FASL polymorphism is associated with response to bacillus Calmette-Guérin immunotherapy in bladder cancer

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ABSTRACT

Objective: Deregulation of FAS/FASL system may lead to immune escape and influence bacillus Calmette-Guérin (BCG) immunotherapy outcome, currently the gold standard adjuvant treatment for high-risk non-muscle invasive bladder tumors. Among other events, functional promoter polymorphisms of *FAS* and *FASL* genes may alter their transcriptional activity. Therefore we aim to evaluate the role of *FAS* and *FASL* polymorphisms in the context of BCG therapy, envisaging the validation of these biomarkers to predict response

Patients and Methods: DNA extracted from peripheral blood from 125 bladder cancer patients treated with BCG therapy, was analyzed by Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphism for *FAS-670A/G* and *FASL-844T/C* polymorphisms. *FASL* mRNA expression was analyzed by real time PCR.

Results: Carriers of *FASL-844* CC genotype present a decrease recurrence free survival after BCG treatment, when compared to *FASL-844* T allele carriers (mean 71.5 *vs.* 97.8 months, p=0.030) and have an increased risk of BCG treatment failure (HR=1.922; 95%CI: [1.064-3.471]; p=0.030). Multivariate analysis shows that FASL-844T/C and therapeutic scheme are independent predictive markers of recurrence after treatment. The evaluation of *FASL* gene mRNA levels demonstrated that patients carrying *FASL-844* CC genotype had higher *FASL* expression in bladder tumors (p = 0.0027). Higher *FASL* levels were also associated with an increased risk of recurrence after BCG treatment (HR=2,833; 95%CI: [1.012-7.929]; p=0.047). *FAS-670A/G* polymorphism analysis did not reveal any association with BCG therapy outcome.

Conclusions: Our results suggest that analysis of *FASL-844T/C* , but not *FAS-670A/G* polymorphisms, may be used as a predictive marker of response to BCG immunotherapy.

Keywords: Bladder Cancer; BCG immunotherapy; Polymorphisms; Fas/FasL; predictive markers.

INTRODUCTION

The Fas/Fas ligand (FasL) system is one of the major pathways of apoptosis and an important regulator of cell proliferation and immune system regulation. During carcinogenesis, tumor cells may alter this mechanisms to subvert the immune system and suppress the antitumor immune response [1].

Fas/FasL further controls T cell apoptosis after immune reaction by the process of activation-induced cell death (AICD) [2]. This mechanism involves the overexpression of both Fas and FasL upon activation by antigen or other stimuli, subsequently promoting cell "suicide" or "fratricide" [3]. Decreased Fas and elevated FasL expression has been found in many types of cancer including bladder cancer [4]. It has been shown that tumor cells may counterattack Fas-sensitive tumor-infiltrating lymphocytes (TILs) using heightened expression of FasL. This mechanism is thought to lead to tumor cell immune escape, thus contributing to cancer formation and progression [5].

Functional polymorphisms in the promoter region of *FAS* and *FASL* genes have been identified and alter the transcriptional activity of these genes, which may have implications in the Fas/FasL pathways [6, 7]. Moreover, they have been associated with a higher risk in various cancer models [8, 9]. In bladder cancer, *FASL-844* CC genotype was associated with a significantly increased risk of bladder cancer [10]. However, the role of Fas/FasL and its functional polymorphisms have not been addressed in the context of bladder cancer treatment.

At the moment, the most effective treatment for high risk non-muscle invasive bladder cancer (NMIBC) consists of an immunotherapy with bacillus Calmette-Guérin (BCG) [11] . Generally it is performed as adjuvant to transurethral resection of bladder tumor (TURBT) in intermediate and high risk patients [11]. However, several studies demonstrated that 30% of the patients fail to respond to this therapeutic and in some cases the tumor may become more

aggressive [12]. Thus, early identification of patients better served by alternative and/or more aggressive approaches such as cystectomy is a critical aspect in the management of high-risk NMIBC [13, 14]. However, at the moment there are no established biomarkers to determine the outcome of BCG immunotherapy. Recently, several authors suggest that immunological predictive markers may yield promising clinical value in the context of predicting BCG immunotherapy outcome [14-16].

One of the main pathways of BCG anti-tumoral response is mediated by BCG and lymphokine-activated killer cells (BAK and LAK) [17]. Although it has been demonstrated that Fas/FasL pathway is not directly involved in BCG anti-tumoral effect [17, 18], BCG effector cells are sensitive to FasL-dependent AICD regulation [2] and possibly to FasL counterattack by tumor cells. Hypothesizing that a deregulation of Fas/FasL pathway may lead to immune escape and influence the efficacy of BCG immunotherapy, in this study we aimed to evaluate the role of *FAS* and *FASL* polymorphisms in the context of BCG therapy response. To the best of our knowledge, this is the first study addressing this subject and may provide important insights about the role of these molecules as predictive markers of BCG treatment outcome.

