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Annexin A2, A7, and A11 expression in head and neck squamous cell carcinoma*

Aim: To investigate annexin A2 (ANXA2), annexin A7 (ANXA7), and annexin A11 (ANXA11) expression in head and neck squamous cell carcinoma (HNSCC). Although overexpression of ANXA2 has been reported in HNSCC, there are no studies on ANXA7 or ANXA11 in the same region.

Materials and Methods: ANXA2, ANXA7, and ANXA11 were immunohistochemically studied in paraffin-embedded sections obtained from 43 patients with HNSCC.

Results: ANXA2 and ANXA11 expression was more intense in the tumor areas in HNSCC (P = 0 for ANXA2, P = 0.05 for ANXA11). Both the tumors and normal mucosae showed membranous staining for ANXA7 and ANXA2, while there was mostly nuclear staining for ANXA11. Another important finding was that tumor differentiation decreased as ANXA2 expression intensity (P = 0.001) and staining score (P = 0) decreased. There was a significant relationship between ANXA7 expression and tumoral growth pattern (P = 0.001).

Conclusions: This is the first study to show ANXA7 and ANXA11 expression in HNSCC. ANXA2 staining severity and score were related to the degree of tumor differentiation in HNSCC. The membranous staining pattern decreased with decreasing differentiation for ANXA7. There was a linear relationship between the expression of ANXA2 and ANXA7.

Key words: Annexin A2, annexin A7, annexin A11, squamous cell carcinoma, head and neck neoplasms

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Baş boyun bölgesi skuamöz hücreli karsinomunda anneksin A2, A7 ve A11 in ekspresyonları

Amaç: Bu çalışmanın amacı baş boyun bölgesi skuamöz hücreli karsinomunda (BBSHK) anneksin A2 (ANXA2), anneksin A7 (ANXA7) ve anneksin A11 (ANXA11) in ekspresyonlarını araştırmaktır. Anneksin A2 nin BBSHK larında ekspresyonuna ilişkin çalışma bulunurken, bu bölge karsinomlarında anneksin A7 ve anneksin A11 ile ilgili data yoktur.

Yöntem ve Gereç: ANXA2, ANXA7 ve ANXA11, 43 BBSHK lu hastanın parafine gömülü materyallerinden elde edilen kesitlere immünohistokimyasal olarak uygulandı.

Bulgular: BBSHK larında ANXA2 ve ANXA11 ile tümörde boyanma daha şiddetli bulundu (ANXA2 için P = 0 ve ANXA11 için P = 0,05). ANXA7 ve ANXA2 hem tümör hem de normal mukozada membranöz paternde boyanırken, ANXA11 de nükleer patern hakimdi. ANXA2 nin ekspresyon şiddeti (P = 0,001) ve yaygınlığı (P = 0) azaldıkça tümörde diferansiyasyonun azalması saptanan diğer önemli bulgulardı. Ayrıca ANXA7 ekspresyonu ile BBSHK larının büyüme paterni arasında anlamlı bir ilişki izlendi (P = 0,001).

Sonuç: İlk defa bu çalışmada ANXA7 ve ANXA11 in BBSHK larında eksprese edildiği gösterilmiştir. ANXA2 nin boyanma şiddetinin ve yaygınlığının BBSHK larında tümörün diferansiyasyon derecesi ile ilişkili olduğu gözlenmiştir. ANXA2 ile ANXA7 ekspresyonları arasında doğrusal bir ilişki saptanmıştır.

Anahtar sözcükler: Anneksin A2, anneksin A7, anneksin A11, skuamöz hücreli karsinom, baş-boyun neoplazileri

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Introduction

Annexins are a protein family sensitive to phosphorylation and dephosphorylation that can bind phospholipids in the presence of Ca++ ions and can suppress phospholipase A2. Annexin is composed of a 70-amino acid domain that consists of 5 alpha helices bound together with short bonds. Each member of the ANX protein family contains 4 annexin repeats in a characteristic manner (1).

