

Pathological roles of c-Met in bladder cancer: Association with cyclooxygenase-2, heme oxygenase-1, vascular endothelial growth factor-A and programmed death ligand 1

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Abstract. c-Met is a receptor tyrosine kinase that binds a specific ligand, namely hepatocyte growth factor (HGF). The HGF/c-Met system is important for malignant aggressiveness in various types of cancer, including bladder cancer (BC). However, although phosphorylation is the essential step required for biological activation of c-Met, pathological roles of phosphorylated c-Met at the clinical and molecular levels in patients with BC are not fully understood. In the present study, the expression levels of c-Met and the phosphorylation of two of its tyrosine residues (pY1234/pY1235 and pY1349) were immunohistochemically examined in 185 BC tissues. The associations between these expression levels and cancer cell invasion, metastasis, and cyclooxygenase-2 (COX-2), heme oxygenase-1 (HO-1), VEGF-A and programmed death ligand 1 (PD-L1) levels were investigated. c-Met was associated with muscle invasion ($P=0.021$), as well as the expression levels of HO-1 ($P=0.028$) and PD-L1 ($P<0.001$), whereas pY1349 c-Met was associated with muscle invasion ($P=0.003$), metastasis ($P=0.025$), and COX-2 ($P=0.017$), HO-1 ($P=0.031$) and PD-L1 ($P=0.001$) expression. By contrast, pY1234/1235 c-Met was associated with muscle invasion and metastasis ($P=0.006$ and $P=0.012$, respectively), but not with the panel of cancer-associated molecules. Furthermore, COX-2 and PD-L1 expression were associated with muscle invasion and metastasis, respectively ($P=0.045$ and $P=0.036$, respectively). Hence, c-Met serves important roles in muscle invasion by regulating HO-1 and PD-L1, whereas its phosphorylation at Y1349 is associated with muscle invasion and metastasis via the regulation of COX-2, HO-1 and PD-L1 in patients with

BC. Furthermore, phosphorylation at Y1234/1235 may lead to muscle invasion and metastasis via alternate mechanisms associated with c-Met and pY1349 c-Met.

Introduction

Bladder cancer (BC) is a common urological cancer, and is recognized as an inflammatory and immunogenic disease (1). Patients with advanced or metastatic BC have a poor prognosis despite treatment with standard chemotherapies including combination therapy with gemcitabine and cisplatin (GC therapy) (2). Furthermore, in patients with muscle-invasive BC, the 2-year recurrence-free survival rate was reported to be 60.9% if pT0 was not obtained by neo-adjuvant GC chemotherapy (3). Accordingly, information regarding the pathological characteristics of BC cells is important to improve outcomes for patients with BC. Specifically, understanding the molecular mechanisms underlying cancer cell growth, invasion, and metastasis is essential to formulate new treatment strategies for these patients.

c-Met is a receptor tyrosine kinase that binds a specific ligand, namely hepatocyte growth factor (HGF). In many types of cancer, the HGF/c-Met system is important for malignant potential, cancer cell invasion, metastasis, and determining clinical outcomes (4,5). In fact, cancer cell proliferation, cell cycle, migration, and angiogenesis were previously suggested as HGF/c-Met-related pathological mechanisms, based on *in vivo* and *in vitro* studies (6-8). Furthermore, c-Met is closely associated with the regulation of various cancer-related molecules such as cyclooxygenase (COX)-2, heme oxygenase (HO)-1, and vascular endothelial growth factor (VEGF)-A in various types of malignancies (9-12). In recent years, the HGF/c-Met system has also been reported to promote carcinogenesis and cancer cell progression by regulating the immune system in various types of cancers (10,13). Specifically, programmed cell death ligand 1 (PD-L1) is a representative immune checkpoint inhibitor expressed on various types of cancer cells that has been reported to downregulate the immune response (14,15). Interestingly, a study has reported that c-Met promotes cancer cell survival through the regulation of PD-L1 expression in renal cell carcinoma (RCC) cells (10); however, several other reports have supported the positive correlation between c-Met

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and PD-L1 expression in cancer tissues (12,16). Thus, c-Met is recognized as a key modulator of various malignant behaviors that functions by regulating cancer-related molecules and the immune system via PD-L1.

As it relates to BC, c-Met has been shown to be positively associated with malignant cell behavior and poor prognosis (5,17). Furthermore, COX-2, HO-1, and VEGF-A were reported to be closely associated with carcinogenesis, malignant potential, and prognosis for BC (7,18,19). Recent studies have also reported that PD-L1 expression in BC cells has important roles in malignancy, progression, chemo-resistance, and disease outcome in patients with BC (20,21). However, little information is available regarding the relationships between c-Met and COX-2, HO-1, VEGF-A, or PD-L1 in human BC tissues.

