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Stemness-Related Transcriptional Factors and Homing Gene Expression Profiles in Hepatic Differentiation and Cancer

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Stem cell transcriptional signature activation is an essential event in the development of cancer. This study aimed to investigate the differential expression profiles of three pluripotency-associated genes, *OCT4*, *NANOG* and *SOX2*, G-protein-coupled chemokine receptor 4 (*CXCR4*) and the ligand *CXCL2*, and alpha-fetoprotein (*AFP*) in hepatogenic differentiated stem cells and in sera of hepatitis C virus (HCV) and HCV-induced hepatocellular carcinoma (HCC) patients. Mesenchymal stem cells derived from umbilical cord blood were differentiated using hepatogenic differentiation media. Serum specimens were collected from 96 patients (32 cirrhotic HCV, 32 early HCC and 32 late HCC) and 96 controls. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed for relative quantification of the six target genes using the Livak method. *In silico* network analysis was also executed to explore the pluripotency and tumorigenetic regulatory circuits in liver cancer. The expression levels of all genes declined gradually during the stages of stem cell differentiation. On univariate and multivariate analyses, *NANOG*, *CXCR4* and *AFP* were significantly upregulated in late clinical stage HCC patients. In contrast, *SOX2* and *CXCL2* were markedly overexpressed in cirrhotic patients and could be used for clear demarcation between cirrhotic and HCC patients in our cases. In conclusion, our data highlight the potential role of the *SOX2* stem cell marker and *CXCL2* chemokine in liver cell degeneration and fibrogenesis in HCV-induced hepatic cirrhosis in our sample of the Egyptian population. In addition, the significant association of *NANOG* and *CXCR4* high expression with late HCC could contribute to the acquisition of stem cell–like properties in hepatic cancer and dissemination in late stages, respectively. Taken together, our results could have potential application in HCC prognosis and treatment.

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INTRODUCTION

Carcinogenesis is a multistep process that transforms differentiated tissues into an immature cell population (1). This neoplastic progression in cancer cells causes an accumulation of genetic and epigenetic alterations by multiple DNA mutations and clonal selection. These changes cause cancer cells to return to immature undifferentiated phenotypes and to acquire novel molecular signatures (2). Recent accumulating evidence suggests a highly relevant overlap between stemness, epithelial-mesenchymal transition (EMT) and cancer (3).

Three basic transcription factors control the expression of several genes and function as differentiation repressors (4): octamer-binding transcription factor 3/4 (OCT3/4), also known as POU domain class 5 transcription factor 1 (POU5F1); sex determining region Y-box 2 (SOX2); and (c) homeobox protein NANOG (named after the Celtic phrase Tír na nÓg, meaning the land of the young). They play critical roles in maintaining the pluripotency and self-renewal (stemness) characteristics of human embryonic stem cells (ESCs) (5) and contribute to the reprogramming of adult somatic cells into an ESC-like state (4). Their gene expression has been reported in cancer stem cell niche and tumor cells in higher levels than in nontumor tissues (1). Overexpression of *OCT3/4*, *SOX2* and *NANOG* has been detected in several cancers, including breast (6), head

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and neck (7), and prostate (8) cancers. They are relevant to tumorigenicity, metastasis (9) and radioresistance (7).

Chemokine (C-X-C motif) receptor 4 gene (*CXCR4*) encodes for a leukocyte-derived transmembrane receptor specific for stromal cell–derived factor-1 (SDF-1), essential for immune surveillance, tissue homeostasis, and tumor growth and metastasis (10). CXCR4 is one of the chemoattractant molecules related to immune cell directed migration (11), gastrointestinal tract vascularization and endothelial progenitor cell recruitment from bone marrow during angiogenesis (12). Its expression is elevated in breast cancer and leukemia (13). Previous studies have reported that *CXCR4* expression in hepatocellular carcinoma (HCC) might correlate with the progress of the cancer and its lymphatic and distant metastasis (14–17).

Chemokine (C-X-C motif) ligand 2 (CXCL2) (i.e., growth-related oncogene [GRO]-2[β]) is a member of the CXC chemokine family, displaying a Glu-Leu-Arg (ELR) amino acid motif that mediates various functions, including attracting and activating neutrophils and stimulating endothelial cell migration, hence participating in tumorigenesis and progression (18). It has diverse effects on carcinogenesis, according to the tumor microenvironment. *CXCL2* relative expression has been found to be low in breast cancer (19) and elevated in an estrogen receptor–positive breast cancer type in another study (20). The same levels of expression have been found in normal and adenocarcinoma samples of the colon (21), while it was significantly elevated in colon cancer compared with normal adjacent tissue, facilitating cancer cell invasion and metastasis, and correlated with an unfavorable outcome in other studies (22,23). However, little is known about the expression of *CXCL2* in HCC and its correlation with the clinical disease outcome.

Taken together, as *SOX2*, *NANOG* and *OCT4* mRNA expression in blood samples of cirrhotic and HCC Egyptian patients has not been studied before, to the authors' knowledge, we propose that altered expression of these stemness genes that are regulators of induced pluripotency and high self-renewal capacity, and those that modulate the epithelial-mesenchymal transition, angiogenesis and immune cell infiltration of the tumor (i.e., *CXCR4* and *CXCL2*), might have tumor-promoting effects in HCC and might correlate with patients' clinical features. It is well known, in addition, that HCV infection is associated with HCC carcinogenesis, especially in the Egyptian population (24). Hence it is important to further detect the potential association between HCV-induced cirrhosis and these biomarkers. The current study was conducted to quantify the relative expression levels of the aforementioned five genes, in addition to the *AFP* gene, in sera of HCV-induced cirrhosis and hepatic carcinoma patients compared to hepatogenic differentiated stem cells and sera of healthy controls, and to correlate these expressions with the clinical parameters.

