Immunohistochemical demonstration of EphA2 processing by MT1-MMP in invasive cutaneous squamous cell carcinoma

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ABSTRACT

Erythropoietin-producing hepatocellular receptor-2 (EphA2) overexpression is prevalent in many types of human cancers, and it has been reported that high EphA2 expression is correlated with malignancy. Recent studies revealed that processing of EphA2 by cleaving off the N-terminal portion by membrane-type 1 matrix metalloproteinase (MT1-MMP) promotes invasion via stimulation of Ras in cancer cells in vitro. The objectives of this study were to investigate the presence and role of EphA2 processing in cutaneous squamous cell carcinoma (SCC) tissues. EphA2 (C-terminal and N-terminal) and MT1-MMP expression patterns and levels were analyzed immunohistochemically in SCC (n=70) and Bowen disease (BD; n=20). Levels of MT1-MMP and EphA2 expression were evaluated using digital image analysis. Proximity between MT1-MMP and EphA2 in cancer cells and its effect on EphA2 processing were investigated using a combination of *in situ* Proximity Ligation Assay (PLA) and western blotting. Immunohistochemical analyses showed that levels of EphA2 N-terminal expression were significantly lower than those of EphA2 C-terminal expression in SCC, whereas levels of EphA2 C- and N-terminal expression were similar in BD. Western blotting showed processed EphA2 fragments in human SCC tissues. Expression levels of MT1-MMP, EphA2, and processed EphA2 fragments were higher in SCC than BD. Proximity between MT1-MMP and EphA2 in SCC was demonstrated by *in situ* PLA. Our results suggest possible involvement of MT1-MMP processing of EphA2 in invasiveness of cutaneous SCC.

Key words: EphA2; MT1-MMP; SCC; digital image analysis

Introduction

Cutaneous squamous cell carcinoma (SCC) is one of the most common types of skin cancer [1]. SCC frequently arises on the sun-exposed skin of middle-aged and elderly persons, and the incidence of SCC has been increasing in recent years. SCC is thought to develop from SCC *in situ*, such as Bowen disease (BD) or actinic keratosis. Degradation of stromal connective tissue and basal membrane components is a key element in tumor invasion and metastasis. Recent studies revealed that the invasion process involves multiple proteolytic enzymes, such as matrix metalloproteinase (MMP) -2, MMP-9, and membrane-type 1 matrix metalloproteinase (MT1-MMP), but the precise mechanism has yet to be elucidated [2-4].

Overexpression of erythropoietin-producing hepatocellular receptor-2 (EphA2) is prevalent in numerous solid tumors, including those of the breast [5], ovary [6], lung [7], brain (e.g., glioblastoma) [8], prostate [9], kidney [10] and skin (e.g., melanoma) [11]. Moreover, high EphA2 expression is reportedly correlated with disease stage, increased tumor metastasis, and poor patient survival, suggesting EphA2 as a candidate prognostic marker for a panel of human malignancies [12-15]. EphA2 is a member of the Eph family of receptor tyrosine kinase, which interact with cell-bound ligands known as ephrins [16,17]. Furthermore, soluble EphrinA1 (sEphrin-A1) can be detected in the culture media of tumor cells and is present in

the serum of patients with liver cancer [18-20]. In normal cells, EphA2 stimulation with EphrinA1 inhibits Ras and its downstream signals, leading to suppression of the EGF-EGFR signaling pathway. In contrast, overexpression of EphA2 in cancer cells promotes invasion and metastasis in a ligand-independent manner [21-23]. The ligand-binding domain of EphA2, which resides in the N-terminal portion of the molecule, was recently shown to be cleaved by MT1-MMP, activating an oncogenic signal that results in enhanced cancer cell motility and invasion, even though soluble Ephrin-A1 is abundantly expressed [24,25].

Upregulation of MT1-MMP expression is frequently observed in many types of human cancers [26]. MT1-MMP is a membrane-bound MMP that plays a critical role in the activation of pro-MMP-2 and the degradation of a number of extracellular matrix (ECM) components as well as in the cleavage of cell-surface bioactive molecules [27-29]. By cleaving adjacent ECM components, MT1-MMP promotes cancer cell invasion through the basement membrane. Furthermore, membrane proteins cleaved by MT1-MMP modulates a variety of biological activities [30].

To our knowledge, there are no published reports evaluating the role of EphA2 expression in cutaneous SCC, particularly with respect to EphA2 processing. Here, we compared the processing of EphA2 in invasive SCC and pre-invasive BD by immunostaining and western blotting analyses. Moreover, mediation of EphA2 processing by MT1-MMP was evaluated through the use

of an *in situ* proximity ligation assay (PLA).

Materials and Methods

Tissue samples

The study material comprised 70 SCC samples from 34 males and 36 females (age range, 46-99 [mean, 75] years) and 20 BD samples from 12 males and 8 females (age range, 64-90 [mean, 77] years) obtained from the skin tumor files of the Department of Pathology, Fukuoka University Hospital, between 1995 and 2013. The study protocol was approved by the Ethics Committee of Fukuoka University School of Medicine. Anonymous use of redundant tissue is part of the standard treatment agreement with patients in our hospitals when no objection has been expressed.

Immunohistochemistry

Surgically resected specimens were fixed in 10 % formalin and processed into paraffin blocks. Tissues were sectioned (3-μm thickness), and the sections were deparaffinized and immersed in 0.3 % hydrogen peroxide in methanol for 10 min at room temperature (RT) to block endogenous peroxidase activity and then heated in 10 mM citrate buffer (pH 6.0) in a microwave

oven (700 W) for 10 min to retrieve epitopes. After nonspecific sites were blocked with 5% nonfat dry milk for 1 h at RT, sections were incubated overnight at 4° C with polyclonal antibodies against MT1-MMP (1:75; Millipore, Temecula, CA) [24], EphA2-C-terminal (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) [25], or EphA2-N-terminal (1:800; Abgent, San Diego, CA, USA) [31]. Sections were then washed and incubated with ChemMate EnVision (Dako, Carpinteria, CA, USA). Immunoreactive proteins were visualized with 3, 3'-diaminobenzidine (DAB; Dako), followed by counterstaining with hematoxylin. In all cases we stained normal control skin sections on the same glass to elucidate the staining pattern and intensity in normal epidermis.

