■ ORIGINAL ARTICLE

Annexin A2 Expression in Head and Neck Squamous Cell Carcinoma

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Objective: Over-expression of annexin A2 (ANXA2) has been reported in various cancers. However, no data are available on the expression of this protein in head and neck squamous cell carcinomas (HNSCC). The objective of this preliminary study is to investigate the expression of ANXA2 in these carcinomas.
Material and method: ANXA2 expression was analyzed by immunohistochemistry in paraffin-embedded sections from 9 patients with premalignant lesions and 21 patients with HNSCC.
Results: All dysplastic tissues showed significantly reduced ANXA2 expression compared to normal tissue. In contrast, ANXA2 expression was observed in all but 1 of the tumours studied. There was a significant correlation of lower ANXA2 expression with a poorer histological differentiation, larger tumours, and nodal metastases.
Conclusions: Our data show for the first time that ANXA2 is expressed in head and neck squamous cell carcinomas and that its expression seems to be related with the degree of differentiation status of these tumours.

Key words: Annexin A2. Cancer. Head and neck. Expression.

Expresión de la anexina A2 en los carcinomas epidermoides de cabeza y cuello
Objetivo: La expresión de la anexina A2 (ANXA2) se ha hallado elevada en varios cánceres. Sin embargo, no hay datos disponibles de la expresión de esta proteína en los carcinomas epidermoides de cabeza y cuello. El objetivo de este estudio preliminar es investigar la expresión de la ANXA2 en estos carcinomas.
Material y método: Se analizó la expresión de la ANXA2 mediante inmunohistoquímica en muestras incluidas en parafina de 9 lesiones premalignas y 21 carcinomas epidermoides de cabeza y cuello.
Resultados: Todas las lesiones con displasia mostraron una reducción en la expresión de la ANXA2 respecto al tejido normal. En contraste, se apreció expresión de la ANXA2 en todos menos uno de los tumores estudiados. La disminución de la expresión de la ANXA2 en los carcinomas se correlacionó de forma significativa con una peor diferenciación histológica, con tumores de mayor tamaño y con metástasis ganglionares.
Conclusiones: Nuestros datos muestran por primera vez que la ANXA2 se expresa en los carcinomas epidermoides de cabeza y cuello e indican que su expresión se relaciona con el grado de diferenciación de estos tumores.

Palabras clave: Anexina A2. Cáncer. Cabeza y cuello. Expresión.

INTRODUCTION

Annexins constitute a family of proteins characterized by their capacity to bind to phospholipids in the presence of the calcium ion, their susceptibility to phosphorylation and dephosphorylation, and their capacity to suppress the phospholipase A2.

Human annexins belong to the subfamily A of annexins of vertebrates, of which 12 members have been identified. They are designated with the symbol ANX followed by a suffix that indicates the number of the annexin, from A1 to A11 and later A13, since there is no annexin A12. Their interactions with other proteins and their apparent role in

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signal-transduction have allowed them to be implicated in the cellular processes related to the maintenance of the cytoskeleton and its interaction with the extracellular matrix, growth and tissue differentiation, inflammation, and blood clotting.[1] Annexin A2 (ANXA2) is 1 of the youngest and most divergent, according to phylogenetic analysis, of the 12 human annexins.[2] Several annexins have been implicated in carcinogenesis, to the point where the altered expression of 1 of them has been associated with the transformation of cell lines,[3] tumour progression,[4] and metastasis.[5,6] This has indicated that annexins could have a possible role as tumour suppressors.

