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Immunohistochemical Evaluation of Annexin A6 in Papillary Thyroid Carcinoma: A Clinicopathological Study

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Abstract

Background Thyroid cancer is the fifth most common cancer in women, worldwide; and the second in Iraqi women according to the statistics of global cancer observatory Iraq fact sheets at 2021. Papillary thyroid carcinoma comprising 90% of thyroid cancers. Annexin A6 is an immunohistochemical marker developed from polyclonal IgG antibody to Annexin A6 protein, which aids in modulating adenosine triphosphate-induced calcium (Ca^{2+}) signaling in thyrocytes.

Objective To evaluate immunohistochemical expression of Annexin A6 in papillary thyroid carcinoma and its correlation with age and sex of patients, stage of cancer and lymph node involvements.

Methods This is a retrospective case control study done at Baghdad, including 30 tissue samples (paraffin block) of papillary thyroid carcinoma, obtained from Al-Yarmouk Teaching Hospital Laboratory and a private laboratory in Baghdad (Dr. Raji Hussein Alhadithi Laboratory) during the period from January 2021 until October 2021. The patients divided into groups according to their age, sex, cancer stage and lymph node involvement, then correlated with Annexin A6 immunohistochemical score of staining. For each sample, 2 serial sections of 4 µm were taken, one stained with Hematoxylin and Eosin and the other stained immunohistochemically with Annexin A6.

Results Annexin A6 expression was positive both membranous and cytoplasmic ??? in (70%) of cases. The intensity was weak in 3 (14.3%), intermediate in 7 (33.3%) and strong in 11 (52.4%) of cases. High, moderate and low expression level was observed in (43%), (38%) and (19%) of patients, respectively. Mean age of patients were 39 years old. Females positive for Annexin A6 represents 77% of patients. Annexin A6 were positive in 75% of patients with lymph node metastasis. Annexin A6 expression more commonly expressed in patient with stage 2 papillary thyroid carcinoma (80% of them).

Conclusion Annexin A6 is highly expressed in thyroid tissue with papillary thyroid carcinoma. No significant correlation between Annexin A6 and any of clinicopathological parameter.

Keywords Annexin A6, ANXA6, papillary thyroid carcinoma, tumor progression

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List of abbreviations: ANXA6 = Annexin A6, ATP = Adenosine triphosphate, BSA = Bovine serum albumin, CISH = Chromogenic in situ hybridization, DAB = 3-3 diaminobenzidine HCl, FISH = Fluorescent in situ hybridization, H&E = Hematoxylin and Eosin, HRP = Horse radish peroxidase, HBME-1 = Human bone marrow endothelial cell marker-1, LNM = Lymph node metastasis, NaN_3 = Sodium azide, PCR = Polymerase chain reaction, PTC = Papillary thyroid carcinoma, TTF-1 = Thyroid transcription factor-1

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid carcinoma (90%) [(1)] and the most common endocrine malignancy [(2)]. It is defined by the presence of a distinctive set of alterations of nuclear morphology, the PTC-type nuclei,

although the presence of either papillae or invasion of the surrounding thyroid parenchyma is generally required for the diagnosis [3].

Until recently, rate of new thyroid cancers was growing faster than for any other cancer in the US. This year, an estimated 44,020 adults (12,500 in men and 31,520 in women) in the United States would be diagnosed with thyroid cancer [4]. Thyroid cancer is the 5th most common cancer in women, worldwide; and the second in Iraq [5].

Globally, in 2020, the age-standardized incidence rates of thyroid cancer were 10·1 per 100 000 women and 3·1 per 100 000 men, and age-standardized mortality rates were 0·5 per 100 000 women and 0·3 per 100 000 men [6]. Women are 3 times more likely to have thyroid cancer than men. However, women and men die at similar rates. This suggests that men have a worse prognosis than women when there is a diagnosis of thyroid cancer (Prognosis is the chance of recovery). Overall, the 5-year survival rate for people with thyroid cancer is 98%. However, survival rates are based on many factors, including the specific type of thyroid cancer and stage of disease. If the cancer is located only in the thyroid, it is called localized thyroid cancer. About two-thirds of cases are diagnosed at this stage. The 5-year survival rate is almost 100% for localized PTC. If thyroid cancer has spread to nearby tissues or organs and/or the regional lymph nodes, it is called regional thyroid cancer, 5-year survival rate for regional PTC is 99% [4].

