

Random Amplified Polymorphic DNA-Based Analysis for Genetic Alterations among Malignant Salivary Gland Tumors and Pleomorphic Adenoma

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Abstract

Objectives: The present study was carried out to analyze the genetic alterations among primary malignant salivary gland tumors and pleomorphic adenoma using RAPD to identify new genetic markers of clinical relevance to these tumors.

Methods: DNA was extracted from paraffin-embedded tissue biopsies of the studied cases. RAPD-PCR was performed using 8 different primers. The isolated genes were sequenced by the automated gene sequencer.

Results: In the current study, the identified genes were collagen type IV, alpha 4, polyprotein, large tumor suppressor (LATS) gene, cytokinin receptor at CKI2-like protein or kinesin family member 3C, isoform and tumor susceptibility gene 101(TSG 101).

Conclusion: The results of the current work explored that retroviruses may play a role in salivary gland tumors. Moreover, there are common gene markers, pathways and themes shared among the various types of salivary gland tumors. In addition, effective drug development should focus on targeting these key players upon which multiple oncogenic pathways converge.

Keywords: RAPD; Salivary gland tumors; Cancer genetics

Introduction

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologists or surgeons. Malignant salivary gland neoplasms are rare, representing approximately 3% to 7% of all head and neck cancers. They can arise in the major salivary glands or in one of the minor salivary glands (predominantly mucus-secreting glands). Most patients who develop malignant salivary gland tumors are in the sixth or seventh decade of life [1]. Pleomorphic Adenoma (PA) is the most common tumor of the benign salivary gland neoplasms and the most common tumor of the parotid gland [2]. It derives its name from the variable architectural appearance seen by light microscopy. It is also known as mixed salivary gland tumor, which describes its pleomorphic appearance as opposed to its dual origin from epithelial and myoepithelial elements [3].

Reliable information regarding the histogenesis and morphogenesis of salivary gland tumors allows the study of the processes that govern the histological characteristics of these tumors, as well as the exact relationships between their various subtypes. There are two theories trying to elucidate the origin of malignant salivary gland tumors [4,5]. The more accepted of the two theories is the Reserve Cell Theory which states that salivary gland neoplasms arise from a reserve or stem cell of the salivary duct system [5]. The second and less accepted theory is the Multicellular Theory which states that salivary tumors arise from differentiated cells along the salivary gland unit [4]. PA is characterized by neoplastic proliferation of parenchymatous glandular cells along with myoepithelial components [3].

Oncogenetic events that follow the initiation of the multistage process that results in neoplastic transformation are partially governed by the type of cell in which neoplastic transformation has occurred, influencing both the biology of the tumor and the pattern of cellular differentiation within it [5]. Previous cytogenetic and molecular genetic

analyses of salivary gland tumors have been limited in scope and size and did not account for their inherent morphological and biological heterogeneity [6,7]. Large-scale gene expression analysis offers a broad approach to explore the genetic alterations of functional significance in salivary gland tumors and identifying potential diagnostic and prognostic markers [8,9]. Most of the differentially expressed genes within salivary gland tumors share functional similarities with members of the adhesion, proliferation, and signal transduction pathways [10].

Random Amplified Polymorphic DNA is a type of PCR reaction, but the segments of DNA that are amplified are random. It is used to analyze the genetic diversity of an individual by using random primers. Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism [11,12]. The identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. Therefore, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel [11,12]. RAPD is based on the amplification of genomic DNA with single primers of the arbitrary nucleotide sequence. These primers

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detect polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers and can be used to construct genetic maps [12].

The present study was carried out to analyze the gene expression profile of the major primary malignant salivary gland tumors using RAPD to identify new genetic markers of clinical relevance to these tumors.

