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GADD45γ Methylation Is More Common In Benign Prostatic Hyperplasia Than In Prostate Cancer

Research Article

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Summary

Prostate cancer (PCa) is one of the most common cancer types worldwide in men. Although *Growth Arrest DNA Damage-Inducible 45* (*GADD45*) family members, *GADD45α*, *GADD45β* and *GADD45γ*, are responsible for the activation of several molecules in certain cellular pathways affecting cell fate, including tumorigenesis, as stress sensors, the role of *GADD45γ* in PCa is still not clear. In this study, our aim was to detect the methylation and protein expression profiles of *GADD45γ* in benign prostate hyperplasia (BPH) (60 patients) and PCa (56 patients). The methylation of *GADD45γ* was determined by methylation-sensitive high resolution melting analysis. Immunohistochemistry was used for evaluating GADD45γ protein expression. The methylation frequency for *GADD45γ* was as low as 1.8% in PCa compared with BPH (*P*=0.000). GADD45γ protein was overexpressed in PCa cases (53.6%) compared with the BPH cases (33.3%), and the difference was statistically different (*P*=0.028). There was no correlation between *GADD45γ* methylation and protein expression in both groups. This study shows that in contrast to hematological malignencies, *GADD45γ* methylation is not one of the common epigenetic changes in PCa. In addition, we suggest that the loss of important regulatory mechanisms involved in *GADD45γ* might play a role in the pathogenesis of BPH.

I. Introduction:

Prostate cancer (PCa) is the disease of males of advanced age and the mean age at diagnosis is 71. It is the most frequent malignancy of males in the developed countries, despite in the other countries lung cancer comes first. At the same time, it is still one of the most frequent causes of death in men (Jemal et al, 2011). In Turkey, PCa is the fourth most frequent cancer type and third most frequent cause of death in men (IARC, 2008). Also, there is an increase in prevalence of the disease over the past 10 years in Turkey according to the Prostatturk 2009 Study carried out by the Turkish Society of Urooncology (Turkish Society of Urooncology, 2013).

The ethiopathogenesis of PCa is still unknown although some hormonal, genetic, epigenetic, and environmental factors appear to be involved in the development and the maintanence of the disease. Since androgens are important for the development and functions of prostate, alterations in the expression of androgen receptors (ARs) and their mutations might be associated with the pathogenesis of PCa (Wang et al, 2009; Koochekpour, 2010; Lonergan and Tindall, 2011). Familial predisposition is also important in PCa with a 2-3 fold increased risk in individuals with a positive family history (Carter et al, 1993). In genome wide association studies some chromosomal loci such as 17q21, Xp11, 10q21, and 8q24 were found to be associated with PCa (Al Olama et al, 2009; El Gammal et al, 2010). Also amplifications in 8q, 1q, 3q, 7p/q, and Xq and deletions of other chromosomal loci like 6q, 8p, 10q, 13q, 15q, 16q, 17p/q, 18q, 19p/q, and 22q have been associated with the disease (Saramaki and Visakorpi, 2007).

DNA methylation is the most frequently studied epigenetic mechanism in PCa. Its role in initiation and progression of the disease was suggested in previous studies (Jeronimo et al, 2011; Chiam et al, 2014). More than 50 genes responsible for various cellular functions such as DNA repair, cell-cycle control, apoptosis, hormonal response, cell signaling and invasion/metastasis are reported to be hypermethylated in the cancer (Chiam et al, 2014; Park, 2010).

