Comparison of the expression of TGF-β1, E-cadherin, N-cadherin, TP53, RB1CC1 and HIF-1α in oral squamous cell carcinoma and lymph node metastases of humans and mice

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Abstract. The aim of the present study was to prove that a mouse model closely simulates human oral cancer progression by comparing the expression levels of transforming growth factor (TGF)-β1, E-cadherin, N-cadherin, tumor protein (TP)53, RB1 inducible coiled-coil (RB1CC)1 and hypoxia inducible factor (HIF)-1α at different stages of oral squamous cell carcinoma (OSCC) in humans and mice. The expression levels of TGF-β1, E-cadherin, N-cadherin, TP53, RB1CC1, and HIF-1α were detected by immunohistochemical staining in normal oral mucosa, oral mucosa dysplasia, OSCC primary tumor and carcinoma tissues from lymph node metastases. Tissue samples were obtained from human specimens and the Balb/c mouse model of lymphatic metastases oral carcinoma, induced by 4-nitroquinoline-1-oxide in drinking water. The results indicated no significant differences in the expression levels of TGF-β1, E-cadherin, N-cadherin, TP53, RB1CC1 and HIF-1α between humans and mice, at any stage of OSCC examined (P>0.05). The expression of TGF-β1, N-cadherin, TP53 and RB1CC1 increased in different stages of OSCC in both humans and mice. The expression of E-cadherin decreased from normal oral mucosa to OSCC, and increased in lymph node metastases in both human and mouse samples. The expression of HIF-1α increased from normal oral mucosa to OSCC, and decreased in lymph node metastases in both human and mouse samples. Additionally, the expression of p53 was positively correlated with that of RB1CC1 in human and mouse samples (r=0.971, P=0.029; r=0.97, P=0.03). Overall, the similar expression of multiple molecules in both human and mouse carcinoma prove that the mouse model of lymphatic metastases from oral carcinoma established in the present study may closely mimic human oral cancer.

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Introduction

The vast majority of oral cancers are squamous cell carcinomas and include cancers in the tongue, cheeks, mouth floor, lips and gingiva. This form of cancer usually originates from normal oral mucosa, which progresses to dysplasia, then to squamous cell carcinoma, and ultimately to metastatic carcinoma (1). Invasion and metastasis occur early in oral cancer; therefore, regional lymph node metastasis is an important factor in the high mortality of oral cancer. Many aspects of the mechanism of lymphatic metastasis from oral cancer are still unknown. Because of ethical reasons, it is very difficult to obtain continuous carcinoma specimens of oral cancer from dysplasia to metastasis from the same patients. Therefore, it is necessary to establish an animal model in which the development and the pathology of oral cancer echo those of the corresponding human cancers, especially in relation to the origin of lymphatic metastases.

Many studies indicate that the administration of 4-nitroquinoline-1-oxide (4NQO) to mice or rats effectively induces oral cancer that resembles oral tumor growth in humans (2-4). However, regional lymph node metastasis from oral cancer has barely been examined in the 4NQO model. In a previous study, we have described the development of a lymph node metastases from oral carcinoma mouse model through the long-term administration of high-dose 4NQO and a prolonged observation period (5). However, the molecular events that, in this model, occur at the different stages of oral tumor carcinogenesis have not yet been investigated. Furthermore, the biological and molecular similarities of oral cancer lymph

node metastasis between this animal model and humans have not been examined. Thus, in the present study, we try to expolre this point by comparing the expressions of TGF (TGF)-β1, E-cadherin, N-cadherin, TP53, RB1CC1 and HIF-1α in different stages of oral squamous cell carcinoma (OSCC) in human and mouse.

The epithelial-mesenchymal transition (EMT) plays an important role in the dissemination and metastasis of oral cancer: It is characterized by the induction of a variety of cytokines and chemokines, which destroy normal cell adhesion, lead to the loss of cell-cell interaction, contribute to the recombination of cytoskeleton, cause cell invasion and eventually result in the dissemination and metastasis of oral cancer cells (6).