Materials and Methods

Sources of genomic DNA

Sixty consecutive cases of tissue biopsies taken for routine diagnosis with patient consent and ethical permission were retrieved from the Department of Oral Pathology archives at Mansoura University. These cases were diagnosed as pleomorphic adenoma, carcinoma ex-pleomorphic adenoma, adenoid Cystic carcinoma, acinic cell carcinoma, mucoepidermoid carcinoma by experienced histopathologists (12 cases for each category). Normal salivary gland tissue was used as a control group. This tissue was obtained from healthy tissue which is available during tumor surgery. DNA was extracted from these paraffin-embedded tissue slides (5-mm thick sections for ethanol-fixed, and between 5-20 mm thick sections for formaldehyde fixation) according to Goelz, et al. [13]. Tissues were deparaffinized by washing twice in xylene (15 min) and in ethanol (100%, 90%, and 70%; 15 min each) and then air dried. Deparaffinized tissue sections were scraped from slides, placed in Eppendorf tubes and rehydrated in TE (250 ml). The samples were then incubated (37°C) after addition of Proteinase K (30 ml of 5 mg/ml; Sigma Chemical, St Louis, MO) dissolved in SDS (10%). After 6 h, additional aliquots of Proteinase K (5 mg/ml) were added until all tissue pieces were visibly digested (48-72 h). The solution was extracted with an equal volume of buffer-saturated phenol (Life Technologies, Gaithersburg, MD) and chloroform/isoamyl alcohol (24:1). Ammonium acetate (100 ml; 10 M) was then added to the supernatant. After addition of MgCl₂ (0.01 M) and glycogen (20 mg; Boehringer-Mannheim, Indianapolis, IN), DNA was precipitated with ethanol (2.5 volume). The pellet was then washed with 70% ethanol, air dried and suspended in distilled water (50 ml).

RAPD PCR for DNA fingerprinting between the examined tissues

The RAPD-PCR was performed using 8 different primers as arbitrary primers (the reverse and the forward primers were used in a separate manner). PCR amplification reactions were performed in 25 µl containing 1X PCR buffer, 1.5 mM MgCl₂, 100 µM of each dNTP, 20 pmoles of each primer (Table 1), 1 unit of *Taq* DNA polymerase and 25 ng genomic DNA. The amplification was performed for 40 cycles in a Thermal-cycler. Each cycle consisted of denaturation at 95°C for 1 min, followed by annealing at 30°C for 1 min and extension at 72°C for 1 min with initial delay for 5 min at 95°C at the beginning of the first cycle and post extension step for 10 min at 72°C after the end of the last cycle (Ting and Manos, 1990). PCR products were separated on agarose gel

electrophoresis using 1.5% (w/v) agarose and 0.5X TBE buffer. The size of each band was estimated by using DNA molecular marker. Finally, the gel was photographed using a gel documentation system.

Scoring and data analysis of RAPDs

The DNA bands were scored for their presence (1) or absence (0) in the RAPD profile of the three flocks. The index of similarity between every two flocks was calculated using the formula: $Bab = 2 Nab / (Na + Nb)$, where Nab is the number of common fragments observed in the individual a and b flocks, and Na and Nb are the total number of fragments scored in a and b respectively [14]. The Band sharing (BS) values were calculated for each primer separately and the average for all primers was carried out with each comparison. The dendrogram was constructed using the average linkage between groups statistical system.

Cloning nucleotide sequence, sequence analysis, and phylogenetic analysis

The resultant PCR product was excised from the gel and purified using a QIA quick gel extraction kit (QIAGEN Inc., Germany). Purified DNAs were ligated into the pGEM-T assay vector Promega Co., USA). Plasmids containing the gene was then directly sequenced using the automated sequencer (Macrogen Company, Korea), with forwarding universal primer. DNA homology searches were carried out with the National Centre for Biotechnology Information (NCBI) databases, using the BLAST network service [15]. Blast search for alignment of the obtained sequence with the published ones was done using a database of NCBI, and they were submitted to be patent at the Egyptian Academy of science. The phylogenetic analysis was carried out using the MEGA4 program according to Tamura et al. [16].

Results

With SDC-1 forward primer

Eighty-two different band patterns were obtained and the molecular size of these bands ranged from 1000 bp to 100 bp. The majority of the obtained bands were polymorphic but only 3 common bands were monomorphic (3 × 5). The monomorphic band's molecular weights were at 700 bp, 250 bp, and 100 bp. In the control sample, 10 different bands were obtained whenever, in the case of sample 1, 19 different bands were shown. This number was decreased in a successive manner starting from samples 5, 3, 4 and 2 (Figure 1A).

