Babeş-Bolyai University Cluj-Napoca

Faculty of Biology and Geology

Bioinformatic strategies in functional genomics applied in cancer biology

- Summary of PhD Thesis -

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Keywords: gene expression, microarray, angiogenesis, cancer, bioinformatics

1 Introduction

Cancer is a genetic disease that occurs due to genetic and epigenetic alterations in oncogenes, tumor supressor genes and other genes that controls, directly or indirectly, cellular proliferation. These alterations determine abnormal activation or inactivation of certain signaling pathways resulting in uncontrolled proliferation of malignant transformed cells [1]. Therefore, understanding of signaling pathways alterations instead evaluation of a single gene could bring vital information for this pathology.

Prostate cancer is one of the most frequently diagnosed oncologic pathologies, representing the second most common cause of death among masculine population in industrialized countries. However, the cure rate can be increased if the disease is early diagnosed. Because of its heterogeneous phenotype, the diagnosis and prognosis of prostate cancer is complicated: being multifocal, it contains more than one histological grade and it is often juxtaposed and combined with benign prostatic hyperplasia. In the case of prostate cancer diagnosis, there is a need for markers in order to appreciate the risk of disease progression, which allows an optimal treatment choice.

Nowadays, a lot of prostate cancer cases are diagnosed in early stages because of the screening and early diagnostic techniques. The screening and diagnosis of prostate cancer are performed by serum prostate-specific antigen (PSA) determination, digital rectal examination (DRE) and histopathological examination of the prostate tissue, taken by biopsy

PSA is considered to be the most important biomarker for the screening and the early detection of prostate cancer [2]. Unfortunately, there are some limitations in the use of PSA because of the lack of specificity in cancer detection. PSA cannot make the distinction between prostate cancer and other benign affection of the prostate (benign prostatic hyperplasia, inflammations and infections), or between irrelevant cancers (microscopic cancers that do not threaten patient's life) and the clinically relevant ones. These false positive findings lead to unnecessary biopsies, or to an over diagnosis and treatment of cancer patients having irrelevant microscopic cancers, who cannot beneficiate of local treatment. According to the results of the "Prostate Cancer Prevention" clinical study [3], 15,2% of the men having the PSA level under 4.0ng/ml were diagnosed with prostate cancer, while only 20 – 25% of the patients having a PSA serum level over this cut off had a histological confirmation of prostate cancer [4].

These findings indicate the necessity of searching efficient biomarkers in prostate cancer, able to detect early disease, or at least before metastasis. The actual progress of genomics and microarray technology facilitated the prostate cancer study at molecular level, in order to identify the relevant genes involved in this pathology. Microarray gene expression profiling for tumoral samples is based on the presumption that gene expression patterns are major determinants of tumour cells' behaviour. By using the microarray technology, it is possible to identify complex molecular aberrations at the level of the entire genome, associated to the tumoral pathology. Despite numerous studies on this subject, the molecular mechanisms underlying the development and progression of prostate cancer are poorly understood.

2 Aim of thesis

The aim of this study was bioinformatics and biostatistics analysis of array data (*microarray, PCR array, Fast Quant array*) for prostate pathology. Due to inter-disciplinary nature of this thesis the objectives were divided in metodological and biological objectives.

Metodological objective:

- Analysis of Agilent microarray data using two bioinformatics approaches

Biological objectives:

- Evaluation of gene expression patterns in human prostate tissues for identifying genes involved in prostate pathology – *microarray study.*

- Evaluation of molecular profile involved in angiogenesis, in blood samples – *PCR array study*.

- Evaluation of a panel of serum proteins involved in angiogenesis, for patients with prostate pathology –*Fast Quant array study*.

3 Genomic profilling of prostate cancer. Microarray study

3.1 Materials and methods

3.1.1 Biological material

For microarray study we used tissue samples from 14 patients, selected on the bases of PSA value > 4 ng/ml, with an abnormal digital rectal examination and a histopathological diagnosis. Thus, we selected: 6 samples of normal prostate tissue (normal group), 4 samples of prostate adenocarcinoma (tumoral group), and respectively 7 samples of benign prostatic hyperplasia (benign group). The tissue samples for the microarray study were taken by macrodissection. All the prostate adenocarcinoma samples used in the study had a Gleason score of 7 (3+4, 4+3). The normal tissue samples were harvested from radical prostatectomy pieces.

3.1.2 Total RNA isolation

Total RNA was isolated with *Tri Reagent® (Sigma Aldrich*), purified with *RNeasy® Mini kit (Qiagen)* and analyzed for quality and quantity with Bioanalizorul 2100 *(Agilent Technologies)*, respectively with the spectrophotometer NanoDrop ND-1000 (*NanoDrop Technologies*). Only the samples that presented a ratio 28S/18S ≥1.6 and a RIN > 7.5 were used for further analysis.

3.1.3 Microarray reaction. Agilent technology

The genomic evaluation at tissue level was performed by the microarray reaction, the Agilent technology. For this study, it was chosen a one-color design. After RNA isolation, the steps of the microarray reaction were the following:

- synthesis and purification of microarray fluorescent probes - *LILAK® (Low Input Linear Amplification Kit®), RNeasy® Mini kit (Qiagen);*
- hybridization of microarray probes on WHG microarray slides (*Whole Human Genome*) 4x44k and the slides washing - *In situ Hybridization Kit Plus® (Agilent Technologies)*;
- slide scanning and image acquisition;

3.1.4 Bioinformatic analysis of microarray data

The images were processed with Feature Extraction® (FE) v.10.5 Agilent software. According to the study objectives, *Gene Spring GX 11 (GS)* and *Limma (Linear Models for Microarray Data)* package were used for pre-processing and differential analysis. The functional analysis of the differentially expressed genes was realized with Ingenuity Pathways Analysis (IPA) software.

3.1.5 RT-PCR reaction

mRNA levels obtained by microarray were validated by RT-PCR.

3.2 Results and discussions

3.2.1 Gene expression analysis using Limma package

The .txt files provided by FE, containing 45015 sequences, were imported in Limma. Data were normalized using *quantile normalization* method. After suppression of positive and negative controls, there were 43376 sequences obtained in Limma. The saturated spots and spots with non-uniform signal were filtered. KNN method was used to replace missing values and the summarization was realized at the probes level. After these analyses, the number of sequences was reduced to 41000. The three defined groups – *normal (N), benign (H) and tumoral (C)* – were clusterized in different positions of the PCA space (figure 1).

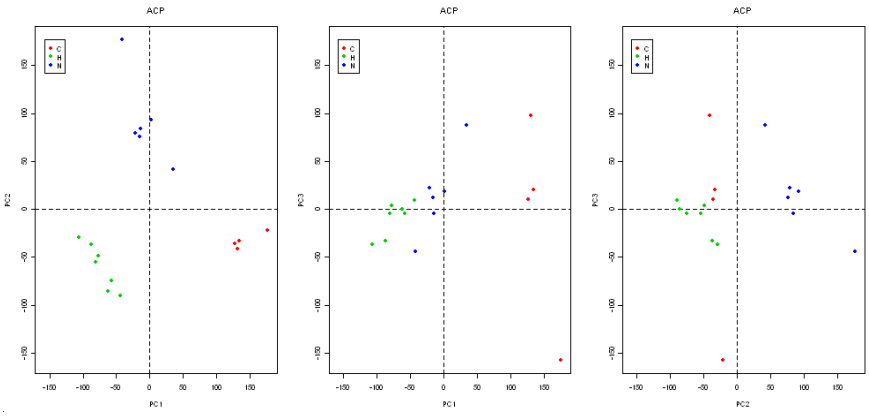

Figure 1. PCA representation in Limma (blue – the normal group (N), red – the tumoral group (C), green – the benign group (H))

The moderated t test and *False Discovery Rate* (*FDR*) correction were applied to identify differentially expressed genes between the studied groups *(tumoral – normal, tumoral – benign, benign - normal*). The cut-off for adjusted p value was established at 0.01 and for fold change value at ± 2. The results are presented in table 1.

Table 1. Differentially expressed genes between the studied groups, obtained with Limma

Comparison	No. differentially expressed genes	Cut-off p	Cut-off Fc
tumoral vs normal	1119	0.01	± 2
tumoral vs benign	3002	0.01	± 2
benign vs normal	1074	0.01	± 2

3.2.2 Gene expression analysis using Gene Spring GX

The number of sequences imported in GS was identical with those imported in Limma (*45015 sequences*). Pre-processing steps in GS are realized by the initial setup of the necessary parameters; this software does not offer the possibility to control each stage of pre-processing. The settings made in GS were similar to the ones in Limma. The data was normalized with the *quantile normalization* method. After suppressing the negative and positive controls, filtering saturated and non-uniform spots, replacing the missing values and summarization, the number of sequences was reduced to 41093, with 93 more sequences than in Limma. The sample evaluation in PCA space underlines a good clusterization of the samples for studied groups: *normal, tumoral, benign* (figure 2).

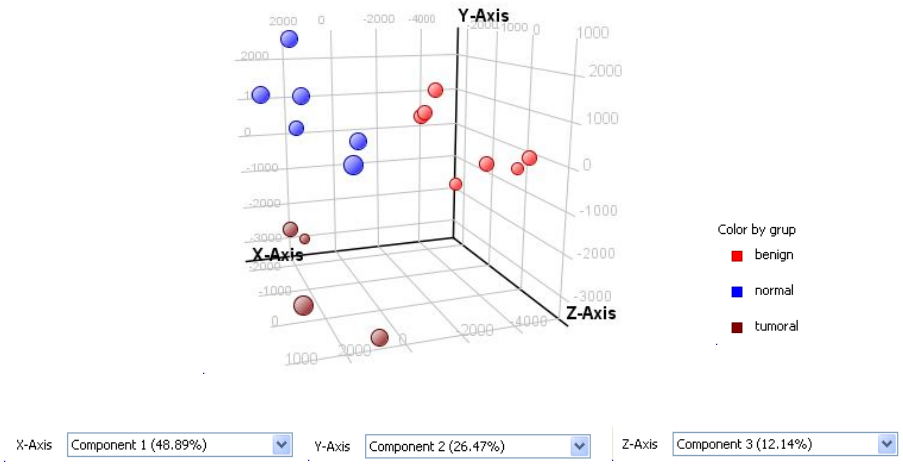

Figure 2 PCA representation in GS (blue – *the normal group*, brown – *the tumoral group*, red – *the benign group*).

To identify differentially expressed genes between the studied groups *(tumoral vs normal, tumoral vs benign, benign vs normal*) unpaired t test and FDR correction, with a cut off of 0.01 for the adjusted p value, were used. The differentially expressed genes with a Fc >2 respectively <- 2 were chosen (table 2).

Table 2. Differentially expressed genes between the studied groups, obtained with GS

Comparison	No. differently expressed genes	Cut-off p	Cut-off fc
tumoral vs normal	454	0.01	± 2
tumoral vs benign	5456	0.01	± 2
benign vs normal	2766	0.01	± 2

3.2.3 The comparision of microarray data obtained with Limma and GS

The results obtained with the two approaches were partially different. 312 differentially expressed genes between tumoral and normal groups were found with both approaches, while 807 differently expressed genes were identified using Limma and just 142 using GS (figure 3).

