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Evaluation of Promoter Hypermethylation, Expression and, Oxytocin Receptor Gene (OXTR) Polymorphism with the Risk of Oral Squamous Cell Carcinoma

[a] Behnaz Hassanpour, Mohammad Ayoub Rigi-Ladiz [b*], Tayebeh Baranzehi[a], Mohammad Javad Ashraf[c], DorMohammad Kordi-Tamandani[*a]

[a] *Departement of Biology, University of Sistan and Baluchestan, Zahedan, Iran; Email: Bz.hassanpour@gmail.com*
[b] *Oral and Dental Disease Research Center, Zahedan University of Medical Sciences, Zahedan, Iran; Email: Dr.rigi@gmail.com*
[a] *Departement of Biology, University of Sistan and Baluchestan, Zahedan, Iran; Email: kiana8588@gmail.com*
[a] *Departement of Biology, University of Sistan and Baluchestan, Zahedan, Iran; Email: dor_kordi@science.usb.ac.ir*
[c] *Department of Pathology, University of Medical Sciences, Shiraz, Iran; Email: M.javad.ashraf@gmail.com*
* *Corresponding author: Email Address: dor_kordi@science.usb.ac.ir*
* *Cocoresponding author: Email Address: Dr.rigi@gmail.com*

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A B S T R A C T

Background: Oral cancer refers to a subgroup of head and neck's neoplasm. It is estimated that about 90% of oral cancer is composed of OSCC. It frequently influences adult men, in particular, consumers of alcohol and tobacco users. OXTR is known as a G-protein receptor with seven transmembrane domains. G-proteins phosphatidylinositol-calcium as second messenger accomplishes its activity. PKC pathway activates through the receptor, in which the pathway has a role in cell proliferation and contraction. **Material and methods:** The present study was done to evaluate methylation, expression, and polymorphism of oxytocin receptor gene in both patients who suffer from OSCC and healthy samples. Promoter methylation status of the OXTR gene was evaluated in 163 samples using MSP-PCR. Also, OXTR mRNA expression profiles were also considered in 23 OSCC cases and 20 controls, by real-time PCR. The present study evaluated the association of the gene polymorphisms, *rs2254298* and, *rs53576*, by using RFLP-PCR technique, with the risk of OSCC.**Results**: Promoter methylation assessment revealed a significant relationship between normal samples and patients. Our data showed that there is no significant linkage between occurrences of these single nucleotide polymorphisms and the risk of OSCC. Regarding allele frequency, no statistically significant differences were observed for A and G of *rs2254298* and *rs53576* genes between OSCC patients and healthy groups. The relative expression of OXTR mRNA in cases showed no significant differences compared to controls The main purpose of this research was to evaluate the correlation of OXTR gene polymorphisms *rs53576 rs2254298* methylation, and expression pattern of this gene with the development of OSCC. **Conclusion**: OXTR methylation gene has an important role in performing OSCC.

Introduction

Oxytocin is a nano peptide hormone and neurotransmitter. This hormone plays a role in producing contractions at childbirth, likewise milk ejection from the lactating breast. i Oxytocin is normally produced by the hypothalamus stored, and released from the neural lobe of the pituitary gland. It is also synthesized in various peripheral tissues; for example, thymus, testis, pancreas, luteum, placenta, amnion, kidney, uterus, and corpus. Henry Dale discovered oxytocin in 1906; but, its molecular

* Corresponding author: Tel.: 09157481534
E-mail address: *dor_kordi@science.usb.ac.ir*

structure was discovered in 1952 (Magon and Karla, 2011). The OXT gene is localized on the long arm of chromosome number 20. Oxytocin has roles in various behaviors such as penile erection, sexual activity, uterine contraction, pregnancy, ejaculation, breast-feeding, maternal behavior, social recognition, pair bonding, and stress, anxiety, which makes oxytocin and OXTR potential candidates as targets for drug therapy (Gimpl and Fahrenholz, 2001, Chatterjee et al. 2016). The functions of OXT are mediated by special oxytocin receptors. These receptor genes, with 3 introns and 4 exons, have been localized on chromosome 3p25 (Kimura et al. 2003). The Oxytocin receptors are GPCR, which activate several different second messenger systems, and belong to the rhodopsin-type group of G-protein-coupled receptors. These receptors function as a receptor for the hormone and neurotransmitter (Bockaert and Pin, 1999). Its receptors are located in the CNS. OXT-OXTR signaling is related to diverse biological functions. Molecular events induced by OXT-OXTR signaling are involved in some processes such as, cell proliferation, neuromodulation, cardiomyogenesis, bone formation, labor induction, and uterine contraction (Cassoni et al. 2006, Kim et al. 2015).

