

Transcutaneous carbon dioxide suppresses epithelial-mesenchymal transition in oral squamous cell carcinoma

EIJI IWATA¹, TAKUMI HASEGAWA¹, DAISUKE TAKEDA¹, TAKESHI UEHA^{2,3}, TERUYA KAWAMOTO⁴, TOSHIHIRO AKISUE⁵, YOSHITADA SAKAI² and TAKAHIDE KOMORI¹

¹Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine;

²Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine;

³NeoChemir Inc.; ⁴Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine;

⁵Division of Rehabilitation Medicine, Kobe University Graduate School of Health Sciences, Kobe, Japan

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Abstract. Oral squamous cell carcinoma (OSCC) is the most common form of oral cancers. Recent studies have shown that the malignant transformation of various carcinomas, including OSCC, is associated with epithelial-mesenchymal transition (EMT), and that expression of the EMT factors are significantly associated with tumor invasion, tumor metastasis, and survival rates in OSCC patients. Hence, there is a possibility that EMT suppression may improve the prognosis of OSCC patients. Hypoxia inducible factor-1 α (HIF-1 α) is a crucial microenvironmental factor in tumor progression, which induces the expression of EMT factors. We previously reported that transcutaneous CO₂ suppresses both human OSCC tumor growth and metastasis to the regional lymph nodes by improving hypoxia in treated tissue. According to this background, we hypothesized that increased EMT with HIF-1 α expression may increase the progression and the metastatic potential of OSCC, and that decreased hypoxia by transcutaneous CO₂ could suppress EMT. In the present study, *in vitro* studies showed that hypoxic conditions increased the expression of HIF-1 α and EMT factors in OSCC cells. In addition, *in vivo* studies revealed that transcutaneous CO₂ increased E-cadherin expression with the decreased expression of HIF-1 α , Snail, Slug, N-cadherin, and Vimentin in tumor treatment. These results suggest that transcutaneous CO₂ could suppress EMT by improving hypoxia, resulting in the reduction of metastatic potential of OSCC. The findings indicate that transcutaneous CO₂ may be able to improve the prognosis of OSCC patients through the suppression of EMT.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common form of oral cancers (1). Despite advances in the diagnosis and the treatment of OSCC, the prognosis remains poor, and metastasis to the cervical lymph nodes and the remote organs is an important issue in determining the prognosis of OSCC patients (2,3). Recent studies have shown that epithelial-mesenchymal transition (EMT) plays an important role in tumor invasion and metastasis (4-8). In addition, many studies have shown that expression of the EMT factors are significantly associated with tumor invasion, tumor metastasis, and survival in patients with SCC, including patients with OSCC (9-19). These findings strongly indicate that EMT suppression may improve the prognosis of patients with OSCC.

Hypoxic microenvironment is a characteristic feature of solid tumors (20). Hypoxia inducible factor-1 α (HIF-1 α), an oxygen-dependent α subunit of HIF which activates the transcription of metastatic genes, is strongly induced under hypoxic conditions (21). Increased expression of HIF-1 α induces tumor invasion and metastasis, and is associated with the activation of EMT factors (22-26). We previously demonstrated that transcutaneous CO₂ suppresses the growth of primary human OSCC and metastasis to the regional lymph nodes by both improving hypoxia and increasing mitochondrial apoptosis in treated tissue (27).

According to the above background, we hypothesized that improving hypoxia by transcutaneous CO₂ could suppress EMT. In the present study, we investigated whether oxygen conditions affect the expression levels of HIF-1 α and EMT factors in OSCC *in vitro* and whether transcutaneous CO₂ affects these factors in OSCC *in vivo*.

Materials and methods

Cell culture. An oral cancer cell line, HSC-3 was used in this study (Health Science Research Resources Bank, Osaka, Japan). HSC-3 cells were established from a metastatic deposit of poorly differentiated OSCC of the tongue in a mid-internal

Correspondence to: Dr Takumi Hasegawa, Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
E-mail: hasetaku@med.kobe-u.ac.jp

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jugular lymph node from a 64-year-old man (28). HSC-3 cells in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1000 U/ml penicillin/streptomycin solution (Sigma-Aldrich) were routinely cultured in an incubator in 5% CO₂ at 37°C. Trypsin (0.25%) and ethylenediaminetetraacetic acid (0.02%) (Sigma-Aldrich) solution were used to isolate cells for subculture, as previously described (27,29).