The *GADD45* gene family plays important roles in various cell functions such as DNA repair (Smith et al, 2000), cell-cycle control (Vairapandi et al, 2002; Liebermann and Hoffman, 1998), and maintenance of cell viability (Gupta et al, 2006). The members of the *GADD45* gene family, *GADD45α*, *GADD45β* and *GADD45γ*, are evolutionary conserved and they share 55% amino acid sequence homogeneity (Schrag et al, 2008). They are expressed in both fetal and adult tissues and their expression increases in the G1 and decreases in the S phase of the cell cycle (Kearsey et al, 1995). All members of *GADD45* family show differential expression in specific tissues. High levels of the GADD45γ expression are seen in skeletal muscle, kidney and liver of mice while low levels of expression are present in heart, brain, spleen, lung and testis (Zhang et al, 1999). The *GADD45* gene family members act as stress sensors that modulate cellular responses against various physical and environmental stress factors (Liebermann and Hoffman, 1998; Zhang et al, 1999). It is also known that they have a suppressor effect on tumor cell proliferation in response to oncogenic stress (Tront et al, 2006). Although all three proteins have similar functions, these functions are not identical, since they have different activation pathways depending on cell type and the source of cellular stress (Zhang et al, 1999; Shaulian and Karin, 1999).

Decreased GADD45γ protein levels were reported in various human cancers, including non-Hodgkin lymphoma (Ying et al, 2005), hepatocellular carcinoma, (Sun et al, 2003), and anaplastic thyroid cancer (Chung et al,

2003). It has been suggested that DNA methylation is involved in the regulation of *GADD45γ* because mutations in *GADD45γ* are extremely rare in human cancers (Ying et al, 2005). However, there was no information about the role of *GADD45γ* in PCa. In this study, we aimed to determine the frequency of *GADD45γ* methylation and the level of its gene expression, and to investigate a relationship between the methylation and the expression status of *GADD45γ* in BPH and PCa.

II. Materials and Methods:

A. Patients with BPH and PCa

We analyzed 56 patients with PCa who have undergone radical prostatectomy and 60 patients with BPH patients who have undergone transurethral resection of the prostate. All patients were diagnosed in the Department of Pathology, Pamukkale University between 2005 and 2012. All PCa cases also were diagnosed as prostate adenocarcinoma. Tissue samples were collected from all patients before treatment. This study was approved by the Institutional Review Board of Pamukkale University and was in compliance with the Declaration of Helsinki. Clinicopathologic data were obtained from patient registries in the Pathology and Urology Departments of Pamukkale University.

Two consecutive sections of formalin-fixed and paraffin embedded (FFPE) tissue were taken for DNA isolation and immunohistochemistry (IHC). DNA was isolated using a commercial kit according to the instructions of the manufacturer (QIAamp DNA Mini Kit, Qiagen) and IHC was performed using polyclonal antibody against GADD45γ as described previously (Zhu et al, 2009).

B. High resolution melting (HRM) analysis

Bisulphite treatment was performed on the isolated DNA samples from FFPE tissues before methylation-sensitive HRM analysis (EZ DNA Methylation-Gold Kit, Zymo Res.). We used the following primer set which is produced a 115 bp DNA fragment within the CpG island in the *GADD45γ* gene: Forward: CGTCGTGTTGAGTTTTGGT and Reverse: TAACCGCGAACTTCTTCCA (Zhang et al, 2010). For the confirmation of Tm degrees, commercially available control DNA samples were used (EpiTect Control DNA Set, Qiagen). All analysis were performed in a LightCycler® 480 (Roche Diagnostics, Germany) instrument.

C. Immunochemistry

For immunohistochemical staining, sections of 5 μm thick sections were cut from FFPE tissues and placed on electrostatic-charged, poly-L-lysine-coated slides (X-traTM, Surgipath Medical Industries, Richmond, IL, USA). Sections were dehydrated at 60ºC for a minimum of 2 hours. All immunostaining procedures including deparaffinization and antigen retrieval processes were performed applying the BenchMark XT® automated stainer (Ventana Medical Systems, USA).

GADD45γ (dilution: 1/200, Bioss Laboratories, Woburn, MA, USA) was used as primary antibody. Larynx squamous cell carcinoma tissue samples were used as positive controls while negative controls were treated with the same immunohistochemical method by omitting the primary antibody. At least 10 fields selected on the basis that they contained immunopositive cells were counted by using a 40x objective lens on the light microscope. A granular cytoplasmic staining was assessed as positive. Scoring counted for representation of the areas of the stains was performed by N.S.T. Immunohistochemical status of GADD45γ was scored as: 0 (less than 25 % positive cells), + (26 to 50 % positive cells), ++ (51 to 75 % positive cells), and +++ (more than 75 % positive cells).

