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ORIGINAL ARTICLE

Impact of WWOX alterations on p73, ΔNp73, p53, cell proliferation and DNA ploidy in salivary gland neoplasms

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OBJECTIVE: *WWOX* gene is altered in a variety of neoplasms. Wwox is pro-apoptotic through interaction with p73 and may be involved in chromosomal stability by interaction with p73 and p53. The aims of this study were to characterize *WWOX* transcription, methylation status and immunoexpression in salivary neoplasms and to determine whether these were associated with p73, p53, cell proliferation and DNA ploidy.

MATERIALS AND METHODS: Seven malignant and 21 benign fresh salivary neoplasms were included. *WWOX* expression was determined by RT-PCR and sequencing of transcripts, quantitative PCR and immunohistochemistry. Methylation-specific PCR was used to assess the methylation of its first exon. For p73, ΔNp73, p53 and ki67 immunohistochemistry and ploidy analysis, 29 malignant samples from archives were included.

RESULTS: No consistent pattern of *WWOX* exon 1 methylation was found, but aberrant and novel transcripts were observed in 17/28 neoplasms; 55% of tumours showed reduced *WWOX* RNA. *WWOX* RNA levels were associated with p53 immunopositivity. Immunohistochemical Wwox expression did not correlate with methylation status, p53 or p73 expression or proliferation. p73, proliferation and DNA ploidy were associated with malignant phenotype.

CONCLUSION: Aberrant *WWOX* transcription and decreased expression are frequent in salivary neoplasms and *WWOX* transcription is associated with p53 staining.
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Keywords: Wwox; p53; p73; salivary gland neoplasms; ploidy; cell proliferation

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Introduction

Salivary gland neoplasms have an annual global incidence of 0.4–13.5 cases per 100 000 individuals (Eveson *et al*, 2005). Although there are no data regarding salivary gland neoplasms incidence in Brazil, it seems that the prevalence of each group of tumours is similar to globally reported (Ito *et al*, 2005; de Oliveira *et al*, 2009). Surgery or multimodality treatment is required and the development of targeted therapy will require a better understanding of their molecular and cellular biology (Chandana and Conley, 2008).

In 2000, the *WWOX* gene was cloned and found to span FRA16D (Bednarek *et al*, 2000; Ried *et al*, 2000). Subsequent studies demonstrated that Wwox expression is lost or reduced in a variety of human malignancies (Ludes-Meyers *et al*, 2003; O'Keefe and Richards, 2006). Other studies demonstrated loss of heterozygosity, aberrant transcription, epigenetic alterations of gene expression (Paige *et al*, 2001; Driouch *et al*, 2002; Kuroki *et al*, 2002; Iliopoulos *et al*, 2005; Pimenta *et al*, 2006, 2008) and a high deletion frequency (Bednarek *et al*, 2000, 2001; Yendamuri *et al*, 2003). Methylation-specific PCR (MSP) of the first exon of the *WWOX* was shown to be a good marker for early lung carcinogenesis and able to differentiate breast cancer from non-neoplastic adjacent tissue and normal mammary tissue (Iliopoulos *et al*, 2005). Experiments *in vivo* suggested that haploinsufficiency of *WWOX* itself predisposed to cancer in spontaneous and chemically induced tumours (Aqeilan *et al*, 2007). In addition, overexpression of Wwox in cell lines resulted in caspase-mediated apoptosis (Fabbri *et al*, 2005; Qin *et al*, 2006; Iliopoulos *et al*, 2007).

p73, a p53 family member is a tumour-suppressor protein, which regulates cell cycle and apoptosis in response to cellular stress, activating the transcription of p53-target genes to various degrees (Melino *et al*, 2002). There are two classes of isoforms that either contain (TA, transcriptionally active) or lack (ΔN, dominant negative) transactivation domain, which is required for

full activation of target genes. Wwox and p73 seem to interact *in vitro* and *in vivo* (Aqeilan *et al*, 2004) leading to redistribution of p73 from the nuclear compartment to the cytosol thus contributing to the proapoptotic activity of Wwox. It was also shown that ΔNp73 has the same ability. Both p53 and p73 act as regulators of mitotic checkpoint proteins and are linked to chromosomal instability (Tomasini *et al*, 2008). Therefore, we examined a link between *WWOX* transcription and expression alterations and p53 immunostaining. We also investigated *WWOX* transcription, expression and methylation status in benign and malignant salivary gland neoplasms. As p73 reportedly interacts with Wwox (Aqeilan *et al*, 2004), we also investigated the immunoexpression of p73 and of ΔNp73.

