Expression of *NFkB1*, *GADD45A* and *JNK1* in Salivary Gland Carcinomas of Different Histotypes

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Abstract. *The class of salivary gland tumours is very heterogenous, both in a histopathological and clinical sense. Since they are uncommon lesions, their clinical management is still problematic. Molecular mechanisms underlying the development of these cancer types may be fundamental for the diagnosis, treatment and prognosis of this disease. In this study, the gene expression of nuclear factor-kappa B (NKkB1/p65), c-Jun N-terminal kinase (JNK1) and growth arrest and DNA damage (GADD45A), which all play an important role in inflammatory and cell survival mechanisms, was assessed in benign and malignant neoplasms of the salivary gland. The absolute mRNA content of paraffin embedded samples of salivary gland cancer was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using specific primers for NFkB1, GADD45A and JNK1. Expression values (relative to HPRT) were statistically evaluated. Among the detected alterations in gene expression, the only difference reaching statistical significance was in the case of NFkB1 in adenocystic carcinomas (p=0.05). Given the importance of these signalling mechanisms in the biology of tumorigenesis, these results may be implemented in further research and these genes might become targets for innovative diagnostic and therapeutic strategies.*

Salivary gland carcinomas are uncommon lesions characterized by varying phenotypic features and diverse clinical outcomes. In our previous study, patients with squamous cell carcinoma and anaplastic carcinoma were found to have the worst (0-1 years and 2 years), and patients with adenoid cystic carcinoma had the best (over 10 years) survival rates (1). Despite efforts to identify new parameters to improve their diagnosis and therapy, little progress in the management of patients with these tumours has been achieved in the past three decades. Such overlapping histologies and variable biological progression pose clinical and differential-diagnostic challenges (2). In the past decades, genomic analysis of tumours underlaid the basis of personalized medicine and molecular target therapy in oncology. The clinical behaviour of a tumour results from interactions at many levels between different genetic alterations and environmental perturbations. Chromosomal, genetic and genomic changes are integrated into networks that act together and lead to the emergence of complex malignant phenotypes. Efforts to identify cancer biomarkers have outstandingly increased in the past two decades (3-6). A further important challenge is to reduce the robust database of candidate cancer biomarkers to a bank of useful predictive, prognostic or diagnostic tools by identifying the biological pathways that are common downstream targets of chromosomal instability, genetic alterations and multiple mutational events.

Studies on genetic changes in different histological subtypes of salivary gland carcinomas are important to better-understand molecular pathogenetic mechanisms and to identify diagnostic and prognostic markers.

Nuclear factor kappa B (NFkB) is a sequence-specific transcription factor and is one of the key regulators of inflammatory and cell survival mechanisms. NFkB consists of a number of closely-related protein dimers that bind to a common sequence motif known as the kB site. Various homo- and heterodimers of p65 (RelA) and c-Rel or p50 protein subunits form the NFkB that acts on the canonical activation pathway dependent on inhibitor of kB kinase (IKK) activation. The pathway is triggered by inflammatory cytokines *via* lipopolysaccharide (LPS) receptors and the Toll-like receptor (TLR) signals induced by microbial, viral and chemical exposure (7-10).

A second, alternative pathway affects NFkB2/p100, dimerized preferentially from RelB subunits and is triggered by the tumor necrosis factor cytokine family (11). Both NFkB dimers have distinct regulatory functions even though

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Table I. *Name and sequence of the primers (5'→3') used in this study.*

Gene name and symbol	Primer sequence
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFkB1*)	Forward: 5'CACTGCTCAGGTCCACTGTC3' Reverse: 5'CTGTCACTATCCCGGAGTTCA3;
Growth arrest and DNA-damage-inducible, alpha (*GADD45A*)	Forward: 5'CTGCCTCCTGGTCACGAA3' Reverse: 5'TTGCCTCTGCTCTCTTCACA3'
Mitogen-activated protein kinase 8 (*JNK1*)	Forward: 5'AACTGTTCCCCGATGTGCT3' Reverse: 5'TCTCTTGCCTGACTGGCTTT3'
Hypoxanthine phosphoribosyltransferase 1 (*HPRT*)	Forward: 5'TCCTCCTCAGACCGCTTTT3' Reverse: 5'CCTGGTTCATCATCGCTAATC3'

their target genes are common, positively regulating the cell cycle, immunregulatory and anti-apoptotic genes. The NFkB signalling and the c-Jun N-terminal kinase (*JNK1*) survival pathways are connected *via* growth arrest and DNA damage (*GADD45A*) and the mitogen-activated protein kinase-4 (MAPK4). *GADD45A* transcript levels increase following stressful growth-arrest conditions and exposure to DNA-damaging agents. GADD family members interact with MAPK4 which is the upstream kinase of *JNK1*, facilitating escape from programmed cell death (8, 9, 11).

