

ORIGINAL ARTICLE

## JAK/STAT pathway interacts with intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) while prostate cancer stem cells form tumor spheroids

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### Summary

**Purpose:** JAK/STAT is an evolutionarily conserved pathway and very important for second messenger system. This pathway is important in malignant transformation and accumulated evidence indicates that this pathway is involved in tumorigenesis and progression of several cancers. It was possible to assume that activation of JAK/STAT pathway is associated with increase in the expressions of ICAM-1 and VCAM-1. In this study we hypothesized that when cells were maintained as spheroids or monolayers, the structure of cancer stem cells (CSCs) could show differentiation when compared with non-CSCs.

**Methods:** DU-145 human prostate cancer cells were cultured using the Ege University molecular embryology laboratory medium supplemented with 10% fetal bovine serum. Clusters of differentiation 133 (CD133)(+high)/CD44(+high) prostate CSCs were isolated from the DU145 cell line by using BD FACSAria. CD133<sup>+</sup>/CD44<sup>+</sup> CSCs were cultured until confluent with 3% noble agar. The expression of these proteins in CSCs and non-CSCs was analyzed

by immunohistochemistry.

**Results :** Different expression profiles were observed in the conventional two-dimensional (2D) and three-dimensional (3D) experimental model system when CSCs and non-CSCs were compared. Human prostate CSCs exhibited intense ICAM-1 and VCAM-1 immunoreaction when compared with non-CSCs. These findings were supported by the fact that VCAM-1 on the surface of cancer cells binds to its counter-receptor, the  $\alpha 4\beta 1$  integrin (also known as very-late antigen, VLA-4), on metastasis-associated macrophages, triggering VCAM-1-mediated activation of the phosphoinositide 3-kinase growth and survival pathway in cancer cells.

**Conclusions:** The results of this study showed that changes in JAK/STAT pathway are related with adhesion molecules and could affect cancer progression.

**Key words:** ICAM, JAK/STAT pathway, prostate cancer stem cell, tumor spheroid, VCAM

### Introduction

The CSC hypothesis proposes that tumors contain only a small subpopulation of cells which is responsible for tumor initiation, growth, and recurrence. During early tumor development, CSCs can not only form tumorigenic daughter CSCs by symmetrical cell division but also generate bulk

populations of non-tumorigenic cells by asymmetrical cell division [1]. The majority of cells in the bulk population of tumor cells has been shown to proliferate at a faster rate than CSCs, but have little tumor-initiating potential. CSCs have been previously isolated and identified using spe-

cific markers such as CD44, CD133, CD24,  $\alpha 2\beta 1$  integrin and aldehyde dehydrogenase1 (ALDH1), among others [2]. Among them, the cell surface protein CD133, also known as prominin1 or AC133 (a glycoprotein comprising of five transmembrane domains), has been described as a marker of stem and progenitor cells in several organs and appears to be the CSC marker for a wide range of tumor types. CD133<sup>+</sup> tumor cells are the prostate stem cells responsible for self renewal, differentiation potential, signal pathway, drug-resistance, recurrence and tumor prognosis [3]. The transmembrane CD44 protein is a ubiquitous multi-structural and multi-functional cell surface glycoprotein involved in cell adhesion, migration, homing, proliferation, survival, apoptosis and stemness regulation of CSCs. The complexity of CD44 alternative splicing and a range of variant isoforms generated (CD44v) reflect the diversity of CD44 function in the niches of many stem cell types [4]. Patrawala et al. Recently showed that CD44<sup>+</sup> prostate cancer cells have stem-like properties of increased tumorigenic, clonogenic, and metastatic potential [5]. In addition, a CD44<sup>+</sup> population from xenograft tumors displayed enhanced proliferative potential and tumor-initiating ability *in vivo*. Collins et al. have demonstrated that prostate cancer tumorigenic cells from patients have CD44<sup>+</sup>/ $\alpha 2\beta 1$ <sup>high</sup>/CD133<sup>+</sup> phenotype [6]. Therefore, CD133<sup>+</sup>/CD44<sup>+</sup> cells could be potential targets of antitumor therapy in the future.

