Expression and significance of Tie-1 and Tie-2 receptors, and angiopoietins-1, 2 and 4 in colorectal adenocarcinoma: Immunohistochemical analysis and correlation with clinicopathological factors

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AIM: There is strong evidence that tyrosine kinases are involved in the regulation of tumor progression, cellular growth and differentiation. Recently, many kinds of tyrosine kinase receptors have been reported, among them Tie-1 and Tie-2 receptors constitute a major class. Angiopoietin (Ang)-1 is known as a ligand of Tie-2 tyrosine kinase receptor. The objective of this study was to establish a comprehensive Tie-1 and Tie-2 and Ang-1, 2 and 4 expression profile in human colorectal adenocarcinomas.

METHODS: We examined 96 cases of surgically resected human colorectal adenocarcinoma by immunohistochemistry and investigated the statistical correlation between the expressions of Ties and Angs and clinicopathological factors.

RESULTS: Among the 96 cases of adenocarcinoma, 87 (90.6%), 92 (95.8%), 83 (86.5%), 89 (92.7%), and 76 cases (79.2%) showed positive staining in the cytoplasm of carcinoma cells for the Tie-1 and Tie-2 and Ang-1, 2 and 4 proteins, respectively. Histologically, the expressions of Ties and Angs were variable. The expressions of Ties and Angs were correlated with several clinicopathological factors, but did not correlate with the presence of lymph node metastasis. Ties and Angs were highly expressed in human colorectal adenocarcinoma cells.

CONCLUSION: These findings suggest that the Tie-Ang receptor-ligand complex is one of the factors involved in the cellular differentiation and progression of human colorectal adenocarcinoma.
The angiopoietin family of growth factors has been recently identified as ligands for Tie-2. Angiopoietin (Ang)-1 activates Tie-2 leading to receptor autophosphorylation upon binding and stimulates endothelial cell migration in vitro\cite{13,14}. Ang-2 appears to be a natural inhibitor of Tie-2 function, binding to Tie-2 with an affinity similar to that of Ang-1 and blocks Ang-1-stimulated receptor phosphorylation in endothelial cells\cite{13,15}. Deletion of the Ang-1 gene\cite{16} or overexpression of Ang-2\cite{18} in transgenic mice results in death in utero due to a broad failure of microvascular morphogenesis similar to that observed in Tie-2 knock-out mice\cite{17}.

The objective of the present study was to evaluate the role of Ties and Angs in the progression and differentiation of human colorectal adenocarcinoma.

**MATERIALS AND METHODS**

**Cases and tissues**

We studied 11 colorectal adenomas and 96 primary human colorectal adenocarcinomas. All specimens of adenoma were resected by endoscopy and all specimens of adenocarcinoma were obtained from patients operated on at Nagasaki University Hospital between 1999 and 2003. Each tumor was assigned a histological type according to the World Health Organization classification\cite{18} and a depth grading of infiltration according to the TNM staging system by the American Joint Commission on Cancer\cite{19}. Fifteen specimens of normal colon mucosal tissue, that were taken from patients without colorectal cancer, were evaluated as the normal control.