Cell experiments. To investigate the effect of changing oxygen conditions on OSCC cells *in vitro*, a total of 1.5×10^5 HSC-3 cells per well was seeded into a 6-well plate and cultured for 24 h. Then, HSC-3 cells were incubated for 96 h in two different conditions: normoxic group (20% O₂, 5% CO₂, 75% N₂) or hypoxic group (1% O₂, 5% CO₂, 94% N₂). After the incubation, the mRNA expression of Snail, Slug, E-cadherin, N-cadherin and Vimentin in the cells were evaluated using quantitative real-time PCR, and the expression of HIF-1 α in the cells was evaluated using immunoblot analysis.

Animal models. Male athymic BALB/c nude mice, aged 7-weeks, were obtained from CLEA Japan (Tokyo, Japan). The animals were maintained under pathogen-free conditions, in accordance with institutional guidelines. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation at Kobe University Animal Experimentation Regulations (permission number: P120602) and were approved by the Institutional Animal Care and Use Committee.

HSC-3 cells were implanted into the back of 24 mice at doses of 2.0×10^6 cells in 500 μ l phosphate buffered saline (PBS), as previously described (27,29). Mice were randomly divided into two treatment groups: a CO₂-treated group (n=12) and a control group (n=12).

Transcutaneous CO₂ treatment. Transcutaneous CO₂ treatment was performed as previously described (27,29). Briefly, the area of skin around the implanted tumor was covered with CO₂ hydrogel. This area was then sealed with a polyethylene bag, and 100% CO₂ gas was administered into the bag (Fig. 1). Each treatment was performed for 20 min. Control animals were treated similarly, replacing the CO₂ with room air.

Treatment commenced one week after HSC-3 cell implantation and was performed twice a week for three weeks. After 24 h of final treatment, treated tumors were removed, and total RNA and cell lysate were extracted from half of the tumor, immediately. The other half of the tumor was formalin-fixed and paraffin-embedded for staining. Serial 10- μ m thick transverse sections were prepared from each block.

Quantitative real-time PCR. The mRNA expression levels of *β -actin*, *Snail*, *Slug*, *E-cadherin*, *N-cadherin* and *Vimentin* were analyzed using quantitative real-time PCR. Total RNA was extracted from the cells and treated tumors by selective binding to a silica-gel-based membrane using an RNeasy Mini Kit, following the manufacturer's instructions (Qiagen, Valencia, CA, USA). The cDNA was synthesized (300 ng total RNA) using the High Capacity cDNA Transcription kit (Applied Biosystems, Foster City, CA, USA).

Quantification of mRNA transcription was performed using a StepOne Real-Time PCR System (Applied Biosystems).

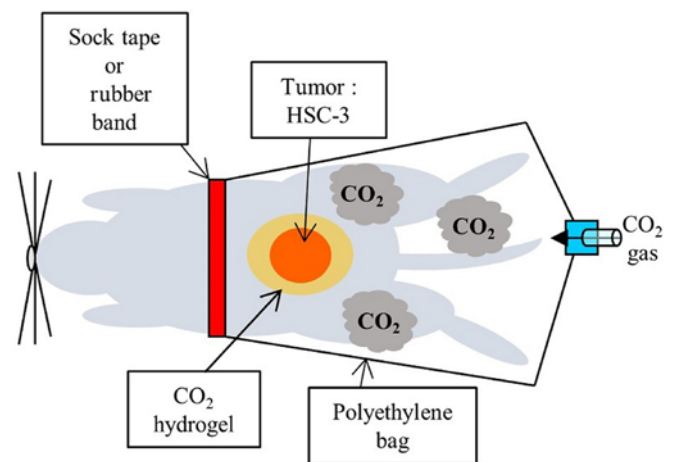


Figure 1. Transcutaneous CO₂ application in an animal model of human OSCC. The area of skin around the implanted tumor was covered with CO₂ hydrogel and sealed with a polyethylene bag through which 100% CO₂ gas was administered.