The JAK/STAT pathway consists of three main components: A receptor, Janus kinase (JAK) and signal transducer and activator of transcription (STAT) families [7]. JAK/STAT signaling plays a central role in principal cell fate decisions, regulating the processes of cell proliferation, differentiation, apoptosis, immune cell development and angiogenesis [8]. In different experimental models tumorigenesis is linked to increased expression and/or activity of JAK1,2 or STAT3,5 [9]. Dysregulation of the JAK/STAT pathway is frequently observed in the development of several types of cancers and leads to enhanced survival of tumors and the metastatic potential of solid tumors [10]. Intercellular adhesion molecule-1 (ICAM-1), is an important molecule in cell-cell and cell-extracellular matrix interactions, and has crucial roles in tumor growth and metastasis. Its expression is transcriptionally regulated by the cytokine IL-6 through the JAK/STAT pathway via the IRE promoter site. Links between JAK/STAT signalling and ICAM-1 have also been associated with increased migratory and invasive potential of can-

cer cells [11]. Gene expression profiling of prostate cancer stem cells (CD44<sup>+</sup>/ $\alpha 2\beta 1$ <sup>high</sup>/CD133<sup>+</sup>) showed that IL-6 and members of the JAK/STAT pathway were overrepresented in CSCs relative to differentiated cells [12]. If the JAK/STAT signalling pathway is indeed important for prostate CSC maintenance, this could represent a potential therapeutic target.

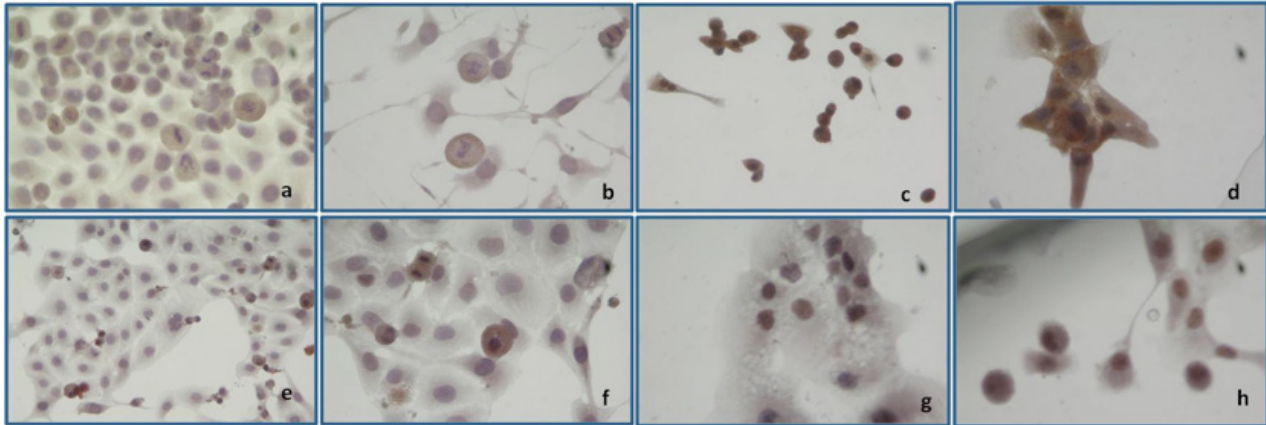
The traditional monolayer two-dimensional (2D) culture, in which only cell-cell flat contact and limited cell-matrix contacts are involved, fails to provide unique perspectives on the behavior of CSCs due to insufficient structural, mechanical and biochemical cues. However, a well-defined three-dimensional (3D) cancer model, which mimics features of the *in vivo* environment and allows cell-cell and cell-matrix contacts, provides a better opportunity to understand crucial cancer mechanisms for a wide variety of diagnostic and therapeutic applications [13]. There is overwhelming evidence that *in vitro* 3D tumor cell cultures more accurately reflect the complex *in vivo* microenvironment than simple 2D monolayer, with respect to proliferation, differentiation, gene expression profiles, signaling pathway activity, drug sensitivity, protein synthesis, morphology and viability [14]. *In vitro*, CSCs in serum-free medium form 3D cellular aggregates, called spheroids, named mammospheres, oligospheres, mesospheres and others; spheroid structures with stem cell-like characteristics contributed to tumor generation, progression and chemotherapy resistance.

