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EVALUATION OF IMMUNOEXPRESSION AND *MDR1* PROMOTER METHYLATION LEVELS IN PROSTATIC TISSUE SAMPLES

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RESUMO

Os objectivos deste estudo foram: (1) Determinar os níveis de metilação do promotor do *MDR1* em tecido prostático com adenocarcinoma (CaP), neoplasia intraepitelial prostática de alto grau (HGPIN), hiperplasia benigna (BPH) e tecido morfologicamente normal (MNP). (2) Correlacionar os níveis de metilação com a imunoexpressão da gp-P.

Os nossos resultados demonstram que a hipermetilação do *MDR1* constitui um mecanismo eficaz de regulação da sua expressão. Estudos futuros permitirão avaliar o impacto destes resultados na terapêutica do cancro da próstata.

PALAVRAS-CHAVE: cancro da próstata ; *MDR1*; gp-P; metilação do promotor; epigénetica.

ABSTRACT

Our aims were: (1) To determine the *MDR1* methylation levels in tissue of Prostate cancer (PCa), high grade prostatic intraepithelial neoplasia (HGPIN), benign prostatic hyperplasia (BPH) and morphologically normal prostate tissue (MNP); (2) to correlate the methylation levels of the *MDR1* promoter with the immunoexpression of P-gp.

Our results demonstrate that *MDR1* hypermethylation constitutes an effective mechanism of P-gp expression regulation. Future studies will be able to evaluate the impact of these results in the treatment of PCa patients.

KEY-WORDS: Prostate cancer; *MDR1*; P-gp; promoter methylation; epigenetics.

1. INTRODUCTION

Prostate carcinoma (PCa) is the sixth most frequently diagnosed cancer in the world and the second leading cause of men death in Europe and USA (Lara *et al*., 2004; Perry *et al*., 2006). The diagnosis of PCa in early stages is crucial for a curative treatment, since in advanced disease the resistance to androgen-ablation therapy results in a high probability of death (Perry *et al*., 2006). As current diagnostic methodologies fail to detect or correctly predict the outcome of a large percentage of prostate carcinomas, the discovery of novel molecular markers with diagnostic and/or prognostic potential is of paramount importance for the clinical management of these patients. Promising results using epigenetic markers have shown the importance of DNA hypermethylation of several genes in prostate cancer development, and the evaluation of methylation level of a small panel of deregulated genes (e.g.: *GSTP1*, *APC*, *RARβ2* and *MDR1*) should thus prove valuable in establishing a correct PCa diagnosis and assess the prognosis of the patients (Enokida *et al*., 2005; Costa *et al*., 2007a).

The *MDR1* gene ("multidrug resistance receptor 1"), mapped at 7q21, encodes for P-glycoprotein (P-gp), a membrane ATP-dependent efflux transporter composed of 1280 amino acids (Ambudkar *et al*., 2003; Rao *et al*., 2005). In normal conditions, *MDR1* is mainly expressed in gastrointestinal, liver and kidney epithelia, and capillary endothelial cells in brain, testis and ovary (Ambudkar *et al*., 2003). It is known that the P-gp protects the body from environmental toxins and xenobiotics, promoting its excretion in bile and urine (Ambudkar *et al*., 2003). Additionally, P-gp has an important pharmacokinetic role by interacting with several drugs, such as *Vinca* alkaloids, topoisomerase II inhibitors and steroid hormones (Ambudkar *et al*., 2003). P-gp was originally discovered as the mediator of the multidrug resistance phenotype (MDR) of cell lines selected *in vitro* for resistance to several anticancer drugs (Kawai *et al*., 2002). Subsequent studies revealed that P-gp is expressed in a tissue-specific manner and that in several malignancies P-gp overexpression is frequent and may constitute an adaptive response to chemotherapy (Bhangal *et al*., 1999). In prostate carcinoma, however, P-gp expression seems to be lower than normal prostatic cells (Bhangal *et al*., 1999; Kawai *et al*., 2002).

