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MCTP2 is a novel biomarker promoting tumor progression and nodal metastasis in oral squamous cell carcinoma

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Head and neck cancer is the sixth most common cancer worldwide. Among them, oral squamous cell carcinoma (OSCC) has remarkable local invasiveness and lymph node metastasis and is frequently found at an advanced stage. The 5-year survival rate of OSCC has remained at approximately 50% for several decades, and there is an urgent need to identify molecular markers that are effective for early diagnosis and treatment. Multiple C2 transmembrane proteins (MCTPs) are C2 domain-containing proteins, with two subtypes in humans: MCTP1 and MCTP2. MCTP1 has been reported to exhibit tumor-promoting activity in several cancer types; however, the role of MCTP2 in cancer remains largely unknown. In this study, we performed a comprehensive analysis using big data from over 500 head and neck cancer cases registered in The Cancer Genome Atlas (TCGA), expression profiling of 63 OSCC samples, and in vitro functional assessment using cell lines to elucidate MCTP2 involvement in OSCC. Compared to normal oral mucosa, MCTP2 expression was elevated in OSCC, and its expression rate was significantly increased at both protein and mRNA levels in cases with lymph node metastasis. In vitro experiments using two OSCC cell lines demonstrated that MCTP2 may be involved in cancer cell migration, invasive capacity acquisition, and epithelial-mesenchymal transition (EMT) phenotype. Furthermore, MCTP2 expression levels were upregulated by TGF-β1 in a concentration-dependent manner. These findings suggest that MCTP2 may serve as a novel marker of invasion and EMT in OSCC, with promising implications for developing new MCTP2-targeted diagnostic and therapeutic approaches for OSCC.

Keywords MCTP2, Oral cancer, Metastasis, Growth, Migration, Invasion, TGFβ1

Globally, oral squamous cell carcinoma (OSCC) is estimated to cause 389,485 new cases and 188,230 deaths in 2022, representing approximately 2.0% of all cancers and 1.9% of cancer-related deaths[1]. OSCC is characterized as a formidable malignancy that exhibits a propensity for deep invasion into adjacent tissues, including jawbone, maxillary sinus, and major nerves, and a propensity for nodal and distant metastases, resulting in poor prognosis. Furthermore, although OSCC occurs in easily observable locations, it often remains undetected until reaching advanced stages, requiring extensive tumor resection. Postoperatively, oral functions such as speech, swallowing, feeding, and occlusion are impaired, resulting in facial alignment changes and decreased patient quality of life[2]. Therefore, control of metastasis and invasion is key to achieving favorable outcomes in OSCC. Surgery is the primary treatment for OSCC, and chemotherapy, radiotherapy, and immunotherapy may complement surgery, although their efficacy remains highly controversial. Hence, unraveling the molecular intricacies underlying cancer progression, invasion, and metastasis is imperative to enhance the prognosis of OSCC through early diagnosis and therapeutic intervention.

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The C2 domain-containing protein has multiple functions in cancer and in vivo, regulating protein kinase C[3]. In association with malignant diseases, mutations in the C2 domain of phosphatidylinositol-3 kinase (PIK3) have been reported to promote proliferation, migration, and invasion of breast cancer cells[4] The C2 domain-containing protein confers chemotherapy resistance in ovarian cancer[5] and promotes cell differentiation and tumorigenesis through Hippo signaling pathway in liver cells[6] Recently, multiple C2 transmembrane proteins (MCTP1) and MCTP2 were identified as novel molecules belonging to the C2 domain-containing protein family[7] Liu et al. reported that MCTP1 increases the malignant potential of androgen-derived prostate cancer by inducing neuroendocrine differentiation and epithelial-mesenchymal transition (EMT)[8] Moreover, in esophageal cancer, MCTP1 has been shown to be involved in regulating DNA methylation and enhancing drug resistance[9] In contrast, little is known about MCTP2, another member of the MCTP subfamily, and there is no research on the relationship between MCTP2 and cancer, except for one report indicating that MCTP2 inhibits cisplatin resistance in gastric cancer[10] This study aims to investigate the role of MCTP2 in OSCC.

Materials and methods

Tumor specimens

Formalin-fixed, paraffin-embedded surgical specimens were obtained from 63 patients with primary OSCC undergoing surgery at the Department of Oral and Maxillofacial Surgery, Kagoshima University Hospital, Kagoshima, Japan from 2021 to 2023. The main inclusion criteria for this study were clinically diagnosed OSCC patients who were not currently receiving or had never received curative treatment for OSCC in the form of radiation therapy, chemotherapy, or other adjuvant therapy; conversely, the absence of histopathological confirmation of OSCC and lack of postoperative follow-up were exclusion criteria. Tumor stage and histological grade were determined according to the Union for International Cancer Control's TNM classification system, 8th edition, and World Health Organization criteria, respectively. Medical records were retrieved from the hospital database. This study involving human samples was approved by the Ethics Committee for Epidemiological Studies at Kagoshima University [Approval No. 213223-1], in accordance with the principles of the Declaration of Helsinki. In addition, the analyses using clinical specimens in this study were performed in principle in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[11].

Immunohistochemistry

Serial Sect. (3-µm thickness) were cut from each block for immunohistochemistry. After deparaffinization, endogenous peroxidase activity was blocked with methanol containing 0.3% H_2O_2 for 30 min. Antigen retrieval was achieved through microwave treatment in Tris-ethylenediaminetetraacetic acid (EDTA) buffer for 1 h. Protein blocking serum was applied for 10 min to block nonspecific antibody reactions, and anti-MCTP2 antibody (Proteintech, Rosemont, IL, USA) diluted 1:100 with Can Get Signal Immunostain (Toyobo, Osaka, Japan) was used as the primary antibody. The specimens were incubated overnight at 4 °C and then treated with horseradish peroxidase (HRP)-conjugated secondary antibody (Medical & Biological Laboratories, Nagoya, Japan) diluted 1:100 for 1.5 h at room temperature. Diaminobenzidine (DAB) solution (DAKO, Carpinteria, CA, USA) was used for color development and counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA).

