Original Article
The expression and clinical significance of Galectin 8 in papillary thyroid cancer

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Abstract: To study the expression of Gal-8 in papillary thyroid cancer, and to explore the relationship between Gal-8 and clinical characteristics of papillary thyroid cancer (PTC) and mean vessel density (MVD). The relationship of MVD among Thyroid diseases was also studied. The relative mRNA expression of Gal-8 in 15 PTC and 15 benign thyroid disease (BTD) were analyzed using real time qRT-PCR (Quantitative Reverse Transcription PCR). The protein expression of 70 Thyroid diseases included 33 PTC (having corresponding normal tissues), 15 follicular adenoma (FA), 10 multi nodular goiter (MNG), 4 medullary carcinoma (MC) and 8 normal thyroid (NT) were analyzed by immunohistochemistry (IHC). MVD was measured focusing on hotspot area of highest vascularization of tumor. The relative mRNA expression of Gal-8 in PTC was 2.6 times higher than BTD. Compared with the BTD and NT, the expression of Gal-8 protein in PTC was significantly increased (P < 0.001). The sensitivity and specificity of Gal-8 in PTC were 96% and 62%, respectively. Gal-8 protein expression was associated with lymph node metastasis in PTC, suggesting that Gal-8 may be transferred from the cytoplasm to the nucleus (P < 0.009) as the tumor metastasized. The expression of Gal-8 was significantly stronger in the low TNM stage (I/II) than in the high TNM stage (III/IV) of thyroid cancer. MVD in FA was significantly higher than PTC and MC, p < 0.001. Gal-8 may be used as a sensitive marker of PTC. The increased expression of Gal-8 in PTC than BTD and in lower TNM stage of carcinoma (I/II) is significant suggesting that Gal-8 may be used as one of diagnostic marker of PTC. Gal-8 also has a certain role in tumor angiogenesis.

Keywords: Galectin 8, CD34, papillary thyroid cancer, angiogenesis, MVD

Introduction

Background

Galectins are a family of animal proteins identified as galactoside binding lectin [1]. Till today 15 galectins have been discovered named according to the order of their discovery. The members of galectins are divided into 3 groups. Prototype galectins contains one carbohydrate recognition domain (CRDs) (Gal -1,-2,-5,-7,-10,-11,-13,-14,-15), tandem repeat type contains two homologous CRDs in a single polypeptide chain (Gal -4,-6,-8,-9,-12) and chimera type contains N-terminal domain connected to a CRD (Gal-3) [2].

Gal-8 is a protein of 35 kDa, belongs to a tandem repeat of structurally different carbohydrate recognition domains within a single polypeptide chain. It was previously isolated from a rat liver cDNA expression library and was found to be structurally homologous to rat Galectin 4 (Gal-4) (34%) in the year of 1995 [3]. PCTA-1, a prostate carcinoma tumor antigen-1 also shares 83% amino acid sequence identity with Gal-8 [4]. Similarly, it is closely related to P066 carbohydrate binding protein (CBP) displaying 82% nucleotide and 98.7% amino acid sequence, isolated from a human lung cancer cells [5].

Functions of galectin

Immobilized Gal-8 functions as a matrix protein similar to fibronectin and promote cell adhesion and migration by ligation and clustering integrin receptors and triggers integrin mediated signaling cascades such as Tyr phosphorylation of FAK and paxillin whereas soluble Gal-8 with interaction with cell surface integrin inhibit cell adhesion [6]. Similarly, Gal-8 interacting with cell surface ligands influences T cell proliferation, induces apoptosis, modulates neutrophil

function, promotes cell spreading and cytoskeletal arrangement [7-11, 12, 13]. Researchers have found that Gal-8 is a potent platelet activator both in soluble and immobilized form [14]. So, it has an important role in thrombosis and hemostasis. Recently studies indicate that Gal-8 acts as a danger receptor intracellularly by inducing antibacterial autophagy [15]. Satelli, et al., studied that secreted Gal-8 undergoes post translation processing as a different molecular form of Gal-8 of ~18 kDa which was immunoprecipitated from the extracellular media [16]. Gal-8 has also been localized to lipid micro domains and interact with GM1 ganglioside and other glycosphingolipid [17]. It also plays an important role in the regulation of vascular and lymphatic angiogenesis [18].

Galectins in thyroid carcinoma

Among the family of Galectins, Galectin 1 (Gal-1) and Galectin 3 (Gal-3) have been mostly focused in Thyroid carcinoma and also extensively studied in other cancers. In the systemic review and diagnostic meta-analysis of thyroid cancer biomarker group, it was reported that Gal-3 had sensitivity of 82% and specificity of 81%. However, a marker to enhance this diagnostic accuracy still seems to be necessary and there are many conflicting results for the expression of Gal-3 in thyroid diseases [19]. Also reported another member of Galectin, a prototype Galectin 7 (Gal-7) which is markedly downregulated in a large proportion of thyroid adenomas when compared with thyroid carcinoma [20]. In a recent publication, it is reported that Gal-1 protein was more strongly expressed than Gal-3 and their protein productions in PTC, was increased when there has been metastasis to the surrounding lymph node [21]. Another prototype Galectin 2 (Gal-2) having nuclear and cytoplasmic localization was shown in PTC, FA and anaplastic carcinoma but their sample size was very small [22].

A single study was done on Gal-8 expression in thyroid diseases but researchers did not show any relationship to the clinical characteristics. They used 41 thyroid tissues samples which included 5 FA, 31 PTC, 5 Follicular carcinoma (FC) together with 36 adjacent hyperplastic or normal thyroid tissue for immunohistochemistry. Gal-8 was expressed in the majority of PTC (87%) positive but weaker staining was also found in some of the FC and FA (40%). The pro-

tein was undetectable in 5 NT. The hyperplastic areas corresponding to tumor were weakly positive (29%). They assumed that Gal-8 can be used as a potential marker for PTC however, it cannot distinguish from FC and FA [23]. This is our first study to define expression of Gal-8 in human PTC, BTD and NT with relationship to the clinical characteristics, metastasis and intracellular localization of this protein. To the best of our knowledge, this is also the first study to see relative mRNA and protein expression in the same series of experiments. Gal-8 expression has been shown to be upregulated and downregulated in various types of normal and cancerous tissues and has its own diagnostic and prognostic values which we will discuss later.