In the present study we hypothesized that when cells were maintained as spheroids or monolayers, the structure of CSCs could show differentiation when compared with non-CSCs. Differentiation of JAK/STAT pathway and its related molecules ICAM and VCAM were investigated and compared in both monolayer and spheroid cultures. By immunohistochemistry, we analysed the expression of these proteins (JAK-1,2, STAT-2,5, ICAM-1 and VCAM) in CSCs and non-CSCs. Different expression profiles were observed in the conventional 2D and 3D experimental model system when CSCs and non-CSCs were compared. These molecules functioning in tumor progression and differentiation may help develop targeted therapies.

## Methods

### Cell culture

The DU145 human prostate cancer cell line was supplied by Ege University Tulay Aktas Oncology Hos-



**Figure 1.** Immunohistochemical staining of JAK1 and JAK2 in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate (CSCs) and non-CSR. **a:** JAK1 immunostaining in monolayer prostate CSCs (200x); **b:** JAK1 immunostaining in monolayer prostate CSCs (400x); **c:** JAK1 immunostaining in monolayer non-CSCs (200x); **d:** JAK1 immunostaining in monolayer non-CSCs (400x); **e:** JAK2 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (200x); **f:** JAK2 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (400x); **g:** JAK2 immunostaining in monolayer non-CSCs (200x); **h:** JAK2 immunostaining in monolayer non-CSCs (400x).

pital Laboratory, Turkey. DU145 cells were grown in monolayer culture for 4 days in RPMI1640 Medium (Lonza, UK) supplemented with 10% fetal bovine serum (Biowest, France), 1% L-Glutamine (Thermo Scientific, USA), and 1% Penicillin/Streptomycin (Thermo Scientific, USA). Once confluent, the cells were harvested using 0.25% Trypsin-EDTA (Gibco, Life Technologies, USA), thereby adding cell culture media to dilute trypsin and centrifuged (Nuve 200, Turkey). After removal of supernatants, the cells were resuspended in culture medium.

#### Fluorescence activated cell sorting (FACS)

DU145 human prostate cancer cells were detached and  $3 \times 10^6$  cells were incubated with 10  $\mu$ l CD133/1 (AC133)-PE (Miltenyi Biotec, USA) and 10  $\mu$ l CD44-FITC (Biolegend, UK) antibodies for 20 min at room temperature, in the dark. After incubation, the cells were washed with FACS washing solution (BD Biosciences, USA) and resuspended for sorting CD133<sup>+</sup>/CD44<sup>+</sup> CSC subpopulation with FACS Aria (BD Biosciences, USA).

#### Formation of spheroids

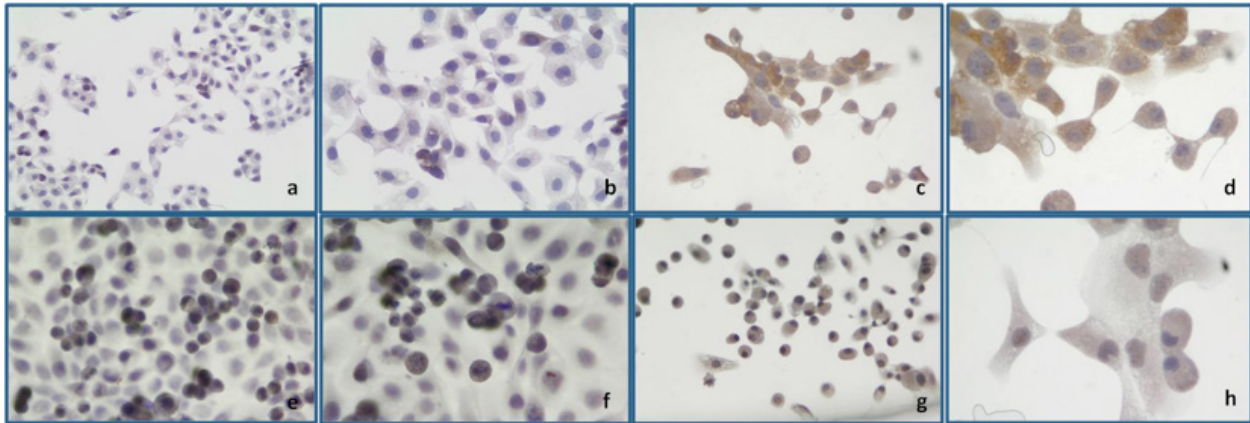
CD133<sup>+</sup>/CD44<sup>+</sup> CSCs were cultured until confluent, counted and  $1 \times 10^4$  cells were planted per well in a 6-well plate (Sarstedt, DE) coated with 3% noble agar (Difco Laboratories, USA). Cells were incubated at 37°C with 5% CO<sub>2</sub> and fresh medium was added every 2 days. Two weeks after plating, the wells were analyzed for spheroid formation.

