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# Requirement of MMP-3 in anchorage-independent growth of oral squamous cell carcinomas

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**BACKGROUND: Matrix metalloproteinase-3 (MMP-3) is expressed in various carcinomas; however, its function is not clearly established. This study was to assess its possible role in oral squamous cell carcinomas (OSCCs).**

**MATERIALS AND METHODS: Specimens of seven oral pre-malignant lesions (OPMLs) and 92 OSCCs were subjected to MMP-3 detection by RT-PCR and Western blot. Antisense oligodeoxynucleotides (AODNs) of MMP-3 were used to transfect OSCC (OECM-1 and SCC-9) and esophageal carcinoma (CE81T/VGH) cell lines, and their growth was subsequently analyzed by XTT and soft-agar colony assay.**

**RESULTS: MMP-3 transcript was preferentially expressed in OSCCs (71 of 92, 77%) than in OPMLs (two of seven, 29%; $P = 0.012$). Both MMP-3 transcript and protein levels were significantly higher in OSCC masses than in neighboring tissues ($P < 0.0001$ and $P = 0.04$, respectively). Growth of the three cell lines was not affected, while the colony numbers of OECM-1 and CE81T/VGH were significantly reduced by the transfection of MMP-3 AODNs ($P = 0.002$ and $P = 0.004$, respectively). SCC-9 did not form colonies in soft-agar/medium.**

**CONCLUSIONS: MMP-3 function may be required in most OSCCs, and it may support the anchorage-independent growth of both OSCC and esophageal carcinoma.**

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**Keywords:** esophageal carcinoma; matrix metalloproteinase-3; oral squamous cell carcinoma; stromelysin-1

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## Introduction

Matrix metalloproteinases (MMPs) are metal ion-dependent proteases capable of degrading essentially all components of the extracellular matrix (ECM; 1, 2). These functions are required for normal tissue reconstruction, e.g. developmental tissue morphogenesis, wound healing, and angiogenesis (3). MMPs are implicated in tumor invasion, migration, and metastasis, and are thought to function throughout tumorigenesis. In addition to degrade ECM, non-matrix proteins have been found to be the substrates for MMPs, such as pro-MMPs, plasminogen, heparin-binding epidermal growth factor, pro-interleukin 1β, insulin-like growth factor-binding protein-3, and E-cadherin (4, 5). Thus, MMPs are generally accepted to orchestrate critical tumorigenic functions through processing of matrix and non-matrix components.

One of the MMP family members, MMP-3 (stromelysin-1/EC 3.4.24.17), is a secretory enzyme whose expression is upregulated by several growth factors, such as epidermal growth factor and transforming growth factor-α, or can be stimulated by the wound-healing process (6, 7). Tissue inhibitor of metalloproteinase-1 (TIMP-1) is the endogenous inhibitor of this proteinase (8). MMP-3 is expressed in a wide variety of tumor types, and is thought to be correlated with the progression and metastasis of breast, skin, and colon tumors (9–11). It is also shown to promote the epithelial-to-mesenchymal transition (EMT) and the carcinogenesis of mammary tumors (12). Recently, the MMP-3-induced EMT and genomic instability has been shown to be mediated by Rac1b and reactive oxygen species (13).

Although some MMPs are expressed in the stromal cells of tumors, MMP-3 protein has been immunohistochemically documented to be exclusively localized at the tumor-invasive front in several different cancers, suggesting a role in cellular invasion and migration (11, 14). It is also shown that MMP-3 specifically hydrolyzes

human plasminogen activator inhibitor-1 (PAI-1). This cleavage may inactivate PAI-1 and decrease the antiproteolytic activity of PAI-1 thus impairing the potential inhibitory effect of vitronectin-bound PAI-1 on cell adhesion and/or migration (15).