In developing countries, Oral Squamous Cell Carcinoma is one of the most broadly prevalent cancers. OSCC occurs in elderly men during the fifth years throughout the eight decades of life, and aging is an important factor, which increases the risk of OSCC. In western countries, alcohol consumption and tobacco use are closely associated with OSCC (Massano et al. 2006, Tsantoulis et al. 2007, Marocchio et al. 2010, Markopoulos 2012). The prevalence of oral cancer in Sistan and Baluchestan province is higher compared to other provinces of Iran. Some studies demonstrated that genetic modifications lead to the development of OSCC. CpG-islands methylation in the promoter region of cancer-related genes may serve as epigenetic biomarkers for oral cancer prognosis and diagnosis. Up-regulation of oncogenes and down-regulation of tumor suppressor genes is related to promote tumorigenesis (Rigi-Ladiz et al. 2019).

Material and Method

Clinicopathologic data were analyzed on 83 patients (43 men, 40 women) of paraffin-embedded tissues of OSCC and 80 normal controls (38 men, 43 women), which was taken from Alzahra hospital in Esfahan and Khalili Shiraz Medical Education Center, Iran, among 2013-2015 (Table1).

Table 1. Demographic Information of Cases and Controls

	Cases (n=83)		Controls (n=80)	
Mean±Sd	59.67±16.08		50.15±16.69	
Age	Male	Female	Male	Female
<41	10(12.04%)	3(3.61%)	13(16.25%)	16(20%)
41-60	12(14.45%)	17(20.48%)	11(13.75%)	7(8.75%)
61-80	19(22.89%)	13(15.66%)	12(15%)	9(11.25%)
> 80	2(2.40%)	7(8.43%)	2(2.5%)	0(0%)
Total	43(51.80%)	40(48.19%)	38(47.5%)	42(52.5%)

The main demographic characteristics of OSCC, including histological grade, tumor size and tumor location, and lymphatic tissue invasion, are described in Table 2.

Table2. Clinicopathologic-demographic Characteristic of OSCC Patients

variable	Male (%)	Female (%)	Total (%)
Histological grade			
Well differentiate	31(37.34)	21(25.30)	52(62.65)
Moderately	8(9.63)	14(16.86)	22(2650)
Poorly	4(4.18)	5(6.02)	9(10.84)
Total	43(51.08)	40(48.19)	83(100)
Tumor size			
< 2.1cm	18(21.68)	15(18.07)	33(27.71)
2.1-4cm	13(15.66)	11(13.32)	24(28.91)
4.1-6.0cm	3(361)	12(14.45)	15(18.07)
> 6.0cm	9(10.84)	2(2.40)	11(13.32)
Tumor position			
Tongue	20(24.09)	28(33.72)	47(57.83)
Oral cavity	18(21.68)	9(10.84)	21(32.52)
Lower lip	1(1.20)	2(2.40)	3(3.61)
Other	4(4.81)	1(1.20)	5(6.02)

DNA extraction, Modification, and Methylation-Specific PCR (MSP)