In case of SDC-1 reverse primer

Eleven band patterns were obtained with the control sample, but 9 bands were obtained in sample 1 with molecular sizes ranged from 2 kbp to 500 bp. Whenever sample 2 contains 17 different bands with molecular weights ranged from 4 kbp to 350 bp. On the other hand, a very low number of the band was observed with the sample 3 (3 bands) with molecular sizes ranged from 700 bp to 450 bp. Sample 4 revealed bands with high molecular weights ranged from 4 kbp to 800 bp (5 bands). Eleven band patterns were observed with the sample 5

Primer	Forward	Reverse
SDC-1	TGG AGA ACA AGA CTT CAC CTT TG	CTC CCA GCA CTT CCT TCC T
GDF3	AAATGTTTGTGTTGCGGTC	TCTGGCACAGGTGTCTTCAG
BCI-2	AGGATTGTGGCCTCTTTGAGTT	
P53		CAGATACTCAGCATACGGATTCC
TNF	TTCTGTCTACTGAACTTCGGGGTGATGGGTCC	GTATGAGATAGCAAATCGGCTGACGGTGTGGG

Table 1: The primers used in the present study.

and their molecular weights ranged from 1500 bp to 450 bp. There is no monomorphic bands were observed with this primer and all the obtained bands are polymorphic (Figure 1B).

With primer GDF3 forward primer

In control sample, 10 different bands with molecular weights ranged from 2 kbp to 300 bp. But 9 bands with molecular sizes ranged from 700 bp to 250 bp were observed with sample 1. In the case of sample 2, 11 band patterns with a molecular weight ranging from 1200 bp to 300 bp, but with sample 3 the number of the obtained bands was decreased into 8 bands. The molecular weights of these bands ranged from 900 bp to 300 bp. seven different bands were observed with sample 4 with molecular weights 800 bp to 400 bp. Finally, sample 5 showed different eleven bands with molecular sizes ranged from 2 kb to 400 bp.

Primer GDF3 forward did not succeed to amplify monomorphic bands between the examined samples (Figure 1C).

With the GDF3 reverse primer

In control sample, about 8 different bands with molecular weight ranged from 800 bp to 300 bp. Whenever 11 different bands were observed in the case of sample 2 and their molecular weights ranged from 1000 bp to 300 bp. Eight different bands with molecular weights ranged from 1000 bp to 300 bp were obtained. In the case of sample 3, only 4 bands were observed and their molecular weights ranged from 700 bp to 400 bp. Sample 4 showed pattern was completely different when compared with the other examined samples, 12 bands with molecular weights 900 bp to 250 bp but 9 bands were observed with the sample 5 and their molecular weights ranged from 800 bp to 300 bp.