The desmoplastic stromal reaction was graded according to the extent of the stromal area involved. It was defined as “slight” (when the fibrous stromal area was less than 25% of the whole tumor), “moderate” (between 25% and 50%), and “extensive” (when it exceeded 75% of the whole tumor) based on the overall pattern\cite{19}. The examination was performed on routine slides to identify lymphatic, venous and perineural invasions. In addition to hematoxylin and eosin staining, we also used elastic van Gieson staining for the invasion was identified with certainty, but defined as “absent” when either was not observed at all or not observed with certainty\cite{20,21}. Lymph node metastasis was defined as “present” only when it was histologically proven. The desmoplastic stromal reaction was graded according to the extent of the stromal area involved. It was defined as “slight” (when the fibrous stromal area was less than 25% of the whole tumor), “moderate” (between 25% and 50%), and “extensive” (when it exceeded 75% of the whole tumor) based on the overall pattern\cite{19}. The examination was performed on routine slides to identify lymphatic, venous and perineural invasions. In addition to hematoxylin and eosin staining, we also used elastic van Gieson staining in all cases. Each parameter was defined as “present” when the invasion was identified with certainty, but defined as “absent” when either was not observed at all or not observed with certainty\cite{20,21}. Lymph node metastasis was defined as “present” only when it was histologically proven. Diagnosis was established by two independent pathologists (TN, IS), and cases of questionable diagnosis were omitted from the study.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Dепaraffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4 °C with an optimal dilution (0.1 μg/mL) of a primary polyclonal rabbit antibody against human Tie-1 (C-18), Tie-2 (C-20), Ang-1 (N-18), Ang-2 (N-18) or Ang-4 (L-18). Each antibody was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). The slides for Tie-1 and Tie-2 were then sequentially incubated with an alkaline phosphatase-conjugated horse antirabbit immunoglobulin antibody, and the reaction products were resolved using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersburg, MD, USA). The slides for Ang-1, 2 and 4 were sequentially incubated with a biotinylated horse antirabbit immunoglobulin antibody, and the reaction products were resolved using diaminobenzidine (DAB; DAKO Ltd., Glostrup, Denmark). Primary antibodies preabsorbed with excess recombinant Tie-1 and Tie-2 and Ang-1, 2, 4 and 4 peptides, respectively (Santa Cruz Biotechnology Inc.) were used as negative controls. Gastric ulcer tissue with capillary proliferation served as the internal positive control for Ties and Angs immunostaining. Analysis of the immunohistochemical staining was performed independently by two investigators (TN, IS). Tie-1 and Tie-2 and Ang-1, 2 and 4 expressions were classified into three categories depending on the percentage of cells stained: -, 0% to 10% positive cells; +, 10% to 50% positive tumor cells; and +++, >50% positive tumor cells.

**Statistical analysis**

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the degrees of Tie-1 and Tie-2 and Ang-1, 2 and 4 expressions were performed by the Mann-Whitney and Spearman’s tests. P<0.05 was taken as significant.

**RESULTS**

Benign colorectal epithelial cells showed focal and patch immunoreactivity of Ties and Angs with faint to mild staining intensity (data not shown). Ties and Angs were also expressed in colorectal adenomas (Table 1). We summarized the immunohistochemical results of colorectal adenocarcinomas in Tables 1, 2 and Figure 1. Among the 96 cases of adenocarcinoma, 87 (90.6%), 92 (95.8%), 83 (86.5%), 89 (92.7%), and 76 cases (79.2%) showed positive staining in the cytoplasm of carcinoma cells for Tie-1 and Tie-2 and Ang-1, 2 and 4 proteins, respectively (Table 1). Histologically, the expressions of Tie-1 and Tie-2 and Ang-1, 2 and 4 were variable. With the exception of mucinous carcinomas, the expressions of Tie-1, Tie-2 and Ang-1, 2 were significantly correlated with the degree of well, moderate and poor histological differentiation (P = 0.000123, P = 0.002209, P = 0.000161, P = 0.008193, respectively). Tie-1 and Tie-2 and Ang-1, 2 and 4 expressions correlated with the depth of tumor invasion (P = 0.000473, P = 0.006137, P = 0.000747, P = 0.0097, P = 0.000949, respectively). The expression of Tie-2 was significantly correlated with the degree of desmoplastic stromal reaction (P = 0.039383) (Table 2). The expressions of Tie-2 and Ang-2 correlated with the degree of venous invasions (P = 0.005992, P = 0.018168, respectively). The expressions of Tie-1 and Tie-2 and Ang-1 correlated with the presence of lymphatic invasion (P = 0.033356, P = 0.001326, P = 0.039066, respectively). There was no correlation between the expression of Angs/Ties and the presence of lymph node metastasis (Table 2).
### Table 1: Histological differentiation, invasion and Tie-1, 2 and Ang-1, 2, 4 expressions in human colorectal adenocarcinoma (n = 96), n (%)