Angiogenesis

Now, we will have brief introduction about angiogenesis. Angiogenesis is the formation of new blood vessels from pre-existing vasculature, capillaries [24] and is essential for tumor growth, metastasis and progression. Endothelial cells are the source of new blood vessels and have a remarkable ability to migrate, proliferate and differentiate. MVD is a quantitative method of assessing angiogenesis and CD34 has been considered an important endothelial cell marker to evaluate angiogenesis. In 1988, researchers introduced the relationship between MVD and metastasis [25]. Thyroid cancers are among the five most frequent cancers in the 2nd, 3rd and 4th decades of life and they have ability to metastasize [26]. Relationship of MVD with thyroid diseases has been shown to be controversial. Herrmann, et al. detected that reduced MVD in PTC was associated with poor differentiation, worst prognosis and reduced survival [27], whereas increased risk of recurrence, worst prognosis and poorer survival were associated with increased MVD in another study by Dhar, et al. [28].

To our knowledge, this is the first study to analyze correlation of Gal-8 protein expression and MVD in PTC. Our purpose of study was to demonstrate whether Gal-8 relative mRNA and protein expression upregulated or downregulated in normal, benign and malignant thyroid diseases and also to see MVD (using CD34) relationship to the tumor size and metastasis. Is Gal-8 a potential diagnostic marker for PTC? Does it show any relationship with clinical characteris-

tics? What is the intracellular localization of Gal-8 in thyroid tissues?

Materials and methods

Patient's information

For Immunohistochemistry, we used paraffin embedded tissue blocks of patients who had undergone thyroidectomy between 2013 and 2015 from pathology department of the Third Xiangya Hospital. A total of 70 samples included 33 PTC with corresponding normal samples, 15 FA, 10 MNG, 4 MC and 8 NT. The study was approved by ethics committee of the Third Xiangya Hospital. All the information about patient's age, gender, tumor size, lymph node metastasis, TNM stages and other clinical characteristics were obtained from pathology department of Third Xiangya Hospital. American Joint Committee on cancer staging system was used for TNM cancer staging. For PTC, patients were 18 to 58 yrs old (mean age 36 yrs). 10 of them were male and 23 were female. The mean tumor sizes of PTC patients were 2.25 cm. 20 of the patients with cervical lymph node metastasis and 13 without lymph node metastasis. At the time of diagnosis, only one patient had lung metastasis. Calcification were positive in 18 cases and negative in 15 cases. Similarly, there were 27 patients with lower stage (stage I/II) and 6 patients with stage (III/IV). Hematoxylin and Eosin stained sections from all cases were examined by two pathologists to confirm the diagnosis.

Immunohistochemistry

Immunohistochemical analysis was performed on 4 µm sections formalin fixed paraffin embedded thyroid tissues. The cut sections were placed on a glass microscope slides. The 4 µm tissue sections were deparaffinized in 100% xylene and rehydrated through a graded series of ethanol. Then it was incubated with 3% hydrogen peroxide in methanol for 10 mins to inhibit endogenous peroxidase activity. All the sections were incubated with 5% bovine serum albumin (BSA) for 20 mins to block non-specific binding. Pretreatment for Gal-8 was done by performing antigen retrieval method. Slides were put in a citrate buffer and was boiled using an electric pressure cooker for 2 mins and were cooled for 10 mins in running water. The sections were incubated with a rabbit poly-clonal anti galectin 8 antibody, {galectin-8 (H-80): 28254, Santa cruz Biotechnologies, USA} at a dilution of 1:100 overnight at 4°C. Slides were washed with PBS solution three times and then treated with the secondary antibody at room temperature for 10 mins. Secondary antibody was diluted at 1:500, peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG (H+C) (code no. ZB-2301).

CD34 was purchased from Maixin Biotech. Company and was diluted at 1:50. For CD34, pretreatment was not done. Again the slides were rinsed with PBS and then reacted with chromogenic substrates for 4 to 6 mins which contain DAB (diaminobenzidine) for the visualization of positive cells. Subsequently the slides were rinsed in a running water and counterstained with Mayer's hematoxylin for 8 to 10 secs. Then the slides were dehydrated and mounted with coverslips.

Both the nucleic and cytoplasmic staining of cells having brown color was considered as positive staining. They were evaluated on a magnification of × 100 and × 400 field by 2 pathologists. The staining intensity of each cells were determined. Score 0 defines no staining of cells, score 1 defines weak staining of cells, score 2 defines moderate staining of cells and finally, score 3 defines strong staining of cells. The percentage of positive staining cells were also graded. Score 0 considering none of the cells were positive, score 1 considering < 10% of both nucleic/cytoplasmic cells were positive, score 2 considering 10-50% of both nucleic/cytoplasmic cells were positive, and score 3 considering > 50% of both nucleic/cytoplasmic cells were positive. Information about patient's clinical characteristics were unknown to us while giving score.

The final IHC score was concluded by adding the score of staining and percentage of cells for each tissue. The IHC score was categorized as follows: 1. Highest score was given 6; 2. Lowest score was given 0; 3. Score greater than 2 was evaluated as positive; 4. Score 2 or lesser than 2 was evaluated as negative.

RNA isolation and real-time qRT-PCR

Fresh tissues were snap frozen immediately in liquid nitrogen and stored at -80°C till RNA extraction. The total RNA from 30 samples

Table 1. The relationship between intracellular localization of Galectin 8 with lymphnode metastasis

Parameters	Intracellular localization of Gal-8		
	Cytoplasm + Nucleus	Nucleus	P-value
Lymphnode metastasis			
Present	6	14	0.009
Absent	10	2	

Gal-8 protein shifted from cytoplasm to nucleus as the tumor metastasized (p<0.009).

Table 2. The relationship between protein expression of Galectin 8 with its clinical characteristics in Papillary thyroid cancer

Parameters	Galectin 8 protein expression		
	Weak expression	Strong expression	P-value
Age			
< 40	5	13	0.46
≥ 40	6	8	
Sex			
Male	3	7	1.00
Female	8	14	
Tumor size			
< 1 cm	2	1	0.54
≥ 1 cm	10	19	
TNM Stage			
Stage I/II	7	20	0.03
Stage III/IV	4	1	
Calcification			
Positive	5	12	0.71
Negative	6	9	
Lymphnode metastasis			
Present	5	14	0.69
Absent	5	8	

*32 cases were positive for Galectin 8 and 1 case was negative. The above features shows the positive protein expression of Galectin 8.

which included 15 PTC and 15 BTD was isolated using trizol reagent following manufacturer's instruction. 1000 ng of total RNA was reverse transcribed to cDNA by using Rever Tra Ace qpcr RT mastermix kit according to the manufacturer's instructions. Real-time qRT-PCR was conducted in an eppendorf mastercycler ep realplex. 1 µl of cDNA was used as a template in a total volume of 10 µl reaction with the help of Thunderbird SYBR qpcr mix without rox (Toyobo Co. LTD, Life Science Department, Osaka, Japan). The mixture contained 5 µl of SYBR green, 0.3 µl of each (reverse and forward) primer, 3.4 µl of distilled water and finally 1 µl of cDNA.