TGF-β1 plays a pivotal role in the activation of EMT. TGF-β1 is a cytokine with multifunctional biological activity, which can induce EMT by a variety of signal transduction mechanisms (7). In addition, TGF-β1 is involved in cell proliferation, differentiation, apoptosis, and plays an important role in immune regulation. The change of the expression and distribution of TGF-β1 in oral pre-cancerous lesions increase the risk of cancer (8).

E-cadherin and N-cadherin are important members of the family of cadherins, which are Ca^{2+}-dependent cell adhesive glycoproteins. E-cadherin is expressed in epithelial tissues, while N-cadherin is expressed in neural tissues (9). During tumor invasion and metastasis, the replacement of E-cadherin with N-cadherin results in the loss of polarity and adhesion in epithelial cells; consequently, the epithelial cells acquire the characteristics of mesenchymal cells, and gain the ability to invade and metastasize. This change is an important process in EMT; therefore, the cadherin switch is considered a critical mechanism in tumor progression and metastasis (10).

The occurrence of many tumors is related to the activation of oncogenes, the inactivation of cancer suppressor genes, or a combination of both. *TP53* is a cancer suppressor gene and is considered the defender of the genome. The inactivation of TP53 (also called p53) causes the loss of p53 tumor suppressor activity, promoting the malignant transformation of the cells. TP53 mutation is related to the pathologic grade, clinical stage and lymph node metastasis of human oral squamous cell carcinoma (OSCC). Changes in TP53 in cancer tissue are an independent factor for poor prognosis in OSCC (11,12).

RB1-Inducible Coiled-coil 1 (RB1CC1) plays a fundamental role in autophagosome formation (13). Studies had shown that autophagy can improve the adaptability of tumor cells: Through degradation of their components, normal cells can provide energy for the tumor cells (14). The evolutionary conserved protein encoded by *RB1CC1* can interact with p53; together, RB1CC1 and p53 regulate multiple signaling pathways in the cells (13), thus controlling the cell cycle and inhibiting cell proliferation (15). A study has pointed that RB1CC1 is associated with early breast carcinogenesis (16).

Hypoxia is an important initiating factor in cancer. In the initial stage of cancer, local hypoxia activates hypoxia-inducible factor-1α (HIF-1α), which upregulates the expression of the downstream vascular endothelial growth factor (VEGF). VEGF induces the formation of blood and lymphatic vessels, and promotes the rapid proliferation of cancer cells (17-19).

By detecting and comparing the expression of the above described molecular biomarkers in normal oral mucosa, dysplasia, OSCC and lymph node metastases samples from patients and mice, the objective of this research had further demonstrated that the mouse model we built closely mimics the human oral carcinogenesis and lymphatic metastases, and to preliminarily explore the function of these biomarkers in the development of oral cancer.

Materials and methods

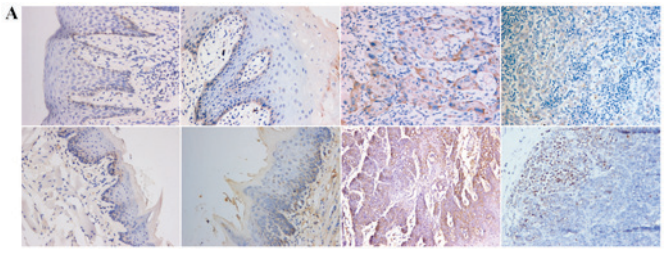
Patients. A total of 72 human samples (12 normal, 24 dysplastic, 24 OSCC and 12 lymph node-metastatic carcinoma) were obtained from the patients of the stomatological hospital affiliated to the Guangxi Medical University, from January 2012 to June 2016. The patients aged between 27 and 78 years and were an even mix of men and women. None of them received chemotherapy or radiation therapy before sample collection. The normal tissues were obtained from the gingiva, the dysplasia and OSCC tissues from the tongue and the lymph node metastasis carcinoma tissues from the neck lymph nodes of the patients. Informed consent was obtained from all the participants. The Human Ethics Committee of Guangxi Medical University, China, approved this study.

