Cancer Stem Cells Stemness Transcription Factors Expression Correlates with Breast Cancer Disease Stage

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Abstract: Cancer stem cell-like cells (CSCs) are cancer cells that have the ability of self-renewal and differentiation into multiple malignant cell types (hierarchy). Thus, can cause relapses and metastasis. CSCs’ phenotype is defined by special transcription factors such as Nanog, Oct3/4, Sox2, Nestin, and CD34. The present study aims to determine the change in gene expression of the above markers in correlation with the stage of the disease in breast cancer patients. Initially, whole blood samples from patients with breast cancer were collected, followed by the isolation and culture of circulating tumor cells (CTCs). This was followed by the quantification of CSCs from the above cultures. CSCs’ molecular analysis was performed with qPCR, with the use of gene specific primers. At the same time of the analysis, the clinical assessments of the patients were requested from their physicians. The results indicated a linear relationship between the gene expression of stemness markers and the stage of the disease, as well as specific expression patterns by stage. It seems that these genes have an important role in the progression of the disease, thus they might be target for new treatment approaches.

Keywords: Breast cancer, cancer stem cells, Nanog, Sox2, Oct3/4, CD34, Nestin.

INTRODUCTION

It is well known both from literature and also from experimental data, that circulating tumor cells are a subset of blood origin cells which have similar size with peripheral blood mononuclear cells (PBMCs) and that cancer stem cells (CSCs) are included in the vast majority of CTCs [1, 2]. The CSCs are proposed to initiate cancer and propagate metastasis. They are also capable of self-renewal and of producing a new tumor with the heterogeneity that is characteristic for the parental tumor [3, 4]. According to literature, there is evidence of the concept that CSCs have specific molecular markers such as Nanog, Oct3/4, Nestin, Sox2 and CD34 genes [5-8]. Each of the above genes has an important role in the functioning of these cells. Cancer stem cell-like cells are the progenitors of tumor and the generators of metastasis. Several theories have been examined about their origin, such as cell fusion or gene transfer during cell development, gene mutation in stem cells, progenitor cells or even in differentiated cells. CSCs are a subpopulation of the heterogeneous tumor mass which has the ability to enter the bloodstream in order to generate metastases and relapses [9-15]. Two reverse processes contribute to this: the epithelial to mesenchymal transition (EMT) and the reverse mesenchymal to epithelial transition (MET). An epithelial cell, by interacting with basement membrane, undergoes multiple biochemical changes and assumes a mesenchymal phenotype. The mesenchymal cell through bloodstream migrates into healthy tissues. The above mesenchymal cell has the ability through MET, to cause a micrometastasis [16-18]. Recent literature data supported that normal and neoplastic nonstem cells can spontaneously convert to a stem-like state [19]. When CSCs are cultivated in semi-suspension they have the ability to form sphere colonies and by this way they evade the cell death. Cancer stem cells are hierarchical populations that are consisted of different subsets of stem cells such as precancerous, primary, migrating and chemoradioresistant cancer stem cells according to a horizontal hierarchy. In another theory, cancer stem cells are placed hierarchically as cancer stem cells, cancer transient-amplifying cells and cancer differentiated cells according to vertical model of hierarchy [20].

Up to now, studies have not identified the change in gene expression of the above markers according to the stage of the disease. This study aims to demonstrate the change in gene expression of the above transcriptional factors in breast cancer in accordance to the stage (TNM Classification) [21]. By having an initial insight into change of gene expression, the question of whether they are responsible for disease progression arises.

The present study indicates the gene expression changes of Nanog, Oct3/4, Nestin, Sox2 and CD34 by stage and suggests a possible expression pattern of markers per stage in CSCs, isolated from breast cancer cell cultures.

MATERIALS & METHODS

Sample Collection

In this study a total of 32 patients were studied, divided equally per stage of the disease according to TNM classification system. From each patient 20ml of peripheral blood were collected and placed in a tube containing EDTA as an-
ticoagulant and rotated to prevent coagulation. The authors have ethical approval for performing the experiments. Each patient has consented writing and has given his permission for storage of samples as for their use in Research and Development purposes. This study is not a clinical trial. All procedures were conducted according to the standards of Safety, Bioethics and Validation [22].