On assessment of immunohistochemical results, almost same expression levels of N- and C-terminals of EphA2 indicated that EphA2 expressed likely retained the N-terminal ligand-binding domain. In contrast, reduced expression levels of EphA2 N-terminal compared with the C-terminal indicated that EphA2 N-terminal was likely cleaved off [25].

Visual and computer supported evaluation of immunohistochemical staining

Immunohistochemical expression of MT1-MMP, EphA2-C-terminus, and EphA2-N-terminus in SCC and BD samples was assessed using Tissue Studio v.2.0 software (Definiens AG, Munich,

Germany). For image analysis, each immunohistochemically stained slide was scanned and converted to a whole-slide image (WSI, also known as a virtual slide) with NanoZoomer 2.0-RS (Hamamatsu Photonics, Hamamatsu, Japan) at 20× magnification. On each WSI, the tumor area was selected utilizing a hand-drawing tool, and tumor cells exhibiting cytoplasmic expression of MT1-MMP and EphA2 were identified with Tissue Studio v.2.0 installed on the server for NanoZoomer 2.0-RS. The DAB color intensity of positive tumor cells in every unit area was measured, and the average value per unit area (/μm^2) was calculated using Tissue Studio v.2.0. The intensity of MT1-MMP and EphA2 staining was also measured in epithelial cells of normal skin, which had been added as control to each sample glass slide with tumor tissues. Furthermore, the ratio of the average staining intensity of tumor cells to that of control epithelial cells was calculated.

Cell culture and stable knockdown of MT1-MMP using shRNA

To assess MT1-MMP-mediated processing of EphA2, we used human HT1080 fibrosarcoma cells (American Type Culture Collection, Manassas, VA) and HT1080-short hairpin (sh)-MT1-MMP (knockdown) cells, which were cultured in DMEM supplemented with 10 % FBS, streptomycin (50 μg/ml), penicillin G (50 U/ml), and blastcidin (50 μg/ml). For stable knockdown of MT1-MMP

by shRNA, MT1-MMP knockdown cells were established using the Virapower Lentiviral Expression System (Invitrogen; Carlsbad, CA) and the cells were maintained in the presence of 10 μg/ml of blasticidin (Invitrogen; Carlsbad, CA). The shRNA sequences used for knockdown of MT1-MMP was 5′ -cagcgatgaagtcttcactta-3′ [25].

Protein extraction and western blotting

All specimens were frozen immediately in liquid nitrogen, embedded in tissue compound, and kept at -80° C until use. Cells in frozen tissues were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40; Millipore, Bedford, MA), sonicated on ice three times for 10 min each, and the resultant lysates were centrifuged at 15000 rpm for 20 min at 4° C. The resultant supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred electrophoretically to an Immobilon membrane (Millipore). Nonspecific sites were blocked with 5% dry nonfat milk in TBS at 37° C for 1 h, and the membrane was then incubated overnight at 4° C with anti-EphA2 (Santa Cruz Biotechnology) [25] and anti-MT1-MMP (AB6004, Millipore) [24] antibodies. After washing with TBS-T (TBS containing 0.05% Tween-20), the membrane was incubated for 1 h with peroxidase-conjugated anti-rabbit IgG. Color was developed

with chemiluminescence reagents according to the manufacturer's instructions (PerkinElmer, Waltham, MA).

In situ Proximity Ligation Assay

Tumor tissues were analyzed using the Duolink PLA (Olink, Uppsala, Sweden), which allows the identification of protein-protein interactions. This technique uses modified secondary antibodies that bind to the Fc portion of two different primary antibodies targeted against candidate proteins of interest. These secondary antibodies are linked to a system that produces oligonucleotides by rolling nucleotide amplification if the proteins to which they are bound are in proximity. Probes that will fluoresce in the presence of oligonucleotides are then added. Thus, each fluorescent signal represents a point of endogenous protein-protein interaction. PLAs were performed according to the manufacturer's instructions. Briefly, sections were pretreated, and primary antibodies against the EphA2 C-terminal (1:200; Santa Cruz Biotechnology) [25], EphA2 N-terminal (1:800; Abgent) [31], and MT1-MMP (1:75) [30] were applied as detailed for immunohistochemistry. For isotype controls, primary antibody was substituted with either rabbit (EphA2 C-terminal or EphA2 N-terminal) or mouse (MT1-MMP) IgG. Sections were then washed twice for 5 min each in Duolink wash buffer A (Olink) before PLA

PLUS and MINUS probes (Olink) were applied for 1 h at 37° C. Following washing (as previously), ligation-ligase solution (Olink) was applied to each sample for 30 min at 37° C. Sections were washed again, and amplification-polymerase solution (Olink) was applied for 100 min at 37° C. Sections were then washed twice for 10 min each in 1× Duolink wash buffer B (Olink) and then for 1 min in 0.01× Duolink wash buffer B before being allowed to air dry. Dried sections were mounted with Duolink II Mounting Medium with DAPI (Olink) before images were captured using the DeltaVision Deconvolution System (Applied Precision, Inc., Issaquah, WA). In paraffin sections of formalin-fixed tissues, dot signals were visualized with DAB. The number of *in situ* PLA signals per cell was determined using Tissue Studio v.2.0.

Statistical analysis

Quantitative data are presented as mean ± standard deviation (SD) and were analyzed using the Student's t-test. A p-value < 0.05 was considered indicative of statistical significance. All data analyses were conducted using StatMate IV for Windows (ATMS, Tokyo, Japan).

Results

Clinicopathological findings

Table 1 summarizes the clinical characteristics of the 70 patients with SCC and 20 patients with BD. In SCC patients, 37% (26 of 70) of the lesions were located on the face and sun-exposed parts, consistent with many reports of SCC on sun-exposed skin, such as the forehead, face, ears, scalp, neck, and dorsum of the hands. Both SCC and BD showed no gender or age predilection.

