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Expression of Secreted Frizzled-Related Protein 1 and 3, T-cell Factor 1 and Lymphoid Enhancer Factor 1 in Clear Cell Renal Cell Carcinoma

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Abstract

Frequency and mortality of renal cell carcinoma (RCC) are increasing for decades. However, the molecular background of RCC tumorigenesis is still poorly understood. In current study we investigated the expression of TCF/LEF and SFRP family members (SFRP1 and SFRP3) to gain a better understanding of biological signaling pathways responsible for epidemiology and clinical parameters of clear cell RCC (cRCC).

36 pairs of paraffin-embedded clear cRCC and adjacent nontumoral tissues samples using immunohistochemistry (IHC) were analyzed and compared with corresponding clinicopathological parameters.

Immunohistochemistry indicated statistically significant decreased SFRP3 expression in tumor tissues but no consistency in SFRP1 expression in analyzed normal and tumor tissue. The TCF1 expression level was significantly weaker in normal tissue compared to tumor samples while LEF1 protein levels were significantly weaker in tumor tissue.

To our knowledge, this is the first report on analysis of the expression of transcription factors TCF1 and LEF1 in clear cell renal cell carcinoma and their comparison with Wnt signal pathway antagonists belonging to SFRP family.

Introduction

Dysregulation of Wnt signaling is common in a variety of human malignancies. Therefore, to further explore the role of Wnt signaling in renal cell carcinoma, we investigated the expression of TCF/LEF transcription factors (TCF1 and LEF1) and SFRP family members (SFRP1 and SFRP3) using immunohistochemistry (IHC). All of these proteins play signaling roles as components of the Wnt signal transduction pathway. In most instances, constitutive signaling through the beta-catenin pathway involves activation of effector molecules or loss of tumor suppressor function downstream of Wnt ligands binding to its cell surface receptors. In the nucleus, beta-catenin relieves inhibition of transcription factors T-cell factor (TCF)/lymphoid enhancer factor (LEF) that was maintained by repressors, leading to transcription of target genes, such as c-myc, matrix metalloproteinase (MMP)-7, cyclin D1, etc. [1, 2]. LEF1 and TCF1 are members of the high mobility group (HMG) DNA binding protein family of transcription factors which consists of the following: Lymphoid Enhancer Factor 1 (LEF1), T Cell Factor 1 (TCF1, also known as TCF7), TCF3 (also known as TCF7L1) and TCF4 (also known as TCF7L2) [3]. LEF1 and TCF1 were originally identified as important factors that act downstream in Wnt signaling regulating early lymphoid development [4]. LEF1 and TCF1 bind to Wnt response elements to provide docking sites for β-catenin, which translocate to the nucleus to promote the transcription of target genes upon activation of Wnt signaling [5]. LEF1 and TCF proteins are dynamically expressed during development and aberrant activation of the Wnt signaling pathway is involved in many types of cancers including colon cancer [6, 7].

The secreted Frizzled-related proteins (SFRP) are the largest family of Wnt inhibitors. SFRPs decrease beta-catenin stabilization and promote cell death even in cells that have downstream mutations in the beta-catenin pathway.

SFRP1 is a 35 kDa secreted glycoprotein that is a prototypical member of the SFRP family and has been reported to bind Wnt ligands and modulate their signaling activity [8, 9]. It acts as a biphasic modulator of Wnt signaling, counteracting Wnt-induced effects at high concentrations and promoting them at lower concentrations [9]. It is located in a chromosomal region (8p12- p11.1) that is frequently deleted in some cancers and is thought to harbor a tumor suppressor gene [10]. Among Wnt antagonist families, secreted frizzled-related protein (SFRP3) is generally thought to be an inhibitor of Wnt signaling in several cancers.

Materials and methods

Tumor specimen

Samples of 36 renal cell carcinoma were collected from the Department of Pathology, University Hospital "Merkur", Zagreb, Croatia. The tumor tissues were formalin fixed paraffin embedded**.** The patients had no family history of RCC tumors. All tumors were studied by pathologists and classified as Clear Cell Renal Cell Carcinoma according to the WHO criteria. The pathohistological classification, TNM stage and histopathological grading are shown in Table 1

Twenty-five patients were male and 11 female. The age of patients varied from 30 to 78 (mean age = 61.4 years). The mean age at diagnosis for males was 59.9, and for females 65 years.