Cells Isolation-Culture

Blood cells were centrifuged with polysucrose solution (Histopaque 10771; Sigma, Histopaque 11191; Sigma) and blood cells were centrifuged with polysucrose solution (Histopaque 10771; Sigma, Histopaque 11191; Sigma) and mononuclear cells, lymphocytes, platelets and granulocytes were collected and washed twice with PBS (P3813; Sigma). Cells were then incubated at 4°C for 30 min with EpCAM magnetic beads (39-EPC-50; Gentaur), an epithelial cell adhesion molecule-associated antigen that is expressed in the vast majority of carcinomas. At the end of the incubation, the samples were placed in a magnetic field, selected and washed with PBS. Isolated breast cancer cells were collected and washed twice with PBS (Histopaque 11191; Sigma) and then mononuclear cells, lymphocytes, platelets and granulocytes were collected and washed twice with PBS (P3813; Sigma). Cells were then incubated at 4°C for 30 min with EpCAM magnetic beads (39-EPC-50; Gentaur), an epithelial cell adhesion molecule-associated antigen that is expressed in the vast majority of carcinomas. At the end of the incubation, the samples were placed in a magnetic field, selected and washed with PBS. Isolated breast cancer cells were then cultured in 25cm² flasks (5520100; Orange Scientific) with STEMPRO® hESC SFM (A10007-01; Invitrogen), which contained DMEM/F12 with Glutamax, Mecaptoethanol, Bovine Serum Albumin 25% and STEMPRO® hESC Supplement. According to the recommended protocol fibroblast growth factor (FGF) (10µg/ml) was added in the cultures. The Human Breast Cancer Stem Cells (36102-29P;Celprogen) were cultured in the appropriate growth medium, provided by the manufacturer (36102-29;Celprogen). The cells were incubated at 37°C in a 5% CO₂ environment [23].

Evaluation of Cells

Based on the ability of CSCs to form microspheres, only the cell cultures in which microspheres were observed were selected. The above cultures have been also evaluated with molecular analysis, including gene expression analysis of specific transcription factors. A Human Breast Cancer Stem Cell Culture (36102-29P;Celprogen) has been used as positive control in both methods. The authentication of the control cell line was tested each time with molecular biology assays, such as Short Tandem Repeats (STRs) profile, which was compared with the manufacture profile. The cultivation of cells was continued for more than 20 passages, to exclude the possibility that might included embryonic stem cells. Cancer stem cells are immortal unlike the embryonic stem cells.

Molecular Analysis

RNA from cell cultures which had the feature of CSCs (total 32 patients, 8 of each stage) was extracted by using TRIZOL reagent (15596-026; Invitrogen) and 1µg of the above was used as a template to cDNA with First strand cDNA synthesis kit (K1612;Fermentas). The above strand was used as a template for the Real-Time PCR reaction (250ng/reaction), which was performed by using the Maxima SYBRGreen qPCR Master Mix (K0221;Fermentas). Specific primers for each marker and for the endogenous control gene (18S rRNA) were designed with Gene Expression 1.1 software. The sequence of primers was run on BLAST to exclude those who amplified undesired genes. The sequence of the primers is the following: 18S rRNA: Forward- 5’GAGAGCTGGTCATGGAGTTGTAC3’, Reverse- 5’TGCCCTATCAACTTTCGATGTGTC3’; Nestin: Forward- 5’TGGGATGTTGTAAGCGTTCACA3’, Reverse- 5’GGTTGTTTGTCCCTTGGAGACTG3’; Sox2: Forward- 5’TGCCCCCACCCTTTGTTGTC3’, Reverse- 5’TCCAGGACATCTAGCAGGATG3’; Sox3: Forward- 5’TCAACGCGAGCTAGCTAGCAGT3’, Reverse- 5’GGCAGCTGATCATGAGTGTACT3’. The PCR reaction program was set as follows: initial denaturation at 95 ºC, 50 cycles of denaturation at 94 ºC for 15 sec followed by annealing at 59 ºC for 15 sec and an extension step at 72 ºC for 30 sec. A final extension step was performed at 72 ºC for 10 min followed by melting curve analysis.

Statistical Analysis

The present study has used random sampling between patients with breast cancer in different stages of disease. The qPCR results were tested according to Kolmogorov-Smirnov test for the distribution and it has been observed that all samples have normal distribution. The correlation between stages was tested according to Pearson’s coefficient correlation. In analysis there were used median values and also performed tests according to Mann-Whitney.