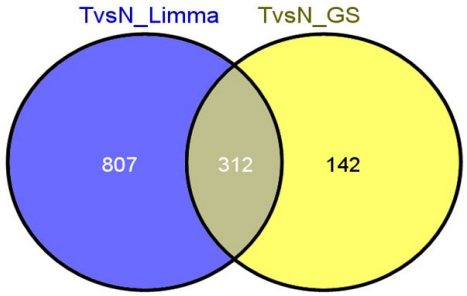

Figure 3. Differentially expressed genes between *tumoral* and *normal* groups, obtained with Limma and GS. Venn diagram

The comparison between the *tumoral* and *benign* groups highlighted a number of 2664 up regulated and down regulated genes in prostate cancer, common in the two approaches. 338 genes were identified using Limma, and 2792 using GS (figure 4).

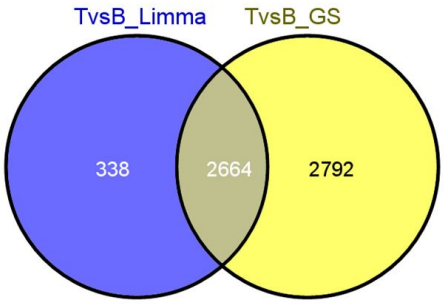

Figure 4. Differentialy expressed genes between *tumoral* and *benign* groups, obtained with Limma and GS. Venn diagram

There were differences also between the *benign* and *normal* groups: 116 genes identified with Limma, 1808 genes identified with GS, and 958 common genes (figure 5).

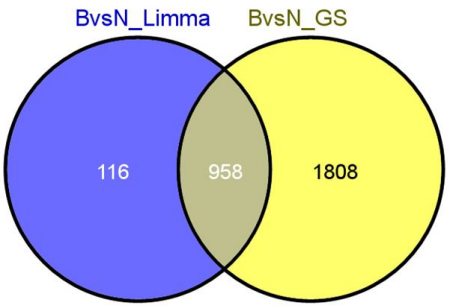

Figure 5. Differentially expressed genes between *benign* and *normal* groups, obtained with Limma and GS. Venn diagram

The comparative functional profile, realized with IPA, for differentially expressed genes between the normal and tumoral tissue, obtained using Limma and GS, showed their involvement in cancer, inflammatory diseases, genetic diseases, and apoptosis. The processes, in which genes obtained with Limma were involved, presented lower p value. (figure 6).

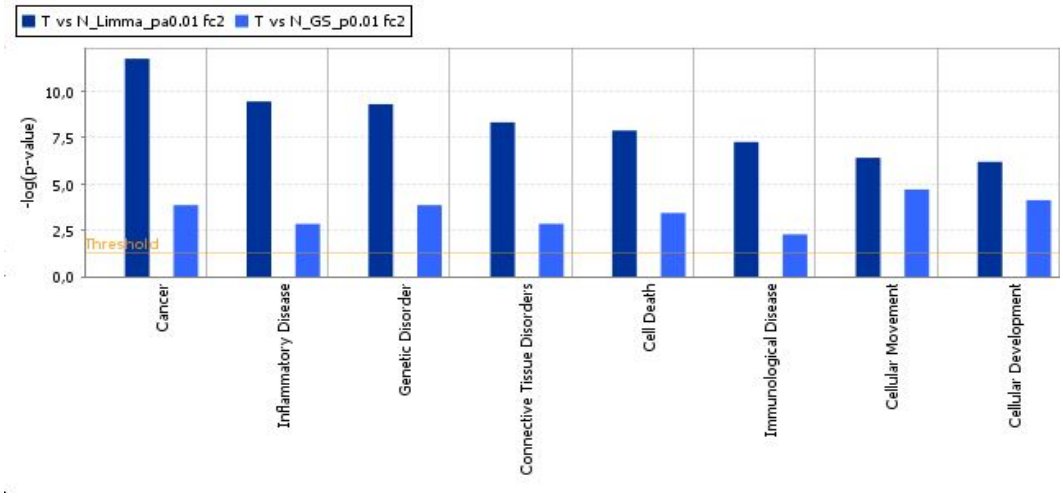

Figure 6. The comparison of functional profiles of differentially expressed genes between the *tumoral* and *normal* groups, obtained using Limma and GS

The results obtained with Limma and GS in the case of the other comparisons (*tumoral* and *benign*, *benign* and *normal*) are presented in the figures 7 and 8.

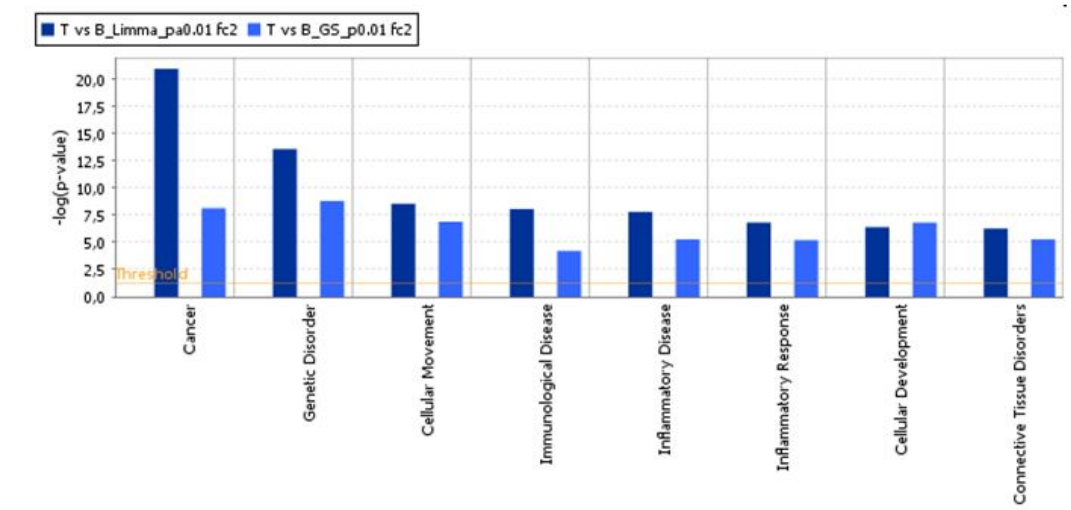

Figure 7. The comparison of functional profiles of differentially expressed genes between the *tumoral* and *benign* groups, obtained using Limma and GS

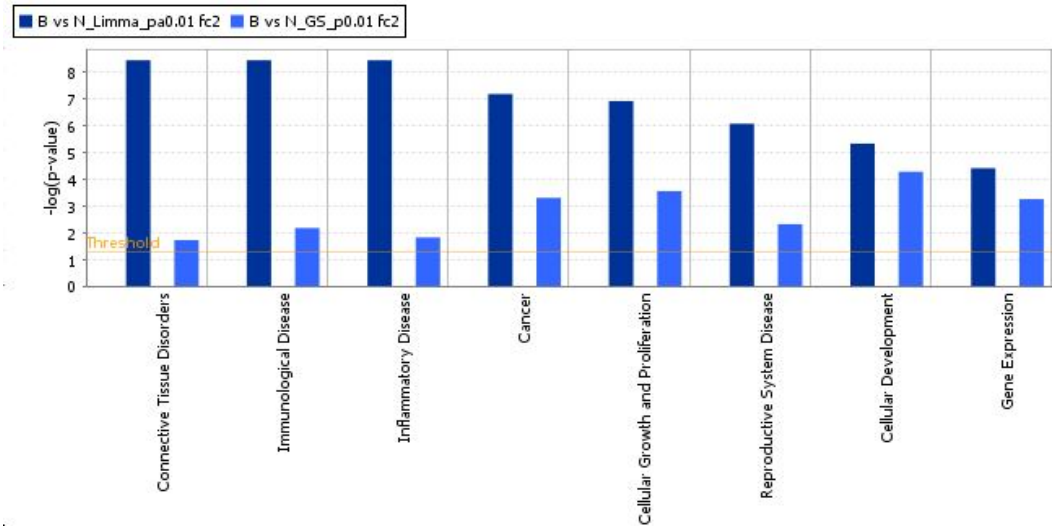

Figure 8. The comparison of functional profiles of differentially expressed genes between the *benign* and *normal* groups, obtained using Limma and GS

3.2.4 Functional profile of differentially expressed genes between tumoral and normal prostate tissues

The functional analysis was realized with IPA for the package of differentially expressed genes identified with Limma, between tumoral and normal prostate tissues. The most important processes in which up- and down-regulated genes were involved are presented in table 3. The genes were grouped in 23 networks, 5 of them having scores higher than 20 and between 25-29 focus genes from 35 possible (figures 9 - 13).

Table 3. Top 5 diseases and genetic disorders in prostate cancer versus normal prostate tissue

Diseases and genetic disorders	p value	No. molecules
Cancer	1,78E-12 - 2,59E-02	252
Inflammatory Disease	3,58E-10 - 2,30E-02	234
Genetic Disorder	5,02E-10 - 2,67E-02	453
Connective Tissue Disorders	4,79E-09 - 2,30E-02	157
Skeletal and Muscular Disorders	2,30E-08 - 2,31E-02	219