|                  | Tie-1 | Tie-2 | Ang-1 | Ang-2 | Ang-4 |
|------------------|-------|-------|-------|-------|-------|
| **Tie-1**        |       |       |       |       |       |
| **Tie-2**        |       |       |       |       |       |
| **Ang-1**        |       |       |       |       |       |
| **Ang-2**        |       |       |       |       |       |
| **Ang-4**        |       |       |       |       |       |
| **Histological differentiation** |
| P                 | 0.00123 | 0.02209 | 0.00061 | NS | 0.008193 |
| **Ga in adenoma** |
| P                 | 0.002209 | 0.000161 | NS | 0.008193 | 0.000943 |
| **Histological differentiation** |
| P                 | 0.000161 | 0.000074 | 0.000747 | 0.00930 | 0.000943 |
| **Tis**           | 0.000473 | 0.006137 | 0.000747 | 0.00930 | 0.000943 |
| **T1**            | 0.000161 | 0.000074 | 0.000747 | 0.00930 | 0.000943 |
| **T2**            | 0.000473 | 0.006137 | 0.000747 | 0.00930 | 0.000943 |
| **T3**            | 0.000473 | 0.006137 | 0.000747 | 0.00930 | 0.000943 |
| **T4**            | 0.000473 | 0.006137 | 0.000747 | 0.00930 | 0.000943 |
| **Depth of tumor invasion** |
| P                 | 0.000473 | 0.006137 | 0.000747 | 0.00930 | 0.000943 |
| **Duke’s classification** |
| P                 | 0.00038 | 0.0037 | 0.015936 | 0.000747 | 0.000943 |

**NS:** not significant.

### Table 2: Ductal infiltrations, lymph node metastasis and Tie-1, 2 and Ang-1, 2, 4 expressions in invasive colorectal adenocarcinoma (n = 76), n (%)

|                  | Tie-1 | Tie-2 | Ang-1 | Ang-2 | Ang-4 |
|------------------|-------|-------|-------|-------|-------|
| **Tie-1**        |       |       |       |       |       |
| **Tie-2**        |       |       |       |       |       |
| **Ang-1**        |       |       |       |       |       |
| **Ang-2**        |       |       |       |       |       |
| **Ang-4**        |       |       |       |       |       |
| **Histological differentiation** |
| P                 | 0.039383 | NS | NS | NS | NS |
| **Slight**       | 13 | 2 (15.4) | 2 (15.4) | 9 (69.2) | 1 (7.7) | 5 (38.5) | 7 (53.8) | 4 (30.8) | 5 (38.5) | 7 (53.8) | 8 (61.5) | 4 (30.8) | 0 (0.0) | 9 (69.2) |
| **Moderate**     | 47 | 2 (4.3) | 11 (23.4) | 34 (72.3) | 1 (2.1) | 11 (23.4) | 35 (74.5) | 4 (30.8) | 11 (23.4) | 35 (74.5) | 35 (74.5) | 2 (4.3) | 11 (23.4) | 35 (74.5) |
| **Extensive**    | 16 | 1 (6.3) | 2 (12.5) | 13 (81.3) | 0 (0.0) | 2 (12.5) | 14 (87.5) | 0 (0.0) | 2 (12.5) | 14 (87.5) | 13 (81.3) | 1 (6.3) | 2 (12.5) | 13 (81.3) |
| **Venous invasion** |
| P                 | 0.059949 | NS | NS | NS | NS |
| **Present**      | 35 | 1 (2.9) | 7 (20.0) | 27 (77.1) | 0 (0.0) | 4 (11.4) | 31 (86.6) | 2 (5.6) | 6 (17.1) | 27 (77.1) | 12 (34.1) | 3 (8.8) | 9 (26.5) | 31 (86.6) |
| **Absent**       | 41 | 4 (9.8) | 8 (19.5) | 29 (70.7) | 2 (4.9) | 14 (34.1) | 25 (61.0) | 4 (9.8) | 11 (26.8) | 26 (63.4) | 7 (17.1) | 10 (24.4) | 23 (56.1) |
| **Lymphatic invasion** |
| P                 | 0.033556 | NS | NS | NS | NS |
| **Present**      | 60 | 1 (1.7) | 12 (20.0) | 47 (78.3) | 0 (0.0) | 11 (18.3) | 49 (81.7) | 3 (5.0) | 12 (20.0) | 45 (75.0) | 5 (8.3) | 12 (20.0) | 45 (75.0) |
| **Absent**       | 16 | 4 (25.0) | 3 (18.8) | 9 (56.3) | 2 (12.5) | 7 (43.8) | 7 (43.8) | 3 (18.8) | 5 (31.3) | 8 (50.0) | 2 (12.5) | 4 (25.0) | 10 (62.5) | 6 (37.5) | 3 (18.8) | 7 (43.8) |
| **Lymph node metastasis** |
| P                 | 0.003126 | NS | NS | NS | NS |
| **Present**      | 27 | 1 (3.7) | 4 (14.8) | 22 (81.5) | 1 (3.7) | 4 (14.8) | 22 (81.5) | 1 (3.7) | 4 (14.8) | 22 (81.5) | 1 (3.7) | 4 (14.8) | 22 (81.5) |
| **Absent**       | 49 | 4 (8.2) | 11 (22.4) | 34 (69.4) | 1 (2.0) | 14 (28.6) | 34 (69.4) | 5 (10.2) | 9 (18.4) | 35 (71.4) | 3 (6.1) | 11 (22.4) | 35 (71.4) |