Immunochemical staining of EphA2 and MT1-MMP

Expression of EphA2 and MT1-MMP was examined immunohistochemically in SCC and BD, and representative examples are shown in Figure 1. Both EphA2 and MT1-MMP were expressed on the cell membrane and in the cytoplasm. In SCC and BD tissues, EphA2 was expressed diffusely throughout the tumor in most cases. MT1-MMP immunolocalization tended to be uneven with more intensities in infiltrative portions in SCC, while BD cells almost evenly expressed MT1-MMP. Quantitative assessment of immunoreactivity is illustrated in Figure 2. In invasive SCC, levels of EphA2 N-terminal expression were significantly lower than levels of EphA2 C-terminal expression ($p < 0.001$). In contrast, expression levels of the C- and N-terminals of EphA2 were similar in BD ($p = 0.2244$). Expression of MT1-MMP was observed in both SCC and BD, and its expression levels were higher in BD than in SCC ($p < 0.001$). Furthermore, within invasive SCC,

we examined the correlation between expression levels of EphA2 and MT1-MMP and clinicopathological parameters (n = 70). Regardless of tumor stratification such as differentiation, Clark level and lymph node metastasis, expression levels of EphA2 N-terminal were significantly lower than those of EphA2 C-terminal (Table 2). As for tumor thickness (< 2 mm vs ≥ 2 mm; according to NCCN guidelines version 2.2012 [32]), tumors with invasion ≥ 2 mm showed significantly lower expression levels of EphA2 N-terminal than those of EphA2 C-terminal, while no significant difference was found in tumors with invasion < 2 mm. MT1-MMP expression levels showed no significant difference according to the tumor stratification (data not shown).

Detection of EphA2 and MT1-MMP protein in human cutaneous SCC and BD tissues

We examined the expression of EphA2 and MT1-MMP protein using western blotting analysis of frozen human tissue samples (2 SCC and 2 BD tissue samples). Bands representing processed EphA2 fragments (50-65 kDa) were detected in extracts of human SCC tissue. EphA2 was expressed as a 130-kDa band, and MT1-MMP appeared as a 65-kDa band. EphA2 and MT1-MMP were noted in extracts of both BD and SCC tissue, although band intensity was higher in SCC than BD samples (Figure 3).

Expression and interaction of EphA2 and MT1-MMP in HT1080 cells

Next, we examined whether the protein-protein interaction between EphA2 and MT1-MMP occurs using *in situ* PLA with human HT1080 fibrosarcoma cells *in vitro*. In MT1-MMP-expressing HT1080 cells, signals indicating interaction between the C-terminal of EphA2 and MT1-MMP were observed (Figure 4a, 34.8 ± 2.53 signals/cell (mean ± SE, n = 10)), and processed EphA2 fragments were detected by western blotting analysis (Figure 4b). In contrast, in HT1080 MT1-MMP-knockdown cells, few signals indicative of interaction were observed (3.1 ± 0.35 signals/cell (mean ± SE, n = 10), $p < 0.001$), and fewer processed EphA2 fragments were detected (Figure 4a, b).

EphA2 and MT1-MMP interaction in human cutaneous SCC and BD tissues

To examine whether EphA2 and MT1-MMP interact *in vivo*, we performed an *in situ* PLA using human cutaneous SCC and BD tissues. Signals indicating interaction between EphA2 (both the C- and N-terminals) and MT1-MMP were observed in both SCC and BD tissues (Figure 5a). In SCC tissues, fewer signals indicative of interaction between MT1-MMP and the N-terminal of EphA2 were observed compared with MT1-MMP and the C-terminal of EphA2, although the difference

was not statistically significant (p = 0.3). There was no significant difference in signal counts in the BD tissues (Figure 5b).

Discussion

To the best of our knowledge, this is the first report that shows presence of EphA2 processing and its possible involvement in invasiveness in human cutaneous SCC tissues. Although the processing of EphA2 by MT1-MMP into a ligand-independent, biologically active form was recently reported by Koshikawa et al. [25] predominantly *in vitro* using cell lines or using animal models, frequent occurrence of this processing was also shown *in vivo* in human SCC tissues in this report. Furthermore, the processing of EphA2 was studied comparatively between invasive SCC and pre-invasive BD tissues. EphA2 was cleaved and processed in invasive SCC but not in pre-invasive BD, because immunohistochemical expression of N-terminal EphA2 was significantly reduced in SCC compared with that of C-terminal EphA2, while this reduction was not seen in BD. We also observed processed EphA2 fragments by western blotting. Moreover, *in situ* PLA results showed protein-protein interactions between EphA2 and MT1-MMP. Our findings suggest that MT1-MMP is likely the cleavage protease of EphA2. Protein quantification using digital image analysis improved the accuracy in this study.

Upon ligand binding, EphA2 is autophosphorylated at cytoplasmic tyrosine residues. As a result, p120Ras-GTP, which inactivates Ras, is recruited and then inhibits the EGF-EGFR signaling pathway [22] [18] [33]. In normal cells, therefore, ligand-dependent EphA2 activation inhibits oncogenic signaling pathways. In contrast, in cancer cells, ligand-independent EphA2 activation enhances malignancy. Ephrin-independent activities of EphA2 do not lead to inhibition of EGFR-associated growth pathways but stimulate Ras/Erk1/2 activated via ErbB-receptor signaling [22]. Moreover, stimulation of ErbB receptors activates the PI3K/Akt pathway, leading to phosphorylation of EphA2 at cytoplasmic serine residues [18]. Consequently, ephexin-4 promotes RhoG activation and recruitment of the RhoG-GTP-binding proteins ELMO2 and DOCK4, resulting in Rac1 activation, which in turn leads to increased cell migration and invasiveness [23] [33]. A recent study demonstrated that the ligand-binding domain of EphA2 is cleaved by MT1-MMP, which enables the conversion of EphA2 into a ligand-insensitive form, which leads to activation of oncogenic signaling pathways such as Ras/Erk1/2 and PI3K/Akt, even though soluble Ephrin-A1 is abundantly expressed [24,25]. In the present study, we showed that EphA2 is processed in invasive SCC, but EphA2 processing was not detected in pre-invasive BD. These findings concur with other reports indicating that processing of EphA2 leads to an increase in invasiveness [24,25]. In MT1-MMP-expressing HT1080 cells, close interaction of EphA2 and MT1-MMP and cleavage of EphA2 by MT1-MMP were clearly

demonstrated by *in situ* PLA and western blotting. In human cutaneous SCC tissues, proximity between MT1-MMP and EphA2 was also demonstrated by *in situ* PLA. These data suggest that MT1-MMP is likely the protease that cleaves EphA2 in cutaneous SCC. However, proximity between MT1-MMP and EphA2 was also demonstrated in BD. We speculate that an additional factor may be responsible for inducing the cleavage of EphA2 by MT1-MMP in invasive cutaneous SCC. In our study, SCC frequently developed in the head and neck region that includes sun-exposed skin, whereas BD occurred frequently in hands and legs, with only 2 cases in the head and neck region. As for development of SCC, exposure to UV in sunlight is generally recognized as the most important extrinsic factor, and other factors include radiation therapy, previous burns, inflammatory lesions and long standing ulcers [34]. The exact underlying causes of BD remain unclear, although multiple factors such as arsenic, coal tar, and HPV infection are likely to be responsible [34]. Regardless of pathogenetic factors and locations of SCC, EphA2 appears to be cleaved and activated in all invasive SCC. The genetical or epigenetic changes related to this processing of EphA2 might be missing in BD.

