Transforming growth factor β-receptor II protein expression in benign prostatic hyperplasia is associated with prostate volume and inflammation.

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OBJECTIVE: • To assess transforming growth factor β-receptor II (TGFBRII) protein expression in benign prostatic hyperplasia (BPH) using immunohistochemistry analysis, and to compare the analysis with phenotypic properties.

METHODS: • TGFBRII protein expression was profiled using three clinical outcome tissue microarrays (TMAs), sampled from 231 patients who underwent Using these TMAs, five inflammatory cell markers surgery for BPH. • The were also assessed, including CD3, CD4, CD8, CD20, and CD163. • surgical procedure was open prostatectomy in 95 patients and transurethral resection of the prostate in 136 patients.

RESULTS: • TGFBRII protein expression was found in BPH epithelium cells for both basal and secretory cells, as well as in fibromuscular stromal cells. TGFBRII staining was also strong in most of the lymphocytes TGFBRII stromal staining was found to be infiltrating the prostate. • significantly associated with prostate volume (P = 0.04), whereas TGFBRII epithelial staining was found to be significantly associated with 5-α-reductase-inhibitor medical therapy received by patients Both stromal and epithelial TGFBRII before surgery (P = 0.004). • staining were found to be associated with CD4 T-lymphocyte infiltrate, independently of prostate volume (P < 0.001 and P = 0.002).

CONCLUSIONS: • TGFBRII protein expression in BPH is associated with prostate gland TGFBRII might be a volume and with CD4 T-lymphocyte prostatitis. • promising therapeutic target to prevent prostate enlargement or even to decrease prostate volume.

TGFBR2 protein expression in benign prostatic hyperplasia is associated with prostate volume and inflammation

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Summary

Background: The aim was to assess transforming growth factor beta receptor 2 (TGFBR2) protein expression in benign prostatic hyperplasia (BPH) using an immunohistochemistry analysis, and to compare it to phenotypic properties.

Methods: TGFBR2 protein expression was profiled on three clinical outcome tissue microarrays (TMAs), including 231 patients operated for BPH. On the same TMAs, five markers of inflammatory cells were also assessed, including CD3, CD4, CD8, CD20, and CD163. Surgical procedure was open prostatectomy in 95 patients and transurethral resection of the prostate in 136 patients.

Results: TGFBR2 protein expression was found either in BPH epithelium cells for both basal and secretory cells, and in fibromuscular stromal cells. Most of lymphocytes infiltrating the prostate also showed a high TGFBR2 staining. TGFBR2 stromal staining was found significantly associated with prostate volume (p=0.04) whereas TGFBR2 epithelial staining was found significantly associated with with 5 alpha reductase inhibitor medical therapy received by patients prior to surgery (p=0.004). Both stromal and epithelial TGFBR2 staining were found associated with CD4 T-lymphocytes infiltrate, independently of prostate volume (p<0.001 and p=0.002).

Conclusion: TGFBR2 protein expression in BPH is associated with prostate gland volume and with CD4 T-lymphocyte prostatitis. TGFBR2 could be a promising therapeutic target to prevent prostate enlargement or even to decrease prostate volume.

Key Words: TGFBR2, BPH, prostate, volume, inflammation.

Introduction

Benign prostatic hyperplasia (BPH) is one of the most common diseases affecting aging men. Clinicians commonly use the term BPH to describe a clinical syndrome consisting of three components: lower urinary tract symptoms (LUTS), benign prostatic enlargement, and bladder outlet obstruction [1]. BPH is histologically defined as overgrowth of the epithelial and stromal cells of the transition zone and periuretral area. So far, a variety of growth factors associated with epithelial/stromal interaction have been described in the pathophysiology of BPH but the cellular and molecular processes underlying the pathogenesis and development of BPH remain poorly understood [2].

TGFBR2, transforming growth factor beta receptor II, is a transmembrane protein that has an intrinsic serine-threonine kinase activity and signals through a heterodimeric complex with another receptor protein (TGFBR1) that binds TGF-beta **[3].** This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation.

