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Highly expressed tumoral emmprin and stromal CD73 predict a poor prognosis for external auditory canal carcinoma

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Abstract

Squamous cell carcinoma of the external auditory canal (SCC-EAC) is rare and has a poor prognosis. The SCC-EAC cases with high-grade tumor budding (TB) or poorly differentiated clusters (PDCs) are associated with shorter survival than those with low-grade TB or PDCs. Extracellular matrix metalloproteinase inducer (emmprin) is a protein expressed in tumor cells that stimulates the production of MMP-2 by stromal fibroblasts to facilitate tumor invasion. Recently, we reported that emmprin forms a complex with CD73 to regulate MMP-2 production from fibroblasts in vitro. Here, we examined the association of emmprin and CD73 expression with TB or PDCs as well as with survival in 34 biopsy specimens of SCC-EAC patients. High tumoral emmprin expression was associated with highgrade TB, whereas high stromal CD73 expression was associated with high-grade PDCs. Furthermore, concurrent elevated expression of tumoral emmprin and stromal CD73 was determined to be an independent poor prognostic factor. In immunoprecipitation analyses, complex formation between emmprin and CD73 was demonstrated in vitro. Production of MMP-2 from fibroblasts was more abundant when cocultured with tumor cells than from fibroblasts cultured alone. Furthermore, MMP-2 production was reduced by the transfection of CD73 siRNA in fibroblasts cocultured with tumor cells. The colocalization of emmprin and CD73 was enhanced in not only the peripheral cells of the tumor cell clusters that interact with fibroblasts but also in the cells of intratumor clusters. Overall, this study provides novel insights into the roles of emmprin, CD73, and MMP-2 in tumor invasiveness.

KEYWORDS

CD73, emmprin, external auditory canal carcinoma, poorly differentiated cluster, tumor budding

Abbreviations: BS3, bis(sulfosuccinimidyl)suberate; CK, cytokeratin; EAC, external auditory canal ; emmprin, extracellular matrix metalloproteinase inducer; IRS, immunoreactive score; MT1-MMP, membrane-type 1 MMP; OS, overall survival; PDC, poorly differentiated cluster; SCC, squamous cell carcinoma; SCC-EAC, squamous cell carcinoma of the external auditory canal; TB, tumor budding.

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1 | INTRODUCTION

The EAC is approximately 2.5-3.0 cm[1] in length and leads from the outside of the head to the eardrum. The EAC is located close to the brain, inner ear, and facial nerve and is lined by stratified squamous epithelium that is unique in its ability to migrate outward.[2] This migratory phenomenon has not been observed in the stratified squamous epithelium of other organs.

Squamous cell carcinoma of the EAC is rare and survival varies greatly depending on disease progression. Each year, approximately 1-6 individuals per million[3] are affected by SCC-EAC. According to the revised University of Pittsburgh TNM staging system, the 5-year survival rate of stage I, II, III, and IV SCC-EAC are 94.8%, 78.9%, 68.3%, and 22.9%, respectively.[4]

Recently, we reported that poor prognosis in SCC-EAC is associated with TB and PDCs.[5,6] Histopathologically, TB and PDCs are nests of invading cancer cells that are dependent on the degradation of the ECM that surrounds tumor cells. This degradation process is catalyzed by MMPs. Matrix metalloproteinase-2 is the most abundant MMP in tumor stroma and contributes to tumor invasion.[7,8] The majority of MMPs are produced by stromal fibroblasts within tumors, rather than the tumor cells.[9] Tumor cells stimulate nearby fibroblasts to produce MMPs through the extracellular matrix metalloproteinase inducer (emmprin), also known as basigin/CD147.[10-13] Emmprin is a cell membrane glycoprotein that belongs to the immunoglobulin superfamily and is composed of two immunoglobulin domains with N-linked glycosylation sites in the extracellular domain.[10,14] The molecular mass of glycosylated emmprin ranges from 31-65 kDa.[10,13,15] Emmprin is expressed more abundantly in tumor cells than in fibroblasts.[16] Possible emmprin-mediated interactions stimulating MMP production are homophilic cis-interactions, homophilic trans-interactions, and heterophilic interactions. Heterophilic interactions occur between tumor cell surface emmprin and a yet unidentified putative emmprin receptor on a fibroblast[11] that has not yet been identified.

Recently, we reported that emmprin forms a complex with CD73 and regulates the production of MMP-2 in fibroblasts cocultured with tumor cells.[17] CD73, also known as ecto-5'-nucleotidase, is a 70 kDa glycosylphosphatidylinositol-anchored cell surface protein that plays a critical role in the regulation of adenosinergic signaling.[18] CD73 is also associated with tumor invasion and poor prognosis.[19-21]

In the current study, we examined whether TB or PDCs are associated with emmprin or CD73 expression. We also used pretreatment biopsy samples to determine if emmprin and CD73 levels affect SCC-EAC prognosis. Moreover, we found that expression of emmprin and CD73 was associated with MMP-2 production in vitro.

2 | MATERIALS AND METHODS

2.1 | Patient selection

We retrospectively reviewed clinicopathologic data for 34 patients with primary SCC-EAC, for whom pretreatment biopsy specimens were available. Patients in this cohort were treated at the Department of Otorhinolaryngology at Fukuoka University Hospital (Fukuoka, Japan) from April 2006 to December 2016 according to the protocol established by Nakagawa et al in 2006.[22] Patients who underwent chemotherapy or radiotherapy prior to biopsy, patients whose biopsy specimen had no stroma, or patients who failed to follow the treatment protocol were excluded from the study. Anonymous use of biopsy samples for research purposes is a part of the standard treatment agreement with patients at Fukuoka University Hospitals. The clinical stage was determined using the University of Pittsburgh TNM staging system modified by Moody et al.[23] Before treatment, the patient disease stage was estimated by physical examination and imaging: data obtained using computed tomography and MRI. Computed tomography and MRI scans were routinely undertaken every 6 months for 3 years after therapy, and annually thereafter. The follow-up period for the complete study ranged from 4-66 months (median, 31 months).