Real-Time PCR was performed in a 20 μ l reaction mixture using SYBR Green Master Mix reagent (Applied Biosystems). PCR conditions were as follows: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The expression of each target gene was normalized to β -actin and relative expression to the control group was calculated (the $\Delta\Delta$ CT method; Applied Biosystems). All primers were obtained from Invitrogen (Carlsbad, CA, USA). Primer sequences were as follows: *β -actin*, forward (5'-GAT GAG ATT GGC ATG GCT TT-3') and reverse (5'-CAC CTT CAC CGT TCC AGGT TT-3'); *Snail*, forward (5'-TGC AGG ACT CTA ATC CAA AGT TTA CC-3') and reverse (5'-GAG GGA TGG CTG CCA GC-3'); *Slug*, forward (5'-GTG TGG ACT ACC GCT GC-3') and reverse (5'-TCC GGA AAG AGG AGA GAG G-3'); *E-cadherin*, forward (5'-ACA GCA CGT ACA CAG ACCC TA-3') and reverse (5'-GCA GAA GTG TCC CTG TCC CAG-3'); *N-cadherin*, forward (5'-TTG GAT CAA TGT CAT AAT CAA GTG CTG TA -3') and reverse (5'-CTC CTA TGA GTG GAA CAG GAA CG -3'); *Vimentin*, forward (5'-AGC CGA AAA CAC CCT GCA AT-3') and reverse (5'-CGT TCA AGG GTC AAG ACG TGC-3').

Immunoblot analysis. Cell lysates were prepared from the cells and treated tumors using a whole cell lysis buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Mammalian Protein Extraction Reagent; Thermo Scientific, Rockford, IL, USA). Protein samples were processed using standard western immunoblot procedures. Membranes were incubated overnight at 4°C with primary antibodies in Can Get Signal[®] Immunoreaction Enhancer Solution 1 (Toyobo, Osaka, Japan): anti-human-HIF-1 α antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA), anti-human Snail antibody (1:1000) (Cell Signaling Technology), anti-human Slug antibody (1:1000) (Cell Signaling Technology), anti-human E-cadherin antibody (1:1000) (Cell Signaling Technology), anti-human N-cadherin antibody (1:1000) (Cell Signaling Technology), anti-human Vimentin antibody (1:1000) (Cell Signaling Technology) and anti-human α -tubulin antibody (1:2000) (Sigma-Aldrich). After washing,

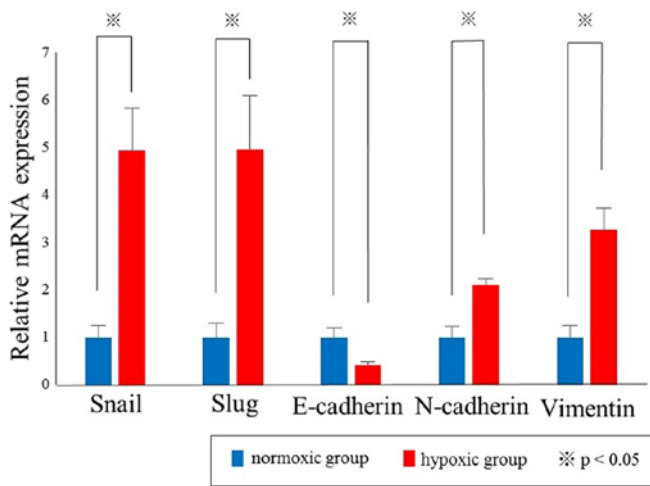


Figure 2. The mRNA expression of *Snail*, *Slug*, *E-cadherin*, *N-cadherin* and *Vimentin* were evaluated using quantitative real-time PCR ($p < 0.05$) *in vitro*.

the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo), and exposed using ECL Prime Plus Western Blotting Detection System Reagent (GE Healthcare Bio-Sciences). The signals were detected using a Chemilumino analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

