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Stemness Gene Profiles of Circulating Tumor Cells

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Abstract

Circulating tumor cells (CTCs) are a population of tumor-derived cells that detach from the primary tumor and initiate metastasis. However, the mechanisms of this process are still unknown. This phenomenon renders CTCs as a valuable resource for prognosis and diagnosis of cancer. The involvement of stemness transcription factors and markers, such as NANOG, OCT3/4, CD34, NESTIN, and SOX2, in metastasis initiation has been studied recently because their abnormally elevated expression in cancer cells may be highly important in understanding tumor initiation. This study analyzed the genetic profiles of the above genes in CTCs derived from patients with different types of cancer. Blood samples were randomly collected from 71 cancer patients with various cancer types. CTCs were isolated using enrichment protocols and RNA was extracted. RT-qPCR was performed in triplicate using ACTB as the reference gene. The statistical analysis was performed among the ΔCts of the samples using parametric and non-parametric methods. The molecular analysis revealed that the expression of each gene was different than the others. When each type of cancer was analyzed separately, the gene expression profile was not always the same. It is noteworthy that, in all cases, the gene expression of NESTIN differed from that of transcription factors. According to the above data, gene expression profiles might be used as a potential biomarker or constitute a gene signature.

Keywords

Stemness, Circulating Tumor Cells, RT-qPCR

1. Introduction

Circulating tumor cells (CTCs) constitute a sub-population of cancer cells that have shed into the vasculature or lymphatics from a primary tumor. CTCs detach from the original tumor and circulate through the blood stream, subse-

quently spreading to other organs where they can initiate tumor metastasis in the new microenvironment. Nevertheless, the mechanisms of detachment from the tumor site and re-establishment at a different site are still unknown.

CTCs were first reported by Thomas Ashworth in 1869 [1]. Since then, CTCs have been the focus of many researchers, because they have proved to have both prognostic and diagnostic value. The existence of CTCs has been reported in most solid cancers, and their use as a diagnostic resource is well established [2]. Another cell subpopulation with EMT properties is cancer stem cells (CSCs). These cells have been proposed to initiate cancer and propagate metastasis, and are capable of self-renewal and regenerating the tumor [3]. Many studies have been performed to identify the gene signatures of CSCs. However, there are no clear data on this issue. NANOG, OCT3/4, SOX2, NESTIN, and CD34 constitute a well-characterized gene group for stem cells [4]. Yu *et al.* have proven that NANOG, SOX2 and OCT3/4 induce pluripotency to somatic cells that do not normally express these factors [5]. Moreover, NANOG, SOX2 and OCT3/4 are overexpressed in the majority of cancer types [6] [7]. On the other hand, NESTIN is a stemness marker of both neurological tumors and tumors of epithelial and mesenchymal origin [8], and CD34 is a common marker or progenitor cell [9].

Pardal *et al.*, reviewing the principles of stem-cell biology to cancer, stated that "several signalling pathways that regulate normal stem-cell self-renewal cause neoplastic proliferation when dysregulated by mutations" [10]. Therefore, the study of stemness markers that have been correlated both to stem-cells and to cancer-derived cells could possibly explain the mechanisms of self-renewal in cancer. Therefore, the present study aimed to identify the gene expression of the above stemness transcription factors and markers in CTCs derived from various types of cancer.

2. Results

The statistical analysis among all cancer types revealed that the expression of each gene was significantly different when all samples were included ($p = 0.0001$). In terms of the gene expression rates, higher expression was observed for OCT3/4, followed by NANOG and SOX2. CD34 was expressed at lower levels, while NESTIN exhibited the lowest gene expression (**Figure 1**). When the analysis was performed in each type of cancer, the data were not the same. In breast CTCs, there was a difference in gene expression among NANOG-OCT3/4 ($p = 0.02$), NANOG-NESTIN ($p = 10^{-9}$), and NANOG-CD34 ($p = 10^{-6}$), but not between NANOG-SOX2 ($p = 0.186$). For the other genes, the difference was statistically significant. In colon CTCs, the gene expression was different for NANOG-CD34 ($p = 0.005$) and NANOG-NESTIN ($p = 0.0001$), but not for NANOG-OCT3/4 ($p = 0.3905$) and NANOG-SOX2 ($p = 0.96$). In addition, there was no significant difference between OCT3/4 and SOX2 ($p = 0.133$). In prostate CTCs, the gene expression differed only for NESTIN ($p = 0.03$) and between OCT3/4 and CD34 ($p = 0.03$). Similar data were obtained for squamous cell