MATERIALS AND METHODS

As mesenchymal stem cells (MSCs) isolated from umbilical cord can be easily obtained, are more primitive than those isolated from other sources such as bone marrow and adipose tissue (i.e., they involve highly invasive procedures for the donors and a significant decrease in quantity and differentiation potential of cells with age), are less immunogenic and have been known to differentiate into hepatocyte-like cells (25), these cells were used in the current work as control cells for stem gene expression.

Collection and Storage of Umbilical Cord Blood

Informed consent was received from the mothers of six full-term newborns for collection of human umbilical cord blood (UCB) samples in the Department of Gynecology and Obstetrics at Suez Canal University Hospital. After baby delivery and placenta separation, UCB was harvested under aseptic conditions by umbilical vein puncture using a 20 mL sterile syringe. UCB (20 mL) was collected by gentle suction and emptied into a sterile 50 mL Falcon tube containing 4 mL citrate phosphate dextrose as an anticoagulant. Consequently, samples were labeled, preserved at room temperature (22 ± 4°C) and processed within 12 h (26).

Separation of Human Umbilical Cord Blood Mononuclear Cells

Mononuclear cells (MNCs) were separated from UCB in 50 mL Falcon tubes by Ficoll density gradient. In brief, 20 mL of fresh cord blood was diluted with phosphate buffered saline (PBS) at a ratio of 1:1 and mixed well. The diluted blood was gently layered drop by drop onto the top of warm Ficoll-hypaque. After centrifugation, the MNC fraction was collected into a sterile conical centrifuge tube. The cells were washed twice in 50 mL cold PBS (26).

Preparation of the Primary Culture of Mesenchymal Stem Cells

The MNC suspensions were seeded at concentrations of 1×10^6 cells/cm^2 and allowed to adhere to 25 cm^2 tissue culture plastic flasks, incubated in 7 mL of the fresh complete nutrient medium (Lonza) containing RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin and 0.5% fungizone. The flasks were incubated in a horizontal position in a humidified incubator at 37°C and 5% CO_2. The media flasks were examined daily, both visually and by an inverted phase-contrast microscope, for assessment of cell morphology and signs of contamination. The media were changed every third day. Splitting was done when 50–70% confluence was reached, and cells were harvested by trypsinization (27).

Hepatogenic Differentiation and Identification of Mesenchymal Stem Cells

At d 0, MSCs were harvested and recultured in tissue culture plastic flasks (25 cm^2). One set was fed with

complete media only, to serve as controls, and another set of flasks were cultured for 3 wks in hepatogenic medium containing the following: 2 mL RPMI with L-glutamine, 20% FBS, 1 mL 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) medium, 20 ng/mL hepatic growth factor (HGF), 100 µl penicillin/streptomycin and 0.5–1% fungizone. The media were examined daily and changed every 4 d. Cells harvested at d 7 and 14 were examined under the microscope for morphological changes (28). Expression profiles of hepatic markers *AFP* and albumin (*ALB*) were examined in differentiated and undifferentiated cells using real-time PCR at d 0, 7 and 14 and normalized with GAPDH.

Study Participants

The study population comprised 96 liver patients and 96 age- and sex-matched healthy controls recruited from blood bank donors without a history of cancer or any other chronic disorders. The patient group included 32 individuals with hepatitis C–related cirrhosis, 32 cirrhosis- and HCV-induced early HCC, and another 32 cirrhosis- and HCV-induced late HCC. Patients had no evidence of metabolic or autoimmune liver disease according to the clinical history and appropriate laboratory test results retrieved from their medical reports (i.e., serum ferritin, iron, iron-binding capacity and transferrin saturation levels for hemochromatosis; serum ceruloplasmin level for Wilson disease; smooth muscle antibodies and/or antinuclear antibodies for autoimmune hepatitis; and antimitochondrial antibodies for primary biliary cirrhosis). Patients were recruited from the general surgery and oncology clinics of Suez Canal University (SCU) Hospital and from the gastroenterology endoscopic ward of Assuit University Hospital, Egypt, during the period of November 2014 to July 2015. Lab work was performed in the Oncology Diagnostic Unit, Institute of Biotechnology for Postgraduate Studies and Research and Center of Excellence in Cellular and Molecular Medicine, SCU. Patients underwent the following: (1) thorough clinical assessment; (2) abdominal ultrasonography and abdominal triphasic computed tomography scan to confirm the presence of cirrhosis, portal hypertension, hepatosplenomegaly, HCC and ascites; (3) quantitative viral load (HCV RNA) assessment by real-time PCR; and (4) prognostic scoring of liver disease by the Child-Turcotte-Pugh classification. Barcelona Clinic liver cancer staging was used to assign patients into early and late HCC groups (29). The study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Medical Research Ethics Committee of the Faculty of Medicine, SCU. Written informed consent was obtained from all participants.

Specimen Collection

Fasting blood samples were collected from patients and controls; 6 mL was withdrawn and subdivided in sodium citrate (200 µL of 3.8%) anticoagulant tubes, plain tubes and trisodium EDTA (1 mg/mL) tubes. Another 2 mL was collected in Vacutainer serum separator tubes (Becton Dickinson) to obtain serum for subsequent genetic analysis. The citrated and clotted blood tubes were centrifuged at 700*g* for 20 min. Part of the plasma was used to immediately measure PT and the rest of the plasma and serum was aliquoted into microcentrifuge tubes (1 mL per aliquot) and stored at –80°C.

Laboratory Investigations

Fasting blood glucose, serum alanine transaminase, serum aspartate transaminase, alkaline phosphatase, serum total bilirubin and albumin were measured by automated enzymatic methods on a Hitachi-912 analyzer (Roche Diagnostics). Serum AFP concentration was measured by chemiluminescent immunometric assay on a Siemens Immulite 2000 (Siemens Healthcare Diagnostics). Hemoglobin estimation, white blood cell count and platelet count were detected by automated cell counter (Cell Dyne-2700, Abbott Lab). Prothrombin concentration was measured using an automated analyzer (Sysmex CA-1500).