The primers were purchased from Sangon Biotech (Shanghai) Co, LTD. The primers were assessed from gene bank, center for medical experiments, the Third Xiangya Hospital. β-Actin was used as an internal reference gene. Primer sequences for real-Time qRT-PCR of Gal-8, Gal-8 and β-Actin are shown in **Table 4**. The samples for relative gene expression were run in triplate. The protocol for PCR cycling condition was 10 min at 95°C, 40 cycles of 15 sec at 95°C, 20 sec at 60°C, 30 sec at 72°C. Melting curve analysis was also performed after amplification for the accuracy of the amplicon and to observe whether there is contamination by non-specific products and primer dimer. Equal amount of RNA was used to avoid genomic contamination of DNA. The relative expression of Gal-8 was calculated using $2^{-\Delta\Delta ct}$ method and β-Actin was used as a reference gene. Hence, the relative expression of Gal-8 at mRNA was detected.

Results

Galectin 8 mRNA expression by real-time qRT-PCR

Gal-8 expression was compared between 15 BTD and 15 PTC tissues. The mRNA expression of Gal-8 was significantly higher in tumor tissues (PTC) in comparison to the BTD. For Gal-8, the mean mRNA expression in PTC comparing BTD was 7.15 ± 2.45 and 5.45 ± 1.46, p < 0.028, (**Figure 1**). PTC expressed Gal-8 at 2.6 fold higher than BTD. The fold changes were concluded as low, normal and high expression giving assumption that changes in fold greater than 2 were considered as high expression, greater than 0.5 and lesser than 2 were considered as normal expression and changes in fold lesser than 0.5 were considered as low expression.

Galectin 8 protein expression by immunohistochemistry

We evaluated Gal-8 expression in 33 PTC with its adjacent normal tissues, 15 FA, 10 MNG, 4 MC and 8 NT. Samples having sum scores of ≤ 4 were considered as low Gal-8 protein expres-

Table 3. The intracellular localization of Galectin 8 in thyroid diseases

Thyroid tissue	Intracellular localization of Galectin 8		
	Cytoplasm	Nucleus	Cytoplasm + nucleus
Papillary thyroid carcioma	6/32	16/32	10/32
Medullary carcinoma	4/4	0	0
Follicular adenoma	2/6	3/6	1/6

Table 4. Primers for Galectin 8 and β-Actin

	Primers Sequence (Forward and Reverse)
Galectin 8	Galectin 8-F1: 5'GGTCCTCTGGGATTAGTTATG3'
	Galectin 8-R1: 5'GTCCTCAGTGATGGGATACGG3'
β-Actin	β-Actin-F1: 5'-CTGGAACGGTGAAGGTGACA-3'
	β-Actin-R1: 5'-AAGGGACTTCCTGTAACAACGCA-3'
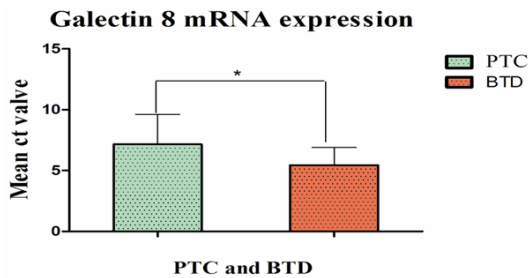
Figure 1. The relative mRNA expression of Galectin 8 comparing between Papillary Thyroid Cancer(PTC) and Benign Thyroid Disease (BTD) by real-Time qRT-PCR. When normalized to β-Actin, the Gal-8 mRNA level in PTC tissues (7.15 ±2.45) is significantly higher than the BTD tissues (5.45 ± 1.46), *P<0.028.

sion and samples having sum scores of ≥ 5 were considered as high Gal-8 protein expression. 9 cases of PTC with Gal-8 expression showed score of 4, only 1 case showed score of 3 and remaining 22 cases showed score of 5. None of these cases showed highest score 6. Only 1 case had a score of 0 which was considered as negative. The immunostaining of Gal-8 for PTC was nuclear, cytoplasmic and nucleocytoplasmic. 30 cases of PTC showed 60% to 90% immunoreactivity of cells while only 2 cases showed 30% to 40% immunoreactivity of cells. Gal-8 expression was positive in 32/33 (96%) of PTC, 6/15 (40%) of FA, 4/4 (100%) of MC, 1/10 (10%) of MNG and negative for NT (**Figures 2** and **3**). Hence, Gal-8 had sensitivity of 32/33 (96%) and specificity of 18/25 (72%) for PTC. The sensitivity and specificity was calculated as defined by Weber, et al. [29].

Out of 32 positive cases of thyroid, 16/32 expressed truly nuclear staining, 6/32 expressed truly cytoplasmic and 10/32 expressed both cytoplasmic and nuclear staining. 4 cases of FA showed score of 4, 1 case showed score of 3 and another 1 case showed score of 5. Similarly among 6 positive cases of FA, 2 cases presented with cytoplasmic localization of weak and moderate staining, 3 cases presented with nuclear localization of weak staining and 1 case presented with both cytoplasmic and nuclear localization of weak staining (**Table 3**). Follicular cells were very weakly expressed in some of the normal tissues of thyroid staining less than 10% cells giving sum score of 2. So, they were considered as negative. The score reading is described in IHC section. In case of some normal tissues the retracted colloid goiter were also weakly positive. Follicular cells were very weakly stained in 1 case of MNG. Both the cytoplasm and nucleus of endothelial cells were positive with weak and moderate expression in 40% to 50% of cancerous tissues and 10% to 20% of normal tissues of thyroid. Some of the lymphocytes and plasma cells also showed weak to moderate expression of Gal-8 having both nucleic and cytoplasmic staining (**Figure 3D**). Gal-8 expression in PTC (4.5 ± 0.97) were significantly strong than FA (1.6 ± 2.04) and MNG (0.3 ± 1.16), p < 0.0001 (Data not shown).