The local Ethical Committee approved our study and patients gave their informed consent.

Immunohistochemistry

Immunohistochemistry was performed in order to establish the levels of expression and cellular localization of SFRP1, SFRP3, TCF1 and LEF1 proteins. The samples were formalin-fixed, paraffin embedded, and 4-μm thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). The sections were immunostained using the biotin–avidin–horseradish peroxidase method. Deparaffinized and rehydrated sections were microwaved in Dako Target Retrieval Solution (Dako Corporation, USA) three times for 5 min at 800 W to unmask epitopes. To block endogenous peroxidase activity, we fixed the cells in methanol containing 3% H_2O_2. Non-specific binding was blocked by the application of normal mouse serum for 30 min in a humid chamber. Slides were blotted and primary antibodies at optimized dilutions were applied for 30 min at room temperature. The antibodies used for protein detection were: rabbit polyclonal anti-human SFRP1 (1:200), rabbit polyclonal anti-human SFRP3 (1:200), mouse monoclonal anti-human LEF1 (1:50) and for TCF1 (1:50) mouse monoclonal anti-human TCF1, all Santa Cruz Biotechnology, USA. After incubation, the slides were washed three times in phosphate-buffered saline/goat serum. Secondary LINK antibody was applied for 25 min. The washing was repeated, and the slides were incubated with streptavidin horseradish peroxidase for another 25 min. All chemicals were from DakoCytomation. Negative controls were samples that underwent same staining

procedure with the exclusion of the primary antibodies. The analysis of the labeling was performed by two independent observers.

Quantitative stereological analysis of numerical density (Nv)

Randomly selected paraffin blocks were used for stereological analysis. Quantitative stereological analysis of numerical density (Nv) was performed by Nikon Alphaphot binocular light microscope (Nikon, Vienna, Austria) using Weibel's multipurpose test system with 42 points (M 42) at magnification of 400x [12]. The area tested (A_t) was 0, 0837 mm^2. For each investigated group the orientation/pilot stereological measurement was carried out in order to define the number of fields to be tested [12]. The numerical density of positive cells was determined according to the point counting method [12]. Numerical density (Nv) was calculated by formula: $Nv=N/A_t \times D$, where N is number of positive cells on tested area [13, 14]. The mean tangential diameter (D) calculated by Ellipse3D for 100 cells were 0, 00917 mm.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.01, (GraphPad Software, Inc., San Diego, CA, USA) and also Principal component analysis (PCA) was done using Matlab Software PLS Toolbox. The stereological data were evaluated by descriptive statistics. Distribution of the data was assessed by Kolomogorov-Smirnov test, Lilliefors test and Shapiro-Wilks W-test. Differences in numerical density of cells in investigated groups were analyzed with Kruskal-Wallis ANOVA. Statistical significance was set at $p < 0.05$.

Results

In this study we analyzed 36 pairs of paraffin-embedded clear cell RCC and adjacent nontumoral tissues samples proved by Department of Pathology Clinical hospital Merkur Zagreb, Croatia.

SFRP1 expression was observed in the nucleus and also occasionally in the cytoplasm. In renal tissue SFRP1 expression was found in tubules and glomeruli cells (Fig 1A, B). Interestingly, we did not find consistency in SFRP1 expression in analyzed normal and tumor tissue. Quantitative analysis revealed 51, 5% of analyzed nontumoral samples showed higher number of SFRP1 positive cells in comparison to tumor tissue. In 18, 2% of analyzed samples number of SFRP1 positive cells was approximately equal in both normal and tumor tissue whereas in 30, 3% of analyzed tumor samples number of SFRP1 positive cells was higher compared to adjacent normal tissue. The percentage of SFRP1 positive tumor tissues was not statistically significant correlated with the degree of tumor differentiation, nor with corresponding clinicopathological parameters.

Subcellular localization of SFRP3 protein in renal tissue was observed in perinuclear region of tubules and glomeruli cells (Fig. 1C, D). We found statistically significant difference in number of SFRP3 positive cells between normal and tumor tissues ($p< 0, 05$) (Fig 2). The amount of SFRP3 protein expression in normal tissues was higher compared to the one observed in tumor tissue. Principal component analysis (PCA) performed with Matlab Software PLS Toolbox confirmed that SFPR3 expression contributes the most to the difference between normal and tumor tissue group (Fig 3 A, B).