Matrix metalloproteinase-3 may also play a role in oral squamous cell carcinomas (OSCCs) as its protein was detected in 30 of 65 (46.2%) or 37 of 65 (56.9%) OSCC specimens by immunohistochemistry (IHC), and its message in 20 of 20 (100%) head and neck carcinomas by RT-PCR (14, 16, 17). Moreover, by cDNA microarray, real-time quantitative PCR, and IHC analyses, this proteinase was shown to be upregulated in OSCC (18). These results suggest that MMP-3 may contribute to the malignancy of OSCC; however, the molecular mechanism has not been illustrated yet. To delineate the functional role of MMP-3 in OSCC, we not only analyzed its expression pattern in seven oral pre-malignant lesions (OPMLs) and 92 OSCCs, but also assessed the effects of transfecting the antisense oligodeoxynucleotides (AODNs) of MMP-3 into two human OSCC cell lines, SCC-9 and Meng-1 (OECM-1) (19), as well as a carcinoma cell line of the upperaerodigestive tract, CE81T/VGH, from human esophagus origin (20, 21). Finally, changes of the cell growth and colony formation in soft-agar were measured.

## Materials and methods

*Tumor specimens*

This study has been reviewed and approved by the Human Experiment and Ethics Committee, Department of Medical Research, Chi Mei Medical Center. Surgical specimens of seven OPMLs and 92 OSCCs were collected with the consent of every patients, and preserved at −80°C for subsequent RNA preparation. Twenty-five of the 92 OSCC specimens as well as their neighboring tissues were simultaneously collected for the comparison of MMP-3 mRNA level between the malignant and the surrounding tissues. The discrimination of the tumor and the neighboring tissues is solely dependent on visional judgment of the operating surgeons. In some histopathologically examined specimens, $< 5\%$ of the collected neighboring tissues contaminated with a few neoplastic cells. Although not all of these neighboring tissues are absolutely tumor-free, they are mainly composed of non-tumor cells, which may provide a valid reference for the comparison of MMP-3 expression pattern with tumor masses.

*Semi-quantitative RT-PCR*

Protocols of tissue homogenate, total cellular RNA preparation and RT-PCR conditions were described in our previous studies (22, 23). The primer sequences and respected sizes of the PCR products of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and MMP-3 genes are listed in Table 1. Briefly, total cellular RNA (4 μg) was reverse-transcribed into the first-strand cDNA, and one-tenth of the reverse-transcribed cDNA was further subjected to PCR amplifications. Cycle numbers of PCR were 24 for GAPDH, and 30 for MMP-3 to insure the generation of submaximal products. Amplified products were separated by electrophoresis with 100 bp DNA Ladder (GeneRuler$^{TM}$, MBI Fermentas, Vilnius, Lithuania) in 1.5% agarose gels and then visualized under UV light after being stained with 0.5 μg/ml ethidium bromide.

*Western blot analysis*

Frozen tissues stored at −80°C were melted on ice, cut into pieces, and suspended in NET buffer (0.5% NP-40, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4) containing 1X protease inhibitor cocktail set I (Calbiochem-Novabiochem, San Diego, CA, USA). The suspensions were sonicated at 25% amplitude for 1 min at 1-s intervals with a sonicator (Sonics & Materials, Newtown, CT, USA). Protein concentrations were then determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA), and 20 μg of homogenate proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose (NC) filter and soaked in TBS buffer (50 mM Tris-HCl, pH 7.35, 0.85% NaCl) containing 5% lipid-extracted milk at room temperature, and then immunoblotted with 1 μg/ml of an anti-MMP-3 monoclonal antibody, recognizing both latent and active MMP-3 protein, in TBS buffer containing 1% lipid-extracted milk at room temperature for another hour. After washing three times with washing buffer (50 mM Tris-HCl, pH 7.35, 0.85% NaCl, and 0.5% Tween 20), the NC filter was further incubated with $1 \times 10^4$-fold diluted goat-antimouse-IgG monoclonal antibody coupled with horseradish peroxidase (CHEMICON International, Inc., Temecula, CA, USA) in the same buffer as the first antibody at room temperature for 1 h. The NC filter was then extensively washed, and signals of MMP-3 protein were detected with Western Lightening$^{TM}$ Chemiluminescence Reagent (Perkin-Elmer Life Sciences, Boston, MA, USA) as instructed by the manufacturer. A control

