EXAMINATION OF TISSUE AND URINE MICROBIOME COMPOSITION AND HUMAN BETA-DEFENSIN PRODUCTION IN BLADDER CARCINOMA PATIENTS

PhD thesis

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1. Introduction

Bladder cancer (BC) ranks as the tenth most frequently diagnosed cancer worldwide. The incidence is the highest in the Southern European region of the world, but when examining the countries individually, the data provided for Greece is outstanding among men, while Hungary has the highest incidence among women. Based on the depth of tumor infiltration, BC is divided into non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). Exogenous exposure to carcinogens - as tobacco smoke, aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons - contributes to the development of BC. The circulating carcinogenic compounds and their metabolites are excreted through the kidneys and spend a shorter or longer time in the urine collected in the bladder. The interaction of the chemicals excreted in the urine with the microbes present in the bladder can lead to the neutralization of carcinogenic substances or the formation of harmful metabolites. Both bacteria and their metabolites present in the bladder are one of the many contributors leading to the development and progression of BC.

The first step of the available treatment options for BC is transurethral resection of bladder tumors (TURBT). The purpose of TURBT is to remove the cancer tissue and take a histological sample to determine the exact diagnosis, staging, and grading. Depending on the results of the histological examination, additional treatment options include neoadjuvant chemotherapy, radical cystectomy, radiation therapy, chemotherapy, the use of Bacillus Calmette-Guérin

(BCG) or checkpoint inhibitors. BCG not only acts against BC by activating the immune system, but also directly reacts to cancerous cells, causing oxidative stress, necrosis or apoptosis. After BCG therapy local or systemic side effects occur in approximately 70% of patients, 25–45% of patients fail treatment in the beginning, and another 40% eventually relapse after initial improvement. The cause of the failure of BCG therapy has been investigated by numerous in vitro and in vivo studies, it has been proven that the increase in the human beta-defensin 2 (hBD2) level in the environment of tumor cells reduces or prevents the uptake of BCG by bladder cancer cells. In the case of cisplatin-ineligible bladder cancer or for patients with metastatic urothelial carcinoma, five immune checkpoint inhibitors (ICI) have been approved to date. Immune checkpoints, as normal part of the immune system, provide protection against the destruction of healthy body cells, checkpoint inhibitors reactivate T cell functions against tumor cells. Unfortunately, only 20–40% of patients respond favorably to ICIs. Several studies have demonstrated the relationship between gut microbiome composition and ICI efficacy.

Prior to our investigations, only a few research results were available on bladder carcinoma and the composition of the microbiome. The majority of the results were about the urinary microbiome related to BC; only one research group at that time characterized the microbiome related to cancerous lesions of the bladder wall. To the best of our knowledge, no one before us compared the microbiome composition of catheterized urine and tumor tissue samples.

Previous research results on the relationship between hBDs and BC were found only for hBD1 and hBD2. In the case of hBD1, exogenous synthetic hBD1 peptide inhibits bladder cancer cell migration and proliferation, and urine-derived hBD1 has been shown to suppress bladder cancer growth. Previous research results on the relationship between hBD2 and BC described that elevated hBD2 levels inhibit the success of BCG treatment.

We did not find any previous research results in the literature that examined the relationship between the microbiome and hBD levels in the case of BC.

2. Objectives

1. Our aim was to develop new and reproducible methods for microbiome analysis from urine and removed bladder mucosal tissue samples from patients with prostatic hyperplasia or bladder cancer.

2. Our goal was to determine whether the microbiome composition is different at the distant points of the tumor tissue sample of a given patient or whether it is characteristic of the given patient.

3. By comparing the tissue and urine microbiome results, our aim was to examine whether the urine sample is a suitable test material for the characterization of the bladder tumor microbiome.

4. Our aim was to determine which bacterial genera are more associated with tissue and which with urine.

5. We set out to compare the microbiome composition of cancerous and healthy histological samples in order to determine

which taxa are associated with all tissue samples and which taxa are associated only with the cancerous tissue samples.

6. Our aim was to determine whether there are differences between the levels of hBD1, hBD2, and hBD3 in the urine of healthy individuals and patients with prostate hyperplasia or bladder cancer.

7. Our aim was to determine the expression rate of hBD1, hBD2, and hBD3 in bladder carcinoma and healthy mucosa tissue samples.

8. Our aim was to find a correlation between the microbiome composition characteristic of tumor tissue samples and the defensin levels belonging to the given sample.

