Research

Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells

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Background

Cancer stem cell hypothesis is the tumoral cells which have stem cell features such as self-renewal, high migration capacity, drug resistance, and aberrant differentiation which constitute the heterogeneous population of tumor [1,2]. Tissue-specific stem cells are defined by their ability to self-renew and to produce the well differentiated and functional cells within an organ. Differentiated cells are

Abstract

Background: The studies on cancer-stem-cells (CSCs) have attracted so much attention in recent years as possible therapeutic implications. This study was carried out to investigate the gene expression profile of CSCs in human lung adenocarcinoma A549 cells.

Results: We isolated CSCs from A549 cell line of which side population (SP) phenotype revealed several stem cell properties. After staining the cell line with Hoechst 33342 dye, the SP and non-side population (non-SP) cells were sorted using flow cytometric analysis. The mRNA expression profiles were measured using an Affymetrix GeneChip® oligonucleotide array. Among the sixty one differentially expressed genes, the twelve genes inclusive three poor prognostic genes; Aldo-keto reductase family 1, member C1/C2 (AKR1C1/C2), Transmembrane 4 L six family member 1 nuclear receptor (TM4SF1), and Nuclear receptor subfamily 0, member 1 (NR0B1) were significantly up-regulated in SP compared to non-SP cells.

Conclusion: This is the first report indicating the differences of gene expression pattern between SP and non-SP cells in A549 cells. We suggest that the up-regulations of the genes AKR1C1/C2, TM4SF1 and NR0B1 in SP of human adenocarcinoma A549 cells could be a target of poor prognosis in anti-cancer therapy.
generally short-lived; in skin and blood for example, they are produced from a small pool of long-lived stem cells that last throughout the life [3-6]. Therefore, stem cells are necessary for tissue development, replacement, and repair [7]. On the other hands, the longevity of stem cells make them susceptible to accumulating genetic damage and thereby representing the growth root for cancer recurrence following treatment [8]. It was reported that some of the tumor stem cells can survive chemotherapy and support re-growth of the tumor mass [9].

Cancer stem cells (CSCs) were first identified in 1990s in hematological malignancies, mainly acute myelogenous leukemia (AML) and also in other subtypes like AML M0, M1, M2, M4, and M5, chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and multiple myeloma [10,11]. CSCs are also known in solid tumors like breast, brain, lung, prostate, testis, ovary, stomach, colon, skin, liver, and pancreas [12-17]. A character of stem cells, termed “side population (SP)”, has been identified using Hoechst 33342 dye. The flow cytometric analysis makes sorting possible either to SP or non-SP cells. The SP cells have been isolated from various types of adult tissue where they demonstrate stem cell activity [18-23]. The findings of these previous studies suggest that the SP phenotype represents a common feature of stem cells.

We performed our work on human lung adenocarcinoma A549 cells (of which SP phenotype revealed several stem cell properties [24]) to identify the genes, which make the CSCs of poor prognostic phenotype and evaluate the gene expression intensities of SP and non-SP cells using oligonucleotide micro-array. The reasons why the A549 cell line was selected, because it has a relatively high proportion of SP cells compared to other cell lines [25] and is more chemo-resistant particularly to platinum drugs [26].

Results

The distinct gene regulations in SP cells

We sorted A549 cell line to SP and non-SP cells (Fig. 1) and compared the gene expression intensities of both cells. Official symbols and gene names were used in accordance with the symbol and name lists approved by HUGO (Human genome organization) Gene Nomenclature Committee (Table 1) [27]. Following data analysis, 12 genes were considered as up-regulated in SP cells (TM4SF1 has 2 probe ID) (fold changes are shown in Table 2), whereas, 49 genes were down-regulated (Fig. 2). Since we focused on distinct gene regulations, the student’s t-test was not employed to prevent loss of up-regulated genes in all of three chip data, though it had large chip variations.