Further, when the pathological significance of c-Met in BC is discussed, we should note that its phosphorylation is essential for its biological effects (17). Briefly, under various physiological and pathological conditions, the phosphorylation of major phosphorylation sites, specifically the kinase domain (Y1234/1235) and the multifunctional docking domain (Y1349/1356), leads to an increase in intrinsic activities and biological functions such as cell motility and transformation (22,23). With respect to the pathological significance of c-Met phosphorylation in cancers, a previous report demonstrated that the expression of phospho-c-Met (Y1349), termed pY1349 c-Met, is positively associated with cancer growth, progression, and poor survival in patients with RCC (18). Likewise, one report indicated that high pY1235 c-Met expression is associated with an increased risk of recurrence for ovarian cancer patients (24); meanwhile, in patients with BC, several reports have shown that phosphorylated c-Met leads to highly malignant disease and poor survival (25,26). However, the precise pathological significance of phosphorylated c-Met in BC is not fully understood. In fact, the relationship between phosphorylated c-Met expression and metastasis in these patients has not yet been characterized. Furthermore, no study has reported the relationships between phosphorylated c-Met and COX-2, HO-1, VEGF-A, and PD-L1 in human BC tissues. Based on these previous findings, herein, we focused on the relationships between c-Met, pY1349 c-Met, and, pY1234/1235 c-Met expression and grade, TNM classification, and the expression of COX-2, HO-1, VEGF-A, and PD-L1 in patients with BC.

Materials and methods

Patients. We investigated 185 formalin-fixed paraffin-embedded BC specimens from patients diagnosed with urothelial cancer via histopathological examination. Patients who received neoadjuvant therapy were excluded. In this study, T stage was also divided into non-muscle invasive BC (Ta and T1) and muscle invasive BC (MIBC; T2-4), as well as into absence of metastasis (N0 and M0) and presence of metastasis (N1-3 and/or M1) groups for multivariate analyses. This study protocol was approved by the Institutional Review Board of Nagasaki University Hospital (12052899), and written informed consent was provided by all patients.

Immunohistochemistry. The expression of all proteins was evaluated by immunohistochemical techniques. An anti-c-Met antibody (Zymed Laboratories Inc.) and two phospho-specific antibodies against human c-Met antibodies (pY1234/1235 and pY1349; Cell Signaling Technology) were used, the specificities of which were previously confirmed to detect the immunoreactivity of phosphorylated c-Met in several malignant tissues (25,27,28). Other primary antibodies included anti-VEGF-A (Santa Cruz Biotechnology), anti-COX-2 (Immuno-Biological Laboratories Co.), anti-HO-1 (Enzo Life Sciences Inc.), and anti-PD-L1 (clone E1L3N, Cell Signaling Technology, Inc.). Immunohistochemical staining and evaluation were performed according to previous reports (19,25,29,30). In short, five-micrometer-thick sections were deparaffinized in xylene and rehydrated in solutions of ethanol. Antigen retrieval was performed at 95°C for 40 min in 0.01 mol/l sodium citrate buffer (pH 6.0) and then immersed in 3% hydrogen peroxide for 30 min. Sections were incubated overnight with the primary antibodies at 4°C. The sections were then incubated with peroxidase using the labeled polymer method with Dako EnVision+ Peroxidase (Dako; Agilent Technologies, Inc.), and the peroxidase reaction was visualized with the liquid 3,3-diaminobenzidine tetrahydrochloride substrate. Sections were counterstained with hematoxylin. As a positive control, RCC tissue was stained for HGFR/c-Met, phosphorylated HGFR/c-Met, and COX-2, and a spleen section was stained for HO-1 and PD-L1. A consecutive section from each sample, processed without the primary antibody, was used as a negative control. Further, save for that of PD-L1, the expression of all molecules was evaluated semi-quantitatively based on staining intensity and the percentage of stained cancer cells, as previously described (19,25). For PD-L1 expression, the percentage of PD-L1-positive cancer cells was above the threshold of 1% according to a previous report (30). Such evaluations were performed using a Nikon E-400 microscope and a digital imaging system (DU100; Nikon). In addition, a computer-aided image analysis system (Win ROOF, version 5.0; Mitani Corp.) was utilized to support this evaluation.

Statistical analyses. The Mann-Whitney U test was used to compare continuous variables. The χ^2 test was used for categorical comparisons of data. The crude and adjusted effects were estimated by logistic regression analysis [described as odds ratios (ORs) with 95% confidence intervals (95% CIs), together with P-values]. All statistical analyses were performed with the statistical package StatView for Windows (version 5.0; Abacus Concept Inc.), and statistical significance of differences was defined as $P < 0.05$.

Results

Immunohistopathological examinations. We previously showed examples of positively stained tissues of c-Met, pY1234/1235 c-Met, pY1349 c-Met, COX-2, HO-1, and VEGF-A in patients with urothelial cancer including BC (18,19,25,31). Their staining patterns in this study were similar to those in these previous reports. Therefore, in this study, we showed representative figures of PD-L1 expression in BC tissues in Fig. 1. PD-L1 was mainly detected at the cell