In a previous published study, our group found TGFBR2 to be upregulated at the RNA level in large prostate glands compared to smaller ones [4]. In another previous publication, inflammatory cells infiltrate within BPH was characterized and the role of inflammation in BPH development was highlighted by the strong correlation between histological inflammation, IPSS, and prostate volume. **[5]**. The goal of the current study was to assess TGFBR2 expression at the protein level in BPH using immunohistochemistry (IHC) analysis, and to compare it to phenotypic properties and inflammation.

Material and Methods

BPH Samples

TGFBR2 was profiled on three clinical outcome tissue microarrays (TMAs), including 231 patients operated for BPH. Surgical procedure was open prostatectomy in 95 patients and transurethral resection of the prostate (TURP) in 136 patients. Characteristics of patients are presented in Table 1. TMAs contained four cores per patient. Prostate tissue was obtained after written consent from each patient and was approved by the local ethics committee.

Out of 231 patients, 217 had symptomatic BPH. Fourteen patients underwent BPH surgery with a diagnostic purpose: they had a suspicion of prostate cancer on the basis of prostate specific antigen (PSA) serum level, but they did not have LUTS requiring surgery. Prostate volume was calculated on transrectal ultrasonography. The presence of a median BPH lobe was assessed during the surgical procedure. Medical BPH treatment was considered when received by patients for at least 3 months before surgery. This treatment was 5 alpha reductase inhibitor (5ARI) alone in 9 patients, alpha blocker therapy in 53 patients, phytotherapy in 6 patients, and 5ARI / alpha blocker association in 10 patients.

On pathology, BPH was associated to inflammation in 78 patients. Inflammation was defined by the presence of more than dispersed lymphocytes, plasma cells, neutrophils or macrophages in the fibromuscular stroma and/or in the glandular ducts and acini.

IHC

TGFBR2 IHC staining was performed on the Ventana Benchmark XT Automated Platform (Ventana Medical Systems, Tucson, AZ, USA) using the Ventana I.View DAB Detection kit, according to the manufacturer's recommended protocol. Briefly, sections of 4-µm thick paraffin-embedded TMA were dewaxed and rehydrated with xylene and ethanol. Slides were pretreated in 10 mM citrate buffer (pH 8.0) for optimal antigen retrieval, before being incubated with the primary antibody against TGFBR2 (TGFBR2 anti-Human Polyclonal Antibody, Lifespan Biosciences, Seattle, WA, USA) in a 1:20 dilution for 32 minutes. Optimal primary antibody concentration was determined by serial dilutions, optimizing for the most discriminating signal without background immunostaining. The antigen-antibody complex was then detected with the I.View DAB Detection kit (Ventana). The slides were counterstained with hematoxylin. Appropriate positive controls were performed.

Five markers of inflammation were also evaluated by IHC on the same TMAs. Antibodies (Ab) decorating T-lymphocytes were CD3. CD4 and CD8 markers were used to differentiate T4-lymphocytes from T8-lymphocytes. B-lymphocytes were marked by CD20, and macrophages were marked by CD163. After antigen retrieval, the sections were processed on a Ventana nexes automated system (Ventana Medical System) using the Ventana Basic DAB detection kit according to the manufacturer's instructions. The list of antibodies used for IHC analyses is presented in table 2.

Scoring of TGFBR2 protein expression and IHC inflammation markers

The intensity of TGFRB2 immunostaining was graded by two independent observers (AD, NW) as absent, low, or high. For each TMA, glandular epithelium and stromal compartments were quoted separately. Both color extent and color intensity were used to grade immunostaining. "Absent" was defined as no staining at all. "Low" was defined as patchy and/or weak staining, and "high" as a strong and diffused staining (figure 1). Reproducibility of the method was tested on 100 samples that were evaluated by each observer twice. Kappa coefficient was 0.91 and 0.93 for observer one and two, respectively. Kappa coefficient between observer one and two was 0.85. IHC inflammatory cells markers were quoted as absent or present by two independent observers (AD, NW). When the two observers disagreed, then the cases were read again to finally find a consensus.