When located on the cell surface, ANXA2 serves as a receptor or binding protein for proteases (cathepsin B, plasminogen, and tissue plasminogen activator) and proteins in the extracellular matrix (collagen and tenascin C). It has been proposed that this relationship between proteases and extracellular matrix proteins through ANXA2 may facilitate the reshaping of the extracellular matrix in physiological and pathological processes, like tumoral invasion.[7] In this sense, overexpression of the ANXA2 has been described in malignant brain, lung, liver, pancreas, colon, and haematological tumours.[8-15] But the diminished expression of this protein has also been described in prostate cancer.[16]

Table 1. Clinical-Pathological Characteristics of the Tumours Studied

Characteristic	
Age, mean (median), y	59 (61)
Sex, n (%)	
Males	20 (95)
Females	1 (5)
Location, n (%)	
Oropharynx	8 (38)
Supraglottis	5 (24)
Glottis	3 (14)
Hypopharynx	5 (24)
pT classification, n (%)	
T1	4 (19)
T2	3 (14)
T3	9 (43)
T4	5 (24)
pN classification, n (%)	
N0	8 (38)
N1-3	13 (62)
Stage, n (%)	
I	3 (14)
II	2 (10)
III	3 (14)
IV	13 (62)
Degree of differentiation, n (%)	
Well-differentiated	11 (52)
Moderately-differentiated	6 (29)
Poorly-differentiated	4 (19)
Total, n	21

The magnitude and specificity of the changes in the expression of ANXA2 in various cancers point to its potential role in carcinogenesis and as a tumoral marker.

The expression of this protein had already been described[17] in the normal epithelium of the upper airways and digestive tracts, but there are no data available on the expression of ANXA2 in epidermoid carcinomas of the head and neck (ECHN). In this preliminary work we use immunohistochemistry to analyze the expression of ANXA2 in a group of precancerous lesions and invasive carcinomas of the pharynx and larynx, together with normal epithelia matched to each patient, to try to establish its potential role in these carcinomas.

MATERIAL AND METHOD

Tissue Samples

Surgical tissue samples were obtained from 21 consecutive patients with epidermoid carcinomas in the head and neck and samples from 9 patients with premalignant laryngeal lesions from the pathology files at our hospital, following the guidelines of the ethics committee.

The patients selected for this study were treated in 2001 and have a follow-up time of at least 2 years. Representative sections of the tissue were selected for the study, and a pathologist confirmed the diagnosis of each lesion. None of the patients had received radiotherapy and/or chemotherapy before the intervention. In all cases there was a histologically normal mucosa adjacent to the tumour.

The characteristics of the patients studied and the clinical-pathological traits of their tumours (location, pT classification, pN classification, sickness stage, and grade differentiation) are shown in Table 1. The disease progression stage was determined after surgical resection of the tumour, in accordance with the TNM system of the International Union Against Cancer (6th edition). The histological grade was determined in accordance with the differentiation of the tumour (Broders classification). All patients were habitual consumers of tobacco and alcohol.

The distribution of the patients with premalignant lesions according to the histological diagnosis was: hyperplasia/hyperkeratosis (4 cases), slight dysplasia (1 case), moderate dysplasia (2 cases), and severe dysplasia/in-situ carcinoma (2 cases).

Immunohistochemical Study

The tissues fixed with formaldehyde and mounted in paraffin were cut into sections of 4 µm and adhered to silicone-coated slides (ChemMate, Dako, Carpinteria, CA, United States). The section was dewaxed with xylene and hydrated in alcohols of different gradation as per the conventional method. For the recovery of the antigen, a citrate buffer heated for 10 min in a pressure cooker was used.

Staining was carried out at room temperature in an automated staining station (TechMate 1000, BioTEK Solutions).

The slides were placed for 20 min in a 3% hydrogen peroxidase blocking medium and later were allowed to react for 10 min with the murine monoclonal antibody IgG anti-ANXA2 (Zymed Laboratories, San Francisco, CA, United States) in a 1:400 dilution for 30 min. Immunodetection was carried out with the Envision system (Envision Plus, Dako, Carpinteria, CA, United States) using diaminobenzedine[18] as a chromogen. The preparation is contrasted with haematoxylin for 1 min as a last step. After staining, the slides were dehydrated with alcohols of different gradation, as per the conventional method, and were mounted with cover slides using a standard medium.

The positive controls utilized were samples of normal laryngeal epithelia obtained from non-oncological surgery. Negative controls were also used, which consisted of samples in which the primary antibody was omitted.