In the present study, we performed a quantitative real-time polymerase chain reaction (qPCR) gene expression analysis of 24 salivary gland carcinomas for *NFkB1*, *GADD45A* and *JNK1* expression, using housekeeper gene (HPRT1) as a reference.

Patients and Methods

Malignant and non-malignant tissue specimens. In our study, 24 formalin-fixed paraffin-embedded samples of salivary gland carcinoma were provided by the archive of the Pathology Institute, University of Pecs, Hungary. Tumour tissue samples were obtained from patients diagnosed between 1987-2006 at the Otolaryngology, Head and Neck Surgery Clinic of Pecs University. All tumours were reviewed by the same pathologist and evaluated according to the WHO classification for salivary gland tumours (12). Comparisons and correlations in the course of statistical analysis were all based on the fact that benign tumours (Warthin's tumour and pleomorphic adenoma) were considered as the 'control' group.

RNA extraction, cDNAsynthesis and quantitative RT-PCR (qRT-PCR). Slides were made from the tumour tissue blocks using sections of 10 μm, de-paraffinised in 1.5 ml reaction volume with xylene and absolute ethanol wash steps according to the advised laboratory protocol given by the High Pure FFPE RNA Isolation kit (Roche, Mannheim, Germany) that was used in the next step for total RNA isolation. The quality of the isolated RNA was checked by absorption photometry at 260/280 nm. The optical density of the RNA was between 1.9 and 2.1. High purity total RNA was used in quantitative real-time PCR using a carousel-based Light Cycler 2.0 PCR instrument (Roche, Berlin, Germany). Reverse transcription and nucleic acid amplification was carried out with one-step Light Cycler RNA Amplification kit (Roche, Berlin) using SYBR green fluorescent labelling. Primers for *NFkB1*, *GADD45A*, *JNK1* and *HPRT* were selected from a primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary) and are listed in Table I.

All RNA samples were run in triplicates in 20 μl optical capillaries. The PCR reaction mix contained: 1 μl of the tissue RNA sample, 2 μl of the primer mix of the forward and the reverse primers at 0.5 μM final concentration, 8 μl of PCR-grade water, 0.4 μl of the RT-PCR Enzyme Mix, 3 μl of the Resolution solution, 1.6 μl of the $MgCl_2$ stock solution and 4 μl of the Light Cycler RT-PCR Reaction Mix SYBR Green I.

Thermal cycling conditions for the PCR were the following: one cycle for reverse transcription at 55˚C for 10 min, denaturation at 95˚C for 30 s followed by amplification of 45 cycles of three steps: denaturation at 95˚C for 0.01 s, annealing at 55˚C for 15 s and extension at 72˚C for 4 s. Melting curves were gained from one cycle of 95˚C for 0.01 s, 55˚C for 30 s and 95˚C for 0.01 s as melting with continuous detection. Fluorometric detection was carried out using a 530 nm channel according to SYBR Green fluorescent labelling. For normalization, the average intensity of the three technical replicates of each PCR reaction was taken. The absolute mRNA content for *GADD45A*, *NFkB p65*, *JNK1* and *HPRT* of the tissues was determined and gene expression alterations were calculated relative that for *HPRT*.