A previous study using cell culture demonstrated that the deletion of all p73 isoforms can only lead to aneuploidy when associated with the loss of p53 (Talos *et al*, 2007). This prompted us to investigate the association between *WWOX* and DNA ploidy status, p73 and p53 expression and the cell proliferation index, an important prognostic marker in many salivary gland neoplasms (Ben-Izhak *et al*, 2008).

Materials and methods

Tumour samples

Fresh tumour samples were obtained from 28 patients (12 males and 16 females; mean age 46 years (range 16–76) who underwent surgical excision of salivary gland neoplasms between March 2007 and May 2008. All diagnoses were reviewed by three experienced pathologists and confirmed as 18 pleomorphic adenomas, one Warthin's tumour, one basal cell adenoma, one mucinous cystadenoma, one cystadenocarcinoma, three polymorphous low-grade adenocarcinomas, two low-grade mucoepidermoid carcinomas and one adenoid cystic carcinoma. Six samples of normal salivary glands obtained from healthy volunteers undergoing surgery for non-neoplastic disease were used as controls. This study was approved by the local Ethics Committee ('Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais', protocol number ETIC 152/07).

For each sample, a section of the resected lesion was immediately snap frozen and stored at −80°C while another section was stored in RNAHolder (BioAgency Biotecnologia, São Paulo, Brazil) at −80°C. A third piece of the tissue was fixed in 10% buffered formalin and paraffin embedded.

For the ploidy and immunohistochemistry assays (p73, ΔNp73, p53 and ki67), formalin-fixed paraffin-embedded tissue from 29 additional archival malignant cases was also included. These were six carcinoma ex-pleomorphic adenomas, six polymorphous low-grade adenocarcinoma, six adenoid cystic carcinomas, five epithelial-myoepithelial carcinomas, four high-grade and two intermediate-grade mucoepidermoid carcinomas.

WWOX gene analysis

Methylation-specific PCR. DNA extraction from microdissected frozen sections was carried out using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Bisulphite modification of DNA was performed as previously reported (Goldenberg *et al*, 2004) and modified DNA was purified using Wizard DNA purification resin (Promega Corp., Madison, WI, USA). The MSP assay was used to examine CpG islands in DNA from the salivary gland neoplasms and normal salivary gland samples using specific primers for methylated or unmethylated *WWOX* exon 1 (Iliopoulos *et al*, 2005). Genomic DNA treated with SssI methylase (New England Biolabs, Ipswich, MA, USA) was used as positive control in the specific reactions for the methylated alleles. DNA from peripheral blood mononuclear cells was used as positive control for unmethylated genes (Brakensiek *et al*, 2005). Three independent MSPs were performed to determine DNA methylation of each sample as reference.

The results were grouped as non-methylated, when only the reactions with the unmethylated target (U) primers showed amplification; partially methylated, when both PCR reactions amplified (with methylated and unmethylated target specific primers); completely methylated, when a positive amplification was obtained only with the methylated target-specific (M) primer pair.

Reverse transcription-PCR. Total RNA was extracted from salivary glands and neoplasms with Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's recommendations and treated with DNAse (Invitrogen Life Technologies). First-strand cDNA was prepared from 1 μg of total RNA treated with DNAse using the Superscript first-strand synthesis system (Invitrogen Life Technologies). After reverse-transcription, cDNA was used as a template for PCR amplification of the human *WWOX* cDNA. The first and second amplifications were performed using nested primers as previously described (Kuroki *et al*, 2002; Pimenta *et al*, 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as a control for cDNA quality. Amplified products were subjected to electrophoresis on 6.5% polyacrylamide gel, silver stained and DNA bands corresponding to normal and abnormal size transcripts purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) and sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The two primer sets of the second PCR amplification that amplify the whole open reading frame were used. For each sample, two different amplification products were sequenced. GenBank accession number NM_016373.1 was used as a reference sequence.

