Identification of differentially expressed genes in oral squamous cell carcinoma TCA8113 cells

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Received December 10, 2015; Accepted May 4, 2017

DOI: 10.3892/ol.2017.7108

Abstract. Previous studies have demonstrated that cancer cells with increased levels of aldehyde dehydrogenase 'bright' activity ($ALDH^{br}$) exhibit stem cell properties compared with cells exhibiting decreased ALDH activity ($ALDH^{low}$). To screen possible biomarkers of cancer stem cells in tongue squamous cell carcinoma, $ALDH^{br}$ and $ALDH^{low}$ cells were isolated from the tongue squamous cell carcinoma TCA8113 cell line, and suppression subtractive hybridization was performed to identify differentially expressed genes in the two subpopulations. A total of 240 positive clones were randomly selected for sequencing and were functionally characterized using bioinformatical tools. The results of the present study identified the differential expression of 104 clones, 62 of which corresponded to known genes and 42 of which corresponded to unknown genes. Cluster analysis revealed that the known genes were involved in the regulation of the cell cycle and cell differentiation. In addition, analysis of 10 signaling pathways revealed that genes were markedly altered in the $ALDH^{br}$ cell subpopulation. Additional study is required to identify the function that these genes serve in the biomolecular regulatory mechanisms of cancer stem cells and to assist in explaining the biological behavior of oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck squamous cell carcinoma (HNSCC), and is among the 10 most prevalent cancer types worldwide (1,2). In spite of improvements in the diagnosis and prognosis of OSCC, long-term survival rates have not improved in the past decade (3). To develop effective therapies, an improved understanding of the biological features and underlying molecular mechanisms of OSCC are required.

In previous studies, it has been suggested that the cancer stem cell (CSC) hypothesis may be applied to a number of types of cancer (4,5). According to the hypothesis, a tumor may be viewed as an aberrant organ initiated by a subpopulation of cells, termed CSCs, which exhibit self-renewing capacities and are responsible for tumor maintenance and metastasis (6). The hypothesis provides a novel insight into the understanding of tumorigenesis and since then, the isolation and identification of CSCs have been studied in depth. Previous studies have supported the validity of this hypothesis in a number of malignant diseases, including breast cancer, brain tumor, colon cancer, melanomas and prostate cancer (7-11). In addition, the existence of CSCs has been identified in HNSCC and has been associated with the expression of aldehyde dehydrogenase (ALDH) (12). Cells with increased ADLH 'bright' activity ($ALDH^{br}$) exhibit CSC-associated properties, including radio-resistance and the ability to produce tumors with a limited number of cells, which is in contrast to cells with decreased ALDH activity ($ALDH^{low}$) (13,14). However, the gene expression profile of the two cell subpopulations remains unknown, which is required to understand the underlying molecular mechanisms of CSCs in HNSCC.

In the present study, $ALDH^{br}$ and $ALDH^{low}$ cells were isolated from the OSCC TCA8113 cell line and suppression subtractive hybridization (SSH) was subsequently performed to identify differentially expressed genes in the two subpopulations. Known and unknown differentially expressed genes were identified in subtracted clones, and the known genes were functionally characterized using bioinformatical tools. The results of the present study suggested that the identified genes may be biomarkers for the identification of CSCs in OSCC.

Materials and methods

Cells and cell culture. The tongue squamous cell carcinoma TCA8113 cell line was obtained from the West China College of Stomatology of Sichuan University (Sichuan, China). Cells

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Key words: oral squamous cell carcinoma, tumor stem cell, aldehyde dehydrogenase, genes, suppression subtractive hybridization

were maintained in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 1% glutamine and 1% penicillin-streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

ALDH assay and cell sorting. An Aldefluor kit (Stemcell Technologies, Inc., Vancouver, BC, Canada) was used to determine ALDH activity in TCA8113 cells, according to the manufacturer's protocol. Cells were suspended in Aldefluor assay buffer, which contained an activated Aldefluor substrate (BAAA, 1 μmol/1x10^6 cells), as recommended by the manufacturer. As a negative control for all samples, an aliquot of 'Aldefluor-exposed' cells (1x10^8 cells) was transfused into the control tube, which contained 5 μl diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Following incubation at 37˚C for 40 min, the cells were centrifuged at 250 x g for 5 min and the supernatant was removed. Subsequently, the cell pellets were resuspended in 0.5 ml ice-cold Aldefluor Assay Buffer, and flow cytometric analysis was performed using FACSAria (BD Biosciences, Franklin Lakes, NJ, USA). Aldefluor staining was determined using a green fluorescence channel. Samples treated with DEAB were used as controls and set the threshold that defined the $ALDH^{br}$ region.

Tumorsphere formation. Since CSCs typically form tumorspheres and non-CSCs die in serum-free medium (15,16), tumorsphere formation in $ALDH^{br}$ and $ALDH^{low}$ cells was investigated in the present study. Cells were plated at a low density (1,000 cells/ml) in RPMI1 640 serum-free medium, supplemented with human recombinant epidermal growth factor (20 ng/ml; PeproTech, Inc., Rocky Hill, NJ, USA), basic fibroblast growth factor (20 ng/ml; PeproTech, Inc.) and B27 serum-free supplements (20 μl/ml; Invitrogen; Thermo Fisher Scientific, Inc.). The formation of tumorspheres was observed daily using an inverted phase contrast microscope (magnification, x100).

