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Cytoplasmic c-ros oncogene 1 receptor tyrosine kinase expression may be associated with the development of human oral squamous cell carcinoma

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Abstract. The c-ros oncogene 1 receptor tyrosine kinase (*ROS1*) gene encodes a proto-oncogenic protein that has been demonstrated to be involved in the pathogenesis of several types of cancer. The present study aimed to analyze the expression of ROS1 in human oral squamous cell carcinomas (OSCCs), and investigate the association between its expression and the clinicopathological parameters of patients with OSCC. Paraffin-embedded OSCC tissues from 31 patients were obtained and the expression of ROS1 was analyzed by immunohistochemistry. The cellular location of ROS1 was determined by immunofluorescence in human oral cancer CAL-27 cells. The association of clinicopathological characteristics and survival rates with ROS1 expression were assessed. The results revealed that ROS1 was exclusively localized in the cytoplasm of the OSCC tissues (24/30, 80.0%), and in the cytoplasm of adjacent dysplastic epithelial tissues (2/15, 13.3%) (P<0.001). The moderate to strong expression of ROS1 in the cytoplasm was higher in OSCC tissues than in the normal epithelial tissues adjacent to the tumor (67.7 vs. 0%, P=0.001). The results of the Kaplan-Meier analysis and multivariate Cox regression analysis indicated that there was no association between the 5-year survival rate of patients and the cytoplasmic (P=0.28 and P=0.60, respectively) or nuclear expression (P=0.90 and P=0.31, respectively) of ROS1. These results suggest that the cytoplasmic expression level of ROS1 may be associated with the development of OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most frequently diagnosed type of cancer worldwide, and accounts for 90% of all oral cancers (1,2). Environmental factors including smoking, regular alcohol consumption, a diet low in fruit and vegetables, and papillomavirus infection are associated with the incidence of OSCC (2,3). A number of genetic syndromes are also associated with OSCC, including Li-Fraumeni syndrome, Fanconi anemia and lupus erythematosus (4). In addition, certain oncogenes have been identified to be activated in OSCC, such as c-Met, c-SRC and Ras (5,6).

Receptor tyrosine kinases have crucial roles in signal transduction during normal and malignant development, and are involved in cellular proliferation, differentiation, migration and apoptosis (7). The c-ros oncogene 1 (ROS1) is an orphan receptor tyrosine kinase proto-oncogene that plays an important role in certain tumor types (7). The mechanisms underlying wild-type ROS1 protein expression and regulation in normal human tissues are yet to be elucidated. In a previous study, ROS1 was undetectable in normal heart, lung, ovary, pancreas, and testis tissues, was expressed at low levels in parathyroid glands, eye, larynx, adrenal gland and skeletal muscle tissues, and was strongly expressed in the cerebellum, peripheric nerves, stomach, small intestine, colon and kidney (8).

A number of point mutations and fusion events have led to high ROS1 expression in a variety of tumor cell lines, including non-small cell lung cancer, gastric carcinoma and glioblastoma (7-11). In preclinical models, ROS1 fusions have been demonstrated to correlate with sensitivity to tyrosine kinase inhibitors, such as crizotinib (12-14).

As a number of receptor tyrosine kinases, including c-MET, vascular endothelial growth factor receptor (VEGFR) and Akt (15-18), are involved in OSCC, it can be hypothesized that ROS1 may also be involved in the development of OSCC. However, the presence of ROS1 in normal oral epithelium tissues remains unknown.

The present study aimed to investigate the expression of ROS1 in OSCC and in adjacent oral epithelium tissue by immunohistochemistry (IHC). The associations between

ROS1 expression and the pathological and clinical characteristics of patients were also examined. Results of the present study may provide new insight into the treatment of OSCC.

