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Basic Science

Immunohistochemical Expression of TGF-B1, SMAD4, SMAD7, TGFβRII and CD68-Positive TAM Densities in Papillary Thyroid Cancer

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Keywords: Thyroid cancer; TGF-β pathway; Immunohistochemical expression; Macrophages; Tumorigenesis

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Abstract

BACKGROUND: Papillary thyroid carcinoma (PTC) accounts for 80% of the thyroid malignancies that are characterised by slow growth and an excellent prognosis. Over-expression of SMAD4 protein restores TGF-β signalling, determines a strong increase in anti-proliferative effect and reduces invasive potential of tumour cells expressing it.

AIM: The study aimed to analyse the immunohistochemical expression of TGF-β1 and its downstream phosphorylated SMAD4, element and of the inhibitory SMAD7 PTC variants and their association with the localisation of TAMs within the tumour microenvironment.

METHODS: For this retrospective study we investigated 69 patients immunohistochemistry with antibodies against TGF-β, TGF – β-RII, SMAD4, SMAD7, CD68+ macrophages.

RESULTS: Patients with low infiltration with CD68+ cells in tumour stroma has significantly shorter survival (median of 129.267 months) compared to those with high CD68+ cells infiltration ($p = 0.034$). From the analysis of CD68+ cells in tumour border and tumour stroma correlated with expression of TGF-β1 / SMAD proteins, we observed that the positive expression of TGF-β1 in tumour cytoplasm, significantly correlated with increased number of CD68+ cells in tumour border ($X^2 = 5,945$; $p = 0.015$).

CONCLUSION: TGF-β enhances motility and stimulates recruitment of monocytes, macrophages and other immune cells while directly inhibiting their anti-tumour effector functions.

Introduction

The transforming growth factor beta (TGF-β) impact on the immune system and tumour progression has been studied in general and in the development of thyroid cancer [1] [2] [3] [4] [5]. TGF-β activation is induced by several mechanisms including the expression of αvβ6 integrin, chymase, elastase, MMP9 etc. [6] [7] [8]. Activated TGF-β, after binding the TGF-β receptor II (TGF-RII) efficiently trans - activates TGF-β receptor I (TGF-RI) and directly promotes tyrosine phosphorylation [9]. Then TGF-β signalling activates R-Smads (SMAD2 and SMAD3 localised in the cytoplasm), through phosphorylation and after that allows the assembly of complexes with SMAD4. These complexes re-localise to the nucleus where they can regulate gene transcription [10]. This is the active TGF–β/SMAD–dependent pathway [2]. On the opposite, SMAD7 antagonises TGF–β/Smad – dependent signalling, and induces TGF-β receptor degradation and thus preventing SMAD2 and SMAD3 phosphorylation**.** SMAD7 leads a loss of TGF–β/SMAD-dependent pathway growth inhibition [10].

Normally and in the early phase of cancer TGF–β/SMAD-dependent pathway signalling inhibits

epithelial or cancer cell proliferation, and sometimes migration and invasiveness. These processes are studied in some cases with papillary thyroid cancer (PTC) [2] [11].

In late phases tumour cells overcome TGF-β-induced suppressor effects on cell proliferation, an on the other hand tumour cells themselves may respond to this cytokine including other effects that contribute to tumour progression such as epithelial-mesenchymal transition (EMT), invasion and metastases [1] [3]. TGF-β modulates the immune response that shields a tumour from immune surveillance [12] [13].

PTC accounts for 80 % of the thyroid malignancies that are characterised by slow growth and an excellent prognosis. However, 10–15 % of cases exhibit aggressive behaviour with hallmarks of local invasion, distant metastases, treatment resistance, and motility [14] [15].