Preparation of total RNA. Total RNA was isolated from $ALDH^{br}$ and $ALDH^{low}$ cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was quantified using a Unico UV-2000 spectrophotometer (Unico Technologies Co., Ltd., Jiangsu, China). The A_{260}/A_{280} ratio was between 1.8 and 2.0. Total RNA (~1 μg) was separated on denaturing agarose (1.2% gel) to confirm integrity.

cDNA synthesis. cDNA was synthesized using the SMART™ cDNA Synthesis kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed (42˚C, 1.5 h) in a 10-μl reaction mixture containing PowerScript™ reverse-transcriptase. Sterile H_2O (24.2 μl), 5X Second-Strand Buffer (8.0 μl), dNTP mix (10 mM; 0.8 μl) and 20X Second-Strand Enzyme Cocktail (2.0 μl) were added to the 10 μl first-strand synthesis reaction tubes and incubated at 16˚C for 2 h in water. T4 DNA polymerase (Clontech Laboratories, Inc.) 2 μl was then added followed by incubation at 16˚C for 30 min in a water bath. Subsequently, 4 μl of 20X EDTA/glycogen mix was added to terminate second-strand synthesis, followed by addition of 100 μl of phenol:chloroform:isoamyl alcohol (25:24:1). Centrifugation was then performed at 2,191.28 x g for 10 min at room temperature. The top aqueous layer was collected and placed in a fresh 0.5-ml microcentrifuge tube. The inter and lower phases were discarded and disposed appropriately. Next, 100 μl of chloroform:isoamyl alcohol (24:1) was added, followed by addition of 40 μl of 4 M NH_4OAc and 300 μl of 95% ethanol. Subsequently, centrifugation was performed at 2,191.28 x g for 20 min at room temperature, and the supernatant was collected. The pellet was overlayed with 500 μl of 80% ethanol, and then centrifuged at 2,191.28 x g for 10 min at room temperature. The supernatant was removed and the pellet was air-dried for ~10 min to evaporate residual ethanol. Precipitate was dissolved in 50 μl of sterile H_2O, and 6 μl was transferred to a fresh microcentrifuge tube. This sample was stored at -20˚C until after *Rsa*I digestion (for agarose gel electrophoresis) to estimate yield and size range of ds cDNA products synthesized.

SSH. Synthesized two-target cDNA was used for SSH, performed with the PCR-select™ cDNA Subtraction kit (Clontech Laboratories, Inc.), according to the manufacturer's protocol. cDNA from $ALDH^{br}$ and $ALDH^{low}$ cells was used as the 'tester' and 'driver', respectively, in the forward subtraction and vice versa for the reverse subtraction. For each subtraction, the 'tester' was ligated to adaptor 1 and adaptor 2R in separate ligation reactions, whereas the 'driver' was not ligated to adaptors. Following ligation, two samples were subjected to hybridization. For the first hybridization, an excess of 'driver' cDNA was added to each adaptor-ligated 'tester' cDNA in the hybridization buffer, heat-denatured (98˚C, 1.5 min) and subsequently annealed (68˚C, 8 h). The two samples from the first hybridization were mixed and fresh denatured 'driver' cDNA was added and annealed at 68˚C overnight. Following the second hybridization, the sample was diluted in 200 μl dilution buffer and incubated at 68˚C for 7 min in a thermal cycler. Subsequently, PCR was performed using the subtracted cDNAs to amplify the desired differentially expressed sequences. The first-round PCR was performed using PCR primer 1 (5'-CTAATACGACTCACTATAGGGC-3') and the cycling parameters were 72˚C for 10 min and 95˚C for 2 min, followed by 25 cycles of 94˚C for 30 sec, 62˚C for 45 sec, 72˚C for 1 min and 72˚C for 6 min. The second-round PCR reaction was performed using nested primer 1 (5'-TCGAGCGGCCGC CCGGGCAGGT-3') and nested primer 2R (5'-AGCGTGGTC GCGGCCGAGGT-3'), and the cycling parameters were 95˚C for 2 min, followed by 29 cycles of 94˚C for 30 sec, 65˚C for 45 sec, 72˚C for 1 min and 72˚C for 6 min.

Cloning of SSH-PCR products. The purified secondary SSH-PCR products were cloned into PMD-18T vector (Takara Bio, Inc., Otsu, Japan) and the ligated products were transformed into *E. coli* DH5α competent cells. Transformed colonies were selected on Luria-Bertani (LB) agar medium (MP Biomedicals, Santa Ana, CA, USA) containing ampicillin (100 mg/l) at 37˚C and ~1,000 positive colonies were obtained, which represented subtraction libraries enriched with differentially expressed genes. A total of 240 positive colonies were selected randomly. A single clone was inoculated in 2 ml LB-ampicillin (100 mg/l) and incubated overnight at 37˚C with gentle agitation at 44.72 x g.

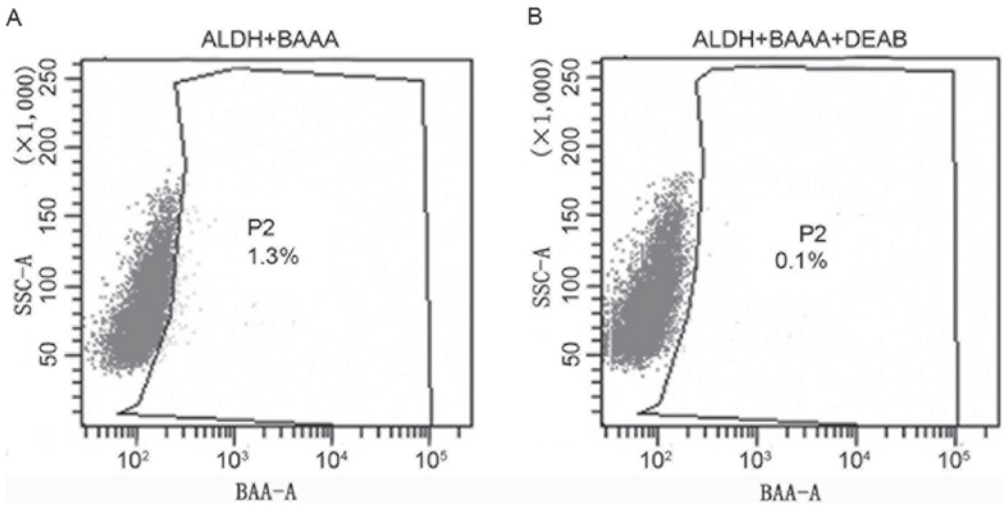

Figure 1. Isolation of ALDH^{br} cells in tongue squamous cell carcinoma TCA8113 cells. (A) Cells incubated with Aldefluor substrate (BAAA) and subsequent fluorescence-activated cell sorting was used to identify cells exhibiting increased ALDH activity (P2 region, 1.3%). (B) With the addition of DEAB, a specific inhibitor of ALDH, the proportion of sorted ALDH^{br} cells was markedly decreased (P2 region, 0.1%). ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; SSC-A, side scatter area.