3. Methods

The whole study was conducted in accordance with the Declaration of Helsinki ethical standards. Protocols for sampling and data management were performed in accordance with the guidelines and regulations of Markhot Ferenc University Teaching Hospital (MFUTH). All research was approved by the Ethics Committee of MFUTH and by the Regional Ethics Committee of Semmelweis University; the permission numbers are SE RKEB 100/2018 and SE-RKEB 100-1/2018/2021. All study participants gave written informed consent to participate in the study and to allow the publication of their anonymized test results.

In the first study period histological and urine samples were collected from 10 patients with BC for microbiome studies. In the

second study period tissue and urine samples were collected from 55 BC patients and from 12 patients with benign prostatic hyperplasia (BPH) for microbiome and defensin measurements. As a negative control for defensin levels, urine samples from 34 healthy volunteers (HV) were also collected. Exclusion criteria in all study groups were an existing infection or antibiotic or probiotic use in the two months prior to the study. Urine and tissue samples were collected from patients with bladder cancer and prostatic hyperplasia during transurethral resection. We took the greatest possible care to avoid contamination when taking samples. From healthy volunteers spontaneously excreted urine was examined.

Urine samples were divided for traditional routine culture, microbiome analysis and hBD tests. The level of detection with traditional routine culture is 100 Colony-forming units per milliliter (CFU/mL).

DNA isolation was performed by the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, USA) from digested tissue and native urine samples. Total RNA was isolated from digested tissue samples by the innuPREP RNA Mini Kit 2.0 (Analytik Jena GmbH, Jena, Germany).

Bacterial DNA was amplified with tagged primers covering the V3-V4 region of bacterial 16S rRNA gene, PCR product libraries were sequenced on Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3. Raw sequencing data were retrieved from Illumina BaseSpace and analyzed using the CosmosId bioinformatics platform

Levels of HBD mRNA expression was measured with qPCR from the BC and BPH tissue samples on qTOWER 3G (Analytic Jena GmbH, Jena, Germany) using the PrimeScript RT reagent kit (Takara Bio, San José, USA). Relative changes in mRNA expression were calculated by using the double delta Ct (ΔΔCt) method, the reference expressed housekeeping gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Urinary amounts of HBD1, 2, 3 were detected with ELISA method using SEB373Hu for hBD1, SEA072Hu for hBD2, and SEE132Hu for hBD3 (Cloud-Clone Corp., Houston, USA) in BC and BPH patients, in addition to 34 healthy volunteers.

To find out the difference between urine defensin levels, defensin expression rate, and bacterial taxa abundances measured in the different cohorts' levels of statistical significance ($p<0.05$) was calculated by the Mann-Whitney U test. Statistical significance between cohorts was implemented by the Wilcoxon Rank Sum test for Chao1 Alpha diversity and PERMANOVA analysis for Jaccard Beta diversity using the statistical analysis support application of CosmosID.

4. Results

Using conventional aerobe culture methods, no bacteria were detected in the urine of any patient. As the detection limit was 10^2 CFU/mL, neither bacteria under this limit nor bacteria that cannot be cultured using the traditional aerobic method (e.g., *Ureaplasma sp*) could be detected in this way. Although no bacteria were cultured from

any urine sample the presence of a current infection could not be ruled out since 1–1 bacterial genera were present in nine BC tissue samples with exceptionally high abundance in the second study. The following genera had high abundance in these samples: BC10: *Streptococcus* (69%), BC17: *Corynebacterium* (93%), BC24, 36: *Gardnerella* (59%, 92%), BC31, 32: *Staphylococcus* (57%, 97%), BC40, 50: *Ureaplasma* (95%, 93%), and BC46: *Lactobacillus* (94%). Samples with an assumed ongoing infection were excluded from further comparative microbiome analyses and defensin expression studies.

When examining the microbiome of urine and tissue samples, comparing alpha diversity values, no significant differences were found when all urine was compared with all tissue samples. In the same way, when comparing all samples from female patients with all male samples, no significant difference in alpha diversity was found at the genus level. The alpha diversity of male and female urine samples also showed no difference, but the microbiome of female and male histological samples was significantly different in the area of Shannon alpha diversity.

The tissue samples of four patients were cut, and two pieces located far from each other were separately digested enzymatically. All the isolation of DNA, PCR, library preparation, sequencing, and analysis procedures were done in duplicate. In the abundance of genera no difference was detected from sites 1 and 2 of a subject's tissue sample, the microbiome analysis results were reproducible, and rigorously characterized the tissue sample of a given person. The abundance of genera in the urine sample of the same patient differs

from the results shown in the tissue. However, at the Jaccard ß diversity Principal Coordinate Analysis (PCoA), the correlated tissue samples from the same patients show nearly the same ß diversity results; they do not cluster with their own urine samples.

