

# Do patients with oral squamous cell carcinoma express receptor tyrosine kinase-like orphan receptor 1? Results of an observational study

Swati Nema<sup>1</sup>, Shreenivas Kallianpur<sup>1</sup>, Ashok Kumar<sup>2</sup>, Rajeev Nema<sup>2</sup>, Supriya Vishwakarma<sup>2</sup>, Sandeep Kumar Nema<sup>3</sup>

<sup>1</sup>Department of Oral Pathology and Microbiology, Peoples College of Dental Sciences Peoples University, <sup>2</sup>Department of Biochemistry, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, <sup>3</sup>Associate Professor of Orthopedics Jawaharlal Institute of Postgraduate Medical Education and Research Pondicherry, Pondicherry, India

## Abstract

**Context:** The receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a transmembrane protein of the receptor tyrosine kinase family. The expression of ROR1 has been linked to cancers.

**Aims:** This study aimed to investigate the expression of ROR1 in oral squamous cell carcinoma (OSCC).

**Settings and Design:** This prospective observational study was conducted at a tertiary referral center for treatment of oral carcinoma from November 2013 to December 2016.

**Subjects and Methods:** One-step quantitative reverse transcription-polymerase chain reaction (30 oral cancer tissues and ten normal oral tissue samples) was performed to characterize the expression of the ROR1 gene in oral cancer.

**Statistical Analysis Used:** Analyses of all tumor samples were carried out at least twice, and the mean value was calculated. The differences in ROR1 messenger RNA (mRNA) expression between OSCC tissue and nontumorous gingival tissue was statistically analyzed using Mann–Whitney U-test. The correlations between the clinicopathological parameters and ROR1 mRNA expression were analyzed using Kruskal–Wallis test  $\chi^2$  value.

**Results:** There were 17, 5, 3 and 1 cases of OSCC of buccal mucosa, tongue and lower alveolus lip, respectively. Nearly 88.5% of cases had a history of tobacco consumption. The most common OSCC type was T2N1M0. There was no difference in ROR1 fold change between controls and cases ( $P = 0.06$ ), but there was a trend for downregulation of ROR1 expression from controls to cases. Subgroup analysis revealed the downregulation of ROR1 expression in controls versus Grade II that was significant ( $P = 0.04$ ).

**Conclusions:** There was no change in the expression of ROR1 between cases and controls. A study involving a larger sample size needs to be formulated and conducted for investigating the relation between expression and regulation of ROR1 in OSCC.

**Keywords:** Mouth neoplasms, real-time polymerase chain reaction, receptor tyrosine kinase-like orphan receptors

**Address for correspondence:** Dr. Swati Nema, DII/24, Dhanvantri Nagar, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.

E-mail: drswati.nema@gmail.com

**Received:** 25-Nov-2018, **Revised:** 25-Sep-2020, **Accepted:** 16-Oct-2020, **Published:** 14-May-2021

### Access this article online

#### Quick Response Code:



#### Website:

www.jomfp.in

#### DOI:

10.4103/jomfp.JOMFP\_293\_18

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**For reprints contact:** WKHLRPMedknow\_reprints@wolterskluwer.com

**How to cite this article:** Nema S, Kallianpur S, Kumar A, Nema R, Vishwakarma S, Nema SK. Do patients with oral squamous cell carcinoma express receptor tyrosine kinase-like orphan receptor 1? Results of an observational study. *J Oral Maxillofac Pathol* 2021;25:105-9.

## INTRODUCTION

Oral cavity is one of the ten frequent sites of cancer.<sup>[1]</sup> Receptor tyrosine kinases (RTKs) are a large family of cell surface receptors that participate in crucial cellular processes. These processes include cell proliferation, differentiation, cell–cell interaction, metabolism, signaling, migration and survival.<sup>[2]</sup> Recently, studies have linked one of the RTKs, RTK-like orphan receptor 1 (ROR1), with different cancers. ROR1 has been extensively studied in hematological malignancies.<sup>[3-8]</sup> To the best of our knowledge, the expression of ROR1 in oral carcinoma has not been studied. We analyzed the expression patterns of ROR1 in malignant and nonmalignant oral tissues using real-time quantitative polymerase chain reaction (qPCR).