TGF-β1 normally expressed and secreted by normal thyrocytes is a potent inhibitor of thyroid cell growth [16] [17]. In tumours, TGF-β expression has been detected in 52-100% of PTC cases [18]. SMAD4 is found to be expressed in 75% of PTC cases [11] [19] and every PTC cell line [19]. **The** SMAD7 expression has been detected in 80% of PTCs and PTC cell lines [11] [20**].** It has been found that TGFβRII mRNA transcripts are mainly expressed in 55% of PTCs compared to other thyroid cancers [21]. However, TGFβRII protein and mRNA expression display reduced levels in thyroid cancer cells [17] [21].

TGF-β is abundant in the tumour microenvironment. It stimulates all cell populations including tumour cells, fibroblasts, and endothelial cells. Moreover, it enhances motility and recruitment of immune cells including monocytes, macrophages, NK cells, dendritic cells and T-cells while directly inhibiting their anti-tumour functions [22] [23].

We decided to analyse the immunohistochemical expression of TGF-β1 and its downstream phosphorylated SMAD4, element and of the inhibitory SMAD7 in PTC variants and their association with the localisation of TAMs within the tumour microenvironment.

Material and Methods

Tumor samples

A series of 69 PTC cases has been retrieved from the Archives of the University Hospital in Stara Zagora, Bulgaria. The group of patients with PTC include cases with lymph node metastases (n = 2 or 2.9%) and without metastases and without metastases (n = 67 or 97.1%). The mean follow-up of 152.58 months; range from 1.64 to 197.07 has been done. PTC tumours were classified by size into tumours 1cm or less in greatest dimension and tumours more than 1cm in greatest dimension. There are 9 (13.0%) men and 60 (87.0%) women with age ranging from 22 to 81 years (mean 51.84 ± 13.756). Among the 69 PTC studied, 6 are ≤ 1 cm in diameter, and 63 tumours are larger. According to AJCC classification [24], 42 of the patients (60.8%) are in stage I, 20 of the patients (29.0 %) are in stage II, 7 of the patients (10.2%) are in stage III, and no patients are in stage IV of the disease, (Table 1).

Table 1: Demographic, clinical data, histological and pathological characteristics of the tumour specimens according to the papillary thyroid tumour type

Characteristics	PTC N (%)
Age (mean ± SD)	54.17 ± 14.48
Gender	
Males	9 (13.0)
Females	60 (87.0)
Pt classification	
T1-T2	64 (92.7)
T3-T4	5 (7.3)
Lymph node metastases	
N0	67 (97.1)
N1	2 (2.9)
Distant metastases	
M0	69 (100)
M1	0 (0)
Ptnm staging	
I stage	42 (60.8)
II stage	20 (29.0)
III stage	7 (10.2)
IV stage	0 (0)
Differentiation	
Well	69 (100)
Poorly	0 (0)
Capsule	
Non	9 (13.0)
Presence	60 (87.0)

* - ANOVA test; ** - $\chi 2$ test.

The study protocol has been approved by the Research Ethics Committee of University Hospital Stara Zagora.

All samples have been fixed in formalin and embedded in paraffin. Clinical data has been collected from the pathology reports, clinical files and Oncology Dispensary. H&E slides are retrieved from the archives, and they have been reviewed independently by two pathologists (MG and KI), and the tumours are classified using the WHO criteria [25]. Cases with doubtful PTC features are excluded.

Immunohistochemistry

Immunohistochemical staining is performed using the streptavidin-biotin technique as previously described [26]. Briefly, the endogenous peroxidase is blocked with 3% hydrogen peroxide in methanol for 10 min. Slides are incubated over night at room temperature with primary antibodies as follows: TGF - β (Clone sc-146) antibody in a dilution 1:50; monoclonal mouse anti-SMAD4 antibody (Clone sc - 7966) in a dilution 1:100; monoclonal mouse anti-SMAD7 antibody (Clone sc-11392) in a dilution 1:100; monoclonal mouse anti-TGFβ RII antibody (Clone sc-400) in a dilution 1:100-all produced from Santa Cruz Biotechnology, CA and monoclonal mouse anti-CD68

antibody (Clone KP11 M0814) ready–to-use, has been purchased from DAKO, Glostrub, Denmark. Then the slides are incubated with biotinylated secondary antibody and streptavidin-peroxidase complex for 2 hours and room temperature. Tissue sections with adequate positive and negative controls are used in every set of straining. Finally, sections are weakly counterstained with Mayer's hematoxylin.