MATERIAL AND METHODS

Population

In this retrospective case-control study, all intermediate and high-risk NMIBC patients who underwent transurethral resection followed by BCG therapy between 1998 and 2006 at the Portuguese Institute of Oncology – Porto were eligible for this study. From a total of 193 patients, 14 had already died. The remaining 179 were invited to participate in the study, 70% of which accepted [19]. Patients blood samples were collected during 2006 and 2008 on patient's follow-up consultation. All patients received intravesical instillation of BCG for 6 consecutive weeks starting 2-3 weeks after surgery, i.e., induction scheme (iBCG) and 56%

underwent further instillations every 3 months for two years, i.e., maintenance scheme (mBCG). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG scheme and patients showing significant intolerance to long BCG treatment. The mean diagnostic age of the patients was 61,54±10,63 (min:36; max:97) years, with a male:female sex ratio of 105:20. The patients were followed by cystoscopy and urinary cytology every 3 months for the first year, every 6 month for the second year and every 12 months thereafter. The median follow-up time was 60 months (from 6 till 135 months). Tumor recurrence was defined as a newly found bladder tumor after the treatment, with at least one tumor-free cystoscopy between. The end point of the study was recurrence-free survival (RFS), defined as the period between the beginning of BCG treatment until the date of the most recent cystoscopy or recurrence date. Non-responders, as opposed to responders, were defined as patients submitted to BCG treatment that showed tumor recurrence. All clinicopathological information was obtained from patients' clinical records. Informed and written consent from each patient was obtained. The institutional ethics committee approved the study.

DNA extraction and genotyping

Peripheral blood samples were collected following standard venipuncture technique in EDTA-containing tubes, and the DNA was extracted from the white blood cell fraction using a salting out protocol [20] and storage at -20ºC.

The *FAS* and *FASL* genetic polymorphisms were genotyped using RFLP-PCR technique. The primers and enzymes used in *FAS-670A/G* and *FASL-844T/C* polymorphisms genotyping were the described by other authors [6, 7]. The PCR-RFLP conditions were the same as used by previously [20]. Genotyping data was read blind to the study endpoint. For quality control, 10% of the samples were randomly selected to a second PCR–RFLP analysis and 100% of concordance was observed.

RNA extraction and FASL mRNA expression analysis

A total of 34 tumor samples were randomly selected from the initial population according to the patient genotype for *FASL-844T/C* polymorphism (17 TT/TC and 17 CC). RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue samples using "Absolutely RNA® FFPE Kit" (Stratagene, La Jolla, CA). Up to 2µg of total RNA was reverse transcribed with random primers, using the "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, Foster City, CA). Real-time PCR amplification of cDNA samples was performed in a StepOne™ Real-Time PCR System (Applied Biosystems) using TaqMan® Gene Expression Master Mix, primers and probes provided by Applied Biosystems. Expression of *FASL* was measured with TaqMan expression assay (ID: Hs00181225_m1) from Applied Biosystems .

The raw -ΔCt was used to analyse the *FASL* expression in *FASL-844* CC or TT/TC genotypes carriers and therefore used as an estimate of the mRNA relative levels. ΔCt stands for the difference between the cycle threshold (Ct) of the amplification curve of the target gene and that of the *GAPDH*. The efficiency of the amplification reaction for each primer-probe is above 95% (as determined by the manufacturer).

Assessment of FasL expression by immunohistochemistry

The same 34 FFPE tissue sections were screened for FasL by immunohistochemistry with the streptavidin/biotin peroxidase method using anti-FasL mouse monoclonal antibody clone 5D1 (Leica Biosystems, Wetzlar, Germany). Tissues were incubated with primary antibody overnight in a 1:35 dilution. A semi-quantitative approach was established to score the immunohistochemical labelling based on the percentage of cells that stained positively. The entire section was screened and the cases were classified as: Low FasL IHC expression when the percentage of positive cells

was above median value of the overall expression (<25%). When the overall expression was above 25%, it was classified as High FasL IHC expression.

Statistical analysis

Statistical data analysis was carried out using the computer software Statistical Package for Social Sciences—SPSS for Windows (version 15.0). Chi-square analysis was used to compare categorical variables. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between genotypes and the risk of recurrence. Kaplan-Meier survival curves were used to evaluate correlation between genotypes and RFS and were compared by log-rank statistical test. Further, multiple Cox regression analysis was used to assess the effect of individual polymorphisms on the time to recurrence in BCG-treated patients and to adjust for potential confounders. Non parametric Mann-Whitney test was used to compare the differences in the mRNA expression between the study groups.

