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Annexin A1 subcellular expression in laryngeal squamous cell carcinoma

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Aims: Annexin A1 (ANXA1) is a soluble cytoplasmic protein, moving to membranes when calcium levels are elevated. ANXA1 has also been shown to move to the nucleus or outside the cells, depending on tyrosine-kinase signalling, thus interfering in cytoskeletal organization and cell differentiation, mostly in inflammatory and neoplastic processes. The aim was to investigate subcellular patterns of immunohistochemical expression of ANXA1 in neoplastic and non-neoplastic samples from patients with laryngeal squamous cell carcinomas (LSCC), to elucidate the role of ANXA1 in laryngeal carcinogenesis.

Methods and results: Serial analysis of gene expression experiments detected reduced expression of *ANXA1* gene in LSCC compared with the corresponding non-neoplastic margins. Quantitative polymerase chain reaction confirmed *ANXA1* low expression in 15 LSCC and eight matched normal samples. Thus, we investigated subcellular patterns of immunohistochemical expression of ANXA1 in 241 paraffin-embedded samples from 95 patients with LSCC. The results showed ANXA1 down-regulation in dysplastic, tumourous and metastatic lesions and provided evidence for the progressive migration of ANXA1 from the nucleus towards the membrane during laryngeal tumorigenesis.

Conclusions: ANXA1 dysregulation was observed early in laryngeal carcinogenesis, in intra-epithelial neoplasms; it was not found related to prognostic parameters, such as nodal metastases.

Keywords: annexin A1, head and neck neoplasm, immunohistochemistry, laryngeal neoplasm

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Abbreviations: ANX, annexin; CI, confidence interval; EGFR, epidermal growth factor receptor; H2G, Hyper and Hypo-expressed Genes; HNSCC, head and neck squamous cell carcinoma; OR, odds ratio; PVDF, polyvinylidene-fluoride; qPCR, quantitative polymerase chain reaction; SAGE, serial analysis of gene expression; SCC, squamous cell carcinoma; SDS, sodium dodecyl sulphate

Introduction

Annexins (ANX) are a family of proteins present in many organisms, from mould to humans, regulated by fluctuations in cellular calcium levels and implicated in multiple molecular and cellular processes. The unique calcium- and lipid-binding properties enable them to associate with negatively charged membrane phospholipids in a calcium-dependent and reversible manner. This property links annexins to membrane-related events such as cytoskeletal organization, transport, ion fluxes and, consequently, to cell differentiation and migration.[1]

ANX is composed of a conserved COOH-terminal with repetitive homologous domains responsible for calcium and phospholipid binding properties. The variable N-terminal region, which is unique in length and sequence, interacts with many cytosolic ligands and is subject to post-translational modification such as myristoylation and phosphorylation. N-terminal tyrosine phosphorylation of some annexins is catalysed by the epidermal growth factor receptor (EGFR) and Src-family tyrosine kinases, which alter their proteolytic sensitivity and calcium affinities (for review, see).[2]

Annexins are soluble and localized in the cytoplasm of cells, moving to membranes when calcium levels are elevated. Different studies have shown that some annexins move from cytoplasm to nucleus or outside the cells, both processes apparently dependent on tyrosine-kinase signalling.[3,4] Interestingly, nuclear retention of ANX by site-directed mutagenesis in the nuclear export signal sequence of the N-terminus results in reduced cell proliferation and increased doubling time of cells.[5] Under conditions of inflammation following their induction by glucocorticoids, human ANX are exported outside of cells and may bind membrane receptors, inhibiting the accumulation of inflammatory cells at sites of injury.[1]

The mammalian subfamily A of annexins encompasses human ANX represented by 12 members and classified from A1 to A13.[6] To date, there is no evidence that loss, mutation, translocation or amplification of human *ANX* genes play a causative role in any disease, although abnormal expression levels or localization might contribute to pathological conditions such as inflammatory processes, cardiovascular disease and cancer.

In fact, different members of the ANX family have been reported to be involved in the neoplastic process with a potential tumour suppressor role. For example, *ANX7* has been implicated as a tumour-suppressor gene in prostatic tumours.[7] Furthermore, overexpression of *ANXA2*,[8] *ANXA4*[9] and *ANXA8*[10] is observed in various tumours.

Annexin A1 (ANXA1), a 37-kDa protein, is claimed to participate in cell transformation as well as in inflammation, signal transduction, keratinocyte differentiation, apoptosis and gene expression modulation.[11–16] The relationship between ANXA1 and the neoplastic process may be derived from the fact that it is a substrate of EGFR and other kinases involved in tumour development.[2]

ANXA1 has been shown to be up-regulated in pancreatic carcinoma,[17] hairy cell leukaemia[18] and skin tumours[19] and down-regulated in prostatic,[20] oesophageal,[21] breast[22] and head and neck neoplasms.[23–26]

Head and neck squamous cell carcinomas (HNSCCs) account for a significant proportion of all new cancer diagnoses worldwide, and their incidence, in particular of those arising from the larynx and oral cavity, is increasing in developed countries. Laryngeal SCC is estimated to have affected 11 295 patients in the USA in 2007.[27] In the early stages, these carcinomas frequently cause few symptoms, resulting in a delay in diagnosis, with a significant impact on patient management and overall survival rates. In this context, molecular markers potentially related to multistep carcinogenesis are urgently needed to assess HNSCC, to further our understanding of the mechanisms of disease formation and for screening for potential therapeutic targets.

In the present study, by using serial analysis of gene expression (SAGE) and real-time quantitative polymerase chain reaction (qPCR), we identified and validated reduced expression of *ANXA1* gene (gene ID 301) in laryngeal SCCs. In addition, we investigated subcellular patterns of immunohistochemical expression of ANXA1 in normal and dysplastic areas, primary neoplasia and lymph node metastasis, searching for

evidence for the role of ANX in laryngeal carcinogenesis.