D. Statistical analysis

The methylation status and protein expression level of *GADD45γ* between the cases with BPH and PCa was compared using the Chi-square test.

Clinicopathologic parameters were also compared across the methylation status and protein expression. $P < 0.05$ was considered to be statistically significant.

III. Results

A. Clinicopathologic parameters

The cases with PCa were between 53 and 76 years old and the mean age was 62.8±5.8 **(Table 1)**. The cases with BPH were between 42-87 years old and the mean age was 68.5±10.1. The majority of PCa cases (66.1%) were ≤64 years while there were 38 (63.3%) cases older than 64 in BPH.

All of the cases with PCa were in T2 and T3 stages. Thirty-six of the cases (64.3%) were in early and 20 (35.7%) were in advanced stage when T1 and T2 stages are considered as early and T3 and T4 are considered as advanced stages **(Table 1)**.

The Gleason score was 6 in 19 cases, 7 in 35 cases, 8 in 1 case, and 9 in 1 case. All of the cases were also grouped as well differentiated (Gleason score ≤6), moderately differentiated (Gleason score of 7), and poorly differentiated tumors (Gleason score of 8-10) according to the American Joint Committee on Cancer (AJCC, 2010) **(Table 1)**.

B. GADD45γ methylation

Informative data was gained from all tissue samples with BPH and PCa. The Tm was 79±0.5°C in the methylated region of *GADD45γ* gene while unmethylated regions had a Tm of 76±0.5°C in the HRM analysis, which was also confirmed by the control DNA samples **(Figure 1)**.

We found *GADD45γ* methylation only in 1 (1.8%) of 56 PCa tissue samples while 15 (25%) of BPH tissue samples showed *GADD45γ* methylation. The difference between the two groups was statistically significant (P= 0.000) **(Table 2)**.

The PCa patient with *GADD45γ* methylation is a 71-years-old man, with early stage and its tumor grade is the well differentiated.

C. GADD45γ protein expression

According to the IHC analysis, 7 patients were 0, 19 patients were 1+, 16 patients were 2+, and 14 patients were 3+ in PCa while the BPH patients with GADD45γ expression scored as 0, 1+, 2+ and 3+ were 10 (%), 30 (%), 15 (%), and 5 (%)patients, respectively. When 0 and 1+ were regarded as low protein expression, 2+ and 3+ were accepted high protein expression, GADD45γ protein was overexpressed in PCa patients (53.6%) compared with the BPH patients (33.3%), and the difference was statistically different (P=0.028) **(Table 2)**. **Figure 2** shows different levels of GADD45γ protein expression in PCa tissues.

We did not observe a significant relation between GADD45γ protein expression and other clinicopathologic parameters including pathologic stage and tumor grade (P=0.129 and P=0.785, respectively) **(Table 3)**.

D. Relation between *GADD45γ* methylation and protein expression

The only patient with PCa who had methylated *GADD45γ* had high GADD45γ protein expression while 6 patients with BPH had *GADD45γ* methylation and high GADD45γ protein expression. Overall, we observed an association between *GADD45γ* methylation and protein expression level in 52 (44.8%) of patients with BPH and PCa, as detected by the HRM analysis **(Table 4)**.

Parameter	Number of cases (%)
Age	
< 59	17 (30.4)
60-64	20 (35.7)
65-69	12 (21.4)
> 70	7 (12.5)
Pathological T staging	
T2	
pT2a	4 (7.1)
pT2b	1 (1.8)
pT2c	31 (55.4)
T3	
pT3a	12 (21.4)
pT3b	8 (14.3)
Tumor grade	
Well differentiated	19 (33.9)
Moderately differentiated	35 (62.5)
Poorly differentiated	2 (3.6)

Table 1. Clinicopathologic parameters of 56 patients with PCa

	GADD45γ methylation (%)			*GADD45γ* protein expression (%)		
	Methylated	**Unmethylated**	***P* value**	**Low**	**High**	***P* value**
BPH	15 (25.0)	45 (75.0)		40 (66.7)	20 (33.3)	
			0.000			***0.028***
PCa	1 (1.8)	55 (98.2)		26 (46.4)	30 (53.6)	

