Gene expression profile of side population cells in human oral cancer cell line SCC-4

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To define the properties of side population (SP) cells of the oral squamous cell carcinoma cell line SCC-4, we compared their gene expression profile with that of main population (MP) cells. SP cells accounted for approximately 1.5% of the cell population. The gene expression profile in SP cells versus MP cells revealed that 15.8% of genes were up-regulated by more than 10%, and 16.6% were down-regulated by more than 10%. Gene Ontology and KEGG pathway enrichment analyses were carried out on the DAVID online tool for 26,879 distinct probes ($p < 0.05$). The terms involved in immune/inflammatory responses, cell migration and angiogenesis were detected as up-regulated genes. The terms related to DNA replication, DNA metabolic process, mismatch repair and base excretion repair were detected as down-regulated genes. Moreover, mRNAs encoding the ABC transporters *ABCG2/BCRP1* and *ABCC2/MRP2,* which mediate the excretion of anti-cancer drugs such as cisplatin and gefitinib, respectively, were expressed at higher levels. In contrast, the level of mRNA encoding the SLC transporter *SLC29A1/ENT1,* which mediates the uptake of anti-cancer drugs such as fluorouracil, was decreased. These results indicate that SCC-4 SP cells might be potentially resistant to anti-cancer drugs. (J Osaka Dent Univ 2015 ; 49 : 205–217)

Key words : Gene expression ; Oral cancer ; Side population ; Anti-cancer drug ; Drug resistance

INTRODUCTION

Cancer stem cells represent a small subset of cancer cells that self-renew, differentiate into multiple lineages, and may generate and maintain the phenotypic heterogeneity of cancer cells.[1,2] Evidence indicates that these cells give rise to tumor cells that are intrinsically resistant to anti-cancer drugs[3–5] and oxidative stress.[6,7] Moreover, they promote neoangiogenesis[8] and maintain a quiescent state by persisting in microenvironments (niches).[9] Cancer stem cells are present in leukemias,[10,11] breast cancers,[12] and colon cancers.[13–16] A novel cancer therapy that targets cancer stem cells may serve as a potentiality radical cancer cure in the future. Cancer stem cells are identified and isolated according to their phenotypic properties as follows : cell-surface antigen expression,[3,6,7,9–16] drug efflux (ABC transporter),[4,5,17,18] sphere formation under specific conditions,[8,19] proliferative potential, and resistance to anti-cancer drugs and radiation. Using fluorescence-activated cell sorting (FACS), we isolated the cells that enhanced excretion of Hoechst 33342 in SCC-4. We performed comprehensive analysis to determine the gene expression profile of these cells.

MATERIALS AND METHODS

Cell culture

The human oral squamous cell carcinoma cell line SCC-4 was purchased from DS Pharma Biomedical, Suita, Japan. Cells were cultured in Gibco DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (MP Biomedicals, Santa Ana, CA, USA), 100 units/mL of Gibco penicillin, 100 μg/mL of Gibco streptomycin, 0.25 μg/mL of Gibco Fungizone (Thermo Fisher Scientific), and 0.4 μg/mL of hydrocortisone (MP Biomedicals) at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of SP cells

Hoechst 33342 dye has the capacity to incorporate into the A–T base sequence of DNA and to stain live cells that have high membrane permeability. When cells are stained with Hoechst 33342 and FACS analysis is performed using UV laser, the Hoechst 33342 is able to excite the cells at two-dimensional wavelengths, 450 nm and 675 nm fluorescence intensities. A total of 7×10^7 cells grown to 70% confluence were analyzed and separated using FACS. Hoechst 33342 was added to a final concentration of 5 μM, and the cells were incubated for 45 min at 37°C with occasional agitation.