**Table 1** Primer sequences and sizes of PCR products

| *Gene* | *Primer sequences* | *Product size (bp)* |
|---|---|---|
| MMP-3 | Forward: 5′-AGCTGGATACCCAAGAGGCAT-3′<br>Reverse: 5′-TCCCTGGAAAGTCTTCAGCT-3′ | 191 |
| GAPDH | Forward: 5′-GTGAAGGTCGGAGTCAACG-3′<br>Reverse: 5′-CAATGCCAGCCCCAGCG-3′ | 895 |

MMP-3, matrix metalloproteinase-3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cytoskeletal protein, β-actin, was re-probed with an anti-β-actin monoclonal antibody (Sigma Diagnostics, Inc., St Louis, MO, USA) by following the same protocols listed above after stripping the above NC filter in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl) 20 min at room temperature and extensively washing by distilled water.

*Transfection of cells with oligodeoxynucleotides*

The phosphorothioate-modified sense oligodeoxynucleotides (SODNs, used as a control) and AODNs of MMP-3 cover from two codons upstream to three codons downstream from the start codon. Their sequences are: SODN: 5′-GTGGAAATGAAGAGTC TTCCAATC-3′, and AODN: 5′-GATTGGAAGACT CTTCATTTCCAC-3′. Cells were seeded at a density of $1 \times 10^6$ cells/well in a 6-well plate in 2 ml Dulbecco's-modified Eagle's medium (DMEM; Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). After 24 h, culture medium was removed and the cells were washed twice with 2 ml PBS, and re-suspended in 1 ml serum-free DMEM. Before the addition of oligodeoxynucleotide–Oligofectamine$^{TM}$ complex into each well, 10 μl of 20 μM AODNs or SODNs were, respectively, diluted into 175 μl serum-free DMEM and 15 μl Opti-MEM® I Reduced Serum Medium (Gibco BRL), mixed gently, and incubated for 10 min at room temperature. For a control purpose, non-transfected parental cells were included in this experiment. All the other transfection procedures were underwent as instructed by the manufacturer.

*Cytotoxicity test*

After the transfection, cells on the 6-well plate were trypsinized and counted. $5 \times 10^3$ cells/well of non-transfected, SODNs, or AODNs transfected OECM-1, SCC-9, and CE81T/VGH cells were seeded onto a 96-well plate. Culture medium (DMEM with 10% FBS) was refreshed after 3 days. After another 3 days, these cells were incubated with XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] labeling reagent, Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IL, USA), as instructed by the manufacturer. Finally, the absorbance of OD450 nm was measured by EL800 Universal Microplate Reader (BIO-TEK Instruments, Inc., Winooski, VT, USA).

*Soft-agar colony-formation assay*

Non-transfected, SODNs, or AODNs transfected OECM-1, SCC-9, and CE81T/VGH cells were plated onto each well of a 6-well plate at a density of $5 \times 10^4$ cells/well in 2 ml DMEM containing 20% FBS and 0.2% agarose (with 0.5% agarose underlay). The same agarose/medium was refreshed after 3 days, and the number of colonies was determined after another 3 days.

*Statistical analyses*

Difference between the MMP-3 incidence in OPMLs and OSCCs was analyzed by Fisher's exact test. Two groups of data presented as mean ± SD were analyzed by Student's *t*-test. A value of $P < 0.05$ was regarded as statistically significant.

## Results

*Expression pattern of MMP-3 transcript in oral tumors*

Among the seven OPML and 92 OSCC specimens, MMP-3 message was preferentially detected by RT-PCR in OSCCs (71 of 92, 77.2%) than in OPMLs (two of seven, 28.5%; $P = 0.012$). On the other hand, MMP-3 incidence was not statistically correlated with tumor stage, thickness, size, and lymph node metastasis (Table 2). In 25 of the 71 MMP-3-positive OSCC specimens, the neighboring tissues of tumors were simultaneously collected. To compare the expression level of MMP-3 message between OSCCs (T1–T25) and their neighboring tissues (N1–N25), MMP-3 signals of the 25 tissue pairs were digitalized and normalized against those of GAPDH by semi-quantitative RT-PCR (Fig. 1) and densitometry. The average MMP-3/GAPDH ratio of T1–T25 was calculated to be 0.980 ± 0.721, which is significantly greater than that of N1–N25 (0.134 ± 0.266; $P < 0.0001$). In most cases, MMP-3/GAPDH ratios of T*x* are remarkably higher than those of N*x* ($x = 1$–5, 7–13, and 15–23); however, in two cases, these two ratios are similar ($x = 6$ and 14), and the ratios of T*x* are evidently lower than those of N*x* ($x = 24$ and 25; Fig. 1). These might reflect the individual differences and resulted in the high SD values of T*av* and N*av*. Taken together, the overall expression pattern still suggested that MMP-3 is preferentially transcribed in the malignant OSCCs than in the benign OPMLs, and the level of MMP-3 transcript is, in most