P-gp is an energy dependent ABC transporter (ATP-Binding Cassette Transporter) whose function is to drive drugs out from cells. This molecule is formed by two homologue domains, each containing six trans-membrane domains (TM) where drug binding occurs, and two ATP-binding sites (Figure 1). They are separated by a flexible binding region, which promotes the communication between the ATP-binding sites. In this intrinsic biochemical process the ATP hydrolysis produces energy for drug transport. (Ambudkar *et al*., 2003).

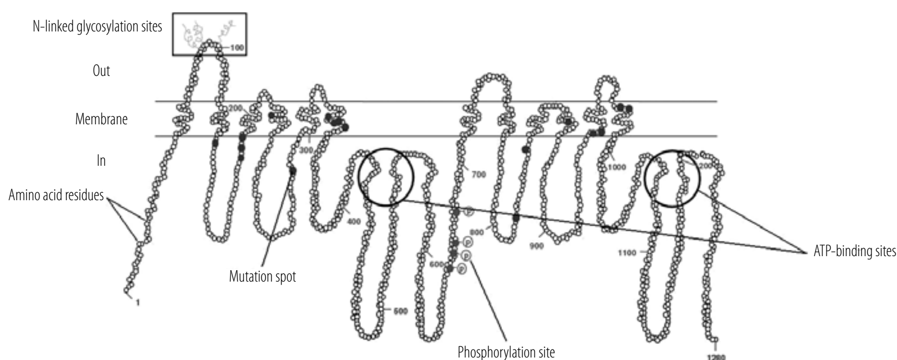

Figure 1. Hypothetical 2-D model of human P-gp. Adapted from Ambudkar *et al.* (2003).

DNA methylation and other epigenetic mechanisms, such as histone modifications, play an important role in regulation of gene expression by altering chromatin structure and hence affecting gene expression in a heritable manner without directly interfering with the genetic code (Kusaba *et al*., 1999; Herman et al., 2003, Perry *et al*., 2006; Esteller, 2007). Methylation of promoter CpG islands results in transcriptional silencing through several mechanisms, as the attraction of proteins that interact with histone deacetylases and chromatin condensation preclude the binding of transcriptional factors to the promoter (Perry *et al*., 2006). Epigenetic alterations modulate gene expression and may modify the tumour phenotype. As a result, the bulk of methylation in a tumour may reflect its course (Perry *et al*., 2006; Dobosy *et al*., 2007). In contrast with genetic alterations, CpG island hypermethylation occurs very frequently in PCa (Cho *et al*., 2007). Hence, this alteration is widely accepted as an important carcinogenic mechanism of PCa and has been suggested by several studies as a tumour marker and prognostic factor (Cho *et al*., 2007). Different surveys have reported a high frequency of *MDR1* promoter hypermethylation in PCa (Enokida *et al*., 2004; Enokida *et al*., 2005; Costa *et al*., 2007a). Although recent investigations suggest that this epigenetic alteration regulates gene expression, the experimental data to sustain this hypothesis is scarce.

2. **OBJECTIVES**

Our aims were: (1) to determine *MDR1* promoter methylation levels in PCa, high grade prostatic intraepithelial neoplasia (HGPIN), benign prostatic hyperplasia and morphologically normal prostate tissue (MNP); (2) to correlate the methylation levels with the immunoexpression of P-gp.

3. **METHODS/EXPERIMENTAL DESIGN**

One hundred and one men with clinically localized PCa, consecutively diagnosed and primarily submitted to radical prostatectomy in I.P.O.F.G – Porto were included in this study [stage T1c and T2, according to TNM system (Hermanek et al., 1997)]. Prostatic intraepithelial neoplasia (PIN) lesions were identified in 39 cases and also collected for further analysis. BPH specimens were collected from 28 patients submitted to transurethral resection and selected for sample controls. All specimens were frozen at -80ºC and then cut with a cryostat for microscopic evaluation and selection of potential areas for analysis. Cut sections were trimmed to maximize target cell content (>70%) and then DNA extraction was performed using phenol-chloroform. From each specimen, parallel fragments were collected, formalin treated and paraffin-embedded for histopathological examination. Gleason score (Gleason et al., 1974) and pathological staging (Hermanek et al., 1997) were evaluated by an expert pathologist. Relevant clinical data, including prostate-specific antigen (PSA), was collected for each patient (Kort et al., 2006). These studies were approved by the Ethics Commission of I.P.O.F.G – Porto.