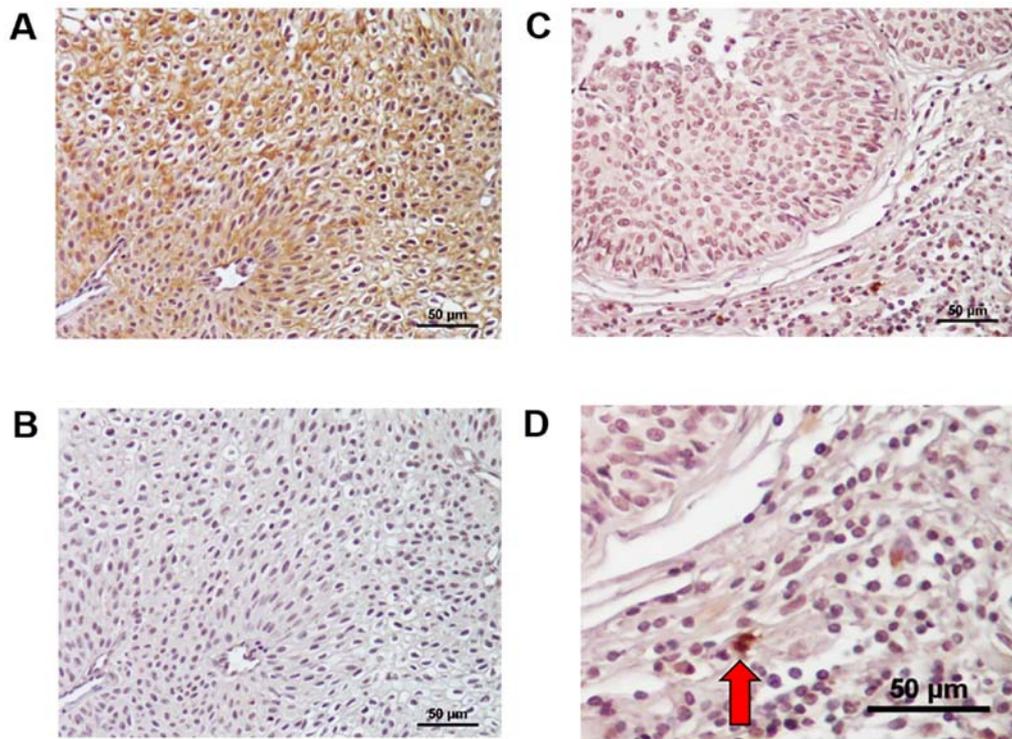


Figure 1. Expression levels of PD-L1 in bladder cancer specimens. PD-L1 was primarily detected at the membranes of bladder cancer cells and was found at lower levels in the cytoplasm. (A) A representative example of a PD-L1-positive tissue. Magnification, x400. (B) Such positive staining of PD-L1 was not detected in the negative control of a consecutive section. Magnification, x400. (C) A representative example of PD-L1-negative tissue. Magnification, x400. (D) In this specimen, a PD-L1-positive infiltrating cell (red arrow) was detected in the peri-tumoral stromal tissue (enlarged image of C). PD-L1, programmed death ligand 1.

membrane and partially in the cytoplasm of BC cells (Fig. 1A), and such positive staining was not detected in negative control of a consecutive section (Fig. 1B). Further, expression was found in infiltrating cells of stromal tissues from some MIBC tissues; however, there were few cancer stromal cells in NMIBC tissues (Fig. 1C and D). Although representative examples of other cancer-related molecules including c-Met, pY1234/1235 c-Met, and pY1349 c-Met were shown in our previous reports (19,25,27), similar staining patterns were confirmed in this study. Among 185 BC specimens, positive c-Met, pY1234/1235 c-Met, and pY1349 c-Met expression was detected in 109 (58.9%), 59 (31.9%), and 82 (44.3%) cases, respectively. Further, 120 (64.9%), 101 (54.6%), 104 (56.2%), and 80 (43.2%) specimens were judged positive for the expression of COX-2, HO-1, VEGF-A, and PD-L1, respectively.

Correlations between clinicopathological features and c-Met, pY1234/1235 c-Met, or pY1349 c-Met expression. As shown in Table I, positive expression of c-Met, pY1234/1235 c-Met, and pY1349 c-Met was significantly associated with high grade ($P=0.004$, $P=0.042$, $P<0.001$, respectively) and T stage ($P=0.013$, $P=0.002$, and $P<0.001$, respectively). Moreover, the expression of pY1234/1235 c-Met and pY1349 c-Met was associated with N stage ($P=0.003$ and 0.006 , respectively) and M stage ($P=0.008$ and 0.027 , respectively); however, such significant associations were not found for c-Met expression. In addition, as shown in Table I, similar relationships were detected between c-Met, pY1234/1235 c-Met, or pY1349 c-Met and muscle invasion (T2-4) or metastasis (N1-3 and/or M1).

The expression of c-Met tended to be positively correlated with metastasis; however, this did not reach statistical significance ($P=0.083$).

Independent roles of c-Met, pY1234/1235 c-Met, or pY1349 c-Met in muscle invasion or metastasis. We next analyzed the independent relationships between c-Met, pY1234/1235 c-Met, or pY1349 c-Met and muscle invasion or metastatic status using multivariate logistic regression analysis models. Similar to the results of univariate analyses, c-Met, pY1234/1235 c-Met, and pY1349 c-Met levels were all independently associated with muscle invasion, whereas only the expression of pY1234/1235 c-Met and pY1349 c-Met was associated with metastasis (Table II). Regarding the relationship between c-Met and metastasis, multivariate analysis showed no significant correlation ($P=0.190$).

