Abstract. It has been reported that the epidermal growth factor receptor (EGFR) expression is associated with the extracellular matrix metalloproteinase inducer (EMMPRIN) in some solid tumors; however, the relationship of EMMPRIN with EGFR in head and neck cancers is not fully understood. To determine the relationship between EMMPRIN and EGFR in head and neck squamous cell carcinoma (HNSCC), HNSCC cells were stimulated with epidermal growth factor (EGF), a ligand of EGFR. EMMPRIN expression in HNSCC cells was upregulated by EGF. In addition, EGF stimulation induced HNSCC cell invasion and MMP-9 expression. This increase in invasion and MMP-9 expression was abrogated by downmodulation of EMMPRIN. Furthermore, to determine the effects of combined EMMPRIN and EGFR targeting in HNSCC, HNSCC cells were treated with an EMMPRIN function-blocking antibody and the EGFR inhibitor AG1478. This combined treatment resulted in greater inhibition of HNSCC cell proliferation and migration compared with the individual agents alone. These results suggest that EMMPRIN mediates EGFR-induced tumorigenicity and that combined targeting of EMMPRIN and EGFR may be an efficacious treatment approach.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common malignancy of the upper aerodigestive tract that is characterized by a propensity for invasion and cervical lymph node metastasis. In spite of advances in surgery, radiation, and chemotherapy, HNSCCs continue to have poor outcomes and are still often fatal. Therefore, new and more efficacious therapies are needed to improve HNSCC survival (1).

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates crucial cellular signaling pathways contributing to tumor progression (2,3) and is frequently amplified and overexpressed in a high percentage of HNSCCs (4,5). Preclinical studies have successfully demonstrated the antitumor effects of EGFR targeting, and the Food and Drug Administration (FDA) has approved the clinical use of EGFR monoclonal antibody cetuximab in HNSCC (6). However, when used alone, EGFR targeting shows insufficient suppression of HNSCCs (7,8); hence, various combination strategies are under investigation with an aim to improve HNSCC treatments and prognosis (9-11).

Accumulating studies have showed that EGFR stimulation induces matrix metalloproteinase (MMP) expression, which degrade extracellular matrix and is critical for tumor development (12,13). Recently, extracellular matrix metalloproteinase inducer (EMMPRIN), also known as CD147, has been identified as a member of the immunoglobulin superfamily. In particular, EMMPRIN has been reported to be highly expressed in malignant tumors (14-16), including HNSCCs (17) and has been found to induce tumor progression through MMP expression (18-20). Although it has been reported that EGFR expression is associated with EMMPRIN in some solid tumors (21,22), the relationship between EMMPRIN and EGFR in oncogenicity is not fully understood, particularly in head and neck cancers.

This study was undertaken to evaluate the relationship between EMMPRIN and EGFR and to test the hypothesis that EMMPRIN mediates EGFR-induced HNSCC tumorigenic behavior to determine if combined inhibition of EMMPRIN and EGFR is a rational treatment approach to improve prognosis in HNSCC.

Materials and methods

Cell and cell culture. The HNSCC cell line SAS, a human tongue squamous cell carcinoma cell line, was used. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37°C in the presence of 5% CO₂.
Western blotting. Protein expression was detected by western blotting. Cells were lysed in detergent, and protein levels were determined using the Bio-Rad protein assay method. Total protein was separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. The blot was incubated with each antibody and developed using the Luminor Regent (Santa Cruz Biotechnology).

Gelatin zymography. Gelatinase expression was detected in conditioned media by gelatin zymography. Cells were cultured for 48 h in serum-free DMEM with or without epidermal growth factor (EGF). This was followed by the detection of gelatinolytic activity in the conditioned media by gelatin zymography. Using a 7.5% separating gel containing 0.3 mg/ml gelatin, the conditioned medium was resolved by SDS-PAGE under non-reducing conditions. The gels were washed and then incubated for 24 h at 37°C in a reaction buffer before being stained. After destaining, gelatinolytic activity on the gel was detected as clear bands on a blue background of undigested gelatin.

Small interfering RNA (siRNA) and siRNA transfection. EGFR siGENOME siRNA, EMMPRIN siGENOME siRNA (Dharmacon RNA Technologies, Lafayette, CO, USA) was transfected into HNSCC cells for each protein silencing. The siGENOME non-targeting siRNA was used as a control. The siRNA transfections were performed using Lipofectamine 2000 (Life Technology Inc.).