**Table 2** Incidence of MMP-3 in seven OPMLs and in 92 OSCCs with distinct tumor characteristics

| *Stage (n = 92)* | *Thickness (n = 90)* | *Size (n = 92)*[b] | *Metastasis (n = 88)*[c] |
|---|---|---|---|
| OPML: 2/7 (28.5%)[a] | 0–5 mm: 11/16 (69%)[a] | T1: 11/14 (79%)[a] | $NLNM^+/MMP\text{-}3^-$: 7/20 (35%) |
| Stage I: 9/12 (75%) | 6–10 mm: 25/32 (78%) | T2: 26/31 (84%) | $NLNM^+/MMP\text{-}3^+$: 28/68 (41%) |
| Stage II: 14/19 (74%) | 11–15 mm: 11/13 (85%) | T3: 6/12 (50%) | |
| Stage III: 9/12 (75%) | >15 mm: 21/29 (72%) | T4: 28/35 (80%) | |
| Stage IV: 39/49 (80%) | | | |

[a]Incidence of MMP-3.
[b]T1–T4: UICC/AJCC TNM classification system (2002).
[c]The involvement of neck-lymph node metastasis ($NLNM^+$) in the presence or absence of MMP-3 ($MMP\text{-}3^+$ or $MMP\text{-}3^-$).
MMP, matrix metalloproteinase; OSCC, oral squamous cell carcinoma; OPML, oral pre-malignant lesion.

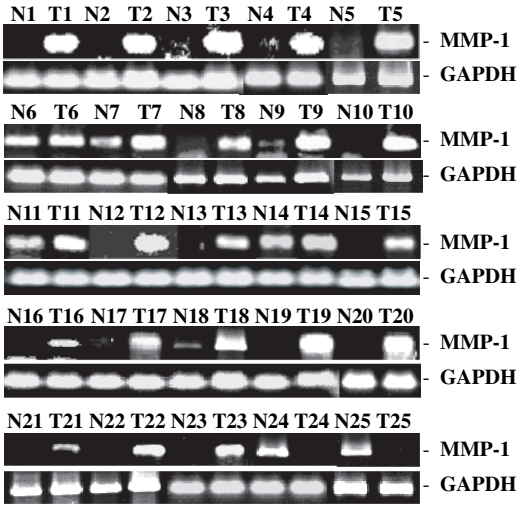


**Figure 1** Comparison of matrix metalloproteinase (MMP)-3 message level between oral squamous cell carcinomas (OSCCs) and their neighboring tissues. Twenty-five surgical specimens of OSCCs (T1–T25) and their neighboring tissues (N1–N25) were subjected to semi-quantitative RT-PCR analysis of MMP-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. Signals of these two genes were digitalized spectrophotometrically and the MMP-3/GAPDH ratio of each specimen was calculated. All of these experiments have been repeated at least twice with similar results.

cases, higher in the OSCC tumor masses than in the neighboring tissues.

*Comparison of MMP-3 protein level between OSCC and its neighboring tissue*

Tissue homogenates from N1T1 to N4T4 were subjected into the Western blot analysis, and the results illustrated the amplified expression of MMP-3 protein in OSCCs (T1–T4; Fig. 2). To achieve a statistical purpose, the control β-actin protein was also detected and density of MMP-3 signals (containing both latent and active MMP-3) was normalized against that of β-actin. The fluctuations of MMP-3/β-actin ratios among these eight specimens (N1T1–N4T4) indicated the individual difference and resulted in the high SD values. However, the average ratio MMP-3/β-actin was significantly higher in the homogenates of T1–T4 (1.016 ± 0.938) than in those of N1–N4 (0.028 ± 0.038; $P = 0.04$). Taken together, the distribution of MMP-3 protein is in consistent with the localization of its mRNA as assessed above.