RESULTS

FAS and *FASL* polymorphisms as predictors of BCG immunotherapy outcome

Regarding the evaluation of *FAS-670A/G* and *FASL-844T/C* polymorphisms, Table 1 presents genotype frequencies and the risk of recurrence after BCG treatment. It was observed that *FASL-844* CC genotype frequency is higher in patients with recurrence, when compared with responders group (37.5% *vs*. 24.7%). An increased risk was noticed (OR=1.8), although, not statistically significant.

Kaplan-Meier function plots and probabilities analysis showed that *FASL-844* CC genotype carriers have a shorter recurrence free survival after BCG treatment (mean 71.5 months) when compared to T allele carriers (mean 97.8 months, p=0.030, Fig. 1). In order to estimate the risk associated to this difference, Cox regression was performed and it was found that *FASL-844* CC genotype carriers have an increase risk of BCG treatment failure (HR=1.897;

95%CI: [1.051-3.424]; p=0.034). Regarding *FAS-670A/G* polymorphism, no association was found with recurrence risk nor RFS.

The relation between clinicopathological characteristics and response to BCG treatment (i.e., responders *vs.* non-responders) is presented in Table 2. It was observed that treatment scheme is associated with recurrence after treatment (35.1% *vs*. 58.3%, p=0.011). Our previous report, with the same sample dataset, showed that iBCG treated patients or patients with multiple tumors have a shorter RFS [19]. In the present study we performed univariate Cox Regression and found that patients treated only with iBCG scheme have 2-fold risk of recurrence after BCG immunotherapy (HR=2.096; 95%CI: [1.177-3.731]; p=0.012). However, the presence of multiple tumors revealed a trend to an increase risk of recurrence (HR=1.760; 95%CI: [0.981-3.159]; p=0.058). There are no association between the evaluated polymorphism and the clinicopathological characteristics.

Therefore, multivariate Cox regression analysis adjusted to treatment scheme and multifocality was performed to assess the individual effect of *FASL-844T/C* on the risk of recurrence in BCG-treated patients. The results showed in table 3, reveal that independently of the treatment scheme adopted, patients carrying *FASL-844* CC genotype as approximately a 2-fold risk of early recurrence and that independently of the patient *FASL-844* genotype, patients treated only with iBCG scheme have approximately 2.5-fold risk of early recurrence. Thus *FASL-844T/C*, BCG scheme and multifocality are independent predictive factors of BCG immunotherapy outcome.

FASL mRNA expression and its relation with FASL-844T/C and treatment response

Of the 125 patients, 34 tumor tissues were available for evaluation of *FASL* mRNA expression levels. As shown in Fig. 2, significantly higher *FASL* mRNA levels were found in tumors from *FASL-844* CC genotype patients than from TT or TC genotype patients (median mRNA relative levels, -7.80 vs. -6.14±0.46, p = 0.0027). In our samples the normalizing gene

(GAPDH) presents stable Ct values (mean±standard deviation: 29.95±2.26), demonstrating that RNA recovered from FFPE is suitable for gene expression analysis.

Individuals were categorized as high expressers (High *FASL*) when the normalized transcripts levels (mRNA relative levels) were above the geometric mean of all cases (> -7.04), while individuals with mRNA relative levels below -7.04 were categorized as low *FASL* expressers (Low *FASL*). From the High *FASL* group 78.6.% individuals were *FASL-844* CC genotype carriers, whereas among Low *FASL* cases, only 30% were CC genotype (p=0.005). Kaplan-Meier analysis (Fig. 3) showed that High *FASL* individuals have a reduced RFS (mean 51.4 months) when compared with Low *FASL* individuals (mean 96.2 months, p=0.030). Univariate Cox Regression analysis demonstrated that High *FASL* individuals have approximately 3-fold risk of recurrence after BCG treatment (HR=2.833; 95%CI: [1.012-7.929]; p=0.047). Immunohistochemistry was used to confirm FasL localization. It was observed that both FasL was present both in tumors and the immune infiltrates, independently of the *FASL-844T/C* genotype. The FasL overall expression was in accordance with the levels of gene expression (p<0.001 – Fig. 4). No association was found between the FasL expression in the tumor and BCG response as well as other clinicopathological characteristics.

DISCUSSION

The management of high-risk NMIBC relies mostly on an adjuvant immunotherapy consisting on intravesical instillations with BCG, performed after transurothelial resection of the tumor [11]. BCG promotes a strong local immune response that results in tumor elimination [13, 21, 22]. In our most recent systematic review on this subject we point out several markers such as CD68, ezrin, HSP90, CD83, IL2 urinary levels related with BCG response [14]. Several functional polymorphisms in genes such as *NRAMP1*, *hGPX1*, *XPA*, *ERCC2* and *6* as well as in genes involved in inflammatory pathways (*IL8*, *TNFA*, *IL6*, *TGFB1*, *COX2*, *IFNG*) [14].