PCR amplification of cDNA inserts. To assess the size of inserts, colony PCR was performed in a 50-μl reaction system containing 12.5 μl 10X buffer (Takara Bio, Inc.), 1 μl 10 mM dNTP (Shanghai CPG Biotechnology Co., Ltd., Shanghai, China), 5 μl $MgCl_2$ (Takara Bio, Inc.), 1 μl 50 pM/μl Nested primer 1 (Clontech Laboratories, Inc.), 1 μl 50 pM/μl 2R primer (Clontech Laboratories, Inc.), and 2.5 U Taq DNA polymerase (Takara Bio, Inc.). The PCR parameters were: 95˚C for 2 min, followed by 35 cycles of 95˚C for 30 sec, 62˚C for 45 sec and 72˚C for 1 min. Colony PCR products (2 μl) were separated using agarose (1.2% gel) to identify the presence and the size of the inserts prior to sequencing. The controls for this protocol included the unsubtracted tester control for the forward subtraction, the unsubtracted tester control for the reverse subtraction and the unsubtracted tester control for the control skeletal muscle tester cDNA [made from the Control Poly A$^+$ RNA (from human skeletal muscle) provided with the kit (the SMART™ cDNA Synthesis kit (Clontech Laboratories, Inc.)]. It serves as control driver cDNA subtraction. All protocols were repeated 3 times.

Expressed sequenced tag (EST) sequencing and bioinformatical analysis. The selected positive clones were sequenced at the Beijing Genomics Institute (Beijing, China) and the sequences were edited to remove the adaptor-primer and vector DNA sequences. ESTs were compared with non-redundant public databases using the Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide to retrieve data from GenBank (www.ncbi.nlm.nih.gov/nucleotide) and BLASTX (blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) algorithms of the National Center for Biotechnology Information (NCBI; blast.ncbi.nlm.nih.gov/Blast.cgi). ESTs with E<0.01 were deemed to exhibit significant homology. Homologies >50 nucleotides that exhibited >90% identity to sequences in the database were considered to have significant homologies, as previously described (17). The physiological functions of these ESTs were classified according to Gene Ontology (www.geneontology.org). Pathway analysis was performed using the Gene Set Analysis Toolkit V2 online system (www.webgestalt.org/option.php).

Results

Isolation of ALDH^{br} cells in tongue squamous cell carcinoma TCA8113 cells. Using the ALDEFLUOR assay and fluorescence-activated cell sorting analysis, the ALDH enzymatic activity in the tongue squamous cell carcinoma TCA8113 cell line was identified to be heterogeneous. As presented in Fig. 1, only a limited proportion (1.3%) of the cells displayed increased ALDH activity (ALDH^{br}; Fig. 1A), whereas the remaining cells expressed decreased levels of ALDH activity (ALDH^{low}). DEAB, the specific inhibitor of ALDH, resulted in a decreased proportion of sorted ALDH^{br} cells (0.1%; Fig. 1B), suggesting the effective isolation of ALDH^{br} cells. The results of the present study revealed that cancer stem cells with ALDH^{br} were successfully isolated. Subpopulation cells were selected for additional analysis.

ALDH^{br} cells form spheres. CSCs may be effectively enriched in serum-free medium (18-20). The majority of cells die in serum-free medium due to a lack of nutritive materials; however, CSCs may survive, proliferate and form three-dimensional spheres. In the present study, ALDH^{br} cells maintained in serum-free medium proliferated and formed spheres within 5 days, whereas ALDH^{low} cells, maintained in the same medium, did not form spheres and were apoptotic (Fig. 2). The results of the present study indicated that the isolated ALDH^{br} cells exhibited typical CSC features.

Constructing the SSH library. Using cDNA from ALDH^{br} cells as 'testers' and that of ALDH^{low} cells as 'drivers' and vice versa, PCR-selected cDNA subtraction for forward and reverse libraries, respectively, was performed. Following subtraction, a pool of putative differentially expressed cDNA fragments was obtained. The cDNA fragments ranged between 200 bp

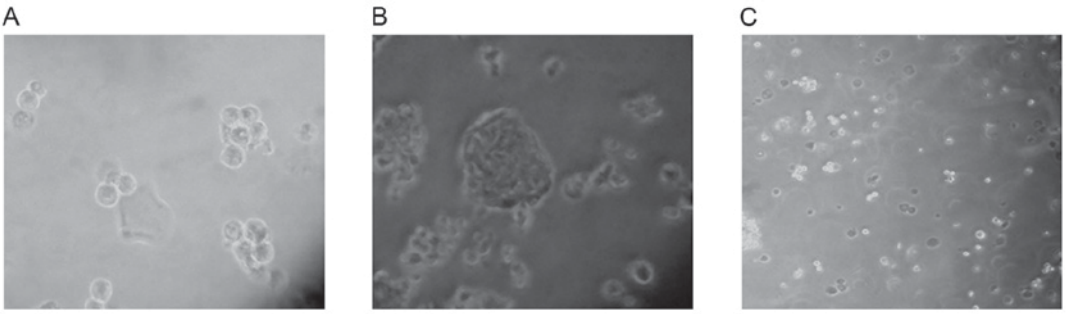

Figure 2. Sphere formation of cells exhibiting increased ALDH activity. Isolated cells exhibiting increased and decreased ALDH activity were maintained in serum-free RPMI 1640 medium. Cells were subsequently inspected using an inverted phase contrast microscope (magnification, x100) at a variety of culture times. Cells exhibiting increased ALDH activity formed spheres on (A) day 5 and (B) day 10; (C) whereas cells exhibiting decreased activity of ALDH died on day 5. ALDH, aldehyde dehydrogenase.