### Aims and objectives

The objectives of this study were:

1. To assess and compare the quantitative expression of ROR1 in oral squamous cell carcinoma (OSCC) and controls
2. To compare the expression of ROR1 in different histological grades of OSCC.

## SUBJECTS AND METHODS

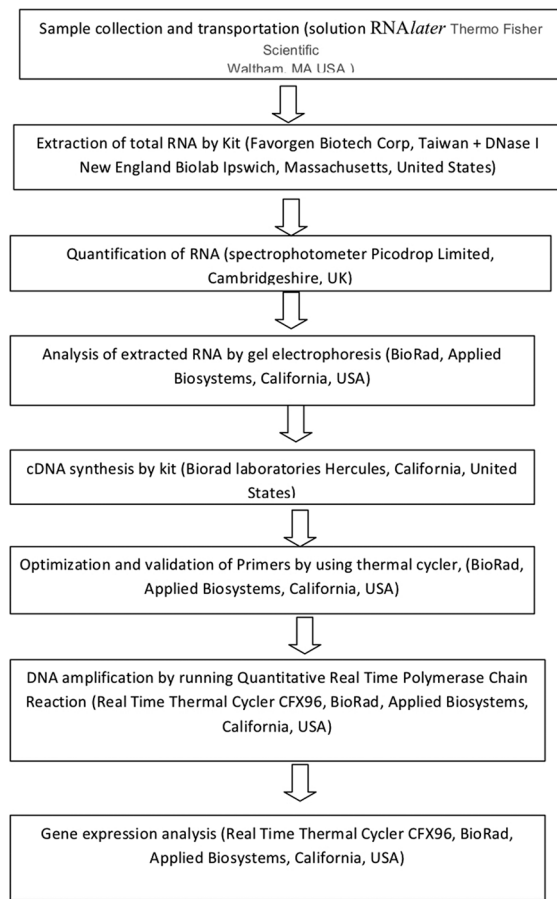
This prospective case–control study was conducted at a tertiary referral center for the treatment of cancers from November 2013 to December 2016. We collected tissue samples from thirty consecutive patients, which were operated for OSCC. Ten samples of normal gingival tissue (controls) were collected from patients presenting to the outpatient department for other reasons. All the samples were collected in RNA later (Thermo Fisher Scientific Waltham, MA USA) from the operating room, transported (at  $-4^{\circ}$  centigrade $^{\circ}$ ) and stored at  $-20^{\circ}$ C. The samples were subjected to qPCR [Figure 1].

- The total RNA was extracted by RNA extraction kit. Quantification of extracted total RNA was done by spectrophotometer and expressed in ng/ $\mu$ l
- Quality and purity of RNA was tested by UV spectrophotometer. The ratio of absorbance at 260 and 280 nm on spectrophotometer was measured, and the ratio above 2 was considered high-quality RNA. The samples were subjected to gel electrophoresis to find out the quality of RNA. The samples not forming any band on gel electrophoresis were excluded from further analysis
- The complimentary DNA (cDNA) synthesis was done from the extracted RNA template using reverse transcriptase. One nonreverse transcriptase was

used with all samples tested to prevent false-positive outcomes

- Before proceeding for qPCR, primers were validated at different annealing temperatures, concentrations and cDNA volume. Annealing temperatures of  $52.1^{\circ}$ C, primer concentration of  $0.5 \mu$ Mol and cDNA volume of  $0.75 \mu$ l were selected for qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a housekeeping gene. The ratio of ROR1 to GAPDH represented the normalized relative levels of ROR1 expression. A nontemplate negative control was also included in each experiment
- The amplification of DNA was done using steps of denaturation, annealing and extension at  $94^{\circ}$ C for 2 min,  $56^{\circ}$ C for 30 s and  $72^{\circ}$ C for 15 s, respectively. Forty-four such cycles were run to get amplified DNA. Figure 2 shows sigmoid-shaped amplification plot of the amplified DNA. Here, cycle threshold ( $C_T$ ) denotes the cycle number at which fluorescence generated by the PCR product (amplified DNA) of a given sample is distinguishable from the background noise. The difference of  $C_T$  of ROR1 and GAPDH of a particular sample of either a case or control was called Delta  $C_T$ , which was given by the formula
  - $\Delta C_T = C_T(\text{ROR1}) - C_T(\text{GAPDH})$
  - An analysis of all samples of cases and controls was carried out at least twice, and the mean value was calculated, which was called average Delta  $C_T$ . Delta  $C_T$  was obtained from Delta  $C_T$  by formula:  $\Delta \Delta C_T = \text{average } \Delta C_T (\text{sample of interest}) - \text{average } \Delta C_T (\text{reference sample})$
  - The reference sample was one, which was having the matched pair of Delta  $C_T$  values in duplicates
  - Relative fold change expression of ROR1 gene was calculated by (Statistical Package for the Social Sciences) SPSS software
  - $\text{Fold gene expression} = 2^{-\Delta \Delta C_T}$