Human annexins belong to the A subfamily of vertebrate annexins and have 12 members. They are shown with the ANX symbol, and A1-A11 and A13 suffixes. Annexin A12 has not yet been defined. Annexins take part in such cellular events as protection of the cytoskeleton and its interaction with the extracellular matrix, growth and tissue differentiation, inflammation, and clotting, thanks to their relationships with other proteins and their role in signal transduction (2). Various annexins have been shown to play a role in carcinogenesis stages, such as cell line transformation (3), tumor progression (4), and metastasis (5,6). This indicates that annexins may play a role in tumor suppression.

Hayes et al. reported in a review of annexinopathies that annexins show both decreased and increased expression in various cancers (but not in the same cancer), and that it is therefore not possible to place them simply in the tumor suppressor gene or oncogene class (7). Musunoor et al. reviewed the potential role of annexins in tumor development and progression (8). They reported a potential role for annexins in tumorigenesis. Changes in annexin expression are associated with some tumor types and diseases (7). Annexins may therefore be useful as biomarkers for clinical usage.

ANXA2 has been found in the normal epithelium of the upper respiratory and digestive systems, and in head and neck carcinomas (9,10); however, there are no data on the expression of ANXA7 and ANXA11 in head and neck carcinomas. We compared the expression of ANXA7 and ANXA11 in head and neck region squamous cell carcinoma (HNSCC) with that in normal epithelium using immunohistochemical methods in this study, and evaluated their relationship with prognostic parameters, such as degree of differentiation, lymph node metastasis, and growth pattern.

Materials and methods

The present study included 43 patients diagnosed with and treated for HNSCC at our hospital between 2005 and 2008. The study conformed with the principles of the Helsinki Declaration and received ethics committee approval.

Histopathological evaluations were performed by evaluating 3-5-µm hematoxylin-eosin stained archive slides that were prepared from paraffin blocks according to routine procedures following fixation with 10% buffered formalin. Distribution according to tumor localization in the patients was as follows: 21 (48.8%) larynx, 11 (25.6%) lower lip, 6 (14%) oral cavity, 4 (9.3%) the tongue, and 1 (2.3%) the tonsils. Mean age of the patients was 57.5 years; 40 (93%) patients were male and 3 (7%) were female. Mean tumor diameter was 2.87 cm (range: 1-7 cm). All the patients had a history of smoking and alcohol use. None of the patients received radiotherapy or chemotherapy before surgery.

All patients contained normal mucosa in addition to the tumor. Histological typing and grading of the tumors were performed according to the World Health Organization (WHO) classification (11), and the tumors were separated into 3 groups: well-differentiated, moderately differentiated, and poorly differentiated. Histopathological analysis of the cases revealed 25 (58.1%) well-differentiated, 15 (34.9%) moderately differentiated, and 3 (7%) poorly differentiated squamous cell carcinomas. The tumor was infiltrative in 11 (25.58%) cases and had an expansile growth pattern in 32 (74.42%) cases. Lymph node metastasis was present (N1-3) in 9 (20.9%) cases and absent (N0) in 34 (79.1%).

Immunohistochemical staining procedure

Annexin 7

1. A paraffin block that best reflected each tumor's general characteristics and also contained normal mucosal epithelium was selected for each case. Sections 3-5-µm thick obtained from paraffin blocks were placed on slides with polylysine.
2. The sections were deparaffinized and then dehydrated.
3. The sections were placed inside a container with 10 mM citrate buffer solution (pH = 6) and

microwaved twice for 5 min at high temperatures, The sections were then cooled at room temperature for 20 min.

4. The sections were then placed in a humidifier and were incubated for 15 min after drops of 0.3% hydrogen peroxide (H2O2) were added to remove endogenous peroxidase activity in the tissues.
5. Large Volume Ultra V Block (Lab Vision, Cat; TA-125-UB) solution was placed on the sections to prevent nonspecific staining and they were incubated for 5 min.
6. A primary antibody (ANXA7, Santa Cruz, monoclonal Mouse) was added and incubation was performed at 1/100 dilution for 1 h at room temperature (18-25 °C).
7. Secondary antibody Biotinylated Goat Anti-Polivalent (Lab. Vision, Cat TP-125-BN) was added and incubated for 20 min. Drops of Large Volume Streptavidin Peroxidase (Lab Vision Cat: TS- 125-HR) were added as a marker and the sections were incubated for 20 min.
8. 3.3'Diaminobenzidine (DAB) solution was used for chromogen application for 10 min.
9. Counterstaining was accomplished with Mayer hematoxylin, while Large Volume Ultra Mount Plus was used for covering.