Relationship of Gal-8 protein with clinicopathological characteristics of PTC patients

We divided all the patients of PTC into weak Gal-8 expression and strong Gal-8 expression category. According to our result we found that there is inverse relation of Gal-8 significantly related to low stage tumor. Cancerous tissues showed Gal-8 protein strong expression in low stage tumor (stage I/II) when compared to high stage tumor (stage III/IV), p < 0.03 (**Table 2**). We didn't find any statistically significant difference of weak and strong protein expression of Gal-8 with other clinical features of patients such as age, sex, tumor size, calcification and lymph node metastasis (**Table 2**). Later we arranged Gal-8 protein expression of PTC into truly nuclear and cytoplasmic + nuclear groups (5 cases of truly cytoplasmic group was also included). We discovered that nuclear reactivity
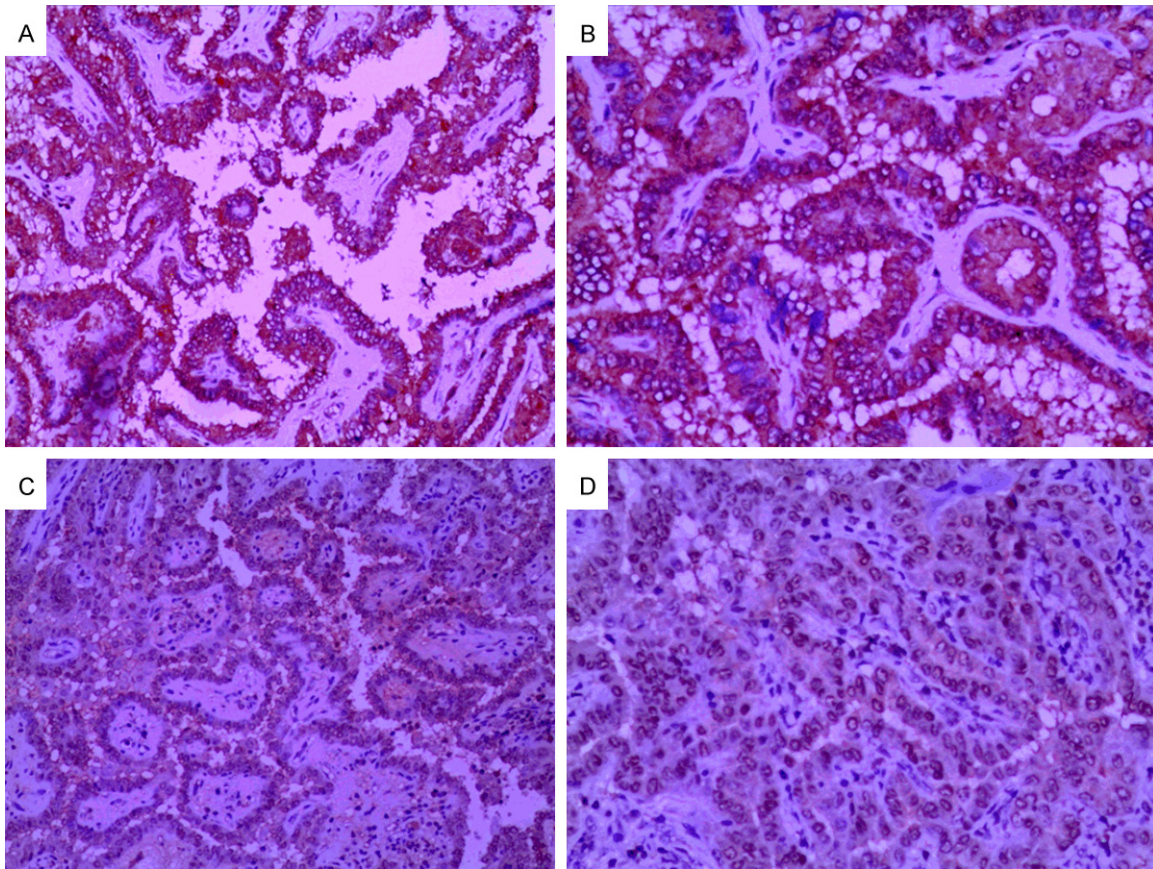
Figure 2. The expression of Galectin 8 in Papillary Thyroid Carcinoma by immunohistochemistry. (A: x 100, B: x 200) Strong expression of Galectin 8 and (C: x 100, D: x 200) Moderate expression of Galectin 8.

of cells significantly increases from cytoplasmic + nuclear staining groups as the cancerous tumor metastasized, p < 0.009 as shown in **Table 1**.

Mean vessel density of CD34

MVD was evaluated by using a low power magnification (× 40) light microscope and areas with the highest neovascularization microvessels (Vascular hotspots) were selected. At (× 200) magnification most highlighted angiogenic areas was examined. Micro vessels near lymphocytes, RBC infiltration and fibrosclerotic area were avoided to count the exact number of vessels (**Figure 4**). Any brown staining endothelial cell or endothelial cell clusters which was clearly separated from adjacent micro vessels, tumor cells and other connective tissue elements was considered a single, countable vessel [30]. Expression of CD34 was quantified by computerized image analysis (**Figure 5**). For each case at least 5 to 10 minutes was given to

assess the hot spots area. The MVD was measured twice for the accuracy of true countable blood vessels using computer imaging and the average of all vessels of thyroid tissue (MVD) was examined and used for statistical analysis.