Association between cancer-related molecules and c-Met, pY1234/1235 c-Met, and pY1349 c-Met. Univariate analyses showed that COX-2 expression was significantly associated with pY1234/1235 c-Met (OR=2.49; $P=0.012$) and pY1349 c-Met (OR=2.98; $P=0.010$); however, multivariate logistic regression analysis, adjusted by grade, muscle invasion, and metastasis, demonstrated that COX-2 expression was only independently associated with pY1349 c-Met (OR=2.30; $P=0.017$; Table III). Further, univariate and multivariate analyses showed that both HO-1 and PD-L1 were significantly associated with c-Met and pY1349 c-Met expression, but not with pY1234/1235 c-Met expression (Table III). In contrast,

Table I. Associations with clinicopathological features.

| Variable | c-Met, n | | pY1234/1235 c-Met, n | | pY1349 c-Met, n | |
|-------------------|----------|----------|----------------------|----------|-----------------|----------|
| | Negative | Positive | Negative | Positive | Negative | Positive |
| Grade | | | | | | |
| Low | 45 | 41 | 65 | 21 | 60 | 26 |
| High | 31 | 68 | 61 | 38 | 43 | 56 |
| P-value | 0.004 | | 0.042 | | <0.001 | |
| T stage | | | | | | |
| Ta | 29 | 24 | 41 | 12 | 39 | 14 |
| T1 | 37 | 47 | 62 | 22 | 50 | 34 |
| T2 | 6 | 20 | 14 | 12 | 8 | 18 |
| T3 | 3 | 12 | 8 | 7 | 5 | 10 |
| T4 | 1 | 6 | 1 | 6 | 1 | 6 |
| P-value | 0.013 | | 0.002 | | <0.001 | |
| MIBC | | | | | | |
| Absence | 66 | 71 | 103 | 34 | 89 | 48 |
| Presence | 10 | 38 | 23 | 25 | 14 | 34 |
| P-value | 0.001 | | 0.001 | | <0.001 | |
| N stage | | | | | | |
| N0 | 74 | 102 | 124 | 52 | 102 | 74 |
| N1-3 | 2 | 7 | 2 | 7 | 1 | 8 |
| P-value | 0.238 | | 0.003 | | 0.006 | |
| M stage | | | | | | |
| M0 | 74 | 99 | 122 | 51 | 100 | 73 |
| M1 | 2 | 10 | 4 | 8 | 3 | 9 |
| P-value | 0.076 | | 0.008 | | 0.027 | |
| Metastasis | | | | | | |
| Absence | 73 | 97 | 121 | 49 | 100 | 70 |
| Presence | 3 | 12 | 5 | 10 | 3 | 12 |
| P-value | 0.083 | | 0.003 | | 0.004 | |

MIBC, muscle invasive bladder cancer.

VEGF-A expression was not associated with the expression of c-Met, pY1234/1235 c-Met, or pY1349 c-Met, even by univariate analysis (Table III).

Correlation between pathological characteristics and COX-2, HO-1, VEGF-A, and PD-L1. As shown in Table IV, univariate analyses showed that COX-2 expression was positively correlated with VEGF-A (OR=2.28; P=0.009) and PD-L1 (OR=3.12; P=0.001), and multivariate analysis adjusted by grade, muscle invasion, and metastasis confirmed these significant correlations (OR=2.08; 95% CI=1.08-4.04; P=0.030 and OR=2.61; 95% CI=1.29-5.27; P=0.008, respectively). In contrast, HO-1 expression tended to be positively associated with PD-L1 expression (OR=1.77, P=0.060); however, an independent correlation was not found by multivariate analysis (Table IV). Furthermore, there was no significant correlation between COX-2 expression and HO-1, HO-1 expression and VEGF-A, or VEGF-A expression and PD-L1. Thus, HO-1 is not significantly correlated with the expression of COX-2, VEGF-A, and PD-L1 (Table IV).

Correlation between cancer-related molecules and muscle invasion or metastasis. The pathological roles of these cancer-related molecules with respect to muscle invasion and metastasis are shown in Table V. Univariate analyses showed that levels of COX-2, HO-1, VEGF-A, and PD-L1 were all associated with muscle invasive disease (OR=3.56; P=0.003, OR=2.23; P=0.024, OR=2.65; P=0.008, and OR=3.71; P<0.001, respectively); however, multivariate analyses demonstrated that only COX-2 expression was independently associated with muscle invasion (OR=2.64; 95% CI=1.02-6.81; P=0.045; Table V). A similar analysis showed that HO-1 and PD-L1 were associated with metastasis (OR=6.06; P=0.020 and OR=9.99; P=0.003, respectively) by univariate analysis; however, only PD-L1 expression was identified as an independent factor by multivariate analysis (OR=5.51; 95% CI=1.12-27.2; P=0.036; Table V).