Reserpine was added to a final concentration of 5 μM as an inhibitor of ABC transporters. Propidium iodide was added to a final concentration of 1 μg/mL to label nonviable cells, excluding the debris in the Hoechst-blue and Hoechst-red channels. In FACS analysis, SP cells have been identified as Hoechst low cells that exist in a darker fraction than the G0/G1 phase. MP cells have been identified as Hoechst high cells that are present in the diploid nuclear fraction. Analysis and sorting were performed using a FACS Vantage SE (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). SP and MP cells were sorted and harvested by ReproCELL, Yokohama, Japan.

Microarray analysis

Total RNA was extracted from SP and MP cells using Invitrogen TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). Gene expression profiles of SP and MP cells were analyzed using the GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) containing 26,879 distinct probes. This procedure was performed at Kurabo Industries, Osaka, Japan. The Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource is made up of integrated biological knowledge and analytic tools to phylogenetically extract biological meaning from output gene lists.[20] In order to extract enrichment Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms by using statistical methods, GO and KEGG pathway enrichment analyses were performed on the DAVID v 6.7 online tool (http : //david.abcc.ncifcrf.gov/).[20]

GO describes biological and functional annotations, such as biological processes, cellular components, and molecular functions. The KEGG pathways are graphical maps of cellular processes, such as metabolism, membrane transport, signal transduction, and the cell cycle.[21–23] The protocol for DAVID Functional Annotation Bioinformatics Microarray Analysis was used to make gene lists.[20] The protocol provides the researcher a detailed account of characteristics that create a good gene list. The good gene list meets seven requirements in the protocol.[20] The list of genes was uploaded using UniGene IDs.

Each up- or down-regulated genetic feature was revealed from the enriched GO and KEGG pathway terms in the DAVID database by specifying a statistically significant threshold of $p < 0.05$. The p-value was tested by the modified Fisher's exact test. To confirm the signaling pathways that were enriched in the SP cells, the up/down expressed groups of probes were classified as either yellow or green using the KEGG Mapper Search & Color Pathway analysis on the KEGG online tool (http : //www.genome.jp/kegg/).[21–23] The list of genes was uploaded in accordance with Entrez Gene IDs that were converted from UniGene IDs.

Semi-quantitative RT-PCR analysis

The cDNAs were synthesized from total RNA using Invitrogen SuperScript VILO (Thermo Fisher Scientific) according to the manufacturer's instructions. PCR amplification of first-strand cDNAs was conducted using KOD FX Neo (Toyobo, Osaka, Japan). The primer sets for RT-PCR were as follows : *ABCC 2/MRP2,* 5'-AGT GAT CAC CAT CGC CCA CA-3' and 5'-GTT CAC ATT CTC AAT GCC AGC TTC-3' ; *ABCG2/BCRP1,* 5'-AGC TGC AAG GAA AGA TCC AA-3' and 5'-TCC AGA CAC ACC ACG GAT AA-3' ; *SLC29A1/ENT1,* 5'-GCC ACT CTA TCA AAG CCA TCC TG-3' and 5'-CCT GCG ATG CTG GAC TTG AC-3' ; *Ribosomal protein S18 (RPS18),* 5'-TTT GCG AGT ACT CAA CAC CAA CAT C-3' and 5'-GAG

CAT ATC TTC GGC CCA CAC-3'. *RPS18* served as an internal control to normalize the semi-quantitative data. The products were electrophoresed through 15% (w/v) polyacrylamide gels (e-PAGEL ; ATTO, Tokyo, Japan) and stained with ethidium bromide. The intensities of the amplicons were estimated using ImageJ software (http : //imagej.nih.gov/ij/). The expression levels are presented as the ratio of values of

SP cells compared with those of MP cells.