**NS:** not significant.
DISCUSSION
Tyrosine-kinase receptors (TKRs) are key molecules in signaling pathways leading to growth and differentiation of normal and carcinoma cells\textsuperscript{[22,23]}. The attenuation of subcellular tyrosine phosphorylation has been reported to induce the differentiated phenotypes in human colorectal cancer cells\textsuperscript{[24]}. Aberrant expression of tyrosine kinases, as reflected by aberrant tyrosine phosphorylation in gastric cancer cells, has been reported\textsuperscript{[25]}. Subversion of different signal pathways may contribute to the progression of different types of colorectal cancer.

Tie-1 and Tie-2 were highly expressed in cancer tissues in this study. One report suggested that Tie-1 expression was restricted to endothelial and hematopoietic cells\textsuperscript{[26]}. However, this study showed that Tie-1 and Tie-2 were expressed in colorectal adenocarcinoma cells as well. The expressed Tie-1 and Tie-2 proteins were localized in the cytoplasm of cancer cells, and the invasive front and/or the peripheral parts of the primary tumor were intensely stained compared to the superficial and central parts of the tumor in almost all cases of invasive carcinomas. The expression of Tie-1 in gastric cancer cells was shown to be associated with reduced survival of the patients and served as an independent predictor of survival\textsuperscript{[27]}. In this study we found some correlations between the expression of Ties and histological differentiation, depth of invasion, Duke's classification and lymphatic invasion of colorectal carcinoma.

In the category of vasculogenesis, a new family of growth factors, termed angiopoietins that are specific for the vascular endothelium has been recently identified\textsuperscript{[13,15,16,28]}. All known angiopoietins bind to Tie-2, but it is still unclear as to whether they use the closely related receptor Tie-1. Transgenic overexpression of Ang-1 could lead to striking hypervascularization\textsuperscript{[29]}. In contrast, transgenic overexpression of Ang-2, a natural competitor of Ang-1 for the Tie-2 receptor, seemed to severely disturb vessel stabilization and remodeling\textsuperscript{[15]}. Ang-3 and Ang-4 were more recently described members of this family that seem to represent the mouse and human counterparts, respectively, of the same genetic locus\textsuperscript{[28]}. Ang-4 seemed to act as an agonist and is expressed at high levels in the lungs\textsuperscript{[28]}. Although the expressions of Ties and Angs have been reported in angiogenesis\textsuperscript{[30]}, the expression of Angs in carcinoma cells has led us to consider the possibility that Ang might be involved in the growth or progression of tumor cells. One report showed that the colon carcinoma cell line, transfected with Ang-1 and Ang-2 expression vector, revealed rapid tumor growth\textsuperscript{[31]}. In this study, both Angs and Ties were expressed simultaneously in carcinoma cells. Therefore, tumor cells should respond to the Angs in an autocrine or paracrine fashion to promote the growth of tumors. Nevertheless, more studies are required to elucidate this potential regulation of signaling pathways by Angs in colorectal carcinoma cells.

