

Methylation Analysis of *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* Promoters in Prostate Biopsies According to Different Degrees of Malignancy

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Abstract. Prostate cancer is the most common cancer among men and the second leading cause of cancer-related deaths in the United States. CpG island methylation causes gene silencing and could be decisive in prostate carcinogenesis and progression. Its role was investigated at multiple gene sites during prostate carcinogenesis. Methylation-specific polymerase chain reaction (MS-PCR) was used to analyze 4 interest gene promoter status in 12 patients with adenocarcinoma, 7 patients with prostate intraepithelial neoplasia, 3 patients with peritumor tissues and 15 healthy patients, so a total of 37 prostate biopsy samples constituted the cohort of the study. Despite the biopsy histology, the results have confirmed that *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* promoter methylation was found in each sample, except two.

Prostate cancer is the second leading cause of cancer-related deaths in the United States. The American Cancer Society estimates that there were over 186,320 cases of new prostate cancer cases in 2008. Prostate cancer is the most commonly diagnosed malignancy among males in Western countries (1) and represented 40,000 new cases in France in 2000 (2). The net risk of developing prostate cancer before the age of 75 years for a patient born in 1943 was multiplied by 3 compared to those born in 1928 (3). Researches have focused on candidate genes to assess their involvement in the predisposition of prostate cancer. Since available screening methods show poor sensitivity and specificity, the development of new molecular markers is warranted.

Epigenetic alterations, mainly promoter hypermethylation of cancer-related genes, are common events in prostate cancer and might be used as cancer biomarkers.

Epigenetic changes, particularly the DNA methylation, are found to be involved in a variety of cancers (4). DNA methylation refers to a covalent chemical modification resulting from the addition of a methyl (CH₃) group at the C-5 position of the Cytosine ring in the DNA (5). The human genome is not uniformly methylated. "CpG islands" are small regions within the genome that are rich in Cytosine and Guanine bases and are largely unmethylated epigenetics target this region thereby affecting gene expression (4). Epigenetic changes are early event in cancer development and can be used to assess the risk of developing cancer. Epigenetic changes in prostate cancer are being studied extensively and genome wide screening will lead to development of novel epigenetic markers. The aim of this study was to determine the methylation promoter status of four cancer-related genes in the prostate according to the degree of malignancy. *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* methylation was explored on prostate biopsies. These four genes of interest are known to be cancer-related genes. The aim was to examine their promoter methylation status and to explore their relationship with histological degree of malignancy.

Patients and Methods

Data collection. This study was conducted with the participation of 37 men which had a prostate biopsy to establish their diagnostic by an urologist. They were hospitalized at the CHU of Clermont-Ferrand and, in a letter of consent, they accepted to give a sample for research. This procedure was advised by the PPC (People Protection Committee) within the framework of the constitution of collection of biological samples. A transrectal ultrasound-guided prostate biopsy was made with a spin and in sextant. This exam was made with local anaesthesia on the recumbent patient. The identity of each patient was

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Table I. Primer sequences of studied genes with MS-PCR (Methyl Specific Polymerase Chain Reaction).