Annexin 2 and 11

The same procedures used for ANXA 7 were repeated, except the solution was not placed in a microwave oven when going through the PBS solution following dehydration in absolute alcohol after the incubation stage, and incubation was for 10 min instead of 15 min after the 0.3% hydrogen peroxide drops were added. We again used 1/100 diluted ANXA2 and ANXA11 (Santa Cruz, monoclonal Mouse) drops as primary antibody and incubated for 1 h at room temperature (18-25 °C).

Immunoreactivity evaluation

We used oral mucosa for Annexin 2, the adrenal gland for Annexin 7, and epididymis tissue sections for Annexin 11 as positive a control in immunohistochemical studies. Membranous staining for Annexin 2, membranous, cytoplasmic, and nuclear staining for Annexin 7, and membranous, cytoplasmic, and nuclear staining for Annexin 11 in control tissues were accepted as positive. Immunohistochemical staining was evaluated in the non-neoplastic mucosal epithelium (squamous epithelium) and areas of least tumor differentiation using 10×, 20×, and 40× oculars. Staining intensity was graded as follows: '–' for no staining; '+' for light yellow granules; '++' for light yellow-brown granules; '+++' for dark yellow-brown dense granules. Staining score was as follows: 100 cells were counted in 5 different fields under 40× magnification and then averaged. A distribution under 5% was '–' (negative), 5% - 25% '+', 25% -75% '++', and over 75% '+++'.

Statistical evaluation

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) v.11.0 for Windows. Clinicopathological and immunohistochemical data were evaluated with the generalized form of Fisher's chi square test for multiple cell tables and marginal homogeneity (McNemar). A P value less than 0.05 was considered significant.

Results

Expression of Annexin 2 in normal epithelium and tumors

There was no difference in the ANXA2 staining pattern between tumors and normal mucosae ($P > 0.05$). Membranous staining was predominant in both fields. There was a significant difference between tumors and normal mucosae in terms of staining intensity ($P = 0.000$). Tumors stained more intensely than normal mucosae. There was diffuse staining in the tumoral areas, while normal mucosae generally only showed staining in the basal and suprabasal regions (Figures 1 and 2). Epithelial cells stained more weakly as they matured.

There were no statistically significant differences in the ANXA2 staining pattern intensity and score between cases with and without lymph node involvement ($P > 0.05$).

There was a significant relationship between the degree of differentiation and the ANXA2 staining pattern ($P = 0.001$). Well-differentiated tumors showed 100% membranous staining, while the rate

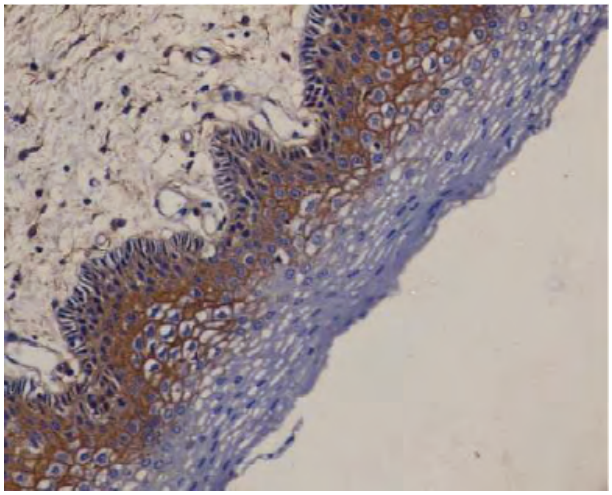


Figure 1. The basal and suprabasal layers are mostly stained membranously with ANXA2 in normal epithelium (400×, immunoperoxidase).

