GSTP1 and prostate cancer

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This study was conducted in the Laboratory for Epigenetics and Molecular Medicine, Department of Medical Biology, School of Medicine, University of Zagreb, Ljudevit Jurak Clinical Department of Pathology and Cytology, and University Clinical Hospital Center Sestre Milosrdnice under the guidance of Asst. Prof. Monika Ulamec, MD, Ph.D., under the research project Epigenetic Biomarkers in Prostate Cancer (epiPro) (Project code: UIP-2017-05-8138) and has been submitted for the Rector's award in the academic year 2020/2021.

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LIST OF ABBREVIATIONS

ASR	Age Standardized Rate
AR	Androgen Receptor
BPH	Benign Prostatic Hyperplasia
CRPC	Castration-Resistant Prostate Cancer
CZ	Central Zone
DRE	Digitorectal Exam
GSTP1	Glutathione S-Transferase Pi 1
HGPIN	High-Grade Prostatic Intraepithelial Neoplasia
IHC	Immunohistochemistry
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate-Specific Antigen
PZ	Peripheral Zone
PCa	Prostate Cancer
SNP	Single Nucleotide Polymorphism
TAT	Tumour-Adjacent Tissue
TBS	Tris-Buffer-Saline
TRUS	Transrectal Ultrasound
TZ	Transition Zone

TABLE OF CONTENTS

1. INTRODUCTION

1.1 ANATOMY, PHYSIOLOGY AND HISTOLOGY OF THE PROSTATE

The prostate is a walnut-sized gland (4 x 3 x 2 cm) that is part of the male reproductive system and weighs 20 to 25 grams (1). Placed low in the pelvis, the organ is situated beneath the bladder between the pubis and the rectum and receives both the urethra and the seminal vesicles as part of its function. Functionally, it is responsible for the production, storage and forceful secretion of seminal fluid during ejaculation, necessary for the survival and motility of sperm. The compositions of these secretions include proteolytic enzymes, phosphatase, fibrinolysin, zinc, and importantly prostate-specific antigen (PSA)—a glycoprotein enzyme that liquefies the thick secretions allowing for free movement of sperm (2).

The structural framework of the prostate is that of stromal and glandular tissue. Connective stromal tissue consists of fibroblasts, myofibroblasts, vascular structures, and nerves, while glandular tissue is composed of a pseudostratified bilayer of basal and luminal epithelial cells and a small subset of neuroendocrine cells (3). The physiological function of this glandular layer depends on the presence of androgen receptors (AR)— nuclear receptors that mediate the function of testosterone initiating male sexual development and phenotypical maintenance (4). The luminal layer is the origin of almost all prostatic cancers (PCa) which are characterized by a complete absence of the basal cell layer and an atypical glandular growth pattern and androgen receptor signalling (5,6,7).

Anatomically, the prostate is stratified into three zones. The posteriormost region is known as the Peripheral Zone (PZ) making up 75% of the organ, the Transition Zone (TZ) surrounding the urethra comprises 20%, and the Central Zone (CZ) makes up the remaining 5-8%. Given its proximity to the rectal wall, the PZ is readily palpated during routine digito-rectal exams (DRE), with its position being clinically significant given that the majority (70-80%) of Prostatic Cancers (PCa) originate in this zone. The anteriormost TZ is the region associated with prostatic growth in adults over 40 known as Benign Prostatic Hyperplasia (BPH). As it is a large internal region that surrounds the urethra, its hypertrophy is associated with urinary problems of old age, and approximately 20% of PCa arise in this zone. The CZ is a small region and rarely the site of origin for PCa (5%).

1.2 BENIGN PROSTATIC HYPERPLASIA

Benign Prostatic Hyperplasia (BPH) is a common diagnosis that emerges in most men after the age of 40. Due to its frequency, it is often considered a physiological change rather than a pathological one and its classification is still a point of discussion today. Regulated by androgen receptors, BPH typically causes the development of lower urinary tract symptoms in older males due to the proliferation of epithelial and stromal cells in the TZ. While this obstructive effect on the urethra may cause voiding problems, there is no evidence to support the notion that this is a precancerous lesion. Nonetheless, the oxidative stress and inflammatory mediators induced in association with BPH can lead to pathological changes in tissue and creating a protumoral microenvironment which is beneficial for PCa development (8,9).

1.3 PROSTATE CANCER

1.3.1 ETIOLOGY AND EPIDEMIOLOGY

PCa is the second most commonly diagnosed cancer in the male population, with adenocarcinomas making up the vast majority of cases (99%). In 2020 1,414,259 newly diagnosed cases represented an 11% increase in incidence from 2018 (10, 11.) While etiology is not entirely understood, a range of causal factors are considered well-established and include older age, genetic predisposition, dietary factors, positive family history and race. The probability of developing PCa increases with age, with the majority of newly diagnosed cases observed in the 65-74 age bracket (median 66). Furthermore, while the average age of death from prostate cancer is 80, metanalyses of post-mortem biopsies reveal that evidence of early prostatic lesions can be found in men as early as their 20's and 30's (12). Men with two first-degree relatives have a 5 times greater risk of developing the disease, with the strong family history component additionally being responsible for younger patient presentation (diagnosis 2.9 years earlier). Studies on the specific oncogenic driver mutations behind these phenomena have been the focus of intense research in recent years with mutations in *BRCA1*, *BRCA2*, *p53* and over 100 Single Nucleotide Polymorphisms (SNPs) having a clear association with both earlier prognosis and castration-resistant strains (13). It is hypothesized that somatic genomic mutations are the link between the disparity inclination in heterogeneous population groups for mutations of specific oncogenic drivers (14). Globally, PCa has an Age-standardized ratio (ASR) of 30.7, with greatly elevated values of 62.1 in Europe (15). Furthermore, in a 2018 report, PCa was the most frequently diagnosed cancer in Croatia, accounting for 21% of newly diagnosed malignancies (16). Correlations to explain vast differences in PCa ASR findings of western countries have recently been associated with the western diet (high intake of red meat, processed meats, fried foods, high-fat dairy) with studies correlating increased PCa frequencies being positively reflected by diets high in saturated fats and milk products, whole milk consumption, and red meat (17, 18,19.)

