Improvement of a Predictive Model of Castration-Resistant Prostate Cancer: Functional Genetic Variants in TGFβ1 Signaling Pathway Modulation

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Abstract

Prostate cancer (PC) is the most frequently diagnosed cancer in men. The acquisition of castration-resistant (CR) phenotype is associated with the activation of signaling pathways mediated by growth factors. The TGFβ1 and its receptors have an important role in tumor progression, being the pro-apoptotic function modulated by the expression of TGFBR2. A single nucleotide polymorphism -875 G > A in TGFBR2 gene has been described, which may influence the expression levels of the receptor. Our purpose was to investigate the potential role of TGFBR2-875G>A in PC risk and in the response to androgen deprivation therapy (ADT). TGFBR2-875G>A polymorphism was studied by allelic discrimination using real-time polymerase chain reaction (PCR) in 891 patients with PC and 874 controls. A follow-up study was undertaken to evaluate response to ADT. The TGFBR2 and SMAD7 mRNA expression were analyzed by a quantitative real-time PCR. We found that TGFBR2-875GG homozygous patients present lower expression levels of TGFBR2 mRNA (AA/AG: ΔΔCt =1.5, P=0.016). GG genotype was also associated with higher Gleason grade (OR=1.51, P=0.019) and increased risk of an early relapse after ADT (HR=1.47, P=0.024). The concordance (c) index analysis showed that the definition of profiles that contains information regarding tumor characteristics associated with genetic information present an increased capacity to predict the risk for CR development (c-index model 1: 0.683 vs model 2: 0.736 vs model 3: 0.746 vs model 4: 0.759). The TGFBR2-875G>A contribution to an early relapse in ADT patients, due to changes in mRNA expression, supports the involvement of TGFβ1 pathway in CRPC. Furthermore, according to our results, we hypothesize the potential benefits of the association of genetic information in predictive models of CR development.

Introduction

Prostate cancer (PC) is an important public health problem that affects the male population. After lung cancer, PC is the most frequently diagnosed cancer in men, the fifth cause of death by cancer worldwide, and nearly three-quarters of the registered cases occur in developed countries [1]. The causes of PC remain poorly understood and many gene products show deregulated functions during cancer progression. At diagnosis, patients with early stages of disease are frequently submitted to prostatectomy, external radiation and/or brachytherapy, which removes or destroys tumoral cells that are confined within the prostate [2]. However, despite recent advances in the early detection of localized PC tumors, there is little effective therapy for patients with locally advanced and/or metastatic disease. Patients diagnosed in advanced stages are currently submitted to androgen deprivation therapy (ADT), due to the androgen dependency of prostate cells for continued...
growth and survival. However, it was found that in most patients the effects of this therapy typically last 18 to 24 months, after which the patients develop resistance to hormonal therapy and develop castration-resistant prostate cancer (CRPC) [3]. Unfortunately, the CRPC treatment is limited, ineffective and the molecular mechanisms of its phenotype progression are not well understood. The CRPC is an invariably lethal condition, which frequently metastasize and is associated with a significant morbidity and mortality [4].

Prostate cells require androgens in the cellular microenvironment to proliferate and differentiate. Nevertheless, PC progression and the acquisition of castration-resistant (CR) phenotypes have been associated with the activation of other signaling pathways mediated by growth factors that modulate the balance between the cell growth rate and apoptosis.

The TGFβ1 and its receptors are key components of the TGFβ signaling pathway, which has an important role in carcinogenesis and tumor progression. The signal transduction initiates with the TGFβ1 activation, then TGFβ1 binds to the type II receptor (TGFβRII), which then phosphorylates the type I receptor (TGFβRI), and activates its kinase. Phosphorylated TGFβRI, in its turn phosphorylates downstream elements of the signaling pathway. However the inhibitory SMAD7 has the capacity to bind to TGFβRII and effectively attenuate pathway activation [5]. In vitro studies have shown that in PC cells, the TGFβ1 signaling pathway has some defects and the restoration of this pathway can suppress tumor growth by inhibiting cell proliferation [6,7]. Reduced TGFβ2 expression levels are correlated with a shorter survival rate of colon cancer patients, as does the reduced expression of the co-receptor betaglycan in breast and PC patients [8,9]. High expression levels of TGFβ2 gene can mediate the pro-apoptotic function of the TGFβ1 signaling pathway and its loss promotes invasion and malignant transformation [10,11].