The diagnosis of papillary carcinoma does not routinely require immunohistochemistry. When this is performed, it is usually to establish the thyroid origin of a tumor at metastatic sites. Thyroglobulin and thyroid transcription factor-1 (TTF-1) are very specific markers, and PAX8 gene can be used to differentiate papillary carcinoma (positive for both TTF-1 and PAX8) from lung adenocarcinoma and other tumors that are positive for TTF-1 but negative for PAX8 [7]. Another situation, considerably more complex, is the use of immunochemistry for the differential diagnosis of papillary carcinoma from other benign and malignant thyroid lesions. Human bone marrow endothelial cell marker-1 (HBME-1) (membrane immunoreactivity), galectin-3 (both nuclear and cytoplasmic immunoreactivity), cytokeratin 19 (cytoplasmic immunoreactivity), and CITED1 gene (both nuclear and cytoplasmic immunoreactivity) are the markers more frequently overexpressed in papillary carcinoma (HBME-1 followed by galectin-3 are considered the most specific) [8], while CD56 is typically negative [9]. While the sensitivity of these markers is high, their specificity is relatively low, thus they are more effective when used in combination (usually HBME-1, galectin-3, cytokeratin 19) [8].

Annexin A6 (ANXA6) is an immunohistochemical marker developed from polyclonal IgG antibody to ANXA6 protein, which is a member of a conserved superfamily of Ca^{2+}-dependent membrane-binding proteins, that display a diverse range of functions in cellular development and differentiation [10].

Over the last several years, ANXA6 (a member of a conserved superfamily of Ca^{2+}-dependent membrane-binding annexin proteins) has been shown to play a major role in the differentiation of chondrocytes [11], migration of neural crest cells [12], growth, adhesion and motility of tumor cells [13] and in membrane repair [14]. Some studies suggest that ANXA6 mediates these cellular processes as a multifunctional scaffolding protein for signal transduction molecules [15]. In the thyroid gland, thyroid stimulating hormone modulated adenosine triphosphate (ATP)-induced Ca^{2+} signaling via ANXA6 in thyrocytes [16]. A range of studies have shown the annexins to be among the genes that are consistently differentially expressed in neoplasia [17]. The expression levels of ANXA6 are closely associated with several malignancies like melanoma, cervical cancer, epithelial carcinoma, breast cancer, gastric cancer,

prostate cancer, acute lymphoblastic leukemia, chronic myeloid leukemia, large‐cell lymphoma and myeloma [10]. ANXA6 exhibits dual functions in cancer, acting either as a tumor suppressor or promoter, depending on the type of cancer and the degree of malignancy [10]. This annexin has also been shown to play a role in the negative regulation of the Ras–Raf–MAPK signaling pathway by targeting the guanosine triphosphate (GTP)ase-activating protein to p120GAP to inactivate ras gene [18]. This is one of the key-signaling pathways in tumorigenesis, and it is a common pathway in development of papillary thyroid carcinoma [19], there is a very limited number of studies to show correlation between ANXA6 and PTC.

The aim of the study was to evaluate immunohistochemical expression of ANXA6 in PTC and its correlation with age and sex of patients, stage of cancer and lymph node involvements

Methods

Patients (Tissue sampling)

Total of 30 tissue samples (paraffin block) included in this retrospective case control study, were obtained from thyroid tissue of patients with PTC, during the period from January 2021 until October 2021, which collected from Al-Yarmouk Teaching Hospital Laboratory and a private laboratory in Baghdad (Dr. Raji Hussein Alhadithi Laboratory). All of these patients had total or subtotal thyroidectomy, clinicopathological parameter records and reports were collected from archives and confirmed and reviewed by prepared tissue with Hematoxylin and Eosin (H&E). An ethical approval for using the specimens were obtained from the Iraqi Board for Medical Specialization at 24th of February 2020. The patients divided into groups according to their clinical conditions regarding age, sex, stage of tumor and lymph nodes involvement; and correlate with ANXA6 immunohistochemical score of staining. For each sample, 2 serial sections of 4 µm were taken, one representative section stained with H&E and the other one stained immunohistochemically with ANXA6.

