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High frequency of p16 and p14 promoter hypermethylation and marked telomere instability in salivary gland tumors

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ABSTRACT

Objectives: to investigate $p16^{INK4a}$ and $p14^{ARF}$ tumor suppressor gene methylation status, determine telomere length and assess the importance of these epigenetic and genetic parameters in the development of pleomorphic adenoma and carcinoma ex pleomorphic adenoma of the parotid salivary glands.
Materials and Methods: Genomic DNA from paraffin-embedded samples of 50 pleomorphic adenomas and 10 carcinomas ex pleomorphic adenoma was subjected to methylation specific polymerase chain reaction for hypermethylation analyses and real time polymerase chain reaction for the relative telomere length calculations.
Results: Promoter hypermethylation of the two genes was a very frequent event in both neoplasms – between 60% and 90% of samples were hypermethylated – but without significant difference between the groups. The mean relative telomere length in the pleomorphic adenoma group was significantly increased in comparison to the control group ($P = 0.00$), and significantly decreased in comparison to the carcinoma group ($P = 0.05$). Telomeres were also longer in myxoid and cellular histological subtypes of adenomas than in the classic type ($P = 0.044$ and $P = 0.018$, respectively). Longer telomeres were more frequent in tumors with hypermethylated $p14^{ARF}$ alleles ($P = 0.013$).
Conclusion: Promoter hypermethylations seems to be an important mechanism of $p16^{INK4a}$ and $p14^{ARF}$ inactivation in parotid gland tumors. Telomeric lengthening appears to be involved in the pathogenesis of both benign and malignant tumors of the parotid glands.

1. Introduction

Pleomorphic adenomas (PA) are the most common salivary gland tumors (Speight & Barrett, 2002) representing 40–74% of all salivary gland neoplasms and the majority of them develop in the parotid salivary glands (Eveson, Auclair, Gnepp, El-Naggar, & Ellis, 2005). Histologically, pleomorphic adenomas are characterized by the presence of both ductal epithelial and myoepithelial cells and mesenchymal elements with a marked morphological diversity (Langman, Andrews, & Weissferdt, 2011). They are classified into three main subtypes: myxoid or "stroma-rich", cellular or "cell-rich" and the so-called classic (both components equally represented) (Torske, 2013). Approximately 5% of PAs undergo malignant transformation giving rise to carcinoma ex pleomorphic adenoma (CXPA), usually a highly infiltrative tumor (Hashimoto et al., 2012).

Because of their histological heterogeneity, salivary gland neoplasms may sometimes represent a diagnostic and therapeutic challenge (Speight & Barrett, 2002). Thus, better understanding of their biological behavior could lead to the identification of new molecular targets crucial for the improvement of treatment outcomes (Bell & Hanna, 2012). Mutation analysis of several oncogenes and tumor suppressor genes has not led to consistent results in terms of their possible prognostic value (Milasin et al., 1993; Kärjä, Syrjänen, Kurvinen, & S.M. Syrjänen, 1997; Gomes et al., 2012), with the exception of HER2/neu oncogene

Abbreviations: PA, pleomorphic adenoma; CXPA, carcinoma ex pleomorphic adenoma; TSG, tumor suppressor gene; RTL, relative telomere length; ALT, alternative lengthening of telomeres.

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amplification which has been related to worse prognosis (Hashimoto et al., 2012; Nikolić et al., 2013). The analysis of chromosomal translocations and their fusion oncogene products, on the other hand, has proved to be a more promising research direction from a diagnostic-prognostic application point of view, reviewed in two excellent papers (Weinreb, 2013; Stenman, 2013).

One not yet fully investigated field of salivary gland biology are epigenetic changes which have also been proposed as important events in the process of neoplastic transformation. It has been well documented that hypermethylation of tumor suppressor gene (TSG) promoters represents a powerful mechanism of transcription silencing and can be found in almost all types of cancers (Jones & Baylin, 2002). The INK4a-ARF locus, comprising two TSGs, $p16^{INK4a}$ and $p14^{ARF}$, is one of the alteration hotspots in human cancer (Sherr, 1996), but also in benign tumors, including epithelial odontogenic tumors (Moreira et al., 2009). It connects and regulates two major pathways of tumorigenesis: the pRb and p53 pathways. The two genes have distinct promoter and exon 1 sequences, but share coding sequences of exons 2 and 3 (Sherr, 2012).