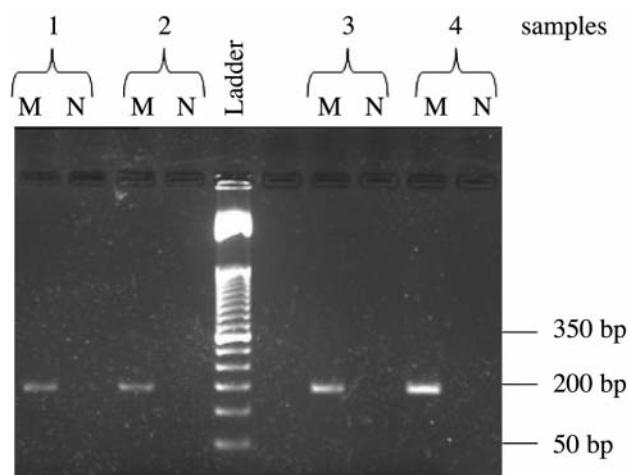
<i>BRCA1 Methylated primer</i>	
Fwd:	AGTTTGATTAACGTGGTGAATTTC
Rev:	ATTTTAAACAATCTCGCTATCG
<i>BRCA1 Unmethylated primer</i>	
Fwd:	TTTGATTAATGTGGTGAATTGTTG
Rev:	TTTTAAACAATCTCACTCTATCACC
<i>RASSF1 Methylated primer</i>	
Fwd:	GGTAGTTAAGGGTAGCGTAGTC
Rev:	TTCAACGATAAAACGAAAATAACG
<i>RASSF1 Unmethylated primer</i>	
Fwd:	GGGGTAGTTAAGGGTAGTGTAGTT
Rev:	TCAACAATAAAACAAAAATAACAAA
<i>GSTP1 Methylated primer</i>	
Fwd:	CGTTTTAGTGTGTGTGAAATTTC
Rev:	TTTAATAAACCCCTCCTACCACGT
<i>GSTP1 Unmethylated primer</i>	
Fwd:	TGTTTAGTGTGTGTGAAATTGTTG
Rev:	TTAAATAAACCCCTCCTACCACATC
<i>EphB2 Methylated primer</i>	
Fwd:	CGTAAGGTTTCGGTATTTTC
Rev:	TACCAATACAGGAAACCACG
<i>EphB2 Unmethylated primer</i>	
Fwd:	GTGTAAGGTTTGTTGGTATTTTT
Rev:	ATACCAATACACAAAACCACCA

confidential and through the anatomopathological exam, the stage of cancer development was diagnosed and could be correlated.

The cohort of 37 patients presented 15 non-malignant (NM) biopsies, 7 prostate intraepithelial neoplasia (PIN), 3 peri tumor tissues (PTT) and 12 adenocarcinomas (ADC).

DNA extraction. Prostate biopsies were disrupted with a scalpel and DNA extraction was performed with Non-Organic DNA Extraction Kit® (Chemicon, International, S4520, USA) according to the manufacturer's protocol. First, the cells were lysed for 15 min in a Wash Solution, after centrifugation at 1000g for 20 min, the tissue sample was resuspended in a 1X Suspension Buffer I and the DNA was deproteinized with 50 µL of Protein Digesting Enzyme and 1 ml of Protein Precipitating solution. Protein contaminants were removed and the DNA was precipitated with two volumes of absolute ethanol and resuspended in 150 µL of Suspension Buffer II. Each sample was assayed with Nanodrop 8000 (LabTech®), measuring optical density ratio at 260/280 nm.

MS-PCR. Interest gene promoter methylation was determined by MS-PCR with bisulfite-converted DNA. The procedure takes advantage of the bisulfite-mediated chemical conversion of cytosine to uracil followed by PCR using primers designed to distinguish methylated DNA from unmethylated DNA (6). Bisulfite modification of DNA to convert unmethylated cytosine residues to uracil was carried out using MethylDetector Bisulfite Modification Kit (Active Motif®) following the protocol from the manufacturer. Sequences of the primers were designed by MethPrimer software (Table I). Each modification was checked using a verification method prescribed by the manufacturer.



M: Modified, N: Not modified

Figure 1. Electrophoresis on a 2.5% agarose gel of Modified (M) and Non-Modified (NM) PCR products in 4 prostate biopsies (The presence of an expected modified band was obtained at 200 bp).

