ERK and *RAF1* genes: analysis of methylation and expression profiles in patients with oral squamous cell carcinoma

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ABSTRACT

The Ras/RAF/MEK/ERK1/2 pathway is important in the control of growth signals, differentiation and cell survival. Over-expression and activation of this pathway have been reported in different types of cancer. This study analyses the promoter methylation and RNA expression profiles of *ERK* and *RAF1* genes with risk of oral squamous cell carcinoma (OSCC) along with the promoter methylation status of *ERK* and *RAF1* genes using a methylation-specific polymerase chain reaction (MS-PCR) in 86 paraffin-wax embedded samples of OSCC and 68 normal control tissues. Furthermore, *ERK* and *RAF1* expression was analysed in 19 cases and 20 normal samples by real-time reverse transcription PCR. Frequency of promoter methylation was detected for *ERK* (93.02% and 6.98%) and *RAF1* (95.35% and 4.65%) genes in cases and controls, respectively. Messenger RNA (mRNA) expression analysis indicated statistically significant difference between cases and controls for *ERK* ($P<0.002$) and *RAF1* ($P<0.006$). The authors believe that this is the first report to show that expression of *ERK* and *RAF1* is involved in risk of OSCC.

KEY WORDS: Carcinoma, squamous cell.
DNA methylation.
ERK.
Gene expression.
Mouth.
RAF1.

Introduction

Oral squamous cell carcinoma (OSCC) is a frequent and very aggressive malignancy.[1] It develops through a multistep process involving genetic and epigenetic variations that disrupt the natural function of tumour suppressors and oncogenesis.[2] Genetic variations in DNA sequence include gene duplications, deletions and mutations, resulting in oncogene activation or tumour suppressor gene inactivation.[3,4]

Epigenetic variations are heritable changes in gene expression that do not occur in the DNA sequence. The major epigenetic variation in mammals is DNA methylation of 5′-cytosine in a CpG dinucleotide. Hypermethylation of promoter CpG islands and repetitive DNA sequences is one of the most significant variations in progression of OSCC.[5,6]

It has been shown that aberrant DNA methylation plays a vital role in oral cancer. Although the precise function of DNA methylation in OSCC is not understood completely, it is well known that gene expression is affected by DNA methylation, while loss of methylation restores gene activation.[7]

One of the mitogen-activated protein kinases (MAPK) is extracellular-regulated kinase (ERK), including RAS, RAF, MEK1/2 and ERK1/2, which mediates different extracellular signals from the cell cytoplasm into the nucleus by activating phosphorylation to control cell proliferation and differentiation.[8]

RAF1 is a cytosolic serine/threonine protein kinase and plays an important part in mitogen and stress-induced signalling responses, proliferation and cell survival. It operates a kinase cascade that includes phosphorylation and activation of MEK, which in turn activates ERK1/2.[9] These are growth factor-activated protein kinases, and the constitutive activation of this pathway occurs following mutations in *RAS* or *RAF* oncogenes in various cancers.[10]

The results of the activation of ERK result in phosphorylation and activation of multiple cytoplasmic substrates such as cytoskeletal proteins or downstream protein kinases. In addition, phosphorylated ERK1/2 can transfer to the nucleus, leading to activation of several transcription factors (e.g., Elk-1, Sp-1 and AP-1), regulating the transcription of different genes.[11]

Over-expression and activation of *ERK* and *RAF* have been reported in different cancers (e.g., hepatocellular carcinoma, renal cell carcinoma, gastric adenocarcinoma, prostate cancer and breast cancer).[12–16] In addition, over-expression of ERK/MAPKs has been shown in OSCC and other cancers.[17]

This study aims to investigate the hypermethylation of *ERK* and *RAF1* genes and their expression profiles in patients with OSCC.

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Table 1. Sociodemographic characteristics of the case and control groups.