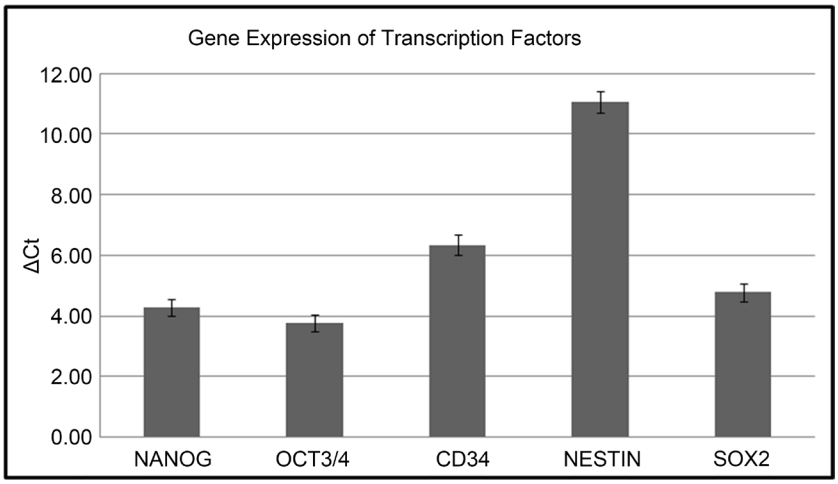

Figure 1. Stemness marker gene expression. qPCR data from the various samples are shown. ACTB served as the reference gene. A lower ΔCt value indicates higher gene expression.

carcinoma (SCC) in which only the gene expression of NESTIN was quite different than that of the other factors ($p = 0.03$). In ovarian and multiple myeloma cancer, we obtained the same expression profiles with differences among NANOG-CD34 ($p = 0.013/p = 0.001$), NANOG-NESTIN ($p = 0.0005/p = 0.0002$), OCT3/4-CD34 ($p = 0.005/p = 0.0003$), OCT3/4-NESTIN ($p = 0.0004/p = 0.0002$), CD34-NESTIN ($p = 0.003/p = 0.0002$), CD34-SOX2 ($p = 0.02/p = 0.002$), and NESTIN-SOX2 ($p = 0.0006/p = 0.0002$). In melanoma and glioblastoma CTCs, except for the former, there were also differences between OCT3/4 and SOX2 ($p = 0.04/p = 0.01$). In cases of primary tumors with an unknown origin, the analysis revealed differences in the gene expression of all markers except for NANOG-OCT3/4 ($p = 0.06$). When the analysis was performed in breast CTCs, according to the state of the disease, there were no significant differences. Figure 2 represents the above data.

Regarding the gene expression among the different types of cancer, NANOG's expression was higher in ovarian, melanoma, sarcoma and multiple myeloma cancer and lower in breast, colon and prostate carcinoma. The lower expression was observed in SCC and in unknown origin of primary tumor cases. In OCT3/4, the higher expression was observed for sarcoma, melanoma and multiple myeloma while the gene expression in the other types was lower with no significant differences among them. Regarding CD34 gene expression, the higher expression was for sarcoma samples followed by caner of unknown origin and colon. The SCC cases had the lower expression, however with no great difference. In NESTIN, the lower expression was for SCC and increasing followed glioblastoma, multiple myeloma, prostate, breast, unknown origin of primary tumor, melanoma, colon and ovarian, while sarcoma demonstrated the higher gene expression levels. Finally, in SOX2 gene, the higher expression observed in ovarian cancer, followed in decreasing rate by myeloma and sarcoma. Breast, colon, prostate and glioblastoma samples exhibited similar gene expression levels, higher that SCC and unknown origin tumor which had the lower SOX2