2.2 | Immunohistochemistry of tissue samples

Biopsy specimens were fixed in 10% formalin, processed into paraffin blocks, sectioned (4-μm thickness), deparaffinized, and hydrated in descending alcohol dilutions. Sections were heated in 10 mmol/L EDTA buffer (pH 8.0) and 10% citrate buffer (pH 6.0) in a microwave (700 W) for 10 minutes to retrieve epitopes before staining. Sections were incubated with an anti-human CK mAb (AE1/AE3, 1:200; Dako) for 1 hour at room temperature and overnight with the human emmprin/CD147 Ab (Clone# 109403, 1:600 dilution; R&D Systems) or the anti-CD73 Ab (ab175396, 1:800 dilution; Abcam) at 4°C. Immunoreactive proteins were visualized with 3,3'-diaminobenzidine (Dako) followed by counterstaining with Mayer's hematoxylin. The immunohistochemical specificity of emmprin and CD73 Abs was confirmed by staining surgical sections of colorectal carcinoma. Detailed experimental procedures were described previously.[17] Two independent pathologists blinded to the clinical data undertook the semiquantitative evaluation of the stained sections.

2.3 | Immunofluorescence in tissue samples and cell lines

Tissue sections were heated in a 10% citrate buffer (pH 6.0) in a microwave (700 W) for 10 minutes. Meanwhile, the cells were permeabilized with 0.1% Triton X-100 in PBS solution for 15 minutes at 4°C. The sections were incubated with human emmprin/CD147 Ab (1:100 dilution) or anti-CD73 Ab (1:200 dilution) at 4°C overnight. Immunoreactive emmprin or CD73 proteins were visualized with Alexa 594 anti-mouse IgG F(ab')2 fragment (red) (Invitrogen) or Alexa 488 goat anti-rabbit IgG F (ab')2 fragment (green) (Invitrogen), respectively. Cell nuclei were counterstained using DAPI (Vector Laboratories). The fluorescent staining pattern was evaluated using a Biozero BZ-8000 (Keyence). Detailed experimental procedures have previously been described.[17]

2.4 | Assessment of TB grade

A single cancer cell or a cancer cell nest comprised of less than 5 cells showing infiltration of the stroma of cancer was assessed as TB in our analysis. After selecting an area of highest TB intensity, TB was counted in a field under a 20× objective lens using both H&E-stained and CK immunostained sections as described previously.[5] The degree of TB was classified as low-grade or high-grade, corresponding to 0-9 or 10 or more budding foci per field, respectively.

2.5 | Assessment of PDC grade

In SCC-EAC, cancer clusters surrounded by stroma and composed of 5 or more cancer cells were defined as PDCs.[6,24,25] To quantify PDCs, the whole tumor was first scanned under low magnification to identify the area with the most PDCs in CK immunostained sections. Next, the clusters within the microscopic field of a 20× objective lens (WHK 10× ocular lens; Olympus) were counted. Tumors with less than 5 or 5 or more PDCs were classified as low-grade or high-grade, respectively.

2.6 | Immunohistochemical assessment of emmprin and CD73 expression

Emmprin expression was evaluated based on the immunoreactivity of the membrane and/or cytoplasm of tumor cells. The percentage of emmprin-expressing tumor cells in the whole tumor was defined as the proportion score, and the average intensity of emmprin expression in tumor cells was defined as the intensity score. The proportion score was evaluated as 0 (0%), 1 (1%-10%), 2 (11%-50%), 3 (51%-80%), and 4 (more than 80%). The intensity score was evaluated as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The product of the proportion and intensity score was assigned as the IRS.[26] Finally, an IRS of 0-6 was considered as low expression, and 8-12 was considered as high expression.

CD73 expression was evaluated by immunoreactivity in the stroma. The evaluation of stromal CD73 expression was carried out according to the following criteria. First, a stromal area with an intensity higher than that of endothelial cells was evaluated as a positive CD73 cell area. Second, the percentage of $CD73^+$ cell areas in the tumoral stroma was divided into 4 grades as 1 (0%-25%), 2 (25%-50%), 3 (50%-75%), and 4 (75%-100%). A final score of 1 or 2 was considered as low expression, and 3 or 4 was considered as high expression.

2.7 | Cell lines and culture

A431 (human skin SCC cell line) and CRL-2095 (human tongue SCC cell line) were purchased from the ATCC. ST353, a human dermal fibroblast cell line, was obtained from nonlesional dermis around nodular fasciitis. ST353 was immortalized by transduction of human telomerase reverse transcriptase to generate ST353i.[27] A431, CRL-2095, and ST353i were cultured in a growth medium consisting of DMEM supplemented with 10% FBS, streptomycin (50 μg/mL), and penicillin G (50 U/mL).

2.8 | Immunoblotting

Sodium dodecyl sulfate-PAGE was undertaken for membrane protein extracts using a 5%-15% gradient gel (Biomate), while immunoblotting of cell lysates and the supernatant was carried out using a 4%-15% Mini-PROTEAN TGX gel (Bio-Rad). The following Abs were used: CD73 Ab (rabbit monoclonal, Clone D7F9A; Cell Signaling Technology), emmprin Ab (mouse monoclonal, Clone #109403; R&D Systems), MMP-2 mAb (Daiichi Fine Chemical), and MT1-MMP (Millipore). The MMP-2 mAb recognizes both the pro-form of MMP-2 (pro-MMP-2) and activated MMP-2.[7,8] Protein bands were visualized with chemiluminescence reagents according to the manufacturer's instructions (PerkinElmer). Detailed experimental procedures were described previously.[17]

2.9 | Immunoprecipitation with cross-linking

Immunoprecipitation was carried out using Abs for CD73 (rabbit monoclonal, D7F9A; Cell Signaling Technology) and emmprin (polyclonal goat IgG; R&D Systems). Bis(sulfosuccinimidyl)suberate was used for amine-to-amine cross-linking. Experiments were carried out as previously described.[17] CD73 or emmprin expression in the samples was analyzed by immunoblotting.