Statistical analysis. Differences in relative expression values of each gene in different groups were assessed by the Kruskall-Wallis non-parametric test, followed by pair-wise comparisons using the Student's *t*-test. The Student's *t*-test was used to assess the statistical significance of the differences in the relative gene expression levels of various histotypes (RT-PCR data). A *p*-value below 0.05 was considered statistically significant. The statistical analyses were performed using the Statistical Package for Social Sciences software, version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Of the 24 investigated salivary gland tumours, the parotid gland was the most common site (n=18), representing 75% of the cases. It was followed by the submandibular gland (n=4)

Table II. *Overview of the clinical specimens under investigation with detailed information on tumour location, histopathological classification, age and gender.*

Tumour site	Histotype	Gender	Age, years
Submandibular gland	Adenocarcinoma	Male	55
Submandibular gland	Adenocarcinoma	Male	33
Submandibular gland	Anaplastic small cell carcinoma	Female	57
Submandibular gland	Adenoid cystic carcinoma	Male	66
Minor salivary gland	Adenoid cystic carcinoma	Male	63
Minor salivary gland	Mucoepidermoid carcinoma	Female	60
Parotid gland	Mucoepidermoid carcinoma	Male	86
Parotid gland	Mucoepidermoid carcinoma	Male	68
Parotid gland	Mucoepidermoid carcinoma	Female	60
Parotid gland	Adenoid cystic carcinoma	Female	56
Parotid gland	Adenoid cystic carcinoma	Female	70
Parotid gland	Adenoid cystic carcinoma	Female	44
Parotid gland	Anaplastic small cell carcinoma	Male	58
Parotid gland	Anaplastic small cell carcinoma	Female	82
Parotid gland	Anaplastic small cell carcinoma	Female	65
Parotid gland	Anaplastic small cell carcinoma	Male	30
Parotid gland	Pleiomorphic adenoma	Male	41
Parotid gland	Warthin tumour	Female	63
Parotid gland	Acinic cell carcinoma	Male	51
Parotid gland	Acinic cell carcinoma	Female	85
Parotid gland	Acinic cell carcinoma	Male	51
Parotid gland	Acinic cell carcinoma	Male	30
Parotid gland	Mucoepidermoid carcinoma	Male	52
Parotid gland	Mucoepidermoid carcinoma	Male	69

with a relative frequency of 16.67% and the intraoral minor salivary glands (n=2), with a relative frequency of 8.33%. The tumours belonged to seven different histological groups, but at the same time their clinical stages were identical (a summary regarding location and histopathological classification of the investigated tumours is shown in Table II). The male to female ratio was 1.4 (58.3% male and 41.7% female), the average age of the examined patients was 58±3.14 (range between 30 and 86) years. The average age for females was 64.2±3.87 years and for males was 53.79±4.37 years.

NFkB1 expression was higher in acinocellular (mean=1.375; a median 1.22-fold increase) and adenocarcinomas (mean=1.47; a median 1.32-fold increase) than in the benign lesions (mean=1.13), while in mucoepidermoid carcinomas, the expression levels (mean=1.127) closely resembled those of the benign group. Anaplastic (mean=1.024, presenting a median 1.10-fold decrease) and adenocystic (with the lowest mean value of 0.928, presenting a median 1.22-fold decrease) carcinomas had lower levels of expression (Figure 1). The difference was statistically significant in the case of adenocystic carcinomas (Student *t*-test: $p=0.05$). Expression of the apoptosis-related gene *GADD45A* was only higher in mucoepidermoid-type carcinomas (mean=1.227, showing a slight, median 1.02-fold increase). Down-regulation was observed in the following histotypes: anaplastic (mean=1.098; median 1.09-fold decrease), adenocystic (mean=0.978, median 1.23-fold decrease) and adenocarcinoma (mean=0.915, median 1.31-fold decrease). In the case of acinocellular carcinoma the expression levels found were very similar (mean=1.205) to those in benign-type neoplasms (mean=1.2) (Figure 2). None of the differences in *GADD45A* expression reached the level of statistical significance. The relative expression level of *JNK1* for non-malignant lesions averaged at 1.2. *JNK1* was overexpressed in adenocystic carcinoma (mean=1.23). Underexpression was registered in the majority of malignant histotypes (in ascending order: adeno-, anaplastic, acinocellular and mucoepidermoid carcinomas). Underexpression in adenocarcinomas was 1.24-fold different from non-malignant lesions (Figure 3). None of the alterations in *JNK1* expression reached the level of statistical significance.