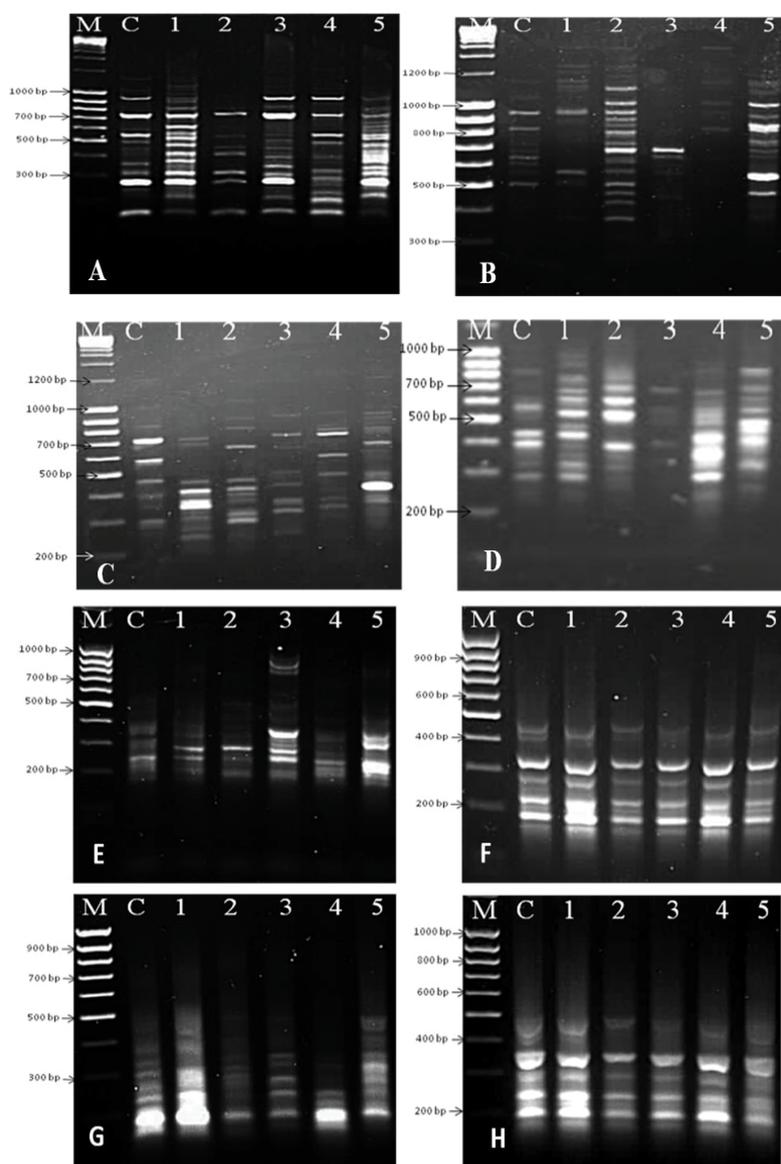


Figure 1: RAPD-PCR for the examined six tissues using (A) SDC-1 forward primer; (B) SDC-1 reverse primer; (C) GDF3 forward primer; (D) GDF3 reverse primer; (E) BCL2 forward primer; (F) P53 reverse primer; (G) TNF forward primer; (H) TNF reverse primer as arbitrary primers. Lanes are as following: M, DNA marker; C-Normal Salivary gland; 1-Pleomorphic adenoma; 2-Carcinoma Pleomorphic adenoma; 3-Adenoid Cystic carcinoma; 4-Acinic cell carcinoma; 5-Mucoepidermoid carcinoma.

There is no monomorphic band was observed between the examined samples (Figure 1D).

In case of BCL2 forward primer

Four bands were observed with the control sample, the molecular weights of these bands ranged from 400 bp to 200 bands. In the case of sample one, 6 different bands were observed and their molecular weights ranged from 400 bp to 200 bp also. Whenever, in case of sample 3, bands ranged from 1 kbp to 200 bp (10 bands), only 6 bands were obtained with sample 4, the molecular weight of these bands ranged from 400 bp to 200 bp. In sample 5 the band patterns with 11 different bands with molecular weights ranged from 800 bp to 200 bp. Only one monomorphic band with a molecular size 200 bp was observed between the examined samples (Figure 1E).

The P53 reverse primer

It gave unique band patterns between all the examined samples including the control. The molecular weights of the amplified bands ranged from 450 bp to 100 bp. No polymorphic bands were observed (Figure 1F).

TNF forward Primer

Revealed different band patterns between all the examined samples. In the control sample 8 different band patterns with a molecular weight ranging from 500 bp to 50bp. In the case of the sample, 16 different band patterns were observed ad the molecular sizes ranged from 500 bp to 50 bp. In samples 2 and 3 the same pattern was observed and the molecular weights of the amplified bands (7 bands) 500 bp to 100 bp. But in sample 4 only 5 bands with molecular weights ranged from 400 bp to 50 bp. Finally, 9 different bands were observed with the sample 5 and the molecular weights ranged from 500 bp to 100 bp (Figure 1G).

The TNF reverse primer gave a unique band pattern. Six monomorphic bands were obtained with all the examined samples including the control. The molecular weights of the obtained bands ranged from 500 bp to 200 bp (Figure 1H).

With the GDF3 reverse primer

In control sample, about 8 different bands with molecular weight ranged from 800 bp to 300 bp. Whenever 11 different bands were observed in the case of sample 2 and their molecular weights ranged from 1000 to 300 bp. Eight different bands with molecular weights ranged from 1000 bp to 300 bp were obtained. In the case of sample 3, only 4 bands were observed and their molecular weights ranged from 700 bp to 400 bp. Sample 4 showed pattern was completely different when compared with the other examined samples, 12 bands with molecular weights 900 bp to 250 bp but 9 bands were observed with the sample 5 and their molecular weights ranged from 800 bp to 300 bp. There is no monomorphic band was observed between the examined samples.

A small set of genes were isolated from all tumor specimens. These isolated genes and their sequences are

>Collagen, type IV, alpha 4

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T T T G C C A A G G A A A G G T -
GCCCTCTCCCCCCTCCCCCCCCAAAAAATTAC-
TACTTGGGGGGGTGTCCGGGTTTTTTTCCCTTTTTTACCA
AAAAAAAAAAAAAAAAACCCCTTTTTTTTCTTTTCCCTTTCC
CCCGCGTGCCCTTCCCCCCCCGGGTGGGTGGGGTCTTT-
GTTCTTTTTTCCCCTAAAAAATAAATAAAAAATATTT-
GTTTTTGGGCAAAAAAAAAAAAAAAAAAAAA
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>Polyprotein