Preparation of tissue section and reagents for H&E staining [20]

1- Sectioning

The tissue blocks were sectioned manually by using rotatory microtome (microtome, Germany); the tissue blocks were fixed in the holder then cut it via a sharp blade into 4 µm thickness. These tissue sections were placed in floating hot water bath preheated to 40-45°C. Clean ordinary slides were marked with a pencil, and then tissue sections were adjusted on a slide surface by immersing the slides in the water bath after placing the paraffin thin section on it.

2- Dewaxing the tissue sections

This step done to allow the water-soluble stains to diffuse by transferring the slides to oven at 65°C for 15 minutes then transferred the slides to three xylene containers for (3 minutes, 2 minutes, 1 minute) respectively. Rehydration is done by passing the slides in descending concentration alcohol (absolute, 90%, 70%), 2 minutes each, then wash with distilled water.

3- Staining tissue sections with H&E stains

Staining resulted into blue color for the negatively charged cellular compartment by hematoxylin stain and red eosin color for the other cellular structures.

4- Mounting

At the end, mounting cover slides were added by using a few drops of clear resin (a mixture of distyrene, a plasticizer, and xylene) to coat the bottom of the cover slide, then the cover was set on the tissue section slowly (this step was done to preserve the stain) later the covered slides were allowed to dry on a slide warmer for 24 hr.

Immunohistochemistry

Principle of staining

Highly sensitive and specific PolyExcel two step detection system is non-biotin, micro-polymer-based detection system, which significantly reduce or shows no back ground on tissues containing high levels of avidin, biotin ex: kidney, liver and lymphoid tissues. This system is based on a horse radish peroxidase (HRP) labeled polymer, which is conjugated with secondary antibodies. The procedure included incubation of the specimen for 5-10 minutes with H_2O_2 quenches any endogenous peroxidase activity. Then the specimen is incubated with respective diluted primary antibody (mouse or rabbit), followed by incubation with the PolyExcel Target Binder for 10 minutes then followed by a PolyExcel HRP labeled polymer using recommended 10 minutes' incubation. Staining is completed by 5-10 minutes' incubation with 3-3 diaminobenzidine HCl (DAB) substrate-chromogen which results in a brown-colored precipitate at the antigen site.

Materials

1. Primary antibody: ANXA6, clone (NM_001155.4), code (MBS9125642), is a rabbit IgG polyclonal antibody, diluted with 1% bovine serum albumin (BSA) and 0.05% sodium azide (NaN_3). The recommended dilution for Immunohistochemistry is 1:50-1:200. The antibody dilution and protocol may vary depending on the specimen preparation and specific application. Positive control slides were prepared from colonic carcinoma known to be positive for each, while negative control slides were prepared from using the antibody diluent only without primary antibody.
2. Secondary detection system: PolyExcel HRP/DAB detection system two steps detection system, HRP labeled polymer and DAB substrate-chromogen. The materials used were peroxidase block, HRB and DAB chromogen.
3. Primary antibody diluents: 1% BSA and 0.05% NaN_3.
4. Antigen retrieval buffers: Sodium citrate buffer acidic solution code (PS007, PS008, and PS009) pH 6 was used.
5. Phosphate buffer solution.
6. Isopropyl alcohol.
7. Xylene.
8. Counterstain: Mayer's hematoxylin (Dako).
9. Distilled water.
10. Aqueous mounting media.

Positive and negative control

1. Positive control: Colonic adenocarcinoma tissue was considered as a positive control.
2. Technical negative control: Negative control slides are done by using the antibody diluent alone and leave the primary antibody, this is done in the same condition (in the same slide).

Immunohistochemistry assessment and scoring of marker positivity

Slides were viewed under light microscope, and they were evaluated with low-power microscopy (10x) to establish the largest staining areas, if no staining seen at low power re-evaluation performed with high power (40x) to assess weak staining region, all areas of every slide were observed and scored semi quantitatively by measuring the percentage of cells that are positively stained over the entire number of malignant cells "percent". The staining level of the tissue samples were assessed by a consultant pathologist.