2.10 | Knockdown of CD73 or emmprin with siRNA

Small interference RNA sequences were used for knockdown of CD73 or emmprin mRNA. Three different CD73 siRNAs (Invitrogen)—NT5EHSS107326, NT5EHSS107328, and NT5EHSS181585—were tested by immunoblotting (data not shown). After analysis, NT5 EHSS107328 (forward, GAUGCAAUGAUUAACAACAA—CCUGA; reverse, UCAGGUUGUUGUUAAUCAUUGCAUC) was selected as CD73 siRNA for further experiments. Additionally, emmprin siRNA (NM_001728_stealth_515, Invitrogen; forward, CGGCACAGUCUU CACUACCGUAGAA; reverse, UUCUACGGUAGUGAAGACUGUG CCG) was used. The siRNAs targeting CD73 were transfected into A431, CRL-2095, and ST353i using Oligofectamine transfection reagent in Opti-MEM (Thermo Fisher Scientific) in the absence of serum and antibiotics according to the manufacturer's instructions. The MMP-2 levels in the conditioned medium were analyzed by immunoblotting.

2.11 | Zymography

Gelatinolytic activity in the conditioned media was determined using gelatin as a substrate, as described previously. We measured the

enzymatic activity of pro-MMP-2 using the commercially available gelatin zymography kit (AK47-COS; Cosmo Bio). The enzyme activity was indicated by a band on the blue background of undigested gelatin.

2.12 | Statistical analysis

The relationships between several clinicopathologic parameters and the levels of tumoral emmprin and stromal CD73 expression were evaluated using Student's *t*-test for the continuous variables and Fisher's exact test for nominal variables. Overall survival and recurrence-free survival curves were plotted using the Kaplan-Meier method, and 2 or 3 groups were compared using the log-rank test or the Bonferroni correction, respectively. Univariate analyses were undertaken for clinicopathologic parameters using the Cox regression model followed by the estimation of their hazard ratios and 95% confidence intervals. Due to inconsistency with the likelihood-ratio test, the log-rank test was adapted for *P* value. The upper limit of the 95% confidence interval for the hazard ratio of high-grade PDCs was numerically a large figure and could not be estimated. Multivariate analysis (Cox proportional hazard model) was used to determine independent prognostic factors. *P* values less than .05 were considered statistically significant; however, *P* values less than .0083 were considered significant in the OS comparison of the 3 groups. SAS version 9.4 (SAS Institute) was used for the Cox proportional hazard model; other analyses were undertaken using JMP10.0.2 for Windows.

3 | RESULTS

3.1 | Clinicopathologic parameters in SCC-EAC

This study cohort included 14 (41.2%) men and 20 (58.8%) women with a mean age of 63 years (range, 38-86 years). The median follow-up period was 28 months (range, 4-66 months). The clinicopathologic characteristics of 34 SCC-EAC patients are summarized in Table S1. Twenty-eight patients (82.4%) showed low-grade TB, and 6 (17.6%) were high-grade. The PDC grade analysis identified 9 (26.5%) patients as low-grade, and 25 (73.5%) as high-grade. Half of the patients (17 cases) had a stage IV tumor in the TNM staging system. Cases positive for lymph node and distant metastasis were 7 (20.6%) and 1 (2.9%), respectively. On therapeutics, lateral temporal bone resection was achieved in 12 cases, subtotal temporal bone resection in 12 cases, and 10 cases received only chemoradiotherapy. Six (25%) of the 24 cases (70.6%) that underwent surgery had postoperative local recurrence.

3.2 | Immunohistochemistry of tumoral emmprin and stromal CD73 expression in pretreatment biopsy samples of SCC-EAC

Immunohistochemically, emmprin expression was mainly seen in the membrane of the tumor cells and the tumor-staining pattern was marginal. Tumoral emmprin expression results indicated 18 (52.9%) patients were low expression (Figure 1A), and 16 (47.1%) patients were high expression (Figure 1B). Cytoplasmic or membranous CD73 expression was found in both stromal and tumor cells. CD73 staining cells of the stroma were endothelial cells or fibroblasts. Stromal CD73 expression levels were low in 14 (41.2%) patients (Figure 1C) and high in 20 (58.8%) patients (Figure 1D).

3.3 | Association between TB or PDCs grade and tumoral emmprin or stromal CD73 expression in SCC-EAC

The association between TB or PDC grade and tumoral emmprin or stromal CD73 expression is summarized in Table 1. High-grade TB was significantly associated with high expression of tumoral emmprin (*P* = .0060), and high-grade PDCs were significantly associated with high expression of stromal CD73 (*P* = .0168). There was no association between tumoral CD73 expression and TB or PDC grade. Representative cases of low-grade and high-grade TB and PDCs are shown in Figure S1.

3.4 | Colocalization of emmprin and CD73 expression in SCC-EAC, detected by immunofluorescent staining

CD73 expression (green) was seen in the cytoplasm or membranes of the stromal and tumoral cells (Figure 1E). Emmprin expression (red) was primarily in the membranes of the tumor cells (Figure 1E). Overlay of CD73 and emmprin expression revealed their colocalization (yellow-to-orange) in tumor cells (Figure 1E).

3.5 | Patient survival in SCC-EAC

High-grade TB or PDC cases had significantly shorter survival times than low-grade TB (*P* = .0296; Figure 2A) or PDCs cases (*P* = .0355; Figure 2B). High tumoral emmprin expression cases showed significantly shorter survival times than low tumoral emmprin expression cases (*P* = .0103; Figure 2C), and high stromal CD73 expression cases showed shorter survival times than low stromal CD73 expression cases, although this difference was not statistically significant (*P* = .298; Figure 2D). Based on the expression of tumoral emmprin and stromal CD73, the prognosis of cases was evaluated after classification into 3 groups: high expression of both tumoral emmprin and stromal CD73 (10 cases), high tumoral emmprin and low stromal CD73 expression (6 cases), and low tumoral emmprin and low or high stromal CD73 expression (18 cases). The shortest survival was seen in cases with high tumoral emmprin and high stromal CD73 expression, followed in order by cases with high tumoral emmprin and low stromal CD73