The slides were studied at random, without clinical data. Staining was predominantly membranous. Given that the majority of the cells, when positive, showed intensive staining, a score was assigned based only on the percentage of cells with membranous staining solution (0%-100%). At least 500 tumoral cells were counted for each case, following a stereological method based on random systematic sampling[19]: within a defined area of the lesion, 1 out of every 5 consecutive fields of vision was systematically included and the positive and negative cells were counted. In each case 5 fields of vision were evaluated, amplified 400 times.

Statistical Analysis

Statistical analysis was carried out with the SPSS version 8.0 statistical package. The mean staining scores were compared within the stratified groups with respect to clinical pathology parameters (T classification, N classification, grade differentiation, location, and recurrence) and within the group as a whole. Variance analysis was used for this comparison.

The survival curves were calculated using the Kaplan-Meier method. Deaths for causes other than the index tumour or its metastases were not considered to be treatment failure and were excluded from the final analysis of specific survival for the illness. The differences between the survival curves were analyzed using the logarithmic ranges method. To analyze the effect of ANXA2 expression on survival, the staining scores were split taking the median as the cut-off point. The survival curves of the staining scores between the different percentiles were obtained before the split. The cut-off point that supplied the best discrimination between the survival curves was chosen. Values of $P<.05$ were considered statistically significant.

RESULTS

Expression of ANXA2 in Normal Epithelia

All the slides selected for the study contained normal and malignant epithelia. As previously described, the expression of ANXA2 was detected in cells of the basal and suprabasal layers of the normal epithelium, with a lack of staining in the majority of the most external and differentiated layers (Figure 1A). ANXA2 staining was preferably membranous in location, although some cytoplasmic staining was observed, especially in cells on the basal layer. We also observed strong staining of endothelial cells and fibroblasts.

Expression of ANXA2 in Premalignant Lesions

Immunohistochemical analysis was carried out on 9 samples of premalignant lesions (distributed as described in "Material and Method") to determine whether ANXA2 expression changes early on in the carcinogenesis of head and neck epidermoid carcinomas. All the slides selected included normal epithelia as an internal control. Hyperplastic epithelium showed the same ANXA2 expression pattern as normal epithelium. However, a loss of ANXA2 expression was detected in dysplastic tissue, in contrast with the strong ANXA2 signal detected in the corresponding normal epithelia (Figure 1B).

Expression of ANXA2 in Epidermoid Carcinomas of the Head and Neck

All except 1 of the 21 samples of carcinogenic tissue studied showed some degree of expression of ANXA2. Just as in normal epithelia, ANXA2 was strongly expressed in the cellular membrane, while an insignificant expression was observed in the cytoplasm of carcinogenic cells. Moreover, ANXA2 was expressed weakly in the fibrous stroma of the tumoral tissue.

Table 2 shows the correlation between the expression of ANXA2 and the clinical-pathological findings in the 21 tumours examined. The expression of this protein correlated significantly with the histopathological grade ($P<.001$). Thus, the expression of ANXA2 in well-differentiated tumours was seen to be greater than in moderate and poorly-differentiated ones. We observed the staining of ANXA2 in almost all the well-differentiated carcinogenic cells in head and neck epidermoid carcinomas (Figure 1C), while in the poorly-differentiated carcinomas the majority of the tumour cells exhibited a negative stain (Figure 1D). Unlike normal epithelia, intense staining was also observed in well-differentiated tumours in most of the differentiated and keratinized areas of the tumours.

Moreover, the expression of ANXA2 correlated significantly with the T classification, particularly in earlier T stages ($P=.023$). There were almost significant differences in the mean ANXA2 staining scores between tumours with metastasis and those without: the lowest scores were associated with lymphatic metastases ($P=.054$). Even though ANXA2 expression was greater in laryngeal than pharyngeal tumours, these differences were not statistically significant ($P=.22$).