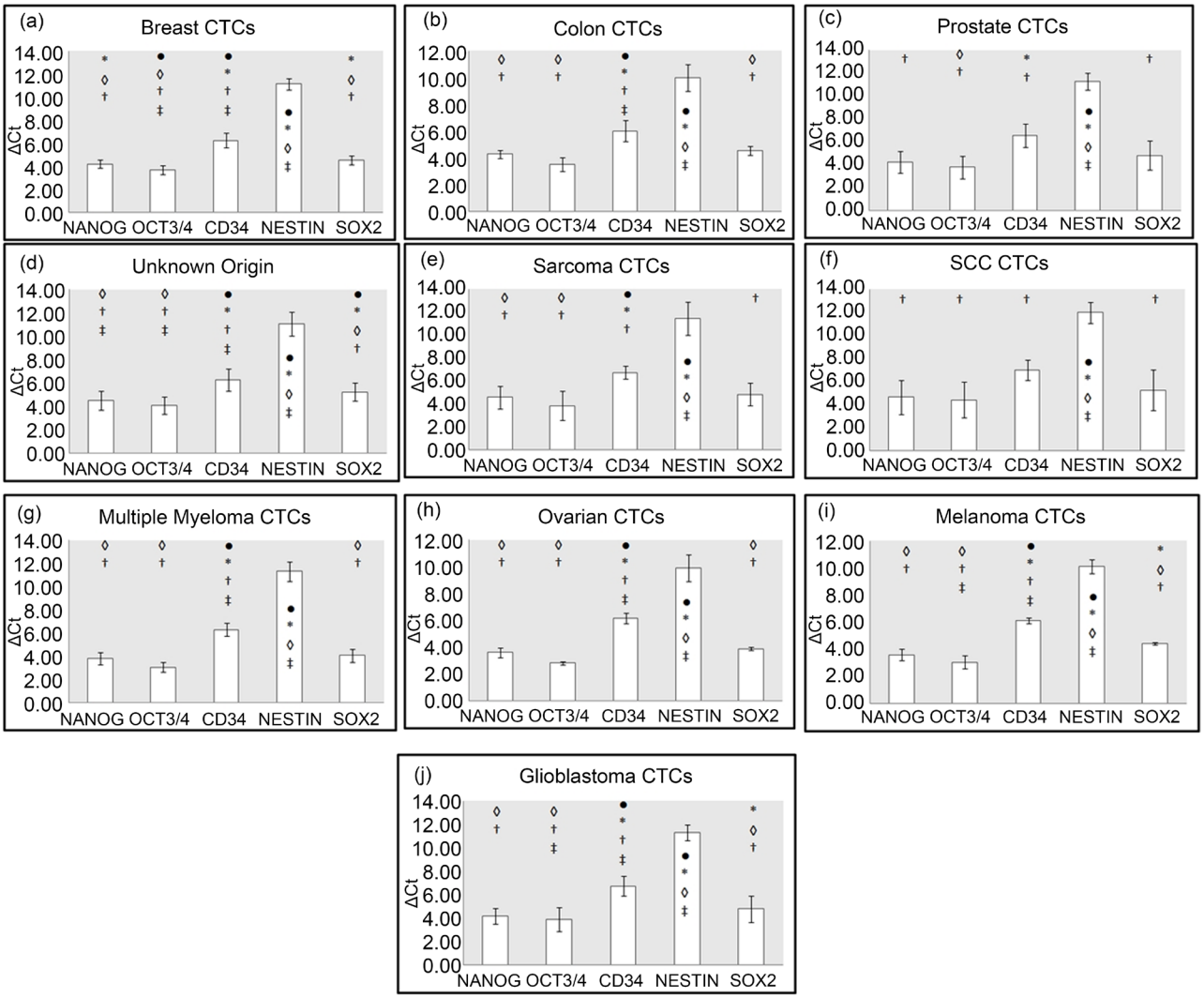

Figure 2. Gene expression differences among NANOG, OCT3/4, CD34, NESTIN, and SOX2 in various cancer types. qPCR data of all samples categorized per cancer type are shown. Symbols indicate statistically significant differences in gene expression: •p < 0.05 compared with NANOG; *p < 0.05 compared with OCT3/4; ◊p < 0.05 compared with CD34; †p < 0.05 compared with NESTIN; ‡p < 0.05 compared with SOX2. The higher the ΔCt, the lower the gene expression and vice versa.

gene expression. Figure 3 represents the above data.