Animals. Mouse samples (9 normal, 20 dysplastic, 20 OSCC and 9 lymph node-metastatic carcinoma) were obtained from the Balb/c mouse model lymphatic metastases from oral carcinoma, induced by 4-NQO. The normal, dysplasia and OSCC tissues were obtained from the tongue and the lymph node metastasis carcinoma tissues were obtained from the submandibular lymph nodes of the mice. The Animal Ethics Committee of Guangxi Medical University, China, approved this study.

Samples. Specimens were fixed in 10% formalin, embedded in paraffin, and sectioned. Each specimen was stained with hematoxylin-eosin (HE) or immunohistochemical staining.

Immunohistochemistry assay (IHC). The antibodies used in the present study were as follows: Mouse monoclonal antibodies for E-cadherin and p53 (ZSGB-Bio, China), rabbit polyclonal antibodies for TGF-β1 and HIF-1α (Boster Biological Technology, China), mouse monoclonal antibody for N-cadherin (Santa Cruz, USA), and rabbit polyclonal antibodies for RB1CC1 (Proteintech Group, China). All antibodies used in the present study were suitable for the detection of proteins in both humans and mouse.

Immunohistochemistry was performed on paraffin sections. Deparaffinized sections were pretreated with 0.4% pepsin for 60 min at 37˚C. Endogenous peroxidase activity was quenched by treatment with 0.2% H_2O_2 for 3 h. The sections were then incubated with the specific antibodies overnight at 4˚C . In addition, sections incubated with 0.01 mol/l phosphate buffer saline (PBS) and tongue cancer tissues incubated with the chosen antibodies were used as negative and positive controls, respectively. The immunostaining was visualized with an SP kit (ZSGB-Bio, China) using a diaminobenzidine-peroxidase substrate. The sections were counterstained with Mayer's hematoxylin and examined using the image analyzer of a light microscope (Leica Leitz DMRB/E, Leica Microsystems, Wetzlar, Germany).



B

Group	No.	Expression of TGF-β1 0	1	2	3	Positive expression rate of TGF-β1 in humans
Normal	12	5	3	2	2	33.3% (4/12)
Dysplasia	24	7	6	6	5	45.8% (11/24)
OSCC	24	2	4	10	8	75.0% (18/24)
Metastasis	12	1	1	5	5	83.3% (10/12)

C

Group	No.	Expression of TGF-β1 0	1	2	3	Positive expression rate of TGF-β1 in mice
Normal	9	4	3	1	1	22.2% (2/9)
Dysplasia	20	5	6	6	3	45.0% (9/20)
OSCC	20	2	2	8	8	80.0% (16/20)
Metastasis	9	0	1	4	4	88.9% (8/9)

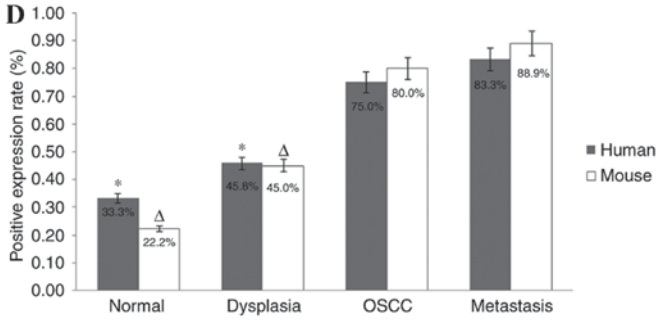
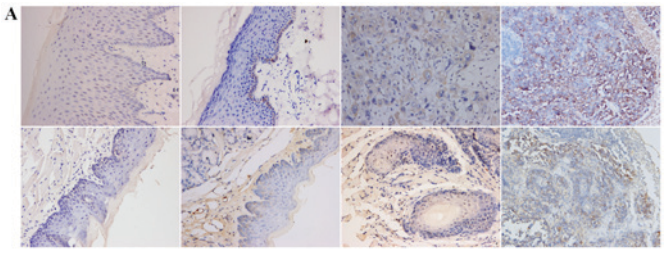