Discussion

NFkB. It is known that NFkB is an important regulator of cell proliferation through its direct role in cell cycle progression (13). NFkB positivity has been reported in many malignancies, such as laryngeal squamous cell (14), breast (15), pancreatic (16) and prostatic (17) carcinomas. Similarly to our results of NFkB expression in salivary gland adenocarcinoma, NFkB was shown to be up-regulated in rat

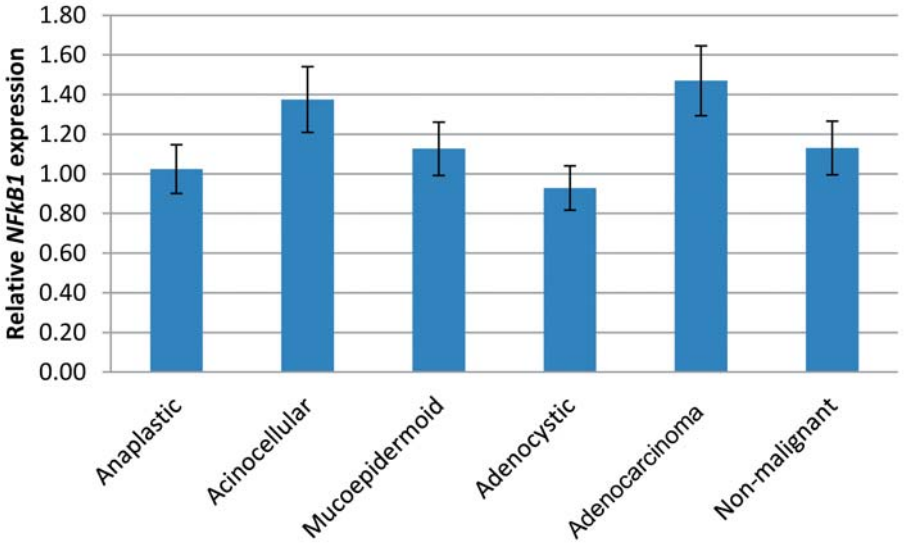

Figure 1. *Gene expression of NFkB (relative to Hprt and normalised) in salivary gland neoplasms of the five studied malignant histotypes.*

colonic adenocarcinomas (18), significantly increased in oesophageal adenocarcinoma (19), and implicated in gastric adenocarcinoma carcinogenesis and survival (20). NFkB inhibition by proteosome inhibition is associated with pre-clinical gastric cancer antitumour activity (20).

JNK1. JNK activity plays a context-dependent role in tumourigenesis. Several studies have linked JNK activity to tumour suppression (21); other studies show pro-tumorigenic roles for JNK activity (22), which may seem contradictory, but they may more likely represent tissue- and cell type-specific differences in the response to different stimuli. JNK1 promotes cell survival in HER2/neu-positive breast cancer (23). Meriin *et al*. had suggested that proteasome inhibitor-induced JNK activation would be pro-apoptotic in lymphoid tumours (24). JNK has been shown to be activated and/or overexpressed in an anti-apoptotic manner in various types of cancer, and JNK inhibition has been shown to increase anticancer effects of proteasome inhibitors in pancreatic cancer cells, suggesting an anti-apoptotic role for JNK in this cell type (25-27). Expression of JNK1 is associated with poor prognosis of patients with H-JNK1 hepatocellular carcinoma (HCC) (28).