All these genetic markers have been identified based on a candidate gene approach using well established polymorphisms. Still, at the moment there are no reliable biomarkers to determine the outcome of this therapeutics, thereby permitting the early identification of patients better served by alternative therapeutics or cystectomy [14, 16]. It has been long thought that such biomarkers may be encountered among molecules/cells involved in the mediation of immune responses at the tumor site [14-16]. Among these putative immune-related biomarkers is the Fas/FasL pathway. Still, its involvement in BCG-mediated tumor cells elimination remains controversial since some authors demonstrated that BCG anti-tumoral effect is not mediated by Fas/FasL [17, 18] while others found higher levels of these molecules in T lymphocytes after treatment [23]. Nevertheless, this pathway is responsible for the regulation of cell apoptosis, namely T cell depletion by increasing AICD rates [2], therefore a deregulation of this pathway may compromise the immune response mediated by BCG immunotherapy and consequently contribute to modulate the therapeutics outcome. Tumor cells are known to modulate and evade Fas-mediated apoptosis, by simultaneously down regulating their own Fas expression and promoting the expression of FasL on their surface [5]. This protects tumor cells against FasL-induced apoptosis and, at the same time, promotes the apoptosis of activated T-cells expressing FasL, in what has been called as the FasL "counterattack" [5]. Functional polymorphisms in *FAS* and *FASL* genes may unbalance the expression of Fas/FasL by tumor cells and contribute to the establishment of an immune suppressive environment that favours tumor proliferation [5]. The unbalanced expression of Fas/FasL was observed in several tumors, including bladder cancer [4]. Moreover, the presence of the *FAS* and *FASL* polymorphism was correlated with an increased risk of developing bladder tumors [10] and with an enhanced T cell apoptosis after immune reaction [24].

Given the putative involvement Fas/FasL pathway on the immune response, one may hypothesize that functional polymorphisms affecting expression levels of this molecules may

deregulate this pathway contributing to the failure of BCG therapeutics [15]. Therefore it may be used as a predictive biomarker of BCG immunotherapy outcome.

Since the *FASL-844T/C* polymorphism was never evaluated in the context of BCG therapy, in this study we have evaluated these polymorphism in a dataset of Portuguese patients and found that those carrying *FASL–844* CC genotype presented an increased risk of recurrence after treatment. In order to corroborate if *FASL–844* CC genotype was associated with higher basal expression levels of FasL, we have also evaluated mRNA levels of *FASL* gene in tumor samples. This showed that patients carrying *FASL-844* CC genotype had higher FASL expression in bladder tumors, as previously reported for others models [7, 24]. We have also demonstrated that higher *FASL* levels were associated with an increased risk of recurrence after BCG treatment. This suggests that patients carrying *FASL-844* CC genotype have higher FASL expression in T-cells membrane or in tumors which may indeed compromise the efficiency of BCG treatment by balancing the immunological boost promoted by the bacillus. Our results suggest that this may not occur by an overexpression of FasL in tumors. More studies are needed to determine whether it may result from enhanced AICD in T-cells.

Herein we opted to extract RNA from FFPE tissues. Although mRNA extracted from FFPE is partial degraded, is possible to use this in real time PCR to obtain accurate and specific gene expression using amplicons under 100bp [25]. Based on these observations we choose amplicons lower than 100bp. We also observed that *GADPH* expression was stable among our samples and that the mean Ct was around 30. According to several authors this is an indicator of mRNA quality and amplification efficiency [25, 26]. Based in these concepts we can say that our RNA samples were suitable for expression analysis.

Regarding *FAS-670A/G* analysis, we could confirm that this polymorphism is not associated with BCG treatment outcome in the Portuguese population, as described for the Indian population [27], suggesting that *FAS-670A/G* may not influence the molecular mechanisms underlying this therapeutics.

Clinicopathological characteristics as predictors of BCG immunotherapy outcome has been long studied, although the findings are controversial [28]. Herein, we evaluated the predictive value of all clinicopathological characteristics and found that only patients treated with iBCG or with multiple tumors present a higher risk of failure, which is in accordance with previous findings [19, 29, 30]. However multivariate analysis showed that this did not influence the biomarker value of *FASL-844T/C* in the context of response to BCG immunotherapy. Still, the main limitation of this study was the low number of mBCG cases, thus the analysis of a larger patient set only treated with mBCG scheme will be required toto evaluate more accurately the value of this polymorphism in the context of the currently adopted therapeutic scheme.

This is the first report addressing the potential role of *FASL-844T/C* polymorphism as a predictive marker of BCG immunotherapy response. Despite the limited number of samples, a significant association with the outcome of the therapeutics was observed. This highlights that *FASL-844T/C* polymorphism could be included in a biomarker panel to guide the management of high-risk bladder cancers.