RNA Extraction of Cultured Cells and Serum

Serum total RNA was stabilized by QIAzol reagent as a preliminary step before extraction (i.e., after addition of QIAzol lysis reagent to the serum; lysates can be stored at –70°C for several months, as recommended by the supplier (Qiagen)). For differentiated and undifferentiated stem cells, the RNeasy Mini Kit (Qiagen) was used to extract total RNA, following the manufacturer's protocol. All samples were subjected to treatment with RNase-free DNase I for 2 h at 37°C. RNA concentration and purity at the absorbance ratio 260/280 nm were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Tech.), followed by an agarose gel electrophoresis check for RNA integrity. The range of the extracted RNA was 20–65 ng/µl.

Reverse Transcription

For the reverse transcription (RT) reaction, a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. For each RT reaction (20 µL), RNA sample (10 ng) was added to 10 µL of 2 × RT reaction mix containing 10 × RT buffer (2 µL), 25 × dNTP mix (100 mM, 0.8 µL), 10 × RT random primers (2 µL), MultiScribe™ Reverse Transcriptase (1 µL), RNase inhibitor (1 µL) and nuclease-free water (3.2 µL). RT was carried out in a Mastercycler Gradient Thermocycler (Eppendorf) at 25°C for 10 min, followed by 37°C for 120 min, and finally 85°C for 5 min, then held at 4°C. Appropriate negative controls were included in each run.

Gene Expression Analysis

Real-time PCR was performed in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. The relative expression of the three pluripotent genes and *CXCL2* was quantified using the TaqMan® assay (Applied Biosystems, assay ID Hs02387400_g1 for *NANOG*, Hs01053049_s1 for *SOX2*, Hs03005111_g for *OCT3/4* and Hs00601975_m1 for *CXCL2*), *GAPDH*

endogenous control assay (TaqMan), and Universal PCR Master Mix II, No UNG (2 ×) (Taqman). PCRs were carried out in a total volume of 20 μL using the StepOne™ Real-Time PCR System (Applied Biosystems), as described previously (30).

Relative expression levels of *CXCR4* and the hepatic markers *AFP* and *ALB* were assessed via SYBR Green qPCR analysis and normalized with *GAPDH*. For *ALB*, *AFP* and *GAPDH*, the primer sequences were retrieved from Doan *et al.* (25), and for *CXCR4*, from Amin *et al.* (31). The reaction mixture (20 μL) contained 10 μL of qPCR Green Master (Jena Bioscience), 0.6 μL (10 μM) forward and reverse primers, 7.8 μL PCR-grade water, and 1 μL cDNA template. The reactions for SYBR Green assay in 48-well plates were performed with the StepOne™ Real-Time PCR System. The thermal cycle conditions were as follows: 95°C for 5 min followed by 15 sec at 95°C, 60 sec at 60°C and 1 min at 72°C, repeated for 45 cycles. PCR amplicons were confirmed to be specific by size and melting curve analysis. Post-PCR agarose gel was performed; the length of the amplicon was 94, 161, 315, and 1182 base pairs for *GAPDH*, *ALB*, *AFP* and *CXCR4*, respectively.

Multivariate Analysis

Ordination and cluster analysis techniques were applied to the data for multivariate analysis to identify the gene combinations that could discriminate different groups of patients. PC-ORD software version 5 (32) was employed to run different analyses. Data were examined for adjustment and to detect potential outliers in the first place. One gene (*POUF5A*) was fractionally too small so the results were multiplied by 100,000 as a transformation while the outliers were kept; trails of deletion or transformation of the outliers didn't show major differences in data representation.

The cluster analysis technique was used to separate groups of patients, and individuals within the groups, based on similarities and dissimilarities. Sørensen coefficient was used as a distance measure, while a flexible β was used as a linkage method, with β value of –0.75 to reach the minimum percentage of chaining (33). NMS ordination technique was run to visualize the gene data of patients along axes according to their resemblances. NMS was adjusted for 50 attempts with the Sorensen coefficient.

In Silico Structural and Functional Network Analysis of Genes

To identify the subcellular locations of the studied transcription factors, proteins and chemokines, the COMPARTMENTS database was used (compartments.jensenlab.org/). It is a Web server that integrates evidence on protein localization based on manually curated literature, high-throughput screens, automatic text mining and sequence-based prediction methods. Confidence scores were assigned as color codes, ranging from light green for low confidence to dark green for high confidence, and presented on a schematic cell (34).

Structural and functional analysis of genes was retrieved from online databases such as Ensemble.org and GeneCards.com. The predicted function of our gene set and their relationships with one another were determined by the GeneMANIA (multiple association network integration algorithm) prediction server (http://www.genemania.org). An automatically selected weighting method displayed 10 related genes and no related attributes. Association data included protein and gene interactions, coexpression, colocalization, pathways and similarities of protein domains (35).

Statistical Analysis

SPSS for Windows version 20.0 was used for data processing. Comparison of categorical variables was done using chi-square (χ^2) or Fisher's exact test where appropriate. Analysis of variance in cases of normally distributed data and Mann-Whitney U and Kruskal-Wallis tests for skewed data were used to compare continuous variables, followed by appropriate multiple comparison tests. Statistical significance was considered at p value (two-tailed) < 0.05. The fold change of mRNA expression in each patient sample relative to the mean of controls was calculated using the Livak method based on the quantitative cycle ($C_q = C_T$) value with the following equation: relative quantity = $2^{-\Delta\Delta CT}$(29).

All supplementary materials are available online at www.molmed.org.