GADD45A. GADD45A expression is critical for c-JUN NH_2-terminal kinase activation and apoptosis in tumour cells (29-31). Recombinant *GADD45A* expression in tumour cell lines by transfection reduces cell proliferation (32). Treatment of cells with genotoxic agents up-regulates *GADD45A* expression, resulting in induction of apoptosis (33). Data are even more scant when dealing with unusual subtypes of salivary gland tumours. In high-grade transformation, adenoid cystic carcinomas (hgACC) microsatellite instability and mutational analysis showed single-nucleotide polymorphism (SNP) missense in rat sarcoma (*RAS*) genes and alterations with allelic instability in cyclin-dependent kinase inhibitor 2A (*CDKN2A/A*) and a double mutation in tumor protein 53 (*TP53*) (34). In a Polish study by Giefing *et al*., cystadenolymphoma-Warthin and adenoma polymorphum located in the parotid and submandibular gland were analyzed with whole-genome comparative genomic hybridization (CGH) to identify recurrent, chromosomal copy number changes possibly indicating novel tumour suppressor genes or oncogene loci. The established copy number profiles of 29 tumour samples were compared in order to assess the smallest common region of gains and losses. Altogether, salivary gland tumours presented a different aberration pattern than these reported for head and neck squamous cell carcinoma (HNSCC), but no significant differences were observed between Warthin and adenoma polymorphum tumours. Several potential tumour suppressor genes and oncogenes were identified in the smallest, commonly altered regions. Frequent deletion of the harakiri gene (12q24.2) in 12/29 tumours and *TP53* gene (17p13.1) were shown in 11/29 tumours as potential tumour suppressors in salivary gland cancer. Moreover, frequent amplification of the 13q22.1-22.2 region in 13/29 cases harbouring the Krüppel-like factor (*KLF5* and *KLF12*) genes were detected (10). Matsuyama *et al*., analyzed gene fusions involving pleiomorphic adenoma gene-1 (*PLAG1*) or high-mobility group AT-hook 2 (*HMGA2*) by RT-PCR using formalin-fixed, paraffin-embedded tumour tissues, derived from a series of pleomorphic adenomas of the salivary gland. In addition, the immunohistochemical expression of

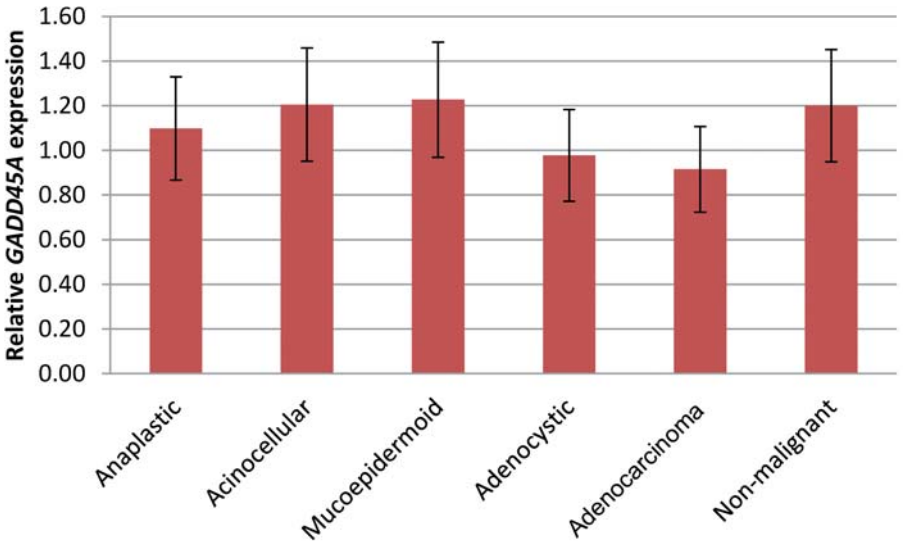

Figure 2. *Gene expression of GADD45A (relative to Hprt and normalised) in salivary gland neoplasms of the five studied malignant histotypes.*

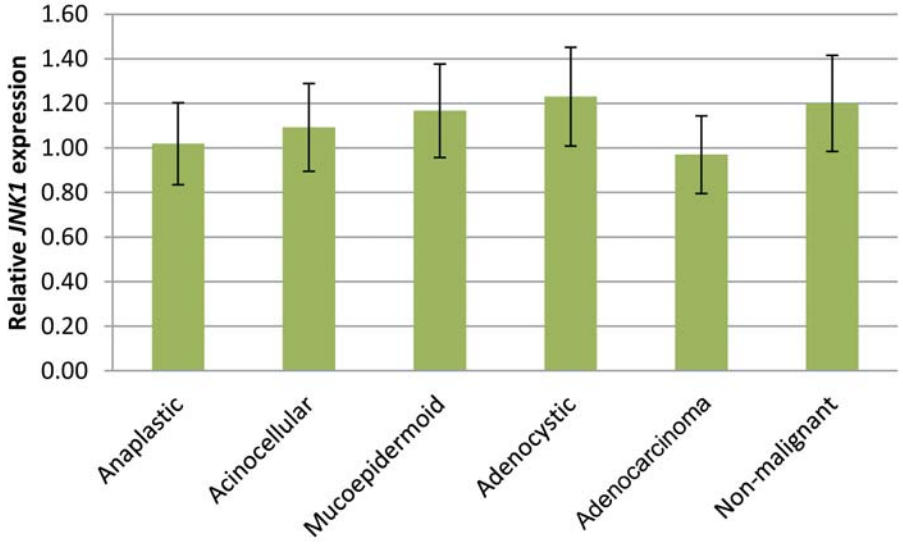

Figure 3. *Gene expression of JNK1 (relative to Hprt and normalised) in salivary gland neoplasms of the five studied malignant histotypes.*