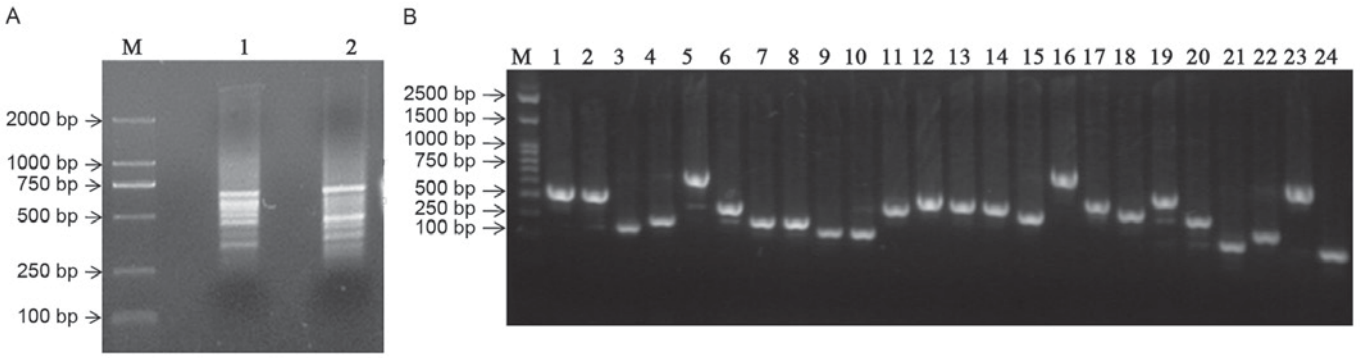

Figure 3. Construction of the suppression substrate hybridization library. (A) Products of PCR-select cDNA subtraction. (B) cDNA fragments of distinct lengths from white clones, amplified using PCR. PCR, polymerase chain reaction; M, DNA marker; 1, forward subtraction products; 2, reverse subtraction products.

and 1 kb, with the majority distributed between 400 and 600 bp (Fig. 3A). Subtracted amplicons were ligated into the PMD-18T plasmid vector and transformed into *E. coli* DH5α competent cells. In total, 240 white colonies were randomly selected and 48 of these clones were subjected to colony PCR, using nested primers. All the recombinants determined revealed amplicons ranging between 200 and 800 bp (Fig. 3B).

Identification of differentially expressed ESTs. All 240 clones were selected and sequenced. Sequences were not obtained for 14 clones and those were omitted from the present study. Comparison of the unique sequences obtained from each library against the GenBank databases identified 104 unique clones, 62 of which corresponded to known genes and 42 of which were unknown genes, while the remaining 122 clones were redundant. Of the known genes, 28 and 34 genes were upregulated and downregulated in $ALDH^{br}$ cells, respectively (Tables I and II). The unknown clones were divided into two groups in the NCBI databases, 28 represented human genomic sequences and 14 were present in the human EST database.

Functional classification of differentially expressed ESTs. On the basis of the functional annotation using Gene Ontology (GO) software, 62 differentially expressed genes were grouped into a number of categories (Fig. 4). In the GO category of biological processes, the highly enriched categories included those associated with metabolic processes (28 genes), biological regulation (21 genes) and developmental processes (16 genes). The molecular functions with those highly enriched genes were associated with protein binding (32 genes) and cellular components in the nucleus (24 genes).

Pathway analysis of differentially expressed ESTs. Signal pathway analysis was performed based on the Wikipathways database and the Pathway Commons database, using the Gene Set Analysis Toolkit V2. The 10 signaling pathways with the most marked alterations in each database are presented in Tables III and IV, and included the transforming growth factor (TGF-) β signaling pathway, the Notch signaling pathway and the c-kit pathway. Typically, ~10 genes were enriched in these pathways and each gene may participate in a number of pathways.

Discussion

CSCs refer to a subset of tumor cells that exhibit the capability to self-renew and generate diverse cells that comprise the tumor (4,21), and have been termed CSCs to reflect the 'stem-like' properties and the ability to sustain tumorigenesis. CSCs share important properties with healthy tissue stem cells, including the capacity for self-renewal and differentiation. An implication of the CSC hypothesis is that cancer cells are hierarchically arranged with CSCs located at the apex of the hierarchy (22). CSCs are the only cells that may maintain tumor viability indefinitely. The remaining cells, although actively proliferating and comprising the majority of the tumor, are

Table I. Characteristics of overexpressed known genes in aldehyde dehydrogenase-positive subpopulation cells.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
1	206	*Drosophila* melanogaster CG4699 (CG4699), transcript variant J	ref|NM_001170153.1|	163/166 (99)	$2.00x10^{-76}$	41911 CG4699	CG4699 (WAH)	Unknown
2	402	*Homo sapiens* solute carrier family 25, member 13 (citrin) (SLC25A13), RefSeqGene on chromosome 7	ref|NG_012247.1|	388/389 (99)	0	10165	SLC25A13	7q21.3
3	303	*Homo sapiens* kelch-like 2, Mayven (*Drosophila*) (KLHL2), transcript variant 3, mRNA	ref|NM_001161522.1|	284/285 (99)	$8.00x10^{-145}$	11275 KLHL2	KLHL2	4q21.2
4	225	*Homo sapiens* Niemann-Pick disease, type C1 (NPC1), RefSeqGene on chromosome 18	ref|NG_012795.1|	174/175 (99)	$3.00x10^{-83}$	4864	NPC1	18q11-q12
5	369	*Homo sapiens* EP300 interacting inhibitor of differentiation 1 (EID1), mRNA	ref|NM_014335.2|	353/354 (99)	0	23741 EID1	EID1	15q21.1-q21.2
6	289	*Homo sapiens* notch 2 (NOTCH2), RefSeqGene on chromosome 1	ref|NG_008163.1|	512/514 (99)	0	4853	NOTCH2	1p13-p11
7	234	*Homo sapiens* BRCA1 associated RING domain 1 (BARD1), RefSeqGene	ref|NG_012047.1|	215/216 (99)	$1.00x10^{-106}$	157266327	BARD1	2q34-q35
8	272	*Homo sapiens* inositol 1,4,5-triphosphatereceptor, type 1 (ITPR1), RefSeqGene on chromosome 3	ref|NG_016144.1|	253/254 (99)	$1.00Ex10^{-127}$	269954693	ITPR1	3p26-p25
9	289	*Homo sapiens* FRG1 (FRG1) gene, complete cds;	gb|AF146191.1| AF146191	264/272 (98)	$2.00x10^{-125}$	AAD46768.1	FRG1	4q35
10	280	*Homo sapiens* methylcrotonoyl-CoA carboxylase 2 (beta) (MCCC2), RefSeqGene on chromosome 5	ref|NG_008882.1|	263/263 (100)	$3.00x10^{-134}$	64087	MCCC2	5q12-q13