TCF1 expression was observed in the nucleus of tubules cells in a renal tissue (Fig. 4A, B). Here also, we revealed statistically significant difference in number of TCF1 positive cells between normal and tumor tissues ($p< 0, 05$) (Fig. 2). The number of TCF1 positive cells was significantly minor in normal tissue compared to tumor samples.

LEF1 protein was also detected in the nucleus of tubules and glomeruli cells in renal tissue (Fig. 4 C, D). We found statistically significant difference in analyzed number of LEF1 positive cells between normal and tumor tissues ($p< 0, 05$) (Fig. 2). The amount of LEF1 protein expression in normal tissues was higher compared to the one found in tumor tissue. We also notice negative correlations between SFRP3 and TCF1 ($r= -0, 46$), LEF1 and TCF1 protein expressions, and positive correlation between SFRP3 and LEF1 protein expressions ($r= 0, 46$), (Fig. 5.) Mean values of numerical density (Nv) all four proteins are presented in Table 2.

Discussion

The SFRP family plays an important role in inhibition of the Wnt signaling pathway. The SFRP family show reduced expression in several types of carcinomas, which is associated with unfavorable clinical outcome [15].

As far as we know our study is a first attempt to analyze expression of transcription factors TCF1 and LEF1 in clear cell renal cell carcinoma and their comparison with Wnt signaling pathway antagonists belonging to SFRP family. SFRP1 competitively binds to Wnt molecules, thereby preventing their binding to the cognate Frizzled receptors and therefore act as a negative modulator of the Wnt pathway.

Loss of SFRP1 has been reported in many human malignancies including RCC [16-19]. Levels of SFRP1 mRNA have been found to be reduced in human cRCC samples taken at different stages of the disease [16].

In their recent study (2009) Saini et al., observed similar downregulation (mRNA level) of SFRP1 expression in primary RCC cell lines. However they also found augmented SFRP1 expression in metastatic RCC cell lines [20].

Immunohistochemical data collected in the present study also indicate lower amount (although statistically insignificant) of SFRP1 expression in 51,5% of analyzed primary cRCC tumor tissue samples. Interestingly, in 30, 3% of primary cRCC analyzed in our study exhibited higher amount SFRP1 expression on a protein level compared to adjacent normal tissue. Since all of these primary tumor samples with higher amount SFRP1 expression were either obtained from patients with detected metastatic dissemination or had high Fuhrman grade we can speculate that registered augmentation of SFRP1 expression, later on in metastatic tumor grades has its potential origine already in primary tumor tissue settings. Although in most studies SFRP1 is considered as tumor suppressor gene there are several reports that offer another view of the activity and regulation of secreted Wnt antagonists in different tumor tissues [19]. Notably, the *SFRP1* gene was up regulated in prostate carcinoma derived from stromal cells and also in prostate carcinoma experimental model in which progressively advanced carcinoma cells acquired the expression of SFRP1 [21].

These results suggested that SFRP1 expression may be subjected to differential regulation during the renal cancer progression and metastasis.

Human FRZB/SFRP3 has been mapped to human chromosome 2q31-33 [11]. SFRP3, another Wnt pathway antagonist, reduces activity of metalloproteinases and activation of β-catenin and thus inhibits epithelial-mesenchymal transition (EMT) seen in several cancer

types [22, 23]. We observed statistically significant decreased of the amount of SFRP3 protein expression in our total renal cancer clear cell tumors compared with normal kidney tissues. Using Principal component analysis (PCA) with Matlab Software PLS Toolbox we discovered SFPR3 expression pattern contributes the most to the difference between normal tissue control and tumor group. This data confirm the tumor-suppressing activities of FRZB/SFRP3. Hiroshi Hirata and his group [24] compared SFRP3 protein expression levels between normal kidney, primary renal cancer, and metastatic renal cancer tissues using tissue microarray. The percentage of samples expressing SFRP3 was lower in primary cancer tissues compared with normal kidney tissues. However, the percentage of samples expressing SFRP3 was significantly higher in metastatic renal cancer tissues compared with primary renal cancer tissues.