		Cases $n=86$	Controls $n=68$	*P* value (χ^2 test)
Gender:	Male	41	27	<0.205
	Female	45	41	
Age (Years)		54.14±12.6	37.07±11.07	<0.000
Grade:	I	19	–	
	II	17	–	
	III	37	–	
	IV	8	–	
Metastasis		5	–	

Materials and methods

Paraffin wax-embedded tissue samples of OSCC ($n=86$) and normal oral mucosa ($n=68$) were collected from individuals referred to the Dental School at Zahedan University of Medical Sciences. Detailed clinicopathological data are summarised in Table 1. Genomic DNA was isolated from tumour and healthy tissue samples using a commercial kit (QIAamp DNA, Qiagen), following the manufacturer's instructions.

Methylation-specific PCR

Bisulphite modification of DNA samples was conducted as described previously.[18] Briefly, the first promoter region of genes was identified through online data (www.ensembl.org), and then methylated and unmethylated specific primers were designed at CpG sites of the promoter region using MatPrime (Table 2).

Amplification reactions were carried out in a final volume of 25 µL containing 2 µL modified DNA as a template, 0.2 µL HotStarTaq, 1 µL dNTP (10 µmol/L), 16.3 µL RNase-free double-distilled water, 2.5 µL buffer (10x), 0.5 µL each primer (10 µmol/L) and 2 µL Mg^{2+} (25 µmol/L).

Amplification was performed using the following conditions: 94°C for 10 min, and then 40 cycles of 40 sec at 94°C and 30 sec at 55°C (methylated [M]) or 57°C (unmethylated [U]) for *ERK* and 30 sec at 55°C (M) or 58°C (U) for *RAF1*, 1 min at 72°C and a final extension at 72 °C for 10 min. Products were electrophoresed on 4% agarose gels and visualised by ethidium bromide staining.

mRNA expression

For expression profile analysis, total RNA from OSCC and normal tissues was extracted using commercial kits (High Pure FFPE RNA Micro Kit [Cat No: 04823125001] and Cinna Pure RNA Purification Kit [Cat No: PR891620], respectively), following the manufacturers' instructions. The cDNA Synthesis Kit (Fermentas, Cat No: K1621) was used to reverse-transcribe 1 mg RNA in a 20 mL final volume. 18S rRNA was used as an internal standard. Real-time PCR of *ERK* and *RAF1* was performed using the primers and annealing temperatures shown in Table 3.

Statistical analysis

Data were analysed using SPSS software (version 19.0). Categorical data were tested using the χ^2 test, and the effect of the methylation of *ERK* and *RAF1* genes on the risk of OSCC was detected by estimating odds ratios (OR) and 95% confidence intervals (95% CI) using the binary logistic regression test. The Mann-Whitney test was used to assess relative gene expression ($2^{-\Delta\Delta CT}$) between healthy subjects and patients. $P \leq 0.05$ was regarded as significant.

Table 2. Primer sequences and annealing temperatures.

Genes	Sequences (5'–3')	Annealing temperature (°C)
ERK (M)	F: ATAGATTCGTAGAGGTTTGAGTTGC	55
	R: AAACACCCAAAAAAACAAACGTA	
ERK (U)	F: ATAGATTTGTAGAGGTTTGAGTTGTG	57
	R: TAAAACACCCAAAAAAACAAACATA	
RAF1 (M)	F: TCGGTCGTTTTGGAAGTC	55
	R: CCCTAAAACGCGAAACG	
RAF1 (U)	F: GGTTTGGTTGTTTTGGAAGTT	58
	R: CACCAAATATAACCACCTCCCACT	

M: methylated; U: unmethylated.

Results

Results of promoter methylation status of *ERK* and *RAF1* in cases and healthy controls, and their relationship to risk of OSCC, are shown in Tables 4 and 5. There was no significant association between *ERK* and *RAF1* methylation status and risk of OSCC. Differences in methylation status between patients and healthy individuals were not significant for either *ERK* or *RAF1*.

The mRNA levels of *ERK* and *RAF1* were evaluated

Table 3. Real-time primer sequences and annealing temperatures.