Semi-quantitative assessment of TGF-β1, SMAD4, SMAD7, and TGFβRII

The TGF-β1 expression is evaluated in the cytoplasm of tumour cells, TGFβRII expression is evaluated in tumour cell cytoplasm and membrane. SMAD4 and SMAD7 are evaluated in tumour cell cytoplasm and tumour cell nucleus. The immunohistochemical expression is evaluated as absent (0) and present (+) in tumour cell cytoplasm and nuclei. The TGF-β1 expression is also evaluated in the centre and periphery of all thyroid cancers (PTC) and in the respective remaining thyroid tissue, which has been used as an internal control. TGF-β1 expression, detected in thyrocytes in the control tissue is considered as the basal expression.

The expression of SMAD4 and SMAD7 is evaluated in all PTCs and in the respective remaining thyroid tissue, which has been used as an internal control tissue and considered as a basal expression. The expression of SMAD4 and SMAD7 has been not evaluated in the centre and periphery since it is considered too difficult to distinguish and therefore assumed as similar. Their expression is also graduated in two categories: absent and present (weak, moderate and intense staining) regarding each subcellular location (nuclear and cytoplasmic) separately.

Macrophage counting

A single pathologist (MG), who is blinded to the clinical assessments of each case, has scored the cases by counting, the number of CD68 TAMs in five independent fields of vision in a tumour and the invasive front under a 400 x magnification. $CD68^+$ cell counts are expressed as the mean ± standard deviation.

Patients' slides with PTC assessed parameters

Clinicopathological parameters. On H&E slides the following parameters are evaluated: capsule formation, capsule infiltration, vascular invasion and from protocols – extra-thyroid extension, metastases, multicentricity and tumour size of micro-carcinomas are checked. Patients are followed up until January 2016.

Statistical analysis

The SPSS 16.0 program for Windows was used for statistical analysis. The chi-squared test and Fisher's exact test were used to compare the immunohistochemical staining and the clinicopathological parameters. ANOVA, Student – t-test, Mann-Whitney U test and Kruskal-Wallis test were applied for comparing the continuous variables depending on the normality of the distribution. Correlations were tested by Spearmen and Person tests. Survival plots were drawn by the Kaplan–Meier test and survival periods were compared by log-rank test. The accepted level of significance was set at $p < 0.05$.

Results

Components of TGF-β1 pathway in tumour tissue

The TGF-β1 expression is detected mainly in tumour cell cytoplasm and is weakly demonstrated in tumour cell membranes. As compared to TGF-β1 expression, TGFβRII immune reaction is weaker in tumour cell cytoplasm (17.8%).

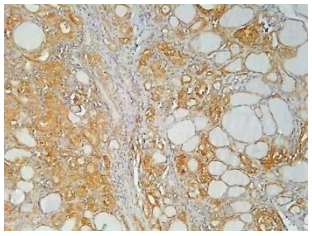

Figure 1: Positive expression of TGF-β1 in tumour border (x 100)

The TGF-β1 expression is not significantly correlated to any other clinical or pathological parameters. The cytoplasmic expression of TGF-β1 is directly proportional to the expression of both SMAD4 and SMAD7.

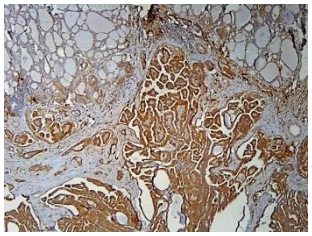

Figure 2: Positive expression of TGF-β1 in tumour cytoplasm and tumour border in micro-papillary thyroid cancer (x 100)

Lack of expression of TGF-β1 and SMAD7 in tumour cell cytoplasm is associated with capsule formations around tumour tissue. The SMAD4 nuclear expression is observed in 8.1% (n=4 patients) of tumours. There is not a significant difference between

TGF-β1 expression at the periphery of each a tumour and in its centre.