Table I. Continued.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
11	410	*Homo sapiens* ribosomal protein, large, P0 (RPLP0), transcript variant 1, mRNA	ref|NM_001002.3|	388/390 (99)	0	6175 RPLP0	RPLP0	12q24.2
12	468	*Homo sapiens* dedicator of cytokinesis 8 (DOCK8), transcript variant 3, mRNA	ref|NM_001193536.1|	449/450 (99)	0	81704 DOCK8	DOCK8	9p24.3
13	365	*Homo sapiens* GLIS family zinc finger 3 (GLIS3), RefSeqGene on chromosome 9	ref|NG_011782.1|	342/347 (99)	$1.00x10^{-172}$	169792	GLIS3	9p24.2
14	412	*Homo sapiens* PARK2 co-regulated (PACRG) on chromosome 6	ref|NG_011525.1|	352/392 (90)	$5.00x10^{-138}$	135138	PACRG	6q26
15	153	*Homo sapiens* collagen, type VII, alpha 1(COL7A1), mRNA	ref|NM_000094.3|	134/135 (99)	$7.00x10^{-63}$	1294 COL7A1	COL7A1	3p21.1
16	226	*Homo sapiens* epidermal growth factor receptor(EGFR), RefSeqGene on chromosome 7	ref|NG_007726.1|	207/208 (99)	$3.00x10^{-102}$	1956	EGFR	7p12
17	450	*Homo sapiens* neuron navigator 2 (NAV2), transcript variant 4, mRNA	ref|NM_001111019.1|	391/399 (98)	0	89797 NAV2	NAV2	11p15.1
18	288	*Homo sapiens* retinoblastoma 1 (RB1), mRNA	ref|NM_000321.2|	269/270 (99)	$1.00x10^{-137}$	5925 RB1	RB1	13q14.2
19	274	*Homo sapiens* tetratricopeptide repeat protein 12 (TTC12) gene, complete cds, alternatively spliced	gb|EF445041.1|	214/257 (84)	$4.00x10^{-58}$	ACA06092.1	TTC12	11q23.1
20	232	*Homo sapiens* nuclear receptor corepressor 1 (NCOR1), transcript variant 3, mRNA	ref|NM_001190440.1|	213/214 (99)	$1.00x10^{-106}$	9611 NCOR1	NCOR1	17p11.2
21	283	*Homo sapiens* SMAD family member 1 (SMAD1), transcript variant 2, mRNA	ref|NM_001003688.1|	262/263 (99)	$1.00x10^{-133}$	4086 SMAD1	SMAD1	7p15
22	646	*Homo sapiens* kelch-like 13 (*Drosophila*) (KLHL13), RefSeqGene on chromosome X	ref|NG_016759.1|	628/630 (99)	0	90293	KLHL13	Xq23-q24

Table I. Continued.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
23	418	*Homo sapiens* PRP39 pre-mRNA processing factor 39 homolog (*S. cerevisiae*) (PRPF39), mRNA	ref|NM_017922.3|	395/399 (99)	0	55015 PRPF39	PRPF39	14q21.3
24	398	*Homo sapiens* septin 9 (SEPT9), transcript variant 4, mRNA	ref|NM_001113495.1|	379/380 (99)	0	10801 SEPT9	SEPT	17q25
25	537	*Homo sapiens* ATPase, Ca^{++} transporting, plasma membrane 4 (ATP2B4), transcript variant 1, mRNA	ref|NM_001001396.1|	186/188 (99)	$5.00x10^{-90}$	493 ATP2B4	ATP2B4	1q32.1
26	392	PREDICTED: *Homo sapiens* hypothetical LOC441072 (FLJ31104), partial miscRNA	ref|XR_113742.1|	203/225 (91)	$3.00x10^{-76}$	441072 FLJ31104	FLJ31104	5q11.2
27	227	*Homo sapiens* CD44 molecule (Indian blood group) (CD44), RefSeqGene on chromosome 11	ref|NG_008937.1|	207/207 (100)	$3.00x10^{-103}$	960	CD44	11p13
28	364	Pongo abelii probable methyltransferase TARBP1-like (LOC100447859), mRNA	ref|XM_002809289.1|	120/124 (97)	$3.00x10^{-51}$	100447859 LOC100447859	LOC100447859	Unknown

Table II. Characteristics of downregulated known genes in aldehyde dehydrogenase-positive subpopulation cells.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
1	410	Pan troglodytes hypothetical protein LOC736141 (LOC736141), mRNA	ref|XM_001135501.1|	322/364 (89)	$3.00x10^{-117}$	736141 LOC736141	LOC736141	X
2	505	*Homo sapiens* claudin domain containing 1 (CLDND1), transcript variant 6, mRNA	ref|NM_001040199.1|	318/320 (99)	$9.00x10^{-163}$	56650 CLDND1	CLDND1	3q12.1
3	383	*Homo sapiens* ankyrin repeat domain 36B (ANKRD36B), mRNA	ref|NM_025190.3|	297/368 (81)	$4.00x10^{-70}$	57730 ANKRD36B	ANKRD36B	2q11.2
4	357	*Homo sapiens* catenin (cadherin-associated protein), alpha 1, 102 kDa (CTNNA1), mRNA	ref|NM_001903.2|	336/337 (99)	$1.00x10^{-174}$	1495 CTNNA1 |	CTNNA1	5q31
5	364	*Homo sapiens* electron-transfer-flavoprotein, alpha polypeptide (ETFA), RefSeqGene on chromosome 15	ref|NG_007077.2|	344/346 (99)	$5.00x10^{-177}$	2108	ETFA	15q23-q25
6	493	Pan troglodytes RAB7, member RAS oncogene family-like 1, transcript variant 1 (RAB7L1), mRNA	ref|XM_001162387.1|	264/296 (90)	$9.00x10^{-98}$	469654 RAB7L1	RAB7L1	1q32
7	635	*Homo sapiens* wings apart-like homolog (*Drosophila*) (WAPAL), mRNA	ref|NM_015045.2|	617/618 (99)	0	23063 WAPAL	WAPAL/WAPL	10q23.2
8	459	*Homo sapiens* KIAA0101 (KIAA0101), transcript variant 2, mRNA	ref|NM_001029989.1|	441/442 (99)	0	9768 KIAA0101	KIAA0101/PAF	15q22.31
9	299	*Homo sapiens* RAN binding protein 10 (RANBP10), mRNA	ref|NM_020850.1|	281/281 (100)	$2.00x10^{-145}$	57610 RANBP10	RANBP10	16q22.1
10	224	*Homo sapiens* mitochondrial ribosomal protein S27 (MRPS27), nuclear gene encoding mitochondrial protein, mRNA	ref|NM_015084.2|	202/204 (99)	$2.00x10^{-99}$	23107 MRPS27	MRPS27	5q13.2
11	426	*Homo sapiens* vacuolar protein sorting 13 homolog A (*S. cerevisiae*) (VPS13A), RefSeqGene on chromosome 9	ref|NG_008931.1|	408/410 (99)	0	23230	VPS13A	9q21