RESULTS

SP cells existed in SCC-4

SP and MP cells accounted for 1.48% and 35.4% of the SCC-4 cell population, respectively (Fig. 1 A). After adding reserpine, the SP cell fraction decreased to 0.150%, while the MP cell fraction remained about the
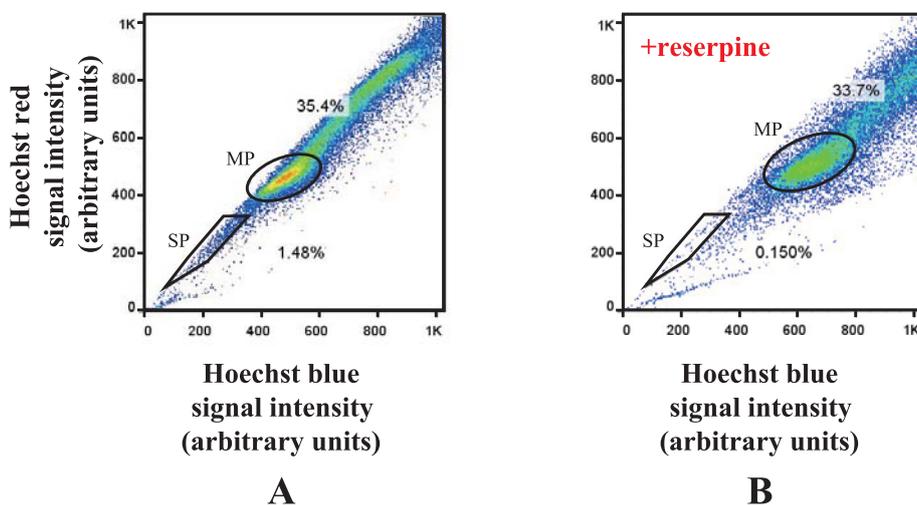

Fig. 1   FACS analysis of SCC-4 cells stained with Hoechst 33342. Hoechst blue and red fluorescence intensities are plotted on the X- and Y-axes, respectively. Reserpine was used as an ABC transporter inhibitor that suppresses excretion of Hoechst 33342. (A) In the absence of 5 μM reserpine, the side population (SP) cell populations were identified as 1.48% Hoechst negative cells and the main population (MP) cell populations were identified as 35.4% Hoechst positive cells. (B) In the presence of 5 μM reserpine of ABC transporter inhibitor, the SP cell fraction decreased to 0.150%, confirming that it was an SP phenotype.
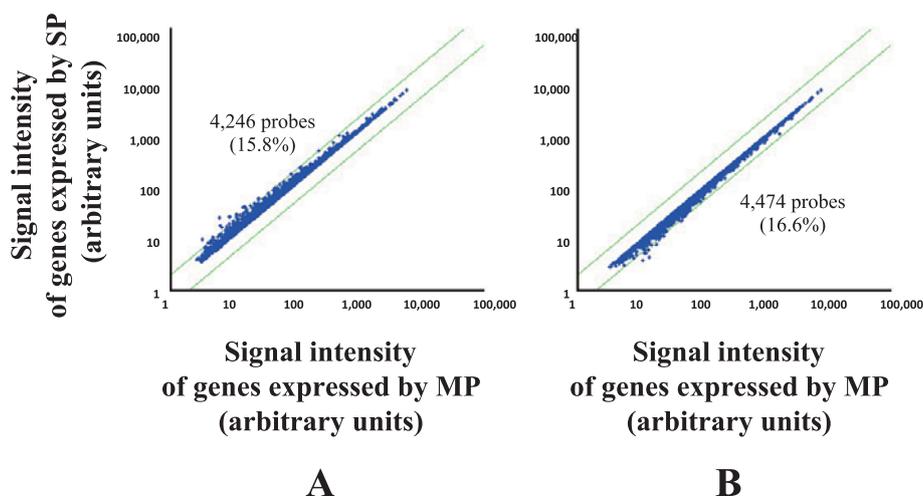

Fig. 2   Scatter-plot analysis. The levels of genes expressed by MP and SP cells are plotted on the X- and Y-axes, respectively. The green lines indicate a 2-fold change in expression level. Genes with more than a 10% difference in the expression by the SP cells were extracted for further analysis. Overall, 15.8% of the genes were up-regulated (Fig. 2 A) and 16.6% were down-regulated (Fig. 2 B) in the SP cells compared with the MP cells.

same at 33.7% (Fig. 1 B).