3.1. **DNA EXTRACTION**

After overnight digestion in lysis buffer and proteinase K at 55ºC, DNA was extracted from all samples with phenol-chloroform. The aqueous phase was transferred to eppendorf tubes and DNA was precipitated at -20ºC overnight by addition of absolute ethanol and 7,5M ammonium acetate. DNA pellets were washed twice with 70% ethanol and finally eluted in distilled water (ddH$_2$O).

3.2. **SODIUM BISULPHITE CONVERSION**

Sodium bisulphite reaction allows for DNA methylation analysis by converting non-methylated cytosine residues to uracil residues, while methylated cytosines residues remain without any modification (Clark et al., 1994). Target DNA was further amplified in quantitative methylation-specific PCR, where all uracils and thymines are amplified as thymines, whereas methylated cytosines appear as cytosines (Olek et al., 1996). Briefly, we start by quantifying DNA concentration in all samples using a NanoDrop spectrophotometer (NanoDrop, USA). As bisulphite conversion only occurs when the template is in a single-stranded conformation, DNA was denatured by incubation with 3M NaOH for 20 minutes at 50ºC. The DNA conversion mixture was prepared using 9,5g of sodium bisulphite (Sigma, USA) dissolved in 12,5mL of water, 2,5mL of 1M hydroquinone (Sigma, USA) and 3,5mL 2M NaOH.

All DNA samples were then gently homogenised and incubated at 70ºC for 3 hours with 450µL of the conversion mixture. Free sodium bisulphite must be removed to provide purified DNA. Therefore, 900µL of Wizard DNA Clean-Up Resin (Promega, USA) was added to all samples. The resulting solution is placed into vertical columns and a vacuum is applied to draw the solution through. The bisulphite and hydroquinone excess are removed by washing the columns with 1,5mL of 80% isopropanol. The filter containing the DNA was transferred to a new eppendorf tube, and 45µL of ddH_2O at 70ºC was added for DNA recovery. The tubes were then centrifuged (13000 rpm for 5 minutes) and the filter discarded. As the DNA must be kept in single-strand, 5µL of NaOH was added. Finally, ammonium acetate and absolute ethanol were used to precipitate the converted DNA, which was further washed with 70% ethanol, dried at room temperature and finally eluted in ddH_2O.

3.3. **QUANTITATIVE METHYLATION-SPECIFIC PCR**

All samples were subjected to two reactions of amplification, one for the quantification of methylated *MDR1* and the other for quantification of an internal reference gene (*β-actin*, which does not contain CpG islands) to normalize DNA input (Costa *et al.*, 2007b). The converted DNA, positive and negative controls, and commercial standards with serial dilutions of fully methylated DNA were amplified in the same run. These standards were used to construct a calibration curve in order to quantify the fully methylated genes in the two reactions. PCR assays were performed in 96-well plate using a reaction volume of 20 µL. The final reaction mixture consisted of 600 nmol/L of each primer (Invitrogen, Carlsbad, CA); 200 nmol/L probe (Applied Biosystems, Foster City, CA); 1 unit of platinum Taq polymerase (Invitrogen, USA); 200 µmol/L concentration each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma; 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; 0.1% DMSO, and 2 µL of bisulfite-converted genomic DNA. Amplification reactions were carried out in 50 cycles of denaturation at 95ºC for 15 seconds and annealing and extension at 60ºC for 60 seconds (Applied Biosystems, USA). For each sample, the relative level of methylation in *MDR1* promoter was obtained by dividing the value of methylated *MDR1* by the respective value of *β-actin*, multiplying by 1000 for easier tabulation.