Materials and methods

Patients and samples. Archived formalin-fixed, paraffin-embedded surgical tissue specimens from 31 OSCC patients were obtained from the Department of Pathology of the Zhongshan Hospital, Fudan University (Shanghai, China). The study population included 22 males and 9 females, with a mean age of 61.8 years (range, 47-88 years), who had undergone surgery between January 2000 and December 2007. The patients had not received chemotherapy, radiation therapy or any other cancer therapy prior to surgery. Written informed consent was obtained from each patient or the patient's family.

The sections were stained with hematoxylin and eosin (H and E). The diagnoses and histological grading were established according to the Broder's classification system (19,20). The patients presented with well- (n=6), moderately- (n=20) and poorly- (n=5) differentiated carcinomas. Details regarding lymph node metastases, according to the tumor-node-metastasis (TNM)/Union for International Cancer Control (UICC) criteria, were obtained from the patients' medical records (21). Follow-up assessment was based upon medical records and interviews with patients/ patients' families. The median follow-up period was 7.6 years, with all patients having at least a 5-year follow-up. The study was approved by the ethics committee of the Zhongshan Hospital, Fudan University (Shanghai, China).

Immunohistochemical analysis. IHC was performed using the EnVision™ peroxidase/3, 3'-diaminobenzidine (DAB) rabbit/mouse detection systems (Dako, Glostrup, Denmark; catalog no. k4065). Initially, 4-μm sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and then treated with 3% hydrogen peroxide for 20 min at room temperature in order to block endogenous peroxidase activity. Following this, the sections were subjected to antigen retrieval by heating in a microwave in citrate buffer (pH 6) for 20 min. The slides were then treated with 2% normal goat serum (Abcam, Cambridge, MA, USA) and incubated overnight with an anti-ROS1 antibody (Abcam; catalog no. ab5512; dilution, 1:80) at 4˚C. Subsequent to washing in phosphate-buffered saline (PBS), the sections were incubated with biotinylated secondary antibodies for 1 h, and staining was performed using ABC reagents and DAB (provided with the EnVision™ kit). The slides were counterstained with H and E for 5 min, and then dehydrated and mounted. Negative controls, which were established by replacing the primary antibody with PBS, showed no immunoreactivity.

Each case was evaluated independently by two experienced pathologists who were blinded to the clinical data. ROS1 immunoreactivity was analyzed by measuring the intensity of staining and the percentage of positivity area. The staining intensity was determined in a semi-quantitative manner as follows: i) 0, negative; ii) 1, weak; iii) 2, moderate; and iv) 3, strong. The area of positivity was calculated as a percentage of the total tumor area as <10%, 10-89% or ≥90%. These two variables were used to establish a final score as follows: i) score 0, negative or intensity 1 staining with a <10% area; ii) score 1, intensity 1 with a 10-100% area or intensity 2 or 3 with a <10% area; iii) score 2, intensity 2 with a >10% area or intensity 3 <90% area; or iv) score 3, intensity 3 with a >90% area (22). The cytoplasmic ROS1 score was then divided into two groups, negative/low: 0-1 and moderate/strong: 2-3, and the nuclear ROS1 expression was divided into two groups, negative or low.

Cell culture and immunofluorescence. The human oral cancer CAL-27 cells were obtained from the Laboratory of Oral Oncology, The Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA), and then cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

The CAL-27 cells growing on coverslips were fixed with 4% (v/v) paraformaldehyde in PBS for 10 min, washed, and then incubated for 1 h at room temperature with the primary anti-ROS1 antibody (dilution, 1:80; Abcam), which was diluted in a staining solution of 0.5% (w/v) bovine serum albumin and 0.2% (w/v) saponin in PBS. Subsequent to washing with PBS, the cells were incubated for 1 h at room temperature with Alexa Fluor 555 goat anti-rabbit immunoglobulin G (dilution, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA; catalog no. 4413), and then stained with 100 ng/ml of 4',6-diamidino-2-phenylindole for 10 min (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were then washed and mounted onto slides with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). All images were captured using a LSM 5 Pascal confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with appropriate filters.