Quantitative PCR. For quantitative PCR (qPCR), wild-type transcripts of *WWOX* were detected using 1× SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) using primers previously described (Kuroki *et al*, 2004). Reactions were performed in duplicate and run on a Step One machine (Applied Biosystems) for 10 min at 95°C followed by 40 cycles at

95°C for 15 s and 57°C for 1 min. This cycling was followed by melting curve analysis to distinguish specificity of the PCR products. *WWOX* expression was normalized to internal control ACTB (beta actin). The average threshold cycle (Ct) for two replicates per sample was used to calculate ΔCt. Relative quantification (RQ) of *WWOX* expression was calculated with the $2^{-\Delta\Delta Ct}$ method calibrated using normal salivary gland samples.

Immunohistochemistry

Immunohistochemistry was performed on 56 samples (27 prospectively collected and 29 archival samples) using the antibodies and conditions shown in Table 1. Anti-Wwox and p73DN staining were performed in all 27 prospectively collected samples (Table 2).

Briefly, 4 μm paraffin-embedded sections were de-waxed in xylene, hydrated with graded ethanol and endogenous peroxidase was blocked in 1% hydrogen peroxidase for 15 min. Antigen retrieval conditions are shown in Table 1. Primary antiserum incubation was performed by diluting in BSA 0.5% for 30 min at room temperature and binding was visualized using a polymer-based system (EnVision; Dako Corporation, Carpinteria, CA, USA) with diaminobenzidine (Sigma, St Louis, MO, USA) as chromogen. For each antibody, positive and negative controls in which the primary antibody was omitted were included. The sections were counterstained with haematoxylin, dehydrated and mounted.

ki67 (Mib1) positive nuclei were counted in 10 fields including the most positive areas (400× magnification). Neoplasms were naturally divided into two groups with five or more positive nuclei per 10 fields, or fewer; p53 stained nuclei were counted in eight fields (400× magnification) and more than 5% positive nuclei were considered positive.

For the other antibodies, the whole sections were analysed, counting at least 1000 cells per case. Wwox, ΔNp73 and p73 staining were scored as described in Table 3 (0, negative; 1, 1–25% positive cells; 2, more than 25% positive cells, regardless of stain intensity). Staining intensity is not always suitable for human tumour samples as they may suffer fixation problems due to different sample sizes, fixative solution, processing procedures, etc. Because of different staining patterns with the two antibodies, ΔNp73 tissue was considered positive only when there was nuclear staining with both antibodies. All scoring were performed by two pathologists independently.

Ploidy analysis

Ploidy analysis was performed as previously reported (Diwakar *et al*, 2005). PAS stained monolayer of nuclei were prepared by protease type XXIV (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) digestion from 4×50 μm sections of formalin-fixed paraffin-embedded tissue from 56 salivary gland neoplasms (27 samples prospectively collected and 29 from archives) (Hedley, 1994). Ploidy analysis was performed on a Fairfield image-based ploidy analyser and a minimum of 300 tumour cell-nuclei were assessed, using lymphocytes as internal diploid controls.

Diagnostic criteria were as previously published by the European Society for Analytical Cellular Pathology (ESACP) (Haroske *et al*, 1998). All analyses were performed, blinded to the histological results.

Statistical analyses

The Mann–Whitney, chi-square test or Fisher exact tests were used when appropriate. *P*-values < 0.05 were considered statistically significant.

Results

WWOX gene

There was no difference in the distribution of methylation status between the benign and malignant salivary gland neoplasms and the control group. The first exon of *WWOX* was partially methylated in 16 of 21 (76%) benign samples and in four of six (66.7%) malignant neoplasms (MSP-PCR results are shown in Table 2). Three of five normal salivary glands showed *WWOX* exon 1 partial methylation, while one showed complete methylation and the other was completely unmethylated.

WWOX transcription alterations were detected in both benign and malignant lesions. All normal salivary glands exhibited only the wild-type transcript (1284 bp), but 17 of the 28 (60.7%) neoplasms showed transcription alterations of *WWOX* including aberrant size

Table 1 Primary antibodies, dilution, antigen retrieval solutions and positive controls for immunohistochemistry

Target protein	*Antibody*	*Supplier*	*Clone*	*Dilution*	*Antigen retrieval solution*	*Positive control*
Wwox	wwox	Non-commercial[c]	Polyclonal	1:100	EDTA pH 8.0	Normal salivary gland
p73 pan isoform	AB4	Calbiochem	Polyclonal	1:10	EDTA pH 9.0	ACC[a]
ΔNp73	OP181	Calbiochem	38C674	1:15	EDTA pH 9.0	ACC[a]
ΔNp73	p73DN	Non-commercial[d]	Polyclonal	1:50	Citric Acid pH 6.0	ACC[a]
ki-67	M7240	DAKO	MIB-1	1:50	Citric Acid pH 6.0	SCC[b]
p53	M7001	DAKO	DO7	1:50	Citric Acid pH 6.0	SCC[b]