To evaluate the immunohistochemistry, two pathologists (TS and KS) and one oral surgeon (YS) discussed each case. MCTP2 immunoreactivity was classified according to Allred's score (AS) based on staining intensity and positivity[12] and divided into four grades: grade 0 (AS = 0), grade 1 (AS = 2–4), grade 2 (AS = 5–6), and grade 3 (AS = 7–8). Cases classified as grade 2 and 3 were considered immunologically positive[13].

Public databases

MCTP2 expression datasets from 519 head and neck squamous cell carcinoma (HNSCC) patients and 44 healthy controls were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov). This database contains clinical information and RNA-seq expression data from 528 patients (72.4% with oral cancer, 21.96% with laryngeal cancer, and 5.64% with oropharyngeal cancer). Patients with a follow-up period of less than 30 days were excluded, and 519 cases were analyzed[14,15]. Furthermore, Gene Set Enrichment Analysis (https://www.gsea-msigdb.org/gsea/index.jsp)was performed to predict signaling molecules regulating MCTP2.

Cell culture

In this study, we used HSC3M3 cells (derived from tongue SCC) and HOC313 cells (derived from floor-of-mouth SCC). The former was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and the latter was kindly provided by Prof. Kudo (Tokushima University, Tokushima, Japan). Both cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Rockford, IL, USA) at 37 °C in 5% CO_2 in air.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using the FastGene RNA extraction kit (Nippon Gene+96+nnnnnnnnnn+\ l90otics, Tokyo, Japan) and from FFPE sections using the RNeasy FFPE Kit (Qiagen, Venlo, Netherlands). Subsequently, cDNA was synthesized using ReverTra Ace qRT kit (Toyobo) with 0.5 µg of total RNA as template. qRT-PCR targeting MCTP2, matrix metalloproteinase 1 (MMP1), MMP2, MMP3, MMP9, MMP11, MMP12, MMP13, E-cadherin (CDH1), alpha-smooth muscle actin (ACTA2), Snail and TGF-β1 (Table 1) was performed using Thermal Cycler Dice Real Time System II (Takara Bio, Kusatsu, Japan) along with THUNDERBIRD SYBR qPCR Mix (Toyobo). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase

Gene	Forward	Reverse
MCTP2	ATTAAAACAGCGGACCAGGC	ACTGCTGCTCTTGGGAAAGAT
CDH1	GAAACTGGCATCCTCACAGC	TCCTCGGACACTTCCACTCT
Snail1	ACCCCAATCGGAAGCCTAAAC	TCCCAGATGAGCATTGGCAG
ACTA2	ACTGCCTTGGTGTGTGACAATC	CCAGTTGGTGATGATGCC
Vimentin	ATGCTTCTCTGGCACGTCTT	AGCCACGCTTTCATACTGCT
MMP1	CCTGAAGAATGATGGGAGGCA	CTCTTGGCAAATCTGGCGTG
MMP2	ATTACCTGGATGCCGTCGT	CCTCCGGGTCCTTCTCTAGT
MMP3	GCAGTTTGCTCAGCCTATCC	GAGTGTCGGAGTCCAGCTTC
MMP9	TCTATGGTCCTCGCCCTGAA	CATCGTCCACCGGACTCAAA
MMP11	TCATGATCGACTTCGCCAGG	CATGGGGTAGCGAAAGGTGT
MMP12	CGTGGCATTCAGTCCCTGTA	CCACGGTAGTGACAGCATCA
MMP13	CTGCTGGCTCATGCTTTTCC	GACCTAAGGAGTGGCCGAAC
TGFβ1	GCAAATTGAGGGCTTTCGCC	AGTGAACCCGTTGATGTCCA
GAPDH	GCATCCTGGGCTACACTGAG	TCCACCACCCTGTTGCTGTA

Table 1. Primer list for qRT-PCR.

Antibody (Cat. No)	Dilution	Host	Company
MCTP2 (17,578–1-AP)	1:1,000	Rabbit/IgG	Proteintech,
E-cadherin (SC-8426)	1:50	Mouse/IgG	Santa Cruz Biotechnology (CDH1)
Snail1 (SC-271977)	1:100	Mouse/IgG	Santa Cruz Biotechnology
Vimentin (SC-6260)	1:500	Mouse/IgG Santa Cruz Biotechnology	
SMA (SC-531422)	1:200	Mouse/IgG	Santa Cruz Biotechnology (ACTA2)
GAPDH (016–25,523)	1:15,000	Mouse/IgG	Fujifilm Wako Chemical

Table 2. Antibody list for immunoblotting.

(GAPDH) and comparisons were made using the ΔΔCt method. Each experiment was performed in triplicate and repeated at least three times independently.

Immunoblotting

Whole cell lysates were obtained using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). For immunoblotting, 20–30 μg of lysates were separated using 5–15% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare, Amersham, UK). These membranes were incubated with primary antibody diluted in Can Get Signal (Toyobo, Osaka, Japan) and peroxidase-conjugated IgG (Santa Cruz Biotechnology, Dallas, TX, USA). The antibodies used are listed in Table 2. The immune complexes were visualized using an ECL Western Blotting Detection System (GE Healthcare). The anti-GAPDH antibody (Fujifilm Wako Chemical, Osaka, Japan) was used as an internal control. After electrophoresis, protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The expression level of each molecule normalized to GAPDH was determined, with the control group set to 1.

Transient SiRNA transfection

Human OSCC cells were seeded at a density of 300,000 cells per well in 6-well plates. After overnight incubation, cells were transfected with 10 nM/well of hsRiMCTP2 for MCTP2 (SASI Hs02 0035-1804: Sigma-Aldrich) and siRNA Universal Negative Control (Sigma-Aldrich). Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific) and 10 nM siRNA. After 48 h of incubation at 37 °C, cells were harvested for subsequent analyses.

Invasion assay

The invasion assay was performed using a Matrigel-coated chamber (Corning, Corning, NY, USA). Approximately 20,000 cells/well were suspended in 500 μL DMEM and seeded into Matrigel-coated inserts, while 500 μL fresh DMEM was added to the lower chamber. After 24–48 h incubation at 37 °C, filters were fixed with 4% paraformaldehyde and stained with hematoxylin (Sigma-Aldrich). Subsequently, filters were examined under a microscope at 100× magnification, and stained infiltrating cells were counted in four randomly selected fields of view.

