

GSK3 β and CREB3 Gene Expression Profiling in Benign and Malignant Salivary Gland Tumors

Nastaran Mohammadi Ghahhari¹, Hamed Mohammadi Ghahhari² and Mehdi Kadivar^{*1}

¹Dept. of Biochemistry, Pasteur Institute of Iran, Tehran, Iran; ²Dept. of Otorhinolaryngology and Head and Neck Surgery, Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran

Received 26 March 2012; revised 2 May 2012; accepted 8 May 2012

ABSTRACT

Background: Salivary gland tumors (SGT) are rare lesions with uncertain histopathology. One of the major signaling pathways that participate in the development of several tumors is protein kinase A. In this pathway, glycogen synthase kinase β (GSK3 β) and cAMP responsive element binding protein (CREB3) are two genes which are supposed to be down regulated in most human tumors. The expression level of the genes was evaluated in SGT to scrutinize their possible under expression in these tumors. **Methods:** Forty eight fresh tissue samples were obtained from patients with benign and malignant SGT, including pleomorphic adenoma, warthin's tumor, mucoepidermoid carcinoma (MEC), salivary duct carcinoma and carcinoma ex pleomorphic adenoma. Eight normal samples were used as controls. Quantitative real-time PCR was used to analyze the expression level of interest genes. **Results:** Data was analyzed by statistical methods. GSK3 β was downregulate in all samples and all results were statistically significant ($P < 0.05$). CREB3 did not show a significant decrease or increase in its mRNA expression, but the results were significant in MEC and salivary duct carcinoma. **Conclusion:** GSK3 β down regulation has been reported in many human tumors. This gene stimulates CREB3, inducing cell proliferation and oncogenesis. Our findings showed GSK3 β down regulation; however, CREB3 expression level was close to normal group. No association between CREB3 expression and inactivated GSK3 β could be postulated in SGT. *Iran. Biomed. J. 16 (3): 140-144, 2012*

Keywords: Gene expression profiling, Salivary Gland neoplasms, glycogen synthase kinase 3 beta (GSK3 β), CREB3 protein

INTRODUCTION

Among rare tumors, salivary gland tumors (SGT) are specified by their different histopathological characteristics. Due to their rare occurrence, fundamental molecular mechanisms related to their heterogeneity and development is unclear [1, 2]. SGT are categorized to benign and malignant tumors. The most frequent benign type is pleomorphic adenoma, compromising 50% of all SGT. PAs grow slowly, but 2-23% of them develop to carcinoma ex pleomorphic adenoma, which is a malignant SGT [3, 4]. Warthin's tumor, also known as cystadenolymphoma, is the second most common type of benign SGT. Warthin's tumor arises from lymph nodes and surgical excision is therapeutic [5]. Mucoepidermoid carcinoma (MEC) includes 10-15% of all salivary gland neoplasms and is the most common malignant SGT. It contains mucus-producing cells and is divided to low-, intermediate- and high-grade subtypes [6, 7]. Salivary duct carcinoma (SDC)

is a very invasive malignant SGT with very poor prognosis for the patients [8]. Another frequent malignant SGT is carcinoma ex pleomorphic adenoma, arising from pleomorphic adenoma showing an invasive behavior [9].

Because SGT are uncommon, very limited studies about molecular events participating in their development have been reported to date. Gene expression analysis would be a useful strategy for investigating new markers and understanding the mechanism of disorder in these tumors. The identification of such genetic differences allows us to further understand human salivary gland tumorigenesis using gene expression analysis. These studies will also allow us to determine whether the deregulation of such genes is an important event in the formation of SGT. Glycogen synthase kinase β (GSK3 β) and cAMP responsive element binding protein (CREB3) genes are parts of the protein kinase A (PKA) signaling pathway. Glycogen synthase kinase-3 (GSK3) decrease or increase the apoptotic threshold and has two isoforms,