Wnt signaling controls the cell behavior by steering the transcriptional properties of DNA binding proteins belonging to the TCF/LEF1 family. In the absence of Wnt signaling TCF/LEF1 associate with corepresssors and blocks expression of Wnt target genes [25]. Since TCF/LEF1 factors cannot activate transcription on their own, they need co-activator, β-catenin, which possesses multiple transactivating elements that can also operate independently of TCF/LEF1. There is a strict correlation between the ability of β-catenin to function in Wnt signaling and its ability to transactivate [26].

Since the discovery of TCF family, the functions of its members have been under immense investigation in the area of cancer biology. Although TCF1 plays an important role in developmental biology, its potential role in cancer progression still remains to be fully investigated. There have been no reports regarding expression of TCF1 and its isoforms in RCC. We revealed that amount of TCF1 expression was significantly weaker in analyzed normal tissue compared to tumor tissue.

The human *LEF1* gene is located at chromosome 4q23-25, the region not known to be involved in clear cell renal cell carcinoma. Nevertheless, we explored the possibility that changes in LEF1 protein level could contribute to the development of clear cell renal cell carcinoma. We observed statistically significant differences in amount of LEF1 expression between normal and tumor tissue. The amount of expression in normal tissues was higher as compared to the amount of expression in tumor tissue. This finding may indicate that LEF1 is not equally important as transcription factor in cRCC. Observed, statistically significant correlation between LEF1 and SFRP3 expression indicate a positive relationship of LEF1 and SFRP3 protein expression. Our result of statistically significant correlation between SFRP3 and TCF1 expression could indicate that in given circumstances SFRP3 downregulation

promotes TCF1 induced β-catenin transactivation of target genes, and that the negative correlation between LEF1 and TCF1 could suggest that in cRCC tumorigenesis exert differential functions.

Reported expression of TCF1 and LEF 1 proteins in clear cell renal cell carcinoma is novel finding necessitating further research in order to establish their exact role in tumorigenesis of cRCC.

Conclusion

Current study represents the first report on TCF1 and LEF1 expression in clear cell renal cell carcinoma compared with Wnt signal pathway antagonists from the SFRP family. Observed differential expression of TCF1 and LEF1 transcription factors as well as SFRP3 in analyzed tumor and normal tissue samples indicates their involvement in cRCC tumorigenesis. However deciphering of their precise role in these processes requires additional studies involving among other more comprehensive methodological approaches and higher number of corresponding tissue samples.

I hereby certify absence of actual or potential conflict of interest in relation to this article.

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Figure description

Table 1. Clinicopathological parameters of 36 clear cell renal cell carcinomas analyzed in this study

Patient number	TNM classification	Fuhrman	age	sex
1.	T3b, N0, M1	II	51	M
2.	T1b, Nx, Mx	I	75	M
3	T1b, Nx, Mx	I	61	M
4.	T3, N0, M1	IV	72	M
5.	T1a, Nx, Mx	II	57	M
6.	T1a, Nx, Mx	II	67	F
7.	T1b, Nx, Mx	II	52	F
8.	T3b, Nx, Mx	III	73	F
9.	T1a, Nx, Mx	II	30	M
10.	T1b, Nx, Mx	III	43	M
11.	T1b, Nx, Mx	II	47	M
12.	T1b, Nx, Mx	II	78	M
13.	T1b, Nx, Mx	III	64	M
14.	T3b, N0, M1	IV	56	M
15.	T1b, Nx, Mx	II	62	F
16	T2, Nx, Mx	III	67	M
17.	T1a, Nx, Mx	III	62	M
18	T3, N0, Mx	IV	59	M
19.	T2, Nx, Mx	III	53	M
20	T1a, Nx, Mx	I	72	F
21.	T2, Nx, Mx	III	42	M
22.	T1a, N0, Mx	I	46	F
23.	T2, Nx, Mx	III	58	M
24	T3b, N0, M1	II	64	M
25.	T3b, Nx, Mx	IV	60	F
26.	T1a, Nx, Mx	II	67	F
27.	T2, Nx, Mx	II	61	M
28.	T1b, Nx, Mx	II	68	F
29	T1b, Nx, Mx	II	64	M
30.	T1b, Nx, Mx	II	70	F
31.	T1a, Nx, Mx	II	77	M
32	T1b, Nx, Mx	III	75	M
33.	T1b, Nx, Mx	I	78	F
34	T2, Nx, Mx	II	57	M
35.	T2, N2, Mx	II	60	M
36.	T3b, Nx, Mx	II	65	M

Table 2. Mean values and standard error of mean (SEM) for numerical densities (Nv) of SFRP3, TCF1, LEF1 and SFRP1 positive cells in tumors and adjacent control tissue.