Alterations in TGFβ2 levels, with impact in the TGFβ1 signaling pathway, might be involved in PC development/progression. A G > A transition in the -875 promoter position of the TGFβ2 gene was reported and it may enhance transcription activity in normal epithelial cells and might increase the expression of TGFβ2 gene [12]. Genetic variants, which modulate TGFβ2 expression, may have impact in PC development and progression. The present study is the first to evaluate the relevance of the TGFBR2-875G>A (rs3087465) functional polymorphism in CRPC patients.

Material and Methods

Ethics statement

The study was conducted according to the principles of the Helsinki Declaration. The study was approved by the local ethics committee at the Portuguese Institute of Oncology of Porto (Portugal). All individuals signed a written informed consent to participate in the study.

Study Population

This case–control study was performed in 891 patients, with a mean age of 66.2 (7.7), with histopathologically diagnosed PC at the Portuguese Institute of Oncology of Porto (Portugal). Patients' disease stage distribution at the time of diagnosis was as follows: 53.2% presented localized disease (T1-T2b), 32.7% had locally advanced disease (T3-T4), and 14.2% had metastatic disease (N+ and/or M+). Cumulatively, a follow-up study (n=428) was undertaken to evaluate response to ADT. The types of hormonal treatment were as follows: anti-androgens plus luteinizing hormonereleasing hormone agonists (aLHRH) combination therapy (64.2%), aLHRH alone (5.4%), and anti-androgens alone (30.4%). Hormone resistance was evaluated through PSA recurrence, which was defined as two consecutive increasing PSA values more than 1.0 ng ml⁻¹ and differing by more than 0.2 ng ml⁻¹ [13]. The median follow-up time was 51 months.

Men older than 40 years of age, without known history of cancer were recruited from the Portuguese Institute of Oncology of Porto Centre Blood Donor's Bank and included in the control group (n=874), with a mean age of 44.2 (13.7). Peripheral venous blood samples (8 mL) were collected from each subject enrolled in the study.

TGFBR2 -875G>A polymorphism (rs3087465) genotyping

After DNA extraction using the QiAamp DNA Mini kit (Qiagen®) according to the manufacturer’s protocol, the TGFBR2-875G>A polymorphism (rs3087465) was analyzed by allelic discrimination using 7300 real-time PCR System (Applied Biosystems®). The reaction was based on a 5' nucleic acid PCR assay, using a TaqMan assay, which includes two allele-specific TaqMan®MGB probes (Applied Biosystems®) containing distinct fluorescent dyes and a PCR primer pair to detect the specific TGFBR2-875 G>A single nucleotide polymorphism (SNP). Real-time PCR was carried out using a 6 µL reaction mixture, containing 1x Master Mix (Applied Biosystems®), with 1x probes (TaqMan® assay C_27491740_10, Applied Biosystems®) and 20 ng of the DNA sample. Thermal conditions were 95°C during 10 minutes for DNA polymerase activation, followed by 45 PCR cycles at 92°C for 15 seconds and 60°C for 1 minute. Quality control procedures implemented for genotype analyses included double sampling in 10% of the samples to assess reliability and the use of negative controls to step-away false positives. Two authors obtained the results independently, and the ambiguous results were reanalyzed.