RESULTS

Transcriptomic Analysis

Gene expression analysis showed upregulation of all the studied genes in undifferentiated stem cells (UDSCs), and these levels dropped dramatically upon differentiation of the stem cells using hepatogenic differentiation media. The mean values fluctuated during stages of liver diseases ranging from cirrhosis to early and late hepatocellular HCC (Figure 1). Detailed relative expression values of each gene are illustrated in Supplementary Table S1.

In brief, the mean and standard deviation of *OCT3/4* was 5.03 ± 2.6 in UDSCs, but the relative expression levels dropped significantly to half (2.84 ± 1.5) in DSCs. However, serum *OCT3/4* expression levels remained constantly underexpressed (0.01E-3 ± 0.0) in HCV cirrhotic (8.6E-3 ± 0.01), early HCC (1.1E-3 ± 0.0) and late HCC patients ($p < 0.001$). Regarding *NANOG* expression, although it followed the same trend as *OCT3/4* expression, stood at 69.0 ± 23.1 in UDSCs, reached a trough of 0.02 ± 0.0 in DSC, then evened out in cirrhotic and early HCC patients (0.13 ± 0.1 and 0.45 ± 0.1, respectively), the levels rose significantly to 5.7 ± 3.8 in the sera of late HCC patients. In contrast, *SOX2* showed the highest expression levels among all the studied genes in UDSCs (1155 ± 1070) and followed a similar pattern of downregulation in DSCs as the previous two pluripotent genes (0.6 ± 0.5). However, the most striking finding in the *SOX2* profile is the highest

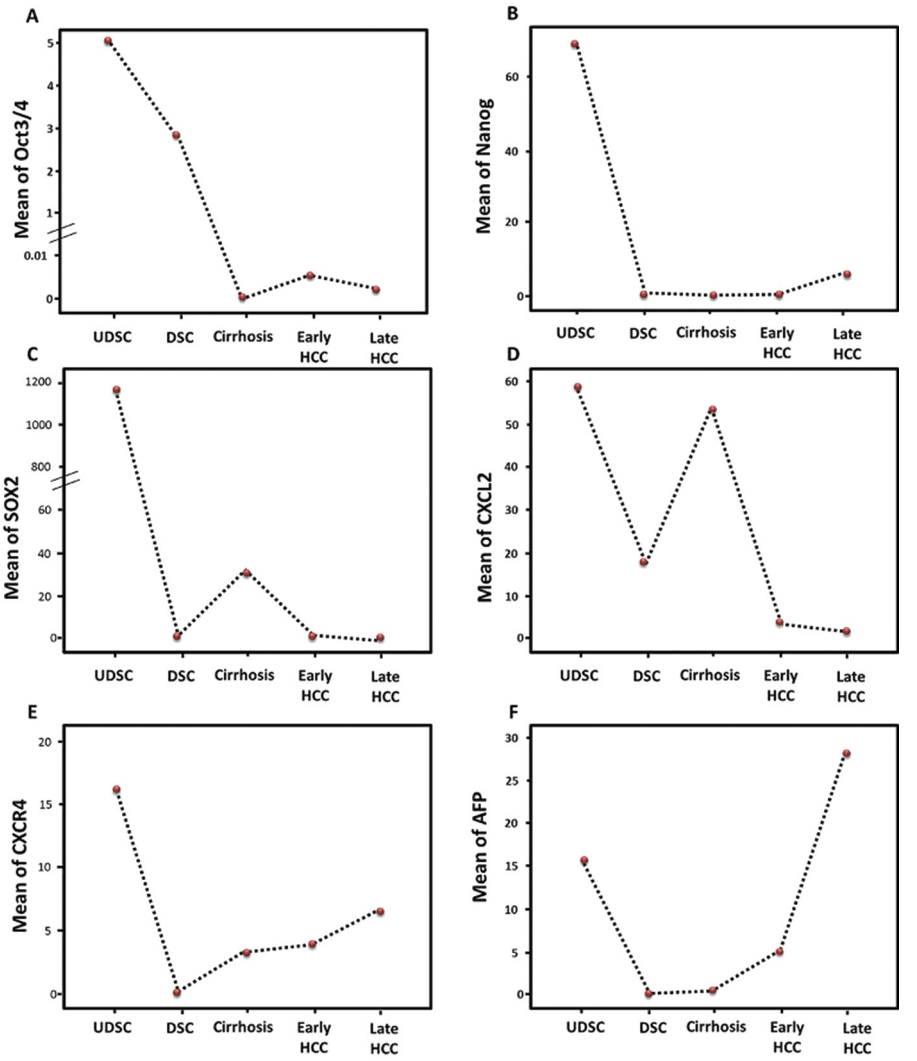

Figure 1. Gene expression levels in cultured stem cells and hepatic patients. Data are presented as means. ANOVA test was used, followed by Newman-Keuls multiple comparison test at 0.05 significance levels. UDSC, undifferentiated stem cells; DSC, differentiated stem cells; HCC, hepatocellular carcinoma.

expression levels in almost all cirrhotic patients enrolled in the study, reaching a mean value of 30.9 ± 12.0 compared with the cancer patient groups (0.63 ± 0.4 and 0.60 ± 0.3 for early and late HCC, $p < 0.001$).

For chemokine genes, *CXCL2* was overexpressed in all of the studied groups, with the highest levels in UDSCs (58.7 ± 3.5) and cirrhotic patients (53.6 ± 1.8). The levels plunged to a low of 4.03 ± 1.3 in the early HCC group and further declined to 1.9 ± 0.8 in late cancer patients ($p < 0.001$). For *CXCR4*, it was overexpressed in all groups except DSCs, and there was a significant increase in the gene transcription level in the late HCC (6.6 ± 5.4) compared with the cirrhotic and early cancer groups (3.23 ± 0.9 and 3.9 ± 1.7, respectively; $p < 0.001$). As for the gold standard hepatic tumor biomarker *AFP*, its level was highly elevated in UDSCs (16.1 ± 11.0), then dropped dramatically to 0.11 ± 0.1 in DSCs. As expected, *AFP* expression levels rose in the sera of cancer patients (4.9 ± 3.4 and 28.3 ± 14.2 for early and late HCC, $p < 0.001$).