EphA2 is frequently overexpressed in many types of cancer, and high EphA2 expression is often associated with aggressive cancer progression and malignancy [12-15]. Although numerous reports have shown that high EphA2 expression correlates with poor prognosis, some reports have shown that high EphA2 expression is not associated with reduced survival [35],

and others have indicated that high EphA2 expression is correlated with reduced survival only in univariate analyses [12]. Moreover, for patients with pathological stage I non-small cell lung carcinoma, higher expression of EphA2 is related to favorable clinicopathologic features [36]. This discrepancy may be explained by the presence or absence of EphA2 processing. In cancer cells, ligand-independent EphA2 activation is critical. Although ligand-dependent EphA2 activation leads to tumor suppression, ligand-independent EphA2 activation has been correlated with malignant transformation. As mentioned above, this ligand-independent activation of EphA2 is induced by MT1-MMP cleavage of EphA2. Upon this cleavage of EphA2, immunoreactivity to anti-EphA2 N-terminal antibody is lost. Therefore, we propose that it is important to evaluate not only with respect to EphA2 expression in general but also with respect to the levels of expression of both the C- and N-terminals of EphA2, for elucidating the role of EphA2.

Upregulation of MT1-MMP, which is frequently observed in numerous types of cancer, promotes cancer cell invasion [29]. Our study revealed that MT1-MMP is expressed in both SCC and BD, with higher expression levels in BD than in SCC. Another study showed that although expression of MT1-MMP was higher in cutaneous SCC and BD compared with normal epithelium, there was no statistically significant difference in MT1-MMP expression between SCC and BD [3]. The authors of that report speculated that a complex involving MT1-MMP, MMP-2, and TIMP-2 mediates

invasiveness in SCC. In addition, analysis of the expression of only one of these molecules or the independent quantification of these molecular markers will not provide sufficient information to enable elucidation of the oncogenic mechanism, because these molecules act in a complex manner. Our results suggest that interaction between MT1-MMP and EphA2 plays a more critical role in the invasive mechanism of SCC than high MT1-MMP expression.

In the present study, protein staining intensity and *in situ* PLA signals were measured objectively using digital image analysis. Several manual scoring systems are used in order to semi-quantify protein expression based on staining intensity. However, manual scoring systems are time-consuming and are subject to significant intra- and interobserver variability. With the rise of advanced digital scanners, image-management tools, and image analysis software, the emphasis on digital pathology has grown substantially. Several studies have shown that quantification of protein expression using digital image analysis is a useful tool that complements manual scoring systems relying on pathologist interpretation [37-39]. We believe that digital image analysis is both reproducible and objective and is a powerful tool for assessing immunohistochemistry data. Quantitative analysis of immunohistochemistry data using digital image analysis improves both the accuracy and reproducibility of the pathologist's interpretations. Thus, these strategies open up a pathway that may lead to more effective future studies.

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Compliance with ethical standards

This study was approved by the Ethics Committee of Fukuoka University School of Medicine (No 14-9-09).

Conflict of interest

The authors declare that they have no conflict of interests.

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Figure legends

Fig. 1 Immunohistochemical analysis of C-terminal (C-ter) of EphA2, N-terminal (N-ter) of EphA2 and MT1-MMP expression in invasive SCC (**a**, **c**, **e**, **g**) and pre-invasive BD tissues (**b**, **d**, **f**, **h**). Immunoreactivity of EphA2 N-ter (**e**) was reduced compared with that of EphA2 C-ter (**c**) in SCC, whereas this difference was not evident in BD (**d**, **f**). This reduced expression levels of EphA2 N-ter compared with the C-ter indicated that EphA2 N-ter was likely cleaved off. Insets show normal epidermis shown as control. **a**, **b**, hematoxylin-eosin; original magnifications: **a**, **c**, **e**, **g** ×20; **b**, **d**, **f**, **h** ×40.

Fig. 2 Quantitative analysis of EphA2 and MT1-MMP expression in SCC and BD using Tissue Studio v.2.0. In SCC, expression levels of EphA2 N-terminal (N-ter) were significantly reduced compared with those of EphA2 C-terminal (C-ter), whereas this reduction was not present in BD. Data are mean ± SD. $*p < 0.001$ by Student's t-test.

Fig. 3 Western blotting detection of C-terminal (C-ter) of EphA2 and MT1-MMP in tissue extracts from cutaneous SCC and BD. Asterisk denotes proteolytic fragments (50-65 kDa). SCC1-2 and BD1-2 indicate two different cases of human SCC and BD, respectively.

Fig. 4 (**a**) Detection of EphA2 and MT1-MMP expression by *in situ* PLA and visualization by fluorescence detection. Red dots indicate proximity between MT1-MMP and EphA2 signals; cell nuclei are stained blue. (**b**) Levels of C-terminal (C-ter) of EphA2 and MT1-MMP protein expression determined by western blotting. Asterisk denotes proteolytic fragments (65 and 60 kDa).

Fig. 5 *In situ* PLA of MT1-MMP and EphA2 expression in human SCC tissues. (**a**) Visualization by DAB staining. Dots show signals indicative of proximity between MT1-MMP and EphA2. (**b**) Quantification of the number of MT1-MMP and EphA2 signals per cell.

Table 1. Characteristics of patients with squamous cell carcinoma (SCC), and Bowen's disease (BD).