Statistical Analysis

IHC grade intensity of each patient was obtained from assessing the maximal stain intensity of all valid cores from the same patient. For example, if only one out of four cores had a "high" score, the patient would be assigned a "high"score. At least one valid core per patient was required. TGFBR2 stain intensity was first compared to clinical and pathological parameters using Fisher test and Kruskall-Wallis test for qualitative and quantitative data, respectively. TGFBR2 was considered as a qualitative ordinal data. Then, TGFBR2 staining was compared to inflammatory cells infiltrate using a Kendall's tau-b non parametric test. Additional statistics were performed to compare binary variables to qualitative and quantitative date using Chi-square test and Student T test,

respectively. Commercially available software was used for statistical analyses (SPSS inc., Chicago, USA).

Results

TGFBR2 staining (figure 1)

Stromal staining was assessed in all 231 patients with a mean valid core per patient of 3.4. Glandular epithelial staining was assessed in 202 patients, with a mean valid core per patient of 2.3. TGFBR2 expression was found either in BPH epithelium cells (for both basal and secretory cells), and in fibromuscular stromal cells. TGFBR2 stromal staining was absent, low and high in 16%, 66%, and 18% of patients, respectively. TGFBR2 epithelial staining was absent, low and high in 7%, 56%, and 36% of patients, respectively (table 1).

Most of lymphocytes showed a high TGFBR2 staining (figure 1d). Lymphocytes' staining was of course not taken in account to assess epithelial and stromal staining.

Association with clinical and pathological parameters (Table 1)

TGFBR2 stromal staining was found significantly associated with prostate volume whereas epithelial staining was not. TGFBR2 stromal staining was also significantly associated with the type of surgery (p=0.003).

TGFBR2 epithelial staining was found significantly associated with inflammation as assessed on pathology. Mean prostate volume was 71 ml (Standard deviation 38) in cases with no inflammation, whereas it was 65 ml (Standard deviation 35) in those with inflammation (p=0.3).

TGFBR2 staining was not found associated to LUTS, presence of median lobe, and serum PSA level.

TGFBR2 protein expression was also compared to medical therapy received by patient prior to surgery. TGFBR2 epithelial staining was significantly associated with 5ARI therapy (p=0.004). No significant association was found between 5ARI therapy and prostate volume (67 ml and 69 ml in the groups with and without 5ARI therapy, respectively, Student T test p=0.8). In addition, no significant association was found between 5ARI therapy received by patients and inflammation on pathology: 47% of patients had inflammation in the 5ARI therapy group versus 32% in the remaining patients group (Chi square test p=0.2). Finally, in multivariable analysis, 5ARI therapy and inflammation on pathology were both associated with TGFBR2 epithelial staining (logistic regression p=0.013 and p=0.002, respectively).

Association with inflammatory cells infiltrate assessed on IHC (Table 3)
TGFBR2 protein expression was also compared to five markers of inflammatory cells infiltrate assessed with IHC. It was found a significant association between TGFBR2 epithelial staining and CD4 expression (p=0.002), and between TGFBR2 stromal staining and CD4 (p=<0.001), CD8 (p=0.046), and CD20 expressions (p=0.003). Prostate volume was associated with CD3, CD4, CD8, and CD20 expressions (p=0.006, p<0.001, p<0.001, and p=0.03, respectively, student T-test, data not shown). In multivariate logistic regression analysis, TGFBR2 staining was associated with CD4 expression, independently of prostate volume (p<0.001 and p=0.001 for stromal and epithelial staining, respectively).

Discussion

TGFBR2 protein expression was assessed in 231 BPH patients using a large scale TMA. It was found a significant association between TGFBR2 stromal staining and prostate volume. It was also found an association between TGFBR2 staining and inflammation in BPH (assessed on standard pathology and using IHC). In particular, cases with CD4 T-lymphocytes infiltrate within the prostate gland were more likely to have stromal and glandular TGFBR2 expression. Finally, we highlighted a significant association between epithelial staining and 5ARI therapy.

Our group previously published a list of genes supposed to be associated with prostate volume [4]. TGFBR2 was one of these. As described by other authors, gene array analyses have major limitations [6], and, therefore, should be validated by analyses using other methods. In the present study, we confirm at the protein level what we found at the RNA level.