Materials and methods

CASE SELECTION

For SAGE experiments, two fresh samples of primary laryngeal cancer, one with lymph node metastasis (N+ status) and one without lymph node metastasis (N− status), and the corresponding non-neoplastic margins were obtained from patients with surgically resected carcinoma at Hospital do Câncer Arnaldo Vieira de Carvalho, São Paulo, SP.

Immunohistochemical analysis was performed on 241 formalin-fixed paraffin-embedded tissue sections from non-neoplastic mucosa, dysplastic epithelium, primary carcinomas and their lymph node metastases. The samples were obtained from 95 patients with surgically resected laryngeal SCC at Hospital das Clínicas and Hospital Heliópolis, São Paulo, SP, Hospital das Clínicas, Ribeirão Preto, SP and Universidade do Vale do Paraíba, São José dos Campos, SP, between 2002 and 2004. The average age of patients was 58.1 years (SD 10.8, range 27–83 years), and the male/female ratio was 7.7:1. Most patients were smokers or former smokers (72.6%) and had a history of chronic alcohol abuse (66.3%). A small subset of 16 samples (eight laryngeal SCCs and eight matched non-neoplastic surgical margins) from the same group of 95 patients was analysed by Western blot.

The expression of *ANXA1* transcripts was validated by qPCR in fresh samples from a different set of 15 laryngeal SCCs and eight non-neoplastic surgical margins.

The study protocol was approved by the ethics committees of enrolled institutions and by the National Committee of Ethics in Research (CONEP 1763/05, 18/05/2005). Tissue samples were taken after obtaining written informed consent from each patient and processed anonymously. Pathological procedures were performed according to protocols approved by the Brazilian Society of Pathology.[28] All histopathological reports and slides were reviewed by senior pathologists (V.A.F.A., P.M.C., E.R.P., C.S-N.), thus confirming the diagnosis and selecting the most representative areas for immunohistochemistry.

RNA AND PROTEIN EXTRACTION AND REVERSE TRANSCRIPTION

Fresh samples of primary laryngeal cancer were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using TRIzol®LS Reagent (Invitrogen Corp., Carlsbad, CA, USA) and treated with RQ1 RNase-Free DNase (Promega Corp., Madison, WI, USA). cDNA synthesis was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. Total protein was extracted by 100% isopropyl alcohol, 0.3 M guanidine hydrochloride in 95% ethanol, 100% ethanol and 1% sodium dodecyl sulphate (SDS).

SERIAL ANALYSIS OF GENE EXPRESSION

SAGE was carried out using the I-SAGE™ Kit (Invitrogen). Clones were checked and sequenced with forward M13 primer in a MegaBACE™1000 sequencer (Amersham Biosciences, Piscataway, NJ, USA) or PRISM® 377 DNA Sequencer (Applied Biosystems) using DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences), or ABI PRISM® BigDye™ Primer Cycle Sequencing Kit (Applied Biosystems), respectively.

For each SAGE library, 6000 sequencing reactions were performed and the SAGE tags were obtained with SAGE™ Analysis 2000 Software 4.0, with minimum tag count set to 1 and maximum ditag length set to 28 bp, whereas other parameters were set as default. The results were analysed with the help of the tools developed by Hyper and Hypo-expressed Genes (H2G) software (http://gdm.fmrp.usp.br/tools_bit.php) and short tags that exhibited at least a twofold change were selected (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). H2G is a bioinformatics tool designed to select over- and down-regulated genes from SAGE datasets and to evaluate differences in gene expression.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Real-time quantification was performed in duplicate using a Primer Express designed TaqMan assay for *ANXA1*. To normalize sample loading, the differences of threshold cycles (ΔCt) were derived by subtracting the Ct value for the internal reference (*GAPDH*) from the Ct values of the evaluated genes. The relative fold value was obtained by the formula $2^{-\Delta\Delta Ct}$ using the median ΔCt value of surgical margin samples as a reference, and ΔΔCt was calculated by subtracting the reference ΔCt from the ΔCt values of the tumour samples. Expression of all samples was measured in a single plate for each gene evaluated. Kruskal–Wallis with Dunn's post test was performed using Prism 4 (GraphPad Software, Inc., San Diego, CA, USA; http://www.graphpad.com).

IMMUNOHISTOCHEMISTRY

Immunohistochemical analyses were performed using the conventional protocol. Sections from representative formalin-fixed paraffin-embedded samples were immunostained with a monoclonal antibody to ANXA1, with amplification by the streptavidin–peroxidase method. Briefly, after deparaffinization in xylene and rehydration in graded ethanol, antigen epitope retrieval was performed using 10 mM citric acid solution, pH 6.0 in a pressure cooker. Endogenous peroxidase activity was blocked with 6% hydrogen peroxide for 20 min.

Primary mouse anti-annexin 1 monoclonal antibody (clone 29, code no. 610067, BD Transduction Laboratories, San Diego, CA, USA), diluted 1:4000, was incubated for 30 min at 37°C followed by overnight incubation at 4°C, and then by addition of biotinylated antimouse secondary antibody and streptavidin–horseradish peroxidase (LSAB+, code no. k0690; Dako, Carpinteria, CA, USA).