#### Immunohistochemical staining

Monolayer cultures of CD133<sup>+</sup>/CD44<sup>+</sup> CSCs were

initiated in 12-well plates. At ~80% confluence, cells were fixed in 4% paraformaldehyde for 30 min at +4°C. Cells were washed with PBS 3 times and permeabilized using 0.01% Triton X-100 for 15 min at room temperature. Cells were incubated with blocking serum (Invitrogen, USA) for 30 min and incubated with primary antibodies (dilution of 1:100, at 4°C overnight) anti-JAK1 (sc-1677, Santa Cruz Biotechnology, USA) anti-JAK2 (sc-294, Santa Cruz Biotechnology, USA), anti-STAT2 (sc-476, Santa Cruz Biotechnology, USA), anti-STAT5 (sc-836, Santa Cruz Biotechnology, USA), anti-ICAM (sc-7933 Santa Cruz Biotechnology, USA) and anti-VCAM (sc-8304, Santa Cruz Biotechnology, USA). After washing with PBS, cells were incubated with secondary antibody and streptavidin complex for 30 min. Substrate solution (DAB) (Invitrogen, USA) was applied, and then Mayer's hematoxylin was used for counter staining.

Spheroids were fixed for 24 hrs in +4°C. After washing with water, spheroids were suspended sequentially in 70, 80, 96 and 100% ethanol for 30 min. each, centrifuged and transferred to 1.5 ml safe lock cup (Eagle, USA). The cup was then filled with 68°C melting paraffin. Samples were carefully sectioned (5  $\mu$ m) with microtome (Leica, DE). This paraffin sections were first incubated for 24 hrs at 65°C, then placed in xylol overnight. After washing in 80, 90 and 100% ethanol each for 2 min, samples were washed with PBS and were incubated with blocking serum (Invitrogen, USA) for 30 min and then with primary antibodies (dilution of 1:100, at 4°C overnight), anti-JAK1 (sc-1677, Santa Cruz Biotechnology, USA) anti-JAK2 (sc-294, Santa Cruz Biotechnology, USA), anti-STAT2 (sc-476, Santa Cruz Biotechnology, USA), and anti-STAT5 (sc-836, Santa Cruz Biotechnology, USA). Following this, sec-



**Figure 2.** Immunohistochemical staining of STAT2 and STAT5 in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate cancer stem cells (CSCs) and non-CSCs. **a:** STAT2 immunostaining in monolayer prostate CSCs (200x); **b:** STAT2 immunostaining in monolayer prostate CSCs (400x); **c:** STAT2 immunostaining in monolayer non-CSCs (200x); **d:** STAT2 immunostaining in monolayer non-CSCs (400x); **e:** STAT5 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (200x); **f:** STAT5 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (400x); **g:** STAT5 immunostaining in monolayer non-CSCs (200x); **h:** STAT5 immunostaining in monolayer non-CSCs (400x).

ondary antibody was added for 30 min. After the samples were washed with PBS again, streptavidin complex (Invitrogen, USA) were added to each sample and incubated for 30 min. After addition of substrate solution (DAB) (Invitrogen, USA) and Mayer's hematoxylin for counterstaining, samples were examined under the microscope.