Figure 1. Expression of transforming growth factor (TGF)-β1 at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of TGF-β1 (scoring 2/3) in normal and dysplastic oral mucosa, OSCC and submandibular lymph node in lymphatic nodes metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of TGF-β1 at the different cancer stages. (D) Positive expression rate of TGF-β1 at different cancer stages in human and mouse samples. *Compared with OSCC or metastatic stages in human samples, P<0.05; Δcompared with OSCC or metastatic stages in mouse samples, P<0.05.



B

Group	No.	Expression of N-cadherin 0	1	2	3	Positive expression rate of N-cadherin in humans
Normal	12	6	4	1	1	16.7% (2/12)
Dysplasia	24	7	6	7	4	45.8% (11/24)
OSCC	24	2	3	11	8	79.2% (19/24)
Metastasis	12	1	1	5	5	83.3% (10/12)

C

Group	No.	Expression of N-cadherin 0	1	2	3	Positive expression rate of N-cadherin in mice
Normal	9	5	3	1	0	11.1% (1/9)
Dysplasia	20	6	5	5	4	45.0% (9/20)
OSCC	20	1	2	9	8	85.0% (17/20)
Metastasis	9	0	1	4	4	88.9% (8/9)

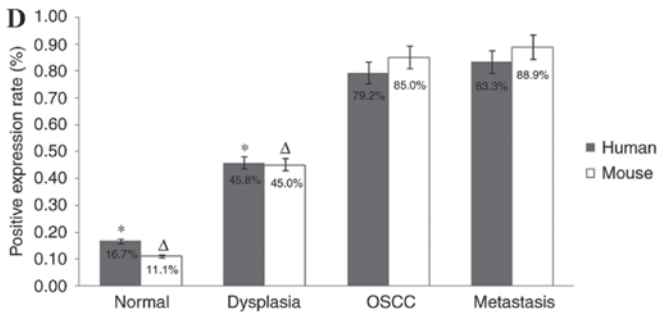


Figure 2. Expression of N-cadherin at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of N-cadherin (scoring 2/3) in oral mucosa in normal oral mucosa, dysplasia, OSCC and submandibular lymph node in lymphatic nodes metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of N-cadherin at the different cancer stages. (D) Positive expression rate of N-cadherin at different cancer stages in human and mouse samples. *Compared with OSCC or metastatic stages in human samples, P<0.05; Δcompared with OSCC or metastatic stages in mouse samples, P<0.05.

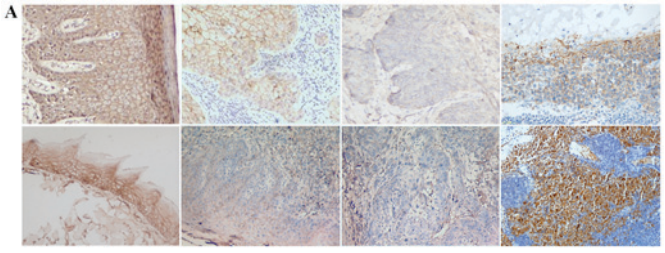
Evaluation of the IHC results. TGF-β1, N-cadherin, TP53, RB1CC1, HIF-1a are expressed in the cytoplasm and E-cadherin is expressed in cytomembrane. IHC staining of these six proteins in the cells was scored subjectively under a light microscope and the percentage of stained tumor cells was expressed according to a previous study (20), with little modification as follows: 0-10% of cells stained, score 0; 11-25% of cells stained, score 1; 26-50% of cells stained, score 2; 51-100% of cells stained, score 3. Cells scoring 0/1 were considered to be negative, and those scoring 2/3 were considered to be positive.