A scoring system was used to determine level of expression in tumor cells from 0 to 3, (0 = no expression, 0.1-1 = low expression level, 1.1- 2 = moderate expression level, 2.1-3 = high expression level) [21], which calculated by multiplying the intensity of expression by the extent of expression (percentage of positive cells) i.e. level of expression = intensity of expression x extent of expression.

The intensity of the stained cells was divided subjectively into four groups from 0 to 3 (0 =

no staining, 1 = weak staining, 2 = intermediate staining, 3 = strong staining).

The extent of expression (percentage of cells with membrane and cytoplasm staining) in the samples were evaluated using 10% as cut-off, (i.e. 10% = 0.1, 20% = 0.2, 30% = 0.3, and so on...). When heterogeneity was observed in tumor samples, then the highest score was used [22].

Statistical analysis

Data was analyzed using statistical package for the social science (SPSS) computer software program version 25. Descriptive statistics presented as frequency tables, continuous variables were expressed as mean ± standard deviation and categorical variables as numbers and percentages. Analytic statistics chi-square test was used to find association between two categorical variables, and unpaired t-test used to find difference in continuous variables between groups. The results were considered statistically significant if the probability P value was <0.05.

Results

It has been found that ANXA6 expression were positive in 21 out of 30 (70%) of samples (Figure 1).

The staining pattern was both membranous and cytoplasmic, and nucleus was negative in PTC cells, and showing varying degree of staining intensity from strong to weak. Staining results were further categorized into low, moderate, and high expression groups, depending on intensity and extent of expression (Figure 2).

The intensity of expression was weak in 3 (14.3%), intermediate in 7 (33.3%) and strong in 11 (52.4%) of positive cases (Figure 3).

Regarding level of expression among ANXA6 positive cases; 9(43%) of them showed high level of expression, 8 (38%) showed moderate expression level and 4 (19%) showed low expression level (Figure 4).