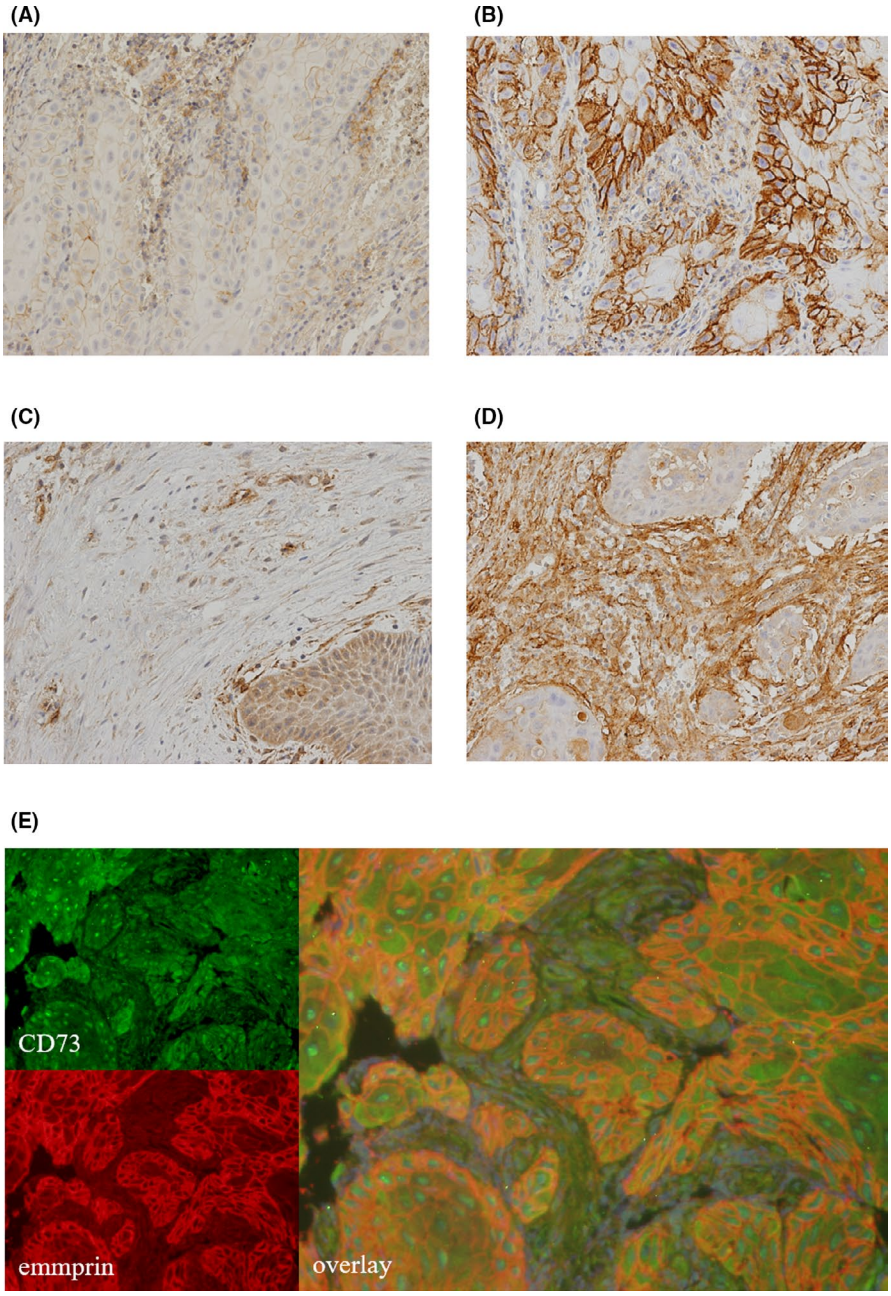


FIGURE 1 Expression patterns and localization of extracellular matrix metalloproteinase inducer (emmprin) and CD73 in squamous cell carcinoma of the external auditory canal. A-D, Immunohistochemical staining for emmprin and CD73. A, Tumoral emmprin: low expression. B, Tumoral emmprin: high expression. C, Stromal CD73: low expression. D, Stromal CD73: high expression. E, CD73 (green) and emmprin (red) expression detected by immunofluorescence: CD73 is green. Emmprin is red. Overlay of emmprin and CD73 is represented by yellow-to-orange fluorescence

TABLE 1 Relationship between invasive morphologies and extracellular matrix metalloproteinase inducer (emmprin)/stromal CD73 expression in squamous cell carcinoma of the external auditory canal

	Tumor budding grade			Poorly differentiated cluster grade		
Tumoral emmprin	**Low-grade (0-2)**	**High-grade (3)**	***P* value[a]**	**Low-grade (1)**	**High-grade (2-3)**	***P* value[a]**
Low expression (IRS: 0-6)	18 (52.9)	0 (0)		7 (20.6)	11 (32.4)	
High expression (IRS: 8-12)	10 (29.4)	6 (17.7)	.0060	2 (5.8)	14 (41.2)	.1250
Stromal CD73						
Low expression (1-2)	11 (32.4)	3 (8.8)		7 (20.6)	7 (20.6)	
High expression (3-4)	17 (50.0)	3 (8.8)	.6722	2 (5.9)	18 (52.9)	.0168

Abbreviation: IRS, immunoreactive score.

[a]Fisher's exact test.