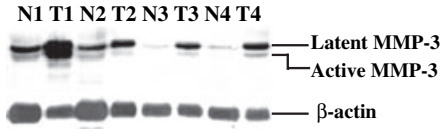


**Figure 2** Comparison of matrix metalloproteinase (MMP)-3 level between tissue homogenates of oral squamous cell carcinomas (OSCCs) and their neighboring tissues. Four OSCC specimens (T1–T4) and their neighboring tissues (N1–N4) were subjected to Western blot. Digitalized signals of MMP-3 protein (containing latent and active MMP-3) were normalized against those of β-actin protein. This experiment has been repeated twice with similar results.

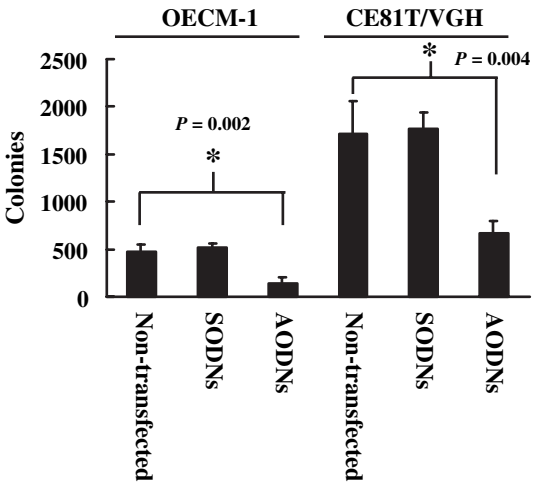


**Figure 3** Effects of the transfection of sense and antisense oligodeoxynucleotides (SODNs and AODNs) of matrix metalloproteinase-3 on colony formation. Colony numbers of non-, SODNs-, or AODNs-transfected OECM-1 and CE81T/VGH cells were scored. All data are presented as mean ± SD. This experiment has been performed in triplicate and repeated three times with similar results.

*Effects of MMP-3 AODN transfection*

The expression pattern of MMP-3 suggested a close correlation with OSCC. Therefore, analysis of various phenotypic changes of tumor cells after the specific inhibition of MMP-3 protein expression by transfecting cells with MMP-3 AODNs was utilized to further examine its possible roles in OSCC. AODNs and the control SODNs of MMP-3 were transfected into OECM-1, SCC-9, and CE81T/VGH, and the consequent changes of the cell viability and the ability of colony formation were measured. The results showed that cell viability measured by XTT-labeling reagent was not affected after SODN or AODNs transfection compared with non-transfected parental cells in these three cell lines (data not shown). With or without the transfection, SCC-9 did not form colonies in the soft-agar/medium (data not shown). On the other hand, colony numbers of both OECM-1 and CE81T/VGH were significantly reduced after MMP-3 AODNs transfection when compared with non-transfected cells ($P = 0.002$ and 0.004, respectively; Fig. 3).

## Discussion

The present study demonstrates the preferential expression of MMP-3 message in malignant OSCCs than in benign OPMLs (Table 2). Transcript level of MMP-3 is also higher in the tumor masses of OSCCs than in their surrounding tissues (Fig. 1). In consistent with its mRNA distribution, MMP-3 protein is concentrated within the tumor masses when compared with their neighboring tissues (Fig. 2). As all specimens used in this study came from Taiwanese patients and the above results are similar to other previous findings from Japan and the USA (14, 16, 17), these evidences collectively indicate that MMP-3 may play a universal and important

role in OSCC. In addition, we have, for the first time, further demonstrated the requirement of MMP-3 in the anchorage-independent growth of both OSCC (OECM-1) and esophageal carcinoma (CE81T/VGH) cells. Thus, not only by providing evidence of a functional role of MMP-3 in OSCC, this study has also extended the involvement of MMP-3 in the malignancy of esophageal carcinoma. It is, therefore, possible for MMP-3 to play a common or similar role in the carcinomas of upper aerodigestive tract.