The reaction product was developed by 3,3′-diaminobenzidine and H_2O_2 and counterstaining was performed with Harris haematoxylin. The primary antibody was omitted for negative controls and endothelial cells of tonsil were used as positive control. Immunoexpression of ANXA1 was assessed independently in the nucleus, cytoplasm and membrane and graded subjectively as 0 (no evidence of immunoreac-

Table 1. List of the 20 most abundant tags in laryngeal tumour serial analysis of gene expression (SAGE) libraries and 20 most abundant tags in non-neoplastic SAGE library

Most abundant tags in laryngeal tumour				Most abundant tags in non-neoplastic laryngeal tissue			
Tags	No.	Unigene	Gene symbol	Tags	No.	Unigene	Gene symbol
TACCTGCAGA	3907	Hs.416073	*S100A8*	TACCTGCAGA	3948	Hs.416073	*S100A8*
GAAATAAAGC	2124	Hs.413826	*IGHG3*	GTGGCCACGG	3758	Hs.112405	*S100A9*
GTGGCCACGG	1515	Hs.112405	*S100A9*	TTTCCTGCTC	1845	Hs.139322	*SPRR3*
GTTGTGGTTA	1278	Hs.48516	*B2M*	AGAAAGATGT	1337	Hs.78225	*ANXA1*
TAAACCAAAT	1063	Hs.105924	*DEFB4*	AAAGCGGGGC	823	Hs.74070	*KRT13*
CTTCCTTGCC	915	Hs.2785	*KRT17*	GGGCTGGGGT	729	Hs.430207	*RPL29*
CCCATCGTCC	792	Hs.193989	*TARDBP*	ATCCTTGCTG	728	Hs.412999	*CSTA*
TTTCCTGCTC	706	Hs.139322	*SPRR3*	GCATAATAGG	596	Hs.458236	*LOC352870*
CTGGGTTAAT	647	Hs.298262	*RPS19*	CCCATCGTCC	559	Hs.193989	*TARDBP*
GATCTCTTGG	621	Hs.38991	*S100A2*	GAGGGAGTTT	556	Hs.76064	*RPL27A*
GAGATAAATG	593	Hs.3185	*LY6D*	GGCAGAGAAG	531	Hs.3235	*KRT4*
GGGCTGGGGT	575	Hs.430207	*RPL29*	GGATTTGGCC	527	Hs.302588	*EST*
CGCCGACGAT	552	Hs.265827	*IFI6*	GAAATAAAGC	499	Hs.413826	*IGHG3*
CCTAGCTGGA	509	Hs.401787	*PPIA*	GATCTCTTGG	498	Hs.38991	*S100A2*
TAGGTTGTCT	476	Hs.401448	*TPT1*	TAGGTTGTCT	483	Hs.401448	*TPT1*
AAAAAAAAAA	466	Hs.0	No unigene cluster	GTGGAAGACG	468	Hs.80395	*MAL*
GGATTTGGCC	454	Hs.302588	*EST*	GGCAAGCCCC	368	Hs.425293	*RPL10A*
AAAGCACAAG	446	Hs.367762	*KRT6A*	TGGGGAGAGG	347	Hs.288998	*S100A14*
GAGGGAGTTT	399	Hs.76064	*RPL27A*	CTCCCCCAAG	341	Hs.366	*MGC27165*
GCATAATAGG	361	Hs.458236	*LOC352870*	TGCACGTTTT	323	Hs.169793	*RPL32*

tivity), grades 1 (5–25% of positive cells), 2 (26–50%), 3 (51–75%) and 4 (>75% of positive cells). Expression differences were evaluated between cases showing (a) negative or grade 1 and (b) grades 2, 3 and 4.

WESTERN BLOT

For Western blot analysis, the antibodies used were polyclonal anti-ANXA1 diluted 1:1000 (Zymed Laboratories, Cambridge, UK), and monoclonal anti-β-actin antibody diluted 1:5000 (Sigma-Aldrich, St Louis, MO, USA). In brief, protein samples (9 μg) were loaded onto 12% resolving gel with 5% stacking gel (SDS–polyacrylamide gel electrophoresis) in denaturing conditions at 130 V for 90 min. The molecular weight ladder was the PageRuler™ Prestained Protein Ladder (Fermentas Life Sciences, Glen Burnie, MD, USA).

The proteins were then transferred electrophoretically (325 mA per blot 70 min; Mini Protean 3 Cell, BioRad, Hercules, CA, USA) to polyvinylidenefluoride (PVDF) paper (Immobilon, Millipore, Billerica, MA, USA) soaked in transfer buffer (25 mM Tris, 0.2 M glycine) and 20% methanol v/v. The PVDF membranes were submitted to chromogenic staining using the Western Breeze kit (Invitrogen). The blots were then scanned and analysed (Gel Logic HP 2200; Carestream Health, Rochester, NY, USA).

STATISTICAL ANALYSIS

To evaluate if the subcellular ANXA1 expression pattern was similar in sections from dysplasia, tumour and metastases from the same individual, different immunohistochemical results were analysed using non-parametric unbalanced repeated measures ANOVA.[29] Differences in immunohistochemical results were also analysed using χ^2 test and Fisher's exact test with Bonferroni correction. The association of ANX immunoexpression with presence or absence of node metastasis was analysed by χ^2 and Fisher's exact test. Statistical significance was set at $P < 0.05$.

Results

SAGE

Approximately 100 000 tags were obtained by sequencing three SAGE libraries, obtained from two samples of SCC of the larynx and from a pool of the corresponding non-neoplastic margins. Excluding redundancy, this approach identified about 17 000 non-redundant tags in each library. The annotation was based on two specific tools, SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE/) and CGAP SAGE Genie (http://cgap.nci.nih.gov/SAGE). The 20 most abundant transcripts for each library are listed in Table 1.