Immunohistochemical analysis. The formalin-fixed and paraffin-embedded tumor sections were pretreated with citrate buffer for 40 min at 95°C, quenched with 0.05% H₂O₂, and incubated overnight at 4°C with the following primary antibodies in Can Get Signal Immunostain Solution A (Toyobo): rabbit anti-human Snail antibody (1:1000) (Cell Signaling Technology), anti-human Slug antibody (1:1000) (Cell Signaling Technology), anti-human E-cadherin antibody (1:1000) (Cell Signaling Technology), anti-human N-cadherin antibody (1:1000) (Cell Signaling Technology), and anti-human Vimentin antibody (1:1000) (Cell Signaling Technology). Following the treatment, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. Signals were developed as a brown reaction product using peroxidase substrate 3,3'-diaminobenzidine (Nichirei Bioscience). The sections were counterstained with hematoxylin and examined with a BZ-8000 confocal microscope (Keyence, Osaka, Japan).

Statistical analysis. Data are presented as the mean value \pm standard error. The results of the two groups were analyzed using the Mann-Whitney U test. The level of statistical significance was set at $p < 0.05$.

Results

Improving hypoxic conditions decreases the expression levels of HIF-1 α and EMT factors in HSC-3 cells *in vitro*. We first examined the *in vitro* effects of changing oxygen levels on HSC-3 cells by culturing the cells in two different oxygen

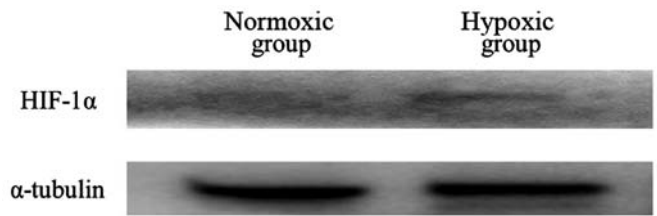


Figure 3. Immunoblot analysis of the expression of HIF-1 α in HSC-3 cells cultured in two different oxygen conditions *in vitro*.

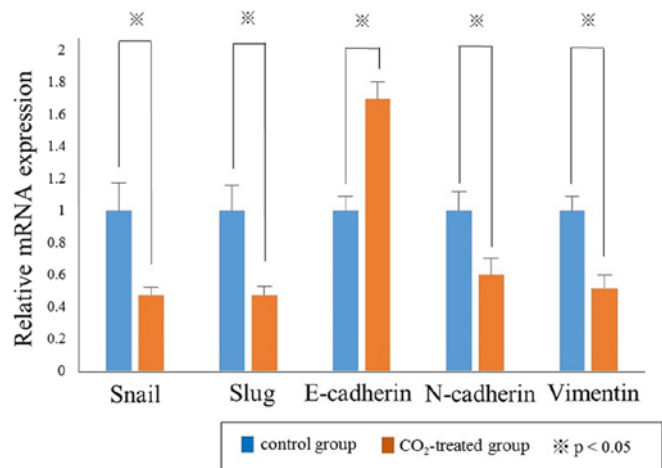


Figure 4. The mRNA expression of *Snail*, *Slug*, *E-cadherin*, *N-cadherin* and *Vimentin* were evaluated using quantitative real-time PCR ($p < 0.05$) *in vivo*.

conditions. Quantitative real-time PCR analyses revealed that the relative mRNA expression levels of *Snail*, *Slug*, *N-cadherin* and *Vimentin* were significantly increased in hypoxic group compared with those in normoxic group, whereas the expression of *E-cadherin* was significantly decreased in hypoxic group compared with normoxic group ($p < 0.05$, Fig. 2). Immunoblot analysis showed that increased protein expression of HIF-1 α was observed in hypoxic group, but not in normoxic group (Fig. 3).