[a]Adenoid cystic carcinoma with known reactivity to the antibody.
[b]Squamous cell carcinoma with known reactivity to the antibody.
[c]Supplied by Dr C.M. Aldaz (MD Anderson Cancer Center/The University of Texas, USA). Non-commercial antibody described by Nunez *et al* (Nunez *et al*, 2005).
[d]Supplied by Professor G Melino (Universita di Rome Tor Vergata, Rome Area, Italy). Non-commercial antibody described by Sayan *et al* (Sayan *et al*,2005).

Table 2 Results of the *WWOX* experiments (RT-PCR, immunohistochemistry and MSP-PCR) in benign and malignant salivary gland neoplasms

Lesion		*WWOX* transcripts	*Loss in the aberrant transcripts*	*IHC Wwox*	*MSP*	
					M	*U*
Benign salivary gland neoplasms						
1	PA	WT		2	–	+
2	PA	WT + 01 aberrant	Exons 1*–7*	2	–	+
3	PA	WT + 01 aberrant	Exons 4*–5*	2	–	+
4	PA[a]	WT + 01 aberrant	Exons 1*–7*	2	–	+
5	PA	WT		2	+	+
6	PA	WT + 01 aberrant	Exons 1*–7*	1	+	+
7	PA	Aberrant	Exons 6–8	2	–	+
8	PA	WT		2	+	+
9	PA	A		2	+	+
10	PA	A		1	+	+
11	PA	WT + 01 aberrant	1* (insertion of 55 nucleotides) –8	1	+	+
12	PA	WT		2	+	+
13	PA	WT + 02 aberrant	Exons 1*–7*/2*–8*	2	+	+
14	PA	WT		2	+	+
15	PA	WT		2	+	+
16	PA	WT + 02 aberrant	Exons 2*–8*/2*–8*	0	+	+
17	PA	WT		2	+	+
18	PA	A		2	+	+
19	MC	WT + 01 aberrant	Exons 6–8	2	+	+
20	BCA	WT + 03 aberrant	Exons 1*–9*/1*–7*/1*–7*	2	+	+
21	WT	WT		2	+	+
Malignant salivary gland neoplasms						
1	ACC	A		1	+	+
2	PLGA	WT + 01 aberrant	Exons 1*–7*	2	+	+
3	PLGA	WT		1	–	+
4	PLGA	WT + 01 aberrant	Exons 3*–8*	2	–	+
5	Cystadenocarcinoma	WT + 02 aberrant	Exons 1*–8*/2*–8*	2	+	+
6	MEC	WT		1	+	+
7	MEC[b]	WT		#	#	#

WT, wild-type; A, absence of transcript; PA, pleomorphic adenoma; MC, mucinous cystadenoma; BCA, basal cell adenoma; ACC, adenoid cystic carcinoma; PLGA, polymorphous low-grade adenocarcinoma; MEC, mucoepidermoid carcinoma, low grade; IHC, immunohistochemistry. M, methylated; U, unmethylated; M+/U+, partially methylated.
Score 0 = negative, 1 = 1–25% cells positive and 2 = more than 25% cells positive.
*Deletion of a variable number of nucleotides within the exon.
[a]A previously described polymorphism in codon c.941G>T was detected.
[b]Paraffin-embedded tissue of this sample was not available.

transcripts or lack of *WWOX* transcription. Of these 17 lesions, 12 showed one to three transcripts with total or partial loss of exons together with the wild-type transcript. One tumour exhibited an aberrant transcript alone and in four samples, no transcript was detected despite GAPDH amplification. Sequence analysis of the RT-products showed partial or complete deletions of exons (Table 2). Interestingly, one pleomorphic adenoma sample showed an insertion of 55 nucleotides encompassing exons 1 and 8. To our knowledge, most of these abnormal transcripts are novel.