The 77.2% (71 of 92) MMP-3 expression frequency in OSCCs as analyzed by RT-PCR in this study is higher than those detected by immunohistochemical staining (14, 16). This may be due to a greater sensitivity of RT-PCR methodology as seen in an independent study showing 100% incidence of MMP-3 in 20 head and neck carcinomas (17). This extremely sensitive method gives positive results, as long as there is a trace amount of MMP-3 message. According to our semi-quantitative RT-PCR (Fig. 1) and Western blot (Fig. 2) results, we thought that MMP-3 incidence might have been correlated with some of the tumor phenotypes, such as stage, thickness, size, and/or metastasis. Surprisingly, the RT-PCR results from 92 OSCC specimens indicate that there is no statistical relevance between MMP-3 incidence and any of these four tumor phenotypes (Table 2). As PCR does not provide the information about the level of gene product, it is still possible that MMP-3 protein level may be correlated with some or any of these tumor phenotypes. Alternatively, if most (or all) of the positive results detected by RT-PCR reflect that MMP-3 protein can be synthesized and is biologically functional in the tested OSCC specimens, our RT-PCR results may also suggest the requirement of MMP-3 function in various OSCCs (stage I–stage IV, shallow to deep invasion thickness, small to large size, and non-metastasis to metastasis), i.e. in the entire OSCC progression process.

The impact of MMP-3 on cell growth may be intricate. There were evidences demonstrating that the basement membrane ECM, but not fibronectin or collagen, suppressed apoptosis of mammary epithelial cells in tissue culture and *in vivo*. The apoptosis could be induced by overexpression of MMP-3, which degrades ECM (24). Thus, overexpressed MMP-3 seemed to kill epithelial cells. To the contrary, another evidence showed that antisense oligonucleotides of MMP-3 inhibited injury-induced proliferation of arterial smooth muscle cells (25). This result in turn suggests that cells cannot divide in the absence of MMP-3. It is possible that functions of MMP-3 may be cell type-specific. In our results, MMP-3 AODNs did not affect the growth of the three different carcinoma cell lines (OECM-1, SCC-9, and CE81T/VGH) indicating the redundancy of MMP-3 in the growth of these cells. Alternatively, the translation of MMP-3 message may not be 100% inhibited by AODNs transfection, and cellular growth could have been maintained as long as functional MMP-3 protein is present. We have tried to verify this speculation; however, only MMP-3 message, but not its protein, is detectable in the three cell lines used (data not shown). In our laboratory, the transfection of MMP-3 AODNs did not interfere the expression of other MMPs, like MMP-2 and MMP-8, which has weakened the possibility of non-specific effect of MMP-3 AODNs on OECM-1 and CE81T/VGH (data not shown). We, therefore, regard that the reduced colony numbers was due to a specific inhibition of MMP-3 translation by its AODNs.

Although MMP-3 was thought to play a role in tumor migration for its localization at the invasive front, the MMP-3 AODNs did not inhibit 2D motility of OECM-1 cells in the Scratching test (data not shown). Similarly, a selective MMP-3 inhibitor (called compound **7**), has been shown to inhibit MMP-3-mediated matrix degradation but not to reduce cellular migration mediated by MMP-1, MMP-2, and MMP-9 (26). Our recent studies show that the 2D migration of OECM-1 and CE81T/VGH is retarded by the treatment of neutralizing antibodies of MMP-1 and MMP-2 (data not shown). Therefore, MMP-3 may play a more important role in colony formation than in cell growth and migration in at least some OSCCs.

MMP-3 has been found to be associated with the malignancy of ESCC. For example, the correlation of MMP-3 with the invasion and metastasis of esophageal carcinomas has been demonstrated (27). A single nucleotide polymorphism in the promoter of MMP-3 gene has also been illustrated to be associated with a risk of ESCC development and its lymphatic metastasis (28). Here, we have presented the first evidence of the participation of MMP-3 in the tumorigenicity of ESCC. Although more studies on ESCC specimens are required before making a more objective conclusion, our findings suggest that MMP-3 may play similar and pivotal roles in both OSCC and ESCC.

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