Table II. Continued.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
12	413	*Homo sapiens* SET binding factor 2 (SBF2), RefSeqGene on chromosome 11	ref|NG_008074.1|	392/396 (99)	0	81846	SBF2	11p15.4
13	613	*Homo sapiens* MT-RNR2-like 2 (MTRNR2L2), mRNA	ref|NM_001190470.1|	562/597 (95)	0	100462981	MTRNR2L2	Unknown
14	423	*Homo sapiens* MT-RNR2-like 8 (MTRNR2L8), mRNA	ref|NM_001190702.1|	373/399 (94)	$9.00x10^{-167}$	100463486 MTRNR2L8	MTRNR2L8	Unknown
15	588	*Homo sapiens* WD repeat domain 7 (WDR7), transcript variant 2, mRNA	ref|NM_052834.2|	133/133 (100)	$2.00x10^{-63}$	23335 WDR7	WDR7	18q21.1-q22
16	261	Pan troglodytes similar to ORF1; putative (LOC745921), mRNA	ref|XR_021946.1|	276/326 (85)	$6.00x10^{-83}$	745921 LOC745921	LOC745921	Unknown
17	459	*Homo sapiens* ornithine decarboxylase 1 (ODC1), mRNA	ref|NM_002539.1|	570/570 (100)	0	4953 ODC1 |	ODC1	2p25
18	411	*Homo sapiens* ataxin 7 (ATXN7), RefSeqGene on chromosome 3	ref|NG_008227.1|	393/394 (99)	0	80145	ATXN7	3p21.1-p12
19	494	*Homo sapiens* zinc finger protein 573 (ZNF573), transcript variant 5, mRNA	ref|NM_001172692.1|	476/476 (100)	0	126231 ZNF573	ZNF573	19q13.12
20	538	*Homo sapiens* bromodomain containing 2 (BRD2), transcript variant 3, mRNA	ref|NM_001199455.1|	297/299 (99)	$4.00x10^{-151}$	6046 BRD2	BRD2	6p21.3
21	353	PREDICTED: Pan troglodytes similar to uracil DNA glycosylase (LOC743143), mRNA	ref|XR_021793.1|	326/332 (98)	$2.00x10^{-163}$	743143 LOC743143	LOC743143	Unknown
22	380	*Homo sapiens* SET nuclear oncogene (SET), transcript variant 1, mRNA	ref|NM_001122821.1|	360/360 (100)	0	6418 SET	SET	9q34
23	205	*Homo sapiens* peroxisomal biogenesis factor 19 (PEX19), transcript variant 4, mRNA	ref|NM_001193644.1|	184/185 (99)	$2.00x10^{-90}$	5824 PEX19	PEX19	1q22
24	386	*Homo sapiens* microphthalmia-associated transcription factor (MITF), RefSeqGene on chromosome 3	ref|NG_011631.1|	369/369 (100)	0	4286	MITF	3p14.2-p14.1

Table II. Continued.

No.	Length, bp	Gene	Accession no.	Identities (%)	E-value	Gene ID	Gene symbol	Chromosomal location
25	449	*Homo sapiens* fatty acid desaturase 1 (FADS1), mRNA	ref|NM_013402.4|	431/432 (99)	0	3992 FADS1	FADS1	11q12.2-q13.1
26	352	*Homo sapiens* protein tyrosine phosphatase type IVA, member 1 (PTP4A1), mRNA	ref|NM_003463.3|	332/335 (99)	$3.00x10^{-170}$	7803 PTP4A1	PTP4A1	6q12
27	323	*Homo sapiens* chromosome X open reading frame 57 (CXorf57), transcript variant 2, mRNA	ref|NM_001184782.1|	303/305 (99)	$2.00x10^{-155}$	55086 CXorf57	CXorf57	Xq22.3
28	214	*Homo sapiens* CREB binding protein (CREBBP), RefSeqGene on chromosome	ref|NG_009873.1|	197/198 (99)	$1.00x10^{-96}$	1387	CREBBP	16p13.3
29	436	*Homo sapiens* mesencephalic astrocyte-derived neurotrophic factor (MANF), mRNA	ref|NM_006010.4|	411/415 (99)	0	7873 MANF	MANF	Unknown
30	263	*Homo sapiens* ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1), mRNA	ref|NM_015161.1|	243/243 (100)	$3.00x10^{-124}$	23204 ARL6IP1	ARL6IP1	16p12-p11.2
31	501	*Homo sapiens* RNA, 18S ribosomal 1 (RN18S1), ribosomal RNA	ref|NR_003286.2|	479/480 (99)	0	100008588 RN18S1	RN18S1	Unknown
32	248	*Homo sapiens* eukaryotic elongation factor-2 kinase (EEF2K), mRNA	ref|NM_013302.3|	227/227 (100)	$3.00x10^{-115}$	29904 EEF2K	EEF2K	16p12.1
33	486	*Homo sapiens* myeloid/ lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*) (MLL), transcript variant 2, mRNA	ref|NM_005933.3|	466/469 (99)	0	4297 MLL	MLL	11q23
34	499	PREDICTED: *Macaca mulatta* ATP synthase subunit a-like (LOC100426362), mRNA	ref|XM_002806045.1|	359/441 (81)	$3.00x10^{-93}$	100426362 LOC100426362	LOC100426362	Unknown