Matrigel invasion and cell migration assays. Cell invasion was evaluated in vitro using Matrigel-coated semipermeable modified Boyden inserts with a pore size of 8 µm (Becton-Dickinson/Biocoat, Bedford, MA, USA). Cell migration was evaluated in vitro using semipermeable modified Boyden inserts with a pore size of 8 µm (Becton-Dickinson/Biocoat). For each assay, cells were plated in duplicate at a density of 5x10^3 cells/well for the invasion assay or 3x10^4 cells/well for the migration assay. Plating was carried out on serum-free DMEM with 100 nM EGF for the invasion assay, or either the control vehicle (DMSO), AG1478 (10 µM), anti-EMMPRIN function-blocking antibody (10 µg/ml), or a combination of AG1478 and anti-EMMPRIN antibody in DMEM with 10% FBS. At selected time-points, cells were trypsinized and stained with trypan blue, and viable cells were counted using a hemocytometer.

Statistical analysis. The statistical significance of differences was assessed using Wilcoxon-Mann-Whitney 2-tailed exact test.

Results

HNSCC cell line SAS expresses EGFR and EMMPRIN. To evaluate the role of the interaction between EGFR and EMMPRIN, both protein expressions were determined by immunoblotting in the HNSCC cell line SAS. FaDu cells, in which the expression of EGFR and EMMPRIN has been reported, were used as the control (23,24) (Fig. 1).

EGF induces EMMPRIN and MMP-9 expression. Coexpression of EGFR and EMMPRIN in clinical samples has been identified for its importance for tumor progression or prognosis in several solid tumors (21,22); however, the relationship between these factors have not been studied completely. Therefore, EGF, a ligand of EGFR, was added to the culture media at various concentrations to stimulate EGFR. EMMPRIN expression was determined by immunoblotting and the expression of gelatinase, MMP-2 and MMP-9, which plays an important role in tumor invasion and metastasis through basement membrane was detected by
EGF increased EMMPRIN expression in a dose- (Fig. 2A) and time- (Fig. 2B) dependent manner. Interestingly, EGF induced MMP-9 expression, and not MMP-2 expression, in culture media in a dose-dependent manner (Fig. 2C).

EGFR mediates EGF-induced EMMPRIN and MMP-9 upregulation. SAS cells were transfected with non-targeting siRNA or EMMPRIN siRNA and cultured with or without 100 nM EGF (A). The MMP-9 expression in HNSCC cells decreased when cells were treated with EMMPRIN siRNA compared with non-targeting siRNA either in the presence or absence of EGF (B). Percent reduction in MMP-9 with EMMPRIN siRNA was calculated from the results shown in (B). The degree of MMP-9 reduction with EMMPRIN siRNA was greater under EGF-treated conditions (C). Cells were also subjected to an invasion assay. Cells were plated on inserts with or without 100 nM EGF in serum-free media with the lower well containing 10% FBS serving as a chemoattractant. EMMPRIN siRNA decreased HNSCC invasion during EGF treatment (D).
that EGF stimulates EGFR and that this interaction resulted in the upregulation of EMMPRIN and MMP-9.

**EMMPRIN plays a critical role in EGFR-induced MMP-9 expression and cell invasion.** The ability of EGFR to promote tumor progression is well documented (2,3,25-27). In addition, accumulating studies have reported that EMMPRIN induces malignant phenotypes such as invasion, proliferation, and MMP expression in some solid tumors including HNSCCs (28-31). Based on these reports and our findings shown in Fig. 3, we hypothesized that EGFR stimulation induces EMMPRIN expression, which induces malignant phenotypes in SAS. To investigate the importance of EMMPRIN in the EGFR-induced tumorigenic process, we analyzed MMP-9 expression and cell invasion in the presence or absence of siRNA-targeting EMMPRIN, with or without EGF in the cell culture media.

The results showed a trend for SAS to decrease MMP-9 expression when EMMPRIN was knocked-down in either the presence or absence of EGF (Fig. 4B). However, the percent decrease in MMP-9 was significantly higher in the presence of EGF (Fig. 4C). This suggests that EMMPRIN plays an important role in MMP-9 expression induced by EGFR stimulation and that both EGFR and EMMPRIN are crucial for MMP-9 expression in SAS. In addition, cell invasiveness was significantly decreased when EMMPRIN was silenced by siRNA under EGFR stimulation by EGF (Fig. 4D), indicating that EMMPRIN has a key role in EGFR-induced HNSCC cell invasion.