1.3.2. PATHOGENESIS

It is widely accepted that PCa exists on a spectrum of both, long indolent disease and treatment-resistant variants with aggressive characteristics (early metastatic events to lymph nodes and distant locations). Research has indicated age-related factors play a role in disease development with a 2017 study elucidating a bimodal distribution of clinically significant PCa. Indications reveal that individuals who contract PCa at younger than 55 and older than 75 were more likely to follow a more dismal clinical course (20). Studies on the aggressive PCa variants have found that patients with treatment-resistant malignancies have a specific pattern of androgen receptor (AR) mutations. The physiological and pathological function of the prostate is a hormone-dependent process subverted by mutations associated with upregulation and novel biosynthesis of AR which plays a critical role in the development of castration-resistant prostate cancer (CRPC) (21). It is well established that malignant developments in the prostate are stratified into precursor lesions that are influenced by both genetic and multifactorial components. Recent developments have identified a strong correlation between certain germline mutations and hereditary forms of prostatic cancer with a 2020 study discovering that 17% of patients with PCa had associated germline mutations (22). While mutations leading to malignancy are frequently complex multigenic interactions, a gene mutation of the *BRCA2* tumour suppressor gene was identified as the most frequent causal genetic mutation driving the development of aggressive treatment-resistant PCa (23). A 3-year study found that individuals who possessed the *BRCA2* mutation were also likely to present at an earlier age (61 years) with a worse prognosis than *BRCA2* negative individuals (24). As such it has been suggested that *BRCA2* mutations should be part of the initial assessment to be followed with PSA measurements. In addition, extensive studies analyzing the *BRCA2* gene in breast cancer found positive associations with elevated cholesterol levels (47% increase)—which interestingly has also been found to have a positive association with prostate cancer (25). This lipid has since been associated with multiple elements leading to elevated risk for aggressive PCa including intratumoral steroidogenesis, increased inflammation, increased proliferation and changes in lipid rafts. Combined pathological factors on cellular and genomic levels as well as lifestyle factors multiply individual risk for development of pathological lesions associated with progression towards PCa.

Two morphological entities have been identified which act as potential precursor lesions for PCa—Prostatic Intraepithelial Neoplasia (PIN) and Intraductal Carcinoma of the Prostate. Considered the main precursor of PCa adenocarcinoma, PIN is a noninvasive epithelial proliferative process that involves the ducts and acini (glands) showing cytological triad of nuclear enlargement, hyperchromatic nuclei, and nucleolar prominence with the relative darkening and thickening of ductal linings (27,28). In 75-80% of instances, the observed changes (often multifocal) are observed in the PZ (29).

1.3.3. Clinical Picture, Diagnosis and Therapy

PCa is a malignancy with an extraordinarily favourable prognosis; with treatment—regional and localized cases possess a 5-year survival of virtually 100% (26). The progression of the disease is variable, with eventual metastatic spread occurring in approximately 80% of cases, and the primary sites of metastasis being the hip, spine and pelvis. Before diagnosis patients typically present with a constellation of symptoms increased urinary frequency, impotence, nocturia, hesitancy, hematuria, and urinary retention. These symptoms however are not unique to PCa with significant overlap presenting with benign conditions such as BPH. As such, bone pain due to metastatic expansion is the initial presenting symptom in many cases. Invasion of lymph and subsequently blood and is not an uncommon phenomenon and poses a fairly grim prognosis. The relative proximity of the lymph nodes means that it is usually the initial site of metastasis, and is of great prognostic significance with 10-year survival rates being significantly worse in patients who have positive lymph nodes (27). Current guidelines suggest that patients over 40 should begin screening for PCa every two years, diagnostic tools being DRE, PSA testing, and Trans-Rectal Ultrasounds (TRUS). In recent years, the use of multiparametric MRI has allowed us to gauge T1-weighted, T2-weighted, diffusion-weighted, and contrast-enhanced images for accurate visualization of suspicious lesions. Although nonspecific, serum PSA values of greater than or equal to 4.0 ng/mL indicate a need for further evaluation (28). Followup assessment of irregular tissue is conducted via the use of an ultrasound-guided needle core biopsy wherein the physician retrieves 10-12 thin cylindrical tissue samples. Tissue samples are evaluated and if confirmed for PCa, graded via the Gleason scoring system. Current guidelines indicate that the two most predominant patterns should be established and their Gleason Scores summed for a value that corresponds to the 2014 International Society of Urological Pathology (ISUP) modified Gleason grading (35,36).

Table 1. Gleason grading patterns, 2014 International Society of Urological Pathology (ISUP), WHO (2016).

Gleason Grade Group	Score	Definition
1	3+3=6	Only individual well-formed glands
2	3+4=7	A predominance of well-formed glands with interspersed fused/cribriform glands
3	4+3=7	Predominantly poorly formed glands with a smaller subgroup of well-formed glands
4	5+3, 3+5, 4+4 (Gleason Score 8)	Only poorly formed glands (>95%), or predominantly well-formed glands with a lesser component lacking glands, or predominantly lacking glands and a lesser component of well-formed glands
5	Gleason scores 9 and 10	Lack of gland formation or with the presence of comedonecrosis (>95%) with or without fused/cribriform glands

While PSA remains a useful tool in assessing PCa progression and recurrence, its use as a mainstay diagnostic tool has been unreliable, fuelling a new direction of research (39).

Current therapeutic endeavours are multimodal combinations of surgery, chemotherapy, radiation, proton beam therapy, cryosurgery and hormone therapy. The treatment modality is largely decided based on whether patients present with localized or metastatic disease. Localized disease constrained to the prostate is treated with a prostatectomy combined with androgen deprivation therapy. Metastatic disease treatment typically follows a course of surgery, chemotherapy and radiation therapy with variable prognosis (29).

1.3.4. PROSTATE-SPECIFIC ANTIGEN AS A DIAGNOSTIC MARKER

Kallikrein-3 is the glycoprotein enzyme commonly referred to as PSA that is coded by the *KLK3* gene. This peptidase is tissue-specific to the prostate and is produced in the glandular tissue and secreted by the epithelial cells as an integral component of ejaculate related to semen motility and liquefaction (30). While its use is generally correlated with metastatic developments, it is not an unequivocal indicator for the disease since elevations in the serum are also linked with other pathological conditions such as prostatitis and BPH (31). The lack of specificity of PSA as a marker for PCa has encouraged research of various diagnostic and prognostic modalities associated with molecular pathways specific to metastatic developments in the prostate.

1.4. *GSTP1* AND HYPERMETHYLATION EVENTS

Gene panel biomarkers such as the Gluthathione S Transferase P1 (*GSTP1*) are currently the focus of intense research as a site of aberrant modification in PCa (32). The GST superfamily of polymorphic enzymes displays incredible versatility of function, responsible for the detoxification of foreign materials, cell proliferation and apoptotic activities (33). Physiologically, GST detoxification involves the addition of glutathione to non-polar carcinogenic compounds which facilitates their elimination as more water-soluble products (34). GSTP1 is overexpressed in solid tumours including renal and urinary carcinomas, breast cancers, and colorectal cancers and with high levels associated with therapeutic failures of chemotherapy, cancer drug resistance and a poor prognosis overall (35). Conversely, the most common alteration events observed in PCa are hypermethylation events with a lack of GSTP1 expression *in vivo* (36). DNA methylation is a genomic regulatory process that involves the covalent addition of a methyl group to the 5’ location on a cytosine ring. In somatic cells, 98% of these methylation events occur in CpG islands—dinucleotide repeats located in proximity to 40% of mammalian gene promoters. Normal developmental processes including genomic imprinting, X-chromosome deactivation, and suppression of transcription factors are all dependent on selective methylation events (37). Prior studies reveal that methylation of *GSTP1* promoter region is observed in 36-100% of tumour tissues

(38,39,40,41,42,43,44)—an occurrence seldom detected in normal prostatic tissue. Furthermore, evidence of *GSTP1* hypermethylation can be detected in urine, serum and ejaculate samples in patients with PCa (45). As such, *GSTP1* hypermethylation shows a 90% PCa specificity, with urine and serum sensitivity values being 18.8-38.9% and 13.0-75.5% respectively (46). This process can also be observed in PIN lesions further solidifying its role as a key precursor in early carcinogenesis, and potentiating the need for further association studies (47). *GSTP1* hyper and hypomethylation states are currently under investigation as a part of different assays associated with various malignancies as a carcinoma-specific process, however, these are all still in a phase of active research and development.