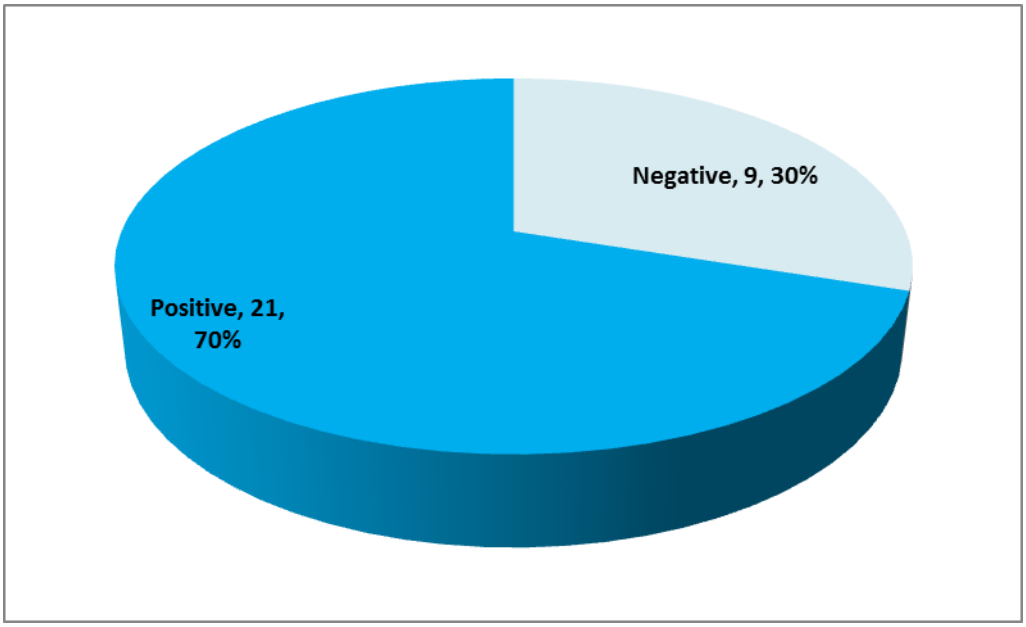

Figure 1. Annexin A6 status in patients with papillary thyroid carcinoma

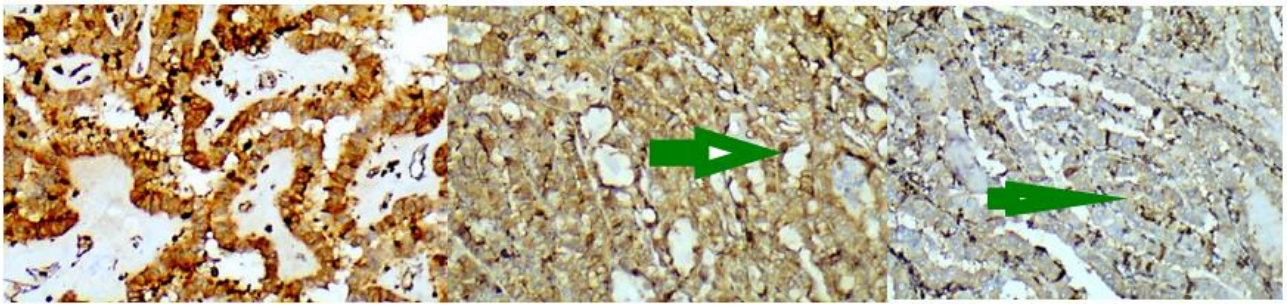

Figure 2. High power view (40x objective) immunohistochemical staining of annexin A6 in patients with papillary thyroid carcinoma, showing A: strong membranous and cytoplasmic staining of cells with high expression level: B; intermediate intensity of stain, both membranous and cytoplasmic staining of cells, C: weak cytoplasmic staining of cells.

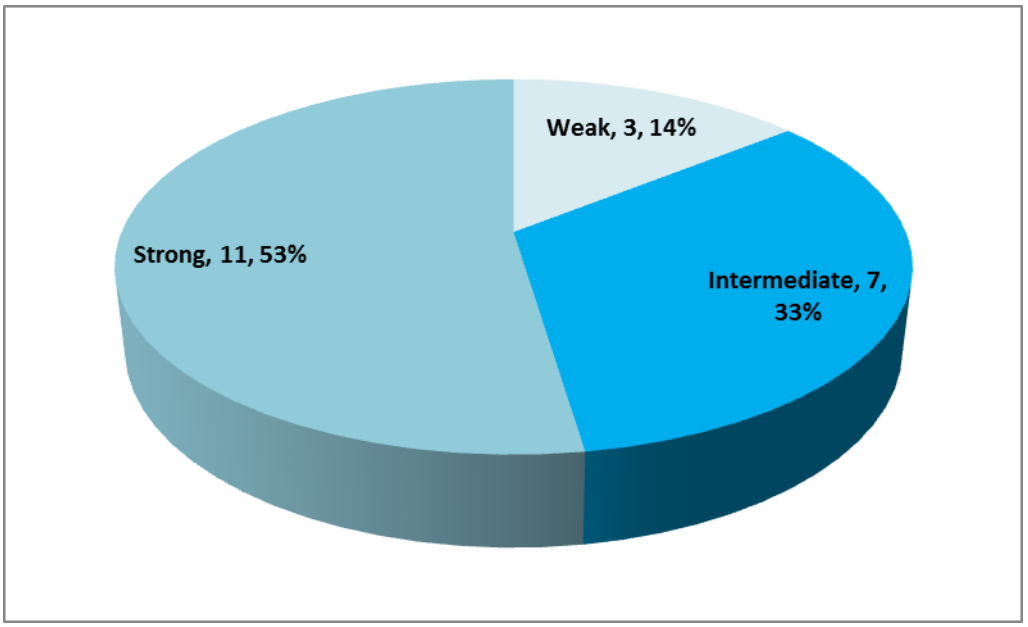

Figure 3. Annexin A6 intensity of expression in patients with papillary thyroid carcinoma