3.4. **IMMUNOHISTOCHEMISTRY**

Sections (3µm thick) from paraffin-embedded samples correspondent to the samples used for methylation analysis were obtained, deparaffinised in xylene and hydrated through a graded alcohol series. Antigen retrieval was accomplished by microwaving the specimens at 800W for 5 minutes with EDTA buffer. After cooling the slides, endogenous peroxidase activity was blocked by incubating the sections in hydrogen peroxide in 3% methanol for 30 minutes. The sections were treated with 5% normal horse

serum (VectaStain, USA) in 1% PBS-BSA for 30 minutes to reduce background interference. The primary mouse monoclonal antibody (C494 clone, ThermoScientific, UK) was applied in 1:50 dilution with 1% PBS-BSA and left at 4ºC overnight. The secondary biotinylated horse antibody (VectaStain, UK) at a dilution of 1:50 was added for 30 minutes. In order to enhance the immunohistochemical staining, the sections were incubated in avidin-biotin complexes for 30 minutes. Then, 3,3'-diaminobenzidine (Sigma, USA) was used for visualization and hematoxilin for counterstaining. Finally, after dehydration and diaphanization the slides were mounted in *Entellan*. Imunohistochemistry results were categorized according to stain intensity into 2^+ (expression similar to normal prostate tissue), 1^+ (expression lower than normal prostate tissue), and 0 (no immunoexpression).

3.5. **STATISTICAL ANALYSIS**

As the analyzed variables did not follow a normal distribution, nonparametric tests were used. In each group of samples, median and interquartile range (p25-p75) of *MDR1* methylation levels were determined, and then compared through Kruskal-Wallis test or Mann-Whitney U-test, depending on the number of categories in each group. The correlation between *MDR1* promoter methylation levels and P-gp immunoexpression was assessed using Kruskall-Wallis test. Statistical analysis was performed in *Statistica for Windows*, *version 6.0* (StatSoft, Tulsa, OK, USA).

4. **RESULTS**

4.1. **CLINICAL AND PATHOLOGICAL CHARACTERISTICS**

The clinical and pathological data of the patients is depicted in Table 1. Although PSA levels are higher in patients with PCa (P=0,003), there is a striking overlap between PSA values in PCa and BPH patients. The median age of patients with BPH was significantly higher than patients with PCa (P=0,00015).

4.2. ***MDR1* PROMOTER METHYLATION IN PROSTATIC TISSUE**

According with a previous study including the same group of patients performed by Baptista et al. (2006), the majority of the samples revealed methylation at *MDR1* promoter region. Kruskall-Wallis showed significant differences in the distribution of methylation levels among all histological subtypes (P<0,00001), in particular between PCa and HGPIN (P=0,00007), PCa and BPH (P<0,00001), and HGPIN and BPH (P=0,00001).

4.3. **P-GP IMMUNOEXPRESSION IN PROSTATIC TISSUES**

The majority of PCa (89%) and HGPIN samples (79%) showed decreased P-gp expression (scores 0 and 1^+), whereas all BPH and MNP exhibited normal expression (2^+, Table 2). Statistical analysis demonstrated significant hypermethylation differences between immunoexpression levels 0 and 2^+, and between 1^+ and 2^+ (P<0,0001), showing that the higher *MDR1* methylation levels are present in PCa and HGPIN (Figure 2), whereas very low methylation is found in BPH and normal prostatic tissue. A representative example of immunoexpression results in PCa and HGPIN is provided in Figure 3.

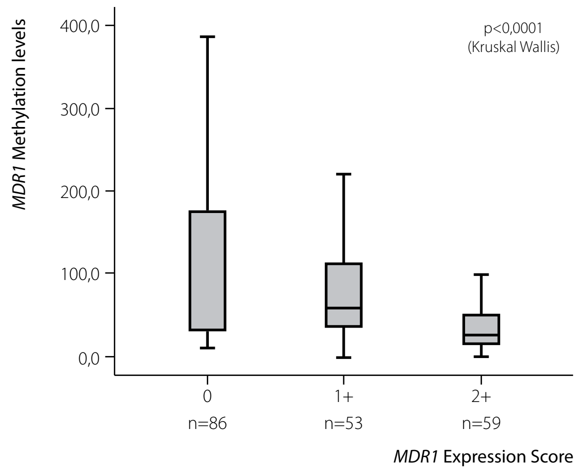

Figure 2. Correlation of *MDR1* promoter methylation levels and P-gp immunoreactivity in prostate tissue samples.