*Corresponding Author; Tel.: (+98-21) 6696 9298; Fax: (+98-21) 6640 2770; E-mail: kadivar@pasteur.ac.ir

α and β . GSK3 β in Wnt signaling pathway regulates the amount of β -catenin to whether promote or repress cell proliferation. Its inactivation leads to abnormal levels of β -catenin, inducing tumorigenesis; however, some studies have been reported its overexpression in several tumors [10, 11]. CREB3 is a transcription factor, participating in tumor suppression [12]. GSK3 β and CREB3 have been reported to be under expressed in many human tumors [11]. No study has investigated the expression levels of GSK3 β and CREB3 in SGT; however, an association between them could be suggested by analyzing their relative expression in tumor cells.

MATERIALS AND METHODS

Patients and tissue sampling. Forty eight fresh tissue samples were used for analysis. Tissues were obtained from surgically resected salivary glands, including pleomorphic adenoma (n = 12), warthin's tumor (n = 7), intermediate grade MEC (n = 9), SDC (n = 7) and carcinoma ex pleomorphic adenoma (n = 7). Seven normal salivary gland tissues from unaffected salivary gland tissues of patients with pleomorphic adenoma were included in the study and used as control group. After surgical resection, tissues were kept in RNA later reagent (Qiagen, Germany) and then stored at -80°C until RNA extraction. Patients consent was obtained previously and the work was approved by Ethical Committee of Pasteur Institute of Iran. All samples were subjected to an experienced pathologist prior to experiments to guarantee their histomorphological credibility. None of the patients underwent radiotherapy or chemotherapy before tissue collection.

Primer design, RNA isolation and cDNA synthesis. Sequences of all primers for quantitative real-time PCR were designed by the Primer Express software (ver. 3.0, Applied Biosystems, CA, USA). To avoid any remarkable homology with other genomic sequences, primers were subjected to a comprehensive search in the NCBI/BLAST databases. The primers were as follow: GSK3 β , 5'- AGTGGTGAGAAGAAAGATG AGGTCTATC-3' and 3'- TGACATAAATCACAGG GAGCGTC-5'; CREB3, 5'- CCTGCATCTTGGTCCTA CTAGTCTC-3' and 3'- GACAACACTCCATGCTCA GCTG-5'. RNA polymerase II (RNA pol II) was used as the endogenous control; 5'- GCTGGTTTTGGTGACGA CTTG-3' and 3'- TCTTCCTCCTCTTGCATCTT GTTC-5'. First, all samples were homogenized by RNXTM-plus reagent (CinnaGen, IRAN). Using the manufacturer's instructions, RNA was isolated from samples. Optical density and concentration were determined using Nanophotometer (Implen, Germany) at 260 and 280

nm for cDNA synthesis. cDNA was constructed in separate tubes and Oligo (dT)s was used as the primer in a total volume of 20 μ L under the following thermal cycle condition: 25°C for 30 s, 45°C for 4 min, 55°C for 30 s repeating 12 cycles and then 95°C for 5 min due to enzyme inactivation.

Quantitative real-time PCR. To calculate the PCR efficiency of each target and reference gene, serial dilutions of one normal tissue cDNA was arranged. Serially diluted normal cDNA was adapted to concentrations of 1000, 100, 10 and 1 ng cDNA for each reaction for drawing standard curves. With the aim of checking the specificity of the reaction and sensitivity of SYBR Green II, melting curve analysis was also performed (Fig. 1). Real-time PCR reactions were performed in triplicate by Rotor-Gene 6000 (Corbett, Australia) and two-step cycling: 95°C for 5 s 60°C for 10 s repeating 40 cycles. Reactions were performed in a total volume of 20 μ L, including 10 μ L SYBR Green II Master Mix (TaKaRa, Japan), 1.2 μ L of mixed primers, 4.8 μ L of cDNA and 4 μ L of ddH₂O. RNA pol II was selected as an endogenous control gene for the assays.