Statistical analysis. Statistical analyses were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). The normally-distributed data were compared using Student's *t*-test, and are presented as the mean ± standard deviation. The categorical variables are expressed as proportions, and were compared using Fisher's exact test or Kruskal-Wallis test, as appropriate. The Kaplan-Meier and log-rank test were used for the survival analysis. The Cox regression analysis was used for the multivariate survival analysis. A value of $P<0.05$ was used to indicate a statistically significant difference.

Results

Expression patterns of ROS1 in OSCC and adjacent epithelium. In total, 67.7% of the OSCC samples were positive for cytoplasmic ROS1 expression (moderately or strongly; Fig. 1C). By contrast, none of the adjacent normal epithelial samples (Fig. 1A) were positive for cytoplasmic ROS1 ($P=0.001$; Table I). A certain amount of staining (2/16, 12.5%) was observed in the adjacent dysplastic epithelia (Fig. 1B and Table I). In total, 80.6% of the OSCC samples, 75% of the adjacent dysplastic epithelial tissues, and 18.8% of the adjacent normal epithelial samples were negative for nuclear ROS1 expression (Table I).

Table I. Immunohistochemical evaluation of ROS1 in OSCC, adjacent dysplastic epithelial tissues and adjacent normal epithelial tissues.

		Cytoplasmic ROS1, n (%)			Nuclear ROS1, n (%)		
Tissue	n	Negative/low	Moderate/strong	P-value	Negative	Low	P-value
OSCC	31	10 (32.3)	21 (67.7)	<0.001	25 (80.6)	6 (19.4)	<0.001
DE	16	14 (87.5)	2 (12.5)		3 (18.8)	13 (81.3)	
NE	8	8 (100.0)	0 (0.0)		6 (75.0)	2 (25.0)	

Kruskal-Wallis test. ROS1, c-ros oncogene 1 receptor tyrosine kinase; OSCC, oral squamous cell carcinoma; DE, dysplastic epithelial tissues; NE, normal epithelial tissues.

Table II. ROS1 localization in OSCC and adjacent dysplastic epithelial tissues.

		ROS1, n (%)			
Tissue	n	Nucleus	Cytoplasm	Nucleus and cytoplasm	P-value
OSCC	30[a]	1 (3.3)	24 (80.0)	5 (16.7)	<0.001
DE	15[a]	1 (6.7)	2 (13.3)	12 (80.0)	

[a]One case, negative for ROS1 expression in the cytoplasm and nucleus, was excluded. Kruskal-Wallis test. OSCC, oral squamous cell carcinoma; ROS1, c-ros oncogene 1 receptor tyrosine kinase; DE, dysplastic epithelial tissues.