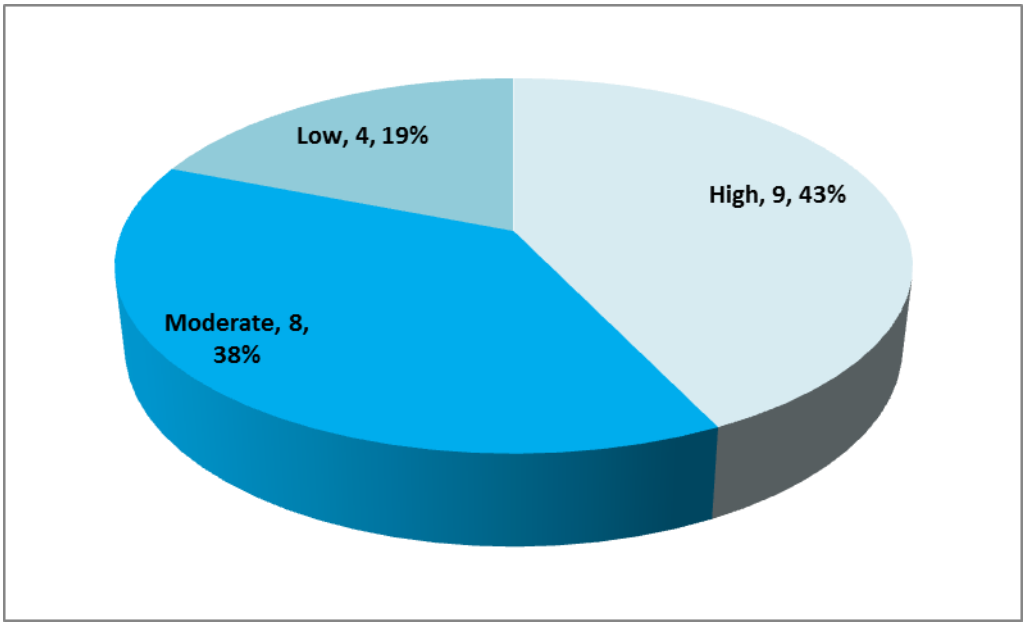

Figure 4. Annexin A6 expression level in patients with papillary thyroid carcinoma

The clinicopathologic characteristics of 30 patients were compared by ANXA6 expression. The age of patients was ranged from 20 to 56 years old (mean age 39 years old). The results showed that there was no statically significant association between ANXA6 expression and the age of patient as shown in table 1.

Table 1. Comparison of Annexin A6 expression according to age of patients with papillary thyroid carcinoma by unpaired t-test

Parameter		Positive ANXA6 N=21	Negative ANXA6 N=9	P value
Age (yr)	Mean±SD Range	39.6 ± 9.9 20-56	38.7 ± 10.7 20-56	0.85

Regarding the sex of patients; 22 (73.3%) of them were females and 8 (26.7%) were males. 17 (77.3%) of females were positive for ANXA6 and 5 (22.7%) were negative, while 4 (50%) of males were positive for ANXA6 and the same 4 (50%) were negative. This was not significant result; p-value (0.15) (Table 2).

Current results showed that there was no correlation between ANXA6 expression and lymph node status of metastasis (LNM) in PTC, as in those with LNM; 6 (75%) of them were positive for ANXA6 and 2 (25%) were negative. While those without LNM showed that 15 (68%) of them were positive for ANXA6 and 7 (32%) were negative. P-value was 0.71 (Table 3).

Table 2. Comparison of Annexin A6 expression according to sex of patients with papillary thyroid carcinoma by Chi square test

Annexin A6 status	Male N=8	Female N=22	P value
Positive	4 (50.0%)	17 (77.3%)	0.15
Negative	4 (50.0%)	5 (22.7%)	

Table 3. Comparison of Annexin A6 expression according to lymph node metastasis in patients with papillary thyroid carcinoma by Chi square test

Annexin A6 status	Lymph node status		P value
	Positive for metastasis N=8	Negative for metastasis N=22	
Positive	6 (75.0%)	15 (68.0%)	0.71
Negative	2 (25.0%)	7 (32.0%)	

ANXA6 expression was also correlated with the pathological stage of PTC according to TNM classification system (where T describes the size of the tumor and any spread of cancer into nearby tissue; N describes spread of cancer to nearby lymph nodes; and M describes metastasis (spread of cancer to other parts of the body) [23]. For those who were at stage 1; 6 (35.3%) were negative and 11 (64.7%) positive. For those who were at stage 2; 2 (20%) were negative and 8 (80%) positive. For those who were at stage 3; 1 (33.33%) negative and 2(66.7%) positive. This was also not a significant finding (Table 4).