Regarding the gene expression among CTCs and normal samples, it has been observed that in NANOG, OCT3/4 and CD34 the changes in gene expression are not quite different, while in NESTIN the gene expression is lower for CTCs. On the contrary, the SOX2 is overexpressed in CTCs. Figure 4 represents the above data. Finally, concerning the validation of CTCs prior to analysis, there were tested with both flow cytometry and PCR for expression of specific markers, depending on the cancer type. Table 1 presents these data.

3. Discussion

CTCs have been of interest to the scientific community for the last 15 years. They have proved to be an invaluable resource for cancer prognosis because their existence has been shown in the majority of malignant tumors. Nevertheless,

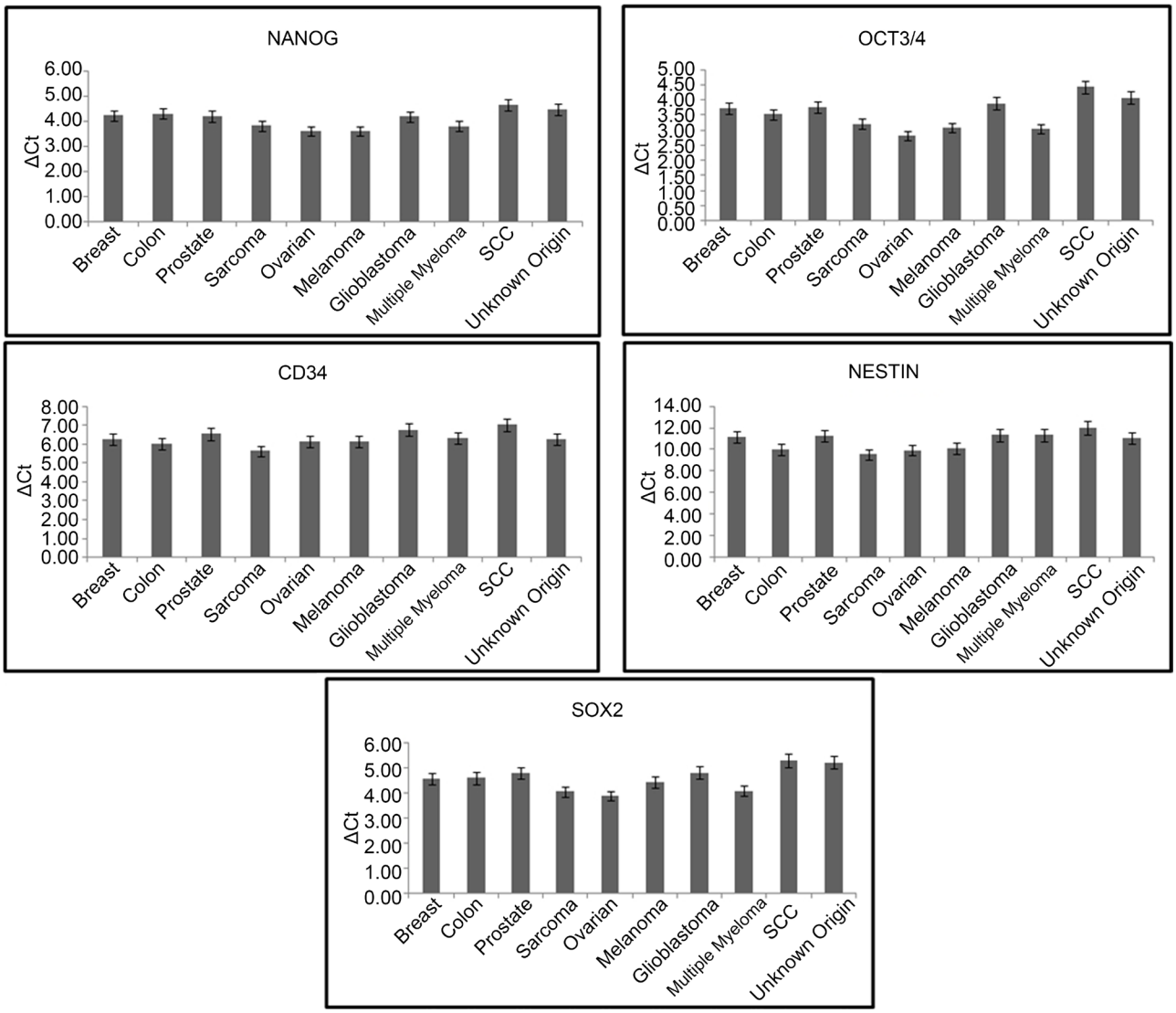

Figure 3. Gene expression differences among NANOG, OCT3/4, CD34, NESTIN, and SOX2 according to the type of cancer. The diagrams represent the ΔCt. The higher the ΔCt, the lower the gene expression and vice versa.

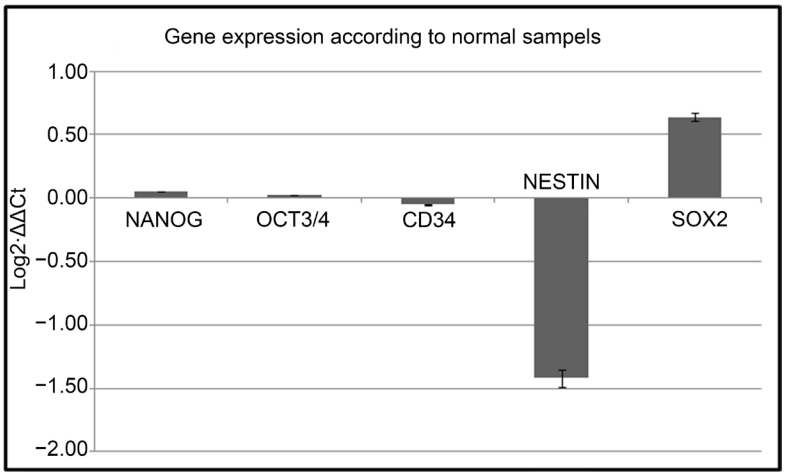

Figure 4. Relative quantification among CTCs and normal samples. The analysis performed according to 2-ΔΔCt method, using the non-cancer samples as the reference group.