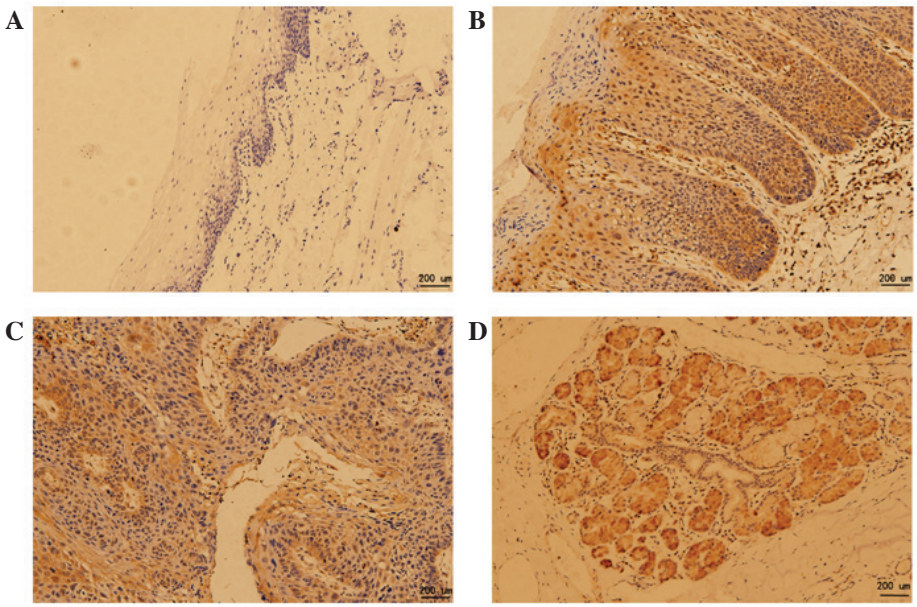

Figure 1. Expression of ROS1 in oral epithelial tissues adjacent to tumor and OSCC tissues. (A) ROS1 was not expressed in normal oral epithelial tissues. However, ROS1 was expressed in adjacent (B) dysplastic epithelial tissues, (C) OSCC tissues and (D) salivary gland tissues. Scale bar, 200 μm. ROS1, c-ros oncogene 1 receptor tyrosine kinase; OSCC, oral squamous cell carcinoma.

Overall, only one of the OSCC samples exhibited no ROS1 IHC staining.

Table II shows that ROS1 was exclusively localized in the cytoplasm in 80.0% of the OSCC samples, in the nucleus in 3.3% of the samples (one case), and in the cytoplasm and nucleus in 16.7% of the samples. This gave a total cytoplasmic localization of 96.7%. In the adjacent dysplastic epithelial tissues, ROS1 was localized in the cytoplasm and nucleus in 80.0% of the samples, in the cytoplasm alone in 13.3% of the samples, and exclusively localized in the nucleus in 6.7% of the samples (one case).

Positive ROS1 staining was observed in the normal salivary gland cells (Fig. 1D). Confocal laser-scanning microscopy revealed nuclear and cytoplasmic ROS1 staining in CAL-27 cells (Fig. 2).

Table III. Correlation between cytoplasmic ROS1 expression and clinicopathological characteristics.

Characteristic	n	Cytoplasmic ROS1, n (%)		P-value
		Negative/low	Moderate/strong	
OSCC, n	31	10 (32.3)	21 (67.7)	
Age, mean ± SD, years		62.70±10.20	61.38±12.70	0.776[a]
Gender, n (%)				
Male	22	8 (36.4)	14 (63.6)	
Female	9	2 (22.2)	7 (77.8)	0.677[b]
Differentiation, n (%)				
Well	6	0 (0.0)	6 (100.0)	
Moderate	20	9 (45.0)	11 (55.0)	
Poor	5	1 (20.0)	4 (80.0)	0.109[b]
Pathological N stage, n (%)				
pN0	23	8 (34.8)	15 (65.2)	
pN1+pN2+pN3	8	2 (25.0)	6 (75.0)	1.000[b]
Clinical stage classification, n (%)				
I+II	23	7 (30.4)	16 (69.6)	
III+IV	8	3 (37.5)	5 (62.5)	1.000[b]

[a]*t*-test; [b]Fisher's exact test. OSCC, oral squamous cell carcinoma; ROS1, c-ros oncogene 1 receptor tyrosine kinase; N, nodal; pN, pathological nodal; SD, standard deviation.

Table IV. Correlations between nuclear ROS1 expression and clinicopathological characteristics.

Characteristic	n	Nuclear ROS1, n (%)		P-value
		Negative	Low	
OSCC, n (%)	31	25 (80.6)	6 (19.4)	
Age, mean ± SD, years		59.24±10.69	72.50±10.77	0.011[a]
Gender, n (%)				
Male	22	17 (77.3)	5 (22.7)	
Female	9	8 (88.9)	1 (11.1)	0.642[b]
Differentiation, n (%)				
Well	6	4 (66.7)	2 (33.3)	
Moderate	20	18 (90.0)	2 (10.0)	
Poor	5	3 (60.0)	2 (40.0)	0.145[b]
Pathological N stage, n (%)				
pN0	23	20 (87.0)	3 (13.0)	
pN1+pN2+pN3	8	5 (62.5)	3 (37.5)	0.161[b]
Clinical stage classification, n (%)				
I+II	23	20 (87.0)	3 (13.0)	
III+IV	8	5 (62.5)	3 (37.5)	0.161[b]