	SFRP3 Nv (mm^{-3}, ±SEM)	TCF1 Nv (mm^{-3}, ±SEM)	LEF1 Nv (mm^{-3}, ±SEM)	SFRP1 Nv (mm^{-3}, ±SEM)
Tumor	30212,8±1431,8	19452±1040,87	11736,5±545,547	37507,2±4250,5
Control	62080,6±2105,59	5524,22±287,554	24405,2±926,49	42758,2±4609,43

Figure 1. Clear cell renal cell carcinoma immunohistochemically stained for protein expression of SFRP1 and SFRP3. Expression of SFRP1 protein in normal renal tissue (A) Expression of SFRP1 protein in cRCC (B) Expression of SFRP3 protein in normal renal tissue (C). Expression of SFRP3 protein in cRCC (D).

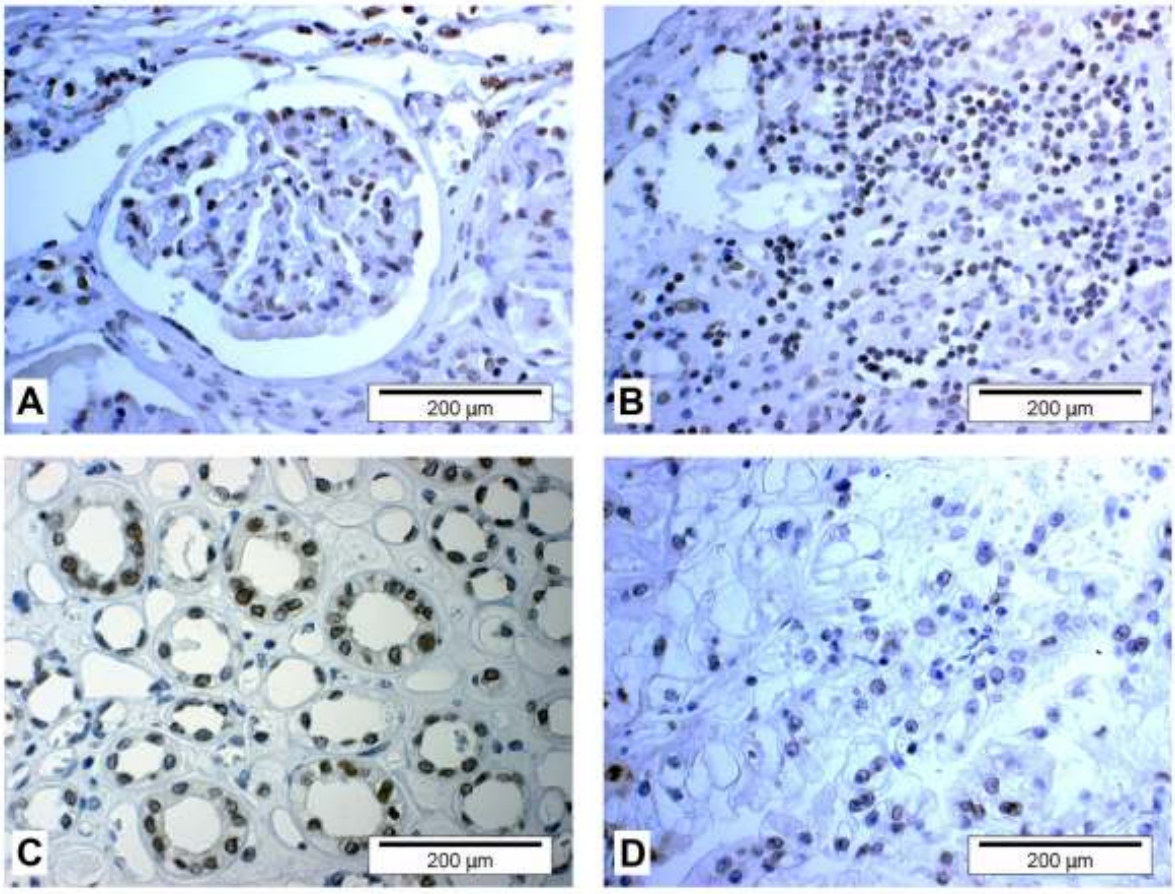


Figure 2. Significant difference in average numerical density (Nv; mean values and standard error of the mean) of SFRP1, SFRP3, TCF1, LEF1 positive cells analyzed between tumor and adjacent nontumoral (control) tissue.