Quantitative analysis was performed by the measurement of CT values during the exponential phase of amplification. Using the formula, gene dosage ratio = $2^{-\Delta\Delta CT}$, the target gene/RNA pol II ratio was calculated for each sample, where $-\Delta\Delta CT = [mCT \text{ Target gene (normal sample)} - mCT \text{ RNA pol II gene (normal sample)}] - [mCT \text{ Target gene (test sample)} - mCT \text{ RNA pol II (test sample)}]$.

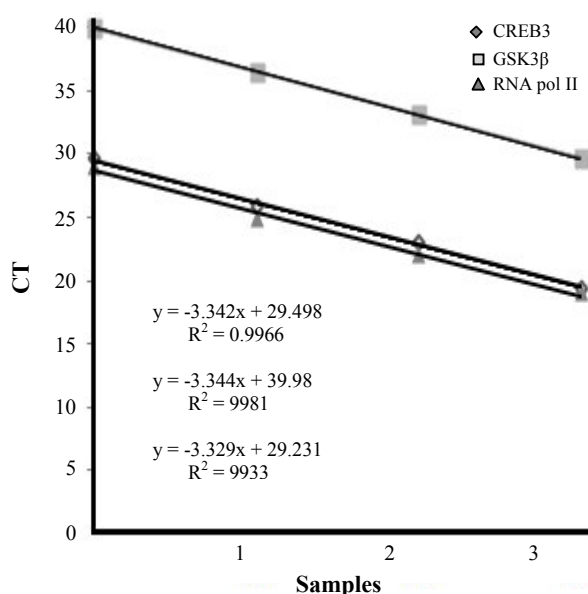


Fig. 1. Optimization of real-time PCR assays for analyzing PCR efficiencies of target and reference genes. Standard curves were designed by plotting ΔCT parameters of each target gene against the amount of serially diluted template DNA per reaction.

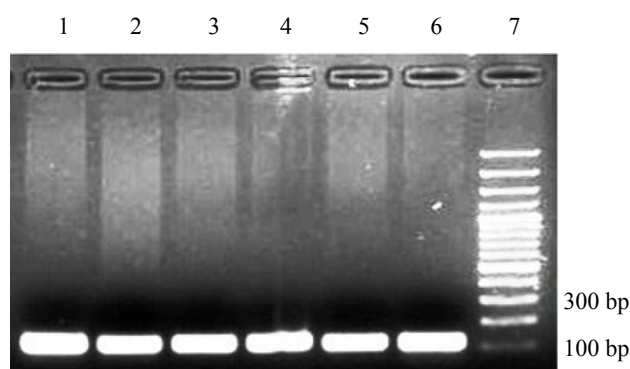


Fig. 2. RNA polymerase II gene gel electrophoresis. The gene showed no variation in its expression level in different normal and salivary gland tumors samples. Lane 1, salivary duct carcinoma; 2, carcinoma ex pleomorphic adenoma; 3, mucoepidermoid carcinoma; 4, Warthin's tumor; 5, pleomorphic adenoma; 6, normal; 7, ladder 100 bp;

The mean gene expression and their related standard SEM for interest genes were compared in normal and SGT samples. One-way ANOVA with Dunnett's post test was performed using GraphPad InStat software (version 3.05, San Diego, California, USA). P value <0.05 was considered significant.

RESULTS

PCR efficiencies for target and reference genes were calculated by standard curve plotting to correlate quantization analysis in gene expression. All best fit trend lines were within the accepted range of $-3.6 < \text{slope} < -3.1$ (Fig. 1). Gel electrophoresis analysis of PCR products showed a single band for each fragment with an expected length of amplicons (Fig. 2). The expression level of two interest genes was analyzed using real-time quantitative PCR. Values were characterized as mean $\pm$ SEM. The statistical significance of results was calculated using Dunnett's multiple comparison tests (Table 1). All results for GSK3 β were significant ($P < 0.05$, Fig. 3A). The gene was significantly down regulated in all benign and malignant subjects. MEC showed the least expression level of the gene among all groups (3.2 ± 0.9). SDC also showed major under expression of the gene (7.8 ± 0.8). For CREB3, the mean gene expressions of all benign and malignant samples were not as remarkable as those measured for the other gene (Fig. 3 and Table 1). Its expression level in benign and malignant SGT samples was close to the control group. Only the results of MEC and SDC group were significant (table 1). GSK3 β was down regulated according to our hypothesis, but it seems that the inactivated GSK3 β cannot induce CREB3 inactivation. However, an interesting relation could be observed in MEC and