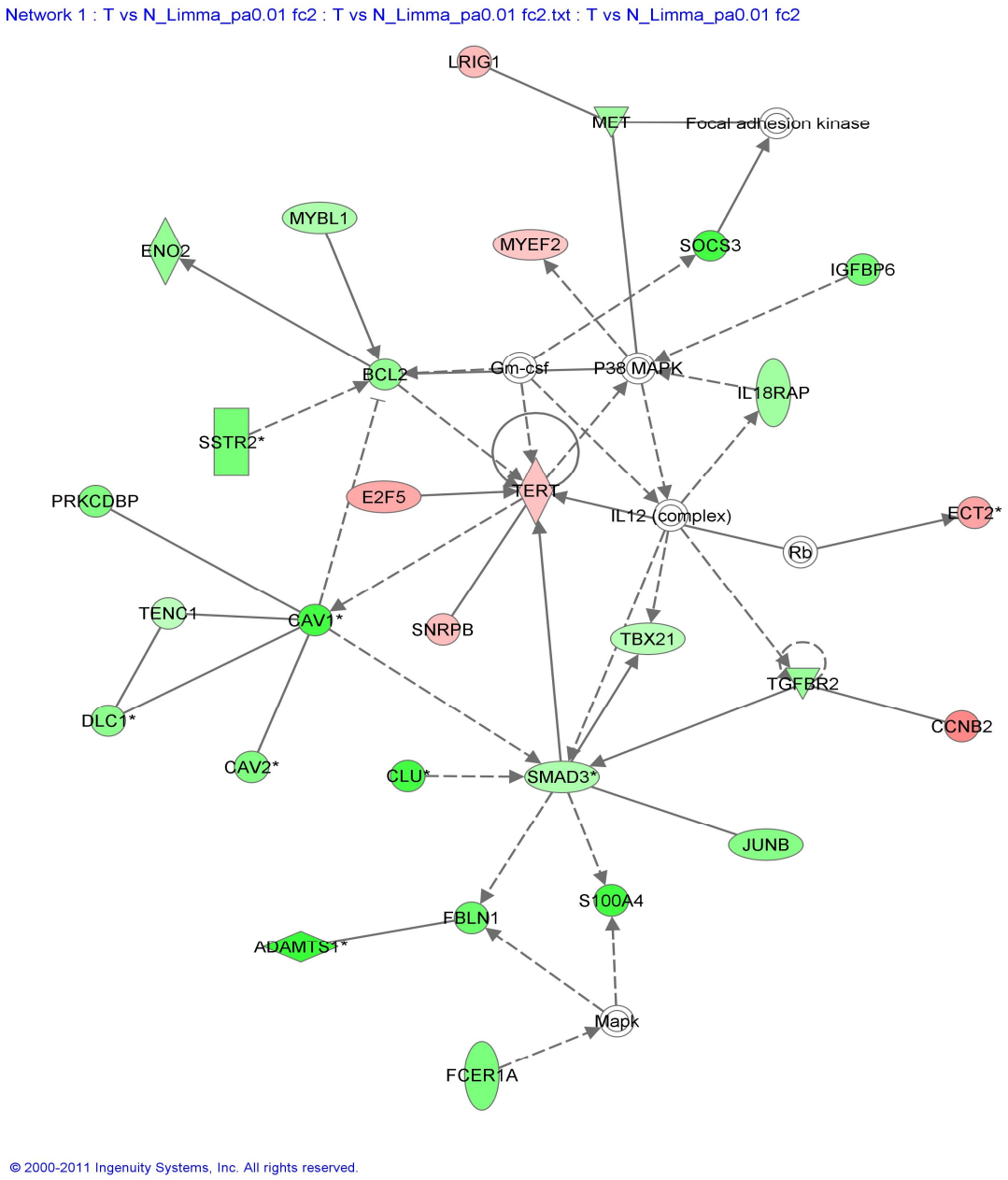

Figure 9. The most important network (network no.1) integrates genes involved in cellular movement, development of cancer, cellular growth and proliferation. The arrows indicate the directions of interaction, which can be direct (a continuous line) or indirect (interrupted line). The colour intensity indicates the degree of regulation: the red represents a gene over-expressed and the green represents under-expressed genes. The colourless genes were not identified in our study; they are part of a pre-established network in which the focus genes that we identified in our microarray experiment were integrated.

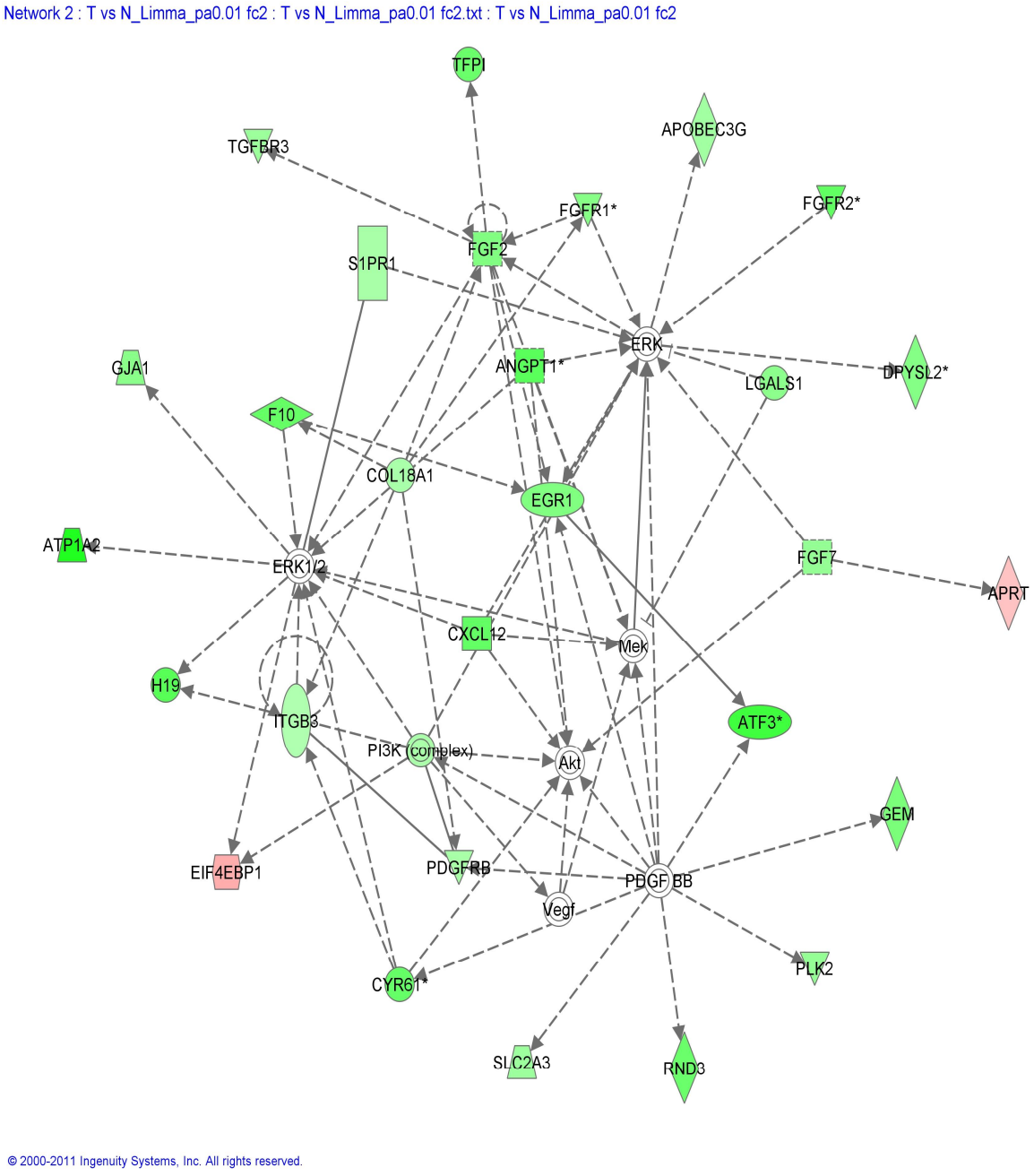

Figure 10. The molecular network (the network no.2) integrates genes involved in cellular movement, cellular growth and proliferation, haematopoiesis.

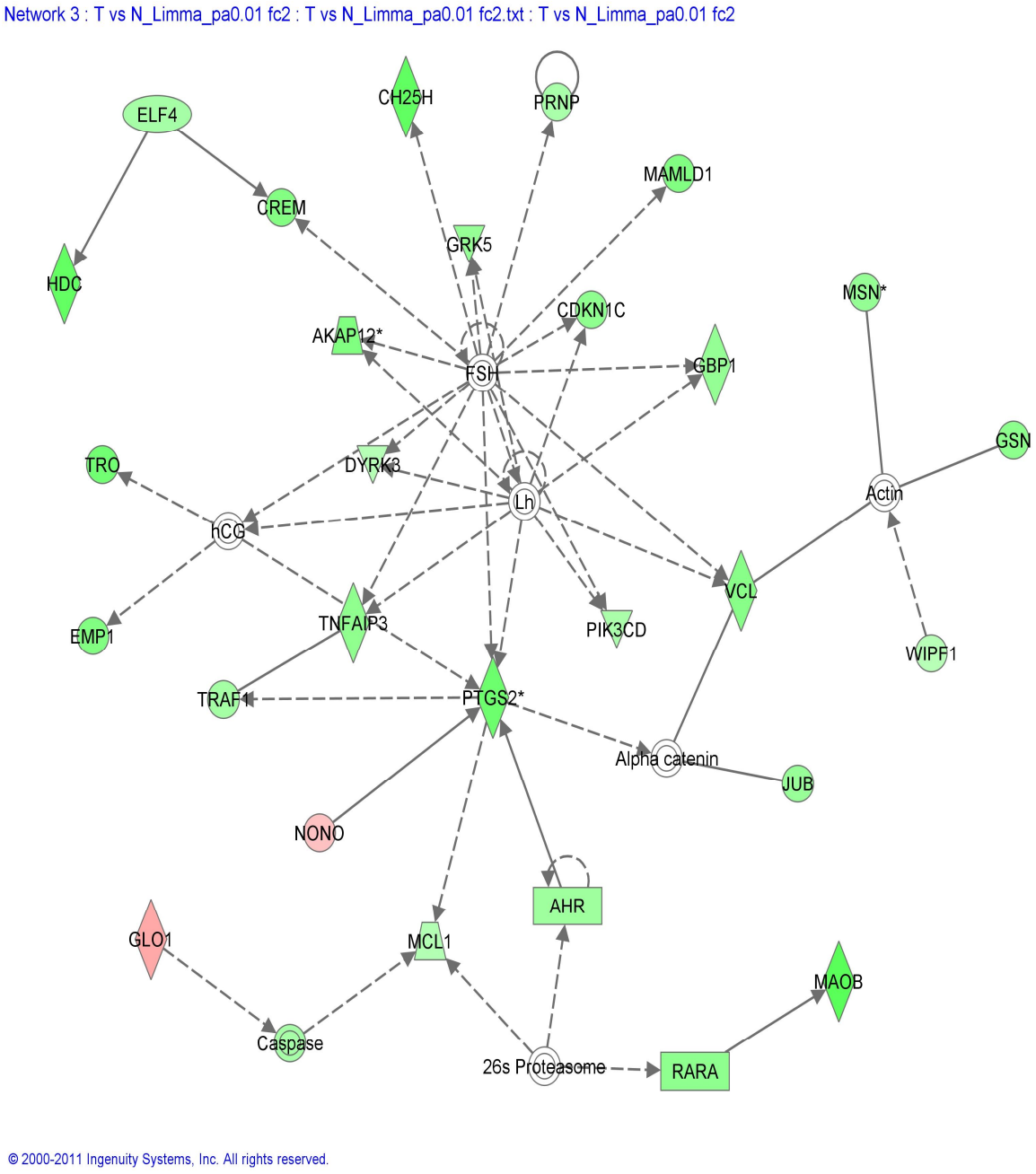

Figure 11. The molecular network (the network no.3) integrates genes involved in genetic disorders, hematologic pathologies or infections.