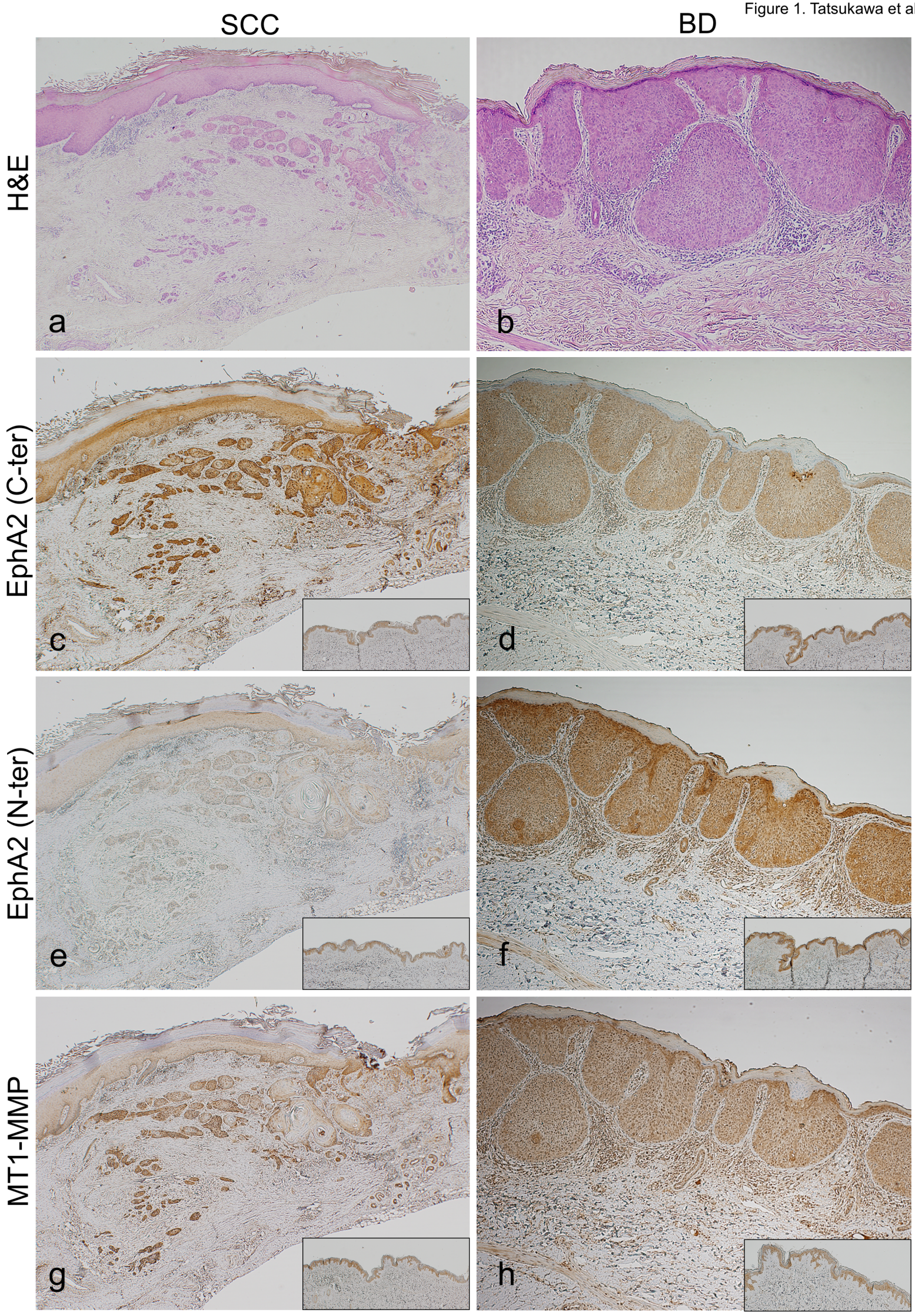
	SCC	BD	*P*-value
n	70	20	
Age (years)			0.53[a]
Mean	75.48	76.95	
Range	46-99	61-90	
Gender			0.37[b]
Male	34	12	
Female	36	8	
Location			
Head	2	0	
Face	26	2	
Arm	3	2	
Hand	11	5	
Body	6	3	
External genitals	7	2	
Leg	8	5	
Foot	7	1	

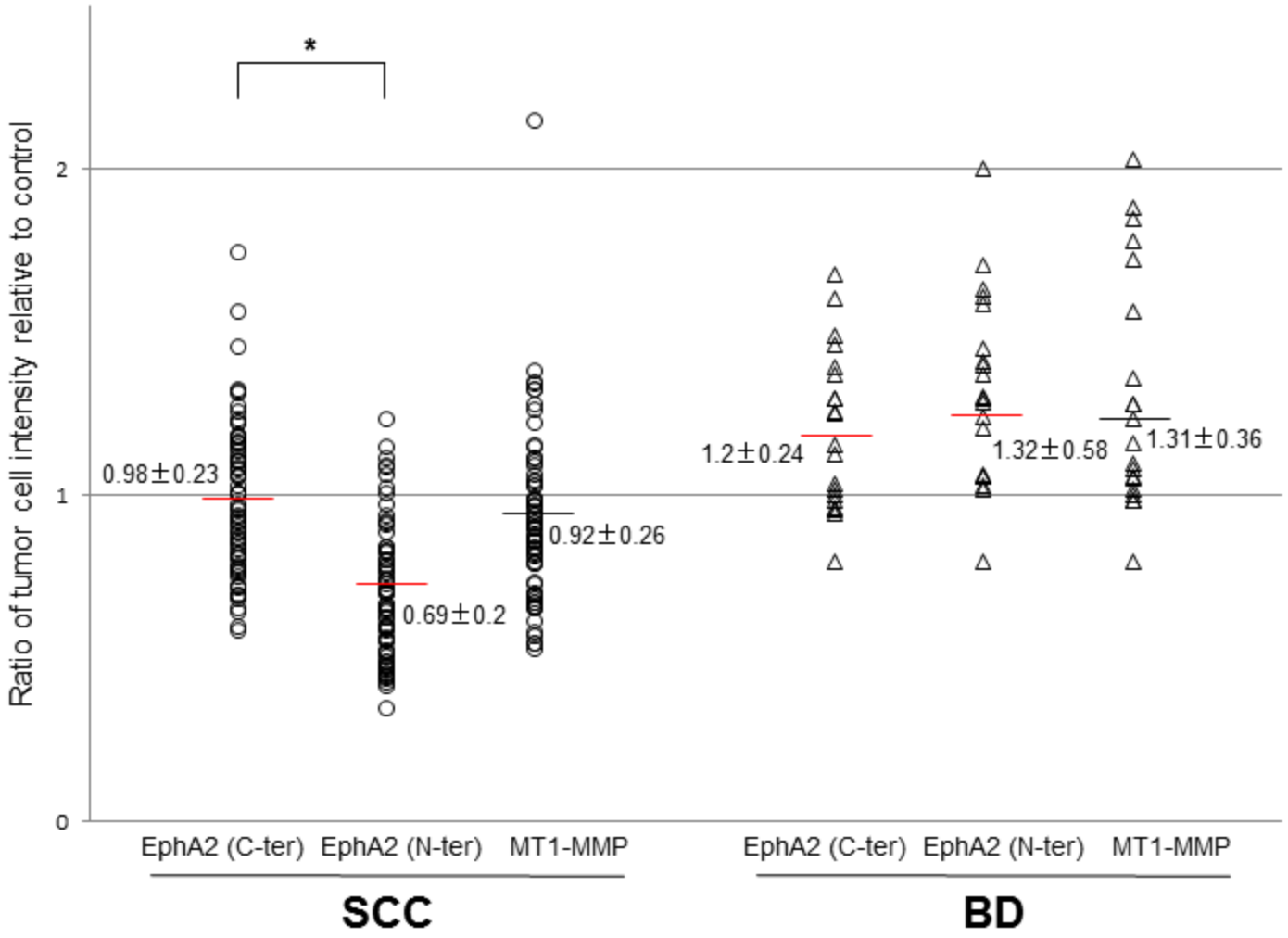
[a] Student's t-tset. [b] χ^2-test.