PLAG1 in pleomorphic adenomas and other types of salivary gland tumours was also examined to evaluate the correlation between PLAG1 protein expression and genetic alterations. Their results suggest that overexpression of PLAG1 is essential for the tumorigenesis of pleomorphic adenomas, although the mechanisms mediating PLAG1 overexpression seem to be variable: alterations involving 8q12, the chromosome region where the *PLAG1* gene is located, are the most common abnormalities that account for 25% of pleomorphic adenomas and 40% of them are characterized by a specific t(3;8)(p21;q12) translocation, which results in catenin (cadherin-associated protein), beta-1 (*CTNNB1*) - *PLAG1* gene fusion. Leukemia inhibitory factor receptor (*LIFR*) - PLAG1 fusion, which is derived from t(5;8)(p13;q12) translocation, has also been identified in some cases of pleomorphic adenomas (35). Published data on several tumour types (intestinal epithelial carcinoma, HCC, HNSCC, Barrett's oesophagus) directly implicate NFkB activation as a key

component in inflammation-based cancer progression (10, 36-39). Additionally, compelling experimentation indicates the role of NFkB in modulating cancer therapy efficacy. Inhibition of NFkB by expression of super repressor inhibitor of kappa B (SR-IkB)-alpha strongly enhanced the apoptotic efficacy of daunorubicin and of irradiation (40). The topoisomerase-I inhibitor CPT-11 activated NFkB in experimental colorectal tumours and administration of adenoviral expressing SR-IkB alpha or proteasome inhibitor PS-341 inhibited NFkB activation and significantly enhanced the apoptotic response of the tumour to CPT-11. Thus, the model was that activation of NFkB in response to chemotherapies and to radiation, functioned to suppress the apoptotic potential of that cancer therapy (39). A number of reports using a variety of chemotherapies and different approaches to block NfkB have supported this model (41-44). Furthermore, one report indicates that NFkB inhibition sensitizes cancer cells to TRAIL-induced apoptosis through the sustained activation of JNK (45). Although there is strong evidence that NFkB often functions in an antiapoptotic manner downstream of its activation by chemotherapy or radiation, data indicate that NFkB activation can also function in a proapoptotic manner after activation by non-traditional cancer therapies. Exposure of cells to retinoid 3-Cl-AHPC blocked the expression of X-linked inhibitor of apoptosis protein (*XIAP*), B-cell lymphoma-extra large (*Bcl-xL*) and cellular inhibitor of apoptosis protein-1 (*cIAP1*), and enhanced the expression of proapoptotic death receptors DR4 and DR5, as well as the expression of Fas receptor (CD95) (19). Perkins and co-workers found that NFkB activation by doxorubicin and daunorubicin in U-2OS osteosarcoma cells promoted cell death. In that study, NFkB activation by these chemotherapeutic compounds led to the binding and repression of antiapoptotic genes such as *Bcl-xL*. Interestingly, the activation of etoposide in the same cells led to the traditional antiapoptotic response and was associated the activation of *Bcl-xL* gene expression (20, 22). It is presently unclear which signalling events determine NFkB activation, but it is reasonable to speculate that the response is determined by the genotypic profile of tumour in combination with the specific cancer therapy.

Conclusion

Salivary gland tumours are not the most common entities in the field of head and neck surgery, yet they are such heterogeneous groups, that overall therapeutical treatment and care still pose challenges. Characterisation of molecular pathways involved in carcinogenesis in benign and malignant salivary gland neoplasms represent the present and the future of personalized patient care and cancer treatment. Our findings lay a foundation for research on the molecular mechanism of gene expression in the pathogenesis of salivary gland carcinomas.

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