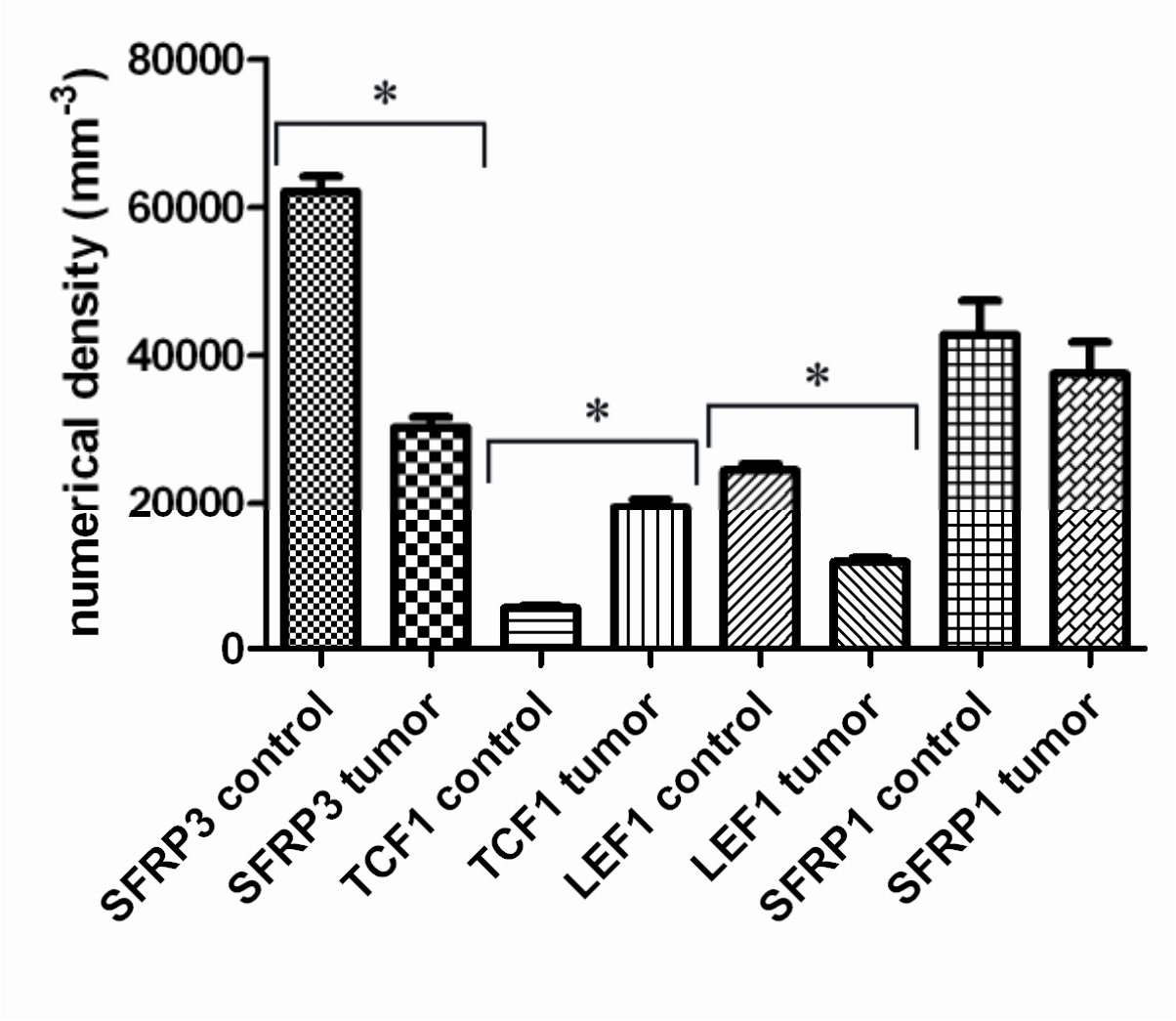
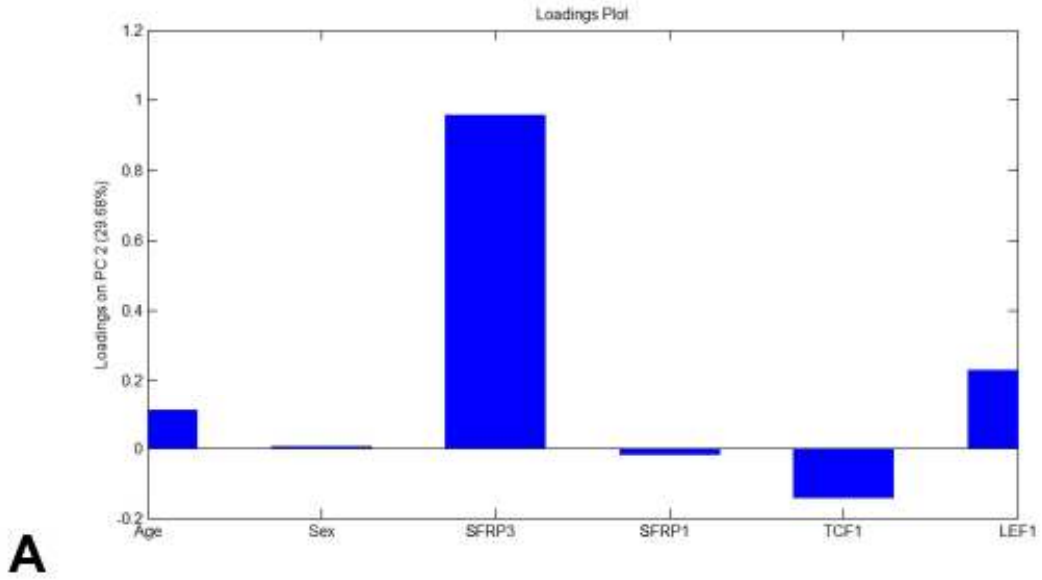
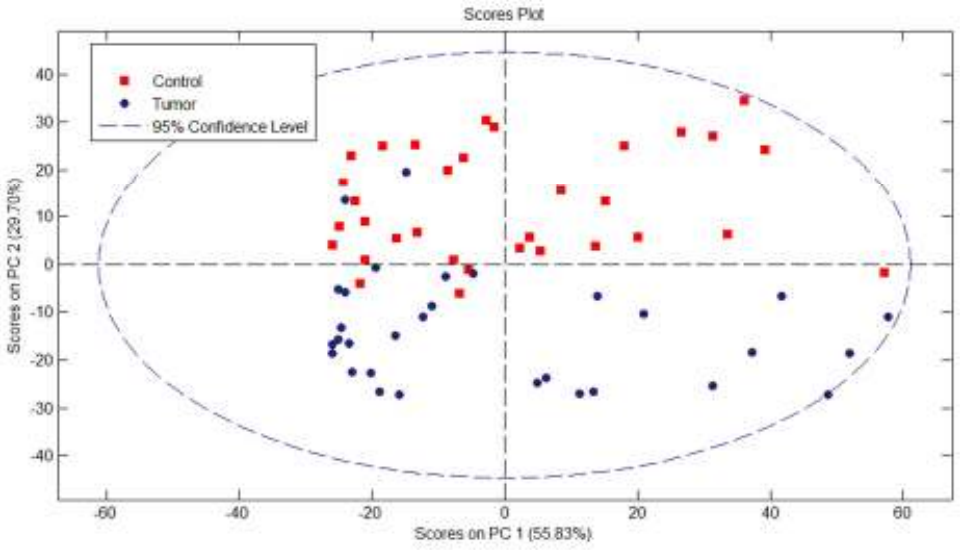


Figure 3. Scores plot showing separation between tumour and control samples (A). Loadings plot showing parameters contribution to the separation of tumor and control (B). Comparing loadings with scores plot it can be seen that control samples have higher values of SFPR3 and LEF1 than tumor while tumor samples have higher values of TCF1 than control samples.