Wound healing assay

For the wound healing assay, transfected OSCC cells were seeded into inserts placed in 24-well plates. The inserts were removed after 24 h of incubation, and images were captured using a phase-contrast microscope at 4× magnification every 4 h. The wound area was subsequently measured using ImageJ software.

TGF-β1 treatment

30,000 cells were seeded in 2 mL of serum-free medium per well in a 6-well plate. After overnight culture, rhTGF-β1 (Proteintech) diluted to 0 ng/mL, 0.1 ng/mL, 1 ng/mL, or 5 ng/mL was added to the medium. The cells were then used for further analysis after 48 h of incubation.

Statistical analysis

Statistical analyses were performed using Student's t-test, unpaired t-test, and Chi-square test. Disease-free survival (11 to 3233 days) and overall survival (22 to 3912 days) were analyzed using the Kaplan-Meier method, and differences between groups were calculated using the log-rank test. All statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, Boston, MA, USA), and $p < 0.05$ was considered statistically significant.

Results

MCTP2 expression in human OSCC cases

First, we investigated the association between clinicopathological features and MCTP2 expression in 63 OSCC patients. Patient age ranged from 37 to 91 years (mean, 69.6 years). MCTP2 immunoreactivity was generally negative or very weak in non-cancerous oral mucosa, but its cytoplasmic localization was confirmed in OSCC cells (Fig. 1A). Table 3 summarizes the association between MCTP2 immunostaining and the clinicopathological features of OSCC. In this study, MCTP2 expression rate in OSCC was 46% (29/63). MCTP2 was detected in 22 of 54 patients (40.7%) without nodal metastasis, but was overexpressed in 77.8% (7/9) of patients with nodal metastasis, showing a statistically significant difference between groups ($P = 0.039$). Furthermore, among the 63 cases used for immunohistochemistry, real-time RT-PCR was performed on 46 cases where total RNA could be extracted from FFPE specimens. As controls, totalRNA was extracted from FFPE specimens of three healthy subjects without OSCC, oral mucosal disease, or systemic disease and subjected to RT-PCR, indicating that OSCC patients express higher levels of MCTP2 ($P = 0.0009$, Fig. 1B). MCTP2 expression levels were also significantly higher in cases with lymph node metastasis than in those without metastasis ($P < 0.0001$, Fig. 1B). Although no statistically significant differences were observed, MCTP2 expression tended to be higher in poorly differentiated OSCCs compared to well-differentiated ones ($P = 0.0539$). In current OSCC cases, no statistically significant difference was found between MCTP2 expression in immunohistochemistry or real-time RT-PCR and other clinicopathological factors. No correlation was observed between MCTP2 expression and overall survival, and although MCTP2-positive patients tended to have a lower disease-free survival rate, the difference was not significant (Fig. S1).

Public dataset analysis of MCTP2 in OSCC

Subsequently, we evaluated MCTP2 expression levels in 519 HNSCC patients and 44 healthy subjects from the TCGA dataset, and as shown in Fig. 1C, MCTP2 expression was significantly higher in HNSCC cases than in healthy controls. Subsequently, we examined the upstream factors regulating MCTP2 by GSEA and found that MCTP2 is positively correlated with TGF-β signaling pathway (Fig. 1D). Since the TGF-β signaling pathway is well known to promote EMT and cancer proliferation and invasion, the following in vitro analyses were performed to determine MCTP2 function in OSCC.

Association of MCTP2 with OSCC cell migration and invasion

The in vitro studies were conducted using OSCC cell lines (HSC3M3 and HOC313 cells) with confirmed MCTP2 expression. In both cell lines, MCTP2 knockdown reduced MCTP2 expression levels (Fig. 2, S2, S4) and time-dependent migration capacity (Fig. 3). Furthermore, MCTP2 knockdown significantly reduced the number of invasive cells traversing type IV collagen-coated Matrigel (Fig. 4). Since multiple changes in gene expression occur during cancer cell proliferation and invasion, further analysis revealed that siRNA treatment of MCTP2 increased expression of epithelial marker CDH1 and decreased expression of EMT-inducing factor Snail, mesenchymal marker vimentin, and ACTA2 (smooth muscle actin) (Fig. 2, S2, S4). We examined changes in stromal connective tissue degradation markers after MCTP2 knockdown and found significantly reduced expression levels of MMP1, MMP3, MMP12, MMP13 (Fig. 5A) and MMP2, MMP9, MMP11, MMP12 in HSC3M3 and HOC313 cells, respectively (Fig. 5B). These findings suggest that MCTP2 may promote OSCC cell migration and invasion by inducing EMT-like changes and stromal destruction.

Changes in MCTP2 expression levels by TGF-β1 in OSCC cells

As mentioned above, MCTP2 expression positively correlates with TGF-β signaling pathway in GSEA public datasets (Fig. 2B), we investigated the effect of rhTGF-β1 treatment on MCTP2 expression in OSCC cells (Fig. S3, S5). Treatment of HSC3M3 and HOC313 cells with rhTGF-β1 increased MCTP2 mRNA and protein expression levels in a dose-dependent manner (Fig. 6). These results indicate that MCTP2 may function as a tumor progression factor that induces EMT in OSCC cells and promotes migration and invasion when regulated by TGF-β1.

Discussion

Despite advances in cancer biology and treatment that have made early-stage cancer curable, OSCC is frequently detected at an advanced stage, and the 5-year survival rate has remained below 50% in recent decades[16] The basic treatment for OSCC is surgery; however, in cases of inoperable advanced cancer, metastasis, or recurrence, chemotherapy and radiotherapy may be indicated or used in combination before or after surgery. However, these treatments often cause severe systemic and local side effects, and drug resistance and radiosensitivity