Pathological roles of c-Met, pY1234/1235 c-Met, and pY1349 c-Met. Finally, we present a schema of the pathological roles of c-Met, pY1234/1235 c-Met, and pY1349 c-Met in Fig. 2.

Table II. Multivariate analyses for muscle invasion and metastasis.

| Clinicopathological features | OR | 95% CI | P-value |
|----------------------------------|------|------------|---------|
| For muscle invasion ^a | | | |
| c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 2.70 | 1.16-6.28 | 0.021 |
| pY1234/1235 c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 2.98 | 1.37-6.46 | 0.006 |
| pY1349 c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 3.27 | 1.49-7.14 | 0.003 |
| For metastasis ^b | | | |
| c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 2.43 | 0.65-9.12 | 0.190 |
| pY1234/1235 c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 4.33 | 1.31-13.50 | 0.012 |
| pY1349 c-Met | | | |
| Negative | 1.00 | - | - |
| Positive | 4.59 | 1.22-17.33 | 0.025 |

^aAdjusted by grade and metastasis. ^bAdjusted by grade and muscle invasion. OR, odds ratio; 95% CI, 95% confidence interval.

Univariate analyses showed that pY1234/1235 c-Met and pY1349 c-Met were associated with both muscle invasion and metastasis and that c-Met was significantly correlated with BC muscle invasion only. Furthermore, complex mechanisms comprising COX-2, HO-1, VEGF-A, and PD-L1 were speculated to be linked to these pathological effects of c-Met signaling. In contrast, multivariate analyses showed similar results regarding the relationship between c-Met pathways and pathological characteristics such as muscle invasion and metastasis (Fig. 2). However, although COX-2 and PD-L1 were thought to play significant roles in muscle invasion and metastasis, respectively, HO-1 and VEGF-A was not associated with either process (Fig. 2). In addition, as shown in Fig. 2, pY1234/1235 c-Met expression was not significantly correlated with any of COX-2, HO-1, VEGF-A, and PD-L1.

Discussion

The present study showed that the levels of pY1234/1235 c-Met and pY1349 c-Met are closely associated with muscle invasion and metastasis in patients with BC. c-Met expression was significantly associated with muscle invasion but not with metastasis based on similar analyses. In contrast, our previous report showed that levels of c-Met, pY1234/1235 c-Met, and pY1349 c-Met were positively associated with pT stage by univariate analyses; however, only pY1349 c-Met expression was independently associated with pT stage based

on a multivariate analysis model (25). Thus, there was a difference regarding the relationship between the expression of c-Met or pY1234/1235 and cancer cell invasion between these two reports. We speculated that this discrepancy was due to differences in clinicopathological features and methodology caused by the study population. In short, the previous study was performed based on 133 patients without metastatic BC, and the multivariate analysis model did not include metastasis. The present study demonstrated, for the first time, that the expression of both pY1234/1235 c-Met and pY1349 c-Met was significantly associated with metastasis, whereas c-Met expression was not, in patients with BC. These results suggest that c-Met phosphorylation is a key process that stimulates cancer cell invasion and metastasis in BC cells and that the kinase domain (Y1234/1235) and multifunctional docking domain (Y1349) are important phosphorylation sites that regulate such malignant behaviors.

One of the most interesting results of the present study was that c-Met was positively associated with the expression of HO-1 and PD-L1. There have been several reports demonstrating positive correlations between c-Met and HO-1 or PD-L1 in a variety of cancers (10,12,16). However, a significant association between c-Met expression and HO-1 and PD-L1 based on multivariate analyses has not been previously reported for patients with BC. Likewise, here, we demonstrated that pY1349 c-Met was closely associated with the expression of COX-2, HO-1, and PD-L1 in BC specimens, for the first time. Unfortunately, besides BC, there are few reports on the relationships between phosphorylated c-Met and cancer-related molecules in human cancer tissues. Therefore, we believe that our results are important to discuss the pathological mechanisms associated with phosphorylated c-Met in malignancies.

In addition to correlations between c-Met pathways and the expression of COX-2, HO-1, VEGF-A, or PD-L1, we clarified the pathological significance of and interrelations among these markers by multivariate analyses. Results showed that COX-2 expression was positively correlated with the expression of VEGF-A and PD-L1. In contrast, HO-1 expression was not significantly correlated with the expression of COX-2, VEGF-A, and PD-L1 in BC tissues. In regard to the relationship between COX-2 and VEGF-A, a positive correlation has been reported for a variety of malignancies such as liposarcoma and gastric cancer (32,33). Furthermore, one report indicated that COX-2 expression is positively correlated with PD-L1 expression in human melanoma tissues (34). However, there have been no studies on such interrelations among COX-2, VEGF-A, and PD-L1 in human BC tissues, but previous results support our findings on the correlations among these cancer-related molecules. Moreover, our univariate analyses suggested that muscle invasion and metastasis in BC are regulated by complex mechanisms comprising COX-2, HO-1, VEGF-A, and PD-L1, and this opinion is supported by many previous reports (7,19,35-37). However, interestingly, our multivariate analyses demonstrated that muscle invasion was independently associated with COX-2 expression and that metastasis was associated with PD-L1 expression, whereas HO-1 and VEGF-A were not associated with either muscle invasion or metastasis. Specifically, we were surprised that VEGF-A expression appeared to play a minimal role in BC