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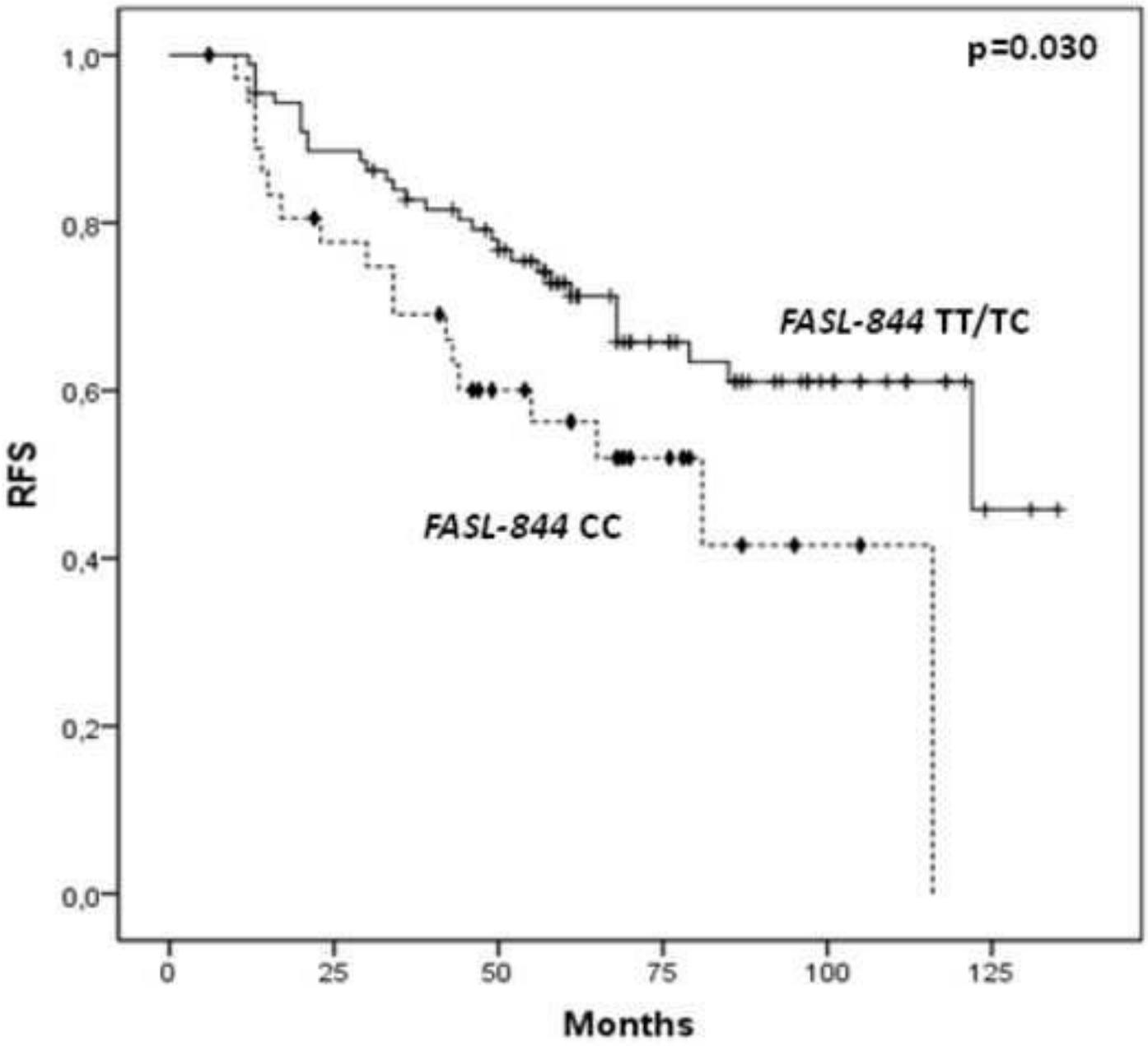