2. HYPOTHESIS

Patients with prostate cancer will have hypermethylated *GSTP1* in tissue compared to patients with benign prostatic hyperplasia which will be reflected at the protein level by the absence of signals in cancerous epithelial tissue.

3. AIMS

3.1. GENERAL AIM

To characterize methylation patterns of *GSTP1* and protein expression of GSTP1 in prostate cancer tissue, peritumoral tissue and benign prostate hyperplasia.

3.2. SPECIFIC AIMS

1. To analyze the immunohistochemical expression of GSTP1 in epithelial and stromal cells of prostate cancer tissue, peritumoral tissue and benign prostate hyperplasia and correlate it with the age, tPSA, stage and grade of the tumours.

2. To analyze *GSTP1* methylation of 5 CpG sites (chr11:67,584,233-67,584,282) of prostate cancer tissue, peritumoral tissue and benign prostate hyperplasia and correlate it with the age, tPSA, stage and grade of the tumours.

3. To correlate *GSTP1* methylation patterns with protein expression in epithelial and stromal cells in prostate cancer tissue, peritumoral tissue and benign prostate hyperplasia.

4. MATERIALS AND METHODS

4.1 HUMAN PROSTATE SAMPLES

Forty Formalin-fixed, paraffin-embedded (FFPE) prostatic needle biopsy tissues of 20 patients diagnosed with BPH (2 blocks from each patient, due to a small amount of tissue in the blocks) and 20 FFPE radical prostatectomy tissue with PCa. Data regarding age, Gleason's score and PSA values (Table 2) were analyzed from the University Hospital Center Sestre Milosrdnice and University Hospital Center Zagreb. The study was conducted in accordance with the ethical principles described in the 1964 Declaration of Helsinki. Patients were informed of the associated ethical principles and had signed an informed agreement approved by the Ethical Council of the University of Zagreb, Faculty of Medicine, University Hospital Center Zagreb and University Hospital Center Sestre Milosrdnice. The study was conducted within the scope of the research of "Epigenetic Biomarkers of prostatic cancer (epiPro)" (Project code: UIP-2017-05-8138 Croatian Foundation of Science). The identities of the volunteers were encrypted and personal data were stored in accordance with the legislation of the Republic of Croatia.

Table 2. Patient characteristics (PCa- prostate cancer, BPH- benign prostatic hyperplasia)

	PCa	***BPH***
All cases	20	20
Age, years	60.1 ± 6.973	60.55± 7.200
tPSA, ng/mL	7.557 ±3.779	8.294±3.914
	n (%)	
< 4 ng/mL	2 (10%)	0 (0%)
4-10 ng/mL	13 (65%)	15 (75%)
> 10 ng/mL	5 (25%)	5 (25%)
	Pathological stage	
T2	14 (70%)	
T3	6 (30%)	
	Gleason score	
3+3	4 (20%)	
3+4	11 (55%)	
4+3	4 (20%)	
3+5	1 (5%)	

4.2 EXPERIMENT WORKFLOW

Radical prostatectomy tissue blocks were sectioned firstly at 4 µm and then at 10 µm, one BPH block was sectioned at 4 µm and another one at 10 µm.

One slide from each patient was stained with hematoxylin and eosin (HE) to assist in identifying the localization of the immunohistochemistry (IHC) signal (PCa, TAT- Tumour-adjacent tissue, BPH, epithelium, stroma) and marking areas (PCa and TAT) for macrodissection. Another slide from each patient was further used for IHC staining while 10 µm thick slides were used for macrodissection, genomic DNA (gDNA) isolation and pyrosequencing (Figure 1 A, B).

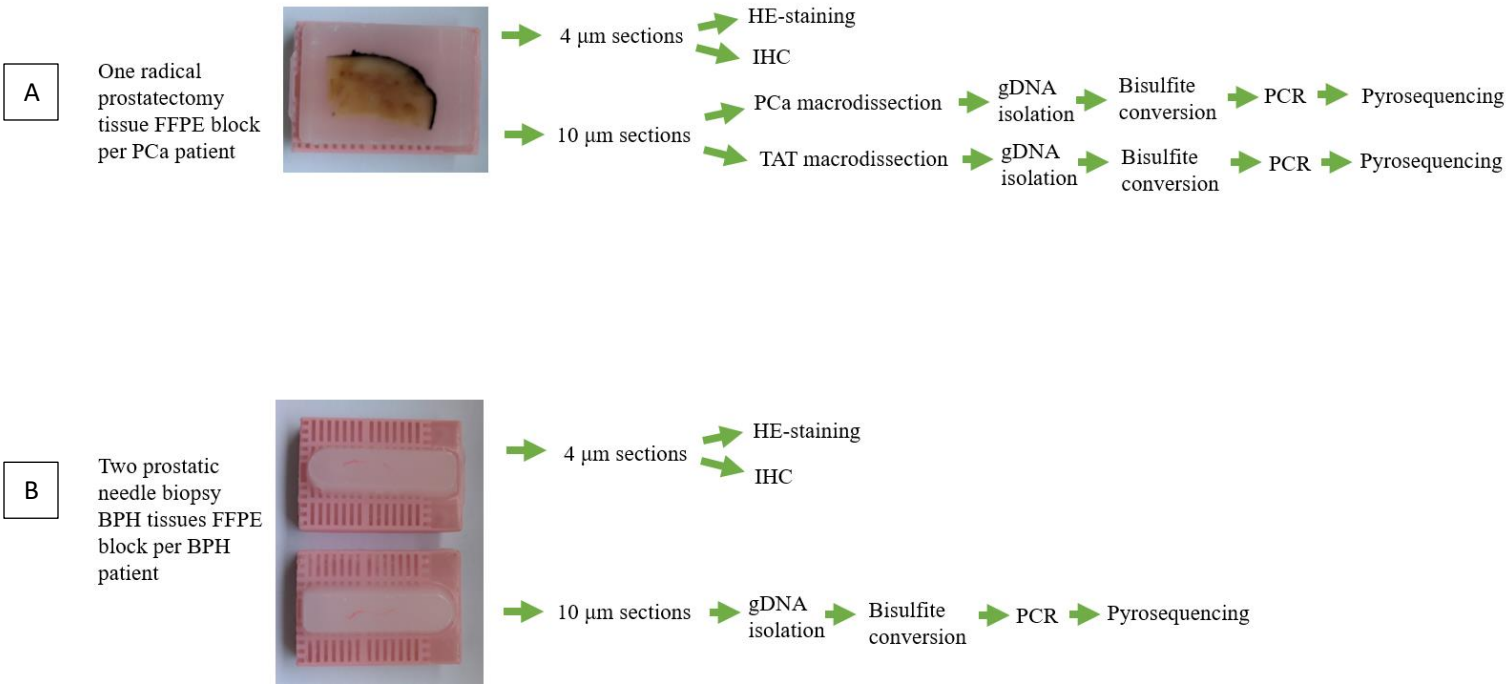

Figure 1. Workflow diagram—(A) Protocol for the preparation of PCa tissue and (B) Protocol for preparation of BPH tissues. (IHC-Immunohistochemistry; TAT-Tumour -Adjacent Tissue; PCa-Prostate Cancer; HE-Hematoxylin & Eosin; gDNA-Genomic DNA)