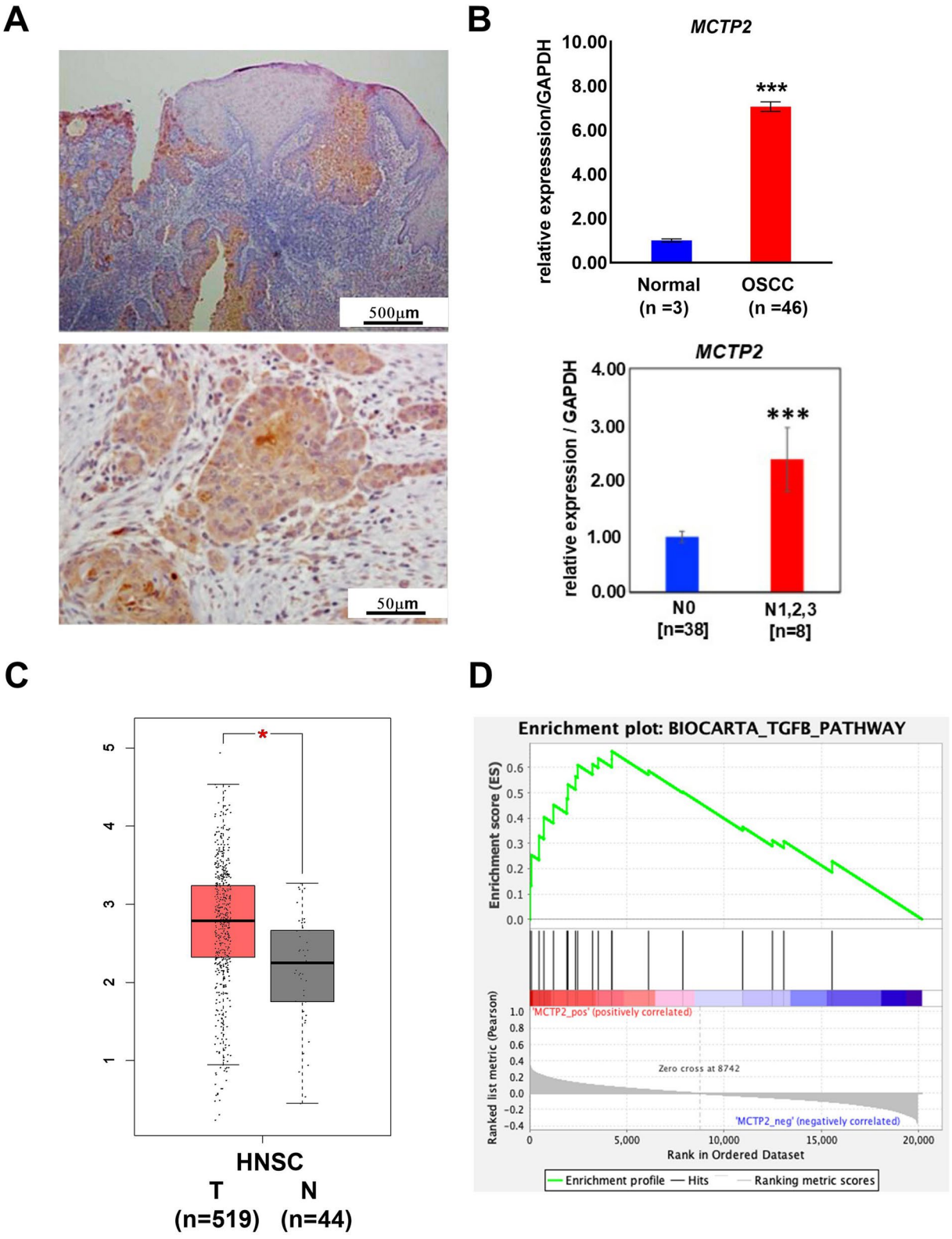

Fig. 1. MCTP2 expression in OSCC. (**A**) Immunohistochemistry showed higher MCTP2 expression in OSCC cytoplasm than non-neoplastic oral mucosa. (**B**) MCTP2 expression levels are higher in OSCC patients compared to healthy controls (upper panel). OSCC patients, MCTP2 mRNA expression levels were significantly higher in cases with lymph node metastasis than in cases without metastasis (lower panel). (**C**) MCTP2 expression was elevated in HNSCC patients compared with the normal group in TCGA database. (**D**) In GSEA, elevated MCTP2 expression in OSCC was associated with TGF-β signaling.
$^{*}p < 0.05$, $^{***}p < 0.001$.

Parameters	MCTP2		*P* value
	Negative	Positive	
Gender			
Male	15	15	
Female	19	14	0.4528
Age			
< 65		13	7
> -65	21	22	0.2309
Site			
Tongue	18		10
Other	16	19	0.1417
Histological differentiation*			
Well	33		24
Mod,Por	1	5	0.0539
T classification			
Tis-2	23	17	
T3, 4	11	12	0.4582
Clinical stage			
I, II	21	17	
III, IV	13	12	0.7993
Nodal metastasis			
Negative	32		22
Positive	2	7	0.0390
Invasion pattern			
YK-1–3	31	24	
YK-4C, 4D	3	5	0.3172

Table 3. Relationship between MCTP2 expression and clinicopathological parameters. Relationship between expression of MCTP2 and each parameter were calculated by Student t-test. T classification and clinical stage were classified according to the TNM classification. *Histological differentiation: Well, well-differentiated squamous cell carcinoma; Mod, moderately differentiated squamous cell carcinoma; Por, poorly differentiated squamous cell carcinoma

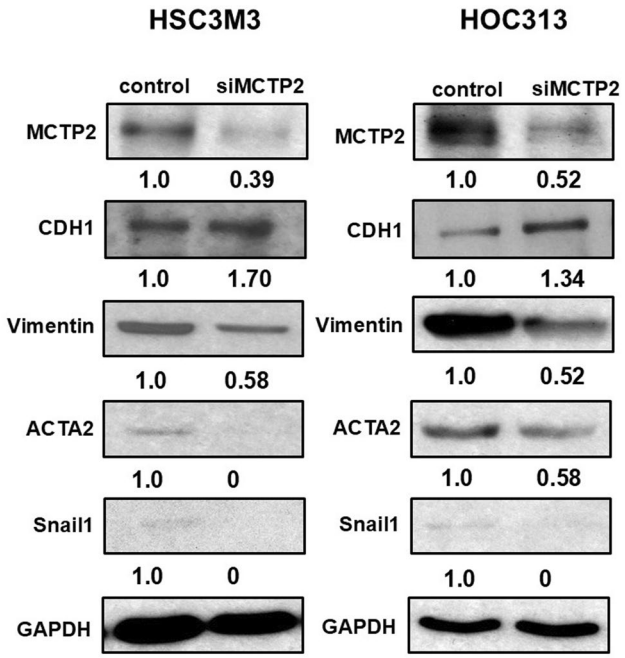

Fig. 2. Changes in the expression levels of various proteins by siRNA treatment of MCTP2 in OSCC cells.