Values were expressed as fold change in expression of ROR1 gene compared to GAPDH. Demographic data, clinicopathologic findings, and results of PCR were recorded. The differences in ROR1 messenger RNA (mRNA) expression between OSCC tissue and nontumorous gingival tissue were statistically analyzed using Mann–Whitney U-test. The correlations between the clinicopathological parameters and ROR1 mRNA expression were analyzed using Kruskal–Wallis Test X2 value. All statistical calculations were carried out using SPSS software (for Windows, Version 20; Chicago Inc., USA). A significant difference was defined at  $P \leq 0.05$ .

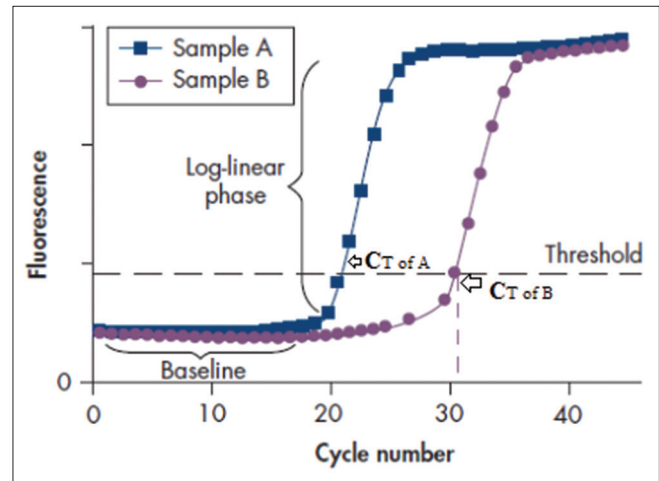


**Figure 1:** Summary of steps in quantitative PCR

## RESULTS

A total of 36 participants were included for analysis through real-time PCR. Four samples from OSCC cases were found inadequate and excluded at the final evaluation. There were 26 cases and ten controls. The mean age of the patients was 47.96 years for cases and 40.0 years for controls. The demographic variables in cases and controls are described in Table 1. The most common site for involvement was buccal mucosa (65.4%). The cases were classified as per TNM classification by imaging, biopsy and Preoperative findings [Table 1]. The most common type was T2N1M0. Around 88.5% of the cases were either tobacco chewers or smokers. Nearly 19.2% of the cases were alcoholics.

The mean weight of the tissue for analysis of qPCR from cases and controls was 30–35 mg. The mean value of total RNA extracted using kit was  $187.89 \pm 30.95$  ng/ $\mu$ l for cases and  $105.09 \pm 64.48$  ng/ $\mu$ l. The quantitative analysis of ROR1 was measured in overall; there was a trend for downregulation in ROR1 expression from controls to cases, which failed to reach statistical significance.



**Figure 2:** Amplification plot of the DNA

However, subgroup analysis showed downregulation in ROR1 expression between Grade II and control which was significant [Table 2]. There was no difference in ROR1 expression between Grade I and III with controls. The analysis of fold change in expression of ROR1 between different grades, tumor size and nodal metastasis was also done, which is given in Table 2. Neither tumor size T1 to T4 ( $P = 0.237$ ) nor nodal metastasis (N0 to N2) (0.32) demonstrated any difference in fold change in ROR1 expression. Comparison of fold change in ROR1 expression among tobacco consumers and nontobacco consumers did not demonstrate any difference between the groups ( $P = 0.42$ ).