4.3 HEMATOXYLIN AND EOSIN STAINING

The 4 µm slides were heated for 1 hour at 55 °C and deparaffinized in xylene, and incubated in 100% alcohol, (2 changes, 5 minutes), 95% alcohol, (2 changes, 5 minutes), 70% alcohol (one change for 5 minutes) and distilled water (1 change for 5 minutes). Slides were then incubated in hematoxylin (BioGnost) for 50 seconds, shortly rinsed in distilled water, then rinsed in running tap water for 5 minutes. Following this, they were placed in distilled water for 5 minutes. Slides were then incubated in

eosin (BioGnost) for 40 seconds, shortly rinsed with 70% alcohol (3 changes), 95% alcohol (2 changes), 100% alcohol (2 changes for 30 seconds), xylene (2 changes for 30 seconds, 1 change for 15 minutes) and were then mounted with BioMount DPX Low (BioGnost).

4.4 IMMUNOHISTOCHEMISTRY

Slides were heated for one hour at 55 °C and deparaffinized in xylene 2 times, for 10 minutes. Incubation was conducted in 100% alcohol, (2 changes, 5 min each), 95% alcohol, (2 changes, 5 min each), 70% alcohol (one change for 5 minutes) and distilled water (1 change for 5 minutes) folowed. Samples were subsequently placed into the antigen retrieval solution (Citrate pH 6, Dako, Agilent Technologies) and steam antigen retrieval was performed for 20 minutes. Samples were then washed with a Tris-Buffer-Saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5) for five minutes, removed from the TBS solution and covered for 20 minutes with 5% Bovine Serum Albumin (BSA)—a blocking solution preventing nonspecific binding. The blocking solution was carefully removed and the rabbit polyclonal anti-GSTP1 primary antibody (NBP1-84748) diluted 1:2 000 with dilution solution (0.1% Tween-20, 1% BSA/TBS) was applied and incubated owernight at 4 °C. The following day samples were washed with TBS five times for 5 minutes. Slides were treated for 20 min with a 3% H_2O_2 in the dark to deactivate endogenous peroxidases. This was followed up with 3 series of five-minute washes with TBS. The next step involved the application of secondary antibody (Dako REAL EnVision Detection System,K5007, Agilent Technologies). This step involved incubation at 37 °C for one hour, followed by a serial TBS wash 3 times for five minutes. The samples were subsequently incubated for 6 minutes in DAB (3,3′-diaminobenzidine-tetrahydrochloride) (Dako REAL EnVision Detection System,K5007, Agilent Technologies) and a brown coloration not soluble in alcohol was produced. Following this, the samples were washed with distilled water for 5 minutes. Finally, the staining procedure involved counterstaining with hematoxylin (BioGnost) for 10 seconds, followed by a distilled and then a subsequent tapwater wash. To allow for a thorough wash we placed the samples under running water for 8 minutes, and thereafter conducted a distilled water wash for 5 minutes. The final step involved a reverse order sequence of the initial step wherein the samples were washed serially in ethanol and finally xylene, allowing for the final biomount and securing of the samples.

4.4.1. IHC ANALYSIS

Analyses of IHC stained slides were performed with the help of a pathologist using an Olympus Bx51 microscope. Staining was assessed in epithelium and stroma separately. The staining proportion was scored as 0 (no signal), 1 (<10% positive cells), 2 (10–50% positive cells), 3 (50-80% positive cells), 4 (> 80% positive cells), while staining intensity was scored from 0 to 3 (none, low, medium or high). The

semi-quantification of protein expression was calculated by immunoreactive score (IRS) by multiplying staining proportion score and staining intensity score, creating a range of 0–12.

4.5. MACRODISSECTION AND GDNA ISOLATION

The pathologist marked the PCa and TAT area on the HE-stained slide which was further used as a guide for macro-dissection and gDNA isolation from the 10 μm thick slide separately from the PCa and the TAT area. A 10 μm thick slide of BPH tissue blocks was used for gDNA isolation without dissection. Since these slides are used for DNA isolation, increased attention was dedicated to avoiding contamination (first slides on the FFPE blocks were not used for gDNA, instruments for cutting were cleaned with 70% EtOH).

DNA was extracted using the in-house protocol. Briefly, the sections were were deparaffinized, cleared in xylene and hydrated to water in graded alcohol solutions following 72 h submersion in TBS. PCa and TAT tissue were scraped separately and homogenized into very small pieces. 500 μl of TES buffer (50mM Tris-HCl pH8 ; 100mM EDTA ; 100mM NaCl ; 1% SDS) and 20 μl proteinase K (10 mg/ml) was added and incubated at 56°C overnight. Samples were vortexed, and 200 μl of 6M NaCl was added and after mixing and centrifugation for 10 min at full speed, the 700 μl supernatant was isolated and transferred to a new tube. 500 ul isopropanol to the supernatant was added, mixed for 2 min and centrifuged for 15 min at full speed. Here, gDNA precipitated and the supernatant was discarded. The pellet was washed with 500 ul 70% ethanol and spun, the ethanol was discarded, and the pellet dried and dissolved in 33 ml of water. DNA concentration was quantified by spectrophotometry (NanoDrop ND-2000, NanoDrop Technologies,) and stored at -20°C.

4.6 BISULFITE CONVERSION AND POLYMERASE CHAIN REACTION

500 ng of isolated gDNA from all three kinds of tissue (PCa, TAT, BPH) was used for bisulphite conversion by EpiTect Plus DNA Bisulfite Kit (Qiagen) and was carried out according to the manufacturer's instructions. PyroMark PCR Kit (Qiagen) was used for polymerase chain reaction (PCR) (Table) with primers:
forward primer: 5′-GGTTGGGGTTGTAGTTTATAGT-3’
and biotinylated reverse primer: 5′-ACAAATTCCTCCCAAAATTTCACACA-3′.

Table 3. PCR conditions

Steps	T (°C)	time	no. of cycles
Activation	95°C	15 min	1
Denaturation	94°C	30 sec	45
Annealing	56°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min	1

4.7. PYROSEQUENCING

GSTP1 methylation of 5 CpG sites (chr11:67,584,233-67,584,282) was measured by pyrosequencing using Pyromark Q24 Advanced System with PyroMark Q24 CpG Advanced Reagents (Qiagen) and the sequencing primer 5′- GGGTTGTAGTTTATAGTTTT -3. Pyrosequencing was carried out according to the manufacturer's instructions and results were analyzed by the PyroMark Q24 Advanced Software.