MVD is the mean of the all neovessels evaluated by quantifying the image of highest vascularized area (hotspots). CD34 positive vessels were analyzed by IHC as described before. They were strongly expressed in all the cancerous tissues and benign tissues and were very diversified varying from lowest 39 vessels per hpf (high power field) to highest 130 vessels per hpf. The MVD score of FA (123.4 ± 24.78) was significantly higher than PTC (78.81 ± 26.15) and MC (46.75 ± 14.64), p < 0.001 and though not statistically but apparently than MNG (101.9 ± 36.61) as shown in **Figure 6**. We delineated that FA had the highest score of MVD whereas MC had the lowest score of MVD.
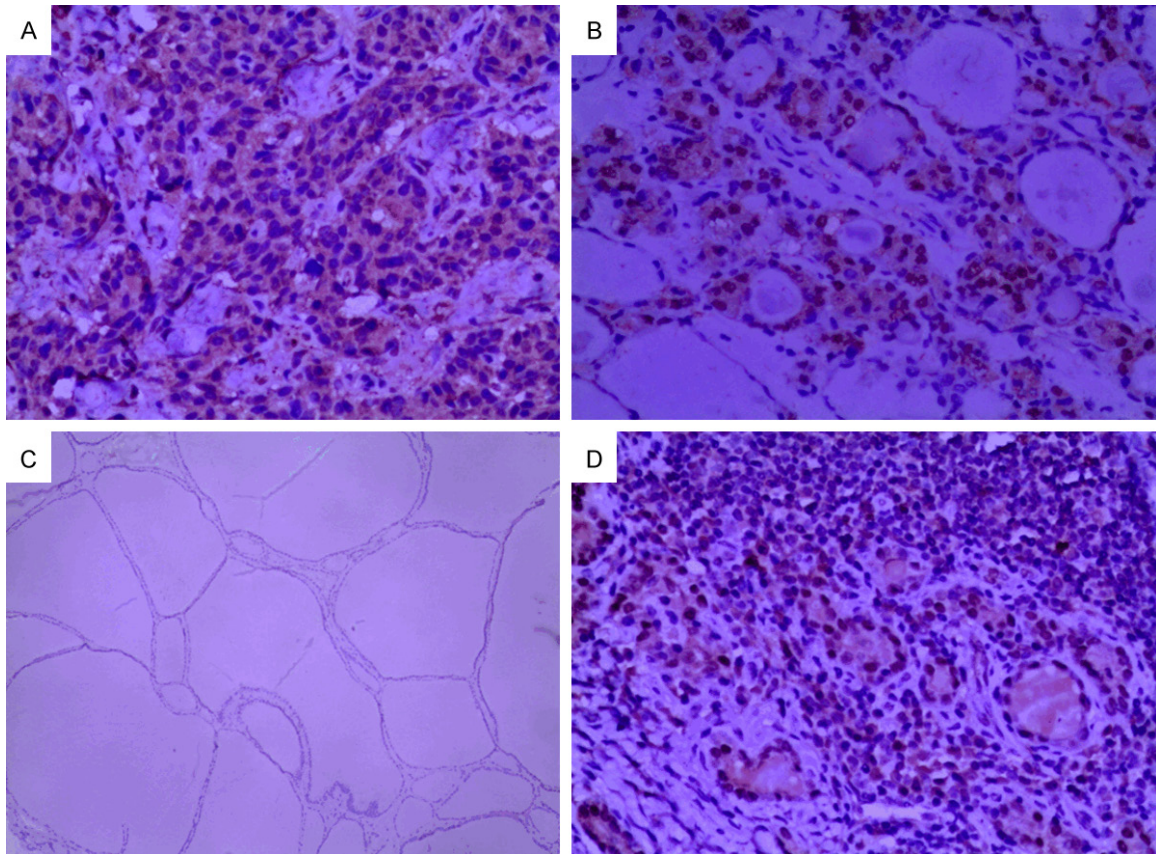
Figure 3. The expression of Galectin 8 by immunohistochemistry. A. Moderate staining of medullary carcinoma of thyroid (x 200). B. Moderate staining of follicular adenoma of thyroid (x 200). C. Negative staining of multinodular goiter of thyroid (x 100). D. Moderate staining of blood vessels and lymphocytes of Papillary thyroid carcinoma (x 200).

We also used CD31 (PECAM), another vascular endothelial marker to evaluate the immunostaining of endothelial cells in all thyroid tissues. We found weak expression of CD31 in 15/33 (45%) of PTC, 2/15 (13%) of FA, 1/10 (10%) of MNG and no expression of cells in MC and NT. Intriguingly, as previously described that CD31 has been shown to evaluate the MVD in many tumors, we detected weakly expressed CD31 in comparison to CD34. Since we detected weaker and negative expression of CD31 in all thyroid tissues, we did not do any statistical analysis regarding MVD in our study for CD31.

Relationship of galectin 8 and MVD in clinical characteristics of PTC

We compared the MVD between the patients of PTC with lymph node metastasis and without lymph node metastasis. We found that there was an apparent increase of MVD in the patient of lymph node metastasis but our data didn't reach any statistical significance (data not shown).

We also compared correlation of expression of Gal-8 to the MVD in PTC group and found that expression of Gal-8 was significantly correlated to MVD, $R^2=0.152$, P < 0.004. There was no relationship regarding MVD to the size of the tumor (data not shown).

Statistical analysis

We used statistical analysis SPSS version 18 for the data collection and analysis. Quantitative data were presented by mean, standard deviation (SD) and median and qualitative data were presented using percentage. Independent t-test or Student t-test was done to evaluate the mean mRNA expression between the two groups. The relationship between clinical characteristics and Gal-8 protein expression was analyzed by fisher exact test or chi square test (x^2 test).
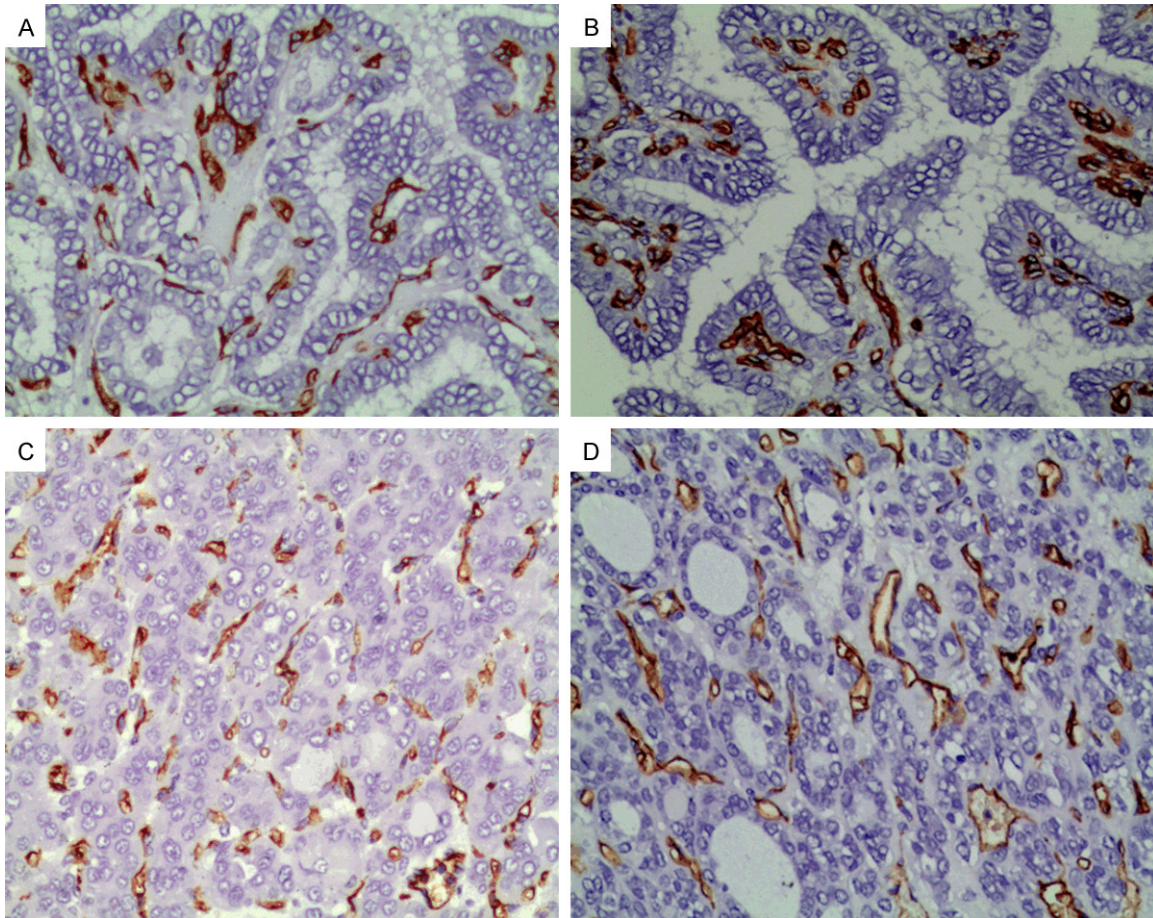
Figure 4. CD34 staining of blood vessels (A, B: x 200) Positive vessel staining of Papillary Thyroid Carcinoma. (C, D: x 200) Dense vascularization of Follicular Adenoma of thyroid.