Phenol chloroform protocol was used for extraction of genomic DNA, and then 1–2 mg of extracted genomic DNA was diluted in 50 µl of DEPC water and was used for DNA modification. Sodium bisulfite modification was done on 2 µg of DNA to treat un-methylated cytosine to uracil, while leaving methylated cytosine unaltered according to the Wizard® DNA Clean-Up System (Promega Corporation, manufacturer,s part No: A7280). Eventually, the modified DNA was kept at -20 °C for the next experiments. Methylated and unmethylated primers for promoter of the gene were used to carry out the MSP. Briefly, reaction for MSP was prepared in a total volume of 25 µL of liquid, including, 1.5 µL DNA (80 ng), 0.5 µL of Hot Star Taq 5 U/mL, 1 µL of each primer (10 mmol/ L), 1 µL of dNTPs mix (10 mmol/L), 2.5 µL of buffer, 16 µL double distilled water, and 2.5 µL of Mgcl2 (25 mmol/L). The PCR program was set at: 95 °C for 5 min, followed by 40 cycles of 95 °C for 40 s, the annealing temperature for Methyl= 55 ºC, Unmethyl= 54 °C for the 30s, extension at 72 °C for 30s ,and Final stage incubation 10 min at 72 °C. 2% agarose was used to separate MSP- PCR products (Figure1).

RNA extraction and quantitative real-time PCR with SYBR green

Pars Tous RNA, extraction kit (cat.No. A101231) was used to extract entire RNA from paraffin-embedded tissues. The first-strand cDNA was synthesized from 1-10µg of total RNA using a 2-step RT-PCR kit (Cat. No. RTPL12, Vivantis Technologies) according to the suppliers' protocol. Quantitative RT-PCR assays were performed with the RT-PCR System (Applied Biosystems) using SYBR green Xuorescence. Samples were assayed in a 20µL reaction mixture containing 2µL of cDNA, 10µL of SYBR green, 1µL of each specific primer, and 6µL of DEPC water. The following optimal thermal condition was applied: 1cycle of 95º C for 15 min; 40 cycles of 95º C for 15s; 60º C for the 30s and 72º C for 40s; finally 1cycle of 72º C 10 min for extension; 1cycle 60-95º C for melting curve. The primer sequences and annealing temperatures for PCR amplifications are listed in table 3.

Table3. Primer sequences and annealing temperatures.

Genes	Oligomers (5' ì 3')	product size	Annealing temp
OXTR(M)	F:GTATTTTTTGTTTTTGGAGGAGTTC R: AATTCTAAAACCCCTAACTTACGCT	120bp	54ºC
OXTR(U)	F: TATTTTTTGTTTTTGGAGGAGTTTG R: AATTCTAAAACCCCTAACTTACACT	120bp	55ºC
OXTR	F:CTGCTACGGCCTTATCAGCTT R:CGCTCCACATCTGCACGAA	242bp	60ºC
RNA 18S	F:GTAACCCGTTGAACCCCATT R:CCATCCAATCGGTAGTAGCG	112bp	60ºC
rs2254298	F:TGAAAGCAGAGGTTGTGTGGACAGG R: AACGCCCACCCCAGTTTCTTC	307bp	60ºC
rs53576	F: GCCCACCATGCTCTCCACATC R: GCTGGACTCAGGAGGAATAGGGAC	340bp	58ºC

The OXTR expression was normalized by rRNA18s and was given by $2^{-\Delta\Delta CT}$.

Restriction Fragment Length Polymorphism-PCR (RFLP-PCR)

Polymorphism of *rs2254298* was studied, and the possibility of mutation in nucleotide A was checked among patients and control group. In addition, *rs53576* polymorphism was investigated and the possibility of mutation in nucleotide G was evaluated between cases and controls. The amplification program was set as follows: 95 °C for 5 min, followed by 40 cycles (95 °C for 30 s), the annealing temperature for SNP1= 60 °C, SNP2= 58 °C for the 30s and extension at 72 °C for 30s. Final incubation was completed at 72 °C for 10 min. After conducting the PCR steps, the samples were affected by BsrI for SNP1 and BamHI for SNP2.

2% agarose was used to separate Real-Time PCR products (Figure 2 and 3).

Statistical Analysis

All statistical analyses were performed using SPSS 20 software version 20.0 (SPSS, Chicago, IL) and Epical Info. The T-test was used to assess the association between clinical parameters (target CT/HK CT) and OXTR genes methylation status. The significant P-value was set less than 0.05. Categorical data were analyzed by Pearson's x2. Mann Whitney test was used to analyze the data.

Results

OXTR gene promoter methylation status by MSP in OSCC cases and control groups are given in Table4.