Figure 1

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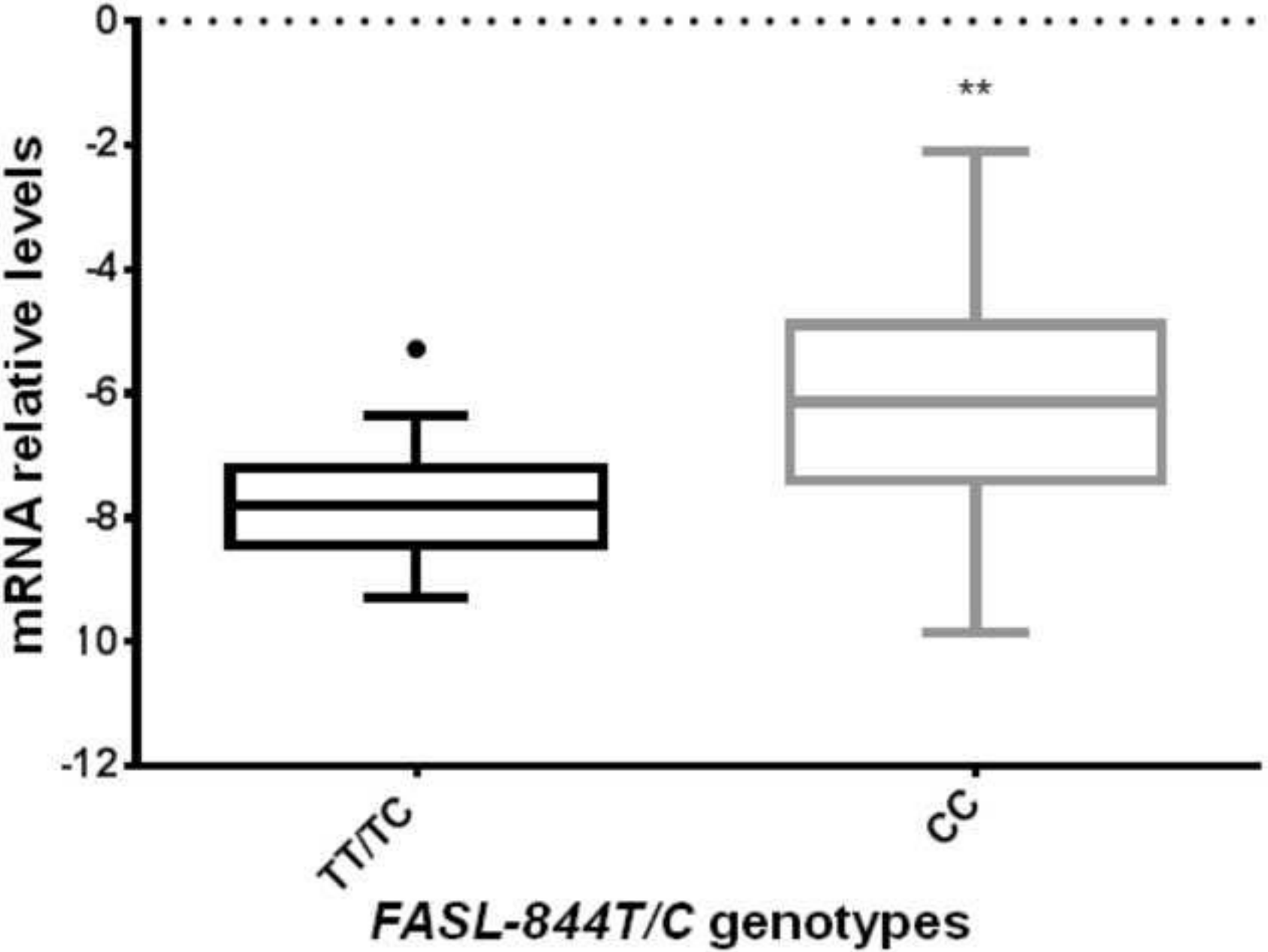

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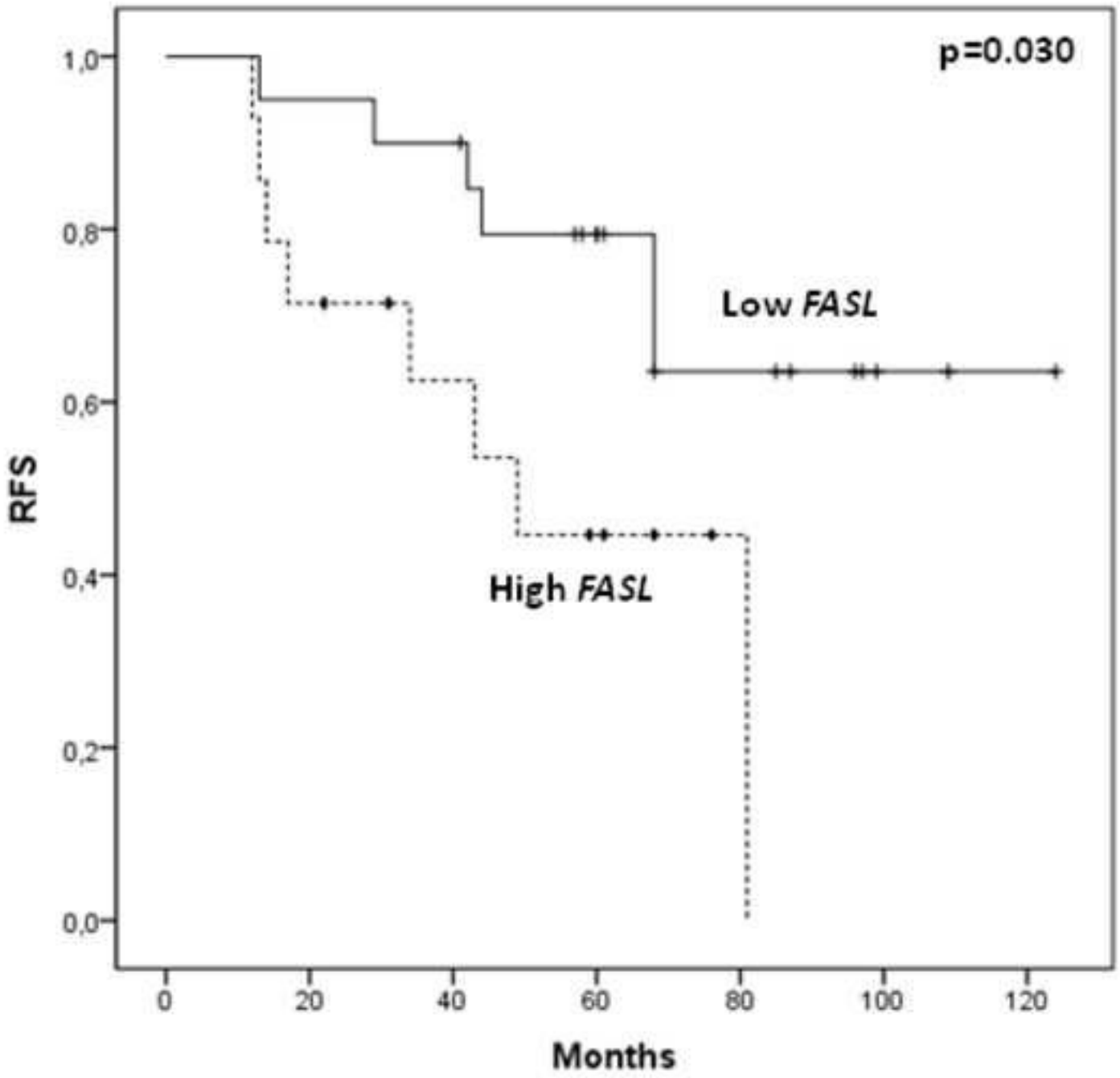

