Identifying molecular features for prostate cancer with Gleason 7 based on microarray gene expression profiles

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Abstract
Prostate cancer represents the first leading cause of cancer among western male population, with different clinical behavior ranging from indolent to metastatic disease. Although many molecules and deregulated pathways are known, the molecular mechanisms involved in the development of prostate cancer are not fully understood. The aim of this study was to explore the molecular variation underlying the prostate cancer, based on microarray analysis and bioinformatics approaches. Normal and prostate cancer tissues were collected by macrodissection from prostatectomy pieces. All prostate cancer specimens used in our study were Gleason score 7. Gene expression microarray (Agilent Technologies) was used for Whole Human Genome evaluation. The bioinformatics and functional analysis were based on Limma and Ingenuity software. The microarray analysis identified 1119 differentially expressed genes between prostate cancer and normal prostate, which were up- or down-regulated at least 2-fold. P-values were adjusted for multiple testing using Benjamini–Hochberg method with a false discovery rate of 0.01. These genes were analyzed with Ingenuity Pathway Analysis software and were established 23 genetic networks. Our microarray results provide new information regarding the molecular networks in prostate cancer stratified as Gleason 7. These data highlighted gene expression profiles for better understanding of prostate cancer progression.

Keywords: gene expression, microarray, prostate cancer.

Introduction
Prostate cancer (PCa) is the most prevalent cancer and the second leading cause of cancer death among western male population [1]. Nevertheless, the cure rate for prostate cancer can be increased if the disease is diagnosed early.

Prostate-specific antigen (PSA) is considered to be the most important biomarker for screening and early detection of PCa [2]. Unfortunately, there are limitations in the use of PSA mainly due to lack of specificity in cancer detection. PSA does not distinguish between PCa and other nonmalignant processes of prostate gland such as benign prostate hyperplasia (BPH), inflammation and infection or between clinically relevant and irrelevant cancers (microscopic cancer that will never cause a problem). These false positive results lead to unnecessary
biopsies or to the overdiagnosis and treatment of many patients with irrelevant cancers. According to the European Randomized Study of Screening for Prostate Cancer (ERSPC) supported by other recent studies, PSA screening reduced prostate cancer mortality with 20% at a median of 9 years follow-up but dramatically increased risk of over-diagnosis [3, 4].

These findings indicate the need to search for effective prostate cancer biomarkers capable to detect cancer at early stages or prior to tumor metastasis. Current advancements in biotechnologies, as microarray technologies, facilitated the study of prostate cancer at molecular level for identifying relevant genes involved in this pathology. New concepts stemming from genomics in the discovery of biomarkers have shown that the pattern of transcription can lead to a better diagnosis [5]. Technologies as microarray can provide data about the whole transcriptome associated with cancer or benign pathology. This technique can be used to study the differences between two different types of cells, like normal and tumoral cells as well as the cellular mechanisms underlying cancer.

Despite of many studies conducted on this topic, the molecular mechanisms underlying PCa are poorly understood, which explains why no targeted therapy has been developed until now. The aim of this study was to explore the changes in gene expression in prostate cancer comparing with normal prostate, based on microarray analysis and bioinformatics approaches. Gene expression profiling could provide potential genes for better understanding of PCa progression.

Materials and Methods

Samples collection

Normal and prostate cancer samples were obtained from patients who underwent surgery at Urology Department of Municipal Clinical Hospital Cluj-Napoca and “Ion Chiricuță” Cancer Institute, Cluj-Napoca, Romania. Approval for this study was obtained from the Institutional Ethics Committee and all patients signed an informed consent.

The patients enrolled in this study had serum tPSA levels ≥4 ng/mL and abnormal digital rectal examination (DRE). The diagnosis for each patient was established by 12 core biopsies using Hematoxylin–Eosin staining. Subsequently, all patients underwent radical prostatectomy surgical procedure. Prostate adenocarcinoma foci could not be macroscopically identified in prostatectomy pieces due to their small size for several samples. Accordingly, for those samples just normal tissues were harvested. We will use through this paper the term “normal” for samples coming from non-cancerous region of the prostate and does not contain tumor cells. Normal and prostate cancer specimens were obtained under control by extemporaneous examination of interest area (Figure 1). Prostate cancer tissues with Gleason score 7 were considered for this study. Finally, we collected four prostate cancer and six normal prostate tissues.