SDC groups. As the expression level of GSK3 β was minimized in MEC and SDC samples, CREB3 showed more significant results.

DISCUSSION

No association between inactive GSK3 β and CREB3 expression could be postulated in the benign and malignant SGT. SGT show heterogeneity; therefore, molecular analysis and finding a significant gene expression profile would be useful to recognize different subtypes. One important feature of SGT is the inactivation of apoptosis pathways [3, 13, 14]. In the present study, the expression level of GSK3 β and CREB3, as important parts of the PKA signaling pathway, was evaluated in the tumors of salivary glands. Both genes should be down regulated in these tumors since they are underexpressed in human tumors [10, 15]. GSK3 functions in several signaling pathways. Its inactivation has been implicated in several tumors, but its paradoxical function in some tumors makes it a target for therapeutic purposes, where it serves as a tumor promoter [16, 17]. Upregulated GSK3 β is reported to play a major role in epithelial cancers [18, 19]. Its function is regulated by site-specific phosphorylation of Tyr216/Ser9 residues. However, upstream kinases like PKA is mainly controlled by c-AMP and phosphorylates GSK3 β . Mediated inactivation of GSK3 β by PKA can be seen in oral tumors [11]. Our finding showed that the gene is down regulated in SGT. Down regulation of GSK3 β also stimulates accumulation of β -catenin and cell proliferation [20].

CREB is a transcription factor and participates in different biological mechanisms and tumorigenesis. CREB subtypes include CREB3. Its activity is promoted by GSK3 phosphorylation. Activated GSK3 promotes CREB phosphorylation to facilitate homeobox-mediated transcription and oncogenesis

Table 1. Expression level of target genes in benign and malignant salivary gland tumors.

Group	Number	GSK3 β	CREB3
NR	7	100.5 $\pm$ 10.3 ^a	100.0 $\pm$ 16.2
PA	12	10.9 $\pm$ 3.1	102.2 $\pm$ 3.3
WT	7	13.3 $\pm$ 2.5	91.5 $\pm$ 7.7
MEC	9	3.2 $\pm$ 0.9	158.2 $\pm$ 12.3
SDC	7	7.8 $\pm$ 0.8	114.7 $\pm$ 7.9
CexPA	7	23.2 $\pm$ 9.2	86.1 $\pm$ 2.3

^aData are represented as mean $\pm$ SEM. Comparing each group independently for GSK3 β and CREB3. GSK3 β is inactivated in all samples; however, CREB3 expression was close to control group. NR, normal; PA, pleomorphic adenoma; WT, Warthin's tumor; MEC, mucoepidermoid carcinoma; SDC, salivary duct carcinoma; CexPA, carcinoma ex Pleomorphic adenoma.