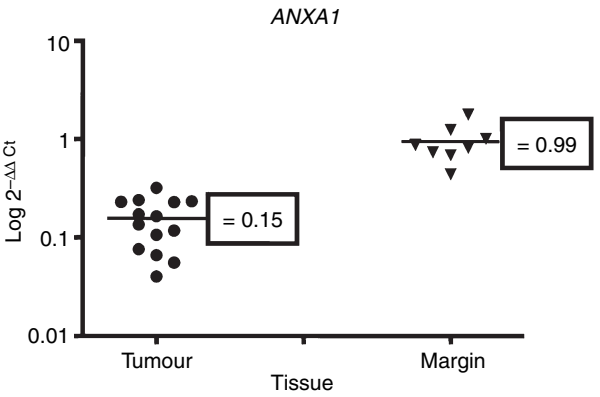

Figure 1. Validation of *ANXA1* gene by real-time polymerase chain reaction (PCR). Quantitative PCR was carried out on 15 squamous cell carcinoma and eight tumour margin samples. Gene expression is shown as log $2^{-\Delta\Delta Ct}$ ($\Delta\Delta$Ct ranged from 0.04053 to 0.70222). Differences between tumour and normal samples were significant ($P < 0.001$).

QUANTITATIVE REAL-TIME PCR

Based on the normalized tag ratios of tumour/non-neoplastic tissues, a set of genes was selected to be validated by real-time PCR, using the H2G tool. The results obtained for *ANXA1* transcripts in 15 laryngeal SCCs and eight non-neoplastic margins are shown in Figure 1. A significant reduction ($P < 0.001$) of ANXA1 transcript levels in tumour samples was observed.

IMMUNOLOGICAL ANALYSIS

Immunohistochemical analysis was performed in 241 samples from 95 patients with laryngeal SCC. Of these, 90 patients had cervical lymph node resection and, thus, had valid pathological information about node metastasis. The immunohistochemistry for ANXA1 in nuclei, cytoplasm and membrane of non-tumour, dysplastic, tumour and metastatic areas of these laryngeal SCCs is presented in Figure 2.

As depicted in Table 2, ANXA1 was detected in nuclei of 88.5% of non-neoplastic squamous epithelial samples, contrasting with only 69.0% of dysplastic samples, 67.0% of primary carcinomas and 62.5% of lymph node metastases. Cytoplasmic ANXA1 immunoreactivity was detected in 98.7% of normal tissues and in 93.1, 86.4 and 87.5% of dysplastic, tumour and

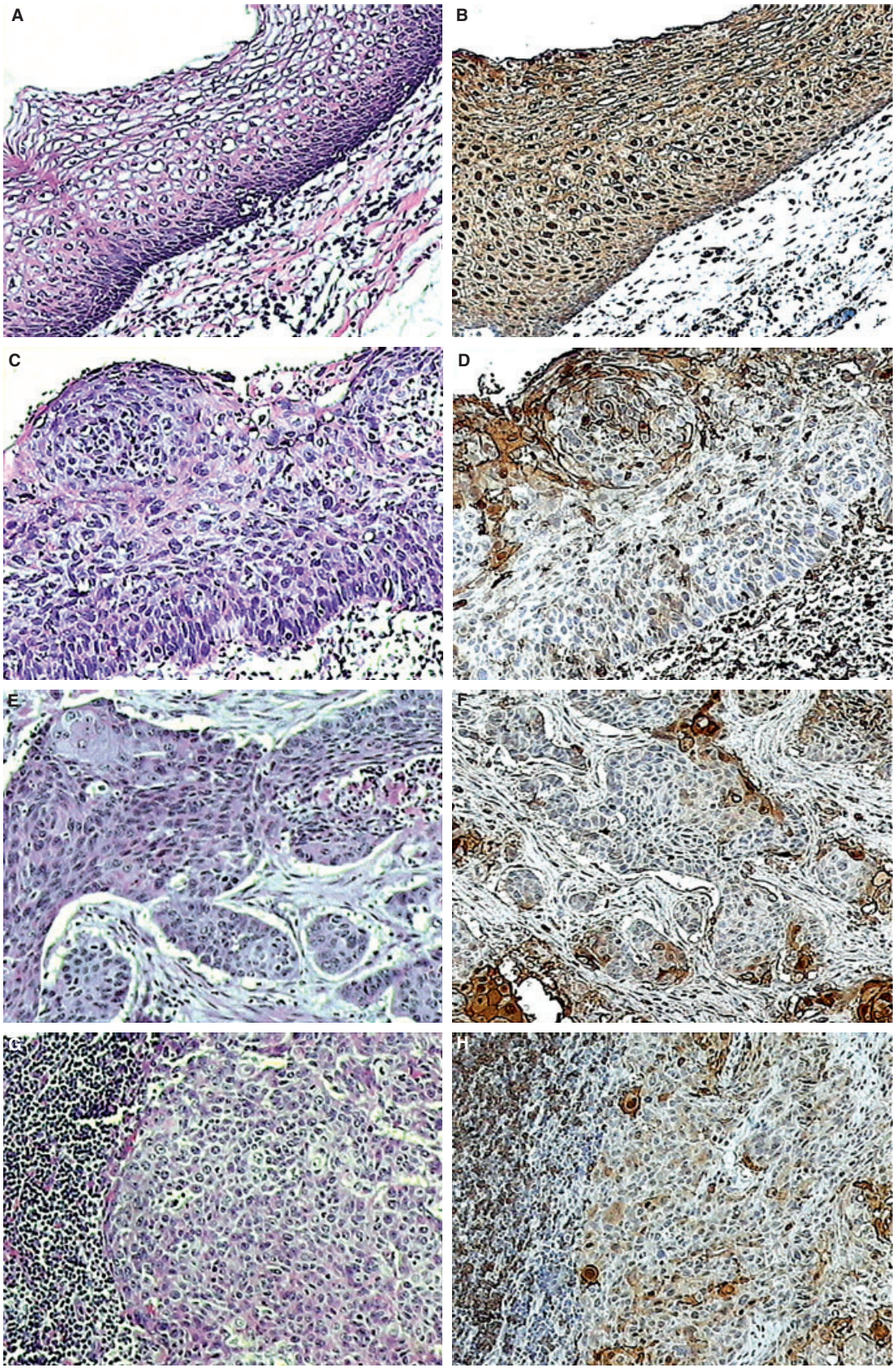

Table 2. Frequency of nuclear, cytoplasmic and membranous immunoreactivity of ANXA1 in normal, dysplastic, primary tumour and metastatic cells from 95 patients with laryngeal squamous cell carcinoma