## DISCUSSION

ROR1 is an embryonal transmembrane glycoprotein, but studies have demonstrated its presence in some adult tissue such as testis, uterus, lung, bladder, colon, pancreas and adipose tissue.<sup>[3]</sup> Recently, the expression of ROR1 has been linked with solid tumors.<sup>[9-15]</sup>

Our study did not demonstrate any difference in ROR1 fold change between controls and cases, but there was a trend for the downregulation of ROR1 expression from controls to cases. Subgroup analysis revealed the downregulation of ROR1 expression in controls versus Grade II that was significant. Liu *et al.* studied ROR1 expression in squamous cell carcinoma of lung.<sup>[12]</sup> The investigators reported downregulation of ROR1 expression in cases of squamous cell carcinoma of lung. Although the results of our study failed to reach statistical significance, there was a downregulation of ROR1 expression in cases. Studies have demonstrated ROR1 as a prognostic factor for different tumors (breast cancer and colorectal cancer).<sup>[11,15]</sup> ROR1 has been linked with tumor aggression

**Table 1: Demographic data between cases and controls**

Parameter	Cases (%)	Controls (%)	P (<0.05)
n	26	10	
Age (years)			
30-40	9 (34.6)	7 (70.0)	
41-50	6 (23.1)	2 (20.0)	
51-60	6 (23.1)	1 (10.0)	
61-70	5 (19.2)	0 (0.0)	
Gender			
Males	23 (88.5)	8 (80.0)	
Females	3 (11.5)	2 (20.0)	
Site			
Buccal mucosa	17 (65.4)		
Tongue	5 (19.2)		
Lower alveolus	3 (11.5)		
Lip	1 (3.8)		
Tobacco consumption	23 (88.5)		0.00
Smoking	9 (34.6)		0.032
Alcohol consumption	5 (19.2)		0.135
Tumor size, node and metastasis			
T1	1 (3.8)		
T2	13 (50)		
T3	4 (15.4)		
T4	8 (30.8)		
N0	10 (38.5)		
N1	11 (42.3)		
N2	5 (19.2)		
M0	26 (100)		

**Table 2: Table demonstrating median receptor tyrosine kinase-like orphan receptor 1 fold change between cases/controls and histopathological grades for different subgroups**

Outcome	ROR1 fold change median (IQR)	P
Cases/controls	0.19 (0.06-0.80)/0.81 (0.23-1.41)	0.06
Grade I/controls	0.25 (0.04-1.20)/0.81 (0.23-1.41)	0.26
Grade II/controls	0.19 (0.08-0.23)/0.81 (0.23-1.41)	0.04
Grade III/controls	0.24 (0.03-0.24)/0.81 (0.23-1.41)	0.27
Grade I/Grade II	0.25 (0.04-1.20)/0.19 (0.08-0.23)	0.89
Grade II/Grade III	0.19 (0.08-0.23)/0.24 (0.03-0.24)	0.91
Grade I/Grade III	0.25 (0.04-1.20)/0.24 (0.03-0.24)	0.70

ROR1: Receptor tyrosine kinase-like orphan receptor 1, IQR: Interquartile range

across several studies. The linking of ROR1 has been studied for chronic lymphoid leukemia, acute lymphoid leukemia, lung adenocarcinoma, ovarian cancer, melanoma and glioblastoma.<sup>[3,4,6,8-10,12-14]</sup> The results of our study suggest that the lower value of the ROR1 expression is indicative of the poor prognosis of patients with OSCC that can be investigated further by studies with a larger sample size. ROR1 might be acting as a tumor suppressor gene. Although the role of ROR1 in cancers is not well understood, it may be governed by the Wnt5A-dependent inhibition of canonical Wnt signaling downstream of beta-catenin stabilization, thereby inhibiting tumor cell growth, inducing cell cycle arrest and apoptosis. Our study is the first on the estimation of ROR1 expression in OSCC. In our study, real-time PCR was performed with optimized values. qPCR is one of the modern methods for studying

gene expression. We used sequence-specific DNA probes with SYBR GREEN fluorescent dye for the optimization of results. One of the limitations of our study is the small sample size of thirty patients of OSCC. Given the incidence of OSCC worldwide, studies with larger sample numbers are required to analyze the role of ROR1 in OSCC. Other methods for the estimation of gene expression such as immunohistochemistry, DNA microarray and Western blotting can be combined with qPCR for increasing the precision of results. Despite the substantial advances in surgery and chemotherapy for oral cancer over the past few decades, therapeutic failure and disease progression are still frequent. For several other cancers such as breast cancer, ovarian cancer and CLL, ROR1 has been studied extensively and found as a biomarker for targeted therapy. This protein also served as an appropriate candidate for designing a cancer vaccine. Thus, we have been searching a novel and valuable marker that associates with oral cancer and could be used as a potential protein for the treatment of oral cancer.