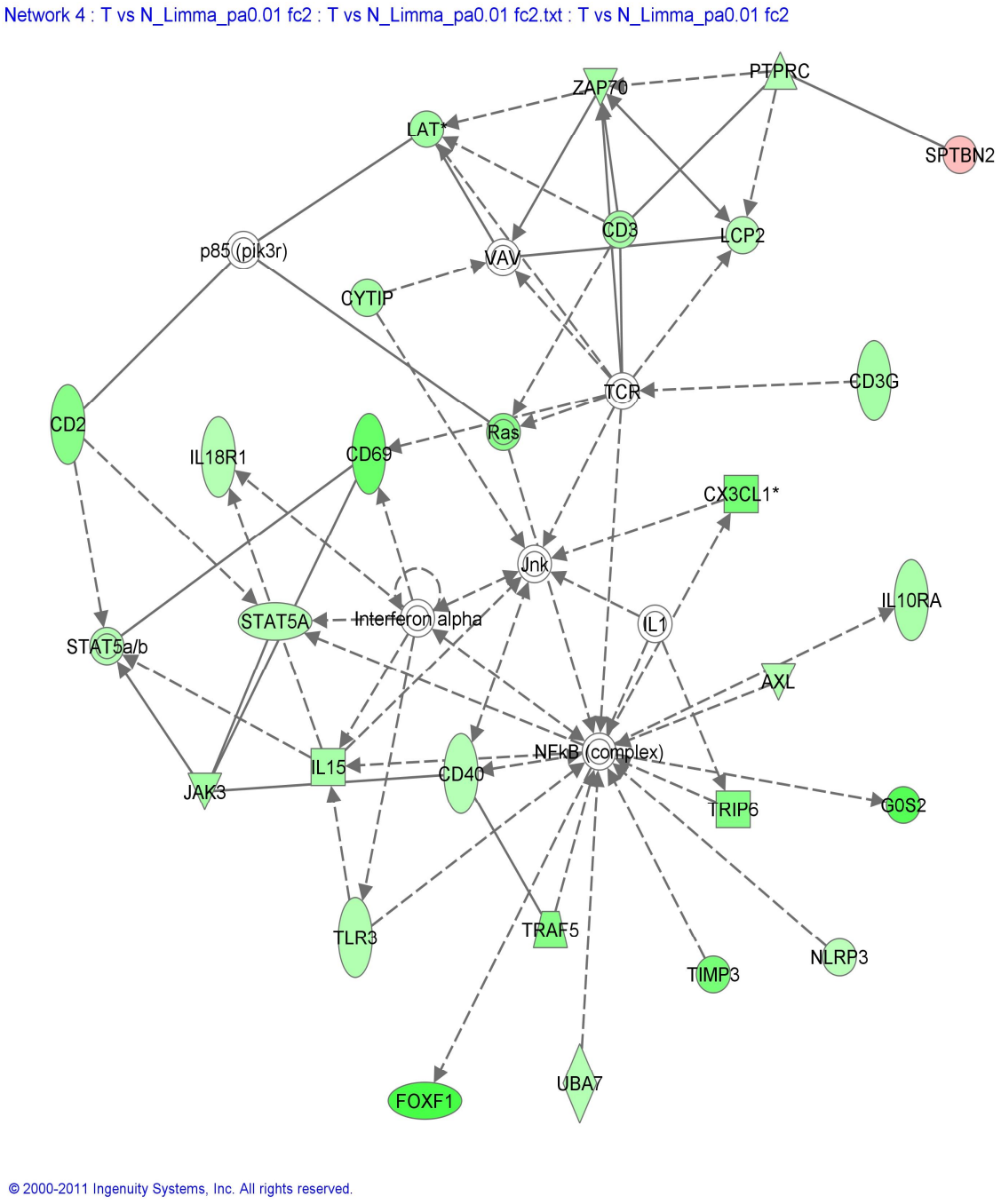

Figure 12. The molecular network (network no.4) integrates genes involved in cellular movement, development and cardiovascular functions, cellular development.

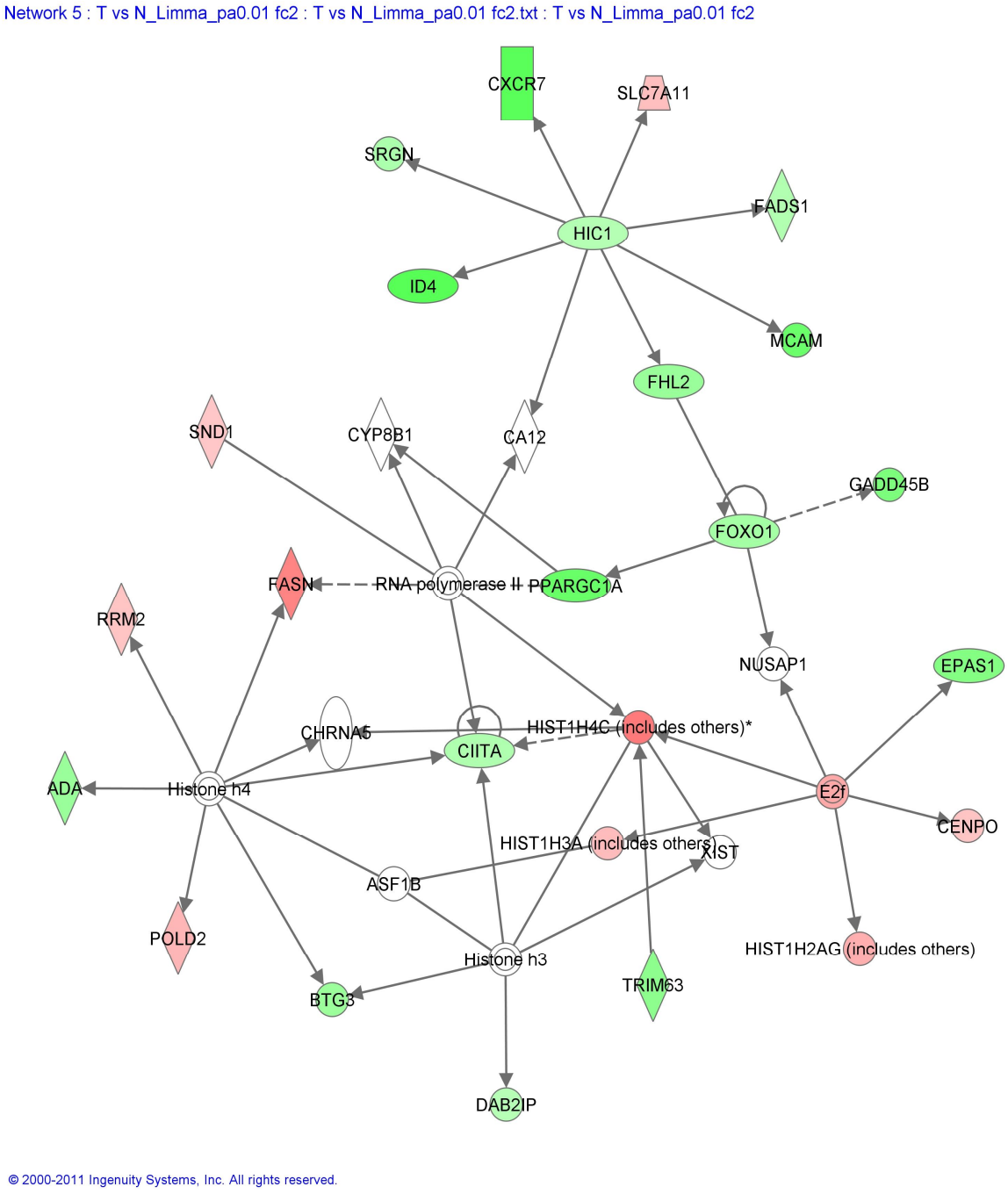

Figure 13. The molecular network (network no.5) integrates genes involved in cellular transport, nucleic acid metabolism and biochemistry of small molecules.

The five molecular networks have nodal genes with implications in prostate cancer: *TERT, BCL2, SMAD3, E2F5, CAV1, FBLN1* (network 1), *FGF2, FGF7, EGR1, PI3K, ITGB3, CXCL12* (network 2), *PTGS2, TNFAIP3* (network 3), *CD69, STAT5a/b* (network 4) *FASN, CIITA, HIC1, E2F1, FOXO1, HIST1H4C* (network 5).

TERT was overexpressed in our study, being a modulator of cellular immortality. Some of the studies in this domain described the activation of this gene in prostate cancer [5, 6]. Our data showed that *TERT* is activated by E2F5 transcription factor. Even though there are data that show that the transcription factor *E2F5* plays an important role in carcinogenesis, there are no studies in the prostate cancer that can confirm the implication of this gene in the initiation and tumor progression [7, 8] . In our study, *CAV1* şi *CAV2* were under expressed in prostate cancer in comparison with the normal prostate tissue. In the majority of studies, *CAV1* şi *CAV2* are over-expressed in prostate cancer comparing with the normal tissue, but our results show low levels of both molecules. This aspect can be explained by the fact that the tissues used in the genomics study were obtained from patients with prostate cancer, having a Gleason score of 7 (3+4 or 4+3). Prostate tumours stratified as Gleason 7 represent a clinical heterogeneous group with a variable biologic potential and a different clinical response [9]. The reduction of tumour suppressor activity of *CAV1* was observed on cancer cell lines, supposing that human tumours can have low expression levels of *CAV1*. *CAV1* can be over-expressed, under-expressed or unchanged, depending on the type of tumor cell. In prostate cancer, *CAV1* is generally over-expressed with the exception of some cases [10]. Expression of CAV1 is positively associated with the Gleason score, the ganglionary implication or positive resection margins [11].

BCL2 is involved in apoptosis, the level of expression in prostate cancer depending on tumoral aggressiveness [12]. In our study, *BCL2* is under expressed, being indirectly blocked by *CAV1*. FBLN1, FBLN4 and FBLN5 are proteins of the extracellular matrix involved in migration and cellular adhesion. The low levels of these proteins were associated with the progression of prostate cancer. *FBLN1* was under expressed in our study, being in accordance with the data found in the literature [13].

The nodal genes identified in the second molecular network: *FGF2*, *FGF7*, *PI3K*, *PDGFRB, ITGB3* and *CXCL12* are involved in the angiogenesis and tumor progression. *EGR1* is a tumour suppressor gene, under-expressed in prostate cancer. Our data show the reduced levels of this gene, in agreement with the literature [14]. The inhibition of *PI3K* signalling pathway can activate androgen receptor signalling pathway. Similarly, the inhibition of *AR* leads to the activation of *AKT*. Both pathways are mutually adjusted by a

feedback loop. The inhibition of one oncogenic pathway leads to the activation of the other one, which maintain the tumor viability [15].

PTGS2 gene was under-expressed in our study, being in direct relationship with *BCL2* [16]. *TNFAIP3* gene is directly regulated by *TNF* gene, being involved in the progression of prostate cancer [17]. The *STAT5a/b* gene is involved in the transduction of molecular signals, having a critical role in tumor growth and viability. The nuclear expression of *STAT5a/b* is associated with a high histology grade, over-expression of these gene being associated with the rapid relapse [18, 19, 20]. Recent data show that the high activity of *STAT5a/b* can be involved in the prostate cancer progression, from the localized form to the metastatic one [21]. The high levels of *FASN* were associated with prostate carcinogenesis. These observations suggest that *FASN* can act as an oncogene in the presence of AR, and the oncogenic effect is realized by inhibition of apoptosis [22].

The genes that are over-expressed in the fifth network: *HIST1H2AG*, *HIST1H3A*, *HIST1H4C* are especially involved in the metabolism of nucleic acids, highlighting a high transcriptional activity, characteristic to carcinogenesis. *E2F1* transcription factor is involved in cancer progression, and it was over-expressed in our study. Some studies showed that *E2F1, Mki67 and TOP2A* could be used as a possible tri-marker, in order to enhance the prognosis and to stratify the prostate cancer treatment [23].