Gene expression profile of SCC-4 SP cells
Among the entire 26,879 distinct probes, microarray analysis detected 4,246 (15.8%) of the probes that were differentially up-regulated and 4,474 (16.6%) of the probes that were down-regulated in SP versus MP cells (Figs. 2 A and 2 B).

Features of functional annotations in SP cells
Using DAVID's Functional Annotation Tool, significant GO terms were extracted from the genes that were up- or down-regulated ($p < 0.05$) (Table 1). There were 242 and 88 significantly enriched GO terms in the up- and down-regulated groups, respectively. The representative GO terms and the corresponding gene

symbols were as follows. Up-regulation : Immune response (147 genes)/inflammatory response (80 genes) − *CXCR4, IL1B, IL6, CXCL8/IL8* and *TNF ;* Integral component to membrane (765 genes) − *ABCC2/MRP2, ABCG2/BCRP1, CD44, PROM1/CD 133* and *SLC7A11/xCT ;* Negative regulation of cell growth (20 genes) − *BCL6, BTG1, CDKN2AIP, TP53 TG5* and *CDKN1A/p21 ;* Positive regulation of NF-kappa B (NF-κB) transcription factor activity (11 genes)/pattern recognition receptor (PRR) signaling pathway (6 genes) − *IRAK2, NOD2, TLR3, TLR4* and *TICAM1 ;* Angiogenesis (27 genes) − *SOX18, ANGPT1, ECSCR, PROK1* and *VEGFC.* Down-regulation : DNA replication (58 genes) − *LIG1, MCM 2,3,6-10, POLD1,2, RFC2,3* and *RPA4 ;* DNA metabolic process (111 genes) − *CDC 45, CHEK1, CDC25*

Table 1   Features of functional annotations in SP cells

Up-regulation				
GO ID	GO term	Count	%	*p*-value
GO : 0007186	G-protein coupled receptor signaling pathway	250	8.9	1.8 E−22
GO : 0007166	Cell surface receptor signaling pathway	339	12.1	1.8 E−15
GO : 0006955	Immune response	147	5.3	1.4 E−11
GO : 0006954	Inflammatory response	80	2.9	1.4 E−09
GO : 0016021	Integral component to membrane	765	27.4	2.7 E−06
GO : 0032760	Positive regulation of tumor necrosis factor production	8	0.3	1.1 E−03
GO : 0002252	Immune effector process	29	1	3.4 E−03
GO : 0001817	Regulation of cytokine production	35	1.3	7.8 E−03
GO : 0016477	Cell migration	48	1.7	1.3 E−02
GO : 0030308	Negative regulation of cell growth	20	0.7	1.6 E−02
GO : 0051094	Positive regulation of developmental process	47	1.7	2.3 E−02
GO : 0051092	Positive regulation of NF-kappa B transcription factor activity	11	0.4	2.4 E−02
GO : 0030155	Regulation of cell adhesion	26	0.9	2.8 E−02
GO : 0001525	Angiogenesis	27	1	3.9 E−02
GO : 0002221	Pattern recognition receptor (PRR) signaling pathway	6	0.2	4.9 E−02

Down-regulation				
GO ID	GO term	Count	%	*p*-value
GO : 0006260	DNA replication	58	1.9	3.3 E−09
GO : 0006259	DNA metabolic process	111	3.6	2.9 E−07
GO : 0006261	DNA-dependent DNA replication	19	0.6	5.0 E−04
GO : 0006281	DNA repair	60	1.9	5.6 E−04
GO : 0005657	Replication fork	11	0.4	8.9 E−03
GO : 0005887	Integral component to plasma membrane	192	6.2	1.1 E−02
GO : 0006270	DNA replication initiation	7	0.2	1.5 E−02
GO : 0010959	Regulation of metal ion transport	19	0.6	2.4 E−02
GO : 0006268	DNA unwinding involved in DNA replication	6	0.2	3.3 E−02
GO : 0030894	Replisome	6	0.2	3.4 E−02
GO : 0000228	Nuclear chromosome	32	1	3