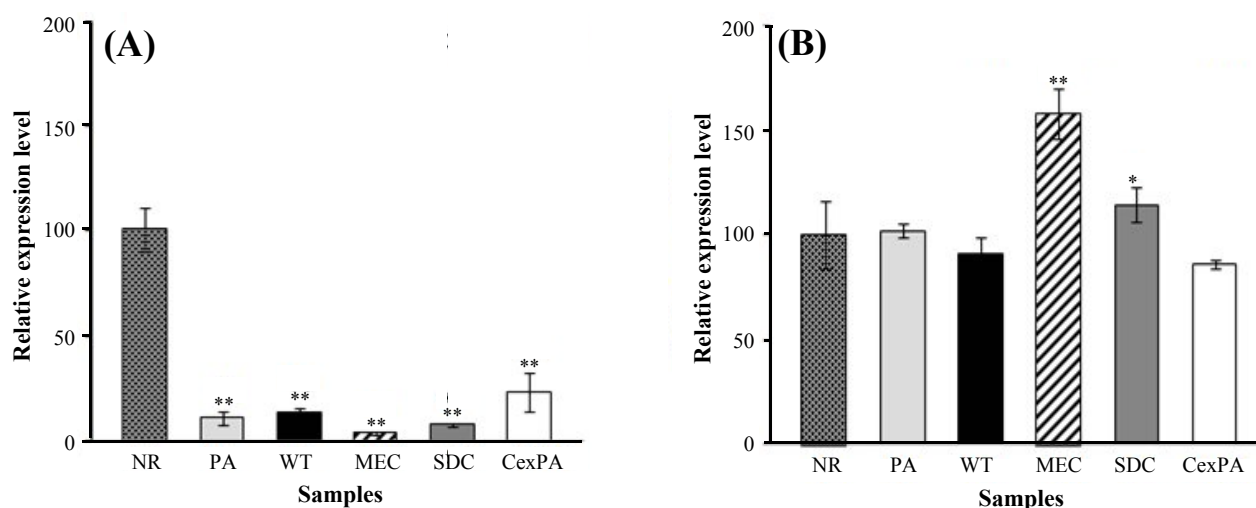


Fig. 3. Expression analysis of GSK3 β (A) and CREB3 (B) in normal salivary gland tissue and benign and malignant SGT. The mRNA levels are illustrated as mean $\pm$ SEM. * P <0.05 and ** P <0.01, as compared with the control group using Dunnett's comparison test. NR, normal; PA, pleomorphic adenoma; WT, Warthin's tumor; MEC, mucoepidermoid carcinoma; SDC, salivary duct carcinoma; CexPA, carcinoma ex pleomorphic adenoma.

[21]. cAMP activates PKA and it is the most important kinase that targets Ser133 of CREB in cell processes. After the phosphorylation, GSK3 activates CREB and its related transcription and oncogenesis. Statistically, CREB3 showed more significant results when GSK3 β was underexpressed in MEC and SDC. Possibly, the reason for this phenomenon is that MEC and SDC are the most malignant and heterogenic types among SGT and CREB3 have been reported to function as a tumor suppressor in some tumors [12, 22]. GSK3 β is also reported to have an inhibitory effect on CREB activity [15]. However, the functional results of the phosphorylation of CREB by GSK3 β is not understood clearly [22]. Our results demonstrated that CREB3 expression in other subtypes was approximately equal to those in normal group. Therefore, we could not suggest any relation between CREB3 expression and inactivated GSK3.

ACKNOWLEDGEMENTS

This project was supported by Pasteur Institute of Iran (No. 510). The authors would like to acknowledge people working in biochemistry Department of Pasteur Institute of Iran (Tehran).