For the follow-up period, local recurrence developed in 7 cases; in 2, regional recurrence and in 1, distant metastasis. The cases presenting tumoral recurrence (grouping together local and regional recurrences, and distant metastasis) had a lower ANXA2 staining score than the cases without recurrence. Moreover, diminished survival was seen in the cases with the least expression of ANXA2 (Figure 2), but this was not significant (log-rank test, $P=.18$), probably due to the insufficient number of cases analyzed.

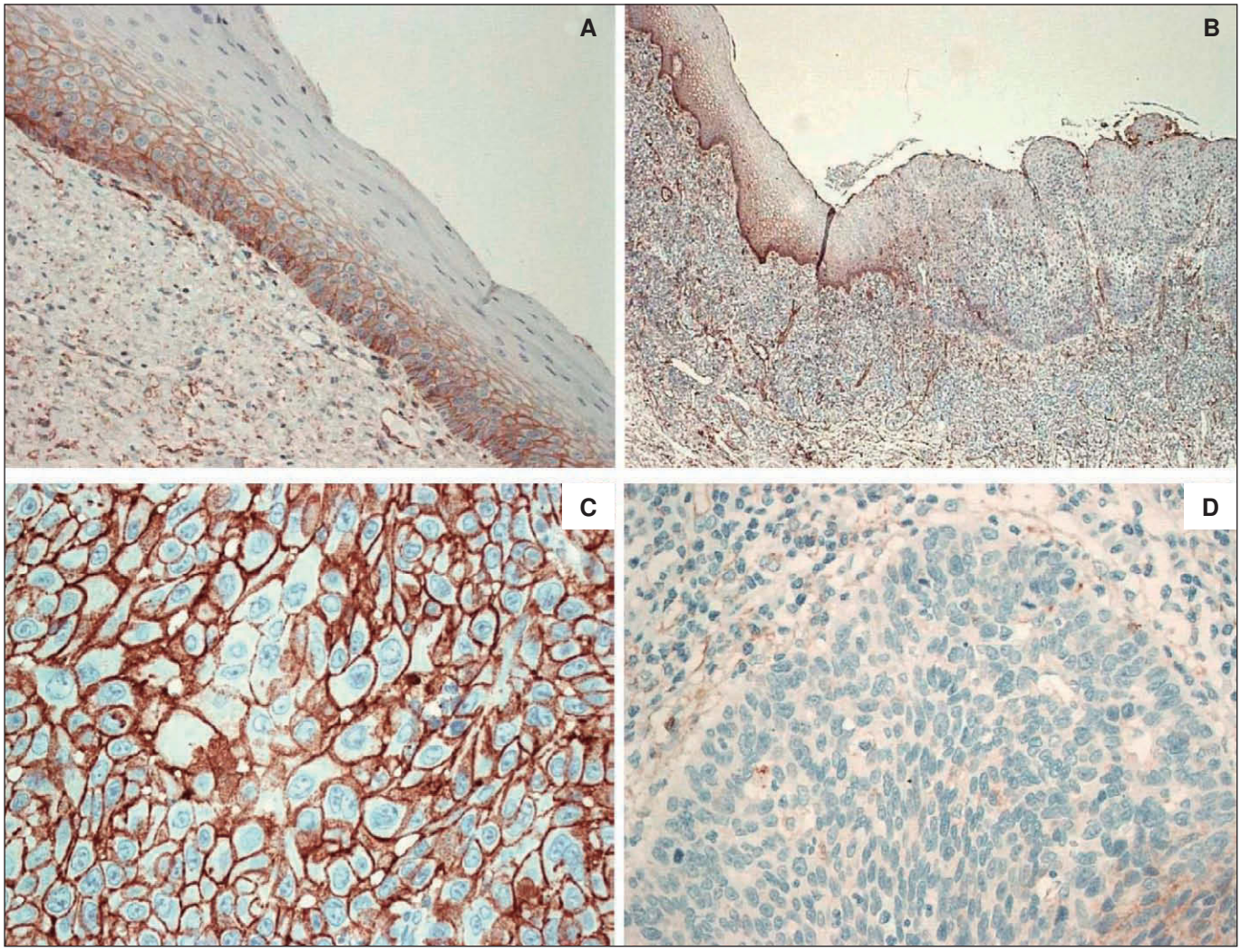

Figure 1. Immunohistochemical analysis of annexin A2 expression. A: normal epithelium (×200). B: dysplastic epithelium; the right side of the image shows a reduction of the expression of the annexin A2 respect to the normal epithelium (left side) (×50). C: well-differentiated tumour (×400). D: poorly-differentiated tumour (×400).