Immunohistochemical reactivity	Normal tissue		Dysplastic cells		Tumour		Metastasis	
	n	%	*n*	%	*n*	%	*n*	%
Nucleus								
Negative	9	11.5	9	31.0	29	33.0	9	37.5
Positive $P < 0.001$	69	88.5	20	69.0	59	67.0	15	62.5
Cytoplasm								
Negative	1	1.3	2	6.9	12	13.6	3	12.5
Positive $P < 0.001$	77	98.7	27	93.1	76	86.4	21	87.5
Membrane								
Negative	36	46.2	14	48.3	28	31.8	11	45.8
Positive $P < 0.001$	42	53.8	15	51.7	60	68.2	13	54.2
Total	78	100.0	29	100.0	88	100.0	24	100.0

metastatic areas, respectively. Neoplastic areas exhibited the highest frequency of membranous immunoreactivity.

Using χ^2 test and Fisher's exact test with Bonferroni correction ($\alpha = 0.05/6 = 0.0083$), we performed a 2×2 comparison (six different comparisons) of nuclear, cytoplasmic and membranous ANXA1 expression among normal, dysplastic, primary tumour and metastatic areas (Table 3). The results showed significantly lower expression in the nucleus and cytoplasm of tumour compared with normal tissues, as well as higher expression in the membrane of tumour versus normal samples. Furthermore, differences in expression were found in the cytoplasm and membrane when comparing dysplasia with tumour and tumour with metastasis.

Statistical analysis of cytoplasmic ANXA1 expression in normal tissue showed significant differences ($P < 0.05$) in relation to dysplastic, tumour and metastatic tissues considering the dependence measurements for each patient (Table 4). Significant differences in cytoplasmic immunoreactivity were also observed between dysplasia and tumour or metastasis and between tumour and metastasis. Membranous and nuclear reactivity were similar between normal and tumour areas and between dysplasia and metastasis, but significantly different in dysplasia or metastasis compared with normal or tumour tissues.

The loss of immunoexpression of ANXA1 was not predictive for the presence of lymph node metastasis. The odds ratios (OR) of having node metastasis in the group showing ANXA1 loss in any subcellular localization relative to the OR of having no metastasis were 0.549 [$P = 0.196$; confidence interval (CI) 0.22, 1.37], 0.44 ($P = 0.309$; CI 0.11, 1.69) and 0.63 ($P = 0.329$; CI 0.25, 1.60) for nuclear, cytoplasmic and membranous ANXA1 immunoreactivity, respectively.

The results of Western blot for ANXA1 expression in 16 samples were quantified and normalized against β-actin. The data were consistent with the immunohistochemical study and showed higher levels of ANX in most surgical margins than in tumours (Figure 3). In all samples, the uncleaved protein (37 kDa) and two cleaved fragments (approximately 33 and 35 kDa) were observed. Pathological features as well as Western blot and immunohistochemistry results are summarized in Table 5.

Discussion

By using SAGE, we observed a significant reduction in gene expression of *ANXA1* in laryngeal SCC. In addition, by immunohistochemistry on a different set of samples, we also detected decreased immunoexpression of ANXA1 in the nucleus and cytoplasm of dysplastic, primary tumour and metastatic lymph node cells of larynx compared with normal tissue. The decreased expression of the protein was observed not in all, but in a significant proportion of cases.

Figure 2. Immunohistochemical features of laryngeal squamous cells. Non-tumoural areas (**A,B**) strongly express ANXA1 in nuclei, cytoplasm and membrane. ANXA1 expression is lower in dysplastic areas (**C,D**), tumoural samples (**E,F**) and in metastatic areas (**G,H**), especially in nuclei and in cytoplasm (**A,C,E,G,** H&E; **B,D,F,H,** annexin A1, LSAB).

Table 3. Results of a 2 × 2 comparison of nuclear, cytoplasmic and membranous ANXA1 expression among normal, dysplastic, primary tumour and metastatic cells from 95 patients with laryngeal squamous cell carcinoma

	P-value		
Areas	Nucleus	Cytoplasm	Membrane
Normal × dysplasia	0.038**	0.178**	0.845*
Normal × tumour	0.002*	<0.001*	<0.001*
Normal × metastasis	0.011**	0.040**	0.978
Dysplasia × tumour	0.937*	0.006*	<0.001*
Dysplasia × metastasis	0.621*	0.649**	0.859*
Tumour × metastasis	0.600*	0.049*	0.001**

*χ^2 test. **χ^2 and Fisher's exact test with Bonferroni correction ($\alpha = 0.05/6 = 0.0083$).

Table 4. Comparison of nuclear, cytoplasmic and membranous ANXA1 immunoexpression among normal, dysplastic, primary tumour and metastatic cells from 95 patients with laryngeal squamous cell carcinoma

	P-value*		
Areas	Nucleus	Cytoplasm	Membrane
Normal × dysplasia	<0.0001	<0.0001	<0.0001
Normal × tumour	0.0589	0.0035	0.1105
Normal × metastasis	<0.0001	<0.0001	<0.0001
Dysplasia × tumour	<0.0001	<0.0001	<0.0001
Dysplasia × metastasis	0.1554	0.0216	0.1784
Tumour × metastasis	<0.0001	<0.0001	<0.0001

P-values were calculated considering non-parametric unbalanced repeated measures ANOVA.