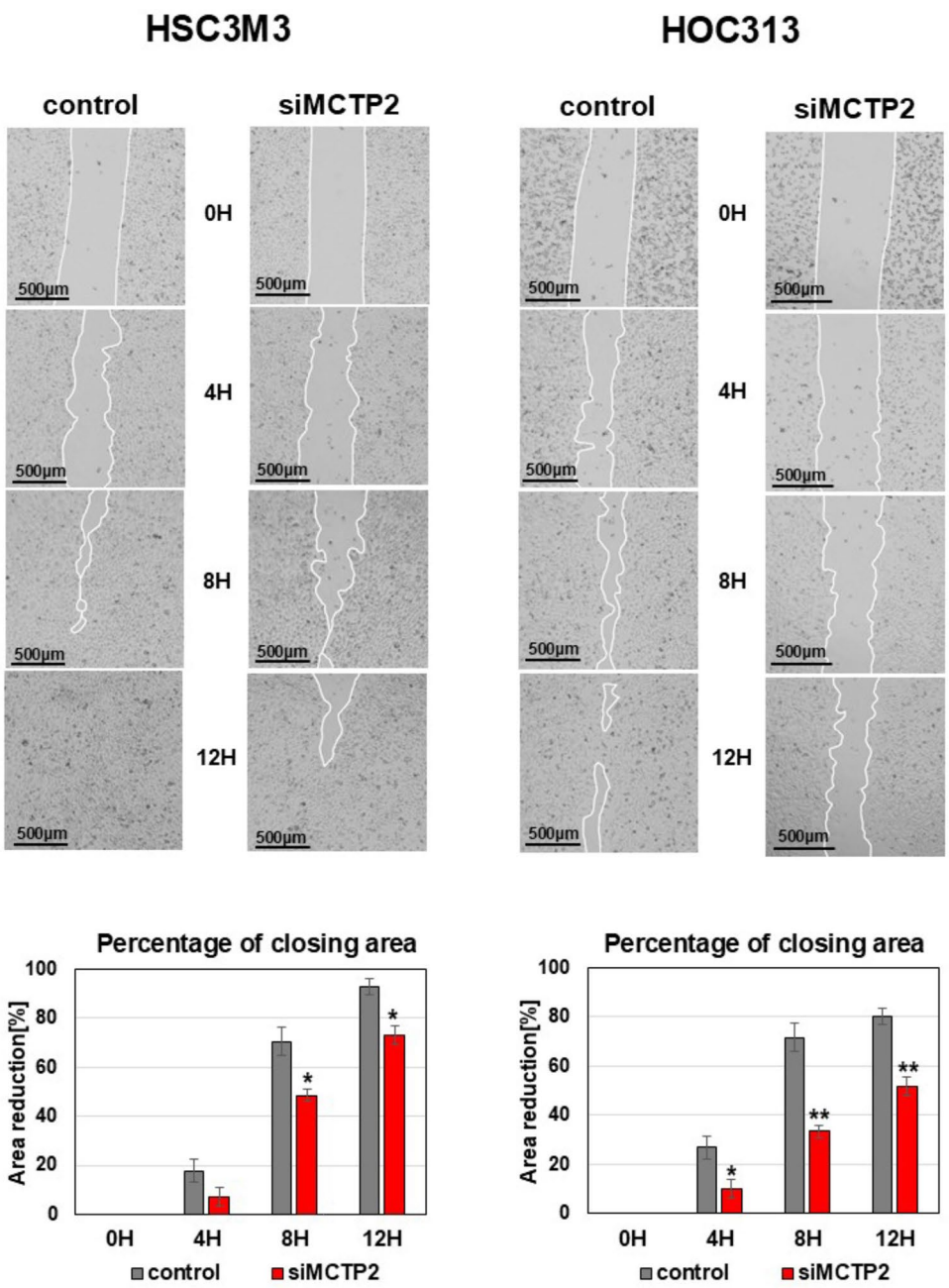

Fig. 3. Association of MCTP2 with migration ability in OSCC cells. Treatment with MCTP2 siRNA reduced the number of invasive cells in HSC3M3 (**A**) and HOC313 (**B**) cells. $^{*}p < 0.05$, $^{**}p < 0.01$.

sometimes pose significant challenges. Although we previously identified several novel molecules conferring drug resistance in OSCC cells[13,17,18], significant knowledge gaps remain regarding the molecular mechanisms underlying acquired resistance to anticancer drugs. Recently, molecularly targeted cancer therapies have gained prominence, though their utility for OSCC patients remains largely limited to cetuximab, a chimeric anti-epidermal growth factor receptor monoclonal antibody, and the anti-programmed cell death 1 (PD-1) antibodies nivolumab and pembrolizumab[19]. Therefore, the development of molecular targets and diagnostics for OSCC remains an urgent priority.

MCTP, a calcium transporter expressed in various cell types, belongs to the C2 domain-containing protein family, and human MCTP consists of two subfamilies, MCTP1 and MCTP2[7]. C2 domain-containing proteins have a variety of functions, including activating membrane-associated signaling pathways[3], and have been reported to affect neuroendocrinology by acting as calcium sensors[20]. However, its role in cancer remains unclear and highly controversial, as some C2 domain-containing proteins have been reported to act as tumor promoters[4–6], while others act as tumor suppressors in lung[21] and breast cancers[22]. Moreover, while multiple studies have documented MCTP1's role in cancer[8,9],evidence for MCTP2 remains limited to circular RNA in gastric cancer[10].