Validation of gene regulations

To confirm the fold changes of AKR1C1 in chip data, quantitative real time – reverse transcriptase PCR was employed. The relative fold changes in SP compared to non-SP cells were 3.11 ± 0.92 and 2.88 ± 0.17 in micro-array and qrt-rtPCR, respectively (Fig. 3).

Discussion

Based on the cancer stem cell hypothesis, we assumed that the up-regulation of certain genes that are related to poor prognosis (high migration capacity or drug resistance) in SP of cancer cells could be a target for therapeutic index. In the present study, we found some genes that are related to drug resistance, such as AKR1C1/C2 and NR0B1, or cancer metastasis, such as TM4SF1, were up-regulated in SP cells of human lung adenocarcinoma A549 cell line. Furthermore, the up-regulated gene, ABCG2, has been noticed to be as an indicator for sorting SP cells by Hoechst 33342 staining [24]. It was reported that ABCG2 pumping out the drugs was associated with multi-drug resistance in many cancers [28,29] and/or effects higher levels of DNA repair and hence lowered the ability to apoptosis [30].

AKR1C belongs to a superfamily of monomeric, cytosolic NADP(H)-dependent oxidoreductases that catalyzes the metabolic reduction and either activate or inactivate several xenobiotics [31,32]. In humans, at least four isoforms of AKR1C (AKR1C1~4) have been identified [33]. AKR1C1/AKR1C3 was known to inactivate progesterone, which could alter the progesterone/estrogen ratio in certain cancers [34,35]. Additionally, AKR1C1/C2 inhibitors were reported as potential anti-neoplastic agents [36,37].
| Gene symbol | Gene name |
|-------------|-----------|
| AKR1C1; AKR1C2 | aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase); aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) |
| TM4SF1 | transmembrane 4 L six family member 1 |
| NR0B1 | nuclear receptor subfamily 0, group B, member 1 |
| LRPRC | leucine-rich PPR-motif containing |
| SFRS3 | splicing factor, arginine/serine-rich 3 |
| ABCG2 | ATP-binding cassette, sub-family G (WHITE), member 2 |
| Unidentified 1 | adult retina protein |
| KRT4 | keratin 4 |
| ZNF567 | zinc finger protein 567 |
| ILE6R | interleukin 6 receptor |
| PAMC1 | peptidylglycine alpha-amidating monoxygenase COOH-terminal interactor |
| ZNF267 | zinc finger protein 267 |
| NEFL | neurofilament, light polypeptide 68 kDa |
| SFMBT2 | Scm-like with four mbt domains 2 |
| FSTL1 | follistatin-like 1 |
| TMEPAI | transmembrane, prostate androgen induced RNA |
| COL5A1 | collagen, type V, alpha 1 |
| SLC6A15 | solute carrier family 6, member 15 |
| COL1A1 | collagen, type I, alpha 1 |
| NTRK3 | neurotrophic tyrosine kinase, receptor, type 3 |
| CDH2 | cadherin 2, type I, N-cadherin (neural) |
| ANX8A | annexin A8 |
| ANX8B | annexin A8 |
| THBD | thrombomodulin |
| RAB3B | RAB3B, member RAS oncogene family |
| ADAM19 | ADAM metallopeptidase domain 19 (meltrin beta) |
| COL4A2 | collagen, type IV, alpha 2 |
| IGFBP7 | insulin-like growth factor binding protein 7 |
| COL4A1 | collagen, type IV, alpha 1 |
| IFI16 | interferon, gamma-inducible protein 16 |
| GLIPR1 | GLI pathogenesis-related 1 (glioma) |
| TGF2 | transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase) |
| COL4A1 | collagen, type IV, alpha 1 |
| IGFBP7 | insulin-like growth factor binding protein 7 |
| MYL9 | myosin, light chain 9, regulatory |
| Unidentified 2 | CDNA FLJ44429 fis, clone UTERU2015653 |
| MATN2 | matrilin 2 |
| TNFAIP6 | tumor necrosis factor, alpha-induced protein 6 |
| FRMD5 | FERM domain containing 5 |
| RUNX2 | runt-related transcription factor 2 |
| TMEPAI | transmembrane, prostate androgen induced RNA |
| GLIPR1 | GLI pathogenesis-related 1 (glioma) |
| NPTX1 | neuronal pentraxin 1 |
| GLIPR1 | GLI pathogenesis-related 1 (glioma) |
| SPARC | secreted protein, acidic, cysteine-rich (osteonectin) |
| COL5A1 | collagen, type V, alpha 1 |
| COL1A1 | collagen, type I, alpha 1 |
| B4GALT1 | UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1 |
| Unidentified 3 | - |
| TNS1 | tensin 1 |
| SPOCK1 | sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1 |
| MOBKL2B | MOB1, Mps One Binder kinase activator-like 2B (yeast) |
| ID2 | inhibitor of DNA binding 2, dominant negative helix-loop-helix protein |
| C4orf18 | chromosome 4 open reading frame 18 |
| COL5A1 | collagen, type V, alpha 1 |
| TAGLN | transgelin |
| CXCR7 | chemokine (C-X-C motif) receptor 7 |
| Unidentified 4 | Transcribed locus, moderately similar to XP_517655.1 PREDICTED: similar to KIAA0825 protein [Pan troglodytes] |
| COL5A1 | collagen, type V, alpha 1 |
| MOBKL2B | MOB1, Mps One Binder kinase activator-like 2B (yeast) |
| TAGLN | transgelin |
| SPARC | secreted protein, acidic, cysteine-rich (osteonectin) |
The high expression of AKR1C was considered as a poor prognostic factor in patients with non-small-cell lung cancer [38], and it was enriched in hepatocellular carcinoma than normal hepatic cells [39]. A previous study evaluated the relationship between the AKR1C and drug resistance and revealed that the over expression of AKR1C1/C2 led to drug resistance in non-small-cell lung cancer cells [26]. A public database (Gene Expression Omnibus (GEO), NCBI) [40] has shown significant up-regulation of AKR1C1 in smokers (Figure 4A). Similar trends were also reported in lung cancer patients [41,42]. This means that smoking can alter the regulation of certain genes related to poor prognosis such as AKR1C1. Moreover, AKR1C1 was suggested as a marker of stem-like cells in thyroid cancer cell lines, though it was not proved by the authors [41].

