

Correlation of *MACC1* and *MET* Expression in Rectal Cancer after Neoadjuvant Chemoradiotherapy

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Abstract. *Background: Metastasis associated in colon cancer 1 (MACC1) is a recently identified gene that plays a key role in regulating hepatocyte growth factor-MET signaling. In this study, we demonstrated the correlation and the clinical significance of MACC1 and MET expression in rectal cancer patients treated with chemoradiotherapy (CRT) followed by surgery. Materials and Methods: The expression of MACC1 and MET mRNA in residual cancer cells from 52 patients after CRT was determined by quantitative real-time polymerase chain reaction. Protein expression was also investigated by immunohistochemical staining. Results: MACC1 and MET expressions were positively correlated. Furthermore, these proteins were also co-expressed in immunohistochemical analyses. High expression of MACC1 or MET was associated with reduced relapse-free survival and the prognosis was worse when both genes were highly expressed. Conclusion: Evaluation of MACC1 and MET expression may be useful for predicting prognosis in patients with rectal cancer treated with CRT followed by surgery.*

Around the world, colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred (1). Among all colorectal cancer cases, nearly 25% of patients are diagnosed with rectal cancer. Although the introduction of total mesorectal excision surgery in combination with preoperative chemoradiotherapy (CRT) has led to significant improvements

in sphincter preservation, local pelvic control and survival (2), 15-20% of patients experience distant recurrent relapse, leading to morbidity and mortality (3-5). Identifying predictive markers is therefore clinically relevant for the refinement of personalized treatment. The hepatocyte growth factor (HGF)-MET signaling pathway has been shown to play a key role in the malignant progression of several human malignancies (6-9). Aberrant activation of HGF-MET signaling is associated with increased cell invasion, correlating with a higher metastatic potential and poor prognosis of several types of cancers, including colorectal cancer (9-11). A recent study analyzing differential gene expression in colon mucosa, primary tumors and metastases of patients with colon cancer, revealed the up-regulation of a novel gene, *metastasis associated colorectal cancer-1 (MACC1)* (12). The expression of *MACC1* in colorectal cancer was suggested to be an independent prognostic indicator of metastasis formation and poor prognosis (12). *MACC1* is also reported to be a key regulator of *MET*, and its expression is positively correlated with that of *MET* (12, 13). In this study, we investigated the relationship between *MACC1* expression and clinical outcome in rectal cancer after CRT, and the correlation with HGF-MET signaling.

Patients and Methods

Patients and specimens. Samples of rectal cancer (clinical stage II/III based on TNM classification criteria from the International Union against Cancer) were obtained from 52 patients who underwent preoperative CRT followed by surgery between 2001-2008. All patients provided written informed consent for their tissues to be used in this study. Samples were provided as formalin-fixed paraffin-embedded (FFPE) specimens.

5-Fluorouracil (5FU)-based CRT regimen. The CRT regimen included four cycles of 5FU given as 600 mg/m² for 24 h by continuous intravenous route, and tegafur-uracil (UFT) given as 400 mg/m² orally for five days, concurrent with 20-45Gy radiation, followed by resection. This regimen is based on the combination of continuous infusion of 5FU and UFT, which was described previously (14, 15). Forty-one patients received short-course radiation at a dose

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Key Words: *MACC1*, *MET*, rectal cancer, preoperative chemoradiotherapy.

Table I. Patient characteristics and association of metastasis associated in colon cancer 1 and MET expression with clinicopathological variables.

| | Number | MACC1 | p-Value | MET | p-Value |
|---------------------------------|--------|-------|---------|-------|---------|
| Age, years | | | | | |
| ≥65 | 26 | 0.147 | 0.564 | 0.278 | 0.295 |
| <65 | 26 | 0.058 | | 0.092 | |
| Gender | | | | | |
| Male | 41 | 0.089 | 0.902 | 0.202 | 0.712 |
| Female | 11 | 0.035 | | 0.092 | |
| T Classification | | | | | |
| T 1/2 | 17 | 0.044 | 0.845 | 0.253 | 0.922 |
| T 3/4 | 35 | 0.089 | | 0.145 | |
| Lymph node metastasis | | | | | |
| Absent | 35 | 0.072 | 0.576 | 0.121 | 0.945 |
| Present | 17 | 0.133 | | 0.253 | |
| Lymphatic vessel invasion | | | | | |
| Absent | 13 | 0.069 | 0.957 | 0.092 | 0.945 |
| Present | 39 | 0.086 | | 0.202 | |
| Vascular invasion | | | | | |
| Absent | 21 | 0.089 | 0.490 | 0.260 | 0.775 |
| Present | 31 | 0.035 | | 0.121 | |
| Histology | | | | | |
| Differentiated | 44 | 0.150 | 0.041 | 0.257 | 0.085 |
| Undifferentiated | 8 | 0.014 | | 0.059 | |
| Mandard TRG | | | | | |
| Non responder: grade 3-5 | 37 | 0.236 | 0.077 | 0.260 | 0.186 |
| Responder: grade 1, 2 | 15 | 0.033 | | 0.066 | |
| Radiation | | | | | |
| Short course: 20 Gy/4 fractions | 41 | 0.044 | 0.622 | 0.202 | 0.797 |
| Long course: 45 Gy/25 fractions | 11 | 0.133 | | 0.077 | |
| Recurrence | | | | | |
| Absent | 37 | 0.069 | 0.333 | 0.107 | 0.222 |
| Present | 15 | 0.272 | | 0.531 | |