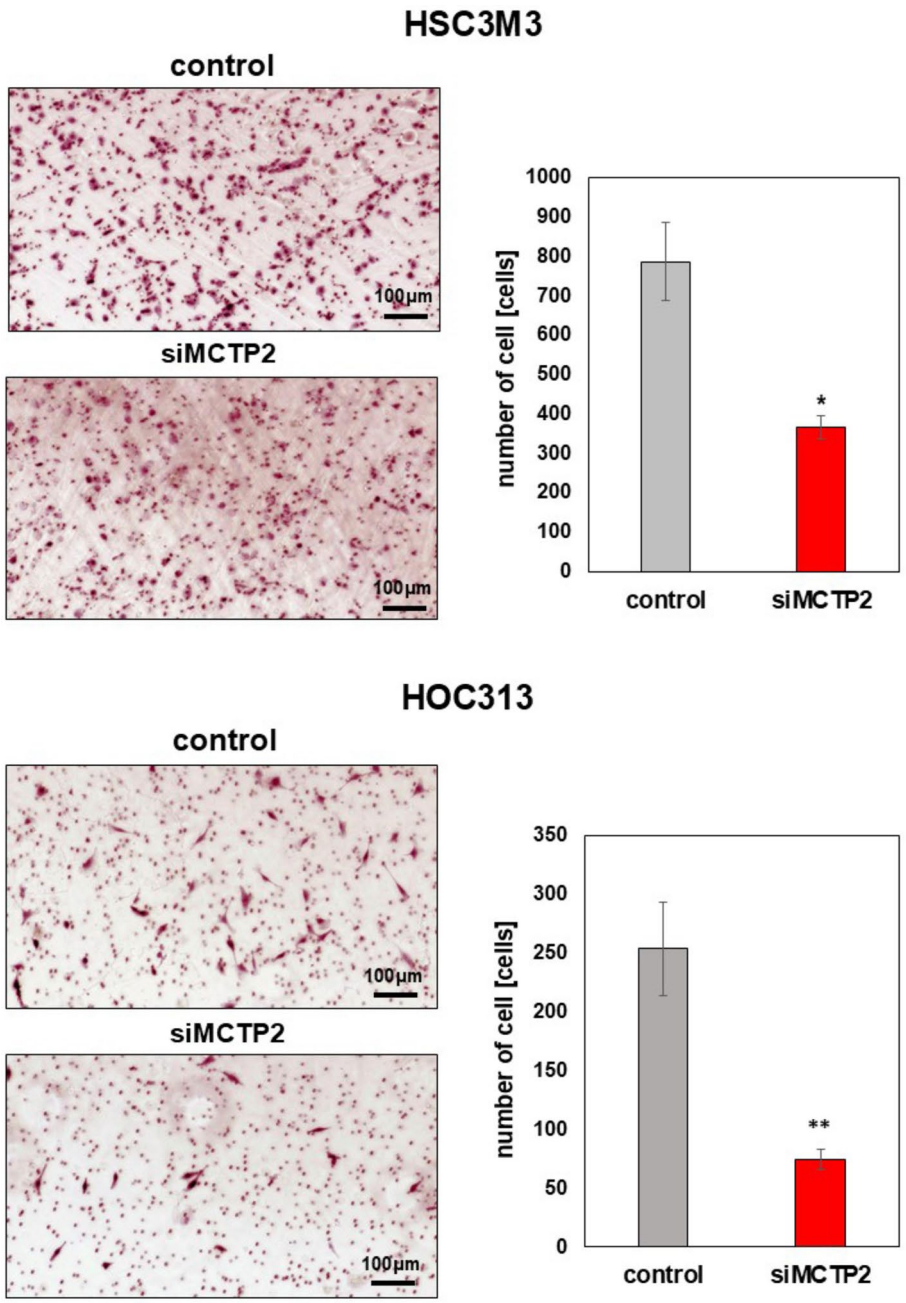

Fig. 4. Effect of MCTP2 on the invasive potential of OSCC cells The invasive potential of HSC3M3 (**A**) and HOC313 (**B**) cells was reduced by MCTP2 knockdown treatment. $^{*}p < 0.05$, $^{**}p < 0.01$.

In the present study, MCTP2 was highly expressed in OSCC compared to normal oral mucosa, both in our patient samples and TCGA dataset, and immunohistochemistry revealed it was significantly correlated with lymph node metastasis. Furthermore, it suggests that MCTP2 induces EMT under TGF-β1 control and may enhance migration and invasion ability of OSCC cells.

Typically, EMT is triggered by various EMT-inducing factors, such as Snail, ZEB, Twist, TGF-β1, and Wnt, which is characterized by decreased E-cadherin expression and increased vimentin expression in cancer cells[23]. Among them, TGF-β1 inhibits the growth of many normal epithelial cells but exhibits a dual role in tumors, acting as a tumor suppressor in early cancer stages while inducing EMT in advanced cancer[24]. In this study, we demonstrated that TGF-β1 upregulates MCTP2 expression. TGF-β1 induces EMT in cancer cells through the Smad signaling pathway and simultaneously promotes MMP secretion from cancer cells, thus contributing to the formation of the cancer microenvironment that facilitates cancer invasion[25]. Our study demonstrated that TGF-β1 acts as an upstream regulator of MCTP2, and both promote migration, EMT, and invasion in OSCC cells. While MCTP1, a homolog of MCTP2, is known to induce EMT in prostate cancer[8], the role of MCTP2 in these processes has remained unexplored. Our data provide the first evidence linking MCTP2 to TGF-β and EMT. The

