: 0 for negative, 1–3 for weak, 4–6 for moderate, and 7–9 for strong. Then, we re-categorized negative & weak positive into low c-Met expression and moderate & strong positive into high c-Met expression.

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expression of c-Met in tumor cells was significantly associated with advanced clinical stage ($P = 0.020$), positive lymph node status ($P = 0.038$), and high expression of VEGF-C ($P = 0.020$). However, no statistically significant association with podoplanin ($P = 0.518$), VEGFR-3 ($P = 0.085$), VEGF-D ($P = 0.203$), or overall survival ($P = 0.360$) was found (Fig. 2, Table 4). We also performed survival analyses after having categorized c-Met was as high and low expression and found no correlation with survival ($P = 0.805$) (Fig. 3).

**DISCUSSION**

In a variety of human cancers, tumor metastasis to regional lymph nodes represents the first step of dissemination and serves as a prognostic implication. The extent of lymph node metastasis is a critical determinant for cancer staging and prognosis, which often guides therapeutic decisions. However, in patients with CRC, the molecular mechanism of lymphatic metastasis is not completely understood, and the role of the c-Met signaling pathway has not been fully elucidated. With this theoretical background, we investigated the expressions of c-Met and several lymphangiogenic factors (VEGF-C, VEGF-D, VEGFR-3, podoplanin) in surgical specimens from 379 patients with CRC to evaluate their clinical significance and the associations between c-
Met and lymphangiogenic factors.

Forced expression of VEGF-C in xenografts and in transgenic tumors results in tumor lymphangiogenesis and increased tumor dissemination to regional lymph nodes [9-11]. In another study, the inhibition of the VEGFR-3 pathway, either VEGF-C/D trap or VEGFR-3 blocking antibodies suppressed approximately 60%–70% of lymph node metastasis in a variety of experimental tumor models [12-15]. In this study, we observed that a high expression of c-Met in tumor cells was significantly associated with high expressions of VEGF-C (P < 0.001) and VEGFR-3 (P = 0.001). From this point of view, we believe that the results from animal studies have been proven by our study using clinical specimen, although we found no correlations with podoplanin and VEGF-D.

c-Met is overexpressed in a variety of carcinomas, including breast, lung, gastric and colon carcinomas [16-18]. In previous reports, the proportion of c-Met expression was 78%. Similar results were found in the present study; c-Met expression in patients with CRC was 79.4% (Table 1). Also, high levels of c-Met and/or HGF expression have been associated with poor survival outcome in a variety of carcinomas, including CRC [17, 19]. However, contrary to our expectation, the present data showed that c-Met expression was not associated with CRC prognosis (P = 0.360) (Fig. 2). This finding may have been due to the fact that our investigation included more patients than the previous studies did [17, 19]. If the correlation of prognosis with c-Met expression is to be accurately determined, an additional study with a large cohort of patients in

### Table 2. Association between clinicopathological features and c-Met expression

| Clinicopathological factors          | c-Met Positive (n = 301) | c-Met Negative (n = 78) | Total | P-value |
|--------------------------------------|-------------------------|-------------------------|-------|---------|
| Median age (yr)                      |                         |                         |       |         |
| 55                                   |                         |                         |       |         |
| 56                                   |                         |                         |       |         |
| Sex                                  |                         |                         |       |         |
| Male                                 | 190 (63.0)              | 29 (36.8)               | 219 (57.8) | 0.191       |
| Female                               | 111 (37.0)              | 49 (63.2)               | 160 (42.2)          |
| pT stage                             |                         |                         |       |         |
| 1                                    |                         |                         |       |         |
| 56 (18.5)                            | 8 (10.5)                | 64 (16.9)               |       | 0.120   |
| 2                                    |                         |                         |       |         |
| 212 (70.3)                           | 59 (75.4)               | 271 (71.5)              |       |         |
| 3                                    |                         |                         |       |         |
| 33 (11.2)                            | 11 (14.1)               | 44 (11.6)               |       |         |
| pN stage                             |                         |                         |       |         |
| 0                                    |                         |                         |       | 0.143   |
| 100 (33.3)                           | 33 (42.2)               | 133 (35.1)              |       |         |
| 201 (66.7)                           | 45 (57.8)               | 246 (64.9)              |       |         |
| Vascular invasion                    |                         |                         |       | 0.252   |
| Absent                               | 256 (85.2)              | 66 (84.2)               | 322 (85.0)          |
| Present                              | 45 (14.8)               | 12 (15.8)               | 57 (15.0)            |
| Lymphatic invasion                   |                         |                         |       | 0.204   |
| Absent                               | 233 (74.1)              | 56 (71.9)               | 289 (76.3)          |
| Present                              | 68 (25.9)               | 22 (28.1)               | 90 (23.7)            |
| Metastasis                           |                         |                         |       | 0.452   |
| 0                                    | 290 (96.3)              | 75 (96.5)               | 365 (96.3)          |
| 1                                    | 11 (3.7)                | 3 (3.5)                 | 14 (3.7)             |
| TNM stage                            |                         |                         |       | 0.143   |
| I, II                                | 100 (33.3)              | 33 (42.1)               | 133 (35.1)          |
| III, IV                              | 201 (66.7)              | 45 (57.9)               | 246 (64.9)          |

Values are presented as number (%).

### Table 3. Association between c-Met and lymphangiogenic markers

| Marker          | c-Met Low expression (L) | c-Met High expression (H) | Total | P-value |
|-----------------|--------------------------|---------------------------|-------|---------|
| VEGF-C          | L                        | 159                       | 130   | 289     |
|                 | H                        | 29                        | 61    | 90      |
| VEGF-D          | L                        | 146                       | 134   | 280     |
|                 | H                        | 42                        | 57    | 99      |
| VEGFR-3         | L                        | 181                       | 166   | 347     |
|                 | H                        | 7                         | 25    | 32      |
| Podoplanin      | L                        | 118                       | 125   | 243     |
|                 | H                        | 70                        | 66    | 136     |

VEGF: vascular endothelial growth factor; VEGFR, VEGF receptor.

![Fig. 2. Kaplan-Meier survival curves for c-Met expression (positive vs. negative) in patients with colorectal cancer (n = 103). No statistically significant association with overall survival was observed (P = 0.360).](image-url)
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whom c-Met expression was observed is necessary. The limitations of the present study include its retrospective design. The study could have been affected by potential selection bias. For example, c-Met did not correlate with tumor stage (n = 379); however, it did correlate with tumor stage (n = 103) in patients for whom a survival analysis was performed. Another limitation is the lack of cancer-specific survival. In conclusion, the major finding of this study was that indirect evidence exists for an association and possible regulatory link of c-Met with the lymphangiogenic markers. Nevertheless, c-Met expression in patients with CRC is not a prognostic indicator for overall survival.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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