Statistical analysis. The statistical analysis of the biological markers was performed using the Chi-square test. The correlation analysis was performed using the Spearman rank correlation test. The results were considered statistically significant if P<0.05.

Results

Positive expression of E-cadherin was mainly observed in the cytomembrane, in both human and mouse samples (Fig. 1A). Positive expression of TGF-β1, N-cadherin, p53, RB1CC1 and HIF-1α was mainly detected in the nucleus and the cytoplasm of cells, in both human and mouse samples (Figs. 2-6A).

Moreover, the positive rates of the above mentioned molecular biomarkers were analyzed and the results indicated



B

Group	No.	Expression of E-cadherin 0	1	2	3	Positive expression rate of E-cadherin in humans
Normal	12	0	0	6	6	100.0% (12/12)
Dysplasia	24	2	2	9	11	83.3% (20/24)
OSCC	24	13	11	0	0	0.0% (0/24)
Metastasis	12	1	1	5	5	83.3% (10/12)

C

Group	No.	Expression of E-cadherin 0	1	2	3	Positive expression rate of E-cadherin in mice
Normal	9	0	0	4	5	100.0% (9/9)
Dysplasia	20	2	2	7	9	80.0% (16/20)
OSCC	20	10	8	1	1	10.0% (2/20)
Metastasis	9	0	0	5	4	100.0% (9/9)

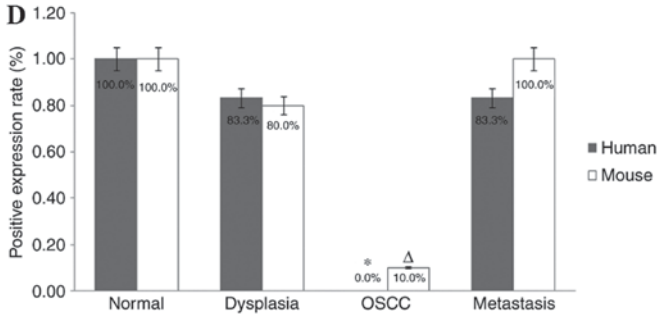
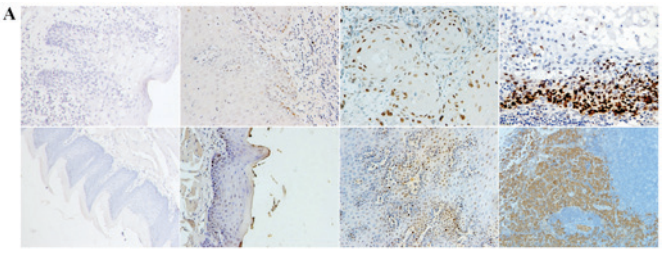


Figure 3. Expression of E-cadherin at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of E-cadherin (scoring 2/3) in normal and dysplastic oral mucosa, OSCC and submandibular lymph node in lymphatic nodes metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of E-cadherin at the different cancer stages. (D) Positive expression rate of E-cadherin at different cancer stages in human and mouse samples. *Compared with normal, dysplasia, or metastatic stages in human samples, P<0.05; Δcompared with normal, dysplasia, or metastatic stages in mouse samples, P<0.05.



B

Group	No.	Expression of TP53 0	1	2	3	Positive expression rate of TP53 in humans
Normal	12	7	5	0	0	0.0% (0/12)
Dysplasia	24	10	6	4	4	33.3% (8/24)
OSCC	24	4	3	8	9	70.8% (17/24)
Metastatic	12	0	1	4	7	91.7% (11/12)

C

Group	No.	Expression of TP53 0	1	2	3	Positive expression rate of TP53 in mice
Normal	9	5	4	0	0	0.0% (0/9)
Dysplasia	20	8	6	3	3	30.0% (6/20)
OSCC	20	3	3	7	7	70.0% (14/20)
Metastatic	9	0	1	4	4	88.9% (8/9)