[a]*t*-test; [b], Fisher's exact test. OSCC, oral squamous cell carcinoma; ROS1, c-ros oncogene 1 receptor tyrosine kinase; N, nodal; pN, pathological nodal; SD, standard deviation.

ROS1 expression and its association with the clinical and pathological characteristics of OSCC patients. Table III shows the clinicopathological characteristics of the patients according to cytoplasmic ROS1 expression (negative/low vs. moderate/strong). No significant associations were identified between cytoplasmic ROS1 expression

Table V. Multivariate Cox regression analysis of the clinicopathological variables and 5-year survival of patients with OSCC.

Covariate	β coefficient	Hazard ratio	95% confidence interval	P-value
Age	0.065	1.068	0.949-1.201	0.276
Gender	-0.674	0.510	0.032-8.006	0.631
Clinical stage classification	16.153	$1.035x10^{7}$	$0.000-3.34x10^{150}$	0.924
Pathological N stage	13.750	936653.769	$0.000-2.96x10^{149}$	0.935
Differentiation	-0.592	0.553	0.079-3.854	0.550
Nuclear ROS1	-2.379	0.093	0.001-9.363	0.312
Cytoplasmic ROS1	-0.540	0.583	0.077-4.436	0.602

OSCC, oral squamous cell carcinoma; ROS1, c-ros oncogene 1 receptor tyrosine kinase; N, nodal.

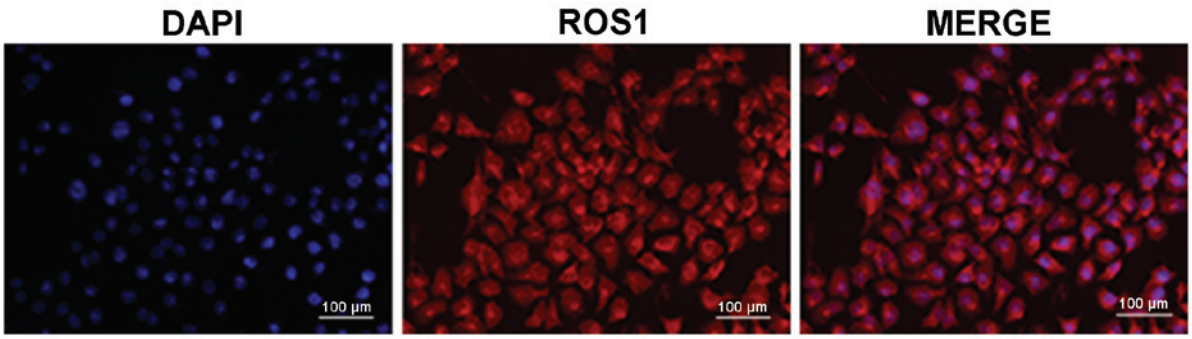

Figure 2. Cytoplasmic and nuclear ROS1 expression detected in the oral squamous cell carcinoma CAL-27 cells by immunofluorescence. DAPI, 4',6-diamidino-2-phenylindole; ROS1, c-ros oncogene 1 receptor tyrosine kinase.