In urine samples, the most abundant phyla detected were *Firmicutes*, with an abundance of 33%, followed by *Proteobacteria* (29%), *Actinobacteria* (23%), *Cyanobacteria* (7%) and *Bacteroidetes* (4%). In contrast, the order in tissue samples was as follows: *Firmicutes* (34%), *Actinobacteria* (23%), *Proteobacteria* (22%), *Bacteroidetes* (15%), and *Cyanobacteria* (8%). The most abundant genera in urine samples were *Corynebacterium* (12%), *Escherichia-Shigella* (8.7%), *Staphylococcus* (7.8%), *Streptococcus* (6.1%), and *Gardnerella* (5.2%). In tissue samples, the most abundant genera were *Lactobacillus* (9.5%), *Bacteroides* (8.8%), *Staphylococcus* (4.2%), and *Akkermansia* (4.0%). *Akkermansia*, *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter* and *Klebsiella* genera showed remarkably higher median compositional abundance in tissue than in urine samples. The co-existence of these five genera is characteristic but not general for all samples.

When examining the microbiome of the BC and BPH tissue samples, the difference was significant both when comparing the alpha diversity values and when comparing the beta diversity values. There is no significant difference between the *Firmicutes* phylum abundances (45% vs. 46%) in the two cohorts, but *Proteobacteria* (23% vs. 16%; $p = 0.006$), and *Actinobacteria* (13% vs. 4%; $p < 0.001$) have significantly higher abundances in the BC group than in the BPH

one. The only phylum that had a higher abundance in the BPH group than in the BC group was *Bacteroidetes* (30% vs. 11%, p< 0.001). Although the *Cyanobacteria* phylum appeared with only a low median abundance in both groups (2% vs. 0,2%), its abundance was significantly higher in the BC group (p = 0.011). At genus level, the most striking differences between BC and BPH groups were in the abundance of *Bacteroides* (3.22% vs. 21.54%; p < 0.001), *Faecalibacterium* (1.92% vs. 7.79%; p < 0.001) *Staphylococcus* (7.89% vs. 0.59%; p < 0.001) and *Corynebacterium* (3.83% vs. 0.63%; p = 0.001). Just as there was a significant difference in the *Cyanobacteria* abundance at the phylum level, *Oxyphotobacteria* belonging to the phylum was not a dominant genus in the samples; however, its abundance was significantly higher in the BC group (2.11% vs. 0.07%; p = 0.024).

On the basis of the patients' clinical data the Jaccard ß diversity PCoA analysis did not confirm significant differences among the microbiome composition of patient groups according to diagnosed hypertension or diabetes mellitus, smoking habits, or tumor grade and stage classification. The only significant difference was observed among the cohorts of male and female patients.

The median hBD1 level was 12.59 ng/mL (IQR: 12.04) in the BPH group, 12.33 ng/mL (IQR: 4.09) in the HV group, and 20.28 ng/mL (IQR: 21.44) in the BC group. There was no relevant difference between the urine defensin levels between the BPH and healthy control groups, with a *p* value of 0.65. The median amount of hBD2 was 30.45 pg/mL (IQR: 21.93) in BPH, 31.59 pg/mL (IQR: 28.88) in

HV, and 151.69 pg/mL (IQR: 560.89) in the BC group. There was no significant difference between the amounts in urinary hBD2 of the BPH and HV groups. hBD3 levels were 151.96 pg/mL (IQR: 202.36), 186.44 pg/mL (IQR: 198.95), and 653.73 pg/mL (IQR: 1321) in the BPH group, HV group, and BC group, respectively. The amounts of urinary hBD3 in the BPH and HV groups did not show significant differences either. Briefly summarized: hBD1-2-3 levels in the urine of BC patients were significantly higher than those of BPH or HV patients, but the results of the latter two groups did not show significant differences from each other.

Based on the fact that the urinary hBD levels of BPH patients were the same as those of HV patients, the hBD expressions of BPH bladder tissues were considered as healthy control expressions. The amount of hBD1 mRNA expression was elevated in 58% of excised cancer tissue. In 2% of the tumor samples, the expression increased to eight times the mRNA level expressed by healthy tissue. The elevated level of hBD1 mRNA expression was not associated with the increase of urinary hBD1 levels, and even with high mRNA expression in tumor tissue, there were low urinary hBD1 levels. In our opinion, although the tumor tissue produces a large amount of hBD1, the surrounding mucous membrane is genetically determined to produce low levels hBD1, so overall, only a moderate increase in the level of hBD1 can be seen in the urine.