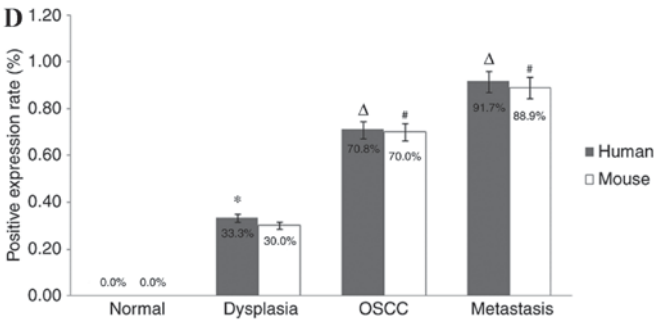


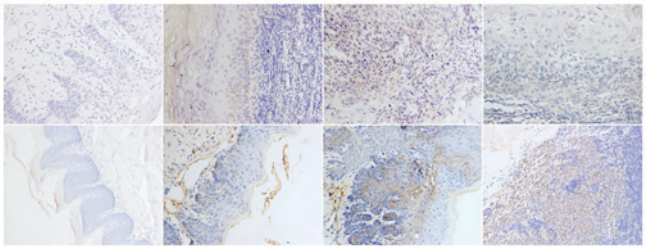
Figure 4. Expression of tumor protein (TP)53 at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of TP53 (scoring 2/3) in normal and dysplastic oral mucosa, OSCC and submandibular lymph node in lymphatic nodes metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of TP53 at the different cancer stages. (D) Positive expression rate of TP53 at different cancer stages in human and mouse samples. *Compared with normal stage in human samples, P<0.05; Δcompared with normal or dysplasia stages in human samples, P<0.05; #compared with normal or dysplasia stages in mouse samples, P<0.05.

that there were no obvious differences between human and mouse samples (P>0.05) (Figs. 1-6B-D). Figs. 1D and 2D point to a significant increase in the expression of TGF-β1 and N-cadherin in both human and mouse samples in the progression from normal mucosa to lymph node metastasis. Fig. 3D shows that the expression of E-cadherin decreases in the progression from normal mucosa to OSCC, but increases almost to its original amount in both human and mouse lymph node metastases. Figs. 4D and 5D indicates a significant increase in the expression of TP53 and RB1CC1 in both human and mouse samples in the progression from normal mucosa to lymph node metastasis. Additionally, the expression of p53 positively correlates with that of RB1CC1 in both human and mouse samples (r=0.971, P=0.029; r=0.97, P=0.03), correlation analysis by Spearman rank correation test. Fig. 6D shows that the expression of HIF-1α increases from normal mucosa to OSCC, but decreases in both human and mouse lymph node metastases.

Discussion

Our study found that samples from the mouse model of lymphatic metastases from oral cancer we built and human oral cancer shared the similar expression in a wide variety of biological molecular markers. Firstly, the expression of TGF-β1 at different stages of oral cancer was similar in samples from

A



B

Group	No.	Expression of RB1CC1 0	1	2	3	Positive expression rate of RB1CC1 in humans
Normal	12	7	5	0	0	0.0% (0/12)
Dysplasia	24	11	9	2	2	16.7% (4/24)
OSCC	24	7	5	6	6	50.0% (12/24)
Metastasis	12	1	1	6	4	83.3% (10/12)

C

Group	No.	Expression of RB1CC1 0	1	2	3	Positive expression rate of RB1CC1 in mice
Normal	9	5	4	0	0	0.0% (0/9)
Dysplasia	20	10	7	2	1	15.0% (3/20)
OSCC	20	6	4	6	4	50.0% (10/20)
Metastasis	9	0	1	3	5	88.9% (8/9)