From the data of GEO, the TM4SF1 gene, which is believed to be involved in cancer invasion and metastasis [44] was also up-regulated in smokers (Figure 4B), and metastatic form of colon cancer patients compared to primary form of colon cancer patients (Figure 4C). TM4SF1 was also suggested as a possible marker of stem-like cells in thyroid cancer cells [41]. However, we could not find out the GEO data that match to tumor genesis of the up-regulated NR0B1, though up-regulated NR0B1 gene was required for the transformed phenotype of certain sarcoma [45].

**Conclusion**

It is still unclear how the cancer stem cells express poor prognostic phenotype in cancer, but we found for the first time that several genes related to chemo-resistance and metastasis are up-regulated in SP cells compared to non-SP cells. Therefore, enrichment of AKR1C1/C2, TM4SF1 and NR0B1 in SP of A549 cells might be a target of poor prognosis in cancer therapy.

**Methods**

**Cell culture**

A549 cells, a representative human lung adenocarcinoma cell line, were obtained from American Type Culture Collection (ATCC, VA, USA). The cells were cultured on F-12K nutrient mixture, Kaighn's modification (1×) liquid (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Genetron Life Technology, INC., CA, USA) and 1% of penicillin/streptomycin (P/S) (Gibco, CA, USA) on standard plastic tissue culture dishes (SPL Life Sciences, Seoul, Republic of Korea) and incubated in an atmosphere of 95% air/5% CO₂ at 37°C.