4.8. STATISTICS

Patient characteristics were analyzed by descriptive statistics. Differences in the IRS, methylation percentage and clinical values between groups were evaluated applying the Mann–Whitney test (unpaired data), Wilcox test (paired data), and Kruskal–Wallis test with Dunn's multiple-comparison post hoc test. The correlation between variables was measured by Spearman's rank-order correlation. P-values < 0.05 were considered significant.

For data analysis, GraphPad Prism software (version 6.0, GraphPad Software) was used.

5. RESULTS

5.1 CHARACTERISTICS OF SELECTED PATIENTS

In table 2 the clinical characteristics of the patients involved in our study—20 patients had prostatic cancer and 20 patients had BPH. The average age of the patients involved was 60 years for both cohorts. The median serum concentration of PSA of patients with PCa was shown to be 7.56 ng/mL, whereas patients with BPH had a serum concentration of 8.29 ng/mL. There was no significant difference in the p values of the patient PSA values nor their ages. Our patients' PSA values fell into an abnormal range for their age (normal values in the 60-70 age bracket: 3.5-4.5 ng/mL), however, the values that fall into the range of 4-10 ng/mL will yield cancer on biopsy only 25% of cases (values over 10 have a 67% probability of cancer on biopsy). According to the TMN PCa staging, the majority of our PCa patients (70%) fall into the T2 category, which reflects a tumour that is still confined to the prostate gland. 5 of our patients were found to be in the T3 stage. 20% of our PCa patients fell into the Gleason score of 6

(Gleason Grade Group 1). The majority (55%) of our patients were found to be in the Gleason Grade Group 2 (3+4=7), carrying a favourable prognosis. The final group of patients had Gleason scores of 4+3 (20%) and 3+5 (5%), belonging to Gleason Grade Group 3 and Group 4 respectively which are unfavourable grades.

5.2 *GSTP1* EXPRESSION ACROSS PCA, TAT AND BPH

The expression of the GSTP1 gene in epithelium and stroma of patients with PCa, BPH, and TAT is shown in Figures 2 and 3 and 4. In our study, we compared staining of BPH, TAT and PCa in both epithelial and stromal prostatic tissue. The expression of GSTP1 in the stroma was approximately the same in all three observed tissue types. Expression in TAT epithelium was higher than in BPH while expression in PCa was completely lost. GSTP1 expression in the epithelium was statistically significantly different across all three tissues.

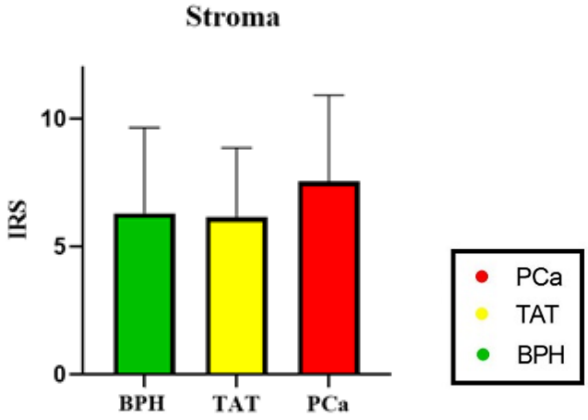

Figure 2: Graphical visualization of *GSTP1* expression in stroma of prostatic biopsies of patients with BPH, TAT and PCa (n=40) (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue; IRS – Immunoreactive score)

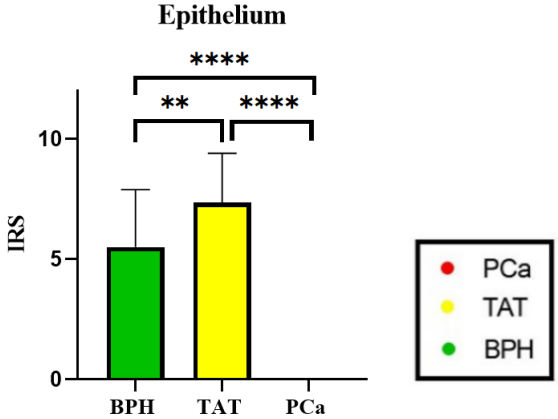

Figure 3: Graphical visualization of *GSTP1* expression in Epithelium of prostatic biopsies of patients with BPH, TAT and PCa (n=40) (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue; IRS – Immunoreactive score)