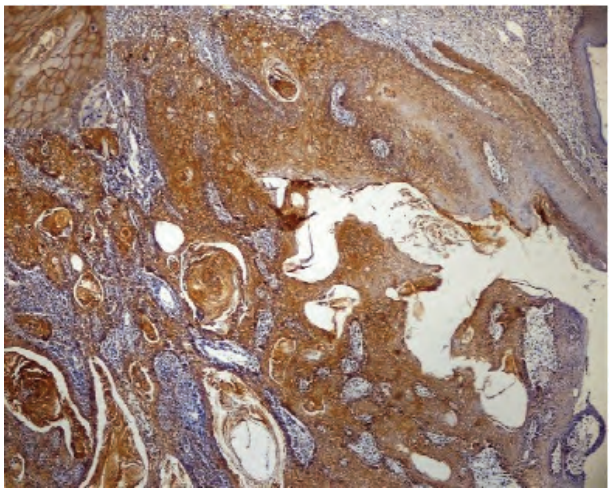


Figure 2. All yields are diffusely stained positive in tumors with ANXA2 (100×, immunoperoxidase). High-power microscopic appearance of the ANXA2 membranous staining pattern is seen in the left upper corner (200×, immunoperoxidase).

was 66.7% for moderately differentiated and 33.3% for poorly differentiated tumors. There was a significant relationship between the degree of differentiation and ANXA2 staining intensity (P = 0.001). ANXA2 staining intensity increased as the tumors differentiated, as can be seen in Table 1 (Figure 3). There was also a significant relationship between the degree of differentiation and ANXA2 staining score (P = 0). Staining became more diffuse as tumor differentiation increased.

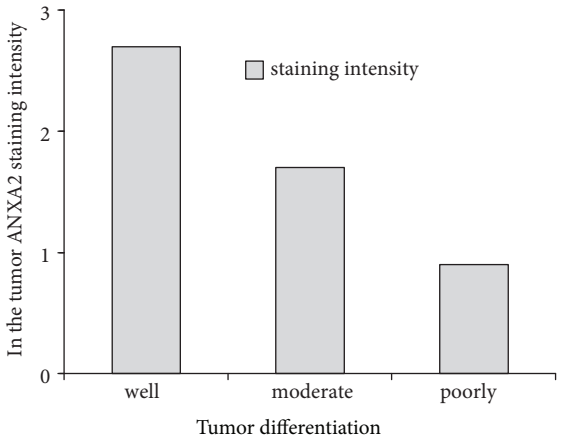


Table 1. The relationship between ANXA2 staining and differentiation in HNSCC.

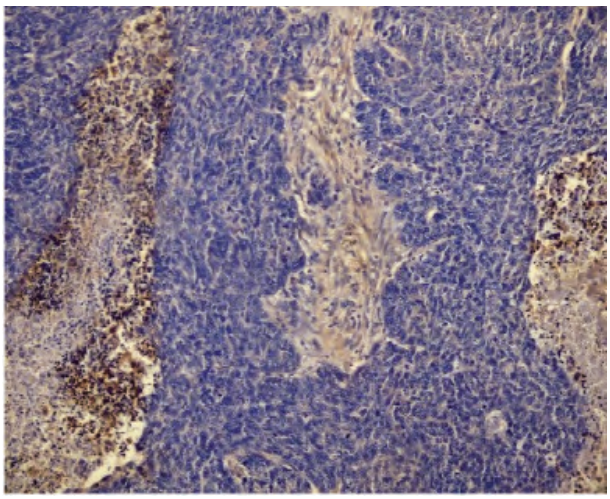


Figure 3. ANXA2 staining in poorly differentiated HNSSC. ANXA2-negative tumor islands are surrounded by ANXA2-positive inflamed cells (100×, immunoperoxidase).

There was no relationship between ANXA2 staining intensity or score, and tumor size (P > 0.05); however, we did observe a significant relationship between the staining pattern and tumor size (P = 0.026). The percentage of membranous staining decreased as tumor size increased (Table 2).

Table 2. ANXA2 membranous immunostaining pattern according to tumor size.

Tumor size	≤ 2 cm (n = 19 cases)	> 2 and < 4 cm (n = 19 cases)	> 4 cm (n = 5 cases)
The percentage of the ANXA2 membranous staining pattern	94.7% (18 cases)	84.2% (16 cases)	40.0% (2 cases)

Expression of Annexin 7 in normal epithelium and tumors

There was no difference between the tumoral and normal mucosal staining patterns with ANXA7 ($P > 0.05$). The tumors showed diffuse staining, while normal epithelium had staining in the lower half. As with ANXA2, the epithelial cells showed decreased staining as they matured.