For statistical analysis, samples were divided into two groups; group 1, with wild-type transcripts only, and group 2, with no or aberrant transcripts. There was no association between the *WWOX* mRNA expression patterns and p53 and p73 immunoexpression or cell proliferation index. However, there was an association between the exclusive presence of wild-type transcripts of *WWOX* and the positive nuclear immunolocalization of ΔNp73 ($P = 0.041$). Figure 1a,b shows nuclear positivity for ΔNp73 in a sample of polymorphous low-grade adenocarcinoma that exhibited only the wild-type *WWOX* transcript.

Quantitative PCR primers detected only the wild-type *WWOX* transcripts. Sample #3 (pleomorphic adenoma) was not examined using qPCR because its aberrant transcript harboured a sequence, which annealed to qPCR primers.

Using qPCR, *WWOX* mRNA levels were associated with p53 immunopositivity ($P = 0.049$) (Figure 1c). Malignant samples did not show decreased *WWOX* mRNA levels as compared with benign samples (0.364), but 4/7 malignant and 11/20 benign demonstrated a reduced *WWOX* RNA levels. Samples exhibiting only the wild-type transcript did not present statistically significant transcriptional levels compared with those carrying the aberrant transcripts ($P = 0.619$) despite showing association with p53 immunopositivity ($P = 0.038$).

Immunohistochemistry

The Wwox immunoexpression data are shown in Table 2. Normal salivary glands stained ductal cells

Table 3 DNA ploidy status and nuclear immunoexpression of p73, ΔNp73, p53 and ki67 in the benign and malignant salivary gland neoplasms

	DNA ploidy		*Nuclear immunoexpression* (n)											
	D	*A*	*p73*			*ΔNp73*					*p53*		*Ki67*	
			AB4			*OP181*			*P73DN*[a]		*DO7*		*MIB1*	
			0	*1*	*2*	*0*	*1*	*2*	*1*	*2*	+	–	*L*	*H*
Benign salivary gland neoplasms														
PA[b] (*n* = 18)	11	0	2	10	6	16	1	1	7	11	5	13	15	3
MC (*n* = 01)	1	0	0	1	0	1	0	0	0	1	0	1	1	0
WT (*n* = 01)	1	0	0	0	1	1	0	0	0	1	0	1	1	0
BCA (*n* = 01)	1	0	1	0	0	1	0	0	0	1	0	1	1	0
Malignant salivary gland neoplasms														
Ca ex PA (*n* = 06)	3	3	0	1	5	5	1	0	–	–	5	1	4	2
MEC (*n* = 07)														
Low[b]	–	–	0	1	0	1	0	0	1	0	0	1	1	0
Intermediate	2	0	0	0	2	2	0	0	–	–	0	2	2	0
High grade	2	2	0	2	2	4	0	0	–	–	0	4	2	2
EMCa (*n* = 05)	3	2	0	2	3	5	0	0	–	–	2	3	3	2
ACC (*n* = 07)	7	0	0	4	3	3	2	2	0	1	6	1	2	5
Cystadenocarcinoma (*n* = 01)	1	0	0	0	1	1	0	0	0	1	0	1	0	1
PLGA[b] (*n* = 09)	6	2	0	3	6	7	1	1	0	3	3	6	3	6

PA, pleomorphic adenoma; MC, mucinous cystadenoma; WT, Warthin tumour; BCA, basal cell adenoma; Ca ex PA, carcinoma ex-pleomorphic adenoma; MEC, mucoepidermoid carcinoma; EMCa, epithelial myoepithelial carcinoma; ACC, adenoid cystic carcinoma; PLGA, polymorphous low-grade adenocarcinoma; D, diploid; A, aneuploid; L, low; H, high.
Score 0 = negative, 1 = 1–25% cells positive and 2 = more than 25% cells positive.
[a]p73DN stain was done only in the samples prospectively collected.
[b]Ploidy could not be analysed in 07 PA and 01 low-grade MEC and 01 PLGA samples.

and mainly the serous acinic cells (score 2). Most of benign samples were score 2 for the Wwox immunoexpression ($n = 17$), whereas three malignant lesions were score 1 and the other three score 2. Although there was no statistical association between the immunoexpression pattern of Wwox and benign or malignant nature ($P = 0.286$), there was evidence of diminished expression of Wwox protein in the neoplasms compared with normal glands. No association was found between the immunostaining of Wwox and the immunoexpression of p53, different patterns of p73 expression, proliferation index or *WWOX* mRNA levels. The methylation status of exon 1 did not exhibit association with the Wwox immunohistochemical pattern. Figure 1d shows the cytoplasmic staining pattern of Wwox (score 2).