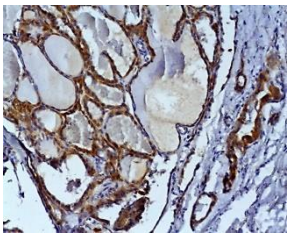

Figure 3: TGFβRII positive, strong expression (x 200)

The expression of SMAD4 and SMAD7 in tumour nuclei is insignificant. The SMAD4 strong cytoplasmic expression is observed in 11 patients (24.4%) and weak positivity in 26 patients (57.8%).

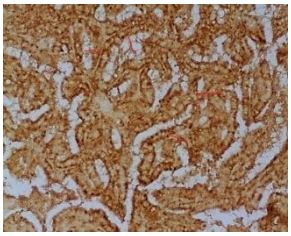

Figure 4: Positive expression of Smad4 in tumour nuclei (x 100)

The SMAD7 strong cytoplasmic expression is observed in 3 patients (6.7%) and weak positivity in 27 patients (60%). TGF-β1, TGFβRII, SMAD4 and SMAD7 cytoplasmic expression is not significantly associated with any clinical or pathological parameters, (Figure 1-5).

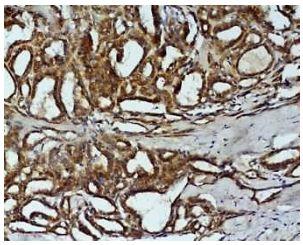

Figure 5: Strong positive expression of Smad7 in tumour cytoplasm (x 200)

Characterization of TAMs in PTC

The CD68 immunohistochemistry effectively stains macrophage cytoplasm. The TAM nuclei in PTC tissue are approximately 1/3 to ½ of the size of the nuclei of tumour cells. TAMs had cellular projections that wrap around tumour cells. TAMs and cancer cells appeared to be in close contact (Figure 6). TAMs formed distinctive canopy-like structures over some tumour cells.

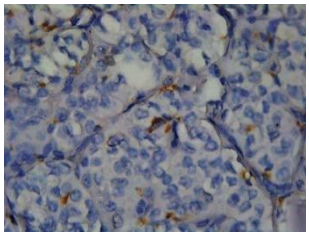

Figure 6: CD68-positive macrophages with canopy-like structures (x200)

Correlations between clinic-pathologic parameters, TGF-β1, TGFβRII, SMAD4 and SMAD7and CD68-positive TAM densities

As histologic grade (type) of thyroid cancer is an important determinant of tumour behaviour and clinical prognosis, it is important to explore the effect of TAMs and TGF-β1 signalling pathway proteins on some clinic-pathologic parameters. There is no statistical correlation between the presence of TAMs (high or low density) and extra-thyroidal extension, capsular invasion and vascular invasion. The intense TGF-β1, SMAD4 and SMAD7 expression in tumour cell cytoplasm correlated with increased CD68+ TAMs number in tumour stroma (and for TGF-β1 in the invasive margin). There were no differences between patients with or without increased CD68- positive TAMs in gender, age, tumour stage and grade (Table 3).

Table 2: Associations between the presence of CD68-positive cells in tumour border and the invasion front of tumours with the expression of molecules involved TGF-β1 signalling pathway