D

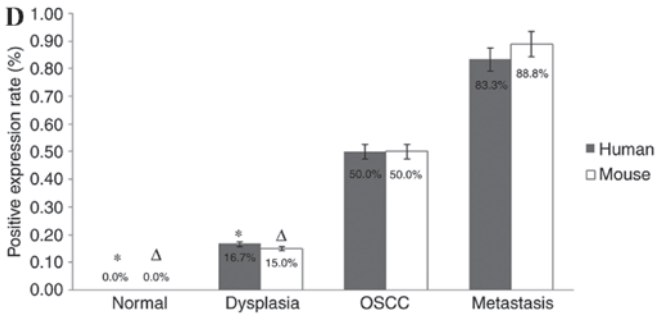
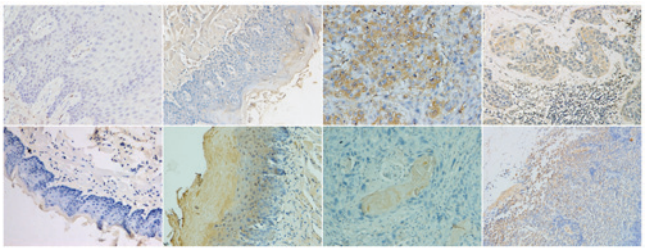


Figure 5. Expression of RB1 inducible coiled-coil (RB1CC)1 at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of RB1CC1 (scoring 2/3) in normal and dysplastic oral mucosa, d, OSCC and submandibular lymph nodes in lymphatic node metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of RB1CC1 at the different cancer stages. (D) Positive expression rate of RB1CC1 at different cancer stages in human and mouse samples. *Compared with OSCC or metastatic stages in human samples, P<0.05; Δcompared with OSCC or metastatic stages in mouse samples, P<0.05.

A



B

Group	No.	Expression of HIF-1α 0	1	2	3	Positive expression rate of HIF-1α in humans
Normal	12	8	4	0	0	0.0% (0/12)
Dysplasia	24	10	9	3	2	20.8% (5/24)
OSCC	24	0	0	8	16	100.0% (24/24)
Metastasis	12	7	4	1	0	8.3% (1/12)

C

Group	No.	Expression of HIF-1α 0	1	2	3	Positive expression rate of HIF-1α in mice
Normal	9	6	3	0	0	0.0% (0/9)
Dysplasia	20	10	6	3	1	20.0% (4/20)
OSCC	20	0	1	7	12	95.0% (19/20)
Metastasis	9	5	3	0	1	11.1% (1/9)

D

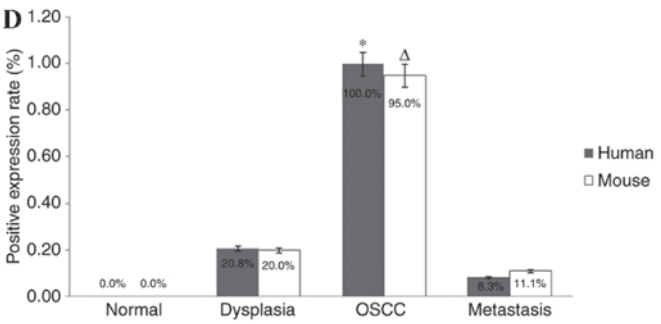


Figure 6. Expression of hypoxia inducible factor (HIF)-1α at different stages in the progression of oral carcinoma in human and mouse samples. (A) Photomicrographs of immunohistochemical positive expression of HIF-1α (scoring 2/3) in normal and dysplastic oral mucosa, OSCC and submandibular lymph nodes in lymphatic node metastatic carcinoma (magnification, x200). (B and C) Data relative to the expression of HIF-1α at the different cancer stages. (D) Positive expression rate of HIF-1α at different cancer stages in human and mouse samples. *Compared with normal, dysplasia, or metastatic stages in human samples, P<0.05; Δcompared with normal, dysplasia, or metastatic stages in mouse samples, P<0.05.