Patients with large prostate glands were more likely to be operated by prostatectomy, whereas those with smaller prostate glands were preferentially operated through a transurethral approach. This might explain the fact that both the operative technique and the prostate volume were associated with TGFBR2 stromal staining.

It was previously shown that TGFB proteins and their receptors might have a role in BPH pathogenesis [7-9]. First, they might be under an androgen influence. They were found downregulated by androgen and upregulated by castration in murine prostate [10,11]. Dhanasekaran et al [12] compared gene expression between adult and pubertal prostates. Change of the prostate gland at puberty is a crucial juncture in prostate development that

is androgen dependent. These authors found TGFBR2 and TGFBR3 to be upregulated at the RNA level in the pubertal prostates compared to adult ones. They also observed a greater stromal to epithelial ratio in the pubertal prostates.

TGFB has been shown to be a regulator of stromal proliferation and differentiation in BPH [13]. Androgen may control the proliferation and differentiation of prostatic stromal cells by regulating the expression of TGFB [14]. Luo et al. [15] compared RNA expression using c-DNA microarrays in 9 BPH samples and 12 normal prostate tissues excised from radical prostatectomy specimen. They supposed that TGFB1 and TGFBR2 were downregulated and TGFB3 and TGFBR3 upregulated in BPH compared to normal prostate tissue. They confirmed that TGFB3 was upregulated in BPH using a semi-quantitative RT-PCR method. They did not confirm their finding on TGFBR2 at the protein level. At the opposite, for Pavelic et al. **[16],** TGFB1 and TGFB2 were upregulated and TGFB3 is downregulated in BPH compared to normal transition zone.

TGFB1 is classically considered to be inhibitory for epithelial cells [17]. In vitro, Untergasser et al **[18]** showed that TGFB1 treated cells compared to senescent cells, morphologically transdifferentiated into myofibroblasts with dense cytoskeletal fibers and increased expression of smooth muscle cells. Prakash et al. **[19]** compared RNA expression using c-DNA Microarrays in 5 samples from patients with asymptomatic BPH to 8 samples from patients with symptomatic BPH. They found a set of 511 differentially expressed genes distinguishing symptomatic and asymptomatic BPH. In particular, TGFB2 was upregulated in symptomatic BPH compared to asymptomatic BPH. Similarly, we compared TGFBR2 protein expression in symptomatic and asymptomatic

BPH cases. We did not observe any significant difference between these two groups. This might be due to the low number of asymptomatic BPH cases (N=14) in our series.

TGFBR2 was found significantly associated to 5ARI therapy. We were concerned by a possible confusing factor due to a possible association between 5ARI therapy and prostate volume, but patients treated by 5ARI had, on average, lower prostate volume than others. In addition, in multivariable analysis, 5ARI therapy and inflammation on pathology were both associated with TGFBR2 epithelial staining. Therefore we made the hypothesis that 5ARI could increase TGFBR2 protein expression in BPH tissue. As 5ARI therapy was previously proven to have an effect on prostate volume and on postoperative bleeding in patients operated for BPH [1, 20, 21], we were not surprised to observe that 5ARI could modify the level of a growth factor such as TGFBR2 within BPH tissue.

To our knowledge, here is the first report assessing an association between TGFBR2 protein expression and inflammation. TGFBR2 epithelial staining was associated with inflammation on pathology. In addition, both stromal and epithelial staining was found associated with epithelial CD4 lymphocyte infiltrate, independently of prostate volume. It means that cases with CD4 lymphocytes infiltrate were more likely to express TGFBR2 protein within BPH tissue. The relationship between BPH and inflammation was previously described [5, 22, 23]. One hypothesis is that inflammation could stimulate the production of growth factors such as TGFBR2, which could lead to BPH development and prostate overgrowth. Further studies should explore the exact role of the relationship between inflammation and TGFBR2 in the pathogenesis of BPH.

Overall, TGFB proteins and receptors, and TGFBR2 in particular, may play an important role in BPH pathogenesis: being under an androgen influence, they might stimulate tissue proliferation and differentiation, and modify stromal/epithelial ratio.