Table 4 presents top ten up regulated and down regulated genes. These genes are involved in tumour development, cellular movement, growth and proliferation, cell to cell interaction and cellular signalling.

Table 4. Top 10 genes with the highest and the lowest expression levels in prostate cancer (Gleason 7) compared to normal prostate tissues.

Up regulated genes		Down regulated genes	
Gene symbol	**Fc**	**Gene symbol**	**Fc**
HPN	**+18.48**	***ATP1A2***	**-12.71**
GOLM1	**+16.79**	***CFD***	**-12.49**
AMACR	**+15.68**	***DPT***	**-12.16**
SIM2	**+13.27**	***ADAMTS4***	**-11.09**
FOLH1	**+10,87**	***FOSB***	**-10.46**
GPR160	**+9.25**	***RNF112***	**-10.13**
TMEFF2	**+8.73**	***MAL***	**-9.43**
CGREF1	**+8.63**	***SMOC1***	**-8.49**
GJB1	**+8.57**	***COL4A6***	**-8.13**
TMSB15A	**+7.89**	***ADAMTS1***	**-7.98**

HPN, AMACR, GOLM1, SIM2 and *FOLH1* have the highest expression level in tumor tissue in comparison to normal tissue and have been described in the literature as possible biomarkers for prostate cancer. *GPR160, TMEFF2* and *TMSB15A* were also described as possible biomarkers in prostate cancer due to their high expression levels in tumor tissue [24, 25, 26, 27]. In our study, *CGREF1* was up regulated in the tumor tissue compared to the normal prostate tissue. Implication of the gene in cellular growth and adhesion was studied for neuronal cell lines and over-expression of *CGREF1* was associated with an increased mortality risk in patients with metastatic melanoma [28, 29]. But information regarding the implication of this gene in prostate cancer was not found in PubMed. *GJB1* is another up regulated gene in our study for which no information was found on PubMed. Its implication in oncogenesis was described for localizations such as breast or liver [30, 31].

3.2.5 Functional profile of differentially expressed genes between tumoral and benign prostate tissues

The most important processes, in which differentially expressed genes between tumoral and benign prostate tissues were identified, are presented in Table 5. 25 networks have been obtained and 11 of them have had the score and the number of focus gene greater than 20. Figures 14-17 present the first four of these molecular networks which integrate these focus genes.

Table 5. Top 5 diseases and genetic disorders in prostate cancer versus benign prostate tissue.

Diseases and genetic disorders	p value	No. molecules
Cancer	1.07E-21 – 1.39E-02	566
Genetic Disorder	2.59E-14 – 1.41E-02	1,034
Dermatological Diseases and Conditions	1.20E-13 – 1.41E-02	249
Reproductive System Disease	1.90E-13 – 5.17E-03	346
Immunological Disease	8.55E-09 – 1.41E-02	463

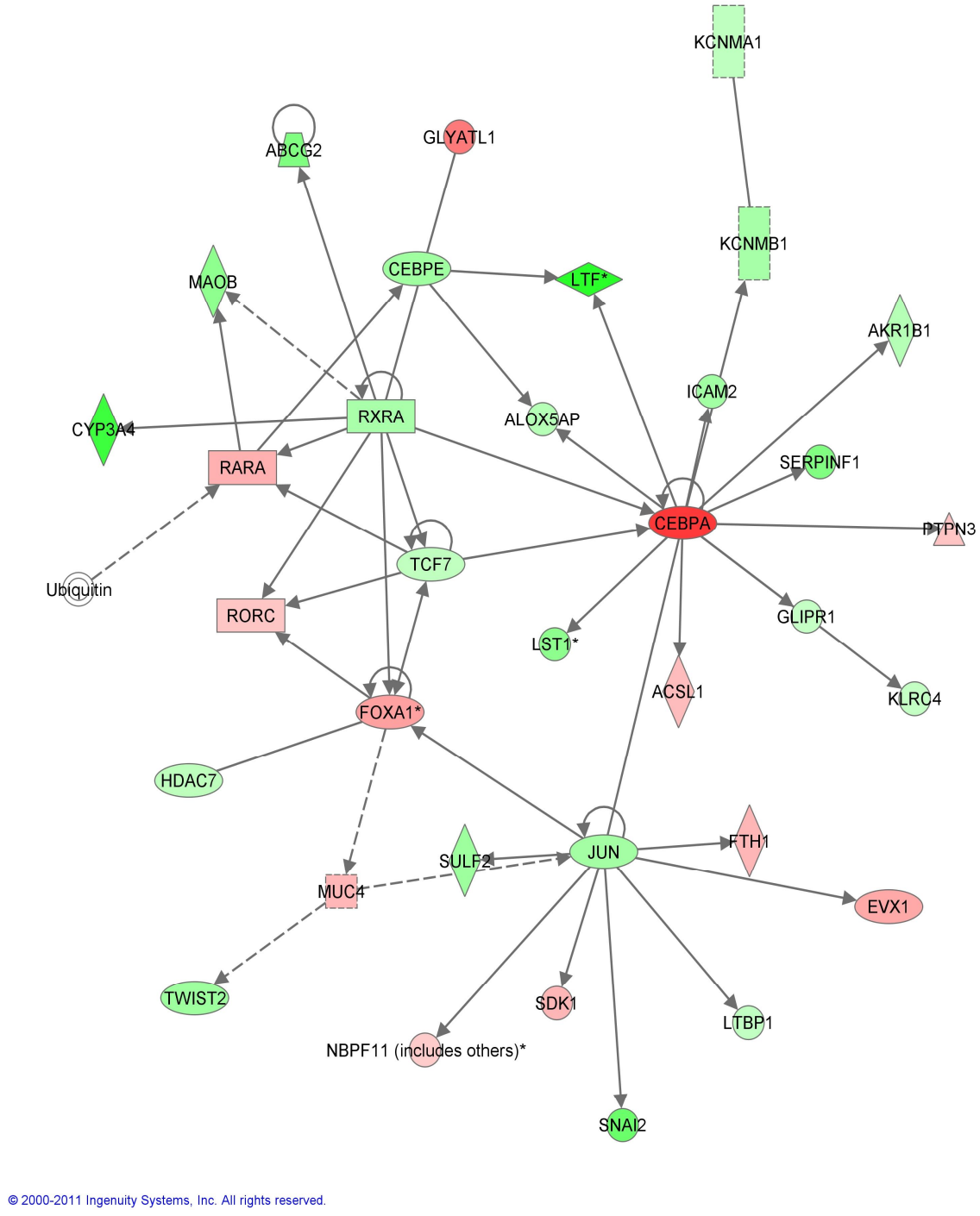

Figure 14. Molecular network (network number 1) integrates genes involved in lipid metabolism, vitamin and mineral metabolism

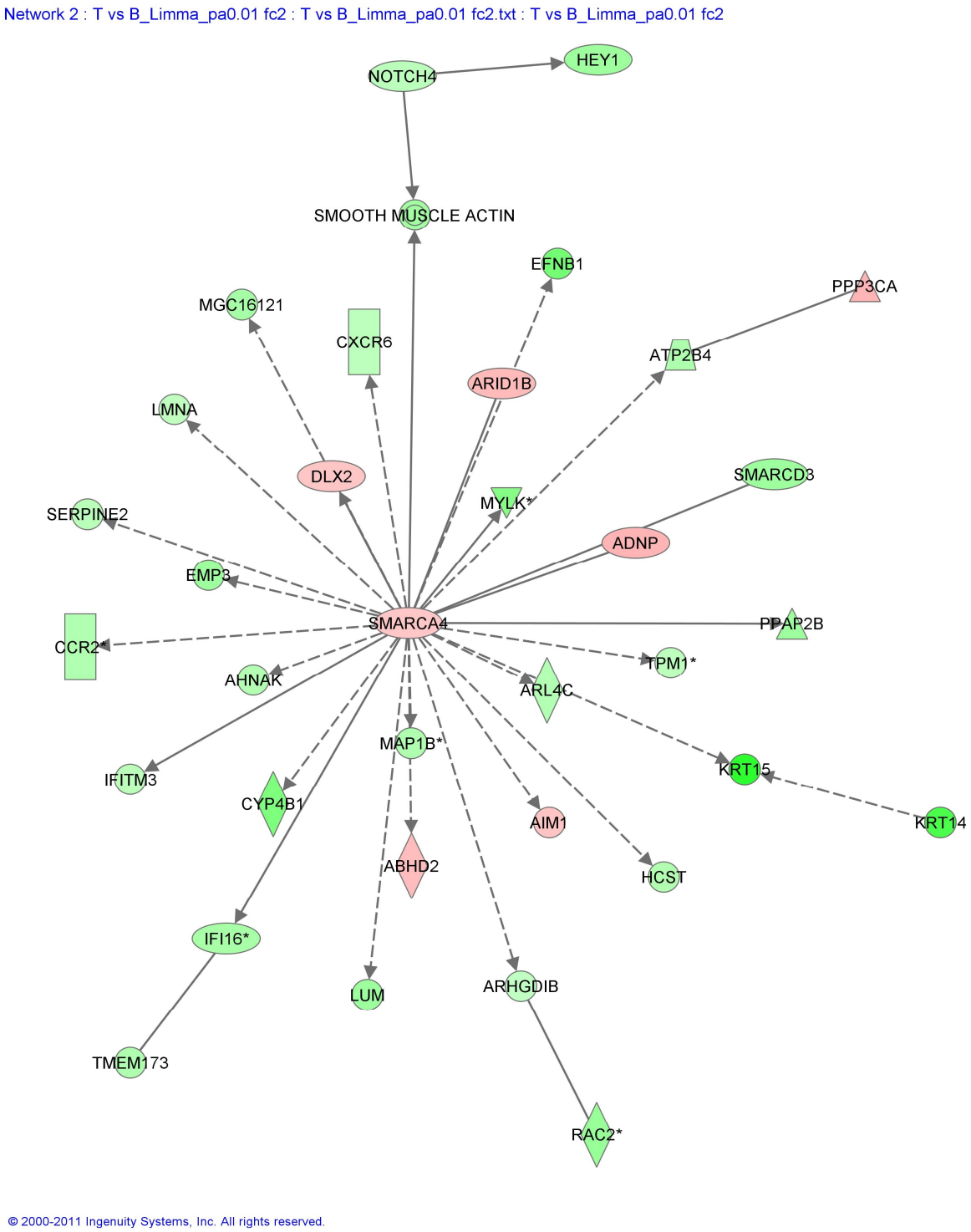

Figure 15. Molecular network (network no. 2) integrates genes involved in cellular movement, dermatological and cardiovascular diseases