DISCUSSION

Many of the complex biological fundamentals of epidermoid carcinomas in the head and neck continue to be poorly known, despite intensive studies. As in other neoplastic epithelia, carcinogenesis of the head and neck seems to develop through a process with multiple steps that involves successive biomolecular changes.[20]

In this way, epithelial carcinogenesis has been divided into 3 phases of initiation, promotion, and progression involving genetic alterations, deregulation of epithelial differentiation, abnormal proliferation, and altered regulatory effects associated with the abnormal expression of cell factors regulating growth and development.

Identifying the molecular alterations associated with these events could be an important step in knowing the mechanisms of initiation and progression of neoplasms and could bring new tools for their diagnosis, treatment and prevention.

Annexins are widely found to be deregulated in cancers.[3] ANXA2 is overexpressed in a variety of tumours and carcinogenic cell lines, like brain astrocytomas, pancreatic cancer, hepatocellular carcinoma, lung carcinoma, breast cancer, colorectal carcinoma, B-cell lymphoma, and promyelocytic leukaemia,[8-15] and its expression in prostate cancer has been found to be diminished. However, the pattern of ANXA2 expression in the normal mucosa of the upper airways and digestive tracts has been studied in only one previous work,[17] and there are no data available on the expression of this protein in epidermoid carcinomas of the head and neck.

As was mentioned in the introduction, ANXA2 has been shown to exist as a monomer, a heterodimer with S100A10 or a heterotetramer.[21] ANXA2 is present in cell cytoplasm as a monomer (heavy chain of 36 kDa or p36) or in a complex with a light chain of 11 kDa (p11), a member of the S100 protein family. Tetrameric ANXA2 is composed of 2 copies of ANXA2 (p36) and 2 copies of p11.[21] Formation of the

Table 2. Expression of Annexin A2 (ANXA2) in Relation to the Clinical-Pathological Findings*

Characteristic	*Cases, n*	*Median Expression of the ANXA2 (% Stained Cells)*	*95% CI*	*P†*
Total Cases	21	58	41-74	
Location				
Pharynx	13	50	28-71	.22
Larynx	8	71	39-101	
pT classification				
T1-T2	7	83	54-111	.023
T3-T4	14	45	25-64	
pN classification				
N0	8	77	52-102	.054
N1-3	13	46	24-68	
Grade differentiation				
Well-differentiated	11	87	74-99	<.001
Moderately-differentiated	6	31	7-56	
Poorly-differentiated	4	18	–15 to 51	

*CI indicates confidence interval.
†ANOVA.

heterotetramer results from the association of tetrameric ANXA2 with the plasma membrane. Even though the physiological function of ANXA2 has not yet been well established, it might participate in calcium-dependent exocytosis, endocytosis, and the adhesion between cells. Moreover, ANXA2 as a tetramer on the cell surface has been shown to serve as a receptor or binding protein for proteases (cathepsin B, the plasminogen tissue activator) and proteins in the extracellular matrix (collagen and tenascin C). In this way, ANXA2 as tetramer can participate in various biological processes like the activation of plasminogen, signal transduction by tenascin C, cellular adhesion, and interactions between the cell and the extracellular matrix.[7]