Gene expression profiling

The TGFBR2 and SMAD7 mRNA expression levels were analyzed by a quantitative real-time PCR. After TGFBR2-875G>A genotyping, 33 patients were randomly chosen among the patients submitted to ADT, and total cellular RNA was isolated. Initially, the RNA was isolated by TriPure reagent (Roche®Applied Science), and after separation of the RNA fraction, the samples were purified using the commercial kit GeneJET™ RNA Purification Kit (Fermentas®). DNA samples were then used as a templates for cDNA synthesis, using a High Capacity RNA-to-cDNA Kit (Applied Biosystems®). Finally, reactions were carried out on a StepOne™One qPCR machine, containing 1x Master Mix (Applied Biosystems®), with 1x probes (TaqMan®Gene Expression assays.
Hs00234253_m1 and Hs00998193_m1, Applied Biosystems®), cDNA sample, and human GUSB (Beta Glucuronidase) endogenous control (Applied Biosystems®) was used to normalize the results, regarding the two biomarkers, since it presents a constant expression level. The data analysis was carried out using the StepOne Software v. 2.2 (Applied Biosystems®) with the same baseline and threshold set for each plate, in order to generate threshold cycle (CT) values for all the genes in each sample. The mRNA quantification was performed in triplicate and the results were confirmed by two independent investigators.

**Statistical analysis**

Data analysis was performed by the computer software IBM®SPSS Statistics for Windows (Version 20.0). The odds ratio (OR) and its 95% confidence interval (95% CI) were calculated as a measurement of the association between TGFBR2-875G>A genotypes and the PC risk. The Hardy–Weinberg equilibrium was tested by a Pearson chi-square analysis to compare the observed versus the expected genotype frequencies. A Cox proportional hazard model was used to analyze the time to CT (determined by the interval of time since the beginning of ADT until CR or the last clinical visit), considering as covariates, tumor stage (clinically localized versus locally advanced versus distant metastases, European Association of Urology (EAU) Guidelines [14]), Gleason Score (≥8), and PSA levels at diagnosis. Cox regression models were used to adjust for potential confounder. The 2ΔΔCT method, along with Student’s t-test, was used in order to evaluate any statistical differences in the normalized expression of the TGFBR2 and SMAD7 mRNA here explored, between the different TGFBR2-875 G>A genotypes. The concordance (c) index was used to compare the predictive ability of the association of well-known prognostic variables with the TGFBR2-875 GG genotype, with c>0.5 being considered with a good prediction ability [15].

**Results**

The TGFBR2-875G>A polymorphism genotype distribution in patients and controls is described in Table 1. The frequencies for AA/AG and GG genotypes were, respectively, 0.37 and 0.63 for PC patients and 0.38 and 0.62 in the control group. Observed versus expected genotype frequencies were calculated, and no deviation from Hardy–Weinberg equilibrium was observed (PC group, P=0.877, control group, P=0.328). We observed no statistical significant association between the TGFBR2-875G>A polymorphism and PC risk (OR=1.05; 95% CI: 0.87-1.28; P=0.645) (Table 1).

Within the group of patients, the TGFBR2-875GG genotype was associated with higher Gleason grades (≥8) (OR=1.51, 95%CI: 1.07-2.16, P=0.019). Additionally, although the association was not statistically significant, there was a trend to an overrepresentation of the GG genotype in the CR group compared with the individuals without CR (OR= 1.41; 95%CI: 0.95-2.08; P = 0.084) (Table 2).

Concerning the time to CR after the beginning of ADT, we observed that the TGFBR2-875G>A polymorphism was associated with CR-free survival. We observed a significantly reduced time-to-CR in TGFBR2-875GG homozygous compared with AA/AG genotypes carriers (86.7 versus 102.4 months) (Log Rank test, P=0.032). Furthermore, multivariate Cox regression model using tumor stage, Gleason ≥8, and PSA levels at diagnosis as covariants, demonstrated a higher risk of earlier relapse in TGFBR2-875GG PC patients following ADT (hazard ratio- HR= 1.47, 95%CI: 1.05-2.05, P= 0.024) (Figure 1).