Expression level of TGF-β1 signalling pathway molecules	№	CD68 in tumour border N (%)		CD68 in tumour stroma N (%)	
		Low numbers (less than 5.57-50th percentile)	High numbers (less than 5.57-50th percentile)	Low numbers (less than 11.80-50th percentile)	High numbers (less than 11.80-50th percentile)
TGF-β1 in tumour stroma					
Negative n (%)	17	3 (17,64)	14 (82,35)	9 (52,94)	8 (47,05)
Positive n (%)	34	21 (52,5)	19 (47,5)	20 (58,82)	14 (41,17)
P value		0.015*		0.689	
TGF-β1 in tumour border					
Negative n (%)	22	8 (36,36)	14 (63,63)	5 (50,00)	5 (50,00)
Positive n (%)	29	16 (55,17)	13 (44,83)	24 (58,53)	17 (41,47)
P value		0.183		0.625	
SMAD4 in tumour stroma					
Negative n (%)	17	7 (41,17)	10 (58,82)	10 (58,82)	7 (41,17)
Positive n (%)	34	17 (70,83)	7 (29,16)	19 (55,88)	15 (44,12)
P value		*0.058***		0.842	
SMAD4 in tumour border					
Negative n (%)	27	15 (40,45)	22 (59,45)	10 (55,55)	8 (44,44)
Positive n (%)	14	9 (64,28)	5 (35,71)	19 (57,57)	14 (42,42)
P value		*0.129****		0.889	
SMAD7 in tumour stroma					
Negative n (%	30	13 (43,33)	17 (56.66)	18 (60,00)	12 (40,00)
Positive n (%)	21	11 (52,38)	10 (47,61)	11 (52,38)	10 (47,61)
P value		0.524		0.589	
SMAD7 in tumour border					
Negative n (%)	25	17 (68,0)	8 (32,0)	15 (60,0)	10 (40,0)
Positive n (%)	26	12 (46,15)	14 (53,84)	9 (34,61)	17 (65,38)
P value		*0.069***		*0.115****	

X^2 - test: * The values in bold shown statistical significance; ** Values in bold italic shown (border) importance; *** The values in italic shown tendency.

From the analysis of CD68- positive cells in tumour border and tumour stroma correlated with expression of TGF-β1/SMAD proteins, we observed that the positive expression of TGF-β1 in tumour cytoplasm, significantly correlated with increased number of CD68-positive cells in tumour border (χ^2 = 5.945; p = 0.015). Our work also revealed marginal significantly of Smad4 in tumor cytoplasm as compared to increased number of macrophages in tumor border (χ^2= 3.606; p = 0.058), and a tendency for Smad7 in tumor border correlated with increased number of CD68-positive cells in tumor stroma (p = 0.115), (Table 2 and 3).

Table 3: Association between various clinic-pathological factors with the number of CD68-positive cells in tumour stroma and tumour border

Characteristics	№	CD68+ cell in tumour stroma mean ± SD	P value	CD68+cell in tumour border mean ± SD	P value*
Age (mean ± SD)					
<53,28	30	22.85 ± 20.27		51.99 ± 9.49	
>53.28	39	16.46 ± 21.30	0.227	34.51 ± 6.76	0.484
Gender					
Males	9	20.77 ± 23.08		15.26 ±18.48	
Females	60	19.71 ± 20.62	0.898	28.17 ± 48.17	0.467
Pt classification					
T1-T2	64	20.82 ± 21.53		31.97 ± 46.31	
T3-T4	5	35.23 ± 24.76	0.177	36.44 ± 71.83	0.565
Lymph node metastases					
No	2	30.55 ± 35.14		30.55 ± 35.14	
Yes	67	22.07 ± 21.87	0.385	22.07 ± 21.87	0.647
Ptnm staging					
I=II	62	20.29 ± 21.20		32.03 ± 47.56	
III-IV	7	33.89 ± 24.90	0.143	34.80 ± 58.76	1.000
Differentiation					
Well	69	26.16 ± 24.84		41.44 ± 54.90	
Poorly			1.000		0.452
Capsule					
None	9	19.50 ± 22.32		29.98 ± 54.91	
Present	60	24.33 ± 22.38	0.177	34.58 ± 49.11	0.937

* Mann-Whitney U test.

Patients with low infiltration with CD68-positive cells in tumour stroma have significantly shorter overall survival (median of 129.267 months) compared to those with high CD68-positive cells infiltration (median is not reached, p = 0.034, Log-rank test).