SAGE is a high-throughput technique that allows measurement of expression levels of a large number of transcripts. Although SAGE is a very powerful method for detecting both known and unknown transcripts, it usually requires independent confirmation at protein level. For this reason, we used Western blot and immunohistochemistry to validate changes in *ANXA1* expression. Similar to SAGE, Western blot requires unfixed tissue, but in contrast to immunohistochemistry, reveals no details of topography or subcellular expression. Therefore, immunohistochemistry was a good choice for visualizing pathological features, semiquantitatively measuring expression of ANX and evaluating its localization.

The loss of ANXA1 protein immunoexpression was found herein as an early event in head and neck oncogenesis, detected in the pre-invasive stages. This important finding confirms and extends previous studies of Garcia Pedrero and collaborators,[23] who observed down-regulation of ANXA1 in 11 out of 16 laryngeal carcinomas. Those authors also reported lower immunoexpression of ANXA1 in eight dysplastic lesions of the head and neck, although no information was provided regarding the sites of these lesions. Vishwanatha and collaborators[30] found loss of anti-inflammatory activity of ANXA1 and up-regulation of proinflammatory cyclooxygenase-2 in oral cells exposed to smokeless tobacco, thus proposing that the dual effect of these regulatory events might lead the cells down the carcinogenic pathway. Silistino-Souza and collaborators[26] have also shown down-regulation of ANXA1 expression in the nucleus and cytoplasm of surgical tissue specimens from 20 patients with laryngeal cancer and a significant increase of the protein in the cytoplasmic granules and nuclei of tumour and peritumoral mast cells. These authors suggested that tumour cells could recruit and activate mast cells to release biological mediators, which may alter the microenvironment and promote or inhibit tumour growth. Taken together, these findings may provide an important link between inflammatory mediators and carcinogenesis in HNSCCs, since tobacco consumption is the commonest aetiological risk factor for the development of these tumours[31] and hyperproliferation of head and neck squamous cells is nearly always found in the context of chronic inflammation.

An important discrepancy between our findings and those of Garcia Pedrero and collaborators[23] is that, in the present study, down-regulation of ANXA1 was not predictive for lymph node metastases. In keeping with the fact that ANX down-regulation is an early event and possibly not strictly related to progression, this result might be site-dependent, since our study was focused specifically on laryngeal SCCs.

The reduced ANX expression in HNSCC as well as in other tumours may be explained by DNA mutations, post-transcriptional/post-translational events or by regulatory mechanisms. Loss of heterozygosity in the 9q12-q21.2 region has been described in HNSCC[32] and may affect the promoter or the coding sequence of the *ANXA1* gene. Otherwise, alterations at RNA level or epigenetic mechanisms are probably uncommon and have not been described. Post-translational events such as ANXA1 phosphorylation are of interest, especially