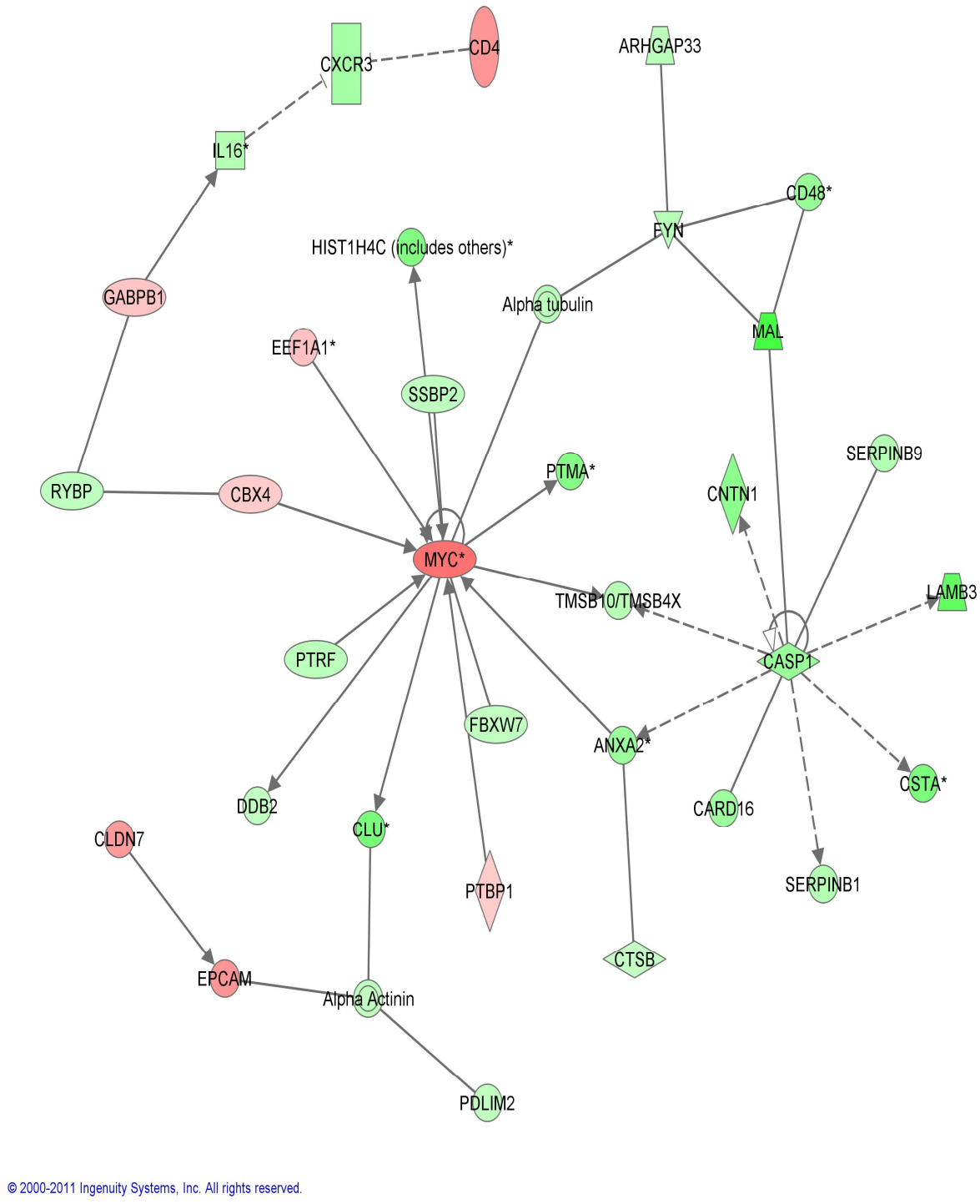

Figure 16. Molecular network (network no. 3) integrates genes with molecular functions involved in tumor development, hematological and dermatological diseases

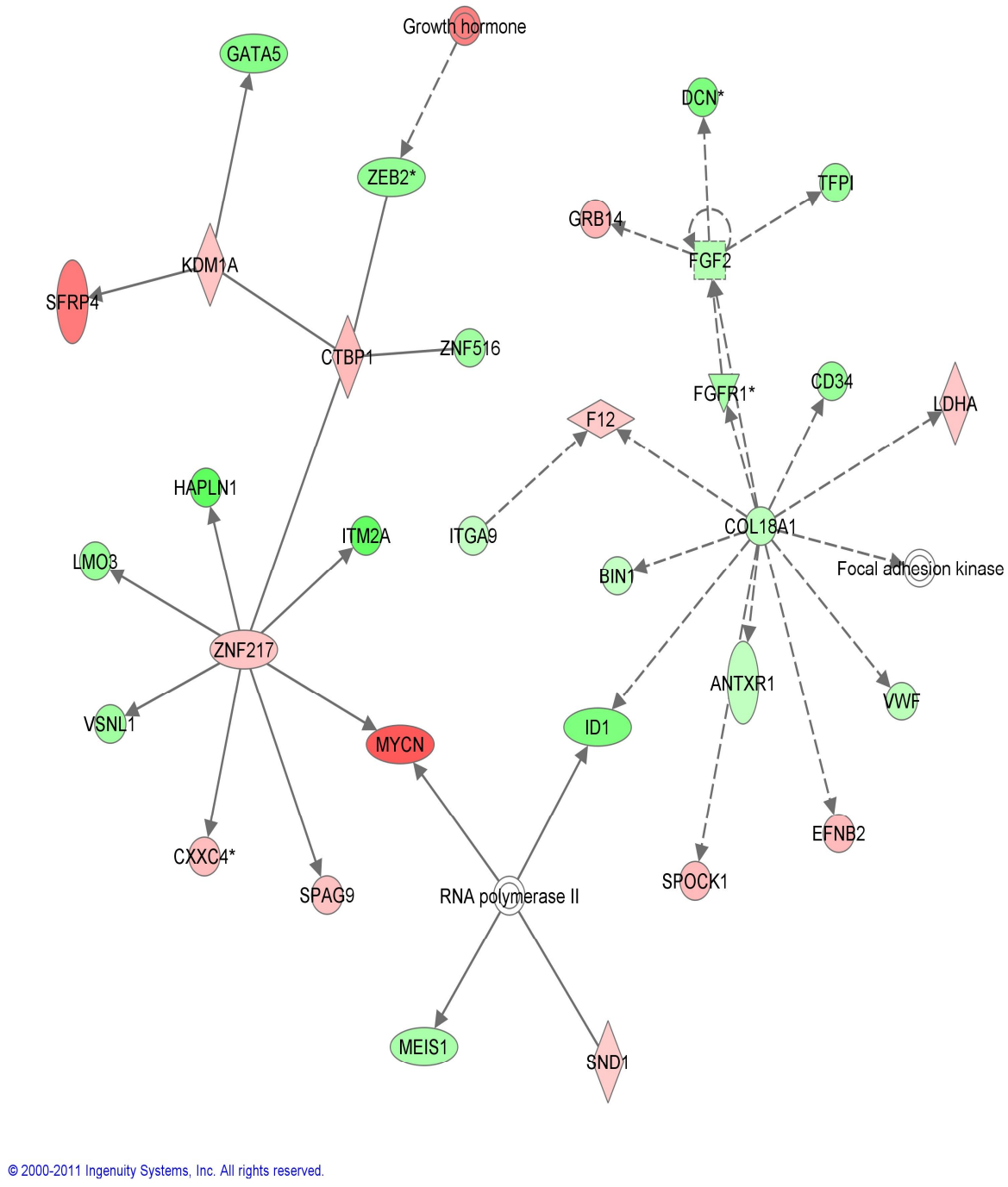

Figure 17. Molecular network (network no. 4) integrates genes involved in cellular movement, cardiovascular diseases.

The nodal genes identified in these networks are: *CEBPA, FOXA1, JUN, MUC 4, RARA, RXRA, RORC* (network 1), *SMARCA4,* (network 2), *MYC, CASP1,* (network 3), *COL18A1, FGF2, ZNF217, MYCN* (network 4).

CEBPA is an important transcription factor whose expression level is incresed in prostate cancer. Our data have also revealed increased levels of expression of *CEBPA,* in accordance to the literature [32]. Over-expression of *FOXA1* is corelated with possibility of metastasis in prostate cancer [33].One of the main transcription factors involved in tumoural development is JUN. Our study revealed a high level of *JUN* expression in prostate cancer in comparison to benign prostate tissue. Data from literature indicate that isoform c-JUN may be involved in carcinogenesis with *NFKB1* [34].Genes identified in our study *RARA, RXRA, RORC* are not described in literature as being involved in the progression of prostate cancer.

SMARCA4 or *BRG1* have a very important role in control of cellular division, abnormal regulations of this gene have been identified in a series of cancers, such as prostate [35, 36]. *MYC* gene codifies another transcription factor whose expression is activated from the early stage or carcinogenesis in prostatic intraepithelial neoplasia [37]. Our data show increased expression levels of *MYC* and isoform *MYCN* in the localized prostate cancer with score Gleason 7 compared to benign prostate tissue. *CASP1* is involved in apoptotic activity, under-expression of this gene have been reported in literature in prostate cancer compared to benign prostate tissue [38]. The results of our study have confirmed the data. *COL18A1* and *FGF2* are involved in the modulation of angiogenesis, beeing directly related to the androgen receptors [39]. *ZNF217* is involved in proliferation and invasion, over-expression of this gene have been revealed in a series of cancers, including prostate cancer [40]. Up regulation of *ZNF217* has been observed in our study too, as this gene is a direct activator of *MYCN*.

Table 6 presents top ten genes with the highest and the lowest level of regulation in localised prostate cancer with score Gleason 7 compared to benign prostate tissue.

Table 6. Top 10 genes with the highest and the lowest expression levels in prostate cancer (Gleason 7) compared to benign prostate tissues.

Over-expressed genes		Under-expressed genes	
Gene symbol	**Fc**	**Gene szmbol**	**Fc**
OUD7A	**+25.05**	***CFD***	**-24.88**
GRIN1	**+23.38**	***CYP3A5***	**-24.01**
OR51E2	**+22.01**	***CXCL13***	**-23.67**
THBS4	**+21.23**	***BMP5***	**-18.46**
ATN	**+18.52**	***NELL2***	**-17.56**
HPN	**+18.18**	***RNF112***	**-17.09**
SOX18	**+17.35**	***PNMT***	**-16.52**
GOLM1	**+15.65**	***NPPC***	**-16.31**
CEBPA	**+15.61**	***MLC1***	**-15.91**
TARP	**+13.54**	***SMOC1***	**-14.68**

HPN, GOLM1, AMACR, SIM2, FOLH1 have been identified with similar levels of expression both in tumoral vs. benign prostate tissue (fc *HPN* = 18.18, fc *GOLM1* = 15.65, fc *AMAC*R = 11.16, fc *SIM2* = 8.93, fc *FOLH1* =11.29) as well as in tumoral vs. normal prostate tissue (fc *HPN* = 18.48, fc *GOLM1* = 16.79, fc *AMAC*R = 15.68, fc *SIM2* = 13.27, fc *FOLH1* = 10.87). In benign prostate tissue compared to normal tissue, these genes have not been differently expressed. These observations sustain that these molecules could be proposed for validation as markers that differentiate benign prostate cancer from normal prostate tissue.