Table III. Association between c-Met-related parameters and cancer-related molecules.

| Variable | Univariate analysis | | | Multivariate analysis ^a | | |
|-----------------------------|---------------------|-----------|---------|------------------------------------|-----------|---------|
| | OR | 95% CI | P-value | OR | 95% CI | P-value |
| For COX-2 | | | | | | |
| c-Met; positive | 1.68 | 0.91-3.09 | 0.985 | 1.31 | 0.69-2.48 | 0.418 |
| pY1234/1235 c-Met; positive | 2.49 | 1.22-5.06 | 0.012 | 1.94 | 0.92-4.08 | 0.080 |
| pY1349 c-Met; positive | 2.98 | 1.56-5.72 | 0.010 | 2.30 | 1.16-4.58 | 0.017 |
| For HO-1 | | | | | | |
| c-Met; positive | 2.37 | 1.30-4.32 | 0.005 | 2.02 | 1.08-3.78 | 0.028 |
| pY1234/1235 c-Met; positive | 1.33 | 0.71-2.48 | 0.378 | 0.99 | 0.51-1.95 | 0.984 |
| pY1349 c-Met; positive | 2.52 | 1.38-4.61 | 0.003 | 2.02 | 1.07-3.84 | 0.031 |
| For VEGF-A | | | | | | |
| c-Met; positive | 1.28 | 0.71-2.31 | 0.412 | 0.99 | 0.53-1.85 | 0.972 |
| pY1234/1235 c-Met; positive | 1.21 | 0.64-2.26 | 0.560 | 0.95 | 0.48-1.86 | 0.870 |
| pY1349 c-Met; positive | 1.08 | 0.60-1.95 | 0.788 | 0.75 | 0.39-1.43 | 0.375 |
| For PD-L1 | | | | | | |
| c-Met; positive | 4.25 | 2.22-8.15 | <0.001 | 3.53 | 1.78-7.00 | <0.001 |
| pY1234/1235 c-Met; positive | 1.74 | 0.93-3.25 | 0.082 | 1.18 | 0.59-2.34 | 0.643 |
| pY1349 c-Met; positive | 4.20 | 2.26-7.80 | <0.001 | 3.17 | 1.64-6.12 | 0.001 |

^aAdjusted by grade, muscle invasion and metastasis. OR, odds ratio; 95% CI, 95% confidence interval; COX, cyclooxygenase; HO, hemeoxygenase; VEGF, vascular endothelial growth factor; PD-L; programmed death ligand.

Table IV. Associations among cancer-related molecules.

| Cancer-related molecules | Univariate analysis | | | Multivariate analysis ^a | | |
|--------------------------|---------------------|-----------|---------|------------------------------------|-----------|---------|
| | OR | 95% CI | P-value | OR | 95% CI | P-value |
| Cox-2; positive | | | | | | |
| HO-1; positive | 1.54 | 0.84-2.82 | 0.166 | 1.26 | 0.67-2.38 | 0.471 |
| VEGF-A; positive | 2.28 | 1.23-4.22 | 0.009 | 2.08 | 1.08-4.04 | 0.030 |
| PD-L1; positive | 3.12 | 1.61-6.03 | 0.001 | 2.61 | 1.29-5.27 | 0.008 |
| HO-1; positive | | | | | | |
| COX-2; positive | 1.54 | 0.84-2.82 | 0.166 | 1.21 | 0.63-2.32 | 0.576 |
| VEGF-A; positive | 1.22 | 0.68-2.18 | 0.509 | 1.01 | 0.54-1.89 | 0.972 |
| PD-L1; positive | 1.77 | 0.98-3.12 | 0.060 | 1.31 | 0.69-2.49 | 0.406 |
| VEGF-A; positive | | | | | | |
| COX-2; positive | 2.28 | 1.23-4.22 | 0.009 | 2.08 | 1.07-4.02 | 0.030 |
| HO-1; positive | 1.22 | 0.68-2.18 | 0.509 | 1.00 | 0.54-1.88 | 0.995 |
| PD-L1; positive | 0.72 | 0.61-1.97 | 0.759 | 0.72 | 0.37-1.39 | 0.323 |
| PD-L1; positive | | | | | | |
| COX-2; positive | 3.12 | 1.61-6.03 | 0.001 | 2.61 | 1.29-5.27 | 0.007 |
| HO-1; positive | 1.76 | 0.98-3.19 | 0.060 | 1.30 | 0.69-2.48 | 0.419 |
| VEGF-A; positive | 1.10 | 0.61-1.97 | 0.759 | 0.72 | 0.37-1.40 | 0.331 |

^aAdjusted by cancer-related molecules, grade, muscle invasion and metastasis. OR, odds ratio; 95% CI, 95% confidence interval; COX, cyclooxygenase; HO, hemeoxygenase; VEGF, vascular endothelial growth factor; PD-L, programmed death ligand.