For non parametric tests, as sample size were not equal, Kruskall-Wallis test was used to see the difference of expression among 3 or more groups which was followed by post-hoc tests (Dunn procedure) to avoid multiple comparison effects. Similarly Mann Whitney U test was done to see the difference between two groups for non parametric tests (for measuring MVD in relationship with tumor size and lymph node metastasis). Pearson correlation coefficient was used to measure the relationship of Gal-8 to MVD in PTC. A p value < 0.05 was considered statistically significant for all the tests.

Discussion

Gal-8 discovered since 1995 from mouse liver cDNA has been recently an interest of subject for researchers. Its role and functions in growth and differentiation of tumors is still lacking. PTC is the most common malignant neoplasm of neck and its incidence has been dramatically increased [31]. Gal-1 and Gal-3 members of

lectin family have been extensively studied in Thyroid cancer but there is a gap in the study of Gal-8 in the field of thyroid diseases. Recently, Gal-8 role has been shown in angiogenesis and hence we developed curiosity towards these complex recombinant tandem repeat type lectin. To the best of our knowledge this is the first study to show both molecular and IHC study of Gal-8 in human thyroid diseases. We investigated Gal-8 relative mRNA and Protein expression in benign and malignant thyroid diseases by real-Time qRT-PCR and IHC. This is also the first study to detect the protein expression of Gal-8 in relationship with the clinical characteristics and to detect its correlation with MVD in PTC.

In the present study we detected that the relative mRNA expression of Gal-8 in tumor tissues versus benign tissues was significantly increased (2.6 fold). We also confirmed these results by IHC and found that protein expression of Gal-8 in PTC significantly increased (p < 0.0001)
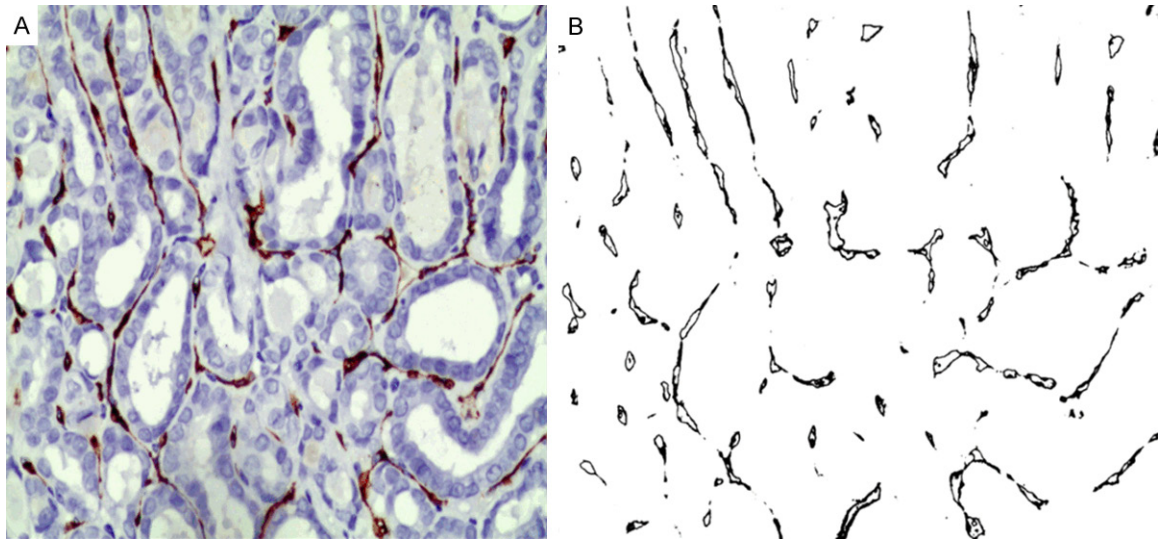
Figure 5. CD34 staining of blood vessels (A) (x 200) Positive vessel staining of Papillary Thyroid Carcinoma (B) Computer image analysis of blood vessels of Papillary Thyroid Carcinoma.
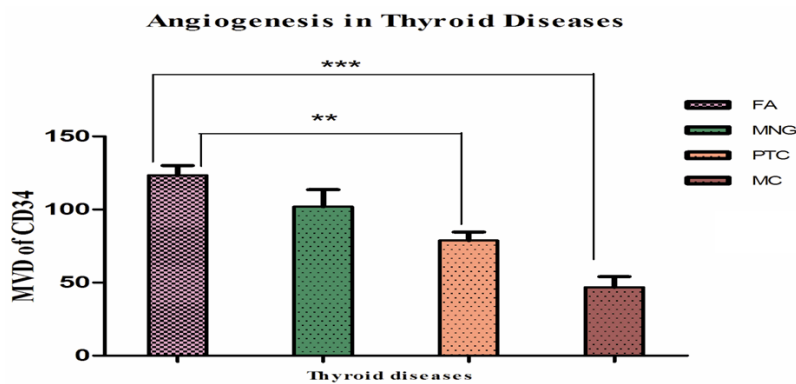

Figure 6. Angiogenesis in Thyroid diseases showing relationship of MVD among Follicular Adenoma (FA) (123.4 ± 24.78), Multi Nodular Goiter (MNG) (101.9 ± 36.61), Papillary Thyroid Carcinoma (PTC) (78.81 ± 26.15) and Medullary Carcinoma (MC) (46.75 ± 14.64), ***$P<0.0001$. Follicular Adenoma showed the highest MVD whereas Medullary carcinoma showed the lowest MVD.

endothelial cells having weak to moderate immunostaining in 40 to 50 % of cancer cells and 10 to 20 % of normal cells. Similar to our results Galectins are reported to be found in the cytoplasm as well as in the nucleus of endothelial cells in other tumor tissues (prostate and breast tissues) [33]. Till today limited reports have been published regarding Gal-8 expression in the endothelial cells of normal and cancerous tissues. Both nuclear and cytoplasmic staining of Gal-8 was observed in colon, kidney, breast, prostate and astrocytic brain cancer [18]. To our knowledge, this is the first study to point out the endothelial expression of Gal-8 in normal and cancerous tissues of thyroid, however, further research in a large group is needed to know actual role of Gal-8 in thyroid tumor angiogenesis. Our data herein focused on Gal-8 expression on thyroid malignancy.