Table4: Promoter Methylation Frequency of OXTR Gene in Cases and Controls

Gene	Methylation status	cases (N=83)	Controls (N=80)	OR	95%*CI*	*P*-value
OXTR	U(ref)	1(1.20%)	8(10%)	-		
	M	23(27.71%	10(12.5%)	18.	52.54-82.75	0.002
	M/U	59(71.08%	62(74.69%	7.6	40.03-57.57	0.03

OR = odds ratio; 95% CI = 95% confidence interval, ref = reference.

[a] Binary logistic regression analysis.

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There was a significant difference in OXTR gene methylation between cases and controls. Results indicate aberrant promoter methylation of OXTR gene (MM: OR= 18.4; 95% CI= 52.54-82.75; p-value= 0.002; MU: OR= 7.61; 95% CI= 40.03-57.57; p-value= 0.03). No statistically significant association was found between OXTR gene methylation, smoking and gender (Table5 and Table6, respectively).

Table5. Association between OXTR Gene Promoter Methylation and Alcohol and Smoking Consumption

			Methylation status			
			M	M/U	U	P value
Smoking	Positive	Male	11(13.2)	17(20.4)	1(1.2)	0.06
		Female	2(2.4)	19(22.8)	0(0)	
	Negative	Male	7(8.4)	8(9.6)	0(0)	
		Female	3(3.6)	15(18)	0(0)	
Alcohol	Positive	Male	10(12.5)	17(20.4)	0(0)	0.024
		Female	0(0)	10(12.5)	0(0)	
	Negative	Male	8(9.6)	8(9.6)	0(0)	
		Female	5(6)	24(28.9)	1(1.2)	

Abbreviations: M, methylation; U, unmethylation used as reference

Table6. Correlation between OXTR Gene Promoter Methylation and Gender

OXTR	Cases			Controls			*P*value
	M	M/U	U	M	M/U	U	
Male	17(20.8)	26(36.3)	0(0)	8(10)	30(36.4)	6(7.5)	0.09
Female	5(6)	34(40.9)	1(1.2)	2(2.5)	6(7.5)	2(2.5)	

Table7. Comparison of Relative Gene Expression for OXTR Gene between Cases and Controls

Gene		No.	Mean±SD	*P*-value
OXTR	Case	23	0.209±0.399	0.4
	Control	20	0.192±0.282	

A Significant association was found between alcohol consumption and OXTR gene methylation (p-value = 0.024, table6).

Analysis of relative gene expression (target CT/HK CT) for OXTR was done, as shown in Table7; the expression profiles difference between the groups (p-value = 0.4).

As Table8 shows, there was no significant difference in the frequency of AG and AA genotypes compared to the GG genotype in the study of *rs2254298* polymorphism between the two groups. Also, in the frequency of AA, AG and GG genotypes, there was no statistically significant difference between healthy and patient groups in *rs53576* polymorphism of the oxytocin receptor gene. The frequency of A and G alleles at the *rs2254298* and *rs53576* in the OXTR gene showed no significant difference between patients and controls (p-value = 0.058 and p-value = 0.07, respectively).

Table8. Genotypes and Alleles Frequencies of (*rs2254298*) and (*rs53576*)

SNP	Genotype/Allele	Cases N=83	Controls N=80	OR	CI	*P*-value
(*rs2254298*)	GG	4(4.8)	2(2.5)	-	-	Ref
	AG	42(50.6)	31(38.57)	0.67	46.09-68.22	0.5
	AA	37(44.75)	47(58.75)	0.44	36.24-57.73	0.3
	G	50(30.12)	35(21.8)			0.058
	A	116(69.87)	125(62.5)			
(*rs53576*)	AA	33(39.75)	48(60)	-	-	Ref
	AG	47(56.62)	28(35)	2.23	51.34-72.67	0.4
	GG	3(3.61)	4(5)	1.09	15.75-75.02	0.3
	A	53(31.92)	36(22.5)			0.07
	G	113(68.07)	114(71.25)			