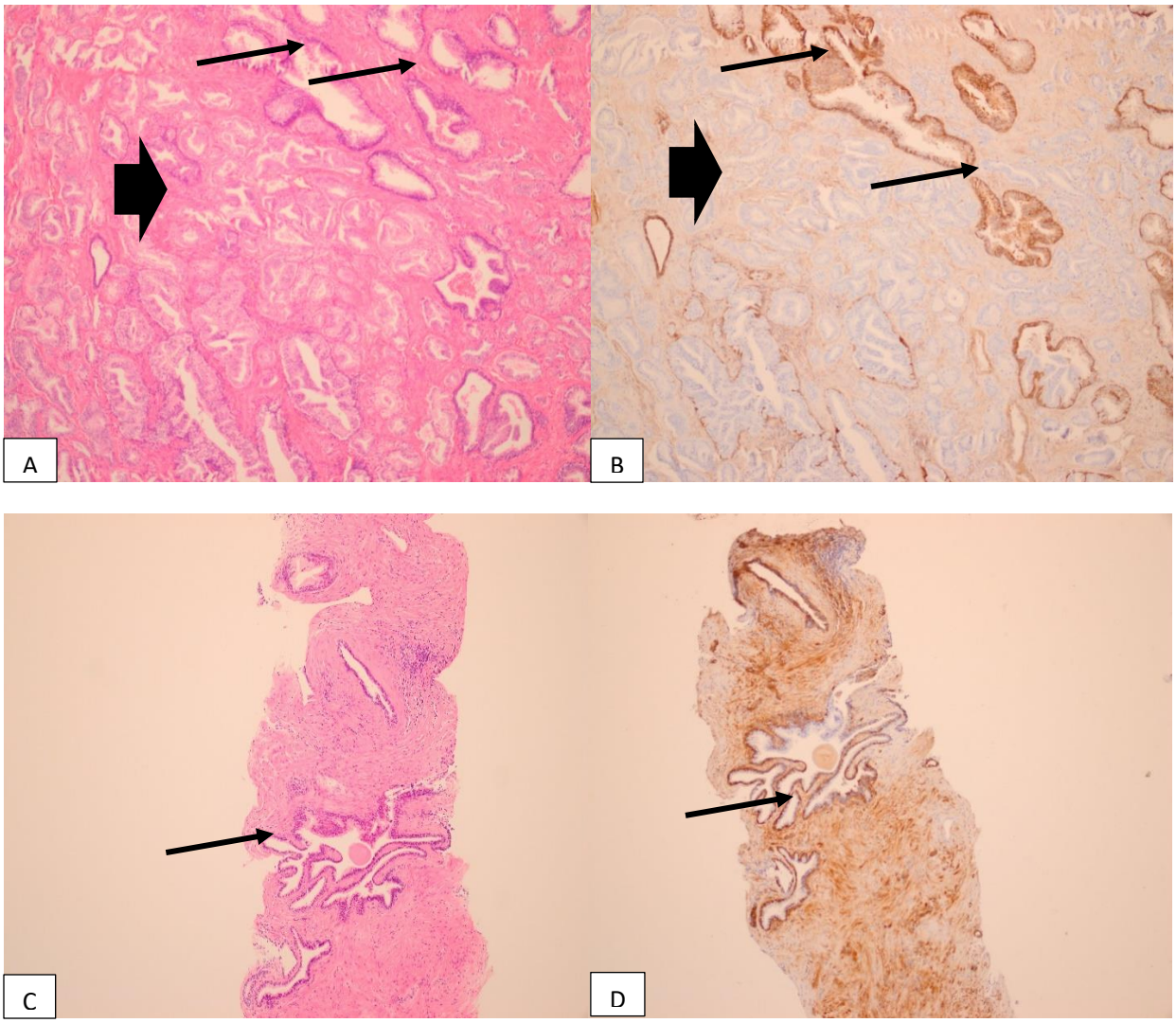

Figure 4. A. Prostate cancer on prostatectomy specimen, fused atypical glands (thick arrow) with several normal glands in the surrounding tissue (thin arrows), (HEx200); B. Immunohistochemically, PCa gland are negative for GSTP1 expression (thick arrow), while normal glands show strong positive reaction in more than 80% of the epithelial cells (thin arrows). Stroma is positive with low intensity (GSTP1x200); C. Benign prostate hyperplasia in core biopsy specimen, arrow showing glandular tissue (HEx100); D. Immunohistochemicaly, Strong expression of GSTP1 in epithelial component, more than 80% of cells (thin arrow), as well as in the stromal tissue (thick arrow), (GSTP1x100).

5.3 Average Methylation State of tissue samples

To determine the viability of *GSTP1* as a potential marker for prostate cancer, we assessed the methylation status of BHP, TAT and PCa. Looking at Figure 5, it is evident that there is a significant process of hypermethylation associated with the development of PCa, otherwise not seen in BPH and

TAT. Overall methylation of the tissues was a mean of 21.54% for PCa, 5.29% for TAT and 3.36% for BPH.

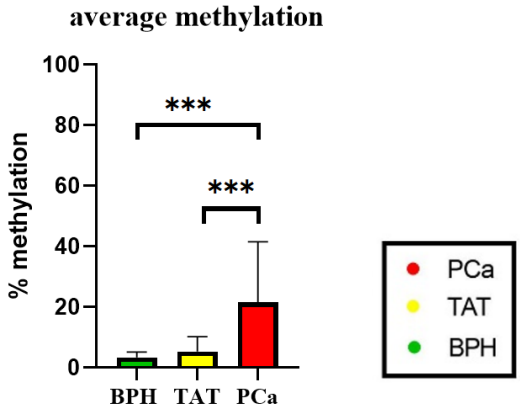

Figure 5: Average methylation values of patient tissue samples including BPH, TAT and PCa (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue)

5.4 Methylation Averages relative to 5 Selected CpG Sites

We conducted a Kruskal–Wallis test with Dunn's multiple-comparison post hoc test to investigate the difference between selected groups of specific CpG sites across 5 selected islands. The TAT methylation group showed statistically higher average methylation values than the BPH group. Each analyzed individual CpG site, as well as average methylation of all 5 sites, was the lowest in BPH tissue, slightly higher in TAT tissue and the highest in PCa tissue. CpG sites 1, 3 and 5 showed statistically significant higher methylation comparing to BPH and TAT (Figure 6).

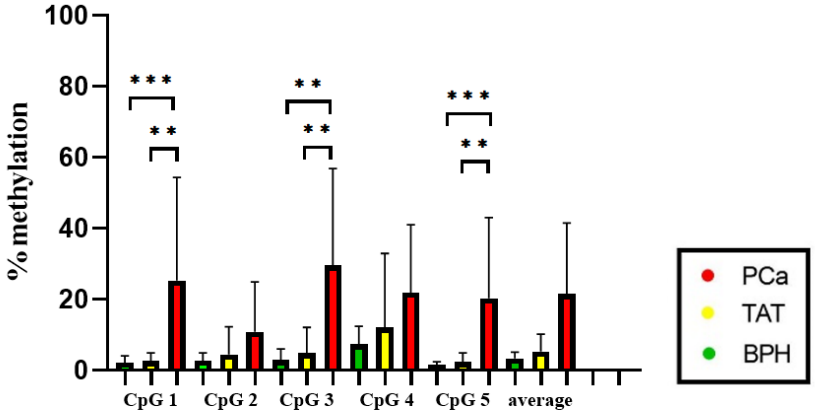

Figure 6: Comparison of methylation averages across selected CpG islands for PCa, TAT and BPH with their comparative statistical significance (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue)

5.5 Spearmans' Correlation Between tPSA and Methylation

Spearman's rank-order correlation showed a strong relationship between tPSA and methylation of CpG 1, 3, 5 and average methylation in the PCa group which is shown in Table 4.

Table 4. Spearman's rank-order correlation between clinical parameters, *GSTP1* methylation and expression.

(PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue, tPSA – Total Prostate Specific Antigen)

	BPH		Pca		
Spearman r	Age	tPSA	Age	tPSA	T
Epithelium	0,034546	-0,07705			
Stroma	-0,33637	-0,25354	0,163194	0,096247	-0,09069
CpG 1	-0,18684	-0,35548	-0,23219	0,655992	-0,11966
CpG 2	-0,27353	-0,19329	-0,02162	0,173381	-0,04395
CpG 3	-0,2325	-0,31518	-0,13801	0,61863	-0,08744
CpG 4	-0,16806	0,059183	-0,17296	0,43077	-0,15329
CpG 5	0,124208	-0,06003	-0,05239	0,526131	-0,21841
Average methylation	-0,32956	-0,07307	-0,14533	0,619298	-0,17457
Age	1	0,190046	1	-0,14148	-0,14203
tPSA	0,079711	1	-0,14148	1	0,155398
Gleason score			0,547758	0,283256	0,368394
TNM - T			-0,14203	0,155398	1