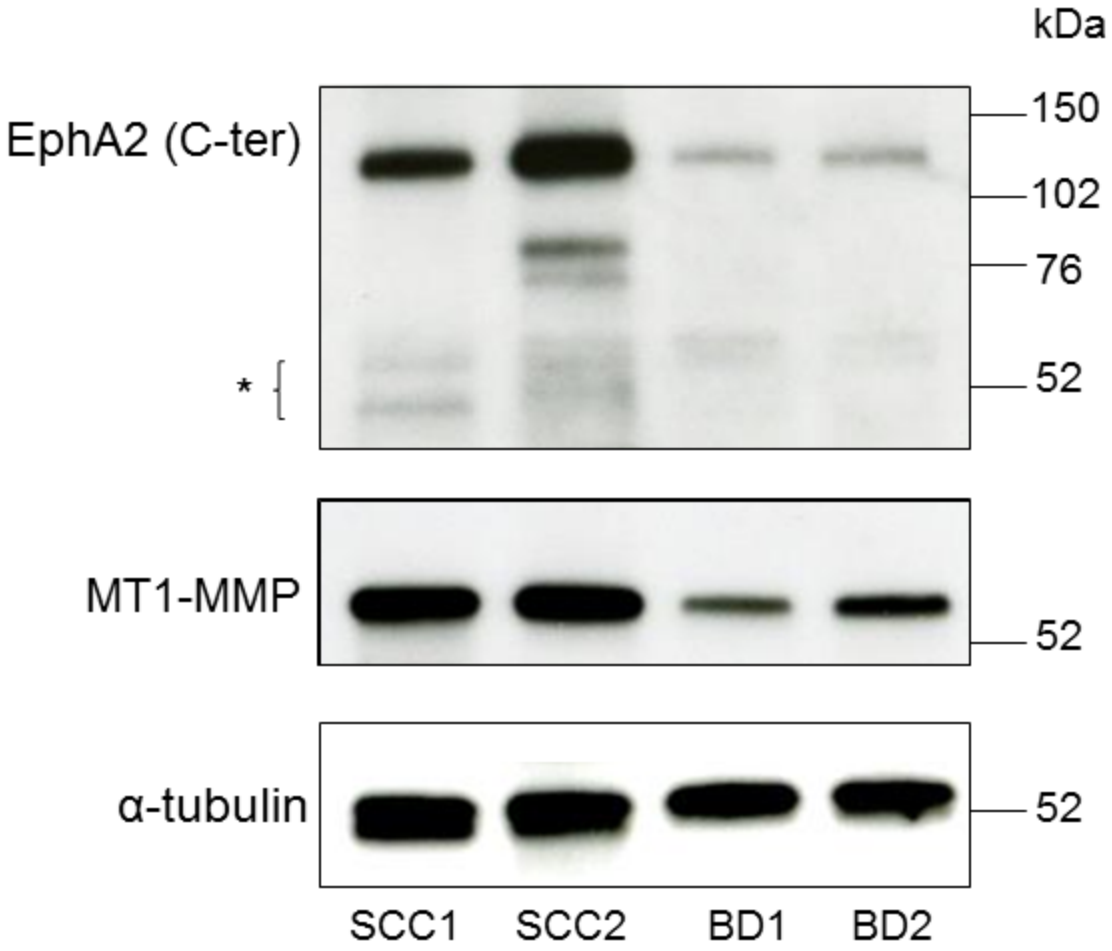
Table 2. Correlation between EphA2 expression levels and clinicopathological parameters of squamous cell carcinoma.