Table 1. Specific markers expression. +: indicates the expression of the specific marker, −: indicates no expression of the above specific marker. NT: the samples were not tested for these markers.

Marker / Cancer Type	Breast	Colon	Prostate	Unknown Origin	Sarcoma	SCC	Multiple Myeloma	Ovarian	Melanoma	Glioblastoma
CK	+	+	+	+	NT	+	NT	+	NT	NT
CD99	NT	NT	NT	NT	+	NT	NT	NT	NT	NT
CD63	NT	NT	NT	NT	NT	NT	NT	NT	+	NT
CD45	−	−	−	−	−	−	+	−	−	−

to truly understand the nature of CTCs and their functional role in cancer development and metastasis, it is imperative to study their genetic expression profile. Similar to CSCs, CTCs express stemness and EMT markers that might implicate them in tumor initiation [11]. The present study revealed the expression of five stemness genes (NANOG, OCT3/4, CD34, NESTIN, and SOX2) in CTCs isolated from patients with various types of cancer.

Stemness involves the ability of cell renewal and differentiation during early embryonic development and in cancer development. Transcription factors such as NANOG, OCT3/4 and SOX2 have been implicated in regulation of early embryonic stem cell development [12] [13] [14]. In particular, NANOG inhibits differentiation and maintains stem cell properties. Furthermore, NANOG, SOX2, and OCT3/4 are overexpressed in CSCs [7] [15].