Table 4. Comparison of Annexin A6 expression according to tumor stage in patients with papillary thyroid carcinoma by Yates Chi square test

Annexin A6 status	Tumor stage			P value
	Stage 1 N=17	Stage 2 N=10	Stage 3 N=3	
Positive	6 (35.3%)	2 (20.0%)	1 (33.3%)	0.811
Negative	11 (64.7%)	8 (80.0%)	2 (66.7%)	

Discussion

PTC originates from thyroid follicular cells and is one of the most frequent endocrine malignancies, but fortunately prognosis is good providing that metastasis rarely occur [20]. The role of ANXA6 in PTC and in particular, the mechanisms underlying its contribution to tumor cell growth and/or motility remain not established.

ANXA6 is closely associated with a variety of tumors; it has been implicated as a potential marker for cervical cancer [24], and as a tumor suppressor in melanoma [25], epithelial carcinoma [15], breast cancer [15], gastric cancer

[26], prostate cancer [27] and chronic myeloid leukemia [28]. Furthermore, ANXA6 is considered to act as a promoting factor in the cellular adhesion, motility and invasiveness of breast cancer [29], the progression of acute lymphoblastic leukemia [30], the adhesion of lymphoma [31], and the secretory processes in myeloma cells [32].

A study by Lee et al. [33] showed there were no statistically significant differences between non-tumor and tumor thyroid tissues in the expression levels of the ANXA6 biomarker.

To the best of our knowledge; this is the first study conducted in Iraq and the world that evaluate ANXA6 immunuhistochemical marker expression in PTC.

The present result showed that ANXA6 immunohistochemical marker expressed in malignant PTC by about 70%. Also, it was demonstrated in this study that immunohistochemical expression of ANXA6 was localized in the cytoplasm and plasma membrane of PTC cells. This finding relatively agreed with the finding of Korolkova, et al. [34] on ANXA6 and RasGRF2 expression on rapidly growing and triple negative breast cancer, which showed that ANXA6 was mainly a cytoplasmic protein and with the finding of Jung, et al. [35] on annexin A3 immunohistochemical expression in PTC, which showed that the expression was mainly cytoplasmic in both normal and PTC cells.

There was no correlation between ANXA6 expression with the age and sex of patients in the current study. This the same as a study conducted in Iraq on urothelial carcinoma and in cystitis cases by Majeed and Ahmed study [36], which showed that ANXA6 expression not correlated with either age or sex of patients. Again we have no ANXA6 study on thyroid to compare with.

The same study above by Jung, et al. [35] on ANXA3 immunohistochemical expression in PTC, showed that there is a statically significant differences between males and females in ANXA3 expression in PTC, which was tend to be higher level of expression in females than in males. This difference in result may contribute to the difference in the type of annexin that used.

The current results revealed that there was no difference in ANXA6 expression between cases of PTC with LNM and cases of PTC without LNM. This is in contrast with a proteomic study conducted at Peking University Cancer Hospital and Institute by Xiong et al. [37], which demonstrated that ANXA6 biomarker was decreased in PTC with LNM, indicating a role in lymphatic metastasis. This difference in results could be contributed to difference in the procedure used, this mentioned study was a proteomic study while current study was immunohistochemical study.

It was also demonstrated that there was no correlation between ANXA6 expression and the pathological stage of PTC according to TNM classification system. This is in contrast to many studies, which shows that ANXA6 was correlated with tumor progression, invasiveness and metastasis in many types of cancer including melanoma [25], cervical carcinomas [38], gastric cancers [39], prostatic carcinoma [38] and also some of blood cancers [40].

This difference can be explained by a small sample size included in this study, we have limited number of cases with stage 2 or 3, while most cases were at stage 1, we suggest further studies on same topic but with larger sample size for more accurate results.

In onclusions, ANXA6 expression is not correlated with the age or sex of patients. No difference in ANXA6 expression between cases of PTC with and without lymph node metastasis. ANXA6 expression is not correlated with the pathological stage of PTC according to TNM classification system.

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Author contribution

Dr. Aziz: Collection of data, processing and staining of the samples and writing the

manuscript. Dr. Ahmed: reading the results and putting the conclusions and supervision.

Conflict of interest

None.

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