Table 2. *GADD45γ* methylation and protein expression in BPH and PCa patients

Clinicopathologic parameters	***GADD45γ* methylation (%)**			**GADD45γ protein expression level (%)**		
	Absent	**Present**	***P*-value**	**Low**	**High**	***P*-value**
Age						
≤ 64	37 (100)	-		16 (43.2)	21 (56.8)	
≥ 65	18 (94.7)	1 (5.3)	0.339	10 (52.6)	9 (47.4)	0.505
Pathologic Stage						
Early	35 (97.2)	1 (2.8)		14 (38.9)	22 (61.1)	
Advanced	20 (100)	-	1.000	12 (60)	8 (40)	0.129
Tumor grade						
Well differentiated	18 (94.7)	1 (5.3)		10 (52.6)	9 (47.4)	
Moderately differentiated	35 (100)	-		15 (42.9)	20 (57.1)	
Poorly differentiated	2 (100)	-	1.900	1 (50)	1 (50)	0.785

Table 3. Associations of *GADD45γ* methylation and protein expression with clinicopathological parameters in PCa patients

	Methylated/ High expression	Methylated/ Low expression	Unmethylated/ High expression	Unmethylated/ Low expression
BPH	6	9	14	31
PCa	1	-	29	26
Total	7	9	43	57