OCT3/4 is a transcription factor associated with pluripotency in embryonic stem cells. In particular, expression of OCT3/4 is required to maintain the pluripotent status of human embryonic stem cells by inhibiting expression of human chorionic gonadotropin, a placental marker of embryonic stem cells. Consequently, OCT3/4 is a stemness regulator in human embryonic stem cells [16]. OCT3/4 appears to regulate dedifferentiation of melanoma cells [17]. It also plays an important role in gastric cancer progression and metastasis [18]. Expression of OCT3/4 protein has been found in lung adenocarcinoma stem cells, which correlates to poor clinical outcomes [19]. Moreover, OCT3/4 is detectable in other types of cancer, such as ovarian [20] [21], prostate [22], and rectal [23].

Various reviews [24] [25] have demonstrated that OCT3/4 is an upstream regulator of NANOG expression by binding to its promoter. When the levels of OCT3/4 are raised, it suppresses both its own expression and that of NANOG. This negative feedback allows a cell to stably maintain NANOG levels. In addition, OCT3/4/SOX2 heterodimers induce expression of NANOG, which bind to its promoter and provoke its transcription. Downstream, potential targets of NANOG are cell cycle-related genes such as cyclin D1 that regulates the transition from G0 to G1 phase of the cell cycle [26]. Another study has suggested that, in embryonic stem cells, OCT3/4, SOX2, and NANOG bind to at least 353 genes in total and possibly influence their transcription. NANOG expression has been found in many types of cancer (breast, cervical, oral, kidney, prostate, lung, gastric, brain, and ovarian cancers), and its expression is associated with stemness properties [27]. Nevertheless, the exact role of NANOG in cancer is not es-

tablished yet, because it does not appear to function as a simple oncogene [7].

In addition to its role in NANOG regulation, SOX2 promotes cellular proliferation in various types of cancer, contributes to apoptosis inhibition, and enhances metastasis [28].

The present study demonstrated that NANOG and OCT3/4 have similar expression profiles in CTCs of the majority of the different cancer types, but it is not always the case for the expression of both NANOG and OCT3/4 and SOX2. In particular, colon, sarcoma, SCC, prostate, multiple myeloma, and ovarian cancers have similar expression of all three genes, whereas breast, melanoma, glioblastoma, and cancer of unknown origin have statistically different expression of OCT3/4 and SOX2 genes. These data suggest that the molecular mechanisms of OCT3/4 and SOX2 functions in CTCs are different in some cancer types.

As far as the gene expression levels among the different types of cancer, the OCT3/4 is overexpressed when compared with NANOG and SOX2. In addition, the gene expression level of OCT3/4 and SOX2 are correlated with these of NANOG's, indicating their role in NANOG regulation. In ovarian cancer, where their expression is higher, there has been proved that due to the stem cells the patients can develop recurrent chemoresistant disease that is usually fatal [29].

NESTIN is an intermediate filament protein that is mainly found in rapidly dividing cells of developing and regenerative tissues. Expression of NESTIN has been associated with cytoplasmic trafficking in progenitor cells, but it has not been identified as a stemness marker in many types of cancer [8] [30]. More importantly, NESTIN expression correlates with sphere formation, a characteristic of CSCs. Although NESTIN is also expressed in CTCs, this study has shown that its expression is significantly different in all types of cancer compared with the expression of other stemness-associated genes (NANOG, OCT3/4, SOX2, and CD34).

CD34 is a transmembrane glycoprotein that was originally identified as a marker of hematopoietic stem cells. However, recent studies have suggested that CD34 is a marker of all progenitor cells, because its expression has been shown in multiple cell types [9]. This study revealed CD34 expression in CTCs from various cancer types. However, in most cases, its expression did not appear to be similar to that of any of the other stemness genes (NANOG, SOX2, OCT3/4, and NESTIN).

According to a previous study, CSCs are a subset of CTCs. CTCs have been found to express epithelial, mesenchymal or stemness markers [31]. CSC isolation is more difficult than CTC detection and isolation, which implies an inhibitory factor for cancer treatment. However, CTCs and CSCs exhibit common features including the expression of many genes. In breast cancer, CTCs have mesenchymal or stemness characteristics that can be used at a clinical level [32]. The present study indicates that some of the above mentioned stemness-associated genes might be important to maintain stemness or initiate metastasis.