Genes	Sequences (5'–3')	Annealing temperature (°C)
ERK	F: CCTAAGGAAAAGCTCAAAGA	60
	R: AAAGTGGATAAGCCAAGAC	
RAF1	F: TGTTCCCCTCACAACACACAA	60
	R: CTGGGACTCCACTATCACCAATA	
18S RNA	F: GTAACCCGTTGAACCCCATT	60
	R: CCATCCAATCGGTAGTAGCG	

Table 4. Promoter methylation frequency of *ERK* and *RAF1* genes in patients with OSCC and in healthy controls.

Genes	Methylation status	Controls ($n=68$)	Cases ($n=86$)	*P* value
ERK	M	66 (97.1)	80 (93.02)	0.228
	U	2 (2.9)	6 (6.98)	
RAF1	M	68 (100)	82 (95.35)	0.094
	U	0 (0.0)	4 (4.65	

M: methylated; U: unmethylated.

Table 5. Risk of OSCC based on gene promoter methylation.

		Unadjusted			Adjusted		
Genes		OR	95% CI	*P* value	OR	95% CI	*P* value*
ERK	U (ref)	0.28	0.06–2.22	0.287	0.809	0.122–5.34	0.828
	M						
RAF1	U (ref)	0.99	0.00	0.999	0	0.00	0.999
	M						

M: methylated; U: unmethylated; OR: odds ratio; CI: confidence interval, ref: reference.
*Binary logistic regression analysis.

between cases and healthy controls, and results are shown in Table 6. The results of expression analysis showed statistically significant difference between patients and healthy individuals.

Discussion

In this study, methylation-specific PCR is used as a tool to analyse the methylation status of *ERK* and *RAF1* genes in patients with OSCC. The results indicated a non-significant difference in methylation status between cases and healthy controls; however, expression analysis shows statistically significant difference between cases and healthy controls. Recently, gene expression and other molecular methodologies have been used as biological tools for detecting cancer.[19]

ERK is one of the most important elements of the EGFR/RAS/RAF/MEK/ERK pathway that is involved in the control of growth signals, differentiation and cell survival; over-expression and activation are frequently detected in a number of cancers including OSCC.[20] The results of the present study are in line with other work reporting the over-expression and activation of the ERK pathway in different cancers (e.g., prostate and breast cancer, melanoma, colorectal and gastric carcinoma).[21–23] Likewise, Mishima *et al*. reported that *ERK* mRNA was at a higher level in well-differentiated and moderately differentiated OSCC than in normal mucosa, suggesting that ERK is related to proliferation in OSCC.[24] Also, Mishima *et al*. have suggested that over-expression and activation of *ERK* play a significant role in cancer progression.[17] Wang and colleagues mentioned that there is a meaningful relationship between the expression level of *ERK* and clinopathological features of OSCC.[25] Wang *et al*. showed that in OSCC the expression level of pERK 1/2 in cancerous tissue is higher than in normal tissue.[26] A study undertaken of a Chinese population has shown that the expression of ERK1/2 in OSCC samples is significantly higher than in adjacent normal mucosa.[27]

Table 6. Comparison of relative gene expression for *ERK* and *RAF1* between patients with OSCC and in healthy controls.

Genes		*n*	Mean±SD	*P* value*
ERK	Cases	19	5.457±7.335	<0.002
	Controls	20	1.460±4.288	
RAF1	Cases	19	3.461±5.301	<0.006
	Controls	20	1.413±4.302	

*Mann-Whitney.

RAF1 is another element of the ERK signalling cascade that is over-expressed in epithelial cells and affects the expression of cyclin D1, c-myc and other genes involved in promoting cell proliferation, invasiveness, angiogenesis and cell survival.[28] Its activity has been demonstrated in solid tumours and in haematological malignancy.[29] Hwanga *et al*. found that over-expression of RAF1 took place in 91.2% (52/57) of cirrhosis and 100% (30/30) of hepatocellular carcinoma samples, which suggests that activation of *RAF1* plays a vital role in cancer progression.[30]

While only limited data have been presented here regarding the methylation status of *ERK* and *RAF1* genes with OSCC, it is possible to suggest that difference in expression of these genes might play a critical role in the development of OSCC. However, to validate these results, further study using larger sample sizes in various genetic populations is required. □

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