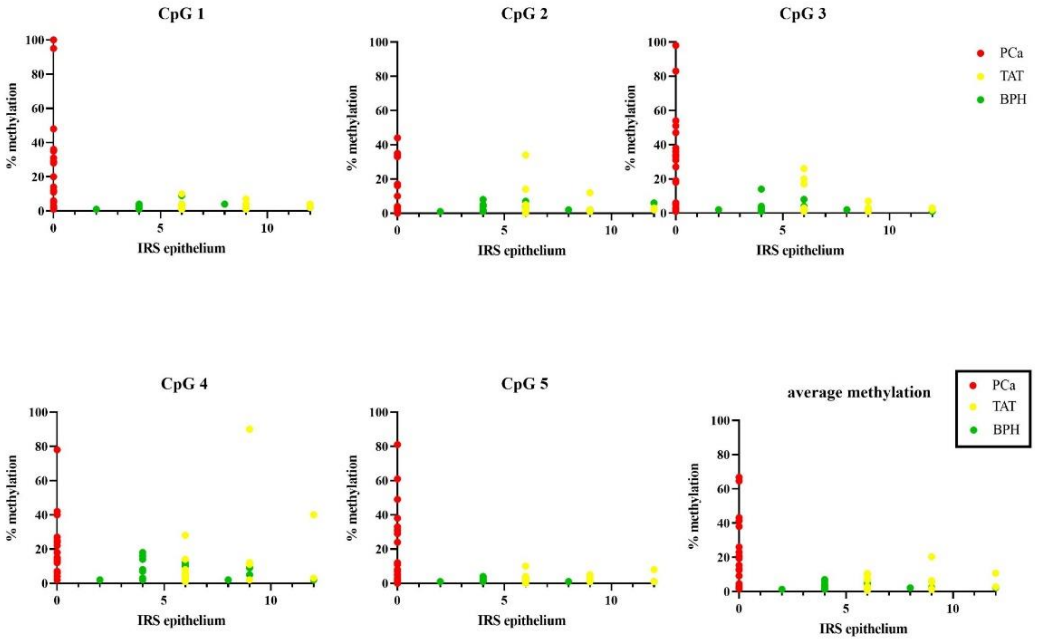

Figure 7: Relationship of *GSTP1* expression in epithelium and individual CpG site methylation. (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue; IRS – Immunoreactive score)

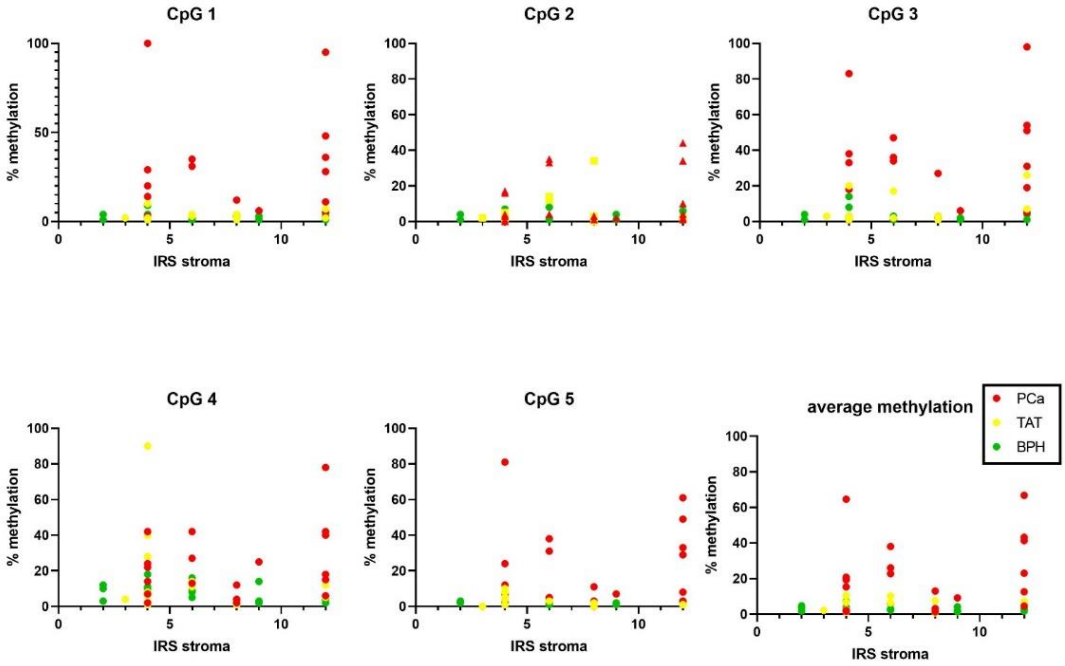

Figure 8: Relationship of *GSTP1* expression in the stroma and individual CpG site methylation (PCa - Prostate Cancer; BPH - Benign Prostatic Cancer; TAT - Tumour-adjacent tissue; IRS – Immunoreactive score)

Correlation of expression and individual site methylation in each group; PCa, TAT and BPH is presented in Table 4 and Figures 7 and 8. Expression in the stroma was strongly negatively correlated with CpG 4 methylation. The highest correlation (r=0.65) was between tPSA and CpG 1. Furthermore, Gleason's score was strongly related to age. When observing correlation of CpG sites within groups, in BPH only CpG 1 and 2 show strong correlation (r=0,53) while in TAT strong correlation exists between CpG 1- CpG 2, CpG 1- CpG3, CpG 1 – CpG 4, CpG1- CpG5, CpG 2 - CpG3, CpG 3 – CpG 4, CpG 4-CpG 5. In the PCa group, a strong positive correlation was noticed between all CpG sites.

6. DISCUSSION

Prostate cancer is a disease that requires an early and accurate diagnosis to facilitate curative therapy, preventing the further progression towards metastatic processes. Unfortunately, current diagnostic tools are outdated and non-specific thus motivating our project to assess a novel biomarker that can be used with greater certainty. In this prospective study, we investigated the expression patterns of GSTP1 in epithelial and stromal tissue, as well as methylation *GSTP1* patterns of Benign Prostatic Hyperplasia, Tumour Adjacent Tissue, and Prostate cancer samples.

In their 2016 study, Martignano et al. showed a complete loss of GSTP1 protein expression compared to the surrounding prostate tissue which was supported by a hypermethylation pattern. Similar results were observed by Zelić et al. where *GSTP1* correlated hypermethylation on a negative biopsy with the risk of prostate cancer on a rebiopsy, especially of high-grade prostate cancer. Consistent results were found only for extremely low Long Interspersed Nuclear Elements (LINE-1) methylation levels (48). Such analyses of multigene assays have been proposed as a means to increase prognostic capacity, increasing the diagnostic power in biopsy negative patients. The 2016 Gurioli et. al study investigated a PCa methylation combination assay (*GSTP1/APC/RASSF1*) with promising results achieving a negative predictive value of 90%.

The results of our research are in concordance with all previous studies. We showed the complete loss of signal in PCa glands due to the hypermethylation of *GSTP1* while additionally analyzing the stromal component in comparison to epithelial tissue, which was not conducted in any other study to date. Expression of GSTP1 is equally distributed in the stromal tissue in BPH, TAT and PCa. PCa values are slightly higher but it is not statistically relevant. While our initial hypothesis anticipated smaller values of GSTP1 activity in PCa tissue, its complete absence was surprising, especially when it was completely

maintained in BPH and TAT glands. A 2009 study conducted by Lovrić et al. corroborates our findings where out of 34 patients with PCa, GSTP expression was detected in only one patient[49].

A recent meta-analysis which pooled 35 studies assessing the *GSTP1* as a potential diagnostic tool found sensitivity values of 81.8%%±8.8% and specificity values ranging from 94.9%±2.4% (50). In our study, the TAT methylation group showed statistically higher average methylation values than the BPH group. Each analyzed individual CpG site, as well as average methylation of all 5 sites, was the lowest in BPH tissue, slightly higher in TAT tissue and the highest in PCa tissue. CpG sites 1, 3 and 5 showed statistically significant higher methylation comparing to BPH and TAT which corroborated our initial hypothesis of methylation states in PCa relative to BPH and TAT in clinically significant values. This progressive rise of methylation in TAT regions confirms a hypothesis of degenerative progression to the eventual development of malignant lesions in a linear fashion.