Correlation Analysis

Gene coexpression analysis based on pairwise correlations of genes across samples is illustrated in Table 1. The expressions of the two pluripotent genes (*OCT3/4* and *NANOG*) and the two chemokine genes (*CXCL2* and *CXCR4*) were strongly correlated (r = 0.764, $p = 0.001$ and r = 0.900, $p = 0.037$, respectively). However, *CXCR4* was moderately correlated with *OCT3/4* and *NANOG*, with a correlation coefficient of 0.407 ($p < 0.05$), while *CXCL2* was strongly associated with *SOX2* expression (r = 0.900, $p = 0.037$).

Prognostic scoring of hepatic patients via the Child-Turcotte-Pugh classification revealed that higher expression of *NANOG* and *AFP* markers was associated with advanced disease stage ($p = 0.011$ and 0.002, respectively), while lower expression levels of *CXCL2* chemokine were significantly associated with Child C stage ($p = 0036$) (Figure 2).

Multivariate Analysis

Multivariate analysis showed separation of patients into three clear, distinct groups based on the expression profiles of the six gene combinations. This demarcation was mostly caused by two genes: *CXCL2* (r = 0.610 with axis 1 and 0.763 with axis 2 in the positive direction) and *AFP* (r = –0.455 with axis 1 and –0.413 with axis 2 in the negative direction) (Figure 3). Cluster analysis showed a clear cut between the cancer and noncancer patient groups, with 100% separation. However, a less clear cut was found between early and late cancer patients, with 70% differentiation (Supplementary Figure S1).

In Silico Data Analysis

Gene ontology and functional analysis of the pluripotent and chemokine analyzed genes are illustrated in Supplementary Figures S2 and S3, respectively. Subcellular localization of all the pluripotent genes was highly expressed in the nucleoplasm (Supplementary Figure S4). However, unlike the OCT3/4 transcription factor, which was confined

Table 1. Correlation analysis matrix of gene coexpression, shown as *p* values.

	Oct3/4	NANOG	SOX2	CXCL2	CXCR4	AFP
Oct3/4	1.0	0.764 **(0.001)**	0.006 (0.977)	–0.30 (0.624)	0.407 **(0.048)**	0.678 (0.150)
NANOG		1.0	0.060 (0.772)	–0.60 (0.285)	0.407 **(0.031)**	0.692 **(0.013)**
SOX2			1.0	0.900 **(0.037)**	0.163 (0.372)	–0.49 (0.106)
CXCL2				1.0	0.900 **(0.037)**	–0.24 (0.164)
CXCR4					1.0	0.531 (0.075)
AFP						1.0

Values shown in bold are statistically significant at $p < 0.05$. Pearson and Spearman's rho correlation tests were used.