## Results

In our study we investigated that the expression levels of JAK/STAT pathway and the adhesion molecules VCAM and ICAM regulated by JAK/STAT pathway in prostate CSCs, non-CSCs and CSCs maintained as spheroids.

The results of the immunohistochemical analyses revealed that non-CSCs exhibited strong (+++) JAK1, but no JAK2 immunoreactivity. On the other hand, CSCs revealed faint (+) JAK1 and strong (+++) to moderate (++) JAK2 immunoreactivity (Figure 1).

STAT2 immunostaining was more intense in non-CSCs when compared with the CSCs. Furthermore, STAT5 immunoreactivity was weak in both groups (Figure 2).

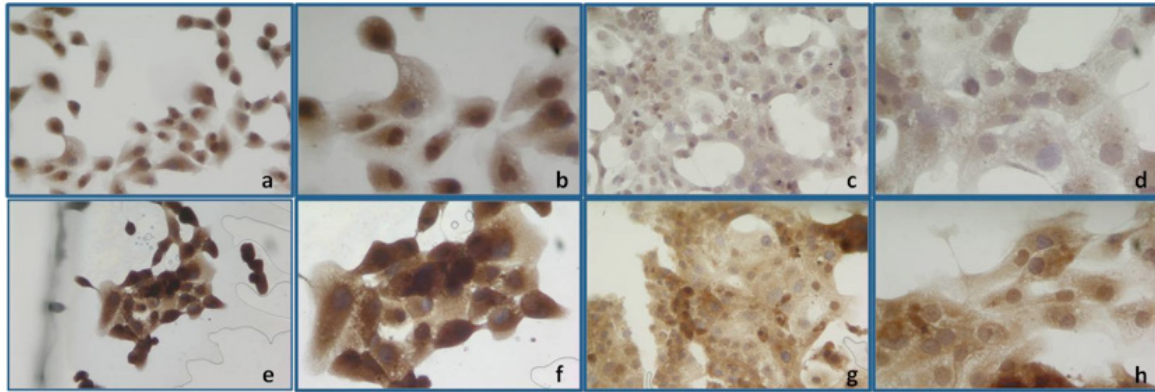
Epithelial cells derived from human prostate CSCs exhibited intense (+++++) ICAM-1 and VCAM-1 immunoreaction whereas non-CSCs samples showed a faint (+) to moderate (++) ICAM-1 and moderate (++) to strong (+++) VCAM-1 expression (Figure 3).

In CSCs maintained as spheroids, JAK2 immunoreactivity was stronger (+++) than the JAK1