Furthermore, these results confirm on gene expression level the study of Gril-

let *et al.* that in colorectal cancer the CTCs express cancer stem cell phenotype and the observation that in ovarian cacer the CTCs can be positive for stem cell markers [33] [34].

Comparing the expression of stemness transcription factors with normal samples, it is noteworthy that NESTIN is under-expressed in CTCs, while SOX2 is overexpressed. For the rest markers, there is observed no significant difference. The under expression of NESTIN may be correlated with its role, which is particularly expressed in tumor of epithelial and mesenchymal origin. On the other hand, the over expression of SOX2 might indicate its contribution in metastasis. Its expression may be involved or regulate other transcription factors, which are responsible for propagating metastasis.

The present study should be performed in more samples from different kinds of cancer, since in some of them the number is not sufficient for global interpretation. However, these data are a useful indication for the majority of types.

4. Materials and Methods

4.1. Sample Collection

Blood samples from 71 patients were collected in sterile 50-ml falcon tubes (4440100; Orange Scientific) containing 7 ml of 0.02 M EDTA (E0511.0250; Duchefa Biochemie B.V.) as an anticoagulant. The cancer types of the samples included breast (24), colon (6), prostate (4), sarcoma (5), ovarian (2), melanoma (2), glioblastoma (2), multiple myeloma (3), SCC (4), and cancer with an unknown origin of the primary tumor (19). The samples were collected randomly from physicians in U.S.A. and Europe. Regarding their age the average was 54.13 ± 3.77. Between them, the majority was female samples (68.9%), while the male samples were 31.4%. 65.2% of samples were received from U.S.A. and the rest 34.8% were collected from patients lived in European countries. The volume of collected blood was 20 mL. The samples were applied to a roller for 30 minutes and then sent to the laboratory for analysis. Transit of the samples to the laboratory did not exceed 72 h. The study was performed from January to June 2016. In addition, two normal samples were used as reference group for the qPCR analysis. The samples were collected from a healthy 40-year old man and a 32-year old woman.

4.2. Sample Stability

The period between transportation and analysis did not affect the experimental analysis. To ensure this, blood samples were collected from five healthy donors and placed in five different 50 ml falcon tubes. The tubes were then stored at 4˚C, which is the temperature of transportation package. Each day, starting from 0 h, every sample from each donor, was tested by using molecular and cellular assays. In a time window of 0, 24, 48, 72 and 96 h of storage, the gene expression of many genes correlated with cell cycle, apoptosis, cytoskeleton, stemness, cytokeratins, growth factors, signaling transduction pathways etc were tested. The same procedure was performed with flow cytometry to study the protein level.

Finally, the number of CTCs was measured for every sample each day. Ta data were analyzed and there was not observed any statistically significant difference among the different time periods. Concerning the above experimental data, the transit period, did not affect the analysis of the present study.

4.3. Sample Preparation

Whole blood cells were centrifuged for 20 min at 4000 rpm at 4˚C with polysucrose solution (Biocoll separating solution 1077; Biochrom). Mononuclear cells, lymphocytes, platelets, and granulocytes were collected after centrifugation and washed with phosphate-buffered saline (PBS) (P3813; Sigma). The cells were incubated in lysis buffer [154 mM NH4Cl (31107; Sigma), 10 mM $KHCO_3$ (4854; Merck), and 0.1 mM EDTA in deionized water) for 10 min to lyse the erythrocytes. They were then centrifuged and washed with PBS. Then, the cells were incubated with magnetic beads, pancytokeratin (5c-81714; Gentaur) for breast, colon, prostate, unknown origin, SCC and ovarian types of cancer, CD99 (39-CD99-250; Gentaur) for sarcoma, CD45 (8804-6802-74; Ebioscience) for myeloma and glioblastoma, or CD63 (39-CD63-250; Gentaur) for melanoma, at 4˚C for 30 min. After incubation, the samples were placed in a magnetic field, positively or negatively selected based on the cancer type, and then washed with PBS. CD45 negative selection was performed only for glioblastoma cells. For the rest types of cancer, positive selection was carried out. The cells were isolated and cultured in 12-well plates (4430400N; Orange Scientific) with RPMI-1640 medium (R6504; Sigma). The number of cells was calculated by performing a trypan blue (0.4%) viability test with neubauer chamber. The isolated CTCs were validated with cellular and molecular-based assays. The validation included identification of the above markers with flow cytometry prior to RNA isolation and then endpoint-PCR after isolation and prior to qPCR experiments.