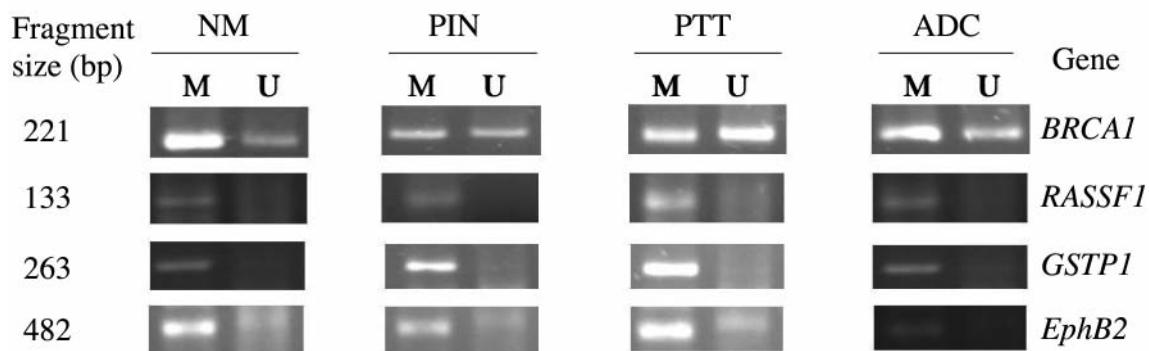
Table II. Repartition of 12 adenocarcinoma cases according to Gleason score.

Adenocarcinoma cases (12)		
Gleason score	6	7
Number of cases	2	9

PCR was carried out in a total volume of 30 µL per reaction containing 0.5 µL of AmpliTaq (Applied Biosystems®), 3 µL of gene probes, 0.5 µL of dNTP, 3 µL of PCR buffer (10X), 19.3 µL of water and 4 µL of DNA sample. Each PCR reaction underwent initial denaturation at 95°C for 10 min, and 40 cycles of the following profile: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The PCR products were then analyzed by electrophoresis on a 2.5% agarose gel and stained with ethidium bromide and visualized by UV transillumination. A 50 bp DNA Ladder (Invitrogen®) was used. DNA was considered methylated if a PCR product using unmethylated-specific primers was absent. When PCR product was present using both unmethylated-specific primers and methylated-specific primers, it meant that between 25% and 75% of the gene promoter could be considered methylated. The assay was successfully completed for the four genes of the 37 subjects.

Results

Grading and pathologic evaluation. Tissue sections fixed in 10% buffered formalin that were routinely processed, whole-mount-embedded, and hematoxylin and eosin-stained were reviewed. The tumor foci was identified, circled in ink, and graded. The cohort of 37 patients presented 15 non-malignant biopsies, 7 prostate intraepithelial neoplasia, 3



M: Methylated, U: Unmethylated

Figure 2. Analyses by Methyl Specific PCR of *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* promoter status in a Non-Malignant biopsy (NM), a Prostatic Intraepithelial Neoplasia (PIN), a PeriTumor Tissue (PTT) and an Adenocarcinoma sample (ADC). (The PCR product of *BRCA1* was obtained at 221bp, *RASSF1* at 133bp, *GSTP1* at 263bp and *EPHB2* at 482bp). When PCR product was present using both unmethylated-specific primers and methylated-specific primers, it meant that between 25% and 75% of the gene promoter could be considered methylated.

Table III. Results of *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* MS-PCR in 37 prostate biopsies according to different histological degrees.

Diagnosis	Number (37)	Promoter status									
		<i>BRCA1</i>		<i>GSTP1</i>		<i>RASSF1</i>		<i>EPHB2</i>			
		M	U	M	U	M	U	M	U	M	U
NM	15	13	15	15	0	15	0	15	0	15	0
PIN	7	7	7	7	0	7	0	7	0	7	0
PTT	3	3	3	3	0	3	0	3	0	3	0
ADC	12	12	12	12	0	12	0	12	0	12	0

NM: non-malignant; PIN: prostate intraepithelial neoplasia; PTT: peritumoral tissue; ADC: adenocarcinoma.

peri-tumor tissues and 12 adenocarcinomas. The Gleason score grading system was used to assign a tumor grade (7). Among the twelve adenocarcinoma, 2 had a Gleason score of 6, 9 had a Gleason score of 7 and 1 had a Gleason score of 8 (Table II).