$p16^{INK4a}$ controls cell cycle progression through inhibition of cyclin-dependent kinase 4 (CDK4), maintaining the Rb protein in an inactive, unphosphorylated state (Serrano, Hannon, & Beach, 1993) which is considered to be critical for establishing a senescence-like cell growth arrest (replicative senescence). In the absence of functional p16 protein, CDK4 binds to cyclin D and phosphorylates Rb protein, initiating the S phase of the cell cycle and extending the replicative lifespan of the cell (Mitra et al., 1999). Different mechanisms of $p16^{INK4a}$ gene inactivation have been described, including promoter methylation (Miracca, Kowalski, & Nagai, 1999; Li, El-Naggar, & Mao, 2005). $p14^{ARF}$ and MDM2 (murine double minute-2) are involved in the regulation of p53 in response to various stressors (Harris & Levine, 2005). $p14^{ARF}$ acts as a mediator in the p53-MDM2 autoregulatory feedback loop, directly interacting with MDM2 and blocking its inhibition of p53. In turn, p53 represses $p14^{ARF}$ expression (Stott et al., 1998; Honda & Yasuda, 1999) and this feedback loop keeps p53 activity in balance, which is important for the preservation of genome integrity and prevention of cancer (Ozenne, Eymin, Brambilla, & Gazzeri, 2010). $p14^{ARF}$-induced growth arrest is therefore p53 dependent and the lack of $p14^{ARF}$ function may significantly affect p53-related tumor suppression and lead to cancer development (Eischen, Alt, & Wang, 2004).

Finally, one area of salivary gland tumor biology that has never been explored before is the behavior of the telomeres during tumor development. Telomeres are protective DNA-protein complexes at chromosome ends, comprised of tandem DNA repeats and associated proteins. Telomeric DNA undergoes progressive shortening with each cell division. When telomeres become sufficiently short, cells enter irreversible growth arrest called cellular senescence and consequently apoptosis. When apoptosis is prevented due to disrupted regulating pathways such as p53 or Rb pathways, continued proliferation leads to dysfunctional telomeres and genomic instability, thus increasing the risk of activation of telomere maintenance mechanisms and oncogenesis (Shay & Wright, 2011). Telomerase is the enzyme that helps to stabilize telomere length and its activity has been found in almost all human tumors but not in adjacent normal cells leading to the hypothesis that maintenance of telomere stability is required for the long-term proliferation of tumors (Shay & Wright, 1996). Some tumors however lack detectable telomerase activity and it is assumed that alternative lengthening of telomeres (ALT) mechanisms are activated (Heaphy et al., 2011).

The aim of this study was to determine (a) $p16^{INK4a}$ and $p14^{ARF}$ methylation status, (b) the relative telomere length (RTL) in pleomorphic adenoma and carcinoma ex pleomorphic adenoma of the parotid salivary glands and (c) to assess their relationship and respective roles in the development, progression and prognosis of these two types of neoplasms.

2. Materials and methods

2.1. Study subjects

A total of 60 patients were analyzed, 50 with pleomorphic adenoma of the parotid gland and 10 patients with carcinoma ex pleomorphic adenoma, treated at the Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade. Genomic DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue using PureLink™ Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, USA), according to manufacturers recommendation. The mean (±SD) patients' age in the PA group was 44.98 ± 14.27 and in the CXPA group 60.82 ± 11.20. A group of 77 healthy individuals were recruited for the control group (mean age (±SD) was 35.12 ± 14.28). In order to assess the changes in relative telomere length, genomic DNA was extracted from blood using the same kit. This study was performed according to the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration (2002 version, www.wma.net/e/policy/b3.htm), and with the approval of the Ethics Committee of the School of Dental Medicine. All patients were informed of all procedures and signed a written informed consent.

2.2. Analysis of $p14^{ARF}$ and $p16^{INK4a}$ promoter methylation status

The promoter methylation status of $p14^{ARF}$ and $p16^{INK4a}$ has been determined by methylation specific PCR (MSP) (Herman, Graff, Myöhänen, Nelkin, & Baylin, 1996). Genomic DNA extracted

Table 1
Primer sequences, product lengths and annealing temperatures for $p14^{ARF}$ and $p16^{INK4a}$ promoter regions, unmethylated (U) and methylated (M) alleles, for telomeres (tel) and human beta globin gene (HBG).

Primer	Primer sequence	Length (bp)	Annealing temperature
$p14^{ARF}$ U 1	TTTTTGGTGTTAAAGGGTGGTGTAGT		
$p14^{ARF}$ U 2	CACAAAAACCCTCACTCACAACAA	132	53 °C
$p14^{ARF}$ M 1	GTGTTAAAGGGCGGCGTAGC		
$p14^{ARF}$ M 2	AAAACCCTCACTCGCGACGA	122	53 °C
$p16^{INK4a}$U 1	TTATTAGAGGGTGGGGTGGATTGT		
$p16^{INK4a}$U 2	CAACCCCAAACCACAACCATAA	151	60 °C
$p16^{INK4a}$M 1	TTATTAGAGGGTGGGGCGGATCGC		
$p16^{INK4a}$M 2	GACCCCGAACCGCGACCGTAA	150	65 °C
tel 1	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT		
tel 2	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT		56 °C
HBG 1	GCTTCTGACACAACTGTGTTCACTAGC		
HBG 2	CACCAACTTCATCCACGTTCACC		54 °C