Discussion

Epigenetics is a constant transform in gene expression without changing the structure of the gene. DNA methylation is an epigenetic mechanism, which has the most significant regulating role in gene expression. Due to its role in carcinogenesis, it has been a considerable subject in the last few years. In various types of tumors, a change in the DNA methylation occurs, especially, hypermethylation of the promoter region of tumor suppressor genes (Das and Singal, 2004). Methylation of specific OXTR CpG sites reduces transcription of the gene (Jack, Connelly and Morris, 2012). DNA methylation within the first exon of OXTR modulates its transcription, and methylation of these regions is variable in the general population. The role of OT in cancer may depend on its concentration, location, and interaction with other hormones. Several studies showed the epigenetic alteration of the OTR gene. For example, when an OTR gene promoter-reporter manufacture was transferred into HeLa cells, with very low levels of OTR mRNA expression, the transcriptional activity was high, usually higher than the activity of an OTR promoter-less construct, which was suggested that, the structure of the OTR gene might be highly modified. In the autism disease methylation of one of these regulatory sites,

OXTR CpG site -934, showed an important role for the actions of DNA methylation on OXTR in behavior (Takayanagi et al. 2005, Nishimori et al. 2008). The results of a study by Aleeca F. Bell and Mary Kimmel, individually, showed the potential importance of OXTR gene DNA methylation as a risk factor for postpartum depression (Bell et al. 2015). In childhood disorders, methylation of a CpG Island in the OXTR gene seems important for the OXTR expression (Kimmel et al. 2016). Extracellular signal-regulated protein kinase2 (ERK2) is a serine/threonine kinase that has an essential role in regulating of cell differentiation and cell growth. ERK2 is involved in MAPK cascades, and activated by a wide variety of extracellular signals. Oxytocin stimulates ERK2 phosphorylation and PGE2 synthesis in Hs578T cells. In the human breast cancer cell line, HS578T, the expression of OTR is up-regulated by serum treatment through a PKC-dependent signaling pathway (Kumsta et al. 2013). As it was mentioned, OTR is GTP binding protein, which stimulates the activity of phospholipase C, activation of PLC leads to IP3 and DAG production, and IP3 causes calcium release from the endoplasmic reticulum and diacylglycerol stimulates PKC, which phosphorylates unidentified target proteins (Copland et al. 1999). Results of Déry MC, by the use of invasion assay, RNA interference, immunofluorescence and pharmacological inhibitors, provided that the OT could effectively enhance invasion properties of HEC cells, through various factors such as matrix-metalloproteinase 14 (MMP14), X-linked inhibitor of apoptosis protein (XIAP), and matrix-metalloproteinase 2 (MMP2). In addition, they showed that OT-mediated invasion depends on both cyclooxygenase 1 (PTGS1) and cyclooxygenase-2 (PTGS2) through the PIK3/AKT pathway (Cattaneo, Lucci andVicentini 2009). OT receptor has been overexpressed in grade I to III endometrial cancer. A result of a study by Cassoni P et al. demonstrated that OT promotes cell proliferation and invasion (Déry et al 2011). Zhong M et al, by the use of a nested PCR strategy, found that OXT induces motility of PC3 and PC3M that is mediated by the Gi-coupled receptor signaling pathway, in prostate cancer cells (Cassoni et al. 2004). In addition, OXTR activation may contribute to prostate cancer invasion and metastasis. OXT has physiological functions and new sites of OXTR expression have been identified in various peripheral organs (Zhong et al. 2010). Under physiologic conditions, OXT can stimulate prostaglandin E2 (PGE2) synthesis in endometrial epithelial and cancer cells; PGE2 influences cancer cells, by activating multiple cellular pathways, to proliferation, survival, and invasion (Blanks and Thornton, 2003). Yu et al. showed the risk of esophageal cancer in women who are breastfed is reduced by 54%. It has been showed that changes in the oxytocin level and changes in the oxytocin receptor gene expression levels exert different effects on breast cancer cells. OXT can inhibit the proliferation of breast cancer cells and the progression of ovarian cancer. Previous findings in patients with breast cancer were indicative of an increase in the concentration of OXT and reduction in the expression of the OXTR gene. It can be concluded that downregulation of the OXTR gene expression may play a role in the development of breast cancer. APPL1 is a protein that interacts with tumor suppressor proteins; oxytocin increased the expression of APPL1. In vivo and In vitro studies showed that oxytocin could increase the proliferation of prostate cancer cells via an increase in the APPL1 expression. The high level of OXT and very high expression of its receptor has been identified in the serum and tissues of patients with prostate cancer (Yu et al. 2011, Lerman et al. 2018, Huan et al. 2017, Zhong et al.2010). Kraii et al. (2019) reported that demethylation leads to an increase in the OXTR gene. Also, their results demonstrated the downregulation of this gene as a consequence of DNA methylation in promoter (Kraaijenvanger et al. 2019). Our results are consistent with previous findings. We observed a significant increase in the methylation of promoter of OXTR gene in the patients with OSCC. This lead to decrease of the OXTR gene expression. *Rs53576* (G→A substitution) is more sensitive to environmental stressful life events. Stress is one of the factors that induce tumorigenesis and promote cancer development. Cortisol, is a stress hormone, OT shows these effects by cross-talk with cortisol, as result, oxytocin reverses tumorogenic effects of cortisol