Table 4. Association between *GADD45γ* methylation and its protein expression

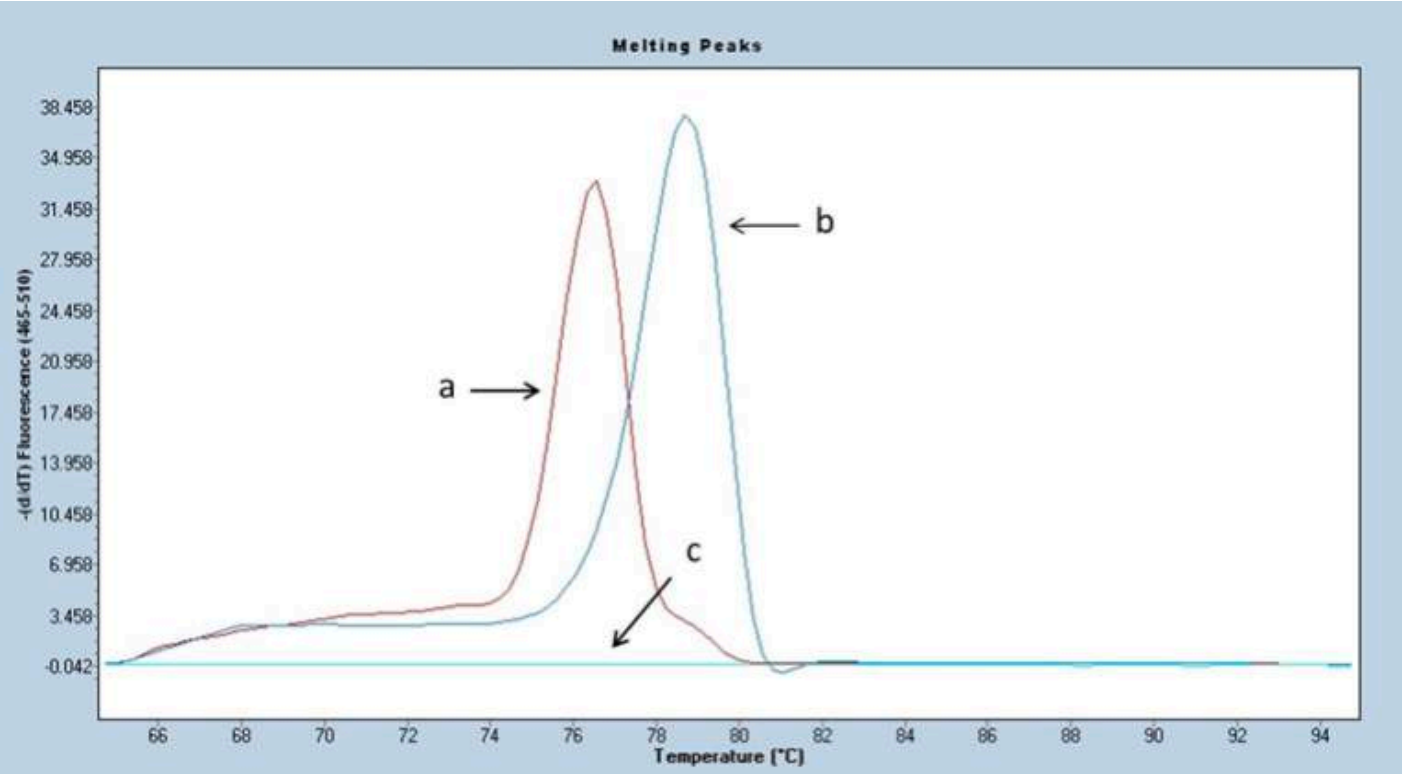

Figure 1. The HRM curves for *GADD45γ* methylation in PCa patients. **a:** Unmethylated *GADD45γ* DNA had a melting peak at 76±0.5°C, **b:** Methylated *GADD45γ* DNA had a melting peak at 79±0.5°C, **c:** Negative control (PCR-grade water was used instead of template DNA).

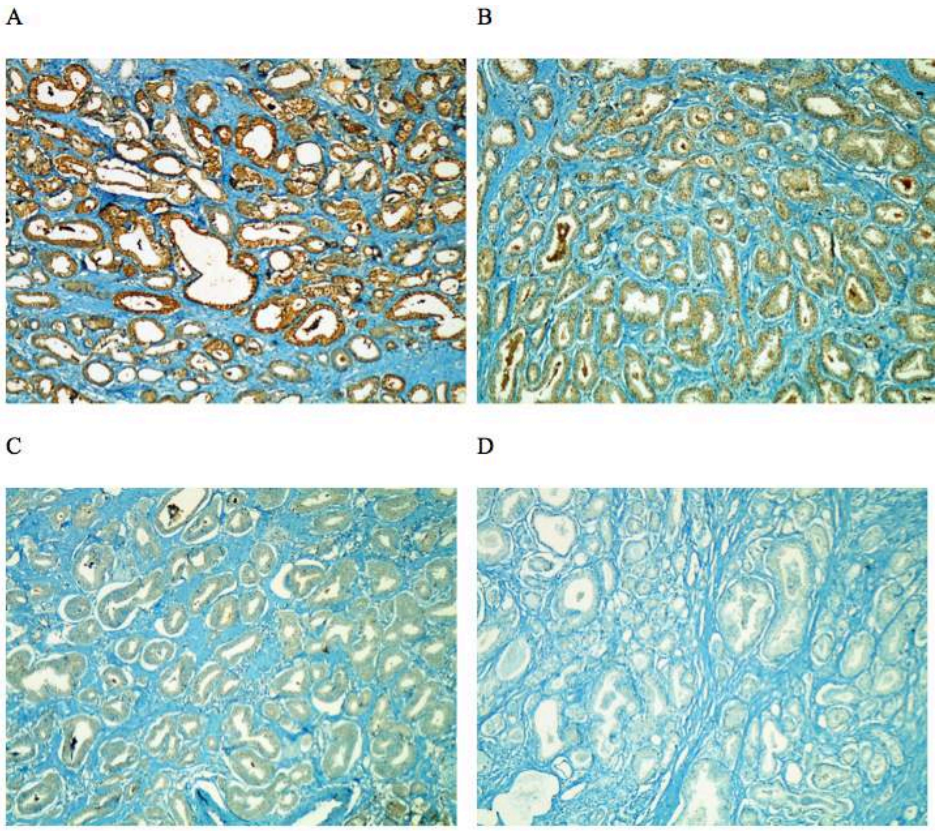

Figure 2: Representative immunohistochemical detection of GADD45γ in PCa (A-D). (A) Tumor with strong GADD45γ staining intensity (Score: +++), (B) Tumor with moderate GADD45γ staining intensity (Score: ++), (C) Tumor with weak GADD45γ staining intensity (Score: +), (D) Tumor with no GADD45γ staining (Score: 0) (original magnification x200).