We did not observe statistically significant differences in ANXA7 staining pattern, intensity, or amount between cases with and without lymph node involvement ($P > 0.05$).

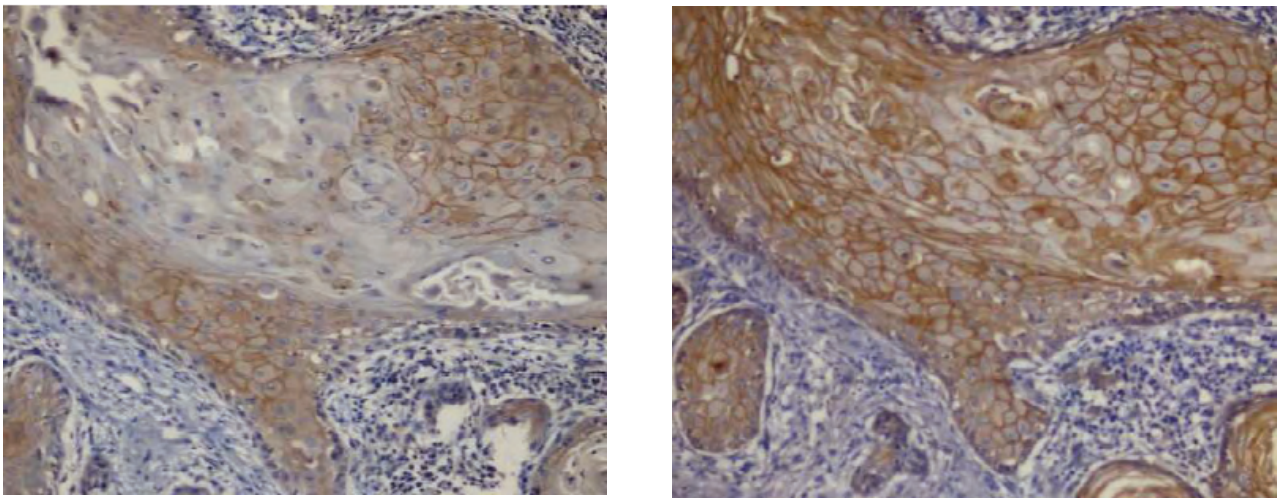
There was an almost significant relationship between the degree of differentiation and ANXA7 staining pattern ($P = 0.056$). There was membranous staining in 84% of well-differentiated tumors and the rate decreased as tumor differentiation decreased. Membranous staining was present in 60% of moderately differentiated tumors and in only 33.3% of poorly differentiated tumors. There was no significant relationship between the degree of differentiation and ANXA7 staining intensity or score ($P > 0.05$).

There was no relationship between the score or intensity of the ANXA7 staining pattern and tumor size ($P > 0.05$); however, there was a significant linear relationship between ANXA2 and ANXA7. ANXA7 produced less intense staining than ANXA2 did, but similar cases showed similar staining (Figures 4 and 5).

When HNSCC was divided into 2 categories according to developmental pattern (expansile and infiltrative) we observed a significant difference in the ANXA7 staining pattern between the 2 groups ($P = 0.001$) (Table 3).

Expression of Annexin 11 in normal epithelium and tumor

Analysis of the expression pattern and intensity of ANXA11 in normal epithelium and tumor areas in 43 cases is presented in Table 4.



Figures 4 and 5. ANXA7 produced less intense staining than ANXA2, but similar cases show similar staining (200×, immunoperoxidase).

Table 3. ANXA7 staining pattern according to HNSCC growth pattern.

ANX A7	No staining	Cytoplasmic	Membranous	Total
Infiltrative	3 27.30%	4 36.40%	4 36.40%	11 100%
Expansile	0 0%	5 15.60%	27 84.40%	32 100%

Table 4. ANX A11 staining pattern and intensity in HNSCC.