The pattern of ANXA2 expression, which differs between normal epithelia, hyperplastic epithelia, dysplastic epithelia, and the invasive epidermoid carcinomas of the head and neck, signals a specific role for this protein in carcinogenesis in the head and neck. The expression of ANXA2 in the invasive tumoral cells is mainly restricted to the cell surface. This membrane-associated ANXA2 may be tetrameric in form. As mentioned above, it has been seen that ANXA2 as a tetramer on the cell surface is a receptor or binding protein for polypeptide ligands, including the proteases and proteins in the extracellular matrix. In this way, ANXA2 as tetramer provides structural bonds not only between the cell and the extracellular matrix, but also between the matrix's proteases and molecules. It has been pointed out that locating the proteases and the components of the matrix on the cell surface through tetrameric ANXA2 might facilitate the reshaping of the extracellular matrix in physiological and pathological processes.[7] On the basis of this function, the overexpression of ANXA2 and plasminogen tissue activator have been demonstrated in pancreatic cancer and they correlate with the invasive potential of the tumour.[9] The overexpression of ANXA2 was also found to be related to invasiveness and poor prognosis in colorectal and gastric carcinomas.[13,22] In contrast, ANXA2 has been linked to

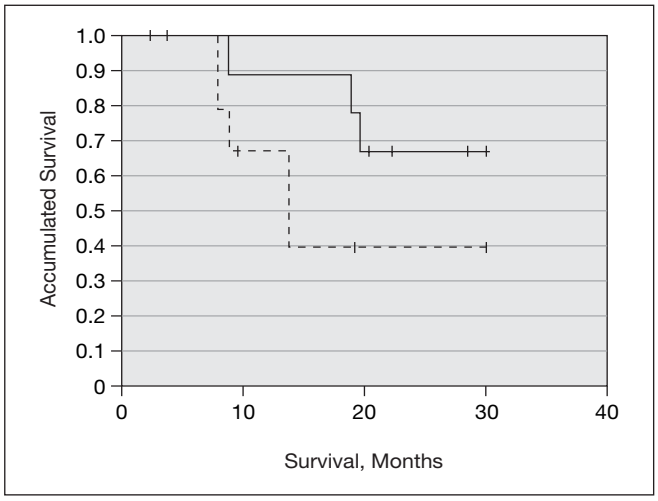

Figure 2. Survival of patients as a function of annexin A2 expression: above the median (continuous line) and below the median (broken line).

tumoral suppression and the inhibition of cell migration in prostate cancer.[16] We have shown that the greatest expression of ANXA2 occurs in the most differentiated and least invasive cases. The poorly-differentiated and most invasive tumours show the least expression of the protein. Moreover, the lower expression of ANXA2 is correlated with advanced tumoral states, lymphatic metastases, and low survival. Taken together, these findings indicate that a reduced rather than an increased expression of ANXA2 is related to invasiveness in head and neck cancers. These results concur with recent data that the expression of ANXA2 diminishes in squamous carcinoma oesophagus, and this diminished expression is related to moderately-differentiated and poorly-differentiated tumours.[23]

Our results raise the question of whether the changes in the expression of ANXA2 play a role in the aetiology of head

and neck carcinogenesis or are a mere consequence of changes in the proliferative index or stages of differentiation of the tumours. The loss of expression of this protein in the dysplastic epithelia and some epidermoid carcinomas of the head and neck are presumably a consequence, rather than an aetiological factor, and this matches the loss of epithelial differentiation and the abnormal proliferation inherent in carcinogenesis. Our observation that a reduced expression of this protein is closely related to histological changes (eg differentiation and proliferation alterations) supports this idea. However, in normal head and neck epithelia, ANXA2 expression was detected only in the least differentiated and proliferative layers of epithelia (basal and suprabasal). It is noteworthy that the patterns of ANXA2 expression seen in the normal epithelia and the carcinomas are similar to those described in some adhesion molecules such as E-cadherin.[24] This shows that ANXA2 could have a function in the cellular adhesion of the epithelia in the upper airway and digestive tract. This would also explain the relationship between the diminishment of ANXA2 in poorly-differentiated and metastatic carcinomas.

In conclusion, in this preliminary study we have described for the first time that ANXA2 is expressed in the cell membrane of the head and neck carcinomas, but not in the dysplastic epithelia, and our results indicate that ANXA2 could have an important role in squamous cell differentiation. Additional studies are on-going to establish the pathogenic role of this protein in epidermoid carcinomas of the head and neck.

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