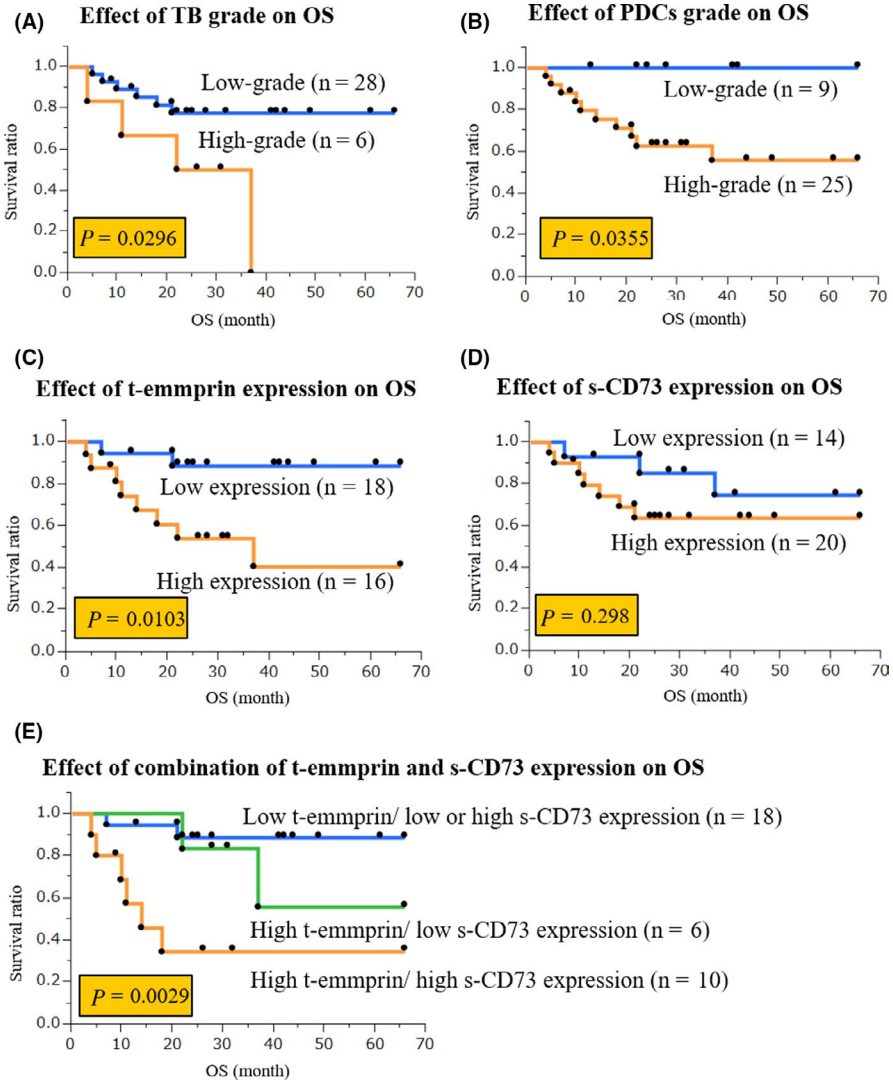


FIGURE 2 Survival analysis in patients with squamous cell carcinoma of the external auditory canal. A, Effect of tumor budding (TB) grade on overall survival (OS). B, Effect of poorly differentiated clusters (PDCs) grade on OS. C, Effect of tumoral extracellular matrix metalloproteinase inducer (t-emmprin) expression on OS. D, Effect of stromal CD73 (s-CD73) expression on OS. E, Effect of the combination of t-emmprin and s-CD73 expression on OS

expression, and low tumoral emmprin and low or high stromal CD73 expression. These differences were statistically significant (P = .0029; Figure 2E).

Univariate and multivariate analyses of clinicopathologic predictors of OS in these SCC-EAC cases were carried out (Table 2). Histopathologic or clinicopathologic parameters strongly related to the prognosis in the univariate analysis were selected as variables in the multivariate analysis. Poorly differentiated type, treatment without surgery, high-grade TB, high-grade PDCs, and high tumoral emmprin/stromal CD73 expression predicted poorer OS in the univariate analysis (P = .0036, .0047, .0296, .0355, and .0009, respectively). High tumoral emmprin and stromal CD73 expression were independent poor prognostic factors for OS in the multivariate analysis (P = .0037).

In 24 advanced cases (stage III and IV), high expression of both tumoral emmprin and stromal CD73 (9 cases) was associated with a poorer prognosis than cases with both/either low tumoral emmprin and/or low stromal CD73 expression (15 cases) (P = .0042; Figure S2A). In 24 surgical therapy cases, high expression of both tumoral emmprin and stromal CD73 (4 cases) was associated with an earlier recurrence than cases with both/either low tumoral emmprin and/or low stromal CD73 expression (20 cases) (P = .0030; Figure S2B).

3.6 | Association between clinicopathologic parameters and high tumoral emmprin and stromal CD73 expression in SCC-EAC

The associations between high tumoral emmprin and stromal CD73 expression and clinicopathologic parameters are summarized in Table S2. High tumoral emmprin and stromal CD73 expression cases were significantly associated with middle cranial fossa invasion and recurrence (P = .0088 and P = .0353, respectively).

3.7 | Localization of emmprin and CD73 expression in fibroblasts cocultured with SCC cells, detected by immunofluorescent staining

In ST353i cells, CD73 expression (green) was detected in the cytoplasm and membrane, and emmprin was subtly expressed (red) (Figure 3A,B, respectively). In contrast, robust expression of emmprin was observed in the membrane of CRL-2095 cells (Figure 3D), while CD73 expression was detected in the cytoplasm and the membrane (Figure 3C).

TABLE 2 Univariate and multivariate analysis of factors affecting overall survival in patients with squamous cell carcinoma of the external auditory canal

	Univariate analysis				Multivariate analysis[d]			
Variable	Hazard ratio[a]	95% CI[a]		*P* value[b]	Hazard ratio	95% CI		*P* value
Age ≥70 y	2.10	0.533	7.40	.2430	—	—	—	—
Poor differentiation type	5.93	1.27	21.6	.0036	—	—	—	—
Patient treated without surgery	5.21	1.48	20.5	.0047	—	—	—	—
High TB grade	3.47	0.95	13.2	.0296	—	—	—	—
High PDC grade[c]	$9.00 \times e^{8}$	2.09	—	.0355	—	—	—	—
High t-emmprin-sCD73	6.94	1.91	28.1	.0009	6.94	1.91	28.1	.0037

Abbreviations: —, invalid value; s-CD73, stromal CD73 expression; TB, tumor budding; t-emmprin, tumoral emmprin expression.

[a]Estimated using Cox regression based on likelihood ratio test.

[b]Log-rank test.

[c]Upper limited confidence interval (CI) was a large figure and could not be estimated numerically. There were no results of fatal cases in low-grade poorly differentiated clusters (PDCs).