A



B

Figure 4. Clear cell renal cell carcinoma immunohistochemically stained for protein expression of TCF1 and LEF1. Expression of TCF1 protein in normal renal tissue (A) Expression of TCF1 protein in cRCC (B) Expression of LEF1 protein in normal renal tissue (C). Expression of LEF1 protein in cRCC (D).

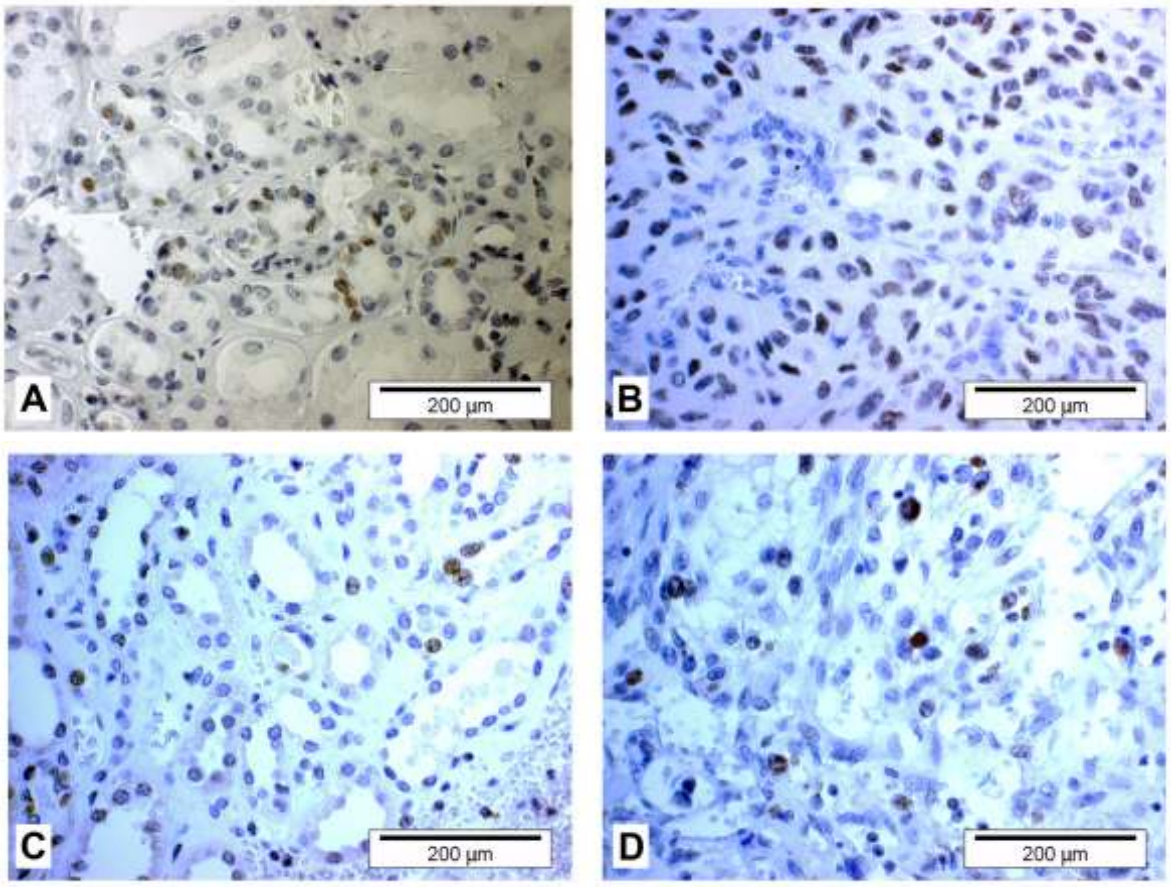


Figure 5. Correlation analysis between values of numerical density SFRP3, TCF1 and LEF1 proteins. Both axes represent protein numerical density (Nv).