Galectin 8 expression in normal and cancerous tissues

Gal-8 is upregulated and downregulated in various normal and cancerous tissues and serum. Nagy, et al. investigated 41 human colon tissue

than FA, MNG and NT, whereas blood vessel density in FA was significantly increased than PTC and apparently but not statistically significant than MNG. The increased expression observed both in mRNA and protein indicates that Gal-8 has a significant role in malignancy of thyroid cancer. We also learned that there is increase in nuclear localization of cancer tissues as the tumor metastasized to nearby lymph nodes. This also points out its role during metastasis of disease. Gal-8 has emerged as an important regulators of tumor angiogenesis and was reported to be expressed in tumor endothelium [32]. We found an altered localization of Gal-8 both in cytoplasm and nucleus of

specimens with 26 carcinomas and found that extensively invasive colon cancers which is associated with a high TNM level exhibited significantly less Gal-8 than locally invasive ones which is associated with a low TNM level [34]. We observed similar result in our study in thyroid cancer in which tumor stage I/II (low stage tumor) showed higher Gal-8 expression compared to the stage III/IV (high stage tumor), p<0.03, but in their study Gal-8 expression was markedly decreased in human colon cancer compared to normal or benign colon tissue while we found a significant increase in Gal-8 expression comparing normal and benign thyroid. The prognostic value of Gal-8 is less likely studied in the literature. It was reported that Gal-8 expression has prognostic value in the late clinical stages (Dukes C and D stages) [35].

Recent report suggested that high mRNA expression of Gal-8 was noticed in 65 PTC at their primary development stage, followed by further increase in metastatic lymph nodes [21]. We herein also discovered that there is significant increase (2.6 fold) in PTC compared to BTD which is consistent to their result. According to Saal, et al. 6 cases of human thyroid tissues (3 normal case and 3 PTC) was examined by qRT-PCR using Gal-8 and found that there was strong expression but the sample size was very small [22].

Danguy, et al. group retrospectively studied 200 paraffin embedded tissues consisting of normal, benign and malignant tissues. They found marked decrease in IHC expression of Gal-8 in colon, pancreas, liver, skin and larynx while comparing malignant tissue to normal tissue and/or benign tumors whereas the relationship was inverse for breast tissues. In the same study Gal-8 expression was also measured significantly in tumors of the central and PNS as well as in skeletal muscle and mesotheliomas. Regarding subcellular localization of Gal-8 in colon cancer authors claimed that nuclei exhibited marked staining in the normal and benign cases of colon while nuclear localization of Gal-8 disappeared in malignant colon [36]. The cytoplasmic and nuclear localization of Gal-8 in normal and benign colon tissues was also reported by Nagy, et al. while its location was exclusively cytoplasmic in malignant colon cells [34].

In our study some of the benign tumor of FA exhibited truly nuclear or truly cytoplasmic localization while some exhibited both nuclear and cytoplasmic localization. In case of PTC the nuclear localization was shifted from (nuclear + cytoplasmic) when cancer cells metastasized to the lymph node of neck. This result suggests that Gal-8 is both a nuclear and cytoplasmic protein and shifting of protein from cytoplasm to nuclear during metastasis may delineate the malignancy of the diseases. However, further research should be done to know the actual functioning of this protein.

Gal-8 previously described as PCTA-1, as it was associated with expression of prostate carcinoma [37]. Gal-8 was expressed at moderate level in several human prostate cancer cell lines by qRT-PCR. The protein expression of Gal-8 was detected in all cell lines. They used IHC on a large samples of prostate tissues having advanced cancer stages and found that Gal-8 was expressed at moderate level in lesions corresponding to all stages including BHP (benign hypertrophy prostate) but discovered no any statistical differences among them. In the same study group they found that Gal-1, a prototype galectin is positively correlated with the number of CD34 positive vessels in advanced human prostate cancer [38]. Similarly, we also found a positive correlation when comparing expression of Gal-8 with CD34 stained positive vessels in PTC (classical type). This result may suggest that Gal-8 may have proangiogenic effects in PTC. Of note further exploration is needed to delineate the angiogeneic effects of Gal-8 in thyroid tumors. Su, et al. shown the upregulation of Gal-8 in prostate cancer. They demonstrated that (pro 1.5 mab) prostate carcinoma 1.5 monoclonal antibody identifying the gene PCTA-1, a human Gal-8 reacted strongly with prostate carcinoma cells and showed some reactivity with PIN (prostate intraepithelial neoplasia) but not with normal or benign prostate [37]. In contrast Gal-8 was expressed constantly compared to benign hyperplasia and adenocarcinoma [1]. So, there is disparity in the expression of Gal-8 regarding prostate cancer. Some researcher discovered constant expression whereas some found increased expression.

Using human breast cancer, colon cancer and glioma cell lines by qRT-PCR Satelli, et al. analyzed that Gal-8 are cancer specific but absence in the non cancer cell lines. When the subcellular fraction were analyzed for the presence of

Gal-8, 36 kDa protein was predominantly detected in the nucleus and to some extent to cytoplasm [16]. We detected 6/32 of Gal-8 protein of cytoplasmic localization, 16/32 of predominantly nuclear localization and 10/32 of both cytoplasmic and nuclear localization.

Similar to prostate cancer there is conflicting result of Gal-8 expression in lungs. Some authors reported that Gal-8 was not expressed in healthy lung but transcripts was found in tumor and embryonic tissues [5]. Some reported high level of Gal-8 in normal and tumoral human lung tissues [36]. Gal-8 was also found to be upregulated in human lung cancer as well as in local and distant metastasis [39]. Gal-8 is very scarcely studied in other tumors with more conflicting results. For bladder cancer it is reported that Gal-8 expression was positively correlated with tumor grading, tumor staging and mortality and a significant upregulation was observed in superficial transitional tumors [40] whereas a high Gal-8 expression was found in normal urothelium and significant decrease of staining was found in association with higher tumor stages [41]. In the latter case a shift from strong to weak staining of nucleus was seen among higher tumor stages.