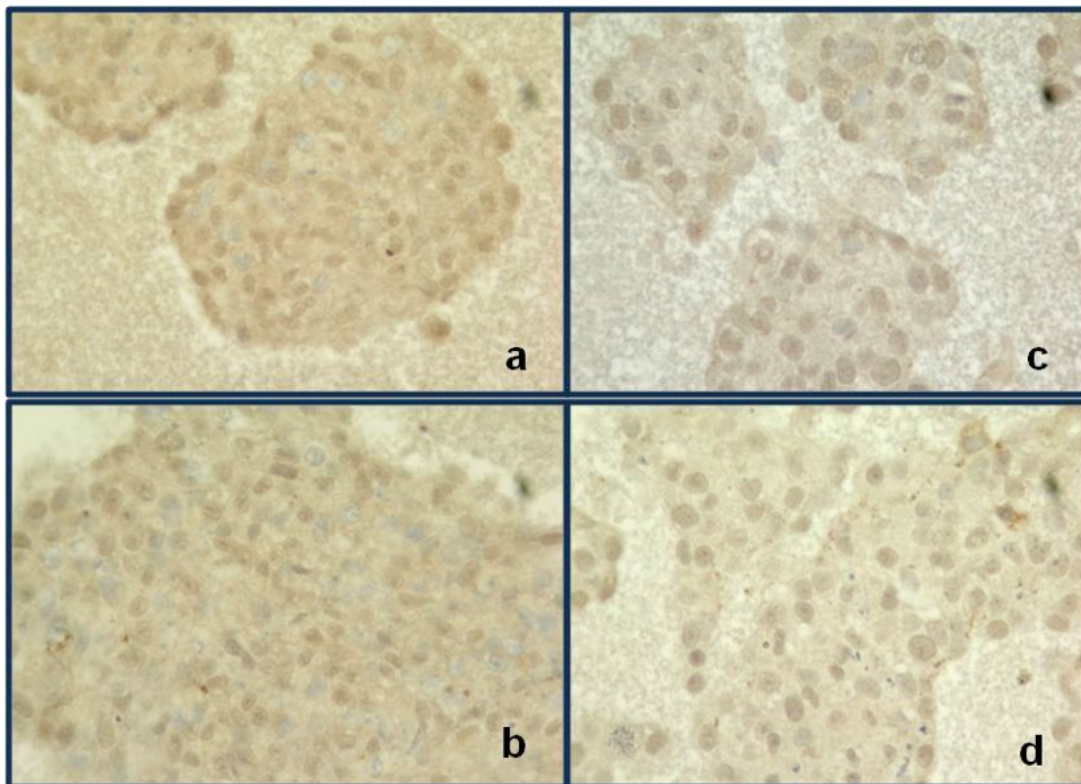
(++) immunoreactivity (Figure 4), whereas STAT2 and STAT5 exhibited only little of no (+/-) immunoreactivity (Figure 5). Furthermore moderate (++) ICAM-1 and strong (+++) VCAM-1 expression was observed in spheroid samples similar to that in monolayer CSC samples (Figure 6).

## Discussion

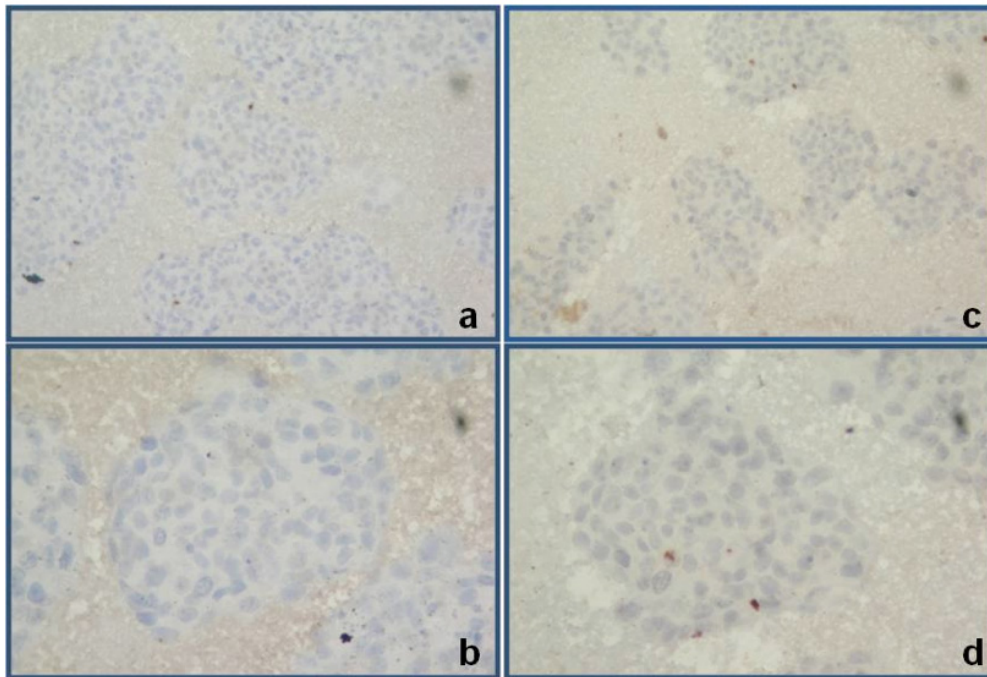
The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is an important pathway in malignant transformation. Recently, accumulating evidence indicates that the JAK/STAT pathways are involved in tumorigenesis and progression of several cancers [10]. The JAK family consists of four members (JAK1, JAK2, JAK3 and TYK2), which play a critical role in tumorigenesis and cancer progression [15]. Herein, we focused on exploring the potential role of JAK1 and JAK2 in prostate CSCs and non-CSCs. In the present study, JAK2 exhibited strong immunoreaction in CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs spheroids when compared to non-CSCs. Barton et al. demonstrated that JAK2 inhibitors may play an important role in the management of patients with prostate cancer [9]. It has also been reported previously that JAK2 inhibitors would be useful in the treatment of patients with prostate cancer [16]. Recent studies have even suggested a role for JAK2 inhibitors in solid tumors [16,17]. In our study, we also showed that the staining intensity of JAK2 was higher in CSCs when compared to that of JAK1.