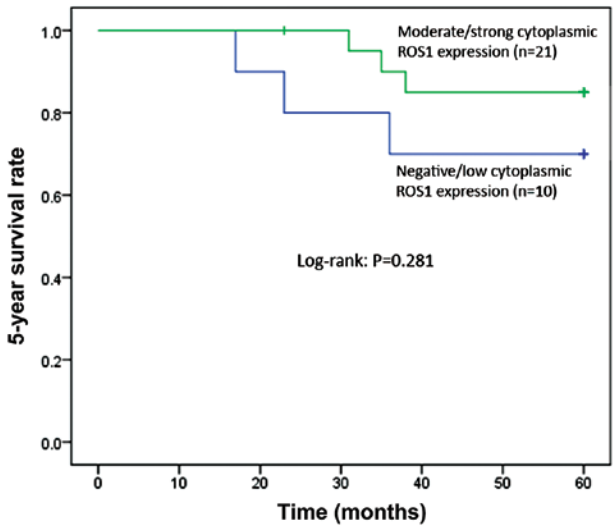

Figure 3. Results of the Kaplan-Meier analysis revealing that the 5-year survival rate of oral squamous cell carcinoma patients with moderate/strong cytoplasmic ROS1 expression was higher than those of patients with negative/low cytoplasmic ROS1 expression. However, this difference was not statistically significant (log-rank test; P=0.281). ROS1, c-ros oncogene 1 receptor tyrosine kinase.

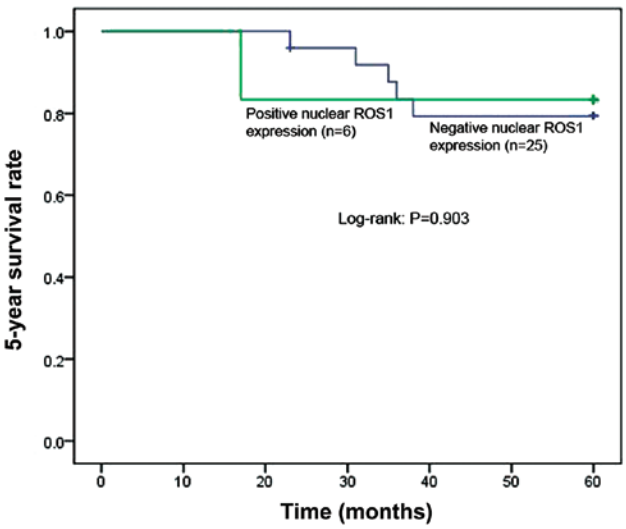

Figure 4. Results of the Kaplan-Meier analysis revealing the association between the 5-year survival rate of oral squamous cell carcinoma patients and nuclear ROS1 expression (log-rank test; P=0.903). ROS1, c-ros oncogene 1 receptor tyrosine kinase.

and the clinical and pathological characteristics of age, gender, differentiation, pathological nodal (N) stage and clinical stage classification. Table IV shows the clinicopathological characteristics of the patients according to the nuclear ROS1 expression (negative vs. low). The mean age was higher in patients with low nuclear ROS1 expression (72.5±10.8 vs. 59.2±10.7 years; P=0.01). No significant associations were identified between nuclear ROS1 expression and the clinicopathological characteristics of gender, differentiation, pathological N stage and clinical stage classification (Table IV).

Survival analysis. No associations were identified between cytoplasmic (P=0.28; Fig. 3) or nuclear ROS1 expression and the 5-year survival rates (P=0.90; Fig. 4). Furthermore, the multivariate Cox analysis indicated that no factor was associated with 5-year survival rates of the patients (Table V).

Discussion

The present study aimed to analyze the expression of ROS1 in OSCC samples, and investigate the association between its expression and the clinicopathological parameters of OSCC patients. ROS1 was predominantly localized in the cytoplasm of the OSCC tissues. The expression of ROS1 was higher in the OSCC tissues than in normal epithelial tissues adjacent to the tumor. No association was identified between the 5-year survival rates and the cytoplasmic or nuclear expression of ROS1.