4.4. Molecular Analysis

Total RNA from cell cultures was extracted using a MagCore Total RNA Cultured Cells Kit (MRC-02; RBC Bioscience). The samples were evaluated spectrophotometrically. Then, 1 μg of each RNA sample was used as a template for cDNA synthesis using a PrimeScript RT Reagent Kit (RR037A; Takara). Real-time qPCR was then performed using KAPA SYBR Fast Master Mix (2×) Universal (KK4618; KAPA Biosystems). Specific primers for each marker and the reference gene (ACTB) were designed using Gene Expression 1.1 software. Primer sequences were evaluated by BLAST searching to exclude those that would amplify undesired genes (**Table 2**). The PCR program was as follows: initial denaturation at 95˚C for 3 min, 40 cycles of denaturation at 95˚C for 10 s followed by annealing at 59˚C for 30 s. Melting curve analysis was performed from 65˚C to 95˚C with 0.5˚C increments for 5 s at each step. The qPCR products were run on agarose gels to validate the results. ΔCt value was used for analysis of experiments. Finally, relative quantification was performed using the normal samples as the reference group according to Livak [35].

Table 2. Primer sequences used in molecular and cytogenetic assays.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Length (bp)	Annealing Temp (°C)
ACTB	GCCCTGGACTTCGAGCAAGAGA	CAGGAAGGAAGGCTGGAAGAGTG	144	84
NANOG	CGTGTGAAGATGAGTGAAACTG	GGATGGGCATCATGGAAA	138	79
OCT3/4	AGGAAGCTGACAACAATG	ACTCGGTTCTCGATACTG	97	79
CD34	CCCATGCTGGAGGTGACATCTC	CCAGGGAGCCGAATGTGTAAAG	130	82
NESTIN	GAGACACCTGTGCCAGCCTTTCTTA	CTGGGCTCTGATCTCTGCATCTACAG	132	83
SOX2	CTCGCCCACCTACAGCAT	GCTGGCCTCGGACTTGAC	91	84

4.5. Statistical Analysis

qPCR results were tested for normality according to the Kolmogorov-Smirnov test. The Kruskal-Wallis test and one-way analysis of variance were performed on the qPCR data depending on their distribution. A significant p-value was defined as less than 0.05. Statistical analysis was performed with PAST version 2.10 [36].

4.6. Ethics Approval

This study is not a clinical trial and does not include intervention in patients. All procedures were conducted according to the standards of Safety, Bioethics and Validation. The study was reviewed and approved by Bioethical Committee of the Research Genetic Cancer Centre Group. Each patient provided informed consent in writing for the use of their sample in the present study. The patients retained the right to withdraw their sample until the date when the sample was received at the laboratory and tested.

5. Conclusion

We demonstrated that CTCs exhibit stemness characteristics and express specific transcription factors. Their expression is different and might be used to delineate either biomarkers or prognostic indicators. The higher expression of some genes may lead to the identification of potential drug targets. The genetic profiles among different types of cancer could also be used as a predictive reference in therapy. However, all the above aspects need to be tested in more samples including other types of cancer.

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Abbreviation List

CTCs: Circulating tumor cells

CSCs: Cancer stem cells

CK: Cytokeratin

EMT: Epithelial-to-mesenchymal transition

qPCR: Quantitative polymerase chain reaction

PCR: Polymerase chain reaction

RT-qPCR: Reverse transcription polymerase chain reaction

SCC: Squamous cell carcinoma