**Fluorescence activated cell sorting (FACS) for SP and non-SP cells**

The cells were detached with trypsin (Gibco, CA, USA), washed with phosphate buffer solution (PBS)/2% FBS, and resuspended at 1 × 10⁶ cells per ml in pre-warmed Hanks' balanced salt solution (HBSS; Gibco, CA, USA)/10% FBS. Hoechst 33342 dye (Sigma, St. Louis, MO, USA) was then added to this portion (final concentration: 5 μg/ml), and incubation continued for 90 min at 37°C. After washing with PBS/2% FBS, the cells re-suspended in ice-cold HBSS/10% FBS were labeled with 2 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) to distinguish live from dead cells prior to analysis. SP analysis and sorting were done using a FACSVantage SE (BD Biosciences, CA, USA). The Hoechst 33342 dye excited at 350 nm using UV laser and the DM610 SP was used to distinguish the red from the blue fluorescence signals. The EF675-LP (Hoechst Red) and BP450/20 nm (Hoechst Blue) filters were then installed in front of the PMT detector.

**RNA isolation and oligonucleotide microarray analysis**

We prepared total RNA from approximately 2 × 10⁶ SP or non-SP cells with Qiagen RNAeasy Mini kit (Qiagen, CA, USA).
Gene clustering of up-regulated genes in SP and non-SP cells. After normalizing each chip to the 50th percentile of the measurements taken that chip, gene-probes scored less than 0.1 either in SP or non-SP were excluded from data analysis. Only matched up-regulated genes in SP compared to non-SP cells are selected in each step of chip data analysis. The 12 genes and 46 genes were considered as up-regulated in SP and non-SP cells, respectively.
USA) according to the instruction of the manufacturer. The RNAs were subjected to GeneChip® expression array in full commercial service with two-cycle target labeling (SeoulLin Bioscience, Seoul, Republic of Korea). Briefly, cDNA were synthesized from total RNA using T7-Oligo (dT) primers. Using that cDNA, biotinylated cRNA was then synthesized. Fifteen μg of the labeled cRNA was hybridized to a Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA). Array image was scanned and analyzed using Genechip operating software (GCOS) (Affymetrix, CA, USA).

Quantitative real-time rt-PCR (qrt-rtPCR)
To validate the fold changes in the expression intensity of SP and non-SP cells in microarray data, we performed qrt-rtPCR using SYBR Premix Ex Taq Perfect Real Time kit (TaKaRa Bio, Ohtsu, Japan) in a SmartCycler (Cepheid, CA, USA) according to the manufacturer’s instruction. The cycle threshold value, which was determined using second derivative, was used to calculate the normalized expression of the indicated genes using Smartcycler Software (Cepheid, CA, USA). The following primer pairs were used: GAPDH (as an internal control); F-primer 5’CGACCACCTTTGGCAAGCTCA3’ and R-primer 5’AGGGGAGATTCAGTGTGGTG3’, AKR1C1;AKR1C2; F-primer 5’GTGGAAAGCTGACCAGGTGT3’ and R-primer 5’AAGCCGTGTTCTTCTGCTG3’.

Microarray data analysis
We used GeneSpring GX 7.3.1 software (Agilent Technologies, CA, USA) to normalize and analyze the microarray data. Following normalization of each chip to the 50th percentile of the measurements taken that chip, probes (genes) scored less than 0.1 both in SP and non-SP cells were excluded from the data analysis. Only matched up-regulated genes in SP and non-SP cells were selected from each step of chip (gene expression microarray) data analysis. More than 2 fold changes in all chip data were considered as up-regulated. Standard curve method was used to analyze qrt-rtPCR for confirmation of fold changes in chip data.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
DS and HS conceived the study. DS, JS, HC and HY carried out the sample preparation, gene expression analyses and quantitative real time RT-PCR. DK contributed to stem cell preparation. KS, IC, JK, AMAE and HS participated in the design, reviewed all data, and contributed in the prep-
oration of the manuscript. All authors read and approved the final manuscript.

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