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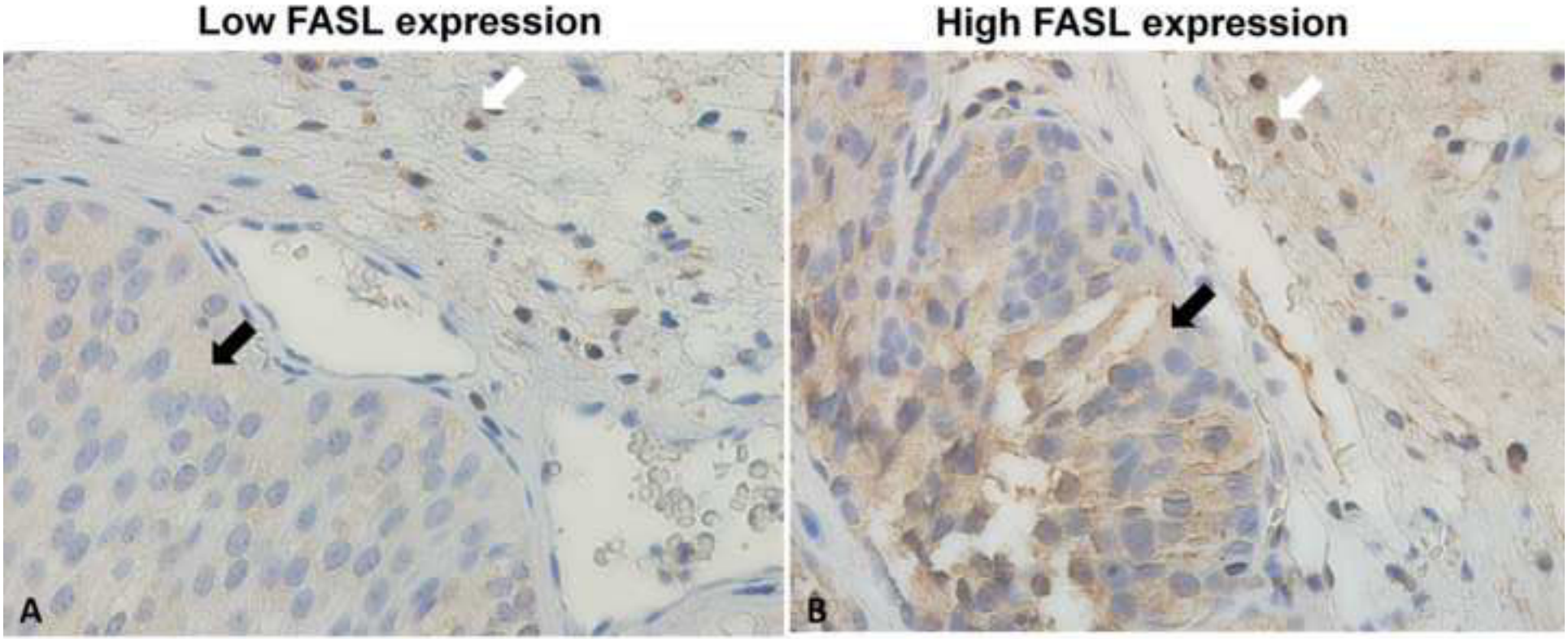