The concordance (c) index was used to compare the predictive ability of different prognostic variables associated with CR development; the predictive value was assessed with Harrell’s concordance indexes, where a c-index of 1 indicates perfect concordance [15]. Tumor stage is a well-known prognostic factor for cancer progression. In our study the predictive value of combined tumor stage (Localized vs advanced) for CR development was 0.683 (Model 1). This predictive value increased to 0.736 with the addition of other variables to the predictive model such as PSA ≥ 20 ng.mL⁻¹ at diagnosis and Gleason score ≥8 (Model 2). Finally, this predictive ability increased with the addition of genetic information regarding the TGFBR2-875G>A polymorphism, with an improvement of the prediction by 6.3% and 1.0% compared with Model 1 and Model 2, respectively (c-index 0.746) (Model 3, Table 3). Furthermore, the predictive value

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**Table 1. TGFBR2-875G>A polymorphism-related odds ratio for PC and genotype frequencies in patients and controls.**

| TGFBR2-875 G>A | PC group | Control group | OR | 95% CI | P |
|----------------|----------|---------------|----|--------|---|
| AA             | 34 (0.04) | 46 (0.05)     |    |        |   |
| AG             | 296 (0.33)| 287 (0.33)    | 1.39| 0.87-2.24| 0.210|
| GG             | 562 (0.63)| 541 (0.62)    | 1.41| 0.89-2.34| 0.179|
| AA/AG          | 329 (0.37)| 333 (0.38)    |    |        |   |
| GG             | 562 (0.63)| 541 (0.62)    | 1.05| 0.87-1.28| 0.645|

PC, Prostate cancer; OR, odds ratio; 95% CI, 95% confidence interval

**Table 2. Aggressive phenotype disease according TGFBR2-875G>A polymorphism.**

| TGFBR2-875 G>A | Gleason Score | OR | 95% CI | P |
|----------------|---------------|----|--------|---|
| AA/AG          | <8            |    |        |   |
| AA/AG          | ≥8            |    |        |   |
| AA/AG          | Localized (T1-T2 stages) |    |        |   |
| AA/AG          | Advanced (T3-T4, N and M+) |    |        |   |
| AA/AG          | No            |    |        |   |
| AA/AG          | Yes           |    |        |   |
| AA/AG          | Yes           |    |        |   |
| AA/AG          | No            |    |        |   |
| AA/AG          | 133 (0.40)    | 55 (0.32) |    |        |   |
| AA/AG          | 196 (0.60)    | 114 (0.68) | 1.41| 0.95-2.08| 0.084|

OR, odds ratio; 95% CI, 95% confidence interval; univariate analysis

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increased to 0.759 considering the covariants tumor Stage (localized vs locally advanced vs distant metastases, EAU), PSA ≥ 20 ng.mL\(^{-1}\) at diagnosis, Gleason ≥ 8, and PSA levels at diagnosis as covariants.

Regarding the TGFBR2 mRNA expression according the TGFBR2-875G>A polymorphism, we observed differences in mRNA expression in GG homozygous carriers compared with AA/AG genotypes carriers. The AA/AG genotype carriers present a 1.5-fold increase in expression levels of TGFBR2 mRNA than GG homozygous (\(2^{-\Delta\Delta CT} = 1.5, P=0.016\)) (Figure 2a). Concerning SMAD7 mRNA expression, we observed no differences in mRNA expression levels between the TGFBR2-875GG homozygous and TGFBR2-875AA/AG genotype carriers (\(P=0.906\)) (Figure 2b).

**Discussion**

Worldwide, PC is the most prevalent malignancy in men. Prostate cancer is a complex, multifactorial and heterogeneous disease. A better understanding of the mechanisms involved in this disease may contribute to early diagnosis and to the establishment of new therapeutic targets to increase survival and improve the quality of patients’ life. The PC progression to CR has been associated with the cell insensitivity to anti-proliferative signals and consequent disruption of the balance...
between cell growth rate and apoptosis. The CRPC is an invariably lethal condition, with chemotherapy being the sole treatment option with only palliative benefits [16,17]. Therefore, it is important to understand the mechanisms involved in CR progression.

In the present study, we describe that the functional TGFBR2 polymorphism influences the development of CRPC. Our results suggest that small changes in TGFBR2 expression levels, due to functional SNPs, may contribute to a disequilibrium in the TGFβ1 signaling pathway activation and thus to a higher risk for an earlier development of resistance to ADT.