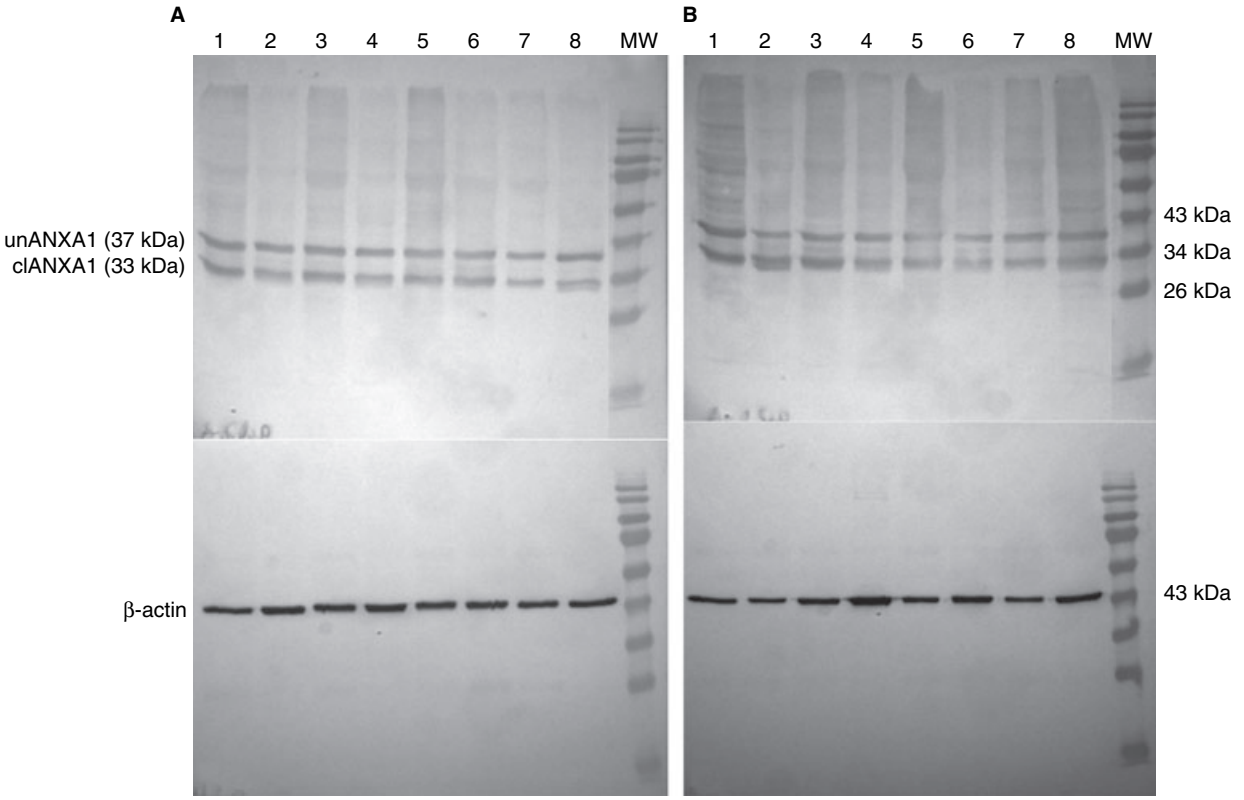

Figure 3. Analysis of ANXA1 protein. Representative Western blots illustrating ANXA1 expression in a subset of (**A**,**B**) eight laryngeal squamous cell carcinomas and eight matched non-neoplastic surgical margins by using anti-annexin A1. **A**, Surgical margins (lanes 1, 3, 5, 7) and tumour samples (lanes 2, 4, 6, 8) from patients 105, 102, 96 and 136, respectively. **B**, Surgical margins (lanes 1, 3, 5, 7) and tumour samples (lanes 2, 4, 6, 8) from patients 35, 31, 19 and 18, respectively. MW, molecular weight marker. Uncleaved ANXA1 (unANXA1): 37 kDa; cleaved fragment (clANXA1): 33 kDa. β-actin was used as an internal control.

because annexin is a substrate of EGFR,[2] which is overexpressed and serves as a tumour growth marker in HNSCC.[33] Phosphorylated ANXA1 is apparently prone to tryptic cleavage at the N-terminal region and may be involved, for example, in controlling and limiting leucocyte emigration into inflamed tissue through ANXA1 N-terminal peptides.[34]

By Western blot, we could observe three ANXA1 fragments at almost the same molecular weight, supporting the fact that ANXA1 has three cleavable sites. Since the annexin N-terminal domain is susceptible to cleavage, tissue storage conditions or protein extraction procedures may influence the levels of peptides. However, the fresh samples of primary laryngeal cancer analysed in the present study, once collected, were immediately snap-frozen and stored at −80°C until protein isolation. Our Western blot results also indicate, in most cases, a satisfactory correlation with ANXA1 expression data obtained by immunohistochemical analysis. Differences can be explained by the use of different antibodies with distinctive binding properties.

Recently, Sakaguchi and collaborators[35] have observed EGF-induced ANXA1 cleavage in normal human keratinocytes and have shown that phosphorylation of ANXA1 occurs before the cleavage event by lysosomal enzymes. The authors also showed that this pathway is constitutively activated in squamous cancer cells.

Garcia Pedrero,[23] Rodrigo[25] and collaborators have detected low ANXA1 expression significantly associated with parameters of aggressiveness in HNSCCs. Since immunohistochemical analysis showed strong positive signals in differentiated areas, whereas poorly differentiated tumours were commonly found to be negative; those authors suggested that ANXA1 expression is in fact linked to epithelial differentiation.

To the best of our knowledge, this is the first study to assess the intracellular distribution of ANXA1 in a large set of laryngeal carcinomas, except for the 20 cases in the above-mentioned study of Silistino-Souza and collaborators.[26] The only other study of such subcompartmentalization that could be found regard-

Table 5. Pathological features of eight patients with laryngeal squamous cell carcinoma as well as the results of Western blot and immunohistochemical analysis

	Patients															
Features	105		102		96		136		35		31		19		18	
Surgical margins	Tumour free		Tumour free		Tumour free		Tumour free		Tumour free		Tumour free		Tumour free		Tumour free	
Differentiation	Moderate		Well				Well		Moderate		Moderate		Moderate		Moderate	
TNM	T4N2cM0		T2N1M0		T4N0M0		T4N2bM0		T4N0M0		T4N2cM0		T4N0M0		T4N2bM0	
Neural invasion	+		–		–		–		–		+		–		–	
Depth of infiltration	Invasive		Cartilage		Cartilage		Cartilage		Invasive		Invasive		Invasive		Cartilage	
Lymphatic infiltration	–		–		–		–		–		–		–		–	
Vascular infiltration	–		+		–		–		–		–		–		–	
Inflammatory peritumoral infiltration	Moderate		Weak		Weak		Weak		Moderate		Moderate		Weak		Moderate	
Immunoreactivity	Tu	M	Tu	M	Tu	M	Tu	M	Tu	M	Tu	M	Tu	M	Tu	M
Nucleus	2	4	2	3	2	4	3	3	1	2	3	3	3	1	3	1
Cytoplasm	3	4	3	4	2	4	4	4	2	4	4	4	3	4	3	2
Membrane	3	1	4	4	3	4	4	2	4	2	3	1	2	2	3	3
Western blot*	1.03	1.48	0.87	1.03	1.02	1.26	1.08	0.93	1.82	1.72	1.12	1.41	1.17	1.32	1.39	1.47

*Relative intensity of the ANXA1 (cleaved and uncleaved protein) signal in Western blot.

ing upper aerodigestive tract tumours was from Liu and collaborators[36] in oesophageal carcinomas. Although those authors did not mention the number of cases studied, they reported a reduction of ANXA1 immunoexpression on the cell membrane and nucleoplasm of neoplastic squamous cells, contrasting with an increase in nuclear membranous immunofluorescence, thus suggesting that translocation of ANXA1 from the cellular to nuclear membrane might be related to oesophageal oncogenesis. Because of differences in technique (primary anti-ANXA1 monoclonal antibody: Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, and indirect immunofluorescence versus clone 29, BD Transduction Labs and streptavidin–biotin LSAB+ Dako) or in the specific organ investigated (larynx versus oesophagus), we were not able to confirm such differences in the intracellular pattern of ANXA1 immunoexpression. In fact, we observed differences in membranous ANXA1 expression (data from Table 4) between normal and dysplasia (or metastasis), but not between normal and invasive tumour from the same individual. Otherwise, applying the χ^2 and Fisher's exact tests, nuclear and cytoplasmic expression were lower and membranous expression higher in primary carcinoma compared with normal tissues (data from Table 3). However, the latter data should be interpreted with caution since the ANXA1 levels in tissue areas and subcellular compartments are interdependent variables.