Increased mRNA expression was confirmed in 74% of cancer tissues, and the amount of hBD2 increased in the urine of 78% of BC patients. The hBD2 mRNA expression increased to a maximum of eight times

higher than the healthy value, but the amount of hBD2 measurable in the urine could be up to 1000 times higher than the average healthy level. The discrepancy between the elevated hBD3 expression of the tumor tissue (up to 40 times compared to healthy) and the hBD3 levels measurable in the urine was even more significant (more than 2500 times the healthy level). Cancer tissue mRNA expression was increased in 50% of the samples, while the amount of hBD3 was multiplied in each urine sample. In our opinion, not only the tumor tissue produced hBD2 and hBD3, but the hBD2-3 production of the other healthy bladder mucosa cells also set the high level of hBD2 and hBD3 measurable in the urine.

The amount of hBD1 production in healthy cells is genetically determined, microbes appearing in the environment do not affect it. At the same time, it depends on their individual sensitivity at which level of hBD1 the microbes are able to survive. Only patients with lower hBD1 amounts contained the *Oxyphotobacteria* genus, which has been proven to be carcinogenic. We did not find any other genus whose abundance depended solely on the abundance of hBD1, hBD2, or hBD3. The production of hBD2 and hBD3 is influenced by the environmental microbiome, but together with several other factors, hBD2 and hBD3 levels select among the colonizing microbes. Based on our study, bacterial genera more characteristic of healthy tissue, such as *Bacteroides, Faecalibacterium* and *Parabacteroides*, appeared in the tumor tissue with decreasing abundance as hBD2 and hBD3 levels increased. We did not find any correlation between the abundance of *Blautia*, *Lachnospira*, or *Oxyphotobacteria* and the

change in hBD2-hBD3 levels. Associated with common high hBD2 and hBD3 levels, the dominant genera of tumor tissue – *Staphylococcus* and *Corynebacterium* – were present in high abundance in the microbiome of BC patients.

5. Conclusions

During the research on the therapeutic failures of BC, both the microbiome composition and differences in hBD levels have already been proven to be important causal factors. With our present research, on the one hand, we were looking for a correct research method for examining the microbiome associated with BC, and on the other hand, by examining the relationship between hBD and microbiome changes, we are trying to gain knowledge that can contribute to the planning of more successful individual therapies in the future. The novel observations of our investigations in response to the objectives are as follows:

1. The method developed by our research group is also suitable for examining the microbiome from urine and tissue samples.

2. The microbiome composition is not different at the distant points of the tumor tissue sample, it is characteristic of the given patient.

3. We found that the urine microbiome does not reflect the composition of the microbiome that can be detected in the tissues, and that the constituent taxa differ not only in their proportions but also in their quality between the two sample types. Thus, we found that, due

to its non-invasive nature, the more favorable urine sampling is not suitable for examining the relationship between BC and microbiome composition.

4. Based on our first study, the most abundant genera in urine samples were *Lactobacillus*, *Corynebacterium, Streptococcus,* and *Staphylococcus*, and in tissue samples, *Akkermansia, Bacteroides, Clostridium sensu stricto, Enterobacter,* and *Klebsiella* genera showed remarkably higher compositional abundance.

5. Our second study, which compared the histological samples of BPH (healthy) and BC patients, found that among the tissue-associated bacteria, the genera *Bacteroides, Parabacteroides, Blautia,* and *Faecalibacterium* were found in the healthy tissue, while the genera *Staphylococcus, Enterococcus, Corynebacterium,* and *Lachnospira* were found in the tumor tissue in higher abundance.

6. The increase in hBD1 levels in the urine of BC patients did not follow the increased expression rate of the tumor tissue; therefore, we concluded that the production of hBD1 in the non-tumorous mucosa in these patients is genetically lower than that of the average healthy person.

7. The expression of hBD2 and hBD3 in the tumor tissue also increased compared to the healthy level, but the levels of hBD2 and hBD3 in the urine of BC patients increased several times more than expected. We conclude that the inducible production of defensin by the non-neoplastic mucosa contributed to this increase.

8. The microbiome composition detectable in the tumor tissue and the defensin levels measurable in the urine are related to each

other. The carcinogenic *Oxyphotobacteria* were present at low defensin-1 levels. Along with the increase in hBD2 and hBD3 levels, the abundance of the bacterial genera that are more common in tumors than in healthy tissue also increased.

6. Bibliography of the candidate's publications

Publications related to the dissertation

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Independent publications

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