TGFβ1 signaling pathway mediates growth, mainly by inhibiting cell cycle progression through G1-arrest, suppressing c-myc, and stimulating cyclin-dependent kinases inhibitors including p21WAF1 and p15Ink4b, but also by inducing apoptosis, through the induction of DAP kinase and others [18]. Several studies demonstrated that TGFβ1 signaling components, including TGFβRII, are often lost in human cancer [5]. In PC, the TGFβ1 is frequently up-regulated and the loss of TGFβRI and RII has been detected. It has been hypothesized, that TGFBR2 repression is responsible for most of the tumor associated TGF β1 resistance observed in vivo [19]. Studies performed by Rojas and co-workers showed that TGFBR2 expression levels can regulate the intensity of activation of the Smad and non-Smad signaling pathways [10]. The inactivation of TGFBR2 gene in mouse fibroblasts has been associated with prostate intraepithelial neoplasia and invasive squamous cell carcinoma of forestomach development, and this alteration can also promote growth and invasion of co-transplanted breast cancer cells [20,21]. Moreover, patients with defective TGFβRII present significantly more colon polyps and preneoplastic lesions than patients with normal TGF βRII [22]. Lu and co-workers, report that the expression of TGF βRII significantly decreased when they observed normal tissue, tissue adjacent to cancer and gastric cancer [23].

Figure 2. Normalized expression among individuals with TGFBR2-875GG vs TGFBR2-875AA/AG genotypes regarding a) TGFBR2 mRNA and b) SMAD7 mRNA. Statistical differences in the TGFBR2 and SMAD7 mRNA levels were evaluated by the Student’s t-Test.

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### Table 3. Predictive models of castration-resistance development after androgen deprivation therapy.

| Model | HR § 95% CI | P | c index |
|-------|-------------|---|---------|
| Model 1 | 3.89 2.67-5.65 | <0.001 | 0.683 |
| Combined Tumor Stage (Localized vs advanced) | 2.61 1.72-3.95 | <0.001 | 0.736 |
| Model 2 | Combined Tumor stage (Localized vs advanced) | 1.47 1.06-2.03 | 0.020 | 1.90 1.40-2.62 | <0.001 |
| PSA ≥ 20 ng.mL⁻¹ at diagnosis | 1.43 1.02-2.00 | 0.045 | 1.64 1.28-2.10 | <0.001 |
| Gleason 38 | 1.39 0.99-1.94 | 0.058 | 1.53 1.10-2.12 | 0.012 |
| Model 3 | Combined Tumor Stage (Localized vs advanced) | 2.70 1.77-4.12 | <0.001 | 0.746 |
| PSA ≥ 20 ng.mL⁻¹ at diagnosis | 1.53 1.02-2.00 | 0.045 | 1.64 1.28-2.10 | <0.001 |
| Gleason 38 | 1.43 1.02-2.00 | 0.045 | 1.53 1.10-2.12 | 0.012 |
| TGFBR2-875GG genotype | 1.39 0.99-1.94 | 0.058 | 1.43 1.02-2.00 | 0.045 |
| Model 4 | Tumor Stage (localized vs locally advanced vs distant metastases, EAU) | 2.28 1.78-2.91 | <0.001 | 0.759 |
| PSA ≥ 20 ng.mL⁻¹ at diagnosis | 1.23 0.87-1.75 | 0.246 | 1.64 1.18-2.29 | 0.004 |
| Gleason 38 | 1.43 1.02-2.00 | 0.045 | 1.53 1.10-2.12 | 0.012 |
| TGFBR2-875GG genotype | 1.44 1.03-2.02 | 0.033 | 1.53 1.10-2.12 | 0.012 |

HR, hazard ratio; 95% CI, 95% confidence interval
TGFβ1 signaling cascade members, show down-regulated levels of TGFBR2, demonstrating the oncogenic potential of TGFβ1 pathway [25].

In the PC3 cell line, which is CR, the AR expression reduces TGFβ1/SMAD transcriptional activity and the growth effects of TGFβ1 (in the absence of DHT), thus preventing growth inhibition and apoptosis. According to Bruckheimer and co-workers, the treatment of LnCaP cells, that overexpress TGFβRII, with TGFβ1 in the presence of DHT, can enhance the cell cycle arrest and apoptosis, through caspase-1 activation and the targeting of BCL-2 [26].