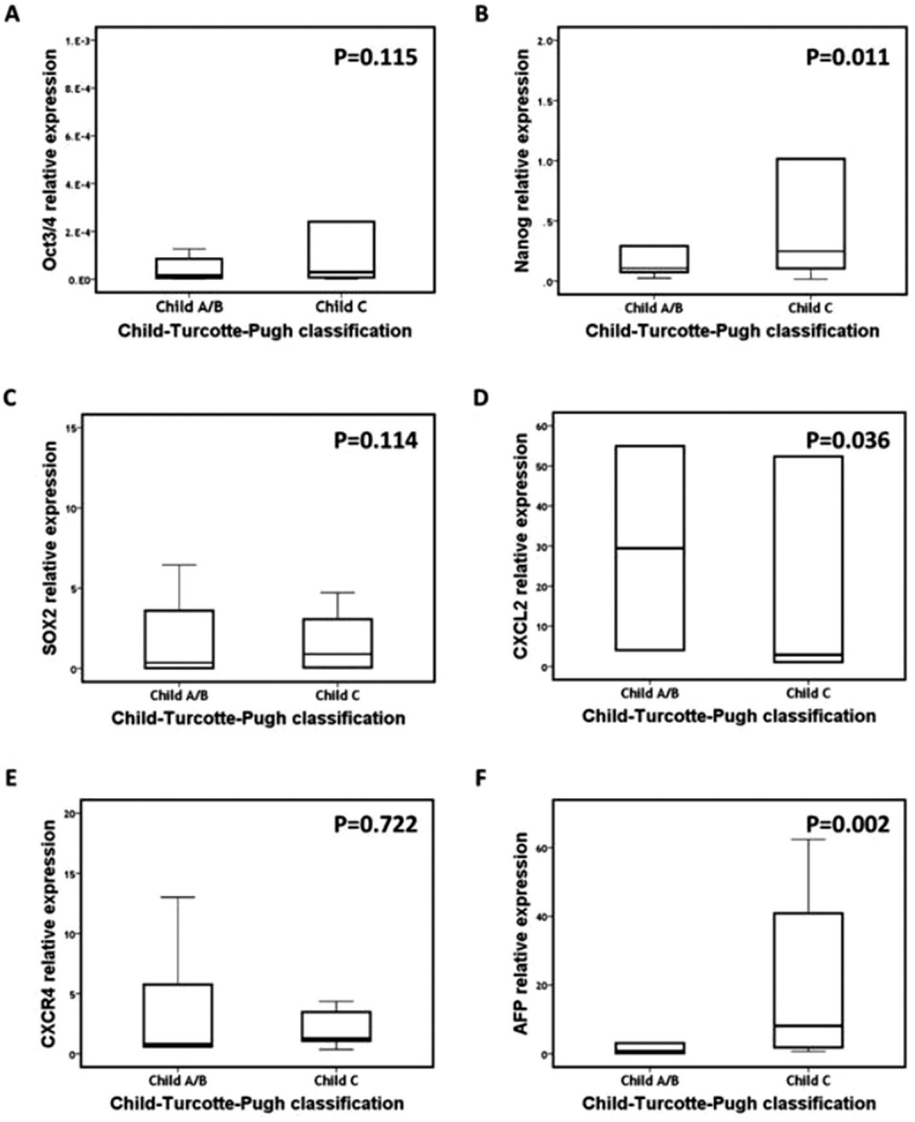

Figure 2. Association between gene expression and clinical characteristics. Child-Turcotte-Pugh classification was used for prognostic scoring in patients with liver disease. Mann Whitney U test was used. Statistical significance at $p < 0.05$.

within the nucleus, NANOG and SOX2 proteins also existed abundantly in the cytosol, cellular cytoskeleton and plasma membrane and could be secreted in the extracellular space as well. CXCL2 and CXCR4 existed in both cytoplasm and nucleoplasm, but were mostly found in the extracellular region. Only CXCR4 receptors existed in membrane-bounded vesicles as lysosomes and endosomes to be secreted at the cell surface. AFP was shown to be involved in all cellular compartments, including endoplasmic reticulum, golgi apparatus, mitochondria and peroxisomes, as well as plasma membrane and extracellular space.

Figure 4 demonstrates the predicted gene functional network of the studied gene set. Association data revealed colocalization of CXCL2 and AFP proteins, shared protein domains and coexpression of *OCT3/4* with both *NANOG* and *SOX2* genes, a physical interaction between SOX2 and OCT3/4 proteins, and genetic interaction between *SOX2* and *CXCR4* genes.

DISCUSSION

To date, various studies have demonstrated that circulating cell-free tumor nucleic acids may reflect the same genetic identity as the primary tumor and are therefore attractive for noninvasive biomarker determination, especially during the course of disease and in patients with no tumor tissue available (36). Growing evidence indicates that *SOX2*, *OCT4* and *NANOG* reexpression might facilitate the growth of tumor cells and metastasis, and modulate signaling pathways to inhibit apoptosis *in vitro* and *in vivo*, therefore playing an essential role in oncogenesis and tumor progression (37,38). Of concern, these markers of ESCs showed elevated expression in multiple cancers, contributing to their aggressiveness and bad outcome (39–42). However, little is known about the relative expression of these transcripts and their significant clinical associations in HCV-induced cirrhosis and related HCC in Egyptian patients.

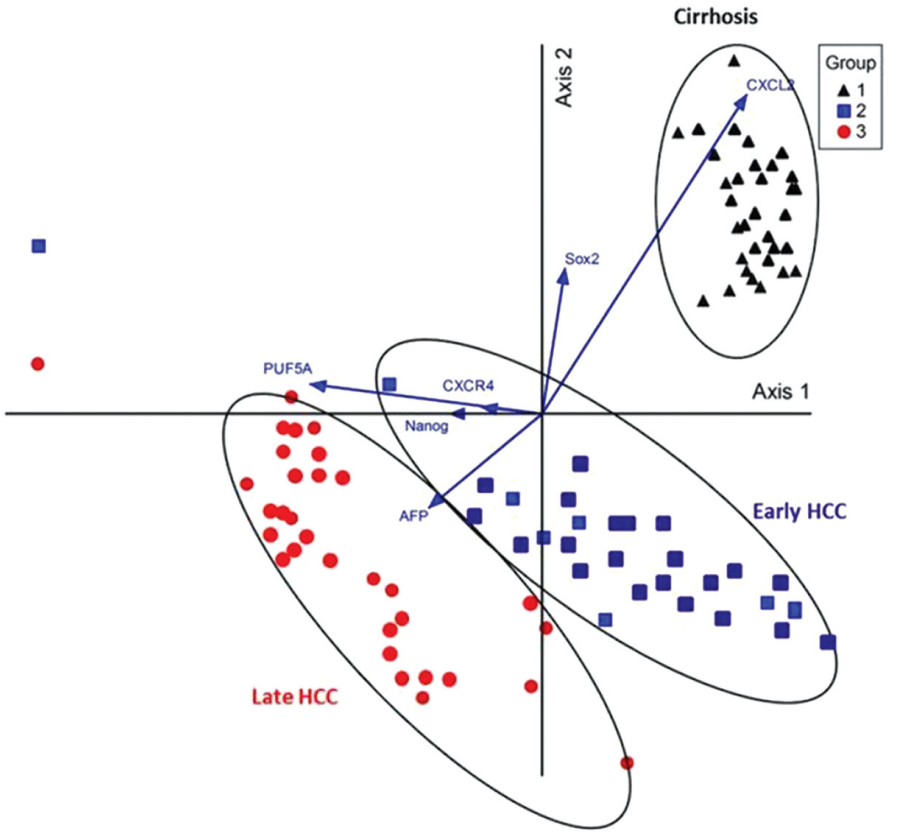

Figure 3. Multivariate analysis by nonmetric multidimensional scaling (NMS) ordination analysis in patients. NMS joint plot showing the separation of three distinct groups of patients along axes 1 and 2 based upon the data collected from gene expression. Different genes' direction and power of correlation with axis 1 and 2 are demonstrated. Both *CXCL2* and *AFP* might be considered as potential genes responsible for the groups' separation. *CXCL2* increased dramatically toward the noncancer group, while much less expression was shown in cancer patients. *AFP* increased dramatically toward the patients with late hepatic carcinoma and much less expression was shown with early cancer patients.