invasion and metastasis. Unfortunately, we cannot describe the reasons for such findings. However, we believe that this result

is logical as nearly all molecular agents with anti-VEGF-A activity have not been found to improve outcomes for patients

Table V. Association between cancer-related molecules and malignant behaviour.

| Cancer-related molecules | Univariate analysis | | | Multivariate analysis | | |
|--|---------------------|-----------|---------|-----------------------|-----------|---------|
| | OR | 95% CI | P-value | OR | 95% CI | P-value |
| For muscle invasion^a | | | | | | |
| COX-2; positive | 3.56 | 1.55-8.18 | 0.003 | 2.64 | 1.02-6.81 | 0.045 |
| HO-1; positive | 2.23 | 1.11-4.48 | 0.024 | 1.36 | 0.59-3.14 | 0.465 |
| VEGF-A; positive | 2.65 | 1.29-5.45 | 0.008 | 2.10 | 0.87-5.04 | 0.098 |
| PD-L1; positive | 3.71 | 1.86-7.43 | <0.001 | 2.20 | 0.97-4.96 | 0.058 |
| For metastasis^b | | | | | | |
| COX-2; positive | 3.83 | 0.84-17.5 | 0.084 | 1.80 | 0.35-9.30 | 0.485 |
| HO-1; positive | 6.06 | 1.33-27.7 | 0.020 | 4.24 | 0.86-20.9 | 0.076 |
| VEGF-A; positive | 1.62 | 0.53-4.93 | 0.398 | 0.83 | 0.23-2.92 | 0.766 |
| PD-L1; positive | 9.99 | 2.19-45.7 | 0.003 | 5.51 | 1.12-27.2 | 0.036 |

^aAdjusted by grade and metastasis. ^bAdjusted by grade and muscle invasion. OR, odds ratio; 95% CI, 95% confidence interval; COX, cyclooxygenase; HO, hemeoxygenase; VEGF, vascular endothelial growth factor; PD-L, programmed death ligand.

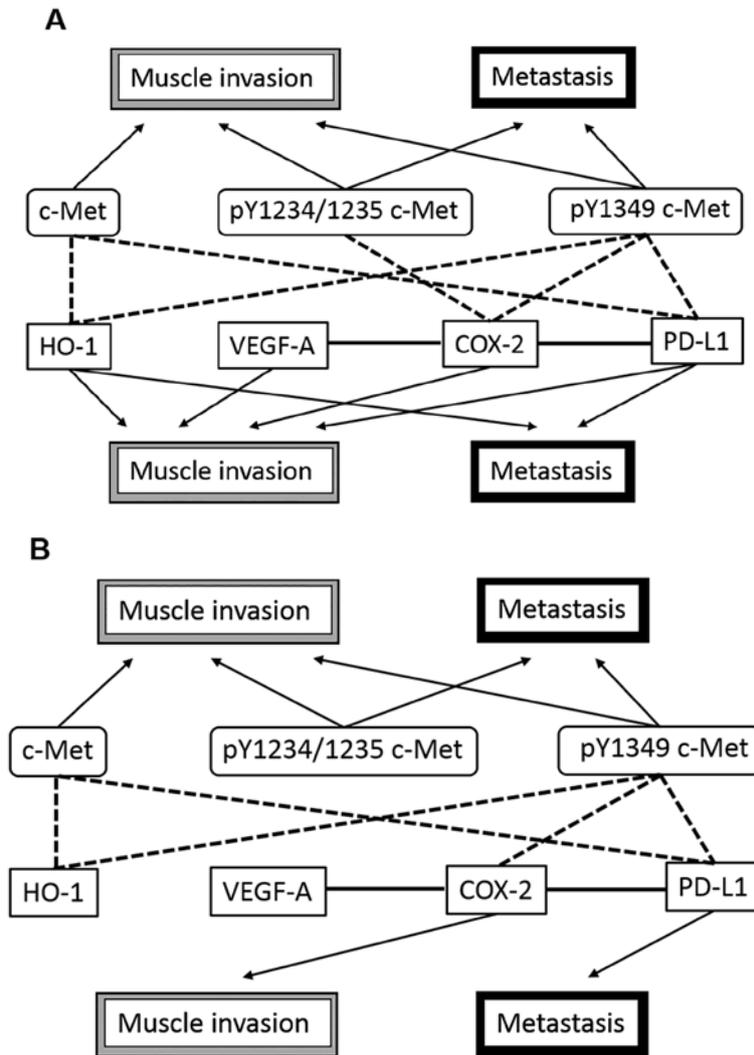


Figure 2. Schematic representation demonstrating associations between c-Met, phosphorylated c-Met, cancer-related molecules and malignant behavior. (A) c-Met and phosphorylated c-Met were suggested to serve important roles in muscle invasion and metastasis; (B) however, such molecular mechanisms did not differ significantly between c-Met, pY1234/1235 c-Met and pY1349 c-Met in bladder cancer. Of note, the present results were obtained via association analyses alone. This schema is useful to design further detailed *in vivo* and *in vitro* studies as these results were based on multivariate analysis models including pathological features. COX-2, cyclooxygenase-2; HO-1, heme oxygenase-1; PD-L1, programmed death ligand 1.

with BC (38). Furthermore, there is a possibility that VEGF-A might modulate cancer cell invasion and/or metastasis through COX-2 or PD-L1 in BC. Finally, as shown in our schematic, we speculate that c-Met and phosphorylation of the multi-functional docking domain (Y1349) play important roles in cancer cell invasion and metastasis by regulating COX-2 and PD-L1 in patients with BC. Therefore, it is possible that these molecules might serve as useful targets to treat these patients.