IV. Discussion

Although the functions of *GADD45α* and *GADD45β* in the maintenance of cellular homeostasis are better understood, *GADD45γ* functions need to be clarified. A number of studies reported the differences in methylation frequency of the genes involved in the response to cellular stress between benign and neoplastic lesions of prostate tissue. The best known example is *GSTP1* methylation which is accepted as an important epigenetic biomarker in PCa because it has a higher specificity than serum PSA level, distinguishes prostate cancer from other prostate disorders such as BPH and high-grade prostatic intraepithelial neoplasia (PIN), and its level is associated with the stage of PCa and recurrence after therapy (Chiam et al, 2014). It is also reported that a hypermethylation profile consists of *GSTP1*, *RASSF1A*, *RARβ2* and *APC* could distinguish PCa cases from nonmalignant cases with 86% sensitivity and 89% specificity (Roupret et al, 2007). However, *SERPINB5*, a putative tumor suppressor gene, was found to be the only gene with significantly higher methylation in BPH although a number of genes including *RARB*, *HIN1*, *BCL2*, *APC* and *GSTP1*, involved in tumor suppression, hormonal response, and cell-cycle control, were highly methylated in PCa (Vasiljevic et al, 2011). These results suggest that different pathways include distinct DNA methylation profiles are involved in the pathogenesis of the major disorders of prostate tissue.

To our knowledge, this is the first study to report the frequency of *GADD45γ* methylation and its protein expression levels in clinical samples of BPH and PCa, which are the two common prostatic disorders in elderly men. We found the frequency of *GADD45γ* methylation was very high in BPH, contrasting to PCa suggesting that *GADD45γ* methylation might play a role in the pathogenesis of BPH. Zhang et al. examined the sensitivity of HRM analysis for *GADD45γ* and they demonstrated that the HRM protocol had high sensitivity which allows the detection of low (1%) amounts of DNA methylation (Zhang et al, 2010). The quality of archival FFPE tissue was assessed for DNA methylation by methylation-specific HRM analysis, which showed that the analysis was successfully used to determine the methylation status of DNA samples extracted from FFPE tissue being up to 30 years old (Kristensen et al, 2009). We obtained informative data in all DNA samples extracted from 2 to 9-year-old FFPE tissues in the present study by the HRM analysis. Therefore, we believe that the HRM analysis could be used to detect the methylation status of *GADD45γ* in prostatic FFPE tissue samples.

We also observed that GADD45γ protein expression in BPH was statistically lower than in PCa cases. It is well known that the functions of GADD45γ proteins could be different depending on the cell type and the source of stress (Liebermann and Hoffman, 1998; Zhang et al, 1999; Tront et al, 2006; Shaulian and Karin, 1999). The difference in *GADD45γ* methylation and protein expression level in both disorders seems to be related with the type of stimulus and the microenvironmental conditions around cells. Therefore, these results may indicated that the *GADD45γ* methylation does not associate with the pathogenesis of PCa, but might show the loss of certain control mechanisms which are under *GADD45γ* regulation in prostate cells inducing the development of BPH.