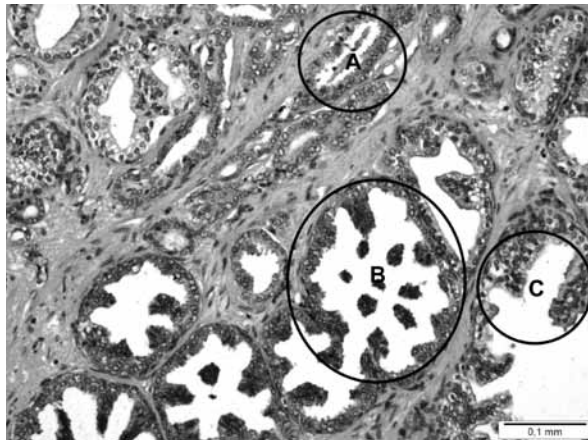

Figure 3. Immunoreactivity of P-gp in three distinct regions: (A) PCa cells; (B) prostatic gland with HGPIN; and (C) normal prostatic gland.

5. **Discussion and Conclusions**

In the present study we evaluated how the promoter methylation and protein expression levels of *MDR1* vary in prostate carcinomas, high grade prostatic intraepithelial neoplasia, benign prostatic hyperplasia and morphologically normal prostate tissue. As reported by others, neoplastic prostate cells have shown lower P-gp expression (Bhangal et al., 1999; Kawai et al. 2002). Not only was this finding confirmed in our series, we could also demonstrate for the first time that higher promoter methylation levels of *MDR1* are significantly correlated with this loss of expression. Our data therefore suggests that *MDR1* hyperme-

thylation is an effective mechanism of P-gp inactivation that may thus explain the frequent loss of *MDR1* expression in prostate cancer.

It is interesting that the "multidrug resistance" role initially assigned to *MDR1* (Ambudkar et al., 2003) represents a contradiction in the prostate carcinoma context. Bearing in mind that hypermethylation generally silences the affected gene, the loss of P-gp expression may be interpreted as an unfavourable change for neoplastic cells. However, researchers on other neoplastic models showed that the expression of this glycoprotein in localised disease is higher than its expression in metastatic stages (Scotlandi et al., 1999), indicating a connection between P-gp loss of expression and tumour progression.

As such, silencing *MDR1* may be considered a therapeutic opportunity for treating patients with prostate cancer. Considering that curative treatment is not possible in locally advanced and/or metastatic PCa, and that this tumor is resistant to conventional chemotherapy, the use of pharmacological agents usually detoxified by P-gp might be a functional alternative in PCa treatment. Biologically, the P-gp role is to drive xenobiotics (*e.g.*, chemotherapeutic agents) out of cells and into urine and bile, thus removing them from the intracellular milieu/environment (Ambudkar et al., 2003). Hypothetically, reducing the expression of P-gp, mediated by *MDR1* hypermethylation, will increase the efficacy of chemotherapeutic agents in treating prostate cancer. This assumption is quite important, since recent studies have shown that hormone-refractory prostate cancer patients treated with taxanes have better survival rates (Mackler et al., 2005).

In conclusion, our study has demonstrated a high prevalence of *MDR1* promoter methylation in malignant prostate lesions, which could be significantly associated with the loss of P-gp expression in the same samples. Due to the role played by this protein in the removal of xenobiotics (*e.g.*, taxanes, Vinca alkaloids), the use of *MDR1* epigenetic silencing as a therapeutic opportunity in the treatment of late stage prostate cancer is promising. However, further studies are needed to evaluate the role of *MDR1* hypermethylation in the treatment with taxanes in prostate carcinoma.

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