To explain why membranous ANXA1 expression does not follow the same pattern as nucleus and cytoplasmic expression in our laryngeal samples, a better understanding of the factors influencing annexin expression dysregulation, translocation and exportation would be necessary. Also, it would be important to know if these factors are related to tumour cell characteristics and activated or inhibited by chronic inflammation in tumours, which is nearly always found in HNSCCs. In this respect, discussion of data on the influence of calcium levels and protein phosphorylation on ANX trafficking may be pertinent.

Different annexins are distributed in a diffuse pattern throughout the cytosol or localized to specific regions or structures in the cell at low free calcium levels. After stimulation, each one assumes a distinct position at

cellular membranes.[37] Monastyrskaya and collaborators[38] have mapped the calcium-induced translocations of annexins in live cells and observed that upon elevation of calcium, ANXA1 becomes associated with intracellular membranes and the nuclear envelope. Because changes in calcium levels regulate signalling events, different annexins may allow a spatially confined, graded response to extra- or intracellular stimuli.[37]

Solito and collaborators[39] have provided molecular, microscopic and pharmacological evidence supporting the view that the trafficking of ANXA1 from the cytoplasm to the cell surface induced by a proinflammatory stimulus is dependent on serine[27] phosphorylation of ANX, and both phosphoinositide-3–kinase and mitogen-activated protein kinase are critical to this event. These kinases are activated in response to different stimuli, including growth factors and environmental stresses, and are involved in various responses such as cell death, survival, cell motility and differentiation. In addition, the authors have suggested that both phosphorylation and lipidation may contribute to ANXA1 export outside the cell. Since ANXA1 is related to cell proliferation and differentiation processes, the analysis of post-translation modifications and subcellular expression may help in understanding the role of this protein in normal and pathological conditions.

In summary, translocation of ANX between cellular compartments in normal cells is affected by calcium levels, and is dependent on phosphorylation or induced by glucocorticoids during inflammation.[1,39–41] In tumour cells, calcium levels and other factors may be impaired and result in abnormal localization of annexins. The explanation for the dysregulation of expression and translocation of annexins in tumours remains to be established and may be cause or effect, linked to inflammation, signal transduction, differentiation, transport or other cell processes.

The importance of the ANX family is emerging and is likely to contribute to our understanding of the link between inflammation, hyperproliferation of epithelial cells and carcinogenesis. Their role in molecular pathways[2,16,42,43] as well as their clinical implications,[44] are just beginning to emerge in the literature. However, much information is still lacking and prospective studies, dealing with diagnosis, prognosis and treatment, are needed.

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Appendix

The GENCAPO (Head and Neck Genome) Project members are the following: Cury PM[3], de Carvalho MB[7], Dias-Neto E[1], Figueiredo DLA[8], Fukuyama EE[5], Góis-Filho JF[5], Leopoldino AM[12], Mamede RCM[8], Michaluart-Junior P[1], Moreira-Filho CA[1], Moyses RA[1], Nóbrega FG[4], Nóbrega MP[4], Nunes FD[6], Ojopi EPB[1], Okamoto OK[11], Serafini LN[8], Severino P[2], Silva AMA[7], Silva Jr WA[8], Silveira NJF[10], Souza SCOM[6], Tajara EH[3], Wünsch-Filho V[9], Zago MA[8], Amar A[7], Arap SS[1], Araújo NSS[1], Araújo-Filho V[1], Barbieri RB[7], Bandeira CM[4], Bastos AU[7], Bogossian AP[4], Braconi MA[4], Brandão LG[1], Brandão RM[8], Canto AL[4], Carmona-Raphe J[3], Carvalho-Neto PB[7], Casemiro AF[7], Cerione M[5], Cernea CR[1], Cicco R[5], Chagas MJ[4], Chedid H[7], Chiappini PBO[7], Correia LA[7], Costa A[9], Costa ACW[7], Cunha BR[3], Curioni OA[7], Dias THG[1], Durazzo M[1], Ferraz AR[1], Figueiredo RO[9], Fortes CS[9], Franzi SA[7], Frizzera APZ[3], Gallo J[1], Gazito D[7], Guimarães PEM[1], Gutierres AP[7], Inamine R[9], Kaneto CM[8], Lehn CN[7], López RVM[9], Macarenco R[4], Magalhães RP[1], Martins AE[7], Meneses C[4], Mercante AMC[7], Montenegro FLM[1], Pinheiro DG[8], Polachini GM[3], Porsani AF[7], Rapoport A[7], Rodini CO[6], Rodrigues AN[9], Rodrigues-Lisoni FC[3], Rodrigues RV[3], Rossi L[7], Santos ARD[8], Santos M[7], Settani F[5], Silva FAM[12], Silva IT[8], Silva-Filho GB[1], Smith RB[1], Souza TB[7], Stabenow E[1], Takamori JT[7], Tavares MR[1], Turcano R[1], Valentim PJ[5], Vidotto A[3], Volpi EM[1], Xavier FCA[6], Yamagushi F[5], Cominato ML[5], Correa PMS[4], Mendes GS[5], Paiva R[5], Ramos O[1], Silva C[1], Silva MJ[5], Tarlá MVC[8].

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