[d]The stepwise method was used for the multiple Cox regression analysis to obtain the best combination of independent variables. Therefore, hazard ratio, 95% CI, and *P* value were not calculated except for High t-emmprin-sCD73, because these variables were not selected in multiple analysis.

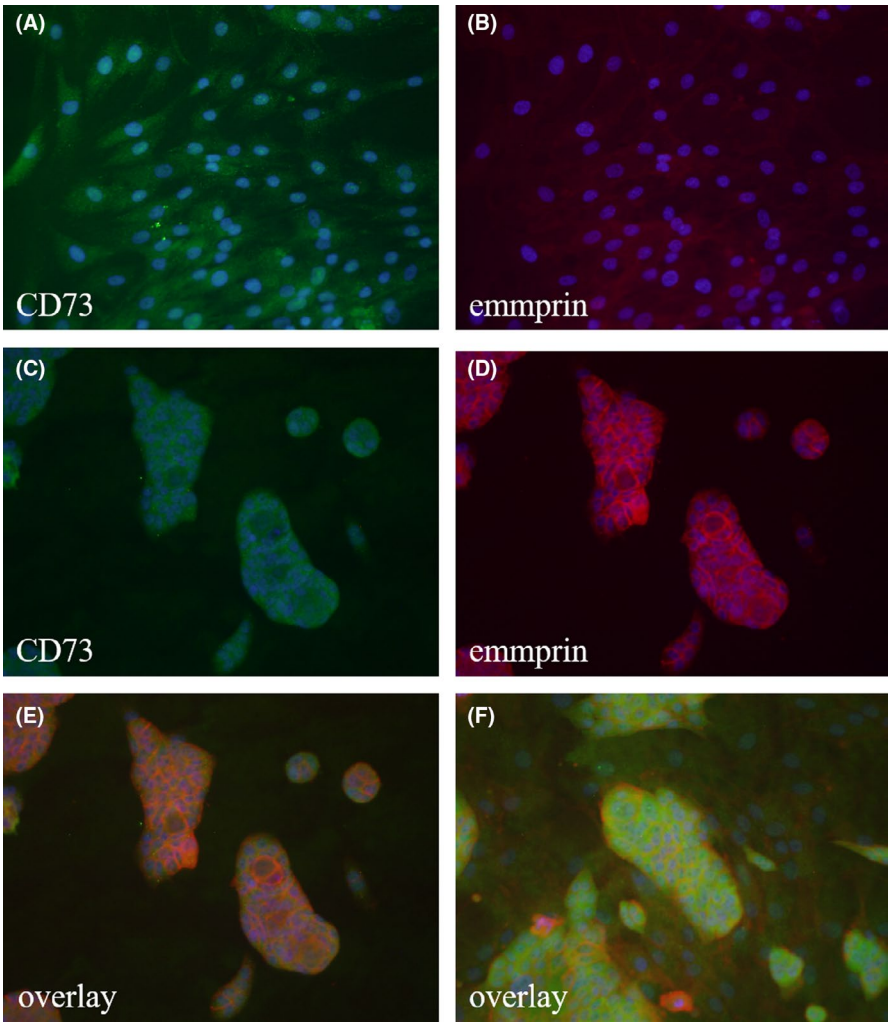


FIGURE 3 Representative immunofluorescence images of fibroblasts and squamous cell carcinoma cells showing CD73 (green) and extracellular matrix metalloproteinase inducer (emmprin) expression (red). A, CD73 expression in ST353i cells. B, Emmprin expression in ST353i cells. C, CD73 expression in CRL-2095 cells. D, Emmprin in CRL-2095 cells. E, Overlay of CD73 and emmprin expression in CRL-2095 cells. F, Overlay of CD73 and emmprin expression in ST353i cells cocultured with CRL-2095 cells

Slight colocalization (yellow-to-orange) of CD73 and emmprin was observed in CRL-2095 tumor cells cultured alone (Figure 3E), whereas the colocalization was increased when tumor cells were cocultured with ST353i (Figure 3F). The colocalization was increased not only in areas where tumor cells neighbored fibroblasts but also in intratumor clusters.

3.8 | Formation of emmprin and CD73 complex in fibroblasts cocultured with SCC cells, determined by immunoprecipitation and immunoblotting

CD73 immunoblotting was carried out following immunoprecipitation with emmprin in ST353i cells cocultured with A431 or CRL-2095 cells (Figure 4A,B). Similarly, emmprin immunoblots were undertaken following immunoprecipitation with CD73 (Figure 4A,B). High-molecular-weight proteins were detected in both CD73 and emmprin immunoblots when proteins were cross-linked with BS3. Immunoblotting for CD73 following emmprin immunoprecipitation and emmprin immunoblotting following CD73 immunoprecipitation were also carried out in A431 or CRL-2095 cells cultured alone. However, only faint or no high-molecular-weight protein expression was seen, even when proteins were cross-linked with BS3, in these conditions (data not shown).

3.9 | Effect of CD73 or emmprin knockdown on MMP-2 production in fibroblasts cocultured with SCC cells when CD73 or emmprin siRNA was transfected, determined by immunoblotting

CD73 immunoblotting was undertaken for ST353i cells cocultured with A431 or CRL-2095 cells (Figure 4C,D), and emmprin immunoblotting was also carried out (data not shown). CD73 or emmprin expression was inhibited by the transfection of CD73 or emmprin siRNA, respectively. Matrix metalloproteinase-2 immunoblotting was carried out on the proteins in the conditioned medium isolated from ST353i cells cocultured with A431 or CRL-2095 cells under condition of CD73 knockdown by siRNA. Similarly, MMP-2 immunoblotting was carried out when emmprin siRNA was transfected. The MMP-2 production from ST353i cells was increased in conditions of coculture with A431 or CRL-2095 cells and was inhibited by transfection of CD73 siRNA (Figure 4E,F) or emmprin siRNA (data not shown).

3.10 | MMP-2 in fibroblasts cocultured with SCC cells investigated by gelatin zymography

Matrix metalloproteinase-2 gelatin zymography was undertaken for ST353i, A431, and CRL-2095 cells in addition to ST353i cells cocultured with A431 or CRL-2095 cells (data not shown). A 68-kDa band in the immunoblotting of MMP-2 corresponded to the molecular mass of the pro-form of MMP-2.[28] ST353i showed a weak gelatinolytic band, whereas A431 and CRL-2095 did not display any detectable gelatinolytic activity. In ST353i cells cocultured with A431 or CRL-2095 cells, gelatinolytic activity was stronger than that in ST353i cells cultured alone.