MACC1 and MET are expressed as median values. TRG: Tumor regression grade.

of 20 Gy in five fractions over one week (low-dose radiation group). The remaining 11 patients received conventionally fractionated radiation at a dose of 45 Gy in 18 fractions for four weeks (high-dose radiation group).

Histopathological tumor regression. The pathological response to CRT was evaluated using the Mandard tumor regression grade (TRG) scale (16). Mandard TRG is separated into five categories: TRG1, complete response with absence of residual cancer and fibrosis extending through the wall; TRG2, presence of residual tumor cells scattered through the fibrosis; TRG3, increase in the number of residual cancer cells, with predominant fibrosis; TRG4, residual cancer outgrowing fibrosis; TRG5, absence of regressive changes. Patients with TRG 1 and 2 were classified as responders, while those with TRG 3-5 categories were classified as non-responders.

Microdissection, RNA extraction and cDNA synthesis from FFPE specimens. FFPE sections (10 µm thick) were stained with nuclear fast red and manually microdissected to collect residual tumor cells with reference to hematoxylin and eosin sections. Microdissected samples were digested with proteinase K in a lysis buffer containing Tris-HCl, ethylenediamine tetraacetic acid, and sodium dodecyl

sulfate, as previously published with minor modifications (17). RNA was purified by phenol and chloroform extraction. cDNA was synthesized with random hexamer primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR analysis was carried out with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Primer sequences for *MACC1*, *MET* and *ACTB* were as follows: *MACC1*: forward: TGATTGACATG GAAGCTGGA, reverse: GGATTTGCAACTTTTGAAGC; *MET*: forward: AGGTGTGGGAAAAACCTGA, reverse: ATTCAGCTGTT GCAGGGAAG; *ACTB*: forward: ACAGAGCCTCGCCTTTGC, reverse: GCGGCGATATCATCATCC. PCR was performed in a final volume of 25 µl with SYBR Green PCR Master Mix, using 1 µl cDNA and 400 nM of each primer for the respective genes. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min each.

Quantitation of relative expression levels of *MACC1* and *MET*. Relative gene expression was determined using the standard curve

method. Standard curves and line equations were generated using five-fold serially diluted solutions of cDNA generated by reverse transcription of qPCR Human Reference Total RNA (Clontech, Mountain View, CA, USA). All standard curves were linear in the analyzed range with an acceptable correlation coefficient (R^2). Target gene expression was calculated from the standard curve followed by quantitative normalization of cDNA in each sample using *ACTB* as an internal control. Assays were performed in duplicate for each sample, and the mean value was used for analysis.

Immunohistochemistry. Immunohistochemical analysis was performed as previously described (18). Sections were stained with primary rabbit polyclonal antibody towards *MACC1* (1:300, HPA020081; Sigma Aldrich, St. Louis, MO, USA) and rabbit monoclonal antibody towards *MET* (1:100, ab51067; Abcam, Cambridge, UK) and proteins were detected using the labeled streptavidin-biotin method (LASB2 kit/HRP; Dako Cytomation, Glostrup, Denmark). Appropriate positive and negative controls were used throughout.

Statistical analysis. Comparisons were performed using the nonparametric Mann-Whitney *U*-test for continuous variables. Analyses of nonparametric receiver operating characteristics (ROC) were performed in order to calculate cut-off values for *MACC1* and *MET* expression according to the most accurate value obtained in all patients. The correlations between variables (levels of *MACC1* and *MET* gene expressions) were assessed with the Spearman rank correlation coefficient. Survival was evaluated by the Kaplan–Meier method. Differences between two groups were determined by log-rank test. Two-sided *p*-values <0.05 were considered statistically significant.