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(J et al. 2019). We assumed that allele frequencies in OXTR gene may demonstrate different distribution between cases and controls. So, spread of alleles of *rs53576* and *rs2254298* polymorphisms may be associated with OSCC development. One SNP in the third intron of OXTR, has been more considered in comparison with other SNPs. The polymorphism (*rs53576*) occurs on the third intron of OXTR in three types: GG, AG, and AA(Cassoni et al. 2001). Numerous studies have investigated the communication between sociality and the polymorphism of OXTR gene. Meta-analyses of Li showed an association between the *rs53576* polymorphism and three phenotype categories and they found genetic variation in the *rs53576* influences general sociality (Smearman et al. 2016). Oxytocin signaling has very importance in targeting and drug therapies due to its different activities (Li et al. 2015).

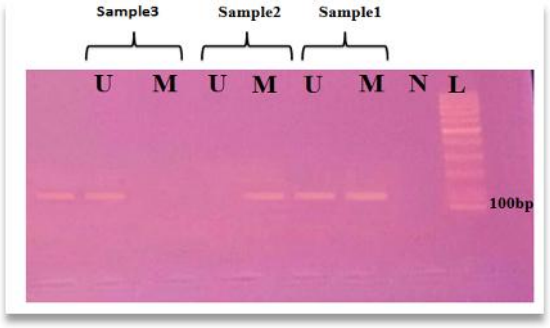

Fig.1- Results of MSP-PCR Analysis of OXTR Gene in OSCC Tissues. M, Methylated; U, UN Methylated.L, Ladder, 100bp Ladder

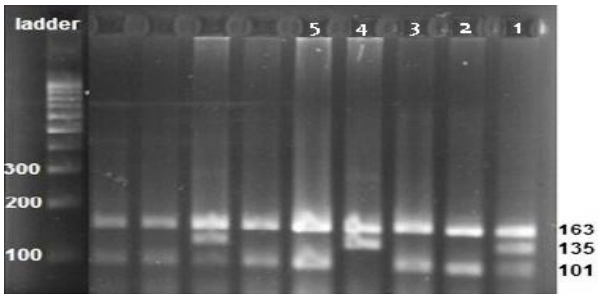

Fig.2- Result of *rs2254298* Polymorphism

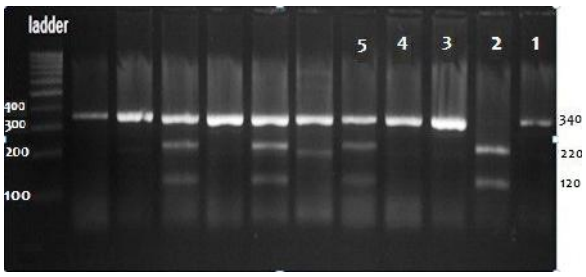

Fig.3- Result of *rs53576* Polymorphism

Conclusions

To our knowledge, this is the first study investigating DNA methylation of the OXTR in OSCC.

The Results obtained in the current investigation showed that OXTR methylation gene has an important role in oral squamous cell carcinoma development. These results suggested that variation at the *rs2254298* and *rs53576* loci may not play a significant role in OSCC develop- pment.

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