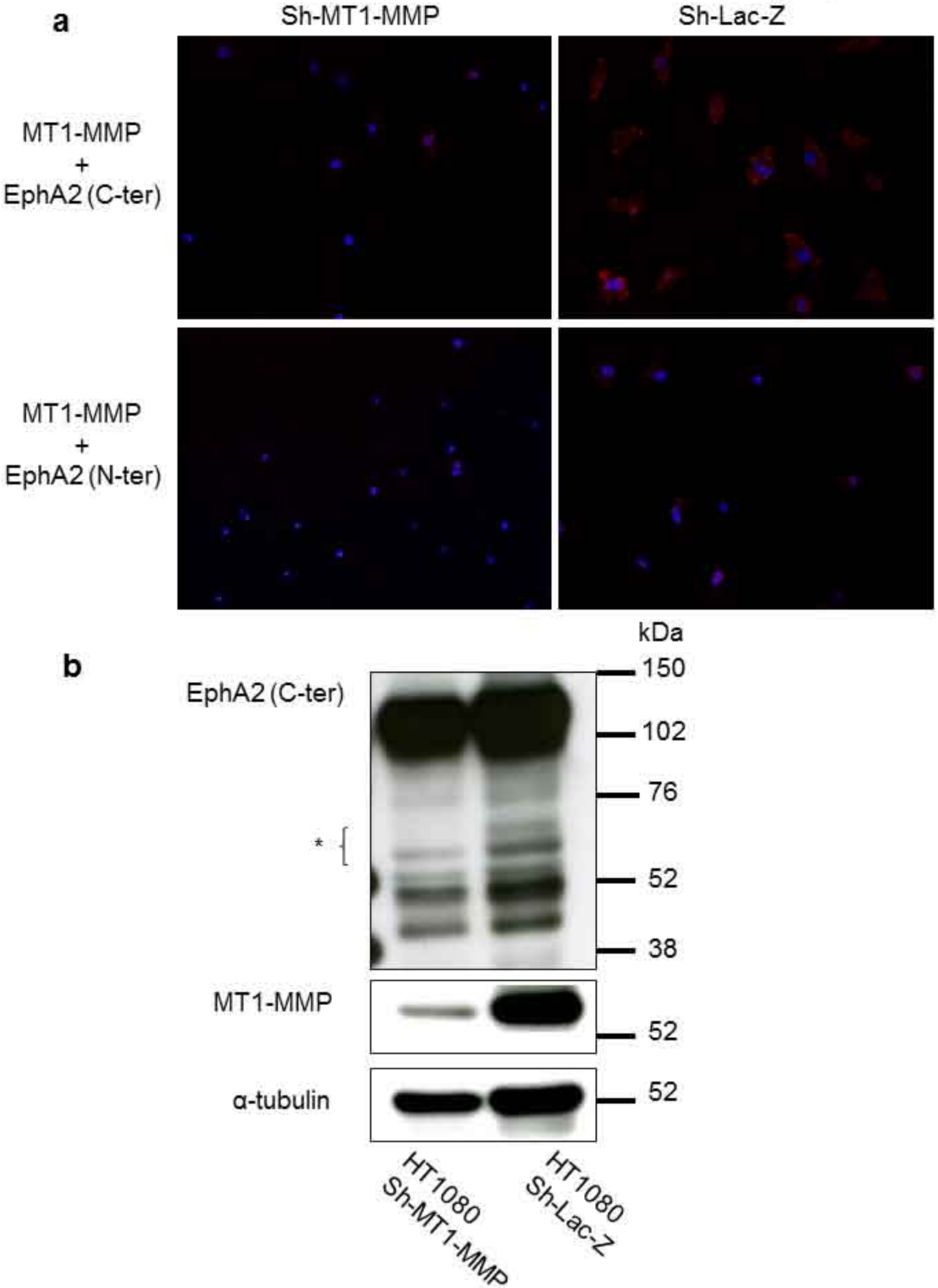
	EphA2		
	C-ter (n = 70)	N-ter (n = 70)	*P*-value [a]
Differentiation			
Well (n = 47)	0.96 ± 0.22	0.69 ± 0.19	< 0.001
Moderate (n = 18)	1.00 ± 0.27	0.76 ± 0.24	< 0.01
Poor (n = 5)	1.10 ± 0.29	0.57 ± 0.2	< 0.01
Tumor thickness			
< 2 mm (n = 8)	1.06 ± 0.23	0.84 ± 0.25	0.08
≥ 2 mm (n = 62)	0.97 ± 0.23	0.68 ± 0.19	< 0.001
Clark level			
< III (n = 39)	0.96 ± 0.23	0.72 ± 0.21	< 0.001
≥ IV (n = 31)	1.01 ± 0.24	0.66 ± 0.2	< 0.001
Lymph node metastasis			
Yes (n = 10)	0.92 ± 0.16	0.68 ± 0.2	< 0.05
No (n = 60)	0.99 ± 0.24	0.69 ± 0.2	< 0.001

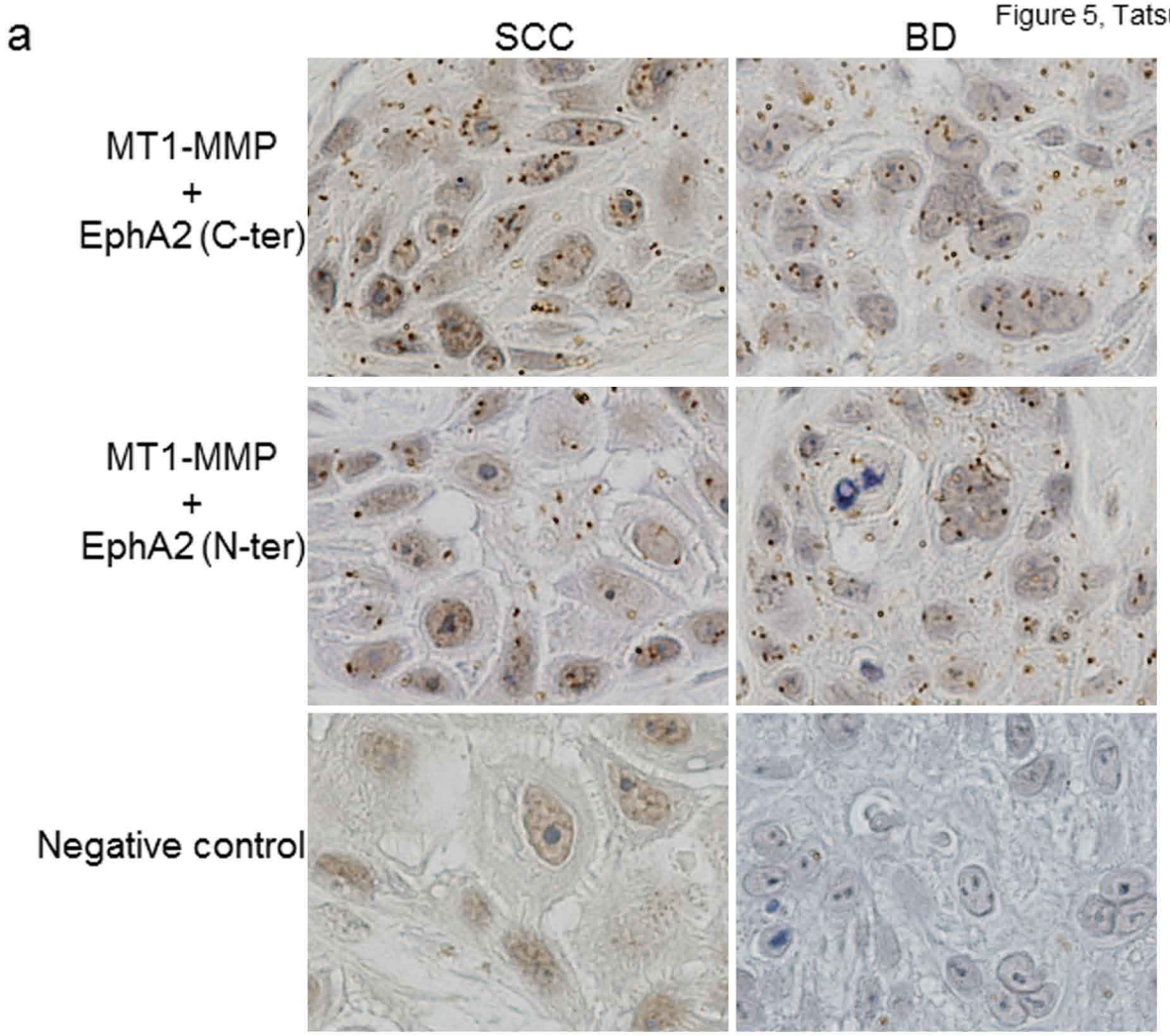
C-ter, C-terminal; N-ter, N-terminal; [a] Student's *t*-test.