An additional key finding in our study is the determination that pY1234/1235 c-Met expression was closely associated with both muscle invasion and metastasis in BC, regardless of the expression of COX-2, HO-1, VEGF-A, and PD-L1. Hence, inhibiting pathways that originate from pY1234/1235 c-Met might lead to the inhibition of malignant behavior and progression via independent mechanisms derived from COX-2, HO-1, VEGF-A, and PD-L1 in BC. Presently, treatment strategies including immune check-point inhibitors such as PD-L1-targeting agents comprise a hot topic in the field of urological oncology (39-41). Importantly, many investigators are interested in pursuing the development of treatments that exploit COX-2, HO-1, and VEGF-A inhibitors for BC (42-44). Further, the anti-cancer effects of inhibiting kinase domain (Y1234/1235) phosphorylation and/or suppressing its activities are speculated to be different from those of COX-2, HO-1, VEGF-A, and/ or PD-L1 inhibitors in patients with BC. Therefore, we hypothesize that pY1234/1235 is a potential therapeutic target for BCs that are resistant to COX-2, HO-1, VEGF-A, and/ or PD-L1 inhibitors.

Our study had several limitations based on methodology. First, the staining patterns and pathological significance of PD-L1 expression in cancer tissues were previously reported to be dependent on the types of antibodies used, such as VENTANA SP142, VENTANA SP263, DAKO 22C3, and DAKO 28-8 (16,44). In this study, we used clone E1L3N of an anti-PD-L1 antibody that was previously used for another study (30). Therefore, we should note that differences in antibody specificities could exist if other anti-PD-L1 antibodies were used. The next limitation is that we evaluated PD-L1 expression in BC cells but not in infiltrating immune cells of stromal tissues despite the fact that this marker was expressed in both cancer cells and infiltrating immune cells in tissues (45). In this study, we focused on PD-L1 expression in BC cells because we wanted to clarify the pathological networks associated with c-Met in BC cells and various cancer-related factors including PD-L1 in patients with BC. It is difficult to evaluate the expression of each molecule in stromal cells that have infiltrated into NMIBC tissues because infiltrating cells within NMIBC tissues were relatively rare. In contrast, it is thought that PD-L1 expression in tumor-infiltrating immune cells has no significant pathological role in tumor growth, metastasis, and prognosis after cystectomy in patients with BC (20). Furthermore, in hepatocellular carcinoma, c-Met expression was found to be positively associated with PD-L1 expression in cancer cells but not in infiltrating cells (16). Based on these facts, we investigated the relationships between c-Met expression and various cancer-related molecules. We also emphasize the importance of clarifying the pathological roles of PD-L1 expression in infiltrating cells within stromal tissues of BC. However, to accomplish these goals, we only performed correlation analysis, rather than a series of *in vitro* and *in vivo*

experiments. Hence, due to our study design, we are unable to provide definitive conclusions regarding the pathological roles of phosphorylated c-Met expression in BC. Nonetheless, we believe that our results will prove useful in advancing future research including the design of *in vitro* and *in vivo* experiments, as c-Met can modulate numerous cancer-related factors via complex mechanisms. In short, our results obtained via multivariate analysis, including that of clinicopathological features, are useful to discuss the pathological roles of the c-Met pathway at the molecular level in patients with BC.

In conclusion, the present study demonstrated that c-Met is positively associated with muscle invasion by regulating HO-1 and PD-L1 and that pY1349 c-Met is associated with muscle invasion and metastasis via the regulation of COX-2, HO-1, and PD-L1 in patients with BC. Alternatively, pY1234/1235 was also found to be associated with muscle invasion and metastasis; however, no correlation was observed with various other cancer-related molecules that were examined. From these results, we postulated that pY1234/1235 might serve as a potential therapeutic target for patients with BC and other diseases that are resistant to inhibitors of COX-2, HO-1, VEGF-A, and/ or PD-L1. However, unfortunately, since our results are solely based on correlation analyses of immunohistochemical expression patterns, further *in vitro* and *in vivo* studies are required to definitively identify the detailed pathological roles of c-Met, pY1234/1235 c-Met, and pY1349 c-Met in BC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YMu performed the experiments and contributed to sample collection and writing of the manuscript. YMi conceived and designed the experiments, generated and analyzed data, and contributed to the writing of the manuscript. KA, YN, TY and AO performed the experiments and analyzed the data. KM, TM and KO contributed to clinical data collection and analyzed the data. HS designed the experiment and was involved in revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The purpose of the present study was explained to the participants, all of whom provided written consent prior to the study.

This study design was approved by the Institutional Review Board of Nagasaki University Hospital (Nagasaki, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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