In the current work, all studied ESC markers were significantly upregulated in UDSCs, supporting their role in these cells. In hESCs, OCT4, NANOG and SOX2 function as differentiation repressors that prevent the ESCs from dividing into the wrong cell type in advance. Each of these transcription factors controls a specific cell fate: e.g., extraembryonic- and epiblast-derived cell destinations are controlled by OCT4; embryonic ectoderm differentiation is repressed by NANOG, which has little effect on other lineages; and mesendoderm differentiation is repressed by SOX2 (43). Subsequently, their levels dropped significantly upon differentiation as expected. In principle, the levels of expression of these pluripotent transcription factors should decrease with cell differentiation. Although our *in silico* analysis revealed that expression of the studied genes had similar colocalization and physical interactions, they seemed to be differentially expressed independently in cancer and cirrhosis. However, we should emphasize that the mechanistic functions of OCT4, NANOG and SOX2 in cancer cells are a little different from their functions in ESCs. Although they share the property of self-renewal, they maintain repression of lineage-specific differentiation in hESCs (38), whereas in cancer-related cells, overexpression of these markers modulates signaling pathways to inhibit apoptosis (4).

Our results revealed the highest expression of *SOX2* mRNA in all cases of cirrhotic patients enrolled in the study. This could emphasize the role of *SOX2* in tissue hyperplasia and tumor initiation rather than correlation with tumor phenotype *per se* in the study cases. According to previous research and bioinformatics prediction tools used in the current study, and validated by previous functional studies, SOX2 regulates cell growth and tumor invasion via multiple signaling pathways, including mitogen-activated protein 4 kinases 4(MAP4K4)-survivin, wingless-related integration site (Wnt)/β-catenin, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), estrogen receptor alpha (ERα), bone morphogenetic protein (BMP) and Notch pathways (44), forming a complex regulatory network, implying that SOX2 plays a critical role in cell and tumor biology. Which of these mechanisms could be related to fibrosis and cirrhosis needs to be further investigated.

Regarding *NANOG* expression in the current work, it was significantly elevated in the sera of late HCC patients compared to cirrhotic and early HCC patients. In recent years, *NANOG* was characterized as a new oncogene by large-scale oncogenomic analysis (45), and its expression has been found to regulate human tumor development, as evidenced by short hairpin RNA transduced cancer cells showing declining long-term clonal growth, decreased proliferation and, in some cases, altered differentiation (46). Our finding was in line with previous observations that linked *NANOG* expression with tumor aggressiveness and bad prognosis in multiple malignancies, including endometrial, gastric, colorectal and hepatocellular carcinomas (47–51). In addition, the current significant association of high *NANOG* expression with late-stage HCC versus other studied stem-related genes has been clarified by multivariate analysis confirming the unique dependency of

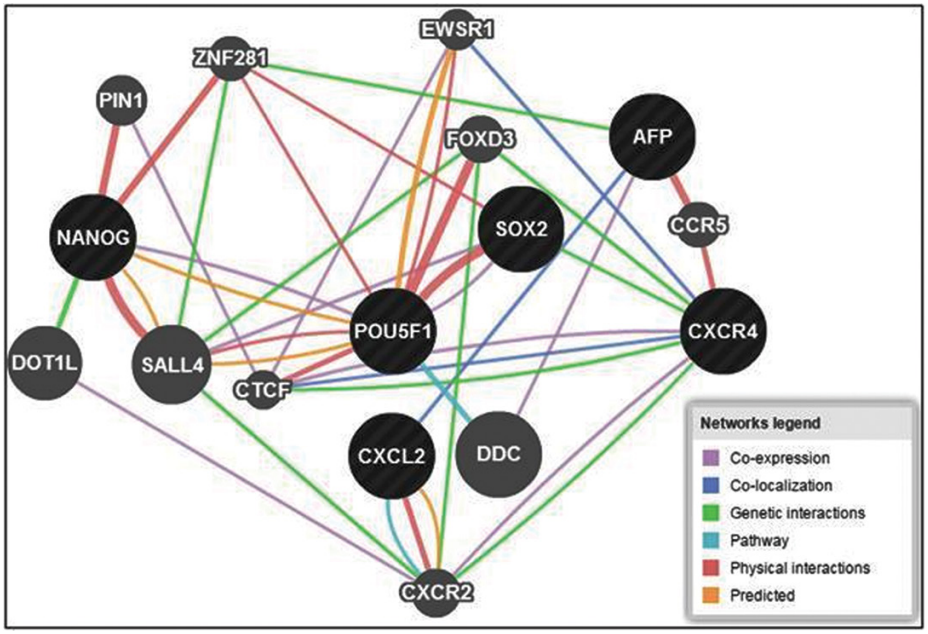

Figure 4. Interactive functional association network of the gene set. The network nodes are proteins. Predicted functional links between the proteins are indicated by edges in color coded lines. GeneMANIA was used (http://www.genemania.org).

SCs on specific pluripotent transcriptional factors for tumor maintenance upon entry into neoplastic, transformed states of self-renewal, as confirmed by Ji and colleagues (52). Later research has found that unlike normal SCs, OCT4 is dispensable for self-renewal, survival and differentiation of transformed cells. There has been direct evidence for the functional divergence of OCT4 from the pluripotent state following transformation, and it has been shown that NANOG is critical in maintaining the undifferentiated state of normal pluripotent SCs while repressing neural differentiation and apoptosis.

Shan *et al.* (53) suggested that NANOG could be a novel biomarker for CSCs in HCC, and that it might play a vital role in maintaining the self-renewal of these cells through the insulin-like growth factor-1 receptor (IGF1R)-signaling pathway. Other researchers (54) have reported that NANOG could facilitate invasion of liver cancer cells by inducing EMT through the NODAL/SMAD3 signaling pathway. In addition, recent studies have shown that *NANOG* could be upregulated by viral oncoproteins (e.g., HCV NS5A accompanied by enforcing expression of phosphorylated stat3 protein that binds directly to the *NANOG* promoter, contributing to regulation of its expression). Subsequently, this promotes cell growth and cell cycle progression (55), indicating that aberrant *NANOG* expression and function is involved in hepatic oncogenesis of HCV-related HCC.