The immunohistochemical p73 results are summarized in Table 3. All neoplasms were positive for p73; the pattern varied using different antibodies. Pan-isoform p73 revealed nuclear localization and this finding was also present using the p73DN antibody

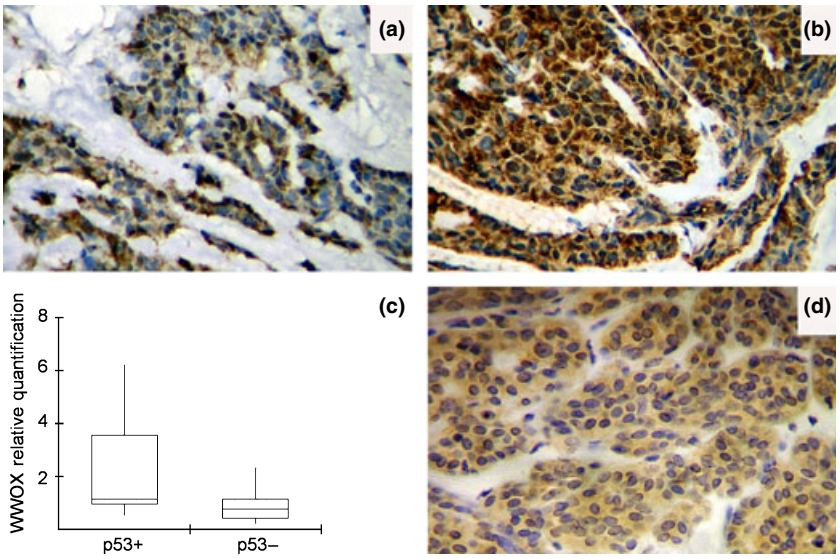

Figure 1 Parameters that showed association with *WWOX* expression pattern. A polymorphous low-grade adenocarcinoma that exhibited only the wild-type transcripts of *WWOX* in the RT-PCR revealed nuclear positivity of ΔNp73 (**a**: antibody OP181; **b**: antibody P73DN). A higher *WWOX* RNA relative quantification was demonstrated in the p53 positive group of salivary gland neoplasms (**c**). A polymorphous low-grade adenocarcinoma showing Wwox immunostaining, score 2 (**d**). Original magnification ×400

against ΔNp73. ΔNp73 staining was also nuclear using antibody OP181, ΔNp73, staining was also nuclear, and cytoplasmic staining was also seen in a few samples.

While all malignant salivary gland neoplasms exhibited p73 immunoexpression, some benign samples were negative. There was an association between the immunoexpression of p73 (pan-isoform antibody) and malignancy ($P = 0.022$) (Figure 2a), and a positive association between p73 staining and a high proliferation index ($P = 0.014$) (Figure 2b). p73 (pan-isoform) immunoexpression was associated with lack of staining for *Δ*Np73 ($P = 0.028$). All samples that showed transcriptional alterations of *WWOX* showed no nuclear expression of ΔNp73 protein ($P = 0.041$).

A high proliferation index (ki67) was present in malignant but not in benign salivary gland neoplasms ($P = 0.012$) and associated with immunopositivity for p73 ($P = 0.014$, see above) and p53 ($P = 0.008$). No other association was demonstrated.

There was a statistically significant association between lack of p53 staining and lack of nuclear *Δ*Np73 ($P = 0.019$). Immunoexpression of p53 did not correlate with any other investigated parameter, but *WWOX* expression, as described above. Figure 3 shows immunostaining patterns of the commercial antibodies used (ki67, p53,AB4, OP181).

Ploidy

Forty-seven neoplasms were successfully evaluated by image cytometry. The mean number of nuclei examined was 899 (range 378–2554). The diploid peak median-CV was 3.76 (range 1.77–5.9). All 14 benign neoplasms were diploid, whereas 9 of 33 (27.2%) malignant neoplasms were aneuploid (Table 3) demonstrating a significant association between aneuploidy and malignancy ($P = 0.046$). Figure 2c shows a representative aneuploid sample histogram. DNA ploidy was not associated with immunoexpression of p53, p73, *Δ*Np73 or cell proliferation index. Twelve of 13 (92.3%) samples that exhibited aberrant *WWOX* transcription alterations were diploid.