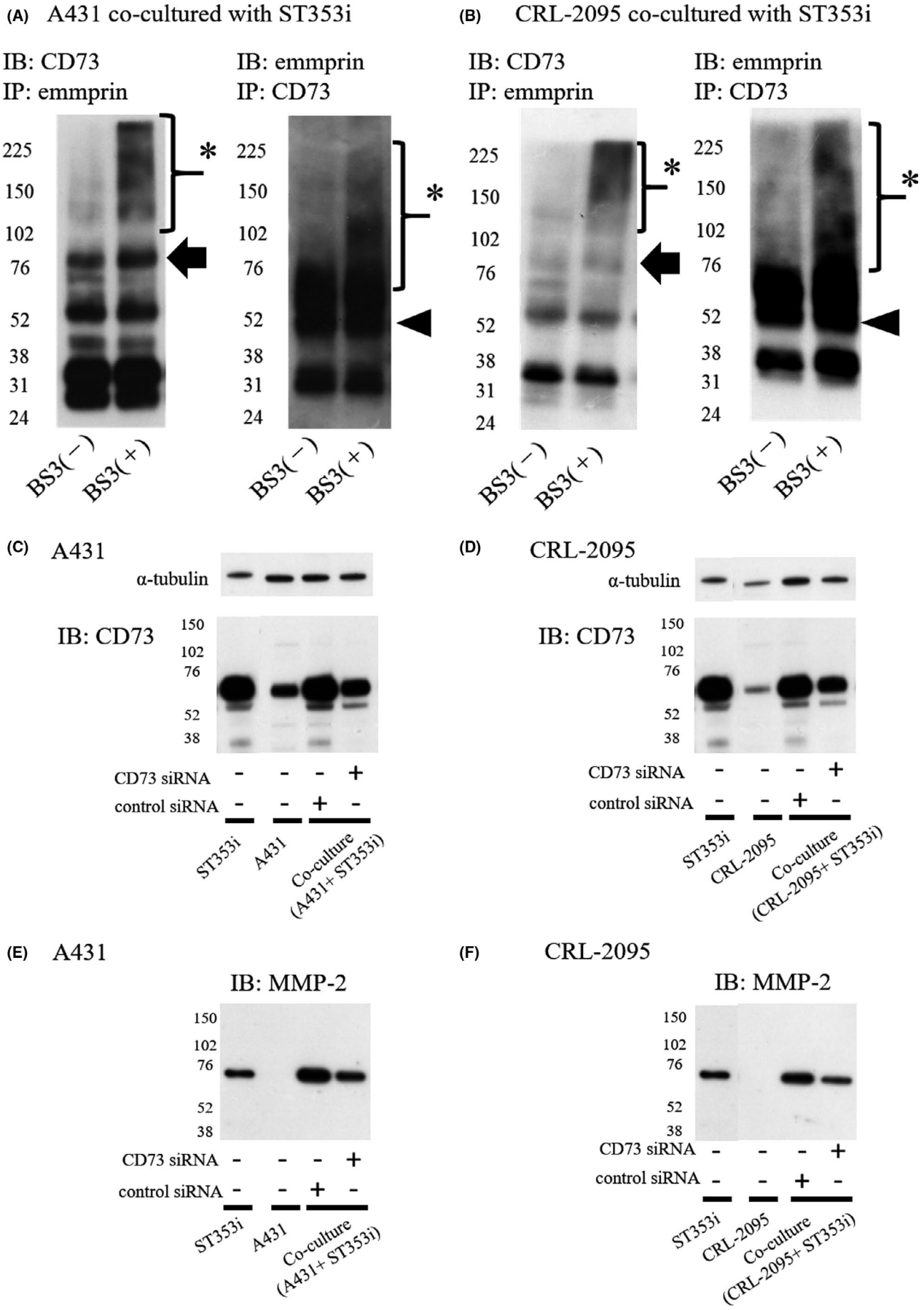
4 | DISCUSSION

Concurrent high expression of tumoral emmprin and stromal CD73 is an independent poor prognostic factor in pretreatment biopsy samples of SCC-EAC, predicts shorter survival in advanced cases, and early recurrence in surgical therapy cases. High-grade TB was associated with high tumoral emmprin expression, and high-grade PDCs were associated with high stromal CD73. Concurrently high expression of tumoral emmprin and stromal CD73 could potentially enhance tumor invasiveness. In vitro, CD73 forms a complex with emmprin and is associated with increased production of MMP-2 from fibroblasts cocultured with SCC cells.

High emmprin expression in the tumor cells is associated with poor prognosis in SCC of the oral cavity, skin, and hypopharynx.[29-31] The expression of CD73 not only in tumor cells but also in stroma affects prognosis. High CD73 expression in tumor cells is related to poor prognosis in colorectal carcinoma and head and neck SCC[21,32]; paradoxically, its expression is associated with favorable prognosis in ovarian cancer.[33] In high-grade serous ovarian cancer, high CD73 intensity of fibroblasts is associated with poor prognosis.[34] Moreover, both tumoral emmprin[35] and CD73[36] expression is predictive of recurrence for some cancers. In the present study, we evaluated CD73 expression in both stroma and tumor cells and found that CD73 expression levels in tumor cells were not associated with the prognosis. Cases with high expression of stromal CD73 had a poorer prognosis than those with low expression of stromal CD73, although this difference was not statistically significant.