**Combined inhibition of EGFR and EMMPRIN prevents the growth and migration of HNSCC cells.** The aforementioned results indicate that EGFR induces HNSCC malignant phenotypes through EMMPRIN. Indeed, it is well known that EGFR induces oncogenicity (32) and that its targeting is effective in managing various solid tumors (33). In addition, both EMMPRIN tumorigenicity and the antitumor effect of EMMPRIN inhibition have been investigated previously (34,35). Hence, we examined the combined effect of EGFR and EMMPRIN in HNSCC cell proliferation and migration.

To inhibit EGFR and EMMPRIN function, cells were treated with or without the EGFR antibody AG1478 and/or the EMMPRIN function-blocking antibody. As shown in Fig. 5B
and C, cell proliferation and migration were significantly decreased by both AG1478 and EMMPRIN function-blocking antibody. Furthermore, combined inhibition showed a more marked and significant reduction in proliferation and migration when compared with single inhibition of EGFR and EMMPRIN.

Discussion

This study demonstrated that in HNSCC, EMMPRIN partly promotes increased MMP-9 expression and cell invasion induced by EGF stimulation through EGFR. Furthermore, combined inhibition of EMMPRIN and EGFR shows effective suppression of HNSCC proliferation and migration. These results suggest the need for further investigation into the mechanism of combined EMMPRIN and EGFR targeting in cancers that express increased levels of these proteins.

HNSCC is the most common malignancy of the upper aerodigestive tract (36), and despite advancements in conventional treatment, the prognosis has remained unchanged over the past several decades with 30% patient deaths every year (37). Therefore, new and more effective therapeutic approaches are needed to improve HNSCC survival (1). Recently, molecular targeting has been introduced for the treatment of malignancy, including HNSCC (38). EGFR is one such target that is thought to be an ideal therapeutic target (39) because it is frequently amplified and overexpressed in high percentage of HNSCCs (4). However, the use of EGFR inhibitors as monotherapy have yielded low response rates in clinical practice (8). Although it has been reported that an EGFR variant is a possible reason for resistance to EGFR targeting in HNSCCs (37,40), the tumor features that contribute to EGFR targeting resistance are incompletely understood. A promising solution to improve the clinical response rate may be through the combination of EGFR inhibitors with other treatment modalities. Some reports have showed promising antitumor efficacy by blocking EGFR with other tumorigenic factors, such as c-Met (11), Src (10), signal transducer and activator of transcription 3 (STAT3) (9) and G-protein-coupled receptor (GPCR) (41).

The importance of EMMPRIN in tumor progression and its association with poor prognosis is widely known in solid tumors including HNSCC. In addition, there are accumulating reports suggesting that EMMPRIN is a negative prognostic factor in malignant tumors (42-44). We previously reported that the EMMPRIN-EMMPRIN hemophilic interaction or EMMPRIN-cyclophilin A interaction play an important role in MMP expression and activation, as well as in invasion and migration (24,28). Recently, several studies have showed that EMMPRIN blocking or silencing is efficient for cancer suppression (35,45), particularly in HNSCCs, where there have been several reports showing the possibility of EMMPRIN targeting therapy for tumor suppression in vivo. Hence, it appears that there is a more detailed mechanism underling EMMPRIN-mediated cancer progression and the relationship with other oncogenic factors that contribute to an improvement in HNSCC progression.

The relationship between EMMPRIN, MMP, and EGFR have been reported in colon (21) and breast (22) cancers. In these studies, increased EGFR expression correlated with MMP-9 and EMMPRIN expression in clinical samples, but details of the interaction mechanisms between EGFR, MMP-9, and EMMPRIN expression were not described. Here we showed that EGFR induces EMMPRIN expression, with EMMPRIN mediating EGFR-induced MMP-9 expression and HNSCC invasion. Our results may account for the expression mechanisms of EGFR, MMP-9, and EMMPRIN in previous studies. However, EGFR silencing did not abolish the expression of EMMPRIN (data not shown). These results suggest that the expression of EMMPRIN is not completely dependent on EGFR expression and that there may be an independent EMMPRIN tumorigenic pathway involved. Therefore, blocking of both EGFR and EMMPRIN may be necessary to regulate HNSCC progression.

In this study, we showed that the combined inhibition of EGFR and EMMPRIN effectively reduces HNSCC cell proliferation and migration. Therefore, complementary blockade of EGFR and EMMPRIN oncogenic pathways might provide a more efficacious treatment approach. Further research into the role of EMMPRIN, and of the apparent synergistic effect, may contribute to improved prognoses in HNSCC.

Acknowledgements

This study was supported by JSPS KAKENHI grant no. 24791740.

References