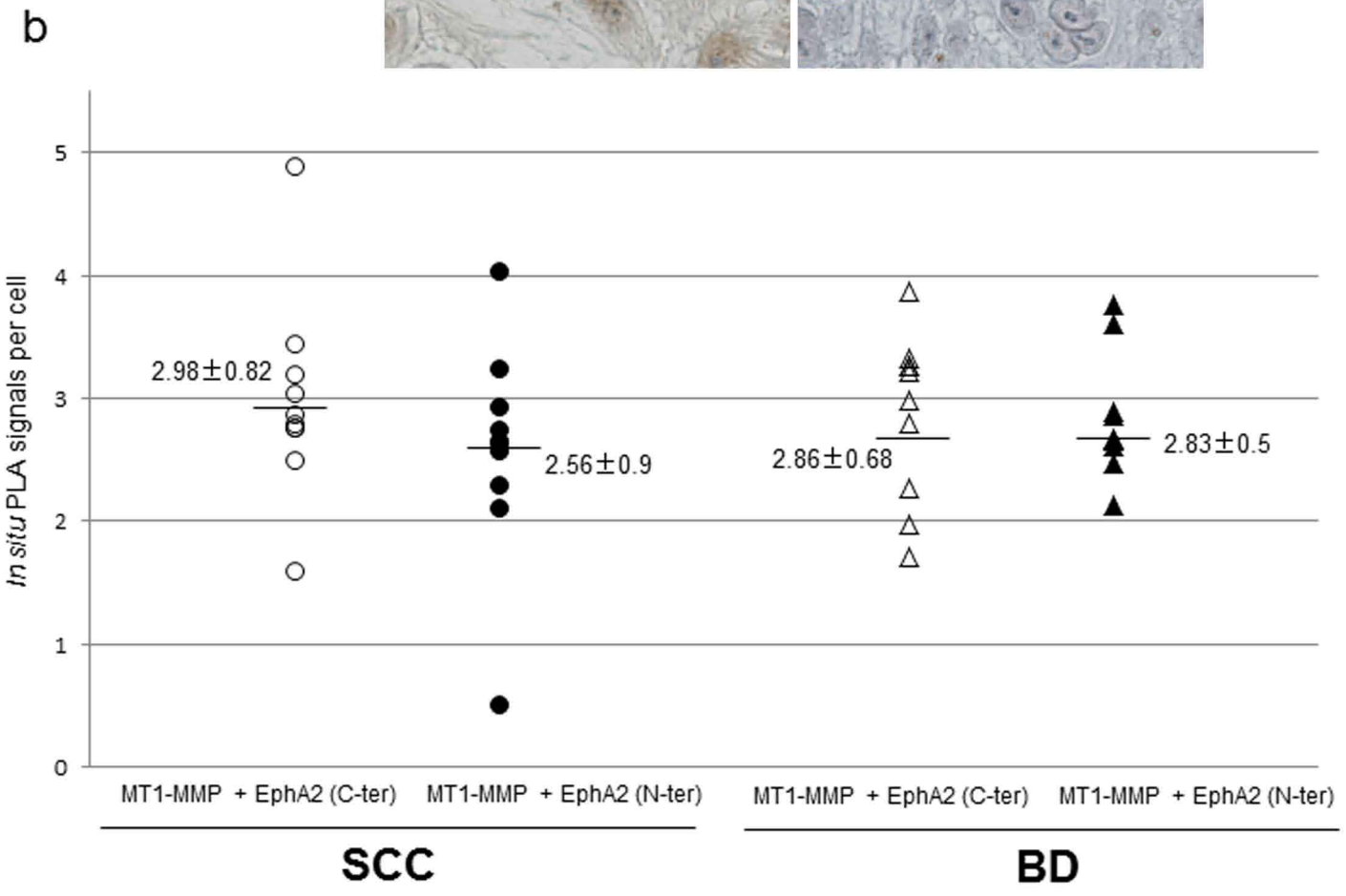

SCC
BD
H&E
EphA2 (C-ter)
EphA2 (N-ter)
MT1-MMP
a
b
c
d
e
f
g
h


*
Ratio of tumor cell intensity relative to control
2
1
0
0.98±0.23
0.69±0.2
0.92±0.26
1.2±0.24
1.32±0.58
1.31±0.36
EphA2 (C-ter)
EphA2 (N-ter)
MT1-MMP
EphA2 (C-ter)
EphA2 (N-ter)
MT1-MMP
SCC
BD


kDa
EphA2 (C-ter)
150
102
76
*
52
MT1-MMP
52
α-tubulin
52
SCC1
SCC2
BD1
BD2


a
Sh-MT1-MMP
Sh-Lac-Z
MT1-MMP
+
EphA2 (C-ter)
MT1-MMP
+
EphA2 (N-ter)
b
kDa
EphA2 (C-ter)
150
102
76
*
52
38
MT1-MMP
52
α-tubulin
52
HT1080
Sh-MT1-MMP
HT1080
Sh-Lac-Z


a
SCC
BD
MT1-MMP
+
EphA2 (C-ter)
MT1-MMP
+
EphA2 (N-ter)
Negative control


b
In situ PLA signals per cell
0
1
2
3
4
5
2.98±0.82
2.56±0.9
2.86±0.68
2.83±0.5
MT1-MMP + EphA2 (C-ter)
MT1-MMP + EphA2 (N-ter)
MT1-MMP + EphA2 (C-ter)
MT1-MMP + EphA2 (N-ter)
SCC
BD