Discussion

We demonstrated aberrant transcription of *WWOX* in most of the benign and malignant salivary gland neoplasms tested, but aberrant transcription was not present in normal salivary glands, consistent with previous studies (Ried *et al*, 2000; Paige *et al*, 2001; Driouch *et al*, 2002; Kuroki *et al*, 2002; Pimenta *et al*, 2008) supporting the concept that aberrant transcription of *WWOX* is found only in neoplastic tissue. Transcriptional alterations of the gene were frequent in salivary gland neoplasms, but whether this transcriptional alteration is an early event in the pathogenesis of such tumours or whether it is just a result from the neoplasia progression remains to be elucidated (Gourley *et al*, 2005). Despite this high frequency of altered transcripts, there was no association with different *WWOX* relative quantification in the present study.

Lost or reduced *WWOX* expression has been reported in several tumour types (Bednarek *et al*, 2000; Ried *et al*, 2000; Paige *et al*, 2001; Ludes-Meyers *et al*, 2003; O'Keefe and Richards, 2006). Our data further extend these findings as we found aberrant transcription of *WWOX* as well as decreased *WWOX* mRNA expression in a great proportion of salivary neoplasms compared with *WWOX* expression in the normal salivary glands (in the qPCR). As in the immunohistochemistry, some tumour samples showed decreased Wwox protein expression compared with normal salivary samples, our results clearly point to a decreased *WWOX* expression in salivary gland tumours. Although methylation status of *WWOX* exon 1 has been shown to be a good marker for early lung and breast cancers (Iliopoulos *et al*, 2005), we did not find any significance of *WWOX* exon 1 methylation in salivary gland neoplasms.

Interestingly, we found a positive and statistically significant association between p53 immunopositivity and increased mRNA expression of *WWOX*. Chang *et al* (2005) demonstrated that Wwox is involved in binding and stabilization of p53 under UV light

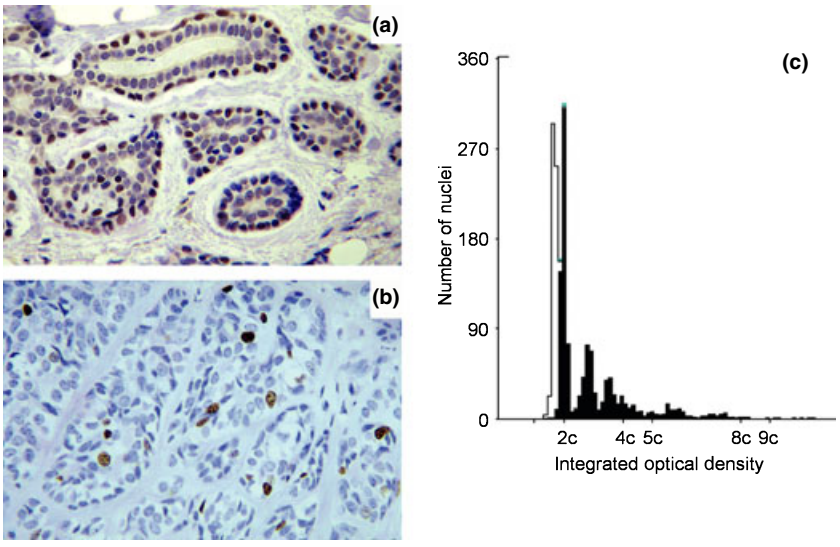

Figure 2 Representative results of the parameters that were statistically associated with malignancy. p73 immunostaining (score 2) in a sample of adenoid cystic carcinoma (**a**); high proliferation index obtained by counting Ki67 positivity in a sample of adenoid cystic carcinoma (**b**); a DNA ploidy histogram of a carcinoma ex-pleomorphic adenoma sample showing aneuploidy (**c**).Original magnification ×400