The primary difference between TB and PDCs is the number of cells that comprise the clusters, and either TB or PDCs branch from the main tumor.[37] The biomolecular or prognostic factors

FIGURE 4 Complex formation of extracellular matrix metalloproteinase inducer (emmprin)-CD73 complex, and the role of CD73 in MMP-2 production when CD73 siRNA was transfected into fibroblasts cocultured with squamous cell carcinoma cells. Arrow, CD73; arrowhead, emmprin; asterisk, emmprin-CD73 complex. The lower-molecular-weight signals in the blots indicate immunoglobulins. A, Blots were probed for CD73 or emmprin expression following immunoprecipitation of emmprin or CD73 in ST353i cells cocultured with A431 cells. B, Blots were probed for CD73 or emmprin expression following immunoprecipitation of emmprin or CD73 in ST353i cells cocultured with CRL-2095 cells. C, The immunoblot (IB) represents CD73 expression when CD73 siRNA was transfected in ST353i cells cocultured with A431 cells. D, The IB represents CD73 expression when CD73 siRNA was transfected in ST353i cells cocultured with CRL-2095 cells. E, The IB represents MMP-2 production when CD73 siRNA was transfected in ST353i cells cocultured with A431 cells. F, The IB represents MMP-2 production when CD73 siRNA was transfected in ST353i cells co-cultured with CRL-2095 cells. BS3, bis(sulfosuccinimidyl)suberate


(A) A431 co-cultured with ST353i
IB: CD73
IP: emmprin
IB: emmprin
IP: CD73
225
150
102
76
52
38
31
24
*
BS3(−)
BS3(+)
(B) CRL-2095 co-cultured with ST353i
IB: CD73
IP: emmprin
IB: emmprin
IP: CD73
(C) A431
α-tubulin
IB: CD73
CD73 siRNA
control siRNA
ST353i
A431
Co-culture (A431+ ST353i)
(D) CRL-2095
α-tubulin
IB: CD73
CD73 siRNA
control siRNA
ST353i
CRL-2095
Co-culture (CRL-2095+ ST353i)
(E) A431
IB: MMP-2
CD73 siRNA
control siRNA
ST353i
A431
Co-culture (A431+ ST353i)
(F) CRL-2095
IB: MMP-2
CD73 siRNA
control siRNA
ST353i
CRL-2095
Co-culture (CRL-2095+ ST353i)

associated with PDCs are similar to those of TB.[38] However, in a previous study we found that, even in cases with low-grade TB, SCC-EAC patients with high-grade PDCs had a poor prognosis.[6] Thus, in this study, we selected both markers of invasive morphology, TB and PDCs (Figure S1), for detailed investigation. Patterns of cancer cell movement seen in PDCs are those of collective cell migration.[39] We, therefore, utilized PDCs as an index to evaluate collective cell migration in tissue sections. In collective cell migration, invading groups of SCC cells are shown to be very closely associated with fibroblasts, and the stromal fibroblasts can promote tumor progression.[40]

Concurrent high expression of tumor emmprin and stromal CD73 could stimulate tumor invasion. Emmprin expression is mainly found in the peripheral cells of invasive tumor clusters, and emmprin plays a role in tumor-stroma interaction and the invasive front of the cancer.[16,41] CD73 expression is positively correlated with signaling pathways of cell junctions, actin cytoskeleton organization and extracellular matrix, and regulation of cell migration.[42] In the present study, emmprin expression was primarily seen in peripheral cells of tumor clusters (Figure 1A,B), and CD73 expression was also predominantly seen in stromal cells surrounding tumor cell clusters (Figure 1C). Coexpression of both emmprin and CD73 was also found in the marginal cells of tumor nests in SCC-EAC (Figure 1E). Furthermore, high stromal CD73 expression was associated with high-grade PDCs. These findings suggest that stromal CD73 in association with tumor cell emmprin regulates/induces the collective cell migration property of PDCs.

CD73 forms a complex with emmprin and regulates the production of MMP-2 in fibroblasts cocultured with SCC cells. Production of MMP-2 from fibroblasts was more abundant when cocultured with tumor cells than in fibroblasts cultured alone,[43-45] and this effect was reduced by the transfection of CD73 siRNA in vitro (Figure 4E,F). Evidence for complex formation between emmprin and CD73 in the coculture of tumor cells and fibroblasts is supported by the findings of the immunofluorescence study (Figure 3F), immunoprecipitation assay (Figure 4A,B), and our previous report.[17] In this study, the 68-kDa band in MMP-2 immunoblotting corresponded to the molecular mass of the pro-MMP-2[28] (Figure 4E,4F). MT1-MMP expressed in tumor cells is known to activate pro-MMP-2 produced by fibroblasts,[17] and MT1-MMP expression was detected in the SCC cells in this study (Figure S3). Higher stromal and tumoral MMP-2 production was associated with high-grade PDCs (Figure S4 and Table S3). We hypothesize that the PI3K/Akt pathway is involved as a downstream effector of the emmprin/CD73 complex. It has been shown that emmprin activates the PI3K/Akt pathway and promotes MMP-2 production in hepatocellular carcinoma.[46] CD73 is also known to promote invasion and metastasis through the PI3K/Akt pathway.[47]

Matrix metalloproteinase-2 production is likely a significant factor in collective cell migration in SCC-EAC. Fibroblasts produce MMP-2 and secrete that into the environment surrounding the tumor cells.[48] Moreover, it is shown that MMP-2 is specifically expressed and localized at the front "pathfinder" cells of the migrating cell sheets and reorganizes the ECM that is essential for collective cell migration.[49] In the present study, PDCs were observed to be associated with stromal and tumor cell MMP-2 expression in SCC-EAC (Figure S4 and Table S3).

It is important to acknowledge the limitations of the present study. The sample size was only 34 cases due to the rarity of SCC-EAC. Surgical specimens could not be included in our cohort because preoperative chemoradiotherapy is carried out for EAC carcinoma patients. Biopsy samples were small because the external auditory canal is narrow. For the in vitro studies, human skin and tongue SCC cell lines were used as no SCC-EAC cell lines have been developed. Despite these limitations, this study had 3 noteworthy strengths. First, we showed that immunohistochemical evaluation of pretreatment biopsy samples can predict the prognosis of SCC-EAC. Second, the study was undertaken by a single surgeon, T. Nakagawa, which eliminated variability in operational procedures. Finally, this study is the first to reveal the confirmatory evidence of the formation of a CD73 and emmprin form a complex that regulates MMP-2 production in SCC cells cocultured with fibroblasts.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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