Our study then focused on identifying and localizing specific genomic regions prone to aberrant changes. CpG site hypermethylation is hypothesized to be an early genomic alteration in prostate cancer which can act as a predictor of progression and grade, which our study was also able to accurately identify and substantiate. We identified five specific CpG sites associated with aberrant DNA methylation patterns linked to the development of PCa and were able to validify that the three tissue sample groups have a significant difference in methylation values across the three-plus average of the five tested CpG sites.

Future studies could benefit from an alternate approach with respect to methodology. Mathieson and Thomas discussed the importance of the material in parrafin embedded samples, stating that formalin fixation could lead to cytosine deamination and subsequent C>T transitions (51). Distinguishing methylated from unmethylated cytosines is based on bisulphite treatment before pyrosequencing which converts unmethylated cytosines to uracil but methylated cytosines are left unmodified. In PCR after bisulphite treatment, uracils are converted to thymines and amplicons are pyrosequenced. In the end, by pyrosequencing, methylated cytosines are read as cytosines and unmethylated ones as thymines. Although *GSTP1* methylation in our and other studies differs between BPH and PCa tissue, formalin fixation-induced thymines imply that methylation research could be improved using fresh samples[52].
Since we know that *GSTP1* expression and methylation in tissue significantly differ amongst benign and PCa tissue, it is possible to consider a more potent biomarker and focus on liquid biopsies. Zavridou et. al. investigated methylation and RNA expression of GSTP1 in EpCAM-positive circulating tumour cells (CTCs) and exosomes of mCRPC patients (53). They showed that *GSTP1* is highly methylated with methylation significantly being correlated with a lower overall surviaval (OS). Contrary to this and many other studies using MSP (Methylation-specific PCR) which provides two kinds of results (methylation positive or methylation negative), pyrosequencing allowed us a precise characterization of

each analyzed CpG. Although in our study we didn't conduct OS data we showed specifically that methylation of CpG sites 1,3 and 5 strongly correlated with tPSA. Further research should include individual CpG methylation to complement our research. Additionally, many research projects quantify expression on an RNA level. Since our result showed complete loss of the GSTP1 protein in the PCa epithelium, it would be interesting to investigate if protein expression instead RNA expression, could more successfully discriminate between prostate patients using liquid biopsies.

Since *GSTP1* shows great potential for distinguishing PCa from BPH, further research with a more considered approach (fresh frozen tissue, individual CpG approach, protein expression) could provide additional data and better resolution.

7: CONCLUSION

Our results showed significantly elevated average methylation values of *GSTP1* in PCa patients compared to BPH and TAT which mutually showed large discrepancies between methylation values. As it was expected, protein expression of GSTP1 was lost in PCa compared to BPH ant TAT.
Correlations were established regarding methylation of specific CpG sites and elevated PSA values, age and Gleason's score as well, specifically the methylation of CpG 1,3 and 5 strongly correlated with tPSA. Therefore, the assessment of *GSTP1* hypermethylation status in patients with suspected PCa could be the foundation of a new method for early detection, diagnosis and screening, in tissue samples as well as so-called liquid biopsies. Larger studies confirming these encouraging results are needed.

8. ACKNOWLEDGMENTS

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9. SAŽETAK

GSTP-1 u karcinomu prostate

Ključne riječi: *GSTP1*, rak prostate, benigna hiperplazija prostate, metilacija, PSA

Rak prostate je maligna bolest s visokom incidencijom i mortalitetom. Istraživanja su usmjerena na pronalazak preciznijih dijagnostičkih i prognostičkih markera. Rano otkrivanje raka prostate je važno zbog liječenje bolesti u početnim fazama kao i za prevencije metastatske progresije. Niska specifičnost biomarkera PSA koji se rutinski koristi u dijagnostici te njegova nemogućnost dobrog razlikovanja stanja poput benigne hiperplazije (BPH), upale prostate i raka dovodi do nepotrebnih biopsija i generiranja troškova zdravstvenog sustava.

Naša studija razmatra mogućnost upotrebe metilacijskog obrasca gena *GSTP1* kao markera za razlikovanje karcinoma od BHP. Analizirali smo tkivo biopsija 20 pacijenata s BPH i 20 biopsija pacijenata s PCa, procjenjujući metilacijski obrazac *GSTP1* na pet CpG mjesta, kao i imunohistokemijsku izraženost proteina GSTP. Naši rezultati pokazuju statistički značajne razlike u obrascima metilacije između BPH i PCa koja je pozitivno povezana i s tPSA te s dobi. U PCa CpG otoci bili su stalno hipermetilirani u odnosu na BPH u tkivu, a zanimljivo je da okolno peritumorsko tkivo (TAT) pokazuje sličan napredak prema hipermetilacijskim obrascima u odnosu na BPH. Ti su rezultati također potvrđeni prilikom procjene ekspresije proteina GSTP1, koji pokazuje potpuni nedostatak izraženosti u PCa, ali je prisutan u BPH i TAT.

Temeljem rezultata naše studije GSTP1 je odličan kandidat za poboljšanje postojećih dijagnostičkih alata u identifikaciji PCa.

10. SUMMARY

GSTP1 and prostate cancer

Key words: *GSTP1*, methylation, prostate cancer, benign prostatic hyperplasia, PSA

Prostate cancer is a widespread disease that currently has no accurate diagnostic markers. Its early identification is important for treatment in its initial stages and prevention of metastatic progression. The inability of the current PSA biomarker to distinguish between benign prostatic hyperplasia and prostate cancer has led to overdiagnosis and unnecessary biopsies, fuelling research to discover novel biomarkers. Our study considers the prospect of using the *GSTP1* gene as a marker to differentiate between prostatic carcinomas and hyperplasia states. In our study, we analyzed biopsies of 20 patients with BPH and 20 biopsies of patients with PCa, assessing *GSTP1* hypermethylation events at five CpG sites. We observed statistically significant differences in methylation patterns between BPH and PCa and were able to positively correlate methylation to tPSA and age. Furthermore, PCa CpG islands were consistently more hypermethylated than BPH tissues, and interestingly, PCa tumour-adjacent tissue (TAT) showed a similar progression towards hypermethylation states compared to BPH. These results were also confirmed when assessing the GSTP1 protein expression, which was absent in PCa, but was present in BPH and TAT. Concerning our results, we think that this marker would be an excellent candidate to enhance current diagnostic tools in PCa identification.

11. CURRICULUM VITAE

Denis Mulabdic was born in Banja Luka, Bosnia and Herzegovina on October 20th 1991. At the age of 7 he moved to Vancouver, Canada where he would stay for 18 years completing a Bachelors's Degree in Molecular Biology and Biochemistry at Simon Fraser University. Upon completing his degree, Denis decided to pursue an education in Medicine, and applied to the University of Zagreb, School of Medicine. It was here that he was able to harbour his love for medicine and his passion for research. Since arriving at the University of Zagreb he has been engaged as a student demonstrator for multiple pre-clinical subjects and is currently involved in multiple research projects.

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