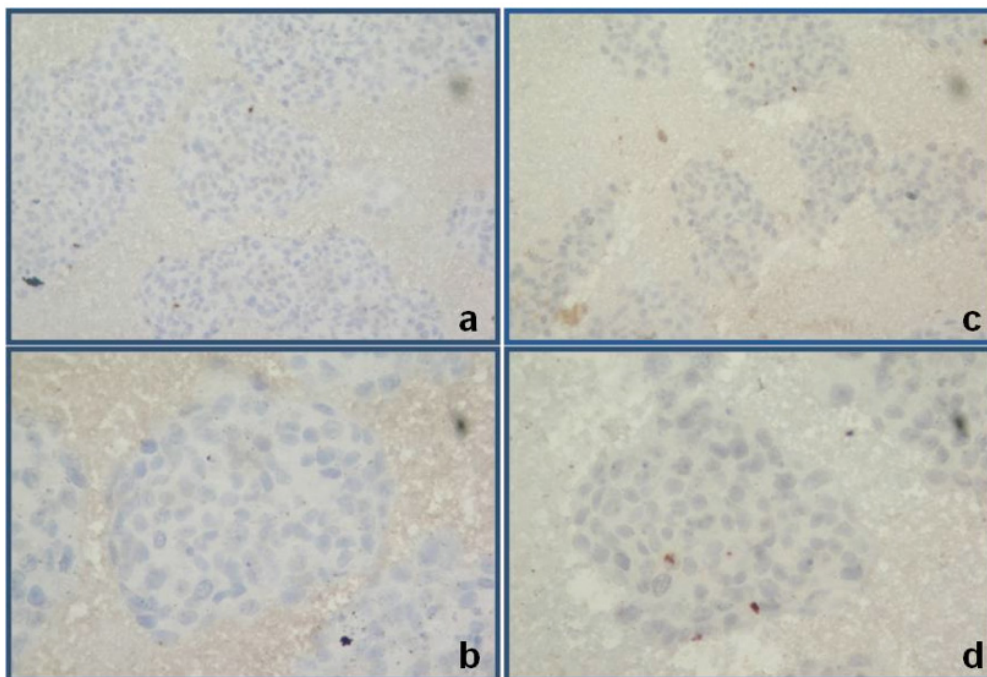
**Figure 3.** Immunohistochemical staining of ICAM1 and VCAM1 in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate cancer stem cells (CSCs) and non-CSCs. **a:** ICAM1 immunostaining in monolayer prostate CSCs (200x); **b:** ICAM1 immunostaining in monolayer prostate CSCs (400x); **c:** ICAM1 immunostaining in monolayer non-CSCs (200x); **d:** ICAM1 immunostaining in monolayer non-CSCs (400x); **e:** VCAM1 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (200x); **f:** VCAM1 immunostaining in monolayer CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSCs (400x); **g:** VCAM1 immunostaining in monolayer non-CSCs (200x); **h:** VCAM1 immunostaining in monolayer non-CSCs (400x).