Prostate volume is known to have a real impact on BPH: baseline prostate size can be considered a strong indicator of BPH progression, particularly for acute urinary retention and BPH-related surgery but also for long-term changes in symptoms, bother, quality of life, and flow rate [20]. As TGFBR2 is associated with prostate volume, it might be a therapeutic target in BPH to prevent prostate enlargement or even to decrease prostate volume.

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Legend of figure 1:
TGFBR2 IHC staining in BPH (x 400). A: Glandular epithelial compartment with high staining. B: Stromal compartment with high staining. C: High staining in epithelial cells and in inflammatory cells. D: Absence of staining in glandular and stromal compartments
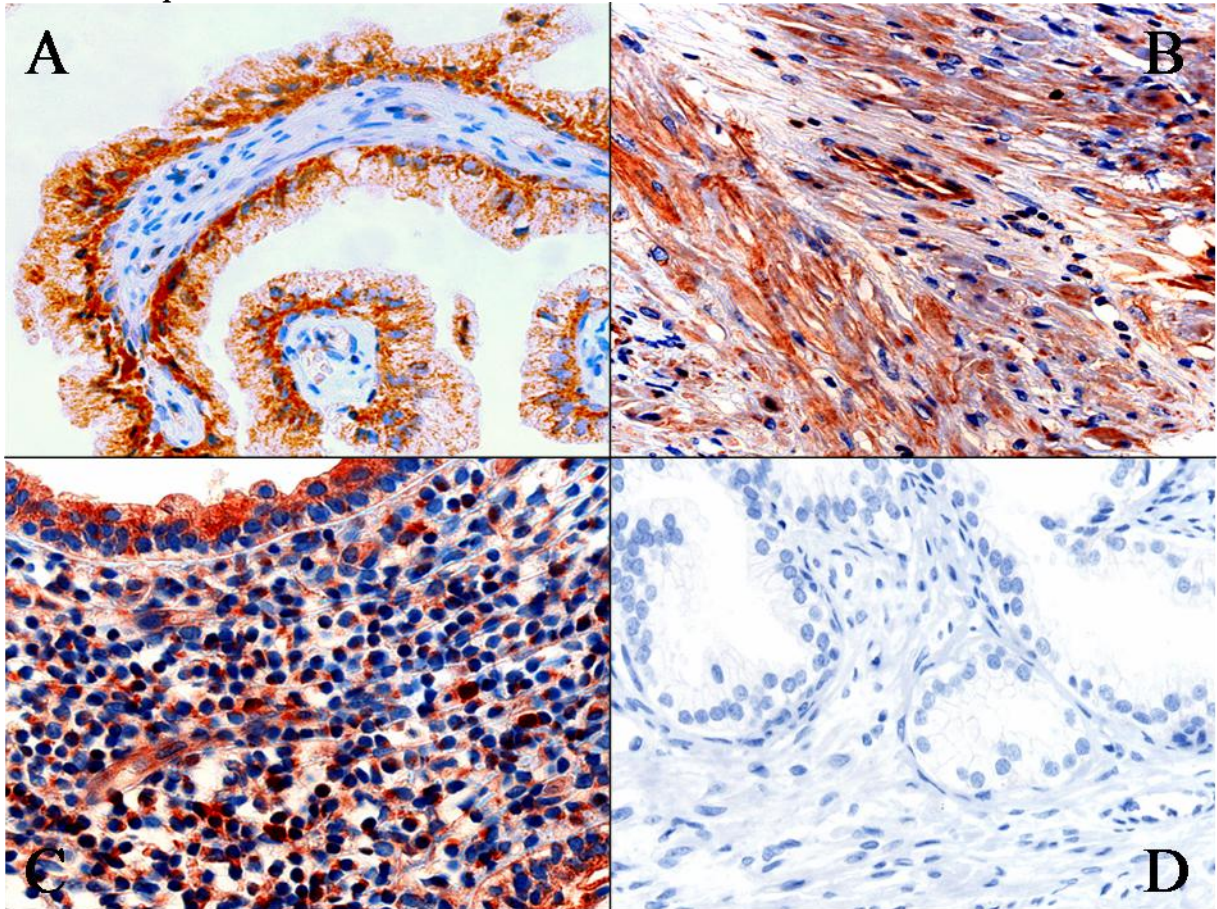
No conflict of interest