The patients’ characteristics are presented in Table 1. The fresh tissues samples collected by macrodissection were snap-frozen and stored in liquid nitrogen until their use for microarray analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Samples code</th>
<th>Group</th>
<th>Age [years]</th>
<th>PSA [ng/mL]</th>
<th>Gleason score</th>
<th>HP exam</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>p21_N</td>
<td>N</td>
<td>55</td>
<td>10.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>4945_N</td>
<td>N</td>
<td>67</td>
<td>13.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3.</td>
<td>4928_N</td>
<td>N</td>
<td>59</td>
<td>10.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>6986_N</td>
<td>N</td>
<td>70</td>
<td>6.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5.</td>
<td>1272_N</td>
<td>N</td>
<td>68</td>
<td>6.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6.</td>
<td>5727_N</td>
<td>N</td>
<td>65</td>
<td>11.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7.</td>
<td>p21_C</td>
<td>PCa</td>
<td>55</td>
<td>10.8</td>
<td>3+4=7</td>
<td>Acinar prostate adenocarcinoma; pT3bN0MxL0V1R1</td>
</tr>
<tr>
<td>8.</td>
<td>4945_C</td>
<td>PCa</td>
<td>67</td>
<td>13.9</td>
<td>4+3=7</td>
<td>Acinar prostate adenocarcinoma; pT3bN0MxL0V0</td>
</tr>
</tbody>
</table>

Figure 1 – Samples obtained by macrodissection: normal prostate (left) and prostate cancer (right) (HE stain, ×200).
samples with RIN greater than 7.5 and 28S/18S ratio more than 1.7 were included in this study.

Microarray hybridizations

Whole Human Genome (WHG) 4x44k oligonucleotide slides (product G4112A) provided by Agilent Technologies was used for gene expression analysis. Microarray probes were synthesized from 500 ng of total RNA using LILAK kit (Agilent Technologies), RNA Integrity Number (RIN) and 28S/18S ribosomal RNA ratio were considered as quality controls. Only samples with RIN greater than 7.5 and 28S/18S ratio more than 1.7 were included in this study.

Real-Time PCR

Real-time PCR was used for validation the microarray data. We used Roche technology (Light Cycler 480) with primers and UPL (Universal Probe Library) probes obtained on in silico studies based on Roche Applied Science software. 500 ng of the total RNA from each sample were used for cDNA synthesis. Changes in gene expression were measured relative to the mean critical threshold (CT) values of 18S housekeeping gene, by the ∆∆Ct method.

Results

It is well known that prostate cancer cells modify their transcriptional profile during cancer progression. To investigate this process, transcriptional profiles for 10 prostate tissues, including four PCa and six normal prostate tissues, were generated using WHG microarray slides. Unsupervised hierarchical clustering of the samples, which was based on Euclidian distance and Ward method, revealed two distinct main clusters – one of them clustering the PCa samples and the second clustering normal prostate tissues (Figure 2). The results showed a good separation between studied groups based on gene expression levels, with a cophenetic correlation coefficient 0.745. Cophenetic correlation was calculated and used as an indicator of degree of similarity among samples. Good separation of PCa samples was supported also by a proper selection of samples using macrodissection procedure and by homogeneity among cancer group based on Gleason Score and PSA values.
We identified 1119 genes more than 2-fold regulated between prostate cancer and normal tissues at a statistical significance level of 0.01. In progression of prostate cancer, 378 up-regulated and 741 down-regulated genes were detected. Using IPA, we further investigated the biological implication of differentially expressed genes. These genes were significantly involved in cancer (n=252), followed by inflammatory disease (n=234), genetic disorder (n=453), connective tissue disorders (n=157) and skeletal and muscular disorders (n=219) (Table 2).