A number of previous studies have investigated ROS1 expression in different tumor types (7-11). *ROS1* gene upregulation and/or mutation were primarily detected in brain and lung cancers, but also in breast fibroadenomas, liver, colon and kidney cancers, and in chemically-induced stomach cancers (13). The aberrant expression, in addition to the various mutant forms of *ROS1*, suggests a role for *ROS1* in tumorigenesis. The present study demonstrated that the ROS1 protein was expressed in the majority of OSCC tissues and in certain adjacent dysplastic epithelial tissues, but not in the adjacent normal epithelial tissues. To the best of our knowledge, the present study is the first to suggest a role for ROS1 in OSCC.

The molecular architecture of the ROS1 protein consists of an extracellular domain, a single transmembrane domain and an intracellular region containing the carboxy-terminal tyrosine kinase domain, which enables ROS1 to transduce signals (23). Previous studies have demonstrated that ROS1 is expressed in a spatial-, temporal- and cell type-specific manner (9). In addition, the staining pattern of ROS1 has been identified to differ in different types of tumors. Cytoplasmic staining patterns were observed in human non-small cell lung cancers (NSCLCs), gastric adenocarcinomas, glioblastomas, HCC78 cells and U118MG cells (11,24); membrane and cytoplasmic patterns were evident in breast carcinomas, and membrane patterns were apparent in hepatocarcinomas (25). The factors responsible for these different localization patterns are yet to be elucidated (24). However, it is conceivable to hypothesize that different subcellular localizations may have different roles during cancer development. The IHC and immunofluorescence results of the present study demonstrated similar diversity in the localization of ROS1. Cytoplasmic staining was present in the majority of the OSCC samples, while nuclear staining was more prominent in the adjacent dysplastic epithelial tissues. By contrast, the adjacent normal epithelial tissues exhibited no cytoplasmic ROS1 expression.

Previous data has established that ROS1 initiates important signaling events during the differentiation of epithelial tissues (9). In addition, ROS1 expression has been shown to be associated with the differentiation of tumors, including gastric adenocarcinomas (11), astrocytomas (26), and invasive breast carcinomas (25). Further studies are required in order to address the function of ROS1 signaling in the context of cell differentiation and transformation.

The present study did not identify any association between the 5-year survival rates of patients and the cytoplasmic or nuclear expression of ROS1. This result is in agreement with the results of a previous study concerning gastric carcinomas (11), but contradict with a study that examined breast carcinomas, which revealed improved survival with increasing ROS1 expression (25), and with a study that investigated cases of NSCLC, which demonstrated poorer survival rates with increasing ROS1 expression (11). Previous studies that analyzed other receptor tyrosine kinases, including c-MET, VEGF and Akt, revealed that these receptors are involved in the development and prognosis of OSCC (15-18). However, further studies are required in order to assess the role of ROS1 expression in the survival of OSCC patients.

The majority of the studies cited in the present study have investigated the rearrangements of *ROS1* genes in human tumors, but not the wild-type *ROS1* gene, or the ROS1 protein. Although several aberrant genomic changes in the *ROS1* oncogene are required in order for it to function as an active oncogene (27), there is an extremely low incidence of *ROS1* gene rearrangement in human tumors. This has been identified to be as low as 1.6% in cases of NSCLC in China (8), and highest in cholangiocarcinomas (8.7%) (10). Further studies are required in order to elucidate the function of *ROS1* without any aberrant genomic change in tumorigenesis.

The present study has certain limitations. Firstly, the sample size was small. A larger number of patients may therefore allow for the identification of further associations between ROS1 expression and clinicopathological factors, in addition to survival and prognosis. Secondly, the samples were obtained from a single population, which could introduce a bias as a result of the genetic background of this population. Finally, due to the retrospective nature of the present study, the data was limited to what was available in the medical charts. Therefore, future studies should include a larger number of patients, and aim to assess a full array of clinicopathological variables.

In conclusion, the present study revealed that there was a high frequency of cytoplasmic ROS1 expression in OSCC samples, whilst no expression in the adjacent normal epithelial tissues. These results suggest that ROS1 is involved in the pathogenesis of OSCC, and may therefore provide a novel therapeutic target and prognostic tool for the treatment of OSCC.

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