## CONCLUSION:

There was no change in expression of ROR1 between cases and controls. A study with larger sample size needs to be conducted for investigating the expression of ROR1 in OSCC.

## Acknowledgment

Dr. Sudhir Goyal, Professor and Head Department of Biochemistry, All India Institute of Medical Sciences Bhopal, Madhya Pradesh.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

- Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol* 2009;45:309-16.
- Rebagay G, Yan S, Liu C, Cheung NK. ROR1 and ROR2 in human malignancies: Potentials for targeted therapy. *Front Oncol* 2012;2:34.
- Baskar S, Kwong KY, Hofer T, Levy JM, Kennedy MG, Lee E, et al. Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia. *Clin Cancer Res* 2008;14:396-404.
- Shabani M, Asgarian-Omran H, Jeddi-Tehrani M, Vossough P, Faranoush M, Sharifian RA, et al. Overexpression of orphan receptor tyrosine kinase Ror1 as a putative tumor-associated antigen in Iranian patients with acute lymphoblastic leukemia. *Tumour Biol* 2007;28:318-26.
- Fukuda T, Chen L, Endo T, Tang L, Lu D, Castro JE, et al. Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. *Proc Natl Acad*

- Sci U S A 2008;105:3047-52.
6. Daneshmanesh AH, Mikaelsson E, Jeddi-Tehrani M, Bayat AA, Ghods R, Ostadkarampour M, *et al.* ROR1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. *Int J Cancer* 2008;123:1190-5.
  7. Tyner JW, Deininger MW, Loriaux MM, Chang BH, Gotlib JR, Willis SG, *et al.* RNAi screen for rapid therapeutic target identification in leukemia patients. *Proc Natl Acad Sci U S A* 2009;106:8695-700.
  8. Choudhury A, Derkow K, Daneshmanesh AH, Mikaelsson E, Kiaii S, Kokhaei P, *et al.* Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells. *Br J Haematol* 2010;151:327-35.
  9. Zhang H, Qiu J, Ye C, Yang D, Gao L, Su Y, *et al.* ROR1 expression correlated with poor clinical outcome in human ovarian cancer. *Sci Rep* 2014;4:5811.
  10. Hojjat-Farsangi M, Ghaemimanesh F, Daneshmanesh AH, Bayat AA, Mahmoudian J, Jeddi-Tehrani M, *et al.* Inhibition of the receptor tyrosine kinase ROR1 by anti-ROR1 monoclonal antibodies and siRNA induced apoptosis of melanoma cells. *PLoS One* 2013;8:e61167.
  11. Zhang S, Chen L, Cui B, Chuang HY, Yu J, Wang-Rodriguez J, *et al.* ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. *PLoS One* 2012;7:e31127.
  12. Liu Y, Yang H, Chen T, Luo Y, Xu Z, Li Y, *et al.* Silencing of Receptor Tyrosine Kinase ROR1 Inhibits Tumor-Cell Proliferation via PI3K/AKT/mTOR Signaling Pathway in Lung Adenocarcinoma. *PLoS One* 2015;10:e0127092.
  13. Jung EH, Lee HN, Han GY, Kim MJ, Kim CW. Targeting ROR1 inhibits the self-renewal and invasive ability of glioblastoma stem cells. *Cell Biochem Funct* 2016;34:149-57.
  14. Henry C, Llamosas E, Knipprath-Meszaros A, Schoetzau A, Obermann E, Fuenfschilling M, *et al.* Targeting the ROR1 and ROR2 receptors in epithelial ovarian cancer inhibits cell migration and invasion. *Oncotarget* 2015;6:40310-26.
  15. Zhou JK, Zheng YZ, Liu XS, Gou Q, Ma R, Guo CL, *et al.* ROR1 expression as a biomarker for predicting prognosis in patients with colorectal cancer. *Oncotarget* 2017;8:32864-72.