ANX A11	Pattern			Severity			Staining none
	Nuclear	Cytoplasmic	Membranous	+ Intensity	++ Intensity	+++ Intensity	
Normal epithelium	23 53.50%	10 23.30%	5 11.60%	19 44.20%	14 32.60%	5 11.60%	5 11.60%
Tumor	24 55.80%	10 23.30%	5 11.60%	5 11.60%	7 16.30%	25 58.10%	6 14.00%

Staining score in the tumoral areas was significantly higher than in normal epithelium.

We did not observe a difference in the ANXA11 staining pattern between tumors and normal mucosae ($P > 0.05$). Tumors frequently stained nuclear, as did normal epithelium (Figure 6). There was a significant difference between tumors and mucosae, in terms of staining intensity ($P = 0.05$). Tumors stained more intensely than normal epithelium, as with ANXA2. There was diffuse staining in the tumoral areas, while epithelium showed staining of the lower half.

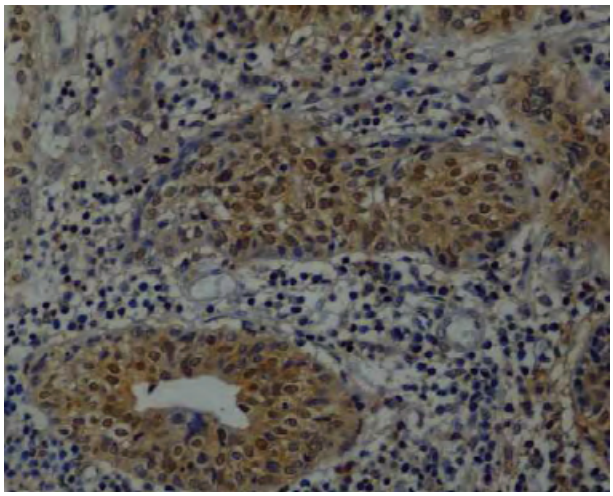


Figure 6. Microscopic appearance of ANXA11 mostly nuclear staining in HNSCC (200×, immunoperoxidase).

There was no statistically significant difference in ANXA11 staining pattern, intensity, or amount between cases with and without lymph node involvement ($P > 0.05$). A significant relationship was not observed between the degree of differentiation, and ANXA11 staining pattern, intensity, or amount ($P > 0.05$). We did not observe a relationship between ANXA11 staining pattern, amount, or intensity, and tumor size ($P > 0.05$).

Discussion

Despite extensive research, many of the complicated biological events in HNSCC remain unclear. Understanding the related molecular changes may be an important step in understanding the diagnosis, ensuring treatment and prevention of the disorder. We observed different immunohistochemical expressions of ANXA2, ANXA7, and ANXA11 in normal mucosal epithelium (squamous epithelium) and HNSCC. These

differences indicate that the relevant proteins may play a role in the carcinogenesis of HNSCC.

Another study reported more dominant ANXA1 staining in the cell membrane in normal oral mucosa and increased membranous staining, together with decreased nuclear staining in oral squamous cell carcinoma cases (12). A similar study reported decreased ANXA1 expression with decreasing differentiation in normal squamous epithelium, dysplasia, and invasive carcinoma spectrum of the head and neck region (13). A 2007 study immunohistochemically defined ANX antibody expression in premalignant and invasive squamous cell lesions of the pharynx and larynx (10). It investigated the potential role of ANXA2 in these tumors by evaluating normal epithelium as well. In that study paraffin blocks from 9 patients with premalignant lesions and 21 HNSCC patients were immunohistochemically stained with ANXA2, and a significant correlation between the expression of this protein and histopathological grade was observed. ANXA2 expression was higher in well-differentiated tumors than in those that were moderately or poorly differentiated. ANXA2 expression was observed in almost all well-differentiated tumors, whereas most cells did not show staining in poorly differentiated tumors. Staining was most dense in the well-differentiated and keratinized areas of well-differentiated tumors. We obtained results that were similar, and ANXA2 expression was strongest in well-differentiated tumors and the most differentiated areas of the tumors. There was weak or no staining in poorly differentiated cases. We did not observe the same significant relationship between ANXA2 immunohistochemical expression and tumor invasiveness as Rodrigo Tapia et al. reported (10). A study on esophageal squamous cell carcinomas reported increased ANXA2 expression associated with moderately and poorly differentiated tumors other than head and neck carcinomas (14).