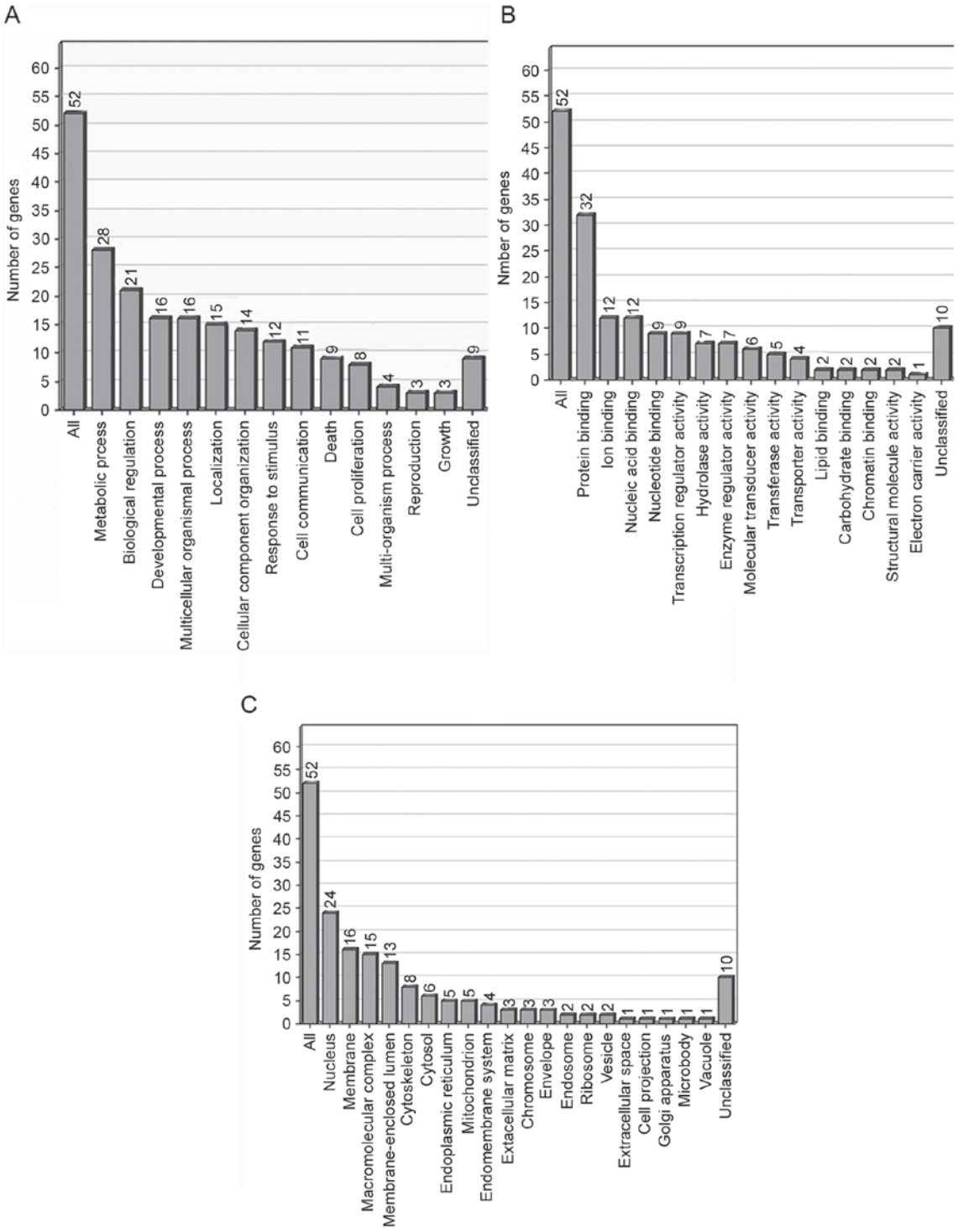

Figure 4. Functional classification of differentially expressed ESTs. (A) Biological process of differentially expressed ESTs. (B) Molecular function of differentially expressed ESTs. (C) Cell component of differentially expressed ESTs. EST, expressed sequence tag.

differentiating and destined to die. The identification of CSCs has marked implications in the study of cancer biology. Previous studies (7-11) have indicated the existence of CSCs in a number of solid tumors and a variety of cell surface makers have been used to isolate CSC subpopulations, including cluster of differentiation (CD)24, CD133 and CD24; however, none of these markers are exclusively expressed by CSCs in solid tumors.

ALDH is a member of the family of $NAD(P)^+$-dependent enzymes involved in detoxifying a variety of aldehydes to the corresponding weak carboxylic acids (23). The use of ALDH activity in flow cytometry-based methods has enabled the isolation of viable CSC subpopulations in a number of cancer types (24-26). In the present study, CSCs were enriched from the tongue squamous cell carcinoma TCA8113 cell line, according to the overexpression of $ALDH^{br}$. $ALDH^{br}$ cells comprised 1.3% of the total cell population, which is consistent with previous studies (13,14). Therefore, $ALDH^{br}$-associated CSCs were successfully isolated for additional investigation.

Table III. Pathway analysis, on the basis of the pathway commons database.

Signaling pathway	Entrez IDs	Enrichment statistics
Notch-HLH transcription	1387, 4853	C=6; O=2; E=0.01; R=290.63; rawP=1.93x10^{-5}; adjP=0.0004
TGF-β receptor	1387, 5925, 960	C=126; O=3; E=0.14; R=20.76; rawP=0.0004; adjP=0.0032
Generic transcription	1387, 4853	C=28; O=2; E=0.03; R=62.28; rawP=0.0005; adjP=0.0032
Microphthalmia-associated transcription factor	1387, 4286	C=51; O=2; E=0.06; R=34.19; rawP=0.0016; adjP=0.0051
Signaling events mediated by stem cell factor receptor (c-Kit)	1387, 9611, 4286, 29904	C=436; O=4; E=0.50; R=8.00; rawP=0.0016; adjP=0.0051
BMP receptor	4086, 4286, 29904	C=189; O=3; E=0.22; R=13.84; rawP=0.0014; adjP=0.0051
NOTCH	4853, 9611	C=58; O=2; E=0.07; R=30.07; rawP=0.0020; adjP=0.0054
Regulation of cytoplasmic and nuclear SMAD2/3	9611, 4286, 29904	C=265; O=3; E=0.30; R=9.87; rawP=0.0035; adjP=0.0066
TGF-β receptor	9611, 4286, 29904	C=265; O=3; E=0.30; R=9.87; rawP=0.0035; adjP=0.0066
Androgen receptor	1387, 5925	C=79; O=2; E=0.09; R=22.07; rawP=0.0038; adjP=0.0066

Table lists the enriched gene sets, the number of Entrez IDs in the user data set for the pathway, the corresponding Entrez ID and the statistics for the enrichment of the pathway. The number of user gene IDs is linked to a table with information about the user IDs, and the Entrez IDs are linked to the Entrez Gene. Notch-HLH, Notch-Helix Loop helix; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; SMAD, mothers against decapentaplegic homolog; C, number of reference genes in the category; O, number of genes in the gene set and in the category; E, expected number in the category; R, ratio of enrichment; rawP, P-value from the hypergeometric test; adjP, P-value adjusted by the multiple test adjustment.