Results

Association of *MACC1* and *MET* expression with clinicopathological variables. Levels of *MACC1* and *MET* were analyzed in 52 patients with rectal cancer after preoperative CRT followed by surgery [male/female, 41/11; median age, 64.5 (range, 38–77 years)]. The median follow-up period was 67 months (range 22–129 months). Out of the 52 patients, 15 were diagnosed with tumor recurrence, including four patients with local recurrence, two with liver and lung metastases, six with lung metastasis alone, one with both local recurrence and lung metastasis, and two patients with peritoneal metastasis. Analysis of *MACC1* and *MET* expression in rectal cancer samples by qRT-PCR, revealed higher levels of *MACC1* and *MET* in patients who developed tumor recurrences compared with patients without recurrence, although this difference was not statistically significant (Table I). Patients with differentiated rectal cancer had significantly higher levels of *MACC1*. There was no significant association between *MET* expression and clinicopathological variables (Table I). As shown in Figure 1, *MACC1* and *MET* expressions were positively correlated in post-CRT tumor specimens (Spearman's ρ : 0.64, $p < 0.0001$).

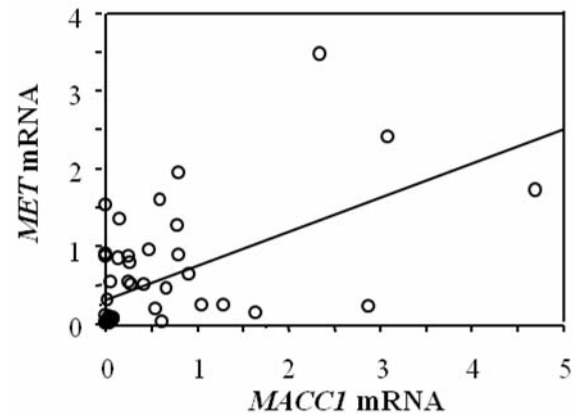


Figure 1. Positive correlation between metastasis associated in colon cancer 1 and *MET* mRNA expression in patients with rectal cancer after chemoradiotherapy ($n=52$, Spearman's $\rho=0.64$ $p < 0.0001$).

Predictive value of *MET* and *MACC1* expression levels for relapse-free survival (RFS). In order to test whether *MACC1* or *MET* expression was associated with RFS, a complete statistical ROC analysis was performed. The cut-off values for *MACC1* and *MET* expression were 0.261 and 0.877, respectively. We observed that patients with higher *MACC1* or *MET* levels had significantly worse RFS (*MACC1*: $p=0.0426$, *MET*: $p=0.0231$) (Figure 2a and b). This association was even more pronounced when both *MACC1* and *MET* were highly expressed ($p=0.0017$) (Figure 2c).

Immunohistochemistry for *MACC1* and *MET* in residual tumor after CRT. We next investigated the expression and cellular localization of *MACC1* and *MET* in residual rectal cancer cells by immunohistochemistry (Figure 3). *MACC1* was diffusely expressed in the cytoplasm, while *MET* was observed in both the cytoplasm and membrane of residual cells compared to negative controls (data not shown).

Discussion

Previous studies have shown that *MACC1* expression is associated with several human malignancies. Over expression of *MACC1* is associated with postoperative recurrence in lung adenocarcinoma (19). *MACC1* is also highly expressed in hepatocellular carcinoma (HCC) tissues and is a prognostic indicator in HCC patients (20). We previously reported the clinical significance of the HGF-MET pathway in patients with rectal cancer who underwent preoperative CRT (21), prompting us to examine the association between *MACC1* and *MET* on these patients. In this study, we identified a positive association between *MACC1* and *MET* expression levels in rectal cancer after CRT. We also showed that high levels of both *MACC1* and *MET* were indicative of significantly worse