We also explored the interactions between genes involved in prostate cancer progression by mapping these genes to genetic networks using information stored in IPKB. There were identified networks and nodal genes, which presented a higher interest for molecular mechanisms understanding. We found 23 genetic networks with a score greater than 3, but we continued to investigate five most important of these networks which had the IPA score higher than 24. Particular functions were associated to each network. The most significantly associated networks functions were related to cellular movement, cellular growth and proliferation, small molecule biochemistry, genetic disorder, hematological disease, molecular transport and nucleic acid metabolism (Table 3).

The nodal genes were identified for every network. In Figure 3 is presented the network with the highest score. The most important nodal gene for this network is TERT, involved in tumor immortality. There are also other important nodal genes: SMAD3, BCL2, CAV1 and FBLN1, all in direct or indirect interaction with TERT. In the same way were established the most important nodal genes for the other four networks: FGF2, FGF7, EGR1, CYR61, PI3K, ITGB3, CXCL12 (network 2), PTGS2, TNFAIP3 (network 3), CD69, STAT5a/b (network 4) FASN, CIITA, HIC1, E2F, FOXO1, HIST1H4C (network 5).

In order to assess validity of microarray data, we chose four genes to evaluate by Real-Time PCR analysis. Table 4 indicates the gene expression levels for TERT, FASN, CLU and CAV1 obtained by Real-Time PCR and microarray. Fold-changes obtained by Real-Time PCR analysis were higher for all genes than those obtained by microarray analysis.
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Discussion

Many molecular methods have been used in attempt to determine the mechanism of development of PCa and to find new diagnostic and prognostic markers [7, 8]. Our study found candidate up- and down-regulated genes involved in prostate cancer progression. To assess gene expression profiles for PCa, we compared them with normal prostate tissues. Using microarray technology, we identified 1119 differentially expressed genes, 378 up-regulated genes and 741 down-regulated genes, involved in 23 genetic networks. It should be kept in mind that there are certain limitations for in silico analysis, not all genes, especially those with unknown symbol, can be integrated into networks.

One important genes network was identified around the TERT, BCL2 and SMAD3 genes (Figure 3). TERT was over-expressed in our study, being a potent modulator of immortality of tumor cells [9, 10]. TERT was co-activated by transcriptional factor E2F5, and in direct relationship with SNRPB which is involved in pre-mRNA splicing. In our study, CAV1 and CAV2 were down-regulated in prostate cancer. Significant down-regulation of CAV1 in PCa vs. normal prostate tissues was also confirmed by Real-Time PCR evaluation (Table 4). Even though in the majority of studies CAV1 and CAV2 are up-regulated, our results revealed low levels of both molecules in tumor samples compared with normal prostate. This could be explained by the fact that all prostate cancer samples used in our study were Gleason 7. It was already supported that the prostate cancer evaluated as Gleason 7 (3+4 and 4+3) represents a clinically heterogeneous group, which present a variable biologic potential and clinical outcomes [11].

Based on the reduction suppressor activity of CAV1 observed in cultured cells, it was predicted that human
tumors would show low levels of CAV1 expression. CAV1 expression depends on tumor cell type and can be down-regulated, unchanged or up-regulated. In prostate cancer, CAV1 is generally up-regulated, with some exceptions [12]. CAV1 expression was associated with increasing Gleason score, lymph node involvement, and positive surgical margins [13].

An important component of prostate cancer progression is based on host-epithelial interaction. Other pathways involved in prostate cancer progression include paracrine regulation of normal stromal-epithelial interactions [14], BCL2 an apoptotic gene was down-regulated in our study being indirectly blocked by CAV1. The fibulins, FBLN1, FBLN4, and FBLN5, represents a class of extracellular matrix proteins involved in cell adhesion and migration. Prostate cancer progression was associated with down regulation of these fibulins [15], our data presented low level of FBLN1 gene expression in prostate cancer compared with normal prostate.

Regarding the second network, we observed that the nodal genes CYR61, FGFR2, FGFR7, PI3K, PDGFBB, ITGB3 and CXCL1 involved in cancer progression and angiogenesis modulation were down-regulated in our study. Was demonstrated that inhibition of PI3K signaling could activate the androgen receptor (AR) and similarly, AR inhibition could activate the AKT signaling by reducing levels of the AKT phosphatase PHLPP [16]. So, these oncogenic pathways could cross-regulate each other by reciprocal feedback, inhibition of one activates the other, contributing to maintaining tumor cell survival.