Karakasiliotis and Mavromara (56) reported that HCV structural and nonstructural protein-induced stemness through parallel pathways underscores its importance in virus proliferation and persistence. Many researchers have suggested that the hypoxia within the HCV-infected cells' microenvironment that is predominantly found in inflamed and malignant areas of the liver could play an essential role in the programming of liver cells and the divergence of multiple pathways induced by HCV toward stemness, mainly due to upregulation of *NANOG*, *OCT4, SOX2*, *c-myc* and *Klf4* (Krupple-like factor 4) (57,58). Remarkably, hypoxic conditions induced HCV replication in Huh7.5 cells, which was associated with increased activity of anaerobic cell energy production and proliferation (59). Thus, the interplay between hypoxia-induced stemness and cell energy machinery activation by HCV, and through that enhancement of HCV replication, may promote HCV spread and tumorigenesis in chronically infected individuals (60).

In the current study, a significant positive relationship ($p = 0.001$, $r = 0.8$) between elevated relative expressions of *NANOG* and *OCT3/4* was found and confirmed by our *in silico* analysis of the interactive functional association network of their genes. This was in line with previous investigations supporting the joint role of both biomarkers in controlling multiple pathways to govern the self-renewal characteristics and pluripotency of ESCs (60) and their positive involvement in tumor invasion and progression of many types of cancer (42,50,61–64). However, the functional impacts of both markers' coexpression in HCC need to be further explored, especially in our case, as a high level of *NANOG* expression was significantly associated with late-stage versus early-stage HCC compared to *OCT3/4*.

For chemokine genes, *CXCL2* was found to be overexpressed in all study groups, with the highest levels in UDSC and cirrhotic patients. Moreover, it was shown to be one of the genes, in addition to *SOX2*, helpful in clear demarcation between cirrhotic and HCC patients by our multivariate analysis. CXCL2 is a classic neutrophil chemoattractant. Binding to its receptor CXCR2 leads to neutrophil recruitment, granule enzyme release and adhesion molecule expression, all of which amplifies the effects of inflammation and subsequently increases the risk of tumorigenesis (23). In line with our results, *CXCL2* relative mRNA expression, in addition, was increased in premalignant adenomas in a previous study (65), which suggests that dysregulation of *CXCL2* may be an early event in tumorigenesis and could partially explain the high expression of

this chemokine in our cirrhotic cases, as HCV-induced cirrhosis is one of the sequential hallmarks of liver disease: steatosis, steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (66). Our results were also in agreement with prior studies that reported an increase in *CXCL2* expression during tumorigenesis of many types of cancers, including esophageal squamous cell carcinoma (67), melanoma (68) and colorectal cancer (23), among others. All these studies support the significance of chemokines in cancer, in addition to having a role in development and inflammation. At the cellular level, CXCL2 has been reported to increase matrix metalloproteinase production, participate in cancer progression of oral squamous cell carcinoma (69) and activate the extracellular signal-regulated kinase-1/2 pathway contributing to esophageal squamous cell carcinoma proliferation (70).

Regarding *CXCR4*, it was overexpressed in all study groups except DSCs and showed significant association with late HCC. This is in concordance with *CXCR4* being expressed in normal stem cells of different organs and tissues and may partially explain why some tumors could express *CXCR4*, as these malignant cells may derive from *CXCR4* expressing normal stem cells (13). CXCR4 seems to govern many aspects of the natural history of cancer, from development to metastasis. Through binding to its chemotactic factor CXCL12, it mediates dissemination of primary tumors to different organs in line with the "homing" theory (14) and induces expression of β-integrin on cancer cells, which is critical for adhesion of these cells to the vascular cellular adhesion molecule, thus facilitating tumor-cell/endothelial-cell attachment (71) and correlating with the advanced state of the cancer (17). In addition, *in vitro* transfection of CXCR4 into a tumor cell was found in a previous study (72) to greatly enhance its metastatic potential. Moreover, CXCR4-expressing cells, oval cells, have been found to migrate to the liver along a CXCL12 gradient established by injured hepatocytes, inducing oval cell–mediated liver regeneration in hepatitis (73). Thus, dissemination of hepatic cancer and regeneration of hepatic cells share common pathways of chemotaxis (14).

In the current study, multivariate analysis confirmed the role of AFP as a tumor biomarker associated with HCC development and progress that could be used for clear demarcation between HCV-induced cirrhosis and HCC in our cases. This role is in line with a number of recent reports proposing a rationale for the ongoing use of AFP to identify and follow patients at higher risk of HCC (74–76).

CONCLUSION

This is the first study, to the best of our knowledge, to indicate that the SOX2 stem cell marker and CXCL2 chemokine could have a potential role in HCV-induced cirrhosis in Egyptian patients. In addition, the current results support previous findings showing that upregulation of *NANOG* and *CXCR4* expression and their significant association with late HCC could contribute to the acquisition of stem cell–like properties in hepatic cancer and dissemination in late stages, respectively. However, one of the limitations that need to be considered is the relatively small sample size in the present study, which might limit the statistical power and could underestimate the weak and potential associations. Hence, additional large sample size studies followed by functional analysis are warranted to validate the current findings and explore the detailed biological mechanisms. Furthermore, future detailed characterization of the studied gene-regulatory networks that are active in HCC is likely to yield powerful diagnostic and prognostic markers and, quite possibly, attractive and specific targets for therapeutic intervention.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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