**Figure 4.** Immunohistochemical staining of JAK1 and JAK2 in CD133<sup>+</sup>/CD44<sup>+</sup> prostate cancer stem cell (CSC) spheroids. **a:** JAK1 immunostaining in prostate CSC spheroids (200x); **b:** JAK1 immunostaining in prostate CSC spheroids (400x); **c:** JAK2 immunostaining in prostate CSC spheroids (200x); **d:** JAK2 immunostaining in prostate CSC spheroids (400x).



**Figure 5.** Immunohistochemical staining of STAT2 and STAT5 in CD133<sup>+</sup>/CD44<sup>+</sup> prostate CSC spheroids. **a:** STAT2 immunostaining in prostate CSC spheroids (200x); **b:** STAT2 immunostaining in prostate CSC spheroids (400x); **c:** STAT5 immunostaining in prostate CSC spheroids (200x); **d:** STAT5 immunostaining in prostate CSC spheroids (400x).



**Figure 6.** Immunohistochemical staining of ICAM1 and VCAM1 in CD133<sup>+</sup>/CD44<sup>+</sup> prostate cancer stem cell (CSC) spheroids. **a:** ICAM1 immunostaining in prostate CSC spheroids (200x); **b:** ICAM1 immunostaining in prostate CSC spheroids (400x); **c:** VCAM1 immunostaining in prostate CSC spheroids (200x); **d:** VCAM1 immunostaining in prostate CSC spheroids (400x).

There is a variety of evidence suggesting that constitutively activated STAT proteins, in particular STAT3 and STAT5, have been demonstrated to directly contribute to oncogenesis, partly by stimulating proliferation and preventing apoptosis in various types of tumor cells [8,9]. The role for STAT3 and STAT5 in the pathogenesis of human breast cancer is becoming increasingly recognized [18]. There is evidence of increased STAT protein binding in the nuclei of breast cancer cells compared to the normal tissue or benign lesions [19]. In addition, a previous study has also shown an association between STAT5 nuclear localization/phosphorylation and improved survival [20]. In our study, a feeble expression level of STAT5 was observed in both CSC and non-CSC groups. However, in the spheroid samples, the immunoreactivity was insignificant.

It is reported that STAT 2 is expressed in MCF-7 cells (an ER+ human breast cancer cell line). Prominent cytoplasmic localization was observed by Uluer et al. [21] and existence of STAT1/STAT2 heteromeric complexes following type I interferon stimulation were reported by Schaber et al. [22]. Gamero et al. identified STAT2 as a novel contributor to colorectal and skin carcinogenesis that may act to increase the gene expression and secretion of pro-inflammatory mediators, which in turn activate the oncogenic STAT3 signaling pathway [23]. At the cellular level, we found that expression of STAT2 protein was more increased in non-CSCs than in CSCs. No expression of this protein in the spheroid samples was detected.

In the present study, it was possible to assume that the activation of JAK/STAT pathway is associated with increase in expression of ICAM-1, which expression is regulated by JAK/STAT pathway. Previously, Yamada et al. demonstrated that JAK/STAT signaling plays an important role in the expression of ICAM-1 [24]. Many studies have revealed that cellular adhesion molecules (CAM) play a critical role in cancer progression and metastasis [25,26]. Here we showed that epithelial cells of human prostate CSCs exhibited intense ICAM-1 and VCAM-1 immunoreaction when compared with non-CSCs. These findings were supported by the fact that VCAM-1 on the surface of cancer cells binds to its counterreceptor, the  $\alpha 4\beta 1$  integrin (also known as very-late antigen, VLA-4), on metastasis-associated macrophages, triggering VCAM-1-mediated activation of the phosphoinositide 3-kinase growth and survival pathway in cancer cells [27]. There was no significant difference between the spheroid and monolayer samples for ICAM-1 and VCAM-1 immunohistochemical staining intensity. Despite significant ICAM-1 and VCAM-1 staining in CSCs, this data does not provide enough relevant information for the use of adhesion molecules as tumor markers [26]. As a next step, RT-PCR analysis would be useful to identify genome-wide levels of these molecules to support our findings. The JAK/STAT is an evolutionarily conserved pathway and very important for second messenger system. The results of this study showed that changes in JAK/STAT pathway are related with adhesion molecules and may be affect cancer progression.

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