Srivastava et al. reported an increased incidence of tumor in mice with the ANXA7 gene removed (knockout) (15). Studies on prostate cancer have shown ANXA7 to be a candidate tumor suppressor gene (16); however, there is no definite consensus that ANXA7 is a tumor-suppressor gene. There are no reports on the expression of ANXA7 in HNSCC. We observed a significant linear relationship between ANXA2 and ANXA7, but the relationship between the ANXA7 expression pattern and the degree of differentiation was on the borderline of significant. We think this may have been due to the low number of tumors that stained with '+++' intensity as a result of weaker staining with ANXA7 and that a larger series may provide more statistically significant data regarding the relationship between ANXA7 and tumor differentiation. Despite the lack of statistical significance for staining intensity, HNSCC cases showed diffuse staining in contrast to the staining limited to the lower half in normal epithelium. The selective staining in the most differentiated areas of the tumors, similar to that of ANXA2 but less intense, was also noted. ANXA7 and ANXA2 showed the staining pattern seen with adhesion molecules, such as E-cadherin, in the normal epithelium and tumor areas of HNSCC. This is also supported by the relationship we observed between the tumor growth pattern and ANXA7. This finding indicates that ANXA7 may be useful for determining tumor invasiveness and prognosis. A 2008 study showed loss of ANXA7 expression in gastric signet ring carcinoma and mucinous adenocarcinoma, while its expression was preserved in glandular and nodular gastric carcinomas (17). The tumors in which they observed expression loss were less differentiated gastric tumors or infiltrative-type tumors. A previous study reported a relationship between decreased ANXA7 expression and high invasive potential of tumors (18). The significant difference between tumors with expansile or infiltrative growth, together with the ANXA7 staining pattern in the present study and the decreased expression in the infiltrative pattern, supports a possible role for ANXA7 in tumor invasiveness, as reported in the mentioned studies. We did not observe a relationship between the growth pattern of HNSCC and ANXA2. These results indicate that ANXA2 and ANXA7 are similar, but have different roles, even though they are both related to HNSCC differentiation and have similar staining features. ANXA7 plays a role in local invasion of HNSCC, in contrast to ANXA2.

It was reported that gastric cancer cases are associated with weak ANXA7 expression and distant metastases (17). We did not find a relationship

between ANXA7, and lymph node metastasis, tumor size, or tumor stage. This may have been due to the small number of cases, and the lack of data regarding follow-up and the development of metastases, as the patients had been operated on recently.

ANXA11 is a nuclear protein during the interphase and then displays a well-regulated localization pattern from the prophase to cytokinesis, the later stages of the cell cycle. This indicates a role for ANXA11 in the terminal phase of cytokinesis (19,20). We observed nuclear staining in the basal and suprabasal layers in the epithelium, with 25%-75% distribution in ~60% of the HNSCC cases.

Proteomic and immunohistochemical analyses have shown increased ANXA11 expression in colorectal carcinoma tumor areas, as compared with normal epithelium, as well as a relationship between this increase and increased tumor stage (21). As with ANXA7, there are no data on the expression of ANXA11 in HNSCC. We did not observe a significant relationship between HNSCC differentiation and ANXA11, as we did with ANXA2; however, the increased expression of ANXA11 in tumors, as compared to normal epithelium, indicates a role in carcinogenesis for these proteins as well.

Previous studies of HNSCC have reported a relationship between stage and ANXA2 expression. We did not observe a statistically significant relationship between the intensity and amount of ANXA2 expression, and tumor size or lymph node metastasis status; as tumor size increased the ANXA7 membranous staining rate decreased and well-differentiated tumors showed increased membranous staining.

Annexins are markers that show tumor type-specific expression characteristics and are indicative of the diagnosis and prognosis (22). Although there has been extensive research on some annexins, the role of most in tumor development remains unclear (23). Staining distribution was different in normal epithelium and tumors; therefore, we think that these markers can be used in the differential diagnosis. ANXA2 and ANXA7 expression is related to differentiation, and we think that ANXA2 and ANXA7 may be used to determine HNSCC prognosis. We would like to note that they may also be used in the differential diagnosis of preliminary HNSCC lesions (dysplasias), based on the observed staining difference in normal epithelium, which has also been reported in other studies.

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