Transcutaneous CO₂ suppresses the expression levels of HIF-1 α and EMT factors in HSC-3 cells *in vivo*. We examined the *in vivo* effects of transcutaneous CO₂ on the expression levels of HIF-1 α and EMT factors in HSC-3 cells. Quantitative real-time PCR analyses revealed that the relative mRNA expression levels of *Snail*, *Slug*, *N-cadherin* and *Vimentin* were significantly decreased in the CO₂-treated group compared with the control group, in contrast the expression of *E-cadherin* was significantly increased in the CO₂-treated group compared with the control group ($p < 0.05$, Fig. 4). Consistent with the results from quantitative real-time PCR, immunohistochemical analysis revealed that decreased expression levels in Snail, Slug, N-cadherin and Vimentin expression with an increased E-cadherin expression was observed in the CO₂-treated tumors compared with the control group (Fig. 5). In addition, a western blot analysis showed that increased protein expression of HIF-1 α , Snail, Slug, N-cadherin and Vimentin were observed in the control group, but not in the CO₂-treated group. In contrast,



Figure 5. Immunohistochemical staining for EMT factors, Snail, Slug, E-cadherin, N-cadherin and Vimentin in implanted tumors after treatment *in vivo*.

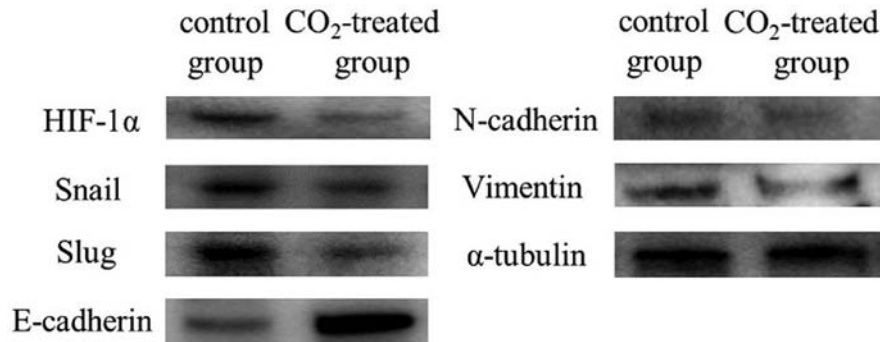


Figure 6. Immunoblot analysis of the expression levels of HIF-1 α , Snail, Slug, E-cadherin, N-cadherin and Vimentin in implanted tumors from both treatment groups *in vivo*.

increased protein expression of E-cadherin was observed in the CO₂-treated group (Fig. 6).

Discussion

OSCC is the most common malignancy in the oral cavity, and is associated with more non-desirable outcomes than any other oral disease because of the highly invasive nature of the tumors and of the cervical lymph node metastasis (30).

Recent studies have demonstrated that the malignant transformation of various carcinomas, including OSCC, is associated with the loss of epithelial differentiation and the gain of the mesenchymal phenotype, a process known as EMT (31,32).

EMT is a physiological phenomenon that involves a loss of polarity and cell-cell adhesion, and that epithelial cells gain characteristics of the mesenchymal cells, which include enhanced motility and matrix resolution (32). EMT itself is an essential cellular function which is involved in tissue construction during normal developmental stages (33,34). Generally, cells undergoing EMT show decreased expression of epithelial markers such as E-cadherin, and increased expression of mesenchymal biomarkers, such as Snail, Slug, N-cadherin and Vimentin (35-38). E-cadherin is one of the major adhesion molecules of epithelial cells. The decreased expression of E-cadherin is an indication of invasion and metastasis, because it liberates carcinoma cells from the primary site and enhances invasion and metastasis (39-41). Thus during the EMT process, E-cadherin is the most important factor, and its expression is a symbol of the occur-

rence of EMT. Snail is a zinc finger protein and the first member of a superfamily identified in *Drosophila* (42-44). Snail 2, known as Slug, has been shown to function in a manner similar to that of Snail; however, Slug displays a distinct tissue distribution (45,46). Both Snail and Slug are considered to be the major transcription factors involved in the modulation of EMT in various types of cancer. They modulate EMT by repressing E-cadherin transcription via binding to the E-cadherin promoter (37). N-cadherin is one of the mesenchymal cadherins, and N-cadherin upregulation has been observed in many types of poorly differentiated tumors (47). In EMT process, 'cadherin switching', the loss of E-cadherin expression and the gain of N-cadherin expression, occurs, upon Snail/Slug expression. Vimentin, which is also promoted by Snail and Slug, is a major protein constituent of the intermediate filaments in normal and neoplastic mesenchymal cells (48). Through the degeneration and production of these factors, the conditions required to generate EMT are established.