PTGS2 was down-regulated in our study. Generally, PTGS2 is over-expressed in prostate cancer but one study noted this gene as being not expressed in prostate cancer comparative with normal prostate tissue [17]. This hypothesis, based on lack of proliferative inflammatory atrophy lesions, could support also the low levels of BCL2 in our samples [18]. TNFAIP3 gene was already presented in prostate cancer, being induced by the TNF and several other stimuli [19]. The protein encoded by this gene is a zinc finger protein, and its role was related to inhibition of NF-kappa B activation as well as TNF-mediated apoptosis [20]. Taken together, low level of BCL2 and TNFAIP3 support the idea that apoptosis modulation by these both molecules is deficient in prostate cancer stratified as Gleason 7.

STAT5 is involved in signal transduction and transcription, being critical for the viability and growth of prostate cancer [21]. STAT5 expression in prostate cancer is associated with high histological grades; the presence of active STAT5 in prostate cancer predicts early disease recurrence [22, 23]. In our study, STAT5 was not expressed, probably due to the low Gleason score. In a recent study was suggested that activation STAT5 activation might be involved in the progression of prostate cancer to metastatic disease [24].

We found in our study high levels of FASN. Over-expression of the fatty acid synthase (FASN) gene has been implicated in prostate carcinogenesis. Some authors suggest that FASN can act as a prostate cancer oncogene in the presence of AR by inhibiting the intrinsic pathway of apoptosis [25].

Some genes up-regulated in network five, HIST1H2AG, HIST1H3A and HIST1H4C, are involved especially in nucleic acid metabolism, explained by a higher transcriptional activity and cell proliferation in prostate cancer than in normal prostate tissue. We did not find literature data about the implications of these genes in prostate cancer progression. We found the transcription factor E2F1 over-expressed in prostate cancer compared with normal prostate. In a previous study, E2F1 was evaluated in association with Ki-67 and TOP2A (DNA topoisomerase II, alpha), being highlighted the potential of these molecules for improving the prognostic and treatment stratification in prostate cancer [26].

HIC1 functions as a regulatory and tumor suppressor gene. The action of this gene was related to suppressor activity for proliferation, tumor growth and angiogenesis. HIC1 could cooperate with p53 to suppress cancer development [27]. In our study, HIC1 was found down-regulated in prostate cancer. FOXO1 has a tumor suppressor role being involved in regulation of transcriptional activity [28]. The protein of this gene represents a key downstream effector of the tumor suppressor PTEN, which could control the expression of genes involved in stress response, apoptosis and cell cycle progression [29]. In our study, the level of FOXO1 gene was down-regulated. CIITA represents a transcription factor, which was moderately methylated in prostate cancer cell lines [30]. We found low levels of CIIT in prostate cancer tissues compared with normal prostate, this finding being in accordance with the methylation stage of this gene on prostate cancer cell lines [30].

The robustness of these results was showed by Real-Time PCR analyses, which were in a good agreement with microarray data (Table 4). Other individual genes presented in Table 3 were found in multiple categories of functions related to cancer development including cell-to-cell signaling and interaction, cell signaling, cell death, cellular growth and proliferation and cellular movement. All these genes taken together could contribute to understanding the molecular mechanisms involved in progression of Gleason 7 prostate cancer.

Conclusions

Our results provide new information regarding the implications of different classes of genes on molecular networks in Gleason 7 (3+4 and 4+3) prostate cancer versus normal tissue. We observed that many genes involved in modulation of apoptosis as CAV1, BCL2, TNFAIP3, HIC1, FOXO1 were down-regulated and genes as HIST1H2AG, HIST1H3A and HIST1H4C, E2F1 or FASN involved in nucleic acid metabolism, transcription and carcinogenesis were up-regulated. These microarray data highlighted some gene expression
profiles for better understanding of the molecular mechanisms for prostate cancer progression stratified as Gleason 7.

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