Statistical analyses of our data showed a correlation only between Tie-1 and Tie-2 and Ang-1, Ang-2, and Ang-4 expressions and histological differentiations. Poorly differentiated adenocarcinomas were strongly positive for each protein in the cytoplasm of carcinoma cells. In gastric cancer, some reports have described a correlation between the expressions of Ties or Angs and histological differentiations\textsuperscript{[31-33]}, but these reports showed variable results. The present study is the only report on correlation between expressions of Ties and Angs and differentiation of colorectal carcinomas. However, more studies are needed to clarify the correlation between expressions of Ties and Angs and histopathological

Figure 1 Positive staining in cytoplasm of colorectal adenocarcinoma cells shown by immunohistochemical staining of Ties and Angs. Immunoalkaline phosphatase staining; Tie-1 (A), Tie-2 (B) and DAB staining; Ang-1 (C), Ang-2 (D), and Ang-4 (E). (magnification: ×100, each).
Prognosis in patients with colorectal cancer has conventionally been determined by the extent of the primary tumor and the presence or absence of metastasis. However, the mechanism of invasion and metastasis of colorectal carcinoma has not been fully elucidated. In our study, Ties and Angs were expressed in normal colorectal epithelium, although these expressions were weak (data not shown). Ties and Angs were also expressed in colorectal adenomas (Table 1). Some reports showed that the expressions of Ties and Angs were correlated with tumor invasion or prognosis of patients with gastric carcinoma. However, in colorectal carcinoma, there was no report of the correlation between expressions of Ties and Angs and prognostic factors. In our study, the expression of Ties and Angs correlated significantly with the depth of tumor invasion, Duke’s classification, venous invasion, lymphatic invasion. It showed no correlation between expressions of Ties and Angs and lymph node metastasis. Further investigations are thus required to establish the details in the correlation between expressions of Ties and Angs and tumor progression and metastasis to lymph nodes in colorectal carcinoma.

Tumor desmoplasia is a common feature in several malignant human tumors, and it has been reported that a well-marked desmoplastic stromal reaction was associated with a poorer prognosis in colorectal cancer. In this study, many cases of colorectal carcinoma expressed Ties and Angs in the cytoplasm of carcinoma cells. However, fibrous stromal cells did not show high expression of Ties and Angs (data not shown). Tie-2 was significantly correlated with the extent of fibrous stromal tissue. Our results suggest that the expression of Tie-2 protein may be used as one of the prognostic factors in colorectal cancer.

In previous reports, Angs were shown to be mainly produced by endothelial cells and pericytes, and their receptor, Tie-2, was also expressed in endothelial cells. Angs were expressed in endothelial cells of tumor-associated vessels, and Ang-1 induced angiogenesis. In this study, Ties and Angs were expressed in vessel cells (data not shown), and the expressions of Tie-2 and Ang-2 correlated with venous invasion. These results suggest that Tie-2 and Ang-2 produced by tumor cells in human colorectal adenocarcinoma induce angiogenesis, which facilitates tumor nutrition. New vessels might be used to make metastasis easy to other organs.

This study demonstrates that Ties and Angs are highly expressed in human colorectal adenocarcinoma cells. It suggests that the Tie-Ang receptor-ligand complex is one of the factors involved in cellular differentiation and progression of human colorectal adenocarcinoma.

ACKNOWLEDGEMENTS
We are grateful to Mr. Toshiyuki Kawada for his excellent immunohistochemical assistance.

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