Many studies have shown the relationship between the expression levels of EMT factors and the survival rates of patients with SCC (including patients with OSCC) (9-19). The decreased expression of E-cadherin was found to be significantly associated with the survival rates of patients with various cancers, including OSCC (9,10), ovarian cancer (11), gastric cancer (12), prostate cancer (13), colon cancer (14), and lung cancer (15). The expression levels of other important EMT factors, Snail (16), Slug (17), N-cadherin (18), and Vimentin (19), are also closely correlated with the prognosis of patients with OSCC. Moreover, the expression levels of

these EMT factors are said to be closely associated with tumor differentiation, tumor stage, lymph node metastasis and recurrence in patients with OSCC (9,10,16-19,49-51). Hence, there is a possibility that EMT suppression may improve the prognosis of patients with OSCC.

Hypoxia, which is a common condition in solid tumors, can increase the invasiveness and the metastatic ability of tumor cells (52). Under hypoxic conditions, HIF-1 α is activated in tumor cells; the activation promotes tumor growth, whereas the loss of HIF-1 α activity markedly decreases tumor growth, angiogenesis, and cellular energy metabolism (53). Recent studies have shown that HIF-1 α is a crucial microenvironmental factor that induces the expression levels of EMT factors such as Snail and Vimentin (22-26).

Based on these facts, we hypothesized that oxygen conditions may affect the expression levels of HIF-1 α and EMT factors in OSCC. We first examined the *in vitro* effects of oxygen levels on HSC-3 cells by culturing the cells in two different oxygen conditions. We revealed that HIF-1 α expression was increased in HSC-3 cells under hypoxic conditions, and in addition, the increased expression of mesenchymal biomarkers with the decreased expression of epithelial markers was observed in hypoxic HSC-3 cells. Consistent with this, Zhang *et al* have reported that HIF-1 α expression was increased under hypoxic conditions in comparison to the expression under normoxic conditions in OSCC *in vitro* (54). These results indicate that hypoxic condition could affect the expression of EMT factors in OSCC cells *in vitro*.

We previously reported that the transcutaneous CO₂ system improves hypoxia in treated tissue, potentially resulting in an 'artificial Bohr effect' (55). Moreover, we have demonstrated that transcutaneous CO₂ suppresses human OSCC growth and metastasis to the regional lymph nodes by both improving hypoxia in treated tissue and increasing mitochondrial apoptosis (27). However, the molecular mechanism of decreased metastatic potential by transcutaneous CO₂ has not been addressed.

Based on previous studies, we hypothesized that transcutaneous CO₂ could decrease the metastatic potential to lymph nodes by EMT suppression via improving hypoxia in treated tissue. We examined the *in vivo* effects of transcutaneous CO₂ on the expression levels of HIF-1 α and EMT factors using human OSCC xenograft models. Our results showed that transcutaneous CO₂ increased the expression levels of epithelial markers with decreased expression levels of HIF-1 α and mesenchymal biomarkers, resulting in EMT suppression.

In the present study, we found that hypoxic conditions increased the expression levels of HIF-1 α and EMT factors in OSCC *in vitro*, and that oxygenation using transcutaneous CO₂ suppressed EMT by improving hypoxia in OSCC *in vivo*. These results suggest that transcutaneous CO₂ could suppress EMT by improving hypoxia and that it may reduce the metastatic potential of OSCC. Our present findings strongly indicate that transcutaneous CO₂ may be able to improve the prognosis of patients with OSCC through the suppression of EMT. In conclusion, this is the first report to show that transcutaneous CO₂ suppresses the expression levels of EMT factors in human OSCC. Our findings indicate that transcutaneous CO₂ suppresses EMT by improving hypoxia and that it could reduce the metastatic potential of OSCC.

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