RESULTS

The microsphere formation in semi-suspension was the first indication about the presence of CSCs in the culture, as CSCs have the ability to form spherical colonies. These formations were compared to those of control cell line and it was observed that they were the same in both cultures. In Figs (1) and (2) are illustrated microsphere formations both in control breast CSCs as well as in breast CSCs derived from a patient. In the next experimental panel the gene expression of representative markers Nanog, Oct3/4, Sox2, Nestin and CD34 was tested by using Real-Time PCR. There were only accepted data with Ct less than 35 cycles. All the above markers were expressed in levels compared to those of control Human Breast CSCs (p<0.05).

The qPCR results indicated that the gene expression levels of each marker were increased according to the stage of the disease. The analysis was performed by using relative quantification, normalized to the gene expression at stage I and using 18S rRNA as reference gene. Changes in the expression of transcriptional factors are presented in Fig. (3). With an exception of Oct3/4, the gene expression levels were increased according to the stage. The rate of increase is much higher between stages I-II and III-IV, while less variation is observed between stages II-III. Oct3/4 seems to be overexpressed at stages I-II, whereas in stage IV Oct3/4, Sox2 and Nestin genes are expressed. Fig. (3) represents the relative expression rates of all molecular markers, in compare with the expression in stage I, according to Livak method [24]. The highest increase has been observed in Nestin, whose expression is about 32 times higher in stage IV than that of the initial stage.
DISCUSSION

Within the heterogeneous cancer population, circulating tumor cells are also included. These cells have been detached from a primary tumor and flew into bloodstream. Recent experimental data have demonstrated that CSCs cells are included in the vast majority of CTCs and circulating tumor cells have many CSCs hallmarks and express related molecular markers [2].

The specific surface marker CD34 was the first that showed to be involved in CSCs, as a CD34⁺ cell subpopulation was able of initiating carcinomas, histological similar to the donor, in mice [8]. Nanog is a pluripotency sustaining factor in embryonic cells and its down-regulation induces differentiation of human embryonic stem cells into other cell types [25-27]. Oct3/4 and Sox2 are also essential regulators of self-renewal and pluripotency of embryonic stem cells. These transcription factors have the ability to form a het-
erodimer and thus bind to DNA together [28, 29]. Nestin is a protein expressed in dividing cells during the early stages of development in nervous system and is down-regulated upon differentiation [30]. All the above transcription factors are also involved in cancer stem cells.

The present study aims to identify the change in the expression of stemness transcription factors that is mentioned above according to the stage of the disease. After the quantification of CSCs, the molecular analysis of relative expression data was followed by using quantitative PCR and the 2^(-ΔΔCT) method [24]. A proportional increase of the expression regarding the stage has been observed, with the exception of Oct3/4 gene. Oct3/4 is expressed more than the other markers in almost all stages, bringing out its significance in stemness. In stages III-IV, Sox2 is increased according to Oct3/4, enhancing the opinion that operates usually as heterodimer. The relative quantification analysis showed that Nestin’s expression is 32-folds increased in compare to the expression in earlier stages. On the contrary, Oct3/4’s expression was 6.5-fold increased, showing the lowest increase in relation to the other markers. All these findings confirm literature data, which demonstrate the above markers as predictors for the prognosis of breast cancers. Nagata et al. demonstrated that Nanog stimulates the growth and metastasis of breast cancer cells [31], while also Liu et al. aimed that Oct3/4 and Nestin are important regulators of the development of breast cancer [32]. In another recent study it was demonstrated that Sox2 is expressed in early stage of breast tumors, whereas Oct3/4 and Nanog were not, suggesting that gene expression is affected by several factors until the level of translation and protein synthesis [33].

According to the above data, it seems that some of the transcription factors are related to the maintenance of the stemness, while others might be responsible for disease progression. Although qPCR data are informative and quantitative, further experiments at protein level need to be performed. More experiments on a larger number of patients will also give us more precise data, in order to draw reliable conclusions.

CONCLUSION

Due to their important role in cancer progression and prognosis, CSCs could be a new therapy approach. The gene expression of several transcription factors changes, according to the stage of the disease, indicating the specific role of each marker in disease progression and in stemness maintenance. Concluding, there are clear indications that the above transcriptional factors are indicators of poor prognosis, as well as that are potential targets for the treatment of breast cancer.

CONFLICTS OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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REFERENCES