Discussion

Our study demonstrates that TGF-β1, SMAD4, SMAD7 and TGFβRII protein expression is observed in PTC tumour cells and these proteins are overexpressed in tumour cells as compared to the surrounding normal thyroid tissue. We couldn't find any difference in the expression of TGF-β1 signalling proteins in tumour centre and periphery as did Eloy et al. (2012). Moreover, the number of patients with PTC is similar in both studies (75 patients in Eloy at al., 2012; 80 patients in our study). Our PTCs are mainly well-circumscribed, and we don't subdivide them to well-circumscribed papillary thyroid cancer (WCPTC) and poorly-circumscribed papillary thyroid cancer (PCPTC), respectively as Eloy et al., 2012 do. These authors establish that in WCPTCs the differences of TGF-β1 expression from centre to the periphery are very insignificant. We detect that TGF-β1, SMAD4 and SMAD7expression in all cases of PTC and control tissues (except in 17 TGF-β1 negative cases) are similar to that reported previously [2] [11] [18]. SMAD4 cytoplasmic/nuclear expression is considered to be indicative of a functioning TGF-β (SMAD-dependent pathway) [11] [19]. In our study, it is demonstrated that all tumours expressed SMAD4 in their cytoplasm to a lesser extent than TGF-β1. It is known that the lower expression of SMAD4 was mainly responsible for the impairment TGF-β signalling [19]. In a previous study, the authors demonstrated that *SMAD4* mutations are frequent in PTC [27] when sequencing of the entire coding part of the *SMAD4* gene was performed. In later study D'Inzeo et al., 2010 hypothesised that the cause of lower expression of SMAD4 could be found in alteration of major components of translational machinery, which are frequently altered in human neoplasms [28]. The molecular mechanism that controls subcellular localisation and activation of Smad proteins is crucial for TGF-β signalling, and it is not yet fully clarified. It has been shown that SMAD4 nuclear expression is reduced in cancer [19]. However, SMAD4 undergoes continuous nucleocytoplasmic shuttling on its own, independently of TGF-β signalling [29]. The levels and the duration of residence in the nucleus of SMAD4 are important events for the response of TGF-β in the cells, and the intensity and duration of the TGF–β-Smad response is important for the signalling specificity. There we demonstrate the reduction of SMAD4 protein expression in PTC tumour cell cytoplasm and in nuclei which may be indicative of a loss of TGF-β cytostatic response (loss of tumour cell growth inhibition). Therefore the reduction of SMAD4 cytoplasmic and loss of nuclear protein expression is associated with the embarrassment of the TGF-β signalling pathway.SMAD7 nuclear expression was associated to loss of TGF–β/Smad–dependent pathway inhibition [2], and its expression is found to be at basal levels and lesser as compared to TGF-β1 and SMAD4 expression of the cases in the presents series-similarly to other reports [11]. TGF-βRII mRNA overexpression was detected in PTC cell lines [17]. In our study, TGF-βRII expression was lower as compared to TGF-β1 expression in tumour cells of PTC.

We demonstrate that all investigated from TGF–β/Smad pathway proteins in our study are associated with increased CD68 TAMs density in tumour stroma and the border of PTCs. It has been shown that large cohorts of cancers including thyroid cancer with high-density TAMs have poor prognoses and poor survival rates [30].

The impact of TGF-β signalling in the immune system was well documented. TGF-β promotes recruitment of monocytes, and it has been hypothesised that TGF-β can promote monocytes to

macrophage differentiation [1]. Moreover, TGF-β stimulation of macrophages had been shown to attenuate macrophages associated suppression of $CD4^+$ T cell proliferation. TGF-β signalling is needed for the alternative activation of macrophages to M2 status. It has been shown that lack of TGF-βRII leads to the defects in the expression of a set of genes that form the hallmark of the M2 polarising program [22].

Therefore, TGF-β enhances motility and stimulates recruitment of monocytes, macrophages and other immune cells while directly inhibiting their anti-tumour effector functions [31]. As a result, TGF-β associated inflammation can promote tumorigenesis due to secretion of growth-factors, cytokines, chemokines, etc. from the recruited immune cells that stimulated cancer cell growth, motility and invasion.

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