REFERENCES

- Maruya S, Kim HW, Weber RS, Lee JJ, Kies M, Luna MA et al. Gene expression screening of salivary gland neoplasms: molecular markers of potential histogenetic and clinical significance. *J Mol Diagn.* 2004 Aug;6(3):180-90.
- Maruyama S, Cheng J, Shingaki S, Tamura T, Asakawa S, Minoshima S et al. Establishment and characterization of pleomorphic adenoma cell systems: an in-vitro demonstration of carcinomas arising secondarily from adenomas in the salivary gland. *BMC Cancer.* 2009 Jul;21(9):247.
- Queimado L, Lopes CS, Reis AM. WIF1, an inhibitor of the Wnt pathway, is rearranged in salivary gland tumors. *Genes Chromosomes Cancer.* 2007 Mar;46(3):215-25.
- Declercq J, Van Dyck F, Van Damme B, Van de Ven WJ. Upregulation of Igf and Wnt signalling associated genes in pleomorphic adenomas of the salivary glands in PLAG1 transgenic mice. *Int J Oncol.* 2008 May;32(5):1041-7.
- Pastore A, Carinci F, Pelucchi S. Genetic expression profiling of parotid neoplasms by cDNA microarrays. *Acta Otorhinolaryngol Ital.* 2005 June;25(3):153-60.
- Rapidis AD, Givalos N, Gakiopoulou H, Stavrianos SD, Faratzis G, Lagogiannis GA et al. Mucoepidermoid carcinoma of the salivary glands. Review of the literature and clinicopathological analysis of 18 patients. *Oral Oncol.* 2007;43(2):130-6.
- Leivo I. Insights into a complex group of neoplastic disease: Advances in histopathologic classification and molecular pathology of salivary gland cancer. *Acta Oncol.* 2006;45(6):662-8.
- Nagao T, Gaffey TA, Visscher DW, Kay PA, Minato H, Serizawa H et al. Invasive micropapillary salivary duct carcinoma: a distinct histologic variant with biologic significance. *Am J Surg Pathol.* 2004 Mar;28(3):319-26.
- Tarakji B, Kujan O, Nassani MZ. Immunohistochemical Expression of p53 in Pleomorphic Adenoma and Carcinoma Ex Pleomorphic Adenoma. *J Cancer Epidemiol.* 2010; doi:10.1155/2010/250606.
- Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A. Glycogen synthase kinase 3: more than a namesake. *Br J Pharmacol.* 2009 Mar;156(6):885-98.
- Mishra R. Glycogen synthase kinase 3 beta: can it be a

- target for oral cancer. *Mol Cancer*. 2010;9(1):144.
12. Audas TE, Li Y, Liang G, Lu R. A Novel protein, Luman/CREB3 reruitment factor, inhibits Luman activation of the unfolded protein response. *Mol cell Biol*. 2008 Jun;28(12):3952-66.
 13. Francioso F, Carinci F, Tosi L, Scapoli L, Pezzetti F, Passerella E et al. Identification of differentially expressed genes in human salivary gland tumors by DNA microarrays. *Mol Cancer Ther*. 2002 May; 1(7):533-8.
 14. Yin HF, Okada N, Takagi M. Apoptosis and apoptotic-related factors in mucoepidermoid carcinoma of the oral minor salivary glands. *Pathol Int*. 2000 Aug;50(8):603-9.
 15. Grimes CA, Joep RS. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J Neurochem*. 2001 Sep;78(6): 1219-32.
 16. Luo J. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett*. 2009 Jan;273(2):194-200.
 17. Green JB, Lkb1 and GSK3-beta: kinases at the center and poles of the action. *Cell Cycle*. 2004 Jan;3(1):12-4.
 18. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. *Mol Cell*. 2005 Jul;19(2):159-70.
 19. Sabbah M, Emami S, Redeuilh G, Julien S, Prevost G, Zimber A et al. Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. *Drug Resist Updat*. 2008 Aug-Oct;11(4-5):123-51.
 20. Miyoshi K, Rosner A, Nozawa M, Byrd C, Morgan F, Landesman-Bollag E et al. Activation of different Wnt/beta-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. *Oncogene*. 2002 Aug;21(36):5548-56.
 21. Wang Z, Iwasaki M, Ficara F, Lin C, Matheny C, Wong SHK et al. GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. *Cancer Cell*. 2010 Jun;17(6):597-608.
 22. Larabee JL, Maldonado-Arocho FJ, Pacheco S, France B, DeGiusti K, Shakir SM et al. Glycogen synthase kinase 3 activation is important for anthrax edema toxin-induced dendritic cell maturation and anthrax toxin receptor 2 expression in macrophages. *Infect Immun*. 2011 Aug;79(8):3302-8.