Gal-8 expression seems to be upregulated in hypopharyngeal and laryngeal tumors. Its expression was positively correlated with the T stages, the nodal and clinical stages in laryngeal squamous cell carcinoma [42, 43]. Danguy, et al. using the small sample size described the decreased expression of this protein in larynx [36]. Expression profile of Gal-8 was also found to be downregulated on Acute myeloid leukemia patients [44] and also in squamous cell carcinoma of nasosinusal diseases [45].

Researchers found that Gal-8 expresssion was abundantly expressed in epithelial and chondriod compartment of pleomorphic adenoma (PA) with both cytoplasmic and nuclear staining and PA formation was associated with shift of Gal-8 from cytoplasm to nucleus. In the same study it was observed excessively in both nucleus and cytoplasm of salivary gland malignancy [46].

Human brain tumor such as Astrocytomas and Glioblastomas [47], placenta [48], Cutaneous T cell lymphoma [49], and cholesteatomas [50] also have upregulation of Gal-8 expression.

Relationship of MVD with thyroid diseases specially PTC

CD34 has been used as a marker to analyze angiogenesis. Conflicting results was discovered in the past for MVD in PTC using different vascular marker such as FVIII and CD31. In the present study, we analyzed that MVD of FA was significantly higher than PTC and MC. We also measured strength of relationship of MVD with tumor size and lymph node metastasis but did not find any statistical difference between them. However, apparently there was increase of MVD in the patient of lymph node metastasis group in comparison to lymph node non metastasis group.

Recently in Chinese study using staining pattern both with CD34 and CD31, they determined that the mean MVD were significantly higher in the benign group (Nodular goiter and adenoma group) comparing PTC [51]. This result was consistent with our results. Similar results were reported by Rzeszutko, et al. who reported that MVD in benign lesions of thyroid (Nodular goiter and FA) was higher than in neoplastic lesions PTC, MC and FC with least MVD in FC and MC [52].

Differentiated thyroid carcinomas are highly vascular usually around the capsular invasion. The MVD of tumor area was significantly related with tumor size greater than 3 cm in diameter according to Dhar, et al. but we found no correlation of MVD with the size of tumor. They noted higher MVD in the area of extrathyroidal invasion and FC had higher MVD than PTC [28]. Using the same antibody (CD34) Wong, et al. also found that MVD of FC significantly higher than FA whereas, apparently FA was greater than PTC but not significantly [53]. Similar to these two study but using different vascular marker (CD31), vascularization was found highest in case of FC than PTC and it became significantly different when they compared well differentiated FC to Anaplastic carcinoma [27]. In contrast to result of another study, they discovered MVD in FA, Grave's disease and PTC was increased than FC and NT [54].

Akslen, et al. analyzed 126 PTC using FVIII and discovered that MVD was increased in PTC comparing surrounding non-neoplastic thyroid tissues and there was inverse relationship significantly shown between MVD and patient's

age, tumor diameter, histological grade and tumor extent. Tumors with grade 2 had reduced MVD than grade 1 and tumors with extrathyroidal extension had lower MVD than intrathyroidal tumors [55] which was in contrast with result of Dhar, et al., however, they had used CD34 as staining marker which may be the cause of different results. Goldenberg and his colleagues analyzed 38 well differentiated thyroid carcinomas and showed it was significantly higher than NT [56]. This result was supported by Akslen, et al. Recent than the two group here visualized 31 PTC blood vessels and they also found that tumor MVD was 1.45 fold higher than in the surrounding normal tissues. PTC had higher MVD than FA and minimally invasive FCs [57]. All these studies showed higher MVD of PTC while comparing NT. In opposite trend to these results taking a large sample it was shown that MVD was decreased in proliferative lesions (benign and malignant) when compared to NT concluding that NT tissues had more angiogenesis than thyroid proliferative lesions. They stated that the lower MVD in some proliferative lesions comparing NT may be due to dedifferentiation of thyroid tissues where it loses the ability of rich vascularization of normal thyroid gland [24]. We found decrease vascularization in PTC and MC in comparison to benign and proliferative diseases of thyroid. In some study the author found no clear relationship between MVD measurement and thyroid pathology [58]. In our present study also we did not find any correlation between blood vessel density and lymph node metastasis but in some study when the PTC group were divided with and without metastasis there was tendency to higher angiogenesis in the metastatic disease group [59]. These results were all in agreement with other three study [30, 55, 56].

In the present study we also compared MVD of higher tumor stage (stage III/IV) to the lower tumor stage (stage I/II) (data not shown) and apparently found that lower stage of PTC had higher MVD than higher stage of PTC but it was not statistically significant. Similar data was found by Gulubova, et al. where patient with advanced PTC (Stage III/IV) had lower level of MVD compared to lower stages (stage I/II) [60]. A similar study detected by Frigugliett, et al. had less vascularization in more advanced stage of carcinoma [61].

In our study we found that MVD was lowest in MC significantly when compared with PTC. Our data was in agreement with Rzeszutko, et al. [52] but not with Fontanini, et al. who found that MVC was higher and have worst prognosis in MC comparing well differentiated and undifferentiated carcinomas [62]. Regarding prognosis of thyroid cancer there is controversial results. Some reported that lower MVD is related with worst prognosis, poor differentiation, advance stages and higher mortality [27, 55, 58, 61] whereas some reported that increased MVD is associated with increased risk of recurrence, shorter disease free survival and poorer survival [28, 59, 63]. We did not analyze any correlation with prognosis of disease.

Conclusion

Gal-8 was expressed in most of papillary thyroid cancer which suggested that Gal-8 may be a sensitive marker for PTC. The expression of Gal-8 in PTC compared to BTD and in the lower stages of carcinoma is significantly increased, this may indicate that Gal-8 may be one of diagnostic marker for PTC. The significant correlation of Gal-8 with MVD suggested that it has some roles in angiogenesis.

However, further exploration is needed to unravel its role in angiogenesis. Angiogenesis is reduced in malignant neoplasm (Papillary carcinoma and medullary carcinoma) compared to benign neoplasm (Follicular adenoma and Multi nodular goiter) in thyroid but we were not able to determine the role of angiogenesis regarding metastasis and tumor size. In the near future prognostic value of Galectin 8 and angiogenesis in thyroid cancer need further exploration.

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Disclosure of conflict of interest

None.

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