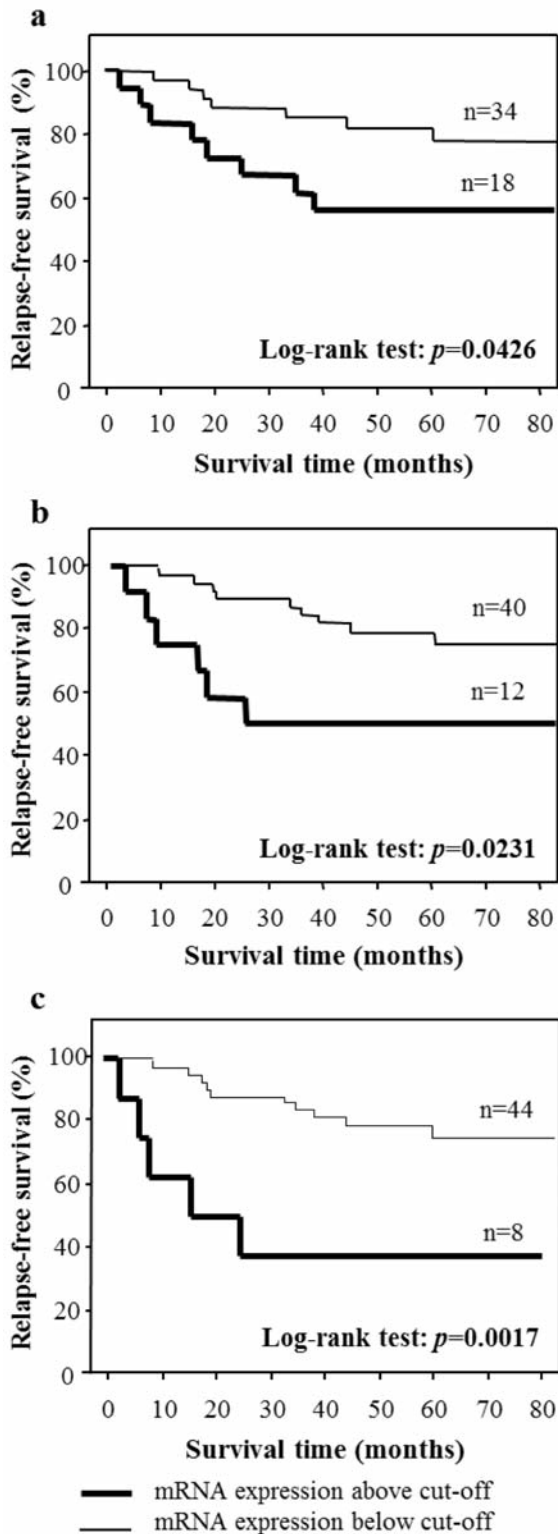


Figure 2. Kaplan-Meier analysis of relapse-free survival according to mRNA expression levels of metastasis associated in colon cancer 1 (a), MET (b), and metastasis associated in coloncancer 1/MET combination (c). The cut-off value was calculated by receiver operating characteristics analysis (cut-off value: $MACC1=0.261$, $MET=0.877$).

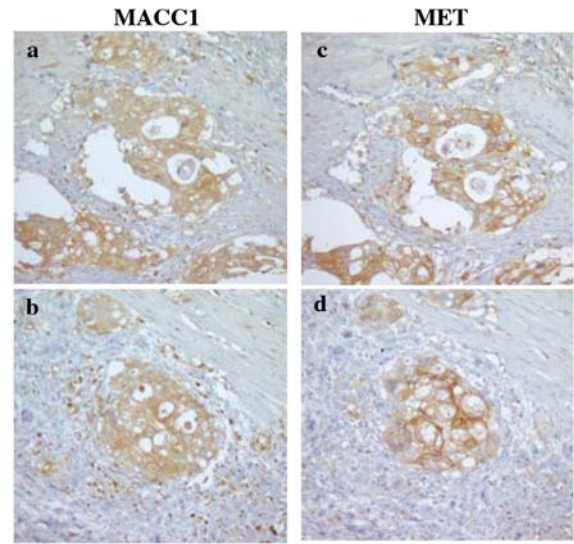


Figure 3. Immunohistochemistry of residual rectal cancer cells from patients after chemoradiotherapy. Representative expression of metastasis associated in colon cancer 1 (a, b), and MET (c, d). Original magnification $\times 200$.

prognosis in these patients. These findings support previous studies showing that *MACC1* promotes cell migration and invasion by regulating the hepatocyte growth factor receptor, *MET* (12). Given the fact that the HGF-MET signaling pathway is activated in many human malignancies, and regulates malignant formation, progression and dissemination, a number of MET pathway inhibitors are currently being evaluated in the clinic (22). Further investigation of the relationship between *MACC1* and HGF-MET signaling will enhance our understanding over the molecular pathogenesis underlying these diseases. Finally, assessment of *MACC1* and *MET* levels in patients with rectal cancer may be useful in order to identify high-risk patients who may benefit from more intensive adjuvant treatment and follow-up care.

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