Analyses of *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* promoter status. It was confirmed that only modified samples presented a band. Each modification was consequently approved and successfully performed (Figure 1) for each sample. In case of *RASSF1*, *GSTP1* and *EPHB2*, each sample revealed a promoter methylation estimated at more than 75%. This was because only the PCR product corresponding to methylated primer was present. For *BRCA1*, 35 out of 37 samples presented two bands. The PCR product was present using both unmethylated-specific primers and methylated-specific primers, and between 25% and 75% of the gene promoter could be considered methylated (Figure 2). The two other samples were detected

as unmethylated and it is worth noting that these 2 samples were non-malignant biopsies (Table III).

Discussion

Epigenetics is one of the most rapidly expanding fields in cancer related research. Recent studies have shown that epigenetics plays an important role in cancer biology, somatic gene therapy, viral infections and genomic imprinting (5). CpG island hypermethylation is an indicator of prostate carcinogenesis and prognostic information increases if multiple gene loci are investigated simultaneously (8-10). In this study, it was attempted to identify the *BRCA1*, *GSTP1*, *RASSF1* and *EPHB2* promoter methylation both in healthy and in malignant prostate tissues with different clinical characteristics including Gleason score.

BRCA1, located on chromosome 17q21, encoded a multifunctional protein involved in DNA repair, control of cell-cycle checkpoints, protein ubiquitination and chromatin

remodelling (11). It was found that in the major samples, between 25% and 75% of the gene promoter could be considered methylated. However, two samples were determined to have an unmethylated promoter status, and both samples were non-malignant. These results were corroborated by Xu *et al.* who demonstrated that *BRCA1* promoter methylation was shown to be associated with increased mortality among women with breast cancer (12). Wilcox *et al.* screened 50 primary epithelial ovarian tumors for *BRCA1* promoter hypermethylation using MS-PCR and the *BRCA1* promoter was hypermethylated in 16% of tumors (13).

Pi-class glutathione-S-transferase (*GSTP1*), located on chromosome 11q13, encodes a phase II metabolic enzyme that detoxifies reactive electrophilic intermediates. *GSTP1* plays an important role in protecting cells from cytotoxic and carcinogenic agents and is expressed in normal tissues at variable levels in different cell types (14). Methylation of CpG islands in the promoter of the Pi class of glutathione S-transferase occurs in prostatic intraepithelial neoplasia and cancer (15). Recent reports indicate that higher levels of *GSTP1* promoter methylation were associated with the transition from prostatic intraepithelial neoplasia to carcinoma (16). It was confirmed that in studied prostate tissues, *GSTP1* promoter was methylated, however differences between the degrees of malignant biopsies could not be detected.

The RAS-association domain family 1, isoform A (*RASSF1A*) is located in the 3p21.3 region (17), and is a well-known tumor-suppressor gene. The alternative transcript has been shown to be inactivated by hypermethylation in several human malignancies, including breast and prostate (18). The purpose of this study was to evaluate the methylation status of *RASSF1* in human prostate tissues according to the degree of malignancy. In this study, it was found that all samples had a positive *RASSF1* methylation status in prostate tissues but with no distinction between histological degrees.

The exact function of the gene Ephrine-B2 (*EPHB2*) is unknown, but evidence suggests that *EPHB2* may be a tumor suppressor gene. The receptor tyrosine kinase *EPHB2* has recently been shown to be a direct transcriptional target of TCF/β catenin. Moreover Alazzouzi *et al.* found *EPHB2* promoter hypermethylation in 53% of colorectal tumors studied (19). All of the studied samples presented an *EPHB2* promoter methylation in prostate tissues.

The *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* methylation promoter status was determined with MS-PCR but it was difficult to estimate differences between the degrees of malignancy for which Methylight will be used in future studies.

This study concluded that *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* promoters were methylated in prostate tissues. As the number of genes known to be hypermethylated in cancer is growing, the detection of aberrant promoter region

methylation, will be a promising approach for using DNA-based markers for the early detection of human cancers. It is quite important to choose a suitable method to minimize the influence of the mentioned limitations on the interpretation of data and its evaluation. MS-PCR lead to the determination of the *BRCA1*, *RASSF1*, *GSTP1* and *EPHB2* methylation promoter status.

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