It is evident that TGFβ1 signaling requires a delicate balance of interactions within the cellular and tumoral microenvironment. Deregulation of TGFBR2 expression levels and of the inhibitory SMAD7 could influence the normal cellular homeostasis and also influence cancer progression. We previously demonstrated that changes in the tumoral microenvironment can modulate PC progression with impact in response to ADT [13,27].

Seijo and co-workers observed, in normal epithelial cells, that the A-to-G transition at the -875 position enhances the activity of TGFβRII, with higher luciferase activity in the presence of the A allele [12]. Our results also suggest that the TGFBR2-875G>A polymorphism could influence the circulating expression levels of TGFBR2 in PC patients. We observed that TGFBR2-875AG/AA patients carriers present a higher expression levels of TGFBR2 mRNA than GG homozygous (2^{ΔΔCT} = 1.5, P=0.016) and that up-regulation may be responsible for the TGFβ1 signaling pathway up-regulation, inducing the cell cycle arrest and apoptosis, and consequently causing these patients to present a higher progression free-interval after the beginning of ADT (AA/AG: 102.4 vs GG: 86.7 months).

In fact we also observed that TGFBR2-875GG homozygous, which present lower circulating levels of TGFBR2 mRNA, present a higher risk for the development of tumors with higher Gleason score (28) (P=0.019). In GG homozygous carriers, the lower expression levels of TGFBR2 may be associated with cellular disruptions in the TGFβ1 signaling pathway and induces the acquisition of aggressive cancer phenotypes.

TGFBR2-875>G/A has been the subject of investigation in several case–control studies involving different types of cancers, with controversial results [28–32].

Several authors, have associated the TGFBR2-875G>A polymorphism with a decreased risk for gastric cancer, esophageal squamous cell carcinoma, breast cancer, and lymph node metastasis, especially in the Asian population [28–33].

However, similarly to other studies in European populations, no association were observed between the TGFBR2-875/A polymorphism and PC risk. In addition, it appears that this polymorphism may have no influence on the onset of PC but instead it can modulate its progression to an aggressive phenotype [34].

In our control population the frequency of the TGFBR2-875G>A polymorphism tended to be similar to those observed in other Caucasian healthy populations like in Finland and Poland. However, the frequencies are also similar to those observed in studies performed in Republic of Korea and North of China [30,33,34]. Additionally, we observed that the frequency of the TGFBR2-875GG genotype was lower than the one of the control group used in the study performed by Seijo and co-workers in an American population and higher than the reported results in the control group that included Cantonese healthy subjects from population in southern China [12,32].

Survival of the PC patients and CR development are related to several variables that include the extent of the initial tumor, PSA levels, patients’ age, which are useful tools for therapeutic decisions [14]. However, due to some predictive limitations, they do not exactly reflect the biologic behavior of PC, and many men can die from aggressive disease. Additional results showed that the definition of profiles that contains information regarding tumor characteristics associated with genetic information have a higher capacity to predict the risk for CR development (c-index from 0.683 to 0.746). Therefore, we hypothesize that the definition of a predictive profile that contains the TGFBR2-875G>A polymorphism information could be useful as molecular marker for predicting the clinical response to ADT in patients with PC. However, future studies should replicate this predictive model in another PC study population submitted to ADT.

In conclusion, the present study suggests that the functional polymorphism TGFBR2-875G>A does not modify the predisposition to PC in our population. However, this SNP has the capacity to change the TGFBR2 mRNA expression levels in PC patients submitted to ADT and influence the CRFI, with a lower time interval to CR in TGFBR2-875GG homozygous compared with TGFBR2-875AG/AA patients carriers. In the future, the identification of these genetic profiles may help to define new effective prognostic models for the follow up of patients submitted to ADT and identifying higher risk groups for an individualized chemoprevention strategy and target therapies.

Author Contributions
Conceived and designed the experiments: ALT RM. Performed the experiments: ALT MG AN AA JA FD JIS. Analyzed the data: ALT RM. Contributed reagents/materials/analysis tools: FL AM JM RM. Wrote the manuscript: ALT MG RM.

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