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GTGGGCGGTTTTTTAAGCCTCCTCCACACAAAATA-
AAAATTTCTCCGCCTTTCTTTTTGGAGAATCTTTTTT-
GTTGGAAAAAAGGGGGATGGAAATTTCTGGCCCG-
GCCTAATAAAAAAAGAAAAAATAGTTGGTATGGAGGG-
GGGGGAGTTCTCCCTGGGGGGTAGTAGGAATAGGGATA-
AGAAAAAAACTTTTATACCCCTGCCTGTCCGCACG-
GATACTTTTGGGAAAGGAATGTAATGGGTGGGGGGGAGG
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>Large tumor suppressor (LATS) gene

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GGACCTCGGT TACGACCGCTAGAGATGTACCCG-
GTGGGGTACCGGGGAGGAAATTTCTCGGGAAGGTTGTAC-
GCGGGGAAGAAAAATTTTTTGGGGGGGGAGGTGGGG-
TAGATATATCCCCCCCCAACACACCTCGGGGGGGGGT-
GGTGGGATTACAAGAAGTATTTTTCAATTCGCTTTCG-
GGGAAGAAAAAGAGAGATAACACCCTGTCTTGGTGGT-
GTTATAGAAGTATTAAGTGGGGTGTTTTTTTTTTTTTCTTT-
GTCTCTCGGAGTAAAATTGGGGCCCGCGCCTTGGGTGAT-
GAAAAAAAAAGGGAATAAAAAAGG
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>Cytokinin receptor at CKI2-like protein or kinesin family member 3C, isoform

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T T A A A A A A A A A A A A A A A T A T C -
CACTTGGGTGGGGGTTGGTTTTTTTTTTTTTCCGCCCC-
GAAAAATTTGTCTCCCCCCCCACTTTGTGGGGGGGGGTT-
GTTGAGGGGGGAGCACCCCTCCTCCAATA-
ATTA A A A A A A A A C C C C G G C C C G C G C -
GGGGGTTTTTTTCTTTTTTTTTTTTTTGTTTTCTTCTCTGT
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GCAAACCCTCGGAAATCTGGGGTATTTTTCTTTG-
GAGGGAATATGTGGCCCGGGGGGGCCCCCTACCCA
GAAGGCCTCCCCCAAACAAGACCCCTGGGCGG-
TAAGGGGGGAAAAACCCCCCCACCCCGGCCCC-
GGGGGGGTCCAAAAATTTTTTTTTTT
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>Tumor susceptibility gene 101(TSG 101)

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AGGGGGGGGAAAAGGTTTGTGGCAAGGAAAGGT-
GCCCTCTCCCCCCTCCCCCCCCAAAAAATTAC-
TACTTGGGGGGGTGTCCGGGTTTTTTTTCTTTTTTACCA
AAAAAAAAAAAAAAAAACCCCTTTTTTTTCTTTTCCCTTTCC
CCCGCGTGCCCTTCCCCCCCCGGGTGGGTGGGGTCTTT-
GTTCTTTTTTCCCCTAAAAAATAAATAAAAAATATTT-
GTTTTTGGGCAAAAAAAAAAAAAAAAAAAAA
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Discussion

In recent years, human cancer genome projects provide unprecedented opportunities for the discovery of cancer genes and signaling pathways that contribute to tumor development [17]. Over the last few decades, many studies have been developed to yield more information about the relation between gene alterations and the process of oncogenesis in several lesions, including salivary glands tumors [18]. Random amplified polymorphic DNA bands are highly reproducible under appropriate PCR conditions. The segments of DNA that are amplified are often variable in size among individuals or populations [19] with the potential to serve as genetic markers [12].

The present study identified a small set of genes that are differentially expressed between normal salivary gland tissues and tumor specimens. These genes included several known and yet to be identified expressed sequence tags. One of these genes was collagen type IV, alpha 4. This coincides with Cutler, et al. [20] and Banerjee, et al. [21] who explained

that epithelial-mesenchymal interactions constitute fundamental phenomena in the development and maintenance of the characteristic branching pattern seen in salivary glands. Furthermore, extracellular matrix (ECM) and basement membrane related genes including the genes for laminin and type IV collagen have been found to be widely expressed in myoepithelium or terminal duct origin salivary gland tumors [22]. Also, Saku, et al. [23] found type IV collagen in the stroma of all tumors included in their study. Moreover, Ratiz, et al. [22] indicated that ECM molecules are increasingly implicated in malignancy through two mechanisms: enzymatic degradation, and the inability of the cell to synthesize and assemble the main ECM components.