	Low FASL mRNA expression	High *FASL* mRNA expression
Low FasL IHC expression	17 (85%)	3 (21.4%)
High FasL IHC expression	3 (15%)	11 (78.6%)

C

p<0.001

Figure legends

Figure 1 – Association between *FASL-844T/C* genotypes and recurrence-free survival (RFS) in the studied patients. Kaplan-Meier analysis to assess the effect and log-rank test (p=0.030); + censored *FASL-844* TT/TC carriers; ♦ censored *FASL-844* CC carriers.

Figure 2 – Real -Time PCR analysis for *FASL* mRNA levels in bladder tumors comparing expression leveles in patients carrying *FASL-844* TT or TC genotypes and CC genotypes. The Box plots represents in the horizontal bar the median value for mRNA in each group, the 25th and 75th percentile have been represented by the boxes. The whiskers represent the maximum and minimum values of the data, respectively. Black dots represent outliers. To compare the transcript levels between groups, non-parametric Mann-Whitney Test was applied. (**) – p = 0,0027.

Figure 3 – Effect of *FASL* mRNA levels in recurrence-free survival (RFS). Kaplan-Meier analysis to evaluate the association between Low or High *FASL* mRNA levels and RFS in the studied patients. Comparison performed by log-rank test (p=0.030); + censored Low *FASL* mRNA levels samples; ♦ censored High *FASL* mRNA levels samples.

Figure 4 – Immunohistochemistry (IHC) using anti-FasL antibody. Representative image of tumor (dark arrow) and immune infiltrate (white arrow) expressing FasL in specimens with low and high expression of *FASL* mRNA (400X). In cases with low *FASL* mRNA the FasL protein expression was lower and less intense (A). In cases with high FASL mRNA expression, FasL was markedly and diffusely expressed in both the tumor and the immune infiltrate (B). Expression of *FASL* mRNA is associated with FasL expression evaluated by IHC, p<0.001 (Chi-square test; C).

Table 1 – Polymorphism genotypes distribution and risk of recurrence after BCG therapy.

	Responders	Non-responders	OR	95%CI	P value[a]
	n (%)	n (%)			
FAS-607A/G					
AA	24 (31.2)	16 (33.3)	1.0		
AG+GG	53 (68.8)	32 (66.7)	0.906	0.419-1.952	0.801
FASL-844T/C					
TT+TC	58 (75.3)	30 (62.5)	1.0		
CC	19 (24.7)	18 (37.5)	1.832	0.839-3.999	0.127

OR: Odds ratio

95%CI: 95% Confidence Interval

[a]**:** Chi-square test

Table 2 – Relation between patients, clinical and tumor characteristics and BCG treatment outcome

Variables	Total	Responders	Non-Responders	P value[a]
	n (%)	n (%)	n (%)	
Stage				
Ta	51 (40.8)	32 (41.6)	19 (39.6)	0.827
T1	74 (59.2)	45 (58.4)	29 (60.4)	
Grade				
Low	26 (22.7)	17 (22.1)	9 (18.8)	0.656
High	99 (77.3)	60 (77.9)	39 (81.2)	
Size (cm)				
<3	77 (69.4)	47 (69.1)	30 (69.8)	0.942
≥3	34 (30.6)	21 (30.9)	13 (30.2)	
Tumor number				
Unifocal	59 (47.2)	40 (51.9)	19 (39.6)	0.178
Multifocal	66 (52.8)	37 (48.1)	29 (60.4)	
CIS				
No	112 (89.6)	67 (87.0)	45 (93.8)	0.230
Yes	13 (10.4)*	10 (13.0)	3 (6.2)	
Recurrence status				
Primary	65 (52.0)	41 (53.2)	24 (50.0)	0.724
Recurrent	60 (48.0)	36 (46.8)	24 (50.0)	
Treatment scheme				
iBCG	55 (44.0)	26 (35.1)	41 (58.3)	0.011
mBCG	70 (56.0)	60 (64.9)	5 (41.7)	

[a]**:** Chi-square test; *: 1.6% of them are pure CIS and 8.8% are concomitant CIS.

Table 3

Table 3 – Multivariate analysis and risk estimation of *FASL-844T/C*, treatment scheme and tumor number on BCG immunotherapy outcome

	HR	95%CI	P value
FASL-844T/C			
TT+TC	1.0		
CC	1.881	1.040-3.402	0.037
Treatment scheme			
mBCG	1.0		
iBCG	2.363	1.316-4.243	0.004
Tumor number			
Unifocal	1.0		
Multifocal	2.092	1.150-3.803	0.016

HR: Hazard ratio

95%CI: 95% Confidence Interval