Among molecules *GPR160* (fc = 9.25), *TMEFF2* (fc = 8.73) and *TMSB15A* (fc = 7.89) identified in the top ten up-regulated genes in prostate cancer compared to normal tissue, only *GPR160* (fc = 3.97) and *TMSB15A* (fc = 4.76) have been upregulated in prostate cancer compared to benign tissue. Over-expression of these genes have not been identified in the benign vs. normal prostate tissue.

CFD , RNF112 and *SMOC1* have been down regulated in the cancer vs. benign prostate tissue (fc *CFD* = -24.88, fc *RNF112* = -17.09, fc *SMOC1* = -14.68) and also in cancer vs.normal prostate tissue (fc *CFD* = -12.49, fc *RNF112* = -10.13, fc *SMOC1* = - 8.49). Likewise, C*GREF1, GJB1* and *E2F5* have been up regulated only in cancer compared to benign tissue (fc *CGREF1* = 6.01, fc *GJB1* = 3.18, fc *E2F5* = 3.33) and normal tissue (fc *CGREF1*= 8.63, fc *GJB1* = 8.57, fc *E2F5* = 2.93). In benign prostate vs normal tissue, these genes have not been identified as being up or down regulated, thus highlighting their

specificity in prostate cancer. In PubMed, studies regarding the implication of these genes in prostate cancer are scarce, one can only find studies concerning some of these genes in animal and cellular lines.

3.2.6 Validation of microarray data

We chosee three genes for validation by RT-PCR: *TERT, CAV1* (network no.1) and *FASN* (network no. 5). RT-PCR results was in a good agreement with microarray data.

3.3 Conclusions

The goal of microarray study has been the transcriptomic evaluation of prostate tissues using bioinformatics strategies. To achieve this goal Gene Spring GX and Limma software have been used. The results indicate relatively high differences between the two approaches, although the analysis algorithm has been the same. In general, with GS has obtained a greater number of differently regulated genes (for *tumour vs. benign, benign vs. normal*). Limma identified a greater number of differently regulated genes only in the *tumour vs. normal* comparison. The first significant difference between the numbers of sequences obtained by the two approaches appears after the pre-processing phase when the number of sequences obtained in GS was higher by 93 than the ones obtained with Limma. Because GS does not ensure access to each phase of pre-processing it is difficult to establish the source of these differences, considering that the algorithm used by the two approaches has been the same. Major differences appear after the differential analysis. In order to identify the differently regulated genes revealed in the studied groups, a t test and FDR correction have been applied using both software. The difference between the classic t test implemented in GS and the improved t tests implemented in Limma is that the latter estimate the variability considering not only the information of the tested genes but also that of other genes with similar variability. Moderate t test implemented in Limma offers solid results even when the distribution of data is not normal.

The differently expressed genes obtained with Limma have been considered to be of interest for the following molecular analysis. 1119 up- and down-regulated genes have been identified in cancer vs. normal prostate tissue, respectively 3002 differently expressed genes in cancer vs. benign prostate tissue. The most important alteration was observed in molecular mechanisms involved in: cellular growth and proliferation, cellular movement, genetic disorders, cellular adhesion, angiogenesis and apoptosis. Our data have put into evidence a

series of genes, up and down regulated, according to data in the medical literature. Among the genes obtained in our study with the highest levels of expression in tumor vs. normal, and respectively benign tissue are: *HPN*, *AMACR*, *GOLM1*, *SIM2* and *FOLH1*, all being recognised as possible biomarkers for prostate cancer.

Up regulated (C*GREF1, GJB1 and E2F5)* respectively down regulated genes (*CFD, RNF112 and SMOC1*) have been identified in tumour vs. normal and benign prostate tissue. For these, the information on PubMed regarding their involvement in prostate carcinogenesis are scarce and based only on animal models and cell lines. None of these genes have been differently expressed in benign tissue compared to normal prostate tissue, thus highlighting their specificity in prostate cancer (Gleason 7). These genes can be considered for validation as markers in prostate cancer. All these genes, with the groups of genes integrated in the molecular networks may contribute to a better understanding of molecular pathology of prostate cancer, Gleason 7.

4 Evaluation of molecular profile involved in angiogenesis. PCR array study

4.1 Materials and methods

4.1.1 Biological material

Thirty-six subjects have been included in this study: 19 patients with localized prostate adenocarcinoma, 11 patients with benign affections of prostate (prostate benign hyperplasia and chronic prostatitis) and 6 healthy subjects. Patients have been selected and diagnosed in the same conditions as in the mircoarray study. The blood samples have been collected before surgery

4.1.2 Total RNA isolation

Extraction of total RNA has been made using the same methods as in the microarray study.

4.1.3 PCR array reaction

PCR array reaction has been used in order to evaluate 84 genes involved in the modulation of angiogenesis mechanism.

4.1.4 Statistical analysis

$\Delta\Delta C_t$ method, t test and FDR correction for multiple testing have been used to establish statistical differences between the studied groups.

4.2 Results and discussions

The aim of PCR array study was to identifiy a molecular profile that would separate patients with prostate cancer from patients with benign pathology, respectively healthy subjects. As a control group, healthy subjects, patients with benign hyperplasia and with chronic prostatitis have been taken in consideration. The molecular signature has been validated by using *training and testing sets*. *The training set* included 13 subjects: 6 healthy subjects from control group and 7 patients with prostate cancer. *The testing set* included 23 subjects: 12 prostate cancer patients and 11 patients with benign prostate hyperplasia.

Using the *training set*, a molecular signature of 28 differently expressed genes has been identified. For all biological replicates, only genes with p value adjusted by FDR correction at 0.05 cut-off and a fold change ≥ 1.5 or ≤ - 1.5 have been taken in consideration. Supervised clusterization revealed two main clusters, one grouping prostate cancer patients and the other one grouping healthy subjects (Figure 18).

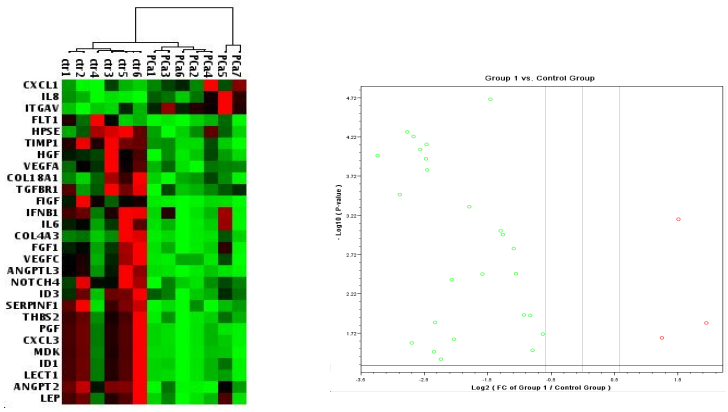

Figure 18. Supervised signature obtained in the *training phase* that separates patients with prostate cancer from healthy subjects. The right side of the figure presents the vulcano plot of the 28 genes with an adjusted p value <0.05 and a fc ≤ -1.5 or ≥ 1.5.

Supervised signature was validated on another set of subjects. The data obtained showed that the molecular signature identified on *training set* separates efficiently the patients from *testing set* as well. Two major clusters have been obtained, one grouping the patients with prostate cancer and the other grouping patients from control group (benign hyperplasia, chronic prostatitis and healthy subjects) (Figure 19).

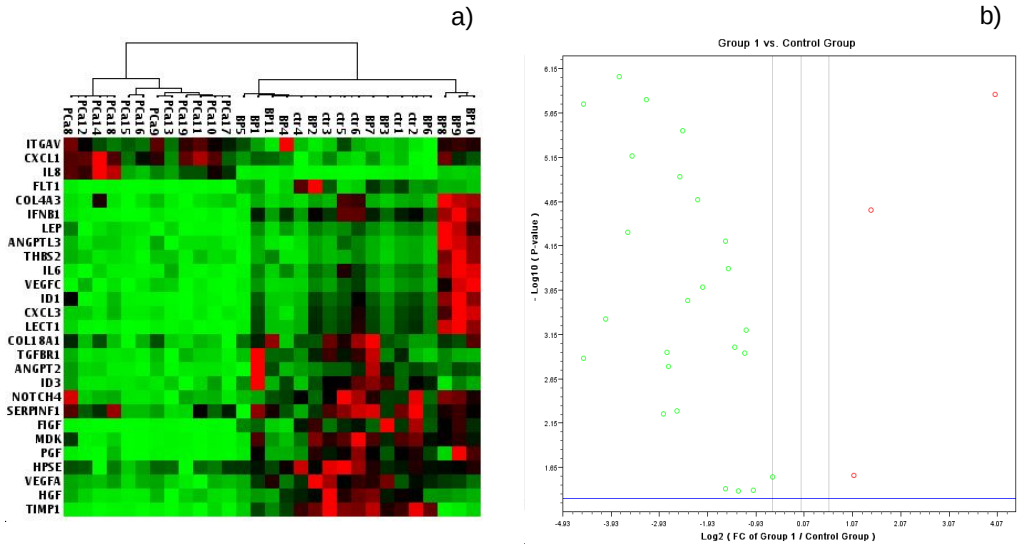

Figure 19. a) Supervised hierarchical clustering of patients from *testing group* (prostate cancer, hyperplasia, chronic prostatitis, and normal prostate tissue). b) Volcano plot for the 28 genes with an adjusted p value < 0.05 and a fc ≤ -1.5 or ≥ 1.5

The specificity of the molecular signature has been tested according to other tumour pathologies as well: cervical cancer (n=5), breast cancer (n=8), cholangiocarcinoma (n=6) and liver carcinoma (n=10). The supervised hierarchical clustering showed that cancer subjects are clustered separately from other tumoral pathologies (Figure 20).

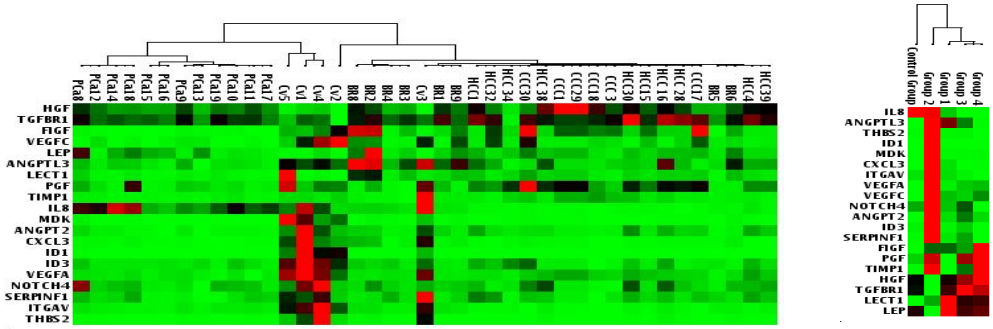

Figure 20. Supervised hierarchical clustering of patients with prostate cancer, cervical cancer (CV), breast cancer (BR), cholangiocarcinoma (CCC), liver carcinoma (HCC).