patients and from the mouse models, and it increased with the progression of the cancer both cases. This finding corroborates a study from Lu *et al* on head and neck squamous cell carcinoma (21) and agrees with the notion that TGF-β1 may promote EMT and accelerate tumor invasion and metastasis. Secondly, with the progression of cancer, the expression of E-cadherin decreased in mouse and human samples, meanwhile, the expression of N-cadherin increased. Hazan *et al* also reported the significant lack of E-cadherin in the most terminal breast cancers and the increased expression of N-cadherin (22). Studies on head and neck cancer, colorectal cancer, pancreatic cancer and esophageal cancer showed similar results (23-25). It is known that the switch from E-cadherin to N-cadherin may cause the loss of adhesion between cells, thus allowing tumor cells to leave the primary tumor and migrate, and eventually induce metastases. Our study also found that E-cadherin was re-expressed in both human and mouse lymph node metastases, similarly to what has been observed in ovarian cancer lymphatic metastases (26). Therefore, we speculate that when the cancer cells reach the targeted metastatic tissue, the re-expression of E-cadherin might promote cancer cell colonization in the invaded tissue: Cancer cells can, in such way, proliferate and invade to ultimately form metastases. There might be a dynamic adjustment of protein expression along with the change of microenvironment. In other words, the expression of E-cadherin is time-dependent

and space-dependent. However, how this process starts and is fine-tuned needs further exploration.

Thirdly, the expression of p53 and RB1CC1 in oral mucosa dysplasia, OSCC and metastatic tissue from human and mouse samples were higher than in oral normal tissue, and the expression of p53 and RB1CC1 increased with progression of the cancer. Nishioka *et al* found that, in human oral squamous cell carcinoma, the expression of p53 increased with the progression of OSCC (27). Suraneni *et al* found that RB1CC1 was over-expressed in prostate cancer cells, and increased with the malignant degree of the cancer (28). Wei and Guan found that RB1CC1 might play a promoting role in early cancer in the MMTV-Pymt mouse model of human breast cancer (29). The studies above are consistent with our results. The possible explanation is that p53 and RB1CC1 play a promoting role in the procession of OSCC.

Our study also found that the expression of p53 correlates with that of RB1CC1. Morselli *et al* (30) found that p53 had a double effect on autophagy. The fraction of p53 located in the nucleus stimulates autophagy-inducing genes, while the fraction of p53 located in the cytoplasm had the opposite effect. Wei *et al* found that RB1CC1 regulated cell growth, division, proliferation, autophagy, and apoptosis through the nuclear cytoplasmic shuttle mechanism and multiple signaling pathways (31). The mutual regulation between p53 and RB1CC1 may be the necessary step to begin autophagy. When the tumor lacks nutrition, during its rapid growth phase, autophagy might allow the tumor to grow more quickly. Probably, the synergistic effect of p53 and RB1CC1 leads to the development of OSCC.

Finally, we found that the expression of HIF-1α at different stages of the oral cancer was similar in human and mouse samples. The positive expression rate of HIF-1α raised from normal to dysplastic oral mucosa, till reaching 100% in OSCC. Many studies suggest that HIF-1α is over-expressed in a variety of malignant human tumors (32). Bos *et al* found that the increased expression of HIF-1α is an independent factor predicting shorter prognosis in breast cancer (33). Aebersold *et al* also found that 94% of nasopharyngeal squamous carcinoma tissues had high expression of HIF-1α (34). However, the positive expression rate of HIF-1α in lymph node metastases decreased in both human and mouse samples. The possible explanation is that even though the primary cancer was anoxic, causing the increased expression of HIF-1α, the metastases of the lymph nodes might have had a good blood supply, therefore justifying the lower expression of HIF-1α.

The results of our comparative study show that the examined molecular markers in our mouse model of lymphatic metastases from oral cancer and in samples from patients had similar expression, and are probably the factors leading to the activation of tumor, blood vessel formation, autophagy, EMT, invasion and metastasis. In conclusion, the mouse model we built may mimic human oral cancer and may be valuable to study the mechanism of lymphatic metastases from oral carcinoma.

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