Table IV. Pathway analysis, on the basis of the Wikipathways database.

Signaling pathway	Entrez IDs	Enrichment statistics
Δ-Notch	9611, 4853, 4086, 1956	C=86; O=4; E=0.10; R=40.55; rawP=3.05x10^{-6}; adjP=3.97x10^{-5}
Senescence and autophagy	5925, 960, 4297	C=60; O=3; E=0.07; R=43.60; rawP=4.65x10^{-5}; adjP=0.0003
Androgen receptor	1387, 5925, 1956	C=115; O=3; E=0.13; R=22.75; rawP=0.0003; adjP=0.0013
B cell receptor	5925, 3708, 493	C=158; O=3; E=0.18; R=16.56; rawP=0.0008; adjP=0.0021
TGF-β receptor	1387, 5925, 960	C=155; O=3; E=0.18; R=16.88; rawP=0.0008; adjP=0.0021
Notch	1387, 4853	C=46; O=2; E=0.05; R=37.91; rawP=0.0013; adjP=0.0026
Id	5925, 4086	C=51; O=2; E=0.06; R=34.19; rawP=0.0016; adjP=0.0026
TGF-β	1387, 4086	C=52; O=2; E=0.06; R=33.53; rawP=0.0016; adjP=0.0026
Estrogen	1387, 9611	C=76; O=2; E=0.09; R=22.94; rawP=0.0035; adjP=0.0051
Wnt and pluripotency	1387, 960	C=98; O=2; E=0.11; R=17.79; rawP=0.0057; adjP=0.0067

Table lists the enriched gene sets, the number of Entrez IDs in the user data set for the pathway, the corresponding Entrez IDs and the statistics for the enrichment of the pathway. The number of user gene IDs is linked to a table with information about the user IDS and the Entrez IDs are linked to Entrez Gene. Id, the inhibitor of DNA binding; TGF-β, transforming growth factor-β; C, number of reference genes in the category; O, number of genes in the gene set and in the category; E, expected number in the category; R, ratio of enrichment; rawP, P-value from the hypergeometric test; adjP, P-value adjusted by the multiple test adjustment.

In order to identify stem cell associated genes differentially expressed in ALDH^{br} and ALDH^{low} cells, SSH was performed. SSH is advantageous compared with other PCR-based techniques as it selectively amplifies target cDNA fragments (differentially expressed), and simultaneously suppresses non-target DNA amplification, to generate a library of differentially expressed sequences (27). The normalization step equalizes the abundance of cDNAs within a target population and the subtraction step excludes the common sequences between the driver and tester populations (27). In addition, the advantage compared with microarrays is that SSH may isolate novel differentially expressed genes (28). In the present study, two SSH libraries were constructed from cDNAs obtained from ALDH^{br} and ALDH^{low} cells, and a total of 240 clones were selected and sequenced. Using GenBank databases, 28 and 34 known genes were identified from the forward and reverse libraries, respectively. A total of 28 of clones revealed homology with chromosome sequences and 14

clones demonstrated homology with ESTs. The known genes were grouped into functional categories on the basis of GO.

In the GO category of biological process, the highly enriched categories included those associated with metabolic processes (28 genes), biological regulation (21 genes) and developmental processes (16 genes). The results of the present study suggested that abnormal stem cell homeostasis associated with the aforementioned processes would result in malignant changes in stem cells.

Signaling pathway analysis identified the 10 pathways that exhibited marked alterations in the Wikipathways database and Pathway Commons database, which included Notch and TGF-β signaling pathways, which have been identified to serve important roles in the regulation of stem cell self-renewal, multi-potency and cell-fate determination (29,30). In addition, one gene may participate in different signaling pathways at the same time; for example, the gene encoding cAMP response element-binding protein (CREB) binding protein (CREBBP/CBP) was involved in 7 of the aforementioned signaling pathways and notably interacted with Wnt signaling to maintain the pluripotency of murine embryonic stem cells in long-term culture (31). A previous study demonstrated that CBP was critical in maintaining an adequate pool of murine hematopoietic stem cells through self-renewal and was important for preventing hematological tumor formation (32), suggesting that CBP was associated with the biological regulation of normal stem cells. There have been a limited number of studies on the expression and function of CBP in CSCs, therefore, whether CBP is a marker of CSCs in tongue squamous cell carcinoma remains unknown. Additionally, nuclear receptor corepressor 1 (NCOR1) was involved in a number of signaling pathways and was initially defined as a regulator of nuclear receptor-mediated repression. NCOR is expressed in the nucleus of neural stem cells (NSCs) and is a regulator of neural stem cells. Following phosphorylation, NCOR translocates to the cytoplasm and induces the astrocytic differentiation of NSCs (33). Furthermore, NCOR has been identified to maintain normal intestinal epithelial cell viability, and silencing of NCOR1 expression in proliferating cells of crypt origin resulted in a rapid viability arrest without associated cell death (34). In glioblastoma multiforme (GBM), NCOR was expressed in the nucleus of undifferentiated CSCs and the nuclear localization of NCOR may function as a marker of GBM stem cells (35).

Differentially expressed genes in tongue squamous carcinoma stem-like cells were profiled using the SSH technique. A total of 62 genes were identified as upregulated or downregulated in tongue squamous carcinoma stem-like cells (termed $ALDH^{br}$ cells), suggesting that distinct gene expression profiles are present in CSCs. CBP and NCOR1 genes were involved in a number of signaling pathways in $ALDH^{br}$ cells. The results of a literature review suggested that CBP and NcoR1 may be CSCs markers (32-35), which is consistent with the results of the present study. Although the results of the present study are preliminary, a group of candidate genes have been identified, which require additional study.

Acknowledgements

The present study was supported by the ChonQing Science and Technology Commission Project (grant no. 2013-1-030). The authors thank Medjaden Bioscience Ltd. (Hong Kong, China) for assisting in the preparation of the original manuscript.

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