4.3 Conclusions

Our results indicate that a molecular signature based on a set of 28 genes involved in the modulation of angiogenesis may separate patients with prostate cancer from patients with benign affection of prostate (benign hyperplasia or hyperplasia associated with chronic prostatitis) and healthy subjects. Supervised signature obtained in this study is independent from PSA value, Gleason score and percentage of tumour cells in prostate tissue. The genes identified in this supervised signature are involved in modulation of angiogenesis, directly by stimulating the proliferation of endothelial cells and indirectly by modulating the interactions between the tumour and the host through an immune response and adhesion molecules [41,42,43,44,45]

5 Evaluation of serum proteins. FastQuant array study

5.1 Materials and methods

5.1.1 Biological material

Forty patients were included in this study: 7 patients with prostate cancer (PCa), 11 patients with benign prostatic hyperplasia (BPH) , 6 patients with chronic prostatitis (CP) and 6 healthy subjects (control group). Serum samples from all of patients were obtained before they received any treatment or underwent biopsies.

5.1.2 Fast Quant array technology

Fast Quant array technology was used for quantifying serum proteins. Fast Quant array combines capabilities of ELISA method with the power of array technology allowing simultaneous evaluation of up to eight proteins from any biological fluid.

5.1.3 Statistical analysis

Data were analyzed with *SPSS (Statistical package for Social Sciences)* software. Differences between studied groups were assessed by using t test; $p < 0.05$ was considered statistically significant.

5.2 Results and discussions

The aim of Fast Quant study was to evaluate the implication of a set of 8 angiogenic molecules (PDGF-BB, VEGF, FGF-b, ANG, KGF, TIMP-1, ICAM-1, ANGPT-2) in prostate cancer.

Four molecules (ANG, ICAM-1,VEGF şi FGF-b), out of eight analyzed, had values outside the standard curve in most of the samples, being imposible to quantify. Shapiro-Wilk test was applied to verify the normality of the distributions for ANGPT-2, KGF, PDGF-BB şi TIMP-1, which were quantified by standard curve. These molecules had normal distribution for all of the studied groups, allowing the use of parametric t test for statistical analysis. The results are presented in table 7.

Table 7. Significant differences for KGF, ANGPT-2 , PDGF-BB şi TIMP-1 ($p<0.05$).

	KGF		**ANGPT-2**		**PDGF-BB**		**TIMP-1**	
	t	p	t	p	t	p	t	p
Control/PCa	1.520	0.193	1.416	0.174	-1.306	0,211	5.077	**0,000**
Control/BPH	0.153	0.881	2.273	**0.038**	-3.198	**0.008**	4.240	**0.001**
Control/CP	0.512	0.621	2.623	**0,037**	-2.179	0.083	3.401	**0,008**
PCa/BPH	-2.447	**0.022**	1.308	0.204	-1.861	0.076	-0.369	0.716
PCa/CP	-2.071	**0.050**	1.944	0.068	-1.786	0.091	-0.277	0.785
BPH/CP	0.425	0.677	0.206	0.839	-0.552	0.591	0.022	0.983

PCa: Prostatic cancer. BPH: Benign Prostatic Hyperplasia. CP: Chronic Prostatitis

Our results revealed that the KGF angiogenic molecule was statistically significantly decreased in PCa (218.96 pg/ml) compared with BPH (371.28 pg/ml) and CP (334.68 pg/ml) (figure 21). KGF is a stromally-derived growth factor important in mediating androgen-induced activities in BPH and PCa. Our results are in concordance with Metha et al. who found lower levels of KGF in PCa than in BPH [46]. In normal prostate, KGF, also known as FGF-7, serves as a paracrine growth factor that is synthesized in stromal cells and acts on epithelial cells through its receptor. Huang et al. demonstrated that KGF exhibited mitogenic and anti-apoptotic effects that correlate with the induction of *CCND1* şi *BCL2* expression in prostate cell lines [47].

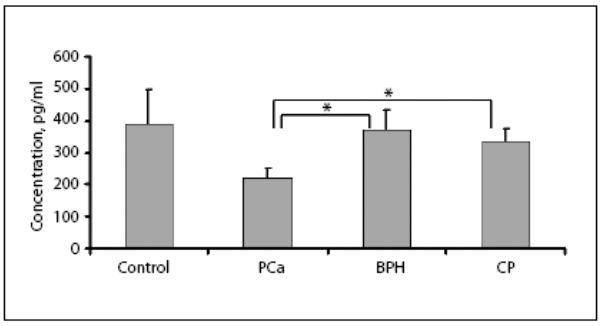

Figure 21. Mean serum concentration values of KGF molecule for Control, PCa, BPH and CP groups, obtained by Fast Quant analysis. (*p<0.05)

Another angiogenic molecule, ANGPT-2, showed statistically significantly decreased values in BPH (4728.05 pg/ml) and CP (4512.80 pg/ml) than in the control group (7632.25 pg/ml) (figure 22). The ANGPT-1 and ANGPT-2 are principal regulators of vascular growth and regression; however, the role of the angiopoietins is unknown in prostate pathology [48]. ANGPT-2 possesses anti-angiogenic properties [49]. ANGPT-2 may also induce endothelial cell apoptosis by disrupting the vascular remodelling ability of ANGPT-1 [50]. Abnormal levels of ANGPT-1, ANGPT-2 and their receptor Tie-2 were reported in PCa [51, 52].

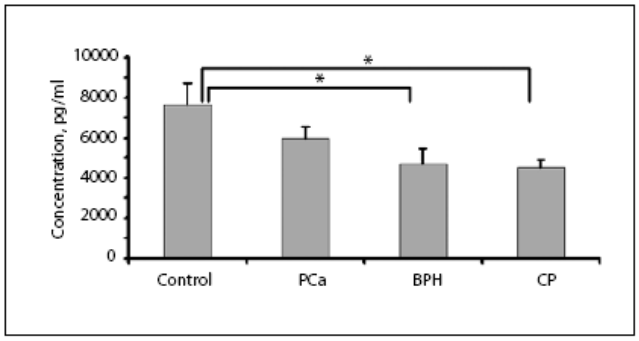

Figure 22. Mean serum concentration values of ANGPT-2 molecule for Control, PCa, BPH and CP groups, obtained by Fast Quant analysis. (*p<0.05)

Our study highlighted low levels of TIMP-1 protein in all groups (BPH (22984.35 pg/ml), CP (22891.10 pg/ml) and PCa (21832.70 pg/ml)) compared to the control group (46172.24 pg/ml) (figure 23). TIMP-1 is reported to inhibit angiogenesis directly by binding MMPs and inhibiting their activity, blocking cell proliferation and downregulating VEGF expression [53]. In PCa, there is an imbalance of MMPs and TIMP-1, with a significant loss of TIMP-1 [54, 55]. TIMP-1 has been shown to have a multifunctional role in PCa for

tumorigenesis, including inhibition of the catalytic activity of MMPs, growth promotion, inhibition of apoptosis and regulation of angiogenesis.

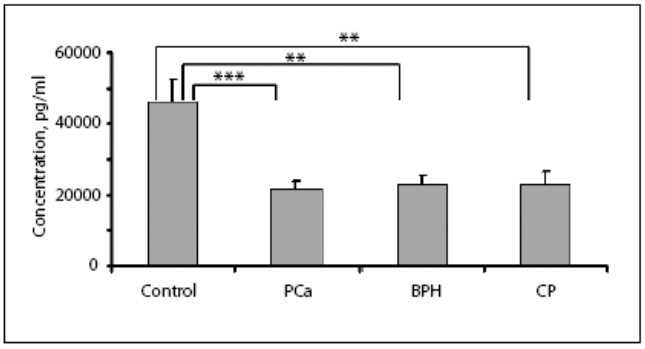

Figure 23. Mean serum concentration values of TIMP-1 molecule for Control, PCa, BPH and CP groups, obtained by Fast Quant analysis. (** $p < 0.01$, *** $p < 0.001$).

Increased levels of PDGF-BB protein was identified in BPH (10800.66 pg/ml) vs. control group (5988.72 pg/ml) (figure 24). PDGF-BB functions as a "competence factor" that induces a set of early response genes expressed in the G1 phase of the cell cycle, including p21WAF1/CIP1 a functional mediator of the tumor suppressor gene *TP53* at the G1/S checkpoint [56].

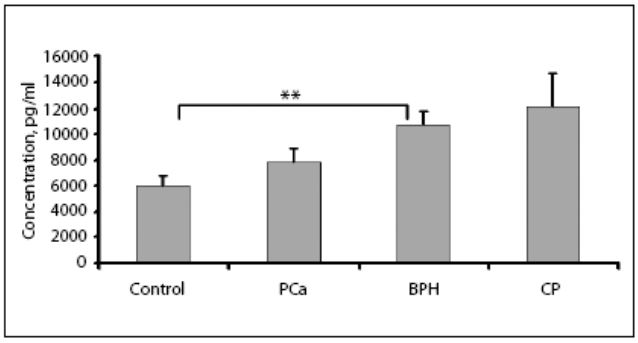

Figura 24. . Mean serum concentration values of PDGF-BB molecule for Control, PCa, BPH and CP groups, obtained by Fast Quant analysis (** $p < 0.01$).

5.3 Conclusions

Fast Quant array study highlighted different concentrations of angiogenic proteins (KGF, PDGF-BB, ANGPT-2 şi TIMP-1) in the serum of patients with prostate pathology. KGF concentration was statistically significant increased in benign prostatic diseases (BPH, CP)

compared to PCa. TIMP-1 were statistically significant decreased in serum of patients with benign and malignant prostatic diseases compared to healthy subjects. Taken together, TIMP-1 and KGF could be used to differentiate between prostate cancer and benign diseases or healthy subjects, but validation of these results requires a study on larger groups of patients.

6 General conclusions

The aim of this thesis was the multiple molecular evaluation on a tissue, blood and serum level using array technologies: *microarray, PCR array and Fast Quant array,* in order to identify the molecular differences in prostate cancer compared to benign affections of prostate and healthy subjects. The results allow to state the following general conclusions:

1) The bioinformatics analysis showed that the use of different software (Limma and Gene Spring GX) for microarray data analysis determines the variability of results even if the same algorithm was followed in both approaches.
2) A set of differently expressed genes has been identified (C*GREF1, GJB1* , *E2F5, CFD, RNF112* and *SMOC1*) in prostate cancer compared to benign and normal prostate tissue, which have been described on PubMed only on animal models, cell lines and other tumour localizations. None of these genes has been differently expressed in the benign tissue compared to the normal prostate tissue, thus highlighting their specificity in prostate cancer. These genes can be considered for validation as markers in prostate cancer.
3) A molecular signature made up of 28 genes, which separate the patients with prostate cancer from the patients with benign affection of prostate and healthy subjects was identified. The signature is independent from PSA value, the Gleason score and the precentage of tumour cells in the prostate. The validation of this molecular signature on a high number of patients can provide new input which can be important in prostate pathology. The confirmation of this signature may represent an alternative to reduce the number of prostate biopsies in patients with benign affection of prostate.
4) Angiogenic proteins evaluated in the serum, taken separately, was not specific to the tumor pathology of prostate but the seric evaluation of a *panel of angiogenic proteins* can provide useful information for this pathology.

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