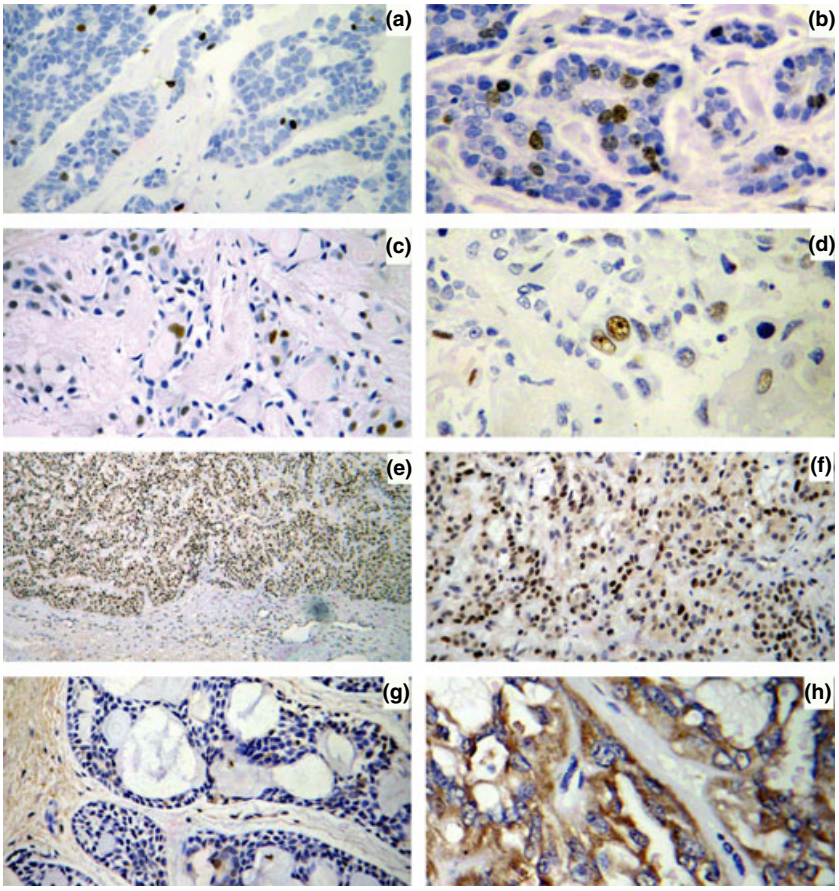

Figure 3 Immunohistochemical photomicrographs showing the expression of the commercial antibodies included in the study. **a** and **b** = KI67 (MIB1), **c** and **d** = p53 (DO7), **e** and **f** = p73 (AB4), **g** and **h** = ΔNp73 (OP181). **a** and **h** = polymorphous low-grade adenocarcinoma, **c** and **g** = adenoid cystic carcinoma, **c**, **e** and **f** = pleomorphic adenoma, **d** = carcinoma ex-pleomorphic adenoma. Original magnification ×400 and ×100 (**e**)

stimulation. On the other hand, Aqeilan *et al*, (2004) were unable to demonstrate p53 binding to Wwox. As p53 may be stabilized when it is mutated or under stress conditions, it is not clear if the p53 stained in our samples is wild-type. In addition, as Wwox antibody did not stain the nuclei, where possibly the interaction with p53 would occur, our study supports the findings of Aqeilan *et al* (2004) showing no clear direct interaction between Wwox and p53, suggesting that Wwox interacts indirectly with p53.

p73 staining was associated with malignancy and with high proliferation index. As all malignant salivary gland neoplasms were positive for p73 immunostaining and all lesions that were negative were benign, negative p73 staining may be used to exclude malignant nature.

DNA aneuploidy was associated with malignancy. Fifty per cent of the samples of carcinoma ex-pleomorphic adenoma were DNA aneuploid, in agreement with Vargas *et al* (2007). Twelve of 13 samples of salivary gland neoplasms that exhibited *WWOX* transcript alterations were diploid, indicating that the molecular alterations of this gene are not responsible for aneuploidy.

It has been suggested that ki67 expression could be a prognostic marker for salivary gland neoplasms (Ben-Izhak *et al*, 2008). This study confirmed a significant relationship between malignancy and high proliferation index and between proliferation and immunopositivity for p73 and p53, consistent with a dysregulated cell cycle.

In conclusion, we have shown that *WWOX* transcription alterations and decreased expression are frequent events in benign and malignant salivary neoplasms, suggesting that *WWOX* alterations are important events in salivary gland tumourigenesis. *WWOX* expression appears independent of p73, proliferation index and chromosomal instability.

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Conflict of interests

All the authors declare that they have no competing interest.

Author Contribution

Gomes CC, Diniz MG and Oliveira CS worked on data acquisition, and worked on literature review. Gomes CC, Gomez RS, De Marco L, Odell EW and Tavassoli M designed

the study. Gomes CC, Diniz MG, Gomes RS, De Marco L, Odell EW and Tavassoli M analysed data. Gomez RS worked on statistical analysis. Gomes CC drafted the paper and all the others revised it.

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