It has been reported that the GADD45γ protein was involved in divergent cellular processes driven by androgens in the rat prostate (Jiang and Wang, 2003). In another study, GADD45γ protein expression was only observed in androgen receptor (AR)-positive human prostate cancer cells, suggesting that the expression of GADD45γ is regulated by androgens in human prostate cancer cells (Jiang and Wang, 2004). It has also been found that the ectopic expression of GADD45γ has effect on growth inhibition and G1 accumulation of AR-positive prostate cancer cells (Flores and Burnstein, 2010). These findings show that GADD45γ may play a role in the negative growth control driven by androgens in the prostate. It was reported that the loss of *GADD45γ* expression at mRNA level in nonfunctioning pituitary adenomas, compared with its counterpart tissue. They also observed an inhibition on cell proliferation in the cells, which had the ectopic expression of GADD45γ protein (Zhang et al, 2002). In another study, *GADD45α* and *GADD45β* expression was found to be similar in normal human thyroid cells and anaplastic tyroid cancer cell lines although *GADD45γ* expression was significantly decreased in anaplastic tumor cell lines (Chung et al, 2003). Also, decreased levels of GADD45γ protein expression was reported in clinical hepatocellular carcinoma samples compared with healthy adjacent tissue (Sun et al, 2003). BPH and PCa are the most known benign and malign disorders of the prostate, respectively. The two disorders have several common characteristics including the slow progression, the presence of inflammation, and the higher prevalence and incidence with increased age. But, the etiopathogenesis of BPH and PCa has not been fully clarified and they mostly arise in different areas of the prostate supporting the

presence of different pathways in the development and the maintanence of the disorders (Elkahwaji 2012; De Nunzio et al, 2011). As we noted before, the *GADD45* gene family members have different functions depending on the cell type and the source of cellular stress. The GADD45 proteins also play a role as important sensors of oncogenic stress, both *in vivo* and *in vitro* (Liebermann and Hoffman, 2008). In the present study, we found that there was a decrease in the protein expression in almost 50% of the PCa patients and this result is consistent with previous studies. But, the loss of expression of GADD45γ (probably due to DNA methylation of the *GADD45γ*) was statistically more frequently observed in BPH which is a benign disease of the prostate. Therefore, we thought that the loss of expression of *GADD45γ* may contribute to the pathogenesis of diorders characterized by increased cell proliferation like BPH in the absence of oncogenic stress. Our study also showed that the two disorders might have at least different cellular mechanisms driven by *GADD45γ*. But, further research may be needed to determine how and when *GADD45γ* may be effective in the pathogenesis of the disorders.

It has been shown that *GADD45γ* methylation is not necessarily related to GADD45γ protein expression. In 58% of human pituitary adenomas *GADD45γ* methylation was present although 9% of these cases also revealed *GADD45γ* transcripts. Likewise, 18% of the cases without *GADD45γ* methylation also showed no *GADD45γ* transcripts (Bahar et al, 2004). In our study, there was discordance between the methylation and protein expression status in both BPH and PCa groups. In the PCa group, although we detected methylation in only one sample the sample had GADD45γ protein expression while 26 other PCa tissues did show neither methylation nor protein expression. Also in the BPH group, 6 tissues with methylation also showed protein expression while 31 unmethylated tissues did not demonstrate protein expression. The discordance between the methylation status and protein expression levels in our study may be explained by the following potential mechanisms: First, our target in *GADD45γ* gene was relatively small because large amplicon sizes are generally unsuitable for HRM analysis. The *GADD45γ* gene has a unique CpG island which contains not only the promoter region but also exons (Ying et al, 2005, Bahar et al, 2004). Searching in whole *GADD45γ* gene should be more accurate to detect the real methylation status. Second, the polyclonal antibody we used for IHC due to unavailability of commercial monoclonal antibody against GADD45γ protein might have crossed reactive with other epitopes in colocalized protein targets. Third, it was reported that a gain of DNA methylation is not always associated with gene silencing (Heyn et al, 2013; Kulis et al, 2012) and it is well known that there are several other mechanisms used by cells for gene silencing other than DNA methylation. Since *GADD45γ* mutation was very rarely detected in primary tumors (Ying et al, 2005) the inhibition of expression might be due to other epigenetic mechanisms than DNA methylation such as small non-coding RNAs and histone modifications.

In conclusion, we found a low level methylation of *GADD45γ* in PCa as well as other solid tumors which is consistent with observations from the literature. We also suggest that the loss of certain control mechanisms which are under *GADD45γ* regulation in prostate cells may be considered as a risk for the development of BPH.

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Conflict of interest

No author of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript. This study was presented in part at the European Human Genetics Conference 2013 - June 8-11 - Paris, France.

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