Table 1: Association between TGFBR2 protein expression and clinical and pathological parameters (*Fisher test, ** Kruskall-Wallis test)

		Total	Sromal staining (N=231)				Epithelial staining (N=202)			
			absent	low	high	p	absent	low	high	p
N		231	37 (16%)	153 (66%)	41 (18%)		15 (7%)	114 (56%)	73 (36%)	
Mean age	Range 50-91	N=70	71.8	69.3	70.8	0.6**	70.2	70.8	68.8	0.6**
Histology	BPH with no inflammation	N= 153	27 (17%)	101 (67%)	25 (15%)	0.3*	13 (10%)	88 (63%)	39 (27%)	0.001*
	BPH with inflammation	N=78	10 (13%)	52 (67%)	16 (21%)		2 (3%)	26 (42%)	34 (55%)	
LUTS	No	N=14	1 (7%)	11 (79%)	2 (14%)	0.7*	0 (0%)	8 (57%)	6 (43%)	0.4*
	Yes	N= 217	36 (17%)	142 (65%)	39 (18%)		15 (8%)	106 (56%)	67 (36%)	
Median lobe	Present	N=37	6 (16%)	24 (65%)	7 (19%)	0.9*	1 (4%)	14 (50%)	13 (46%)	0.2*
	Absent	N=194	28 (15%)	123 (67%)	33 (18%)		12 (7%)	93 (57%)	59 (36%)	
Type of surgery	TURP	N= 136	28 (21%)	90 (66%)	18 (13%)	0.003*	11 (10%)	63 (56%)	38 (34%)	0.3*
	Prostatectomy	N=95	9 (9%)	63 (66%)	23 (24%)		4 (4%)	51 (57%)	35 (39%)	
Medical Therapy	None	N= 153	28 (18%)	94 (61%	31 (20%)		10 (7%)	82 (61%)	43 (32%)	
	Phytotherapy	N=6	0 (0%)	6 (100%)	0 (0%)	NA	0 (0%)	3 (75%)	1 (25%)	NA
	Alpha -	N=63	8 (13%)	49 (78%)	6 (10%)	0.8*	5 (9%)	29 (53%)	21 (38%)	0.8*
	5ARI	N=19	3 (16%)	12 (63%)	4 (21%)	0.4*	0 (0%)	4 (27%)	11 (73%)	0.004*
Mean PSA (ng/ml)	Median 5.4 Range 0.5-43	10.4	7.9	10.5	12.0	0.3**	4.6	12.2	10.0	0.9**
Mean Prostate volume (ml)	Median 60 Range 20-220	69	59,8	69.2	76.9	0.04**	63.3	72.1	71.5	0.7**

Table 2: List of antibodies used for IHC analyses

Target	Antibody	Manufacturer	Dilution
TGFBR2	anti-Human Polyclonal Antibody	Lifespan Biosciences	1:20
CD3	rabbit polyclonal Ab A0452	Dako	1:300
CD4	mouse monoclonal Ab clone 4B12	Novocastra-Menarini	1:20
CD8	mouse monoclonal Ab clone C8/144B	Dako	1:200
CD20	mouse monoclonal Ab clone L26	Dako	1:500
CD163	mouse monoclonal Ab clone 10D6	Novocastra-Menarini	1:800

Table 3: Cross tabulation of TGFBR2 expression and IHC markers of inflammation infiltrate (Kendall's tau-b non parametric test)

IHC marker		Sromal staining (N=268)				Epithelial staining (N=238)			
		Absent	low	high	p	absent	low	high	P
CD3	Absent	15	29	9	0.1	4	25	10	0.07
	Present	23	124	32		12	89	63	
CD4	Absent	32	98	19	<0.001	14	75	37	0.002
	Present	6	55	22		2	39	36	
CD8	Absent	13	26	6	0.046	3	19	10	0.5
	Present	25	127	35		13	95	63	
CD20	Absent	25	68	17	0.03	10	52	29	0.15
	Present	13	85	24		6	62	44	
CD163	Absent	11	29	6	0.12	2	19	16	0.7
	Present	27	124	35		14	95	57	