Polyprotein gene which is a hallmark of retroviral replication [24] was one of the demonstrated genes in the current study. This finding could be supported by Melnick, et al. [25] who stated that retroviruses play an important role in salivary mucoepidermoid carcinoma tumorigenesis. The detection of polyprotein among gene markers of the present work might point to a role for oncogenic viruses in neoplasia of salivary glands. This hypothesis could be supported by Fuentes-Gonzalez, et al. [26] who stated that transforming viruses can change a normal cell into a cancer cell during their normal life cycle. Also, they reported that these viruses have been implicated in the modulation of various biological processes, such as proliferation, differentiation, and apoptosis. Moreover, Gallagher and Gallo, [27] indicated that retroviruses have been isolated from both normal and tumor tissues of humans.

The large tumor suppressor family of serine/threonine kinases control tissue size by regulating cell proliferation and function as tumor suppressor genes in both *Drosophila* and mammals [28,29]. LATS is another cluster of marker genes identified in the present study. This agrees with Lai, et al. [30] who explained that LATS gene is involved in tumorigenesis by regulating cell proliferation, cell growth, and apoptosis. For example, LATS2 might play a role in the tumorigenesis of nasopharyngeal carcinoma by promoting the growth of nasopharyngeal carcinoma cells [31]. This might be attributed to Visser, et al. [32] and Yu, et al. [32,33] who mentioned that disrupting mechanisms that control cell proliferation, cell size, and apoptosis can cause changes in animal and tissue size and contribute to diseases such as cancer. Also, they stated that genetic analysis of Lats/Wts family genes using *Drosophila* and mice models has revealed their role as negative growth regulators and tumor suppressors in the animal. Moreover, the fact that human LATS1 can functionally replace Wts in *Drosophila* supports that LATS may function as a tumor suppressor in human cells. At the same context, an increasing number of mutations in LATS1/2 were detected in human cancer genome projects [17].

Cytokinin receptor at CKI2-like protein or kinesin family member 3C (KIF3C), isoform was one of the demonstrated gene markers in the present study. This is consistent with Hao et al., Stossi et al., Imai et al., Takahashi et al., Taniwaki et al., Zou et al. [34-39] who mentioned that mitotic kinesins are overexpressed in many types of cancers such as lung cancer, pancreatic cancer, and glioma. Moreover, Tan et al., Singel et al. [40,41] reported overexpression of mitotic kinesins in several human cancers including breast cancer. In addition, Yu and Feng, [42] explained that kinesins play important roles in cell division particularly in different stages of mitosis and cytokinesis. Also, they indicated that any errors in cytokinesis could result in cell death, abnormality such as gene deletion, chromosome translocation, or duplication and subsequently cancer.

Tumor susceptibility gene 101 (TSG101) is also identified in the current work. This coincides with Li and Cohen, [43] who

demonstrated that mutations in TSG101 result in reduced expression of the TSG101 protein. Also, this study indicated that implantation of these mutant cells into nude mice, give rise to metastatic tumors. This may be interpreted by Koonin, et al. [44], as well as Ponting, et al. [45] who speculated that the TSG101 protein might act as a transcription factor or regulate ubiquitin-dependent protein degradation. Moreover, previous studies reported that TSG101 is up-regulated in selected human malignancies, and the expression of exogenous TSG101 was suggested to transform immortalized fibroblasts in culture [46]. Also, they observed that, in aging females, the overexpression of TSG101 seemed to increase the susceptibility of mammary epithelia toward malignant transformation. Therefore, they concluded that TSG101 protein has only weak oncogenic properties. But, it plays a more predominant role in the progression of a subset of spontaneously arising breast cancers instead of cancer initiation.

In the current study, the P53 reverse primer gave unique band patterns between all the examined samples including the control. The molecular weights of the amplified bands ranged from 450 to 100 bp. No polymorphic bands were observed. This lack of variations in the single primer based RAPD band patterns might suggest that all or most human genomic DNA is relatively stable with respect to such sequences [47].

Conclusion

Taken together, the results of the current study explored that retroviruses may play a role in salivary gland tumors. Moreover, there are common gene markers, pathways and themes shared among the various types of salivary gland tumors. Therefore, effective drug development should focus on targeting these key players upon which multiple oncogenic pathways converge.

Conflicts of Interest

The authors certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, or company that is present in this article.

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