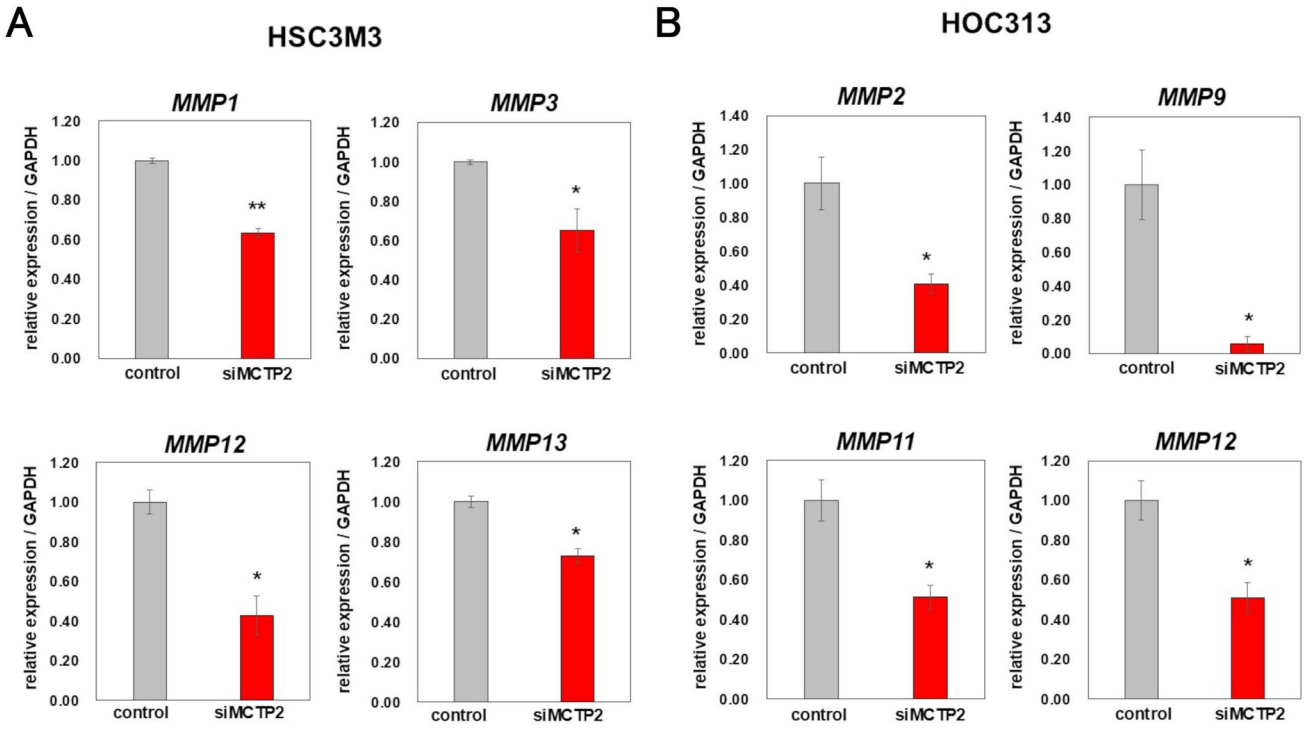

Fig. 5. Influence of MCTP2 on invasion and MMP expression. MCTP2 knockdown treatment reduced various MMP expression levels in HSC3M3 (**A**) and HOC313 (**B**) cells. *$p < 0.05$, **$p < 0.01$.

signaling pathways regulating MCTP2 expression will be further investigated in our laboratory. We also found that MCTP2 expression upregulated MMP expression. Since MMPs expressed in cancer promote invasion by causing stromal destruction[26], MCTP2 may create a microenvironment conducive to cancer invasion. In this study, MMP expression patterns differed between HSC3M3 and HOC313 cells. The reason for this remains unknown; however, the association between MCTP2 and MMPs may vary among cell types. Further studies are needed into how MCTP2-mediated MMP expression contributes to microenvironment remodeling in OSCC.

Recently, the concept of partial EMT (p-EMT), an intermediate state between epithelial and mesenchymal phenotypes in cancer, has garnered significant attention[23,27,28]. Research on p-EMT cell identification, a hybrid intermediate state in EMT's dynamic process, has rapidly advanced through single-cell technology, with mounting evidence linking it to metastasis, chemotherapy resistance, cancer stem cell properties, and poor patient outcomes[27,29]. The elucidation of molecular mechanisms of cancer stemness and therapy resistance in the p-EMT state using cutting-edge technology remains a critical research question, and patient stratification into low- and high-risk groups may open entirely new avenues for treating epithelial tumors[29]. Kudo et al. demonstrated that OSCC cells in the p-EMT state, which maintain epithelial characteristics while exhibiting mesenchymal features, have high malignant potential[30–32], and we recently reported that the transcription factor early growth response protein 1 (EGR-1) may be involved in p-EMT in OSCC[26]. This limited analysis provided no confirmation that MCTP2 is in the p-EMT state. However, given that human cancer cells in vivo are highly heterogeneous, MCTP2-positive OSCC cells may exist in the p-EMT state, and we are also planning to perform single-cell analysis of heterogeneous populations using NGS.

As TGF-β promotes differentiation into regulatory T cells, suppression of TGF-β action can induce immune system activation[33] and shows potential clinical applications in cancer and immune diseases. Recently, intrafusal alfa, a fusion protein of an anti-PD-L1 antibody and a TGF-β inhibitor, was developed as a novel immune checkpoint inhibitor and is now undergoing clinical trials for various cancer types[34,35]. This fusion protein has also been reported to be effective against HPV-positive HNSCC[36,37]. Considering the younger age of diagnosis and diverse sexual practices, the HPV-positive rate in OSCC, as well as cervical and oropharyngeal cancers, will likely increase in the future. Combination therapy with an MCTP2 molecular targeting agent and what is possibly interferon-alpha is anticipated to be a successful treatment approach. TGF-β signaling inhibition suppresses breast cancer[38], bone metastasis, and bone morphogenetic proteins (BMPs), members of the TGF-β family, are involved in cancer bone metastasis[39,40]. Since MCTP2 is regulated by TGF-β, it may represent a novel molecular target candidate not only for HPV-related OSCC but also for HPV-unrelated OSCC and advanced invasive OSCC that destroys jawbone.

Conclusions

A schematic diagram summarizing the findings of this study is shown in Fig. 7. In this comprehensive analysis, including HNSCC big data, primary tumors from 63 OSCC patients, and in vitro studies, we demonstrated that MCTP2 participates in TGF-β-induced OSCC cell migration, invasive ability, and EMT phenotype acquisition.

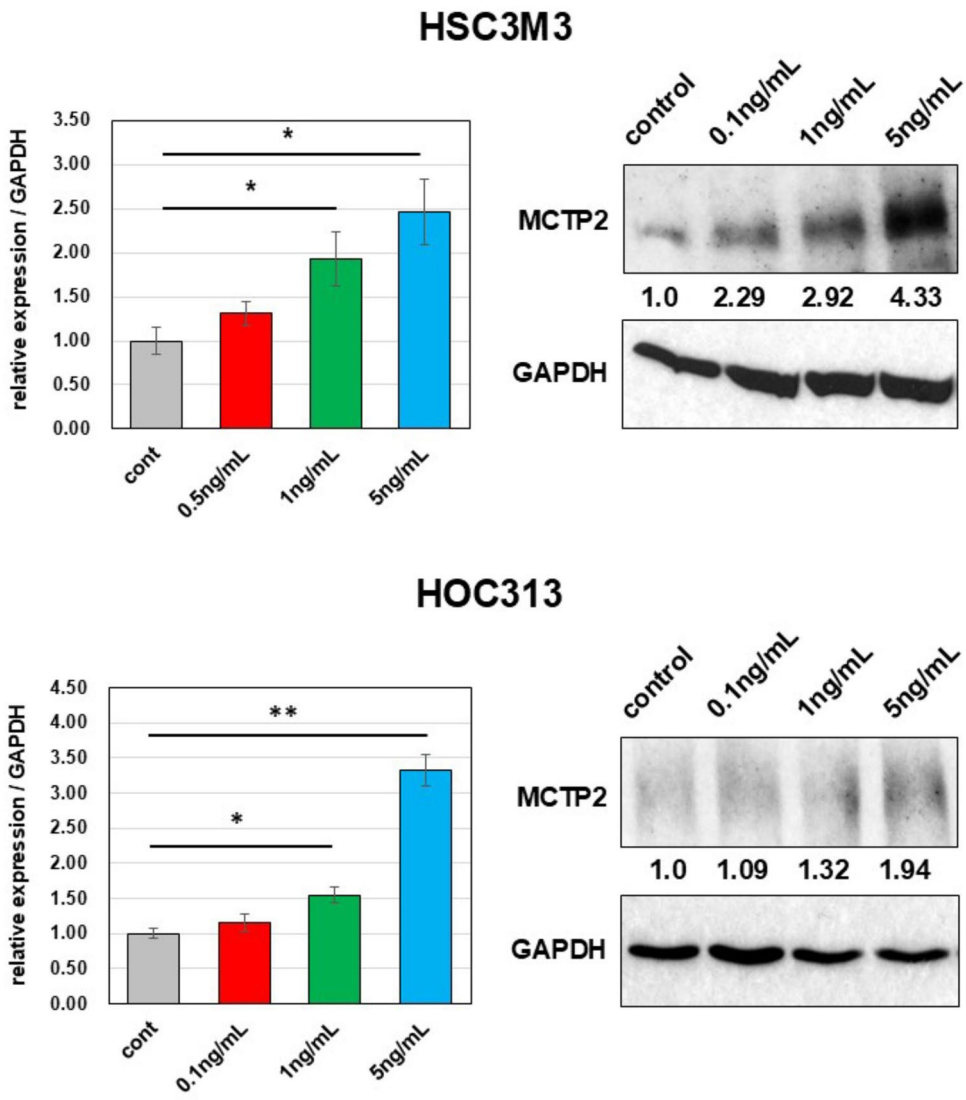

Fig. 6. Relationship between TGF-β and MCTP2 in OSCC cells. TGF-β treatment increased MCTP2 expression levels in HSC3M (**A**) and HOC313 (**B**) cells in a dose-dependent manner.

Although the sample size was limited, MCTP2 protein and mRNA expression levels were notably elevated in patients with lymph node metastasis. However, although MCTP2 regulated the invasive and migratory capabilities in the OSCC cell lines used in this study, no positive correlation was observed between MCTP2 expression level and proliferative capacity (data not shown), and the reason for this remains to be further investigated. One of the main limitations of this study is that cancer cell lines lack the complex interactions of living organisms and do not accurately reflect the heterogeneity and complexity found in human cancers. These limitations suggest that future studies should employ OSCC organoids and spheroids in vitro to better mimic in vivo tumors. Although large-scale clinical studies with a higher number of cases and therapeutic animal experiments are essential for future research development, our findings provide novel insights into OSCC biological dynamics and may lead to new diagnostic and therapeutic targets for OSCC.

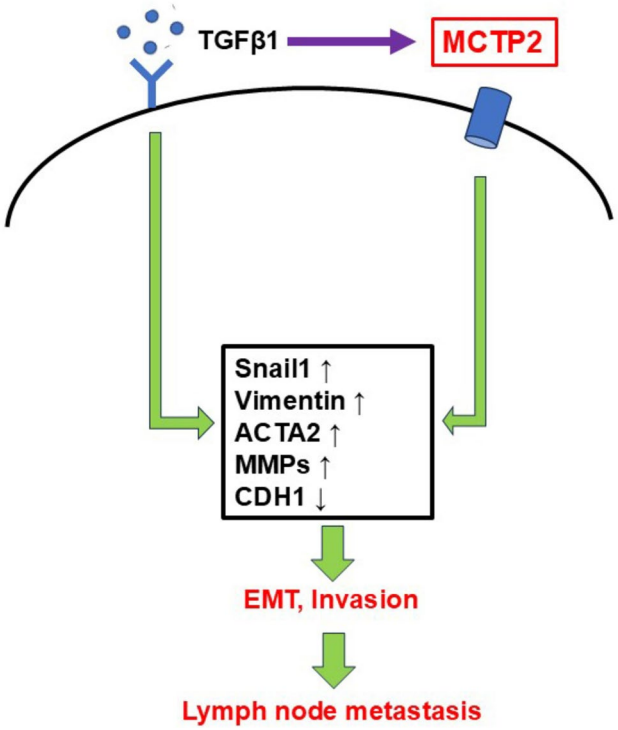

Fig. 7. Schematic representation of the MCTP2 function in OSCC.

Data availability

All data and materials are available from the corresponding author.

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Author contributions

Y.S., A.T., K.S., H.S., and T.S. contributed to conception and designed this study. Y.S., T.A., Y.K., Y.O., K.K., T.T., and K.S. performed experiments, analysis, and statistics. T.I. contributed to advice on experimental methods and statistics. H.S. and T.S. administrated this project. Y.S., A.T., and T.S. wrote the manuscript. All authors have reviewed and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

The present study was in accordance with the Ethics Committee on Epidemiological Studies at Kagoshima University [Approval No. 213223-1].

Competing interests
The authors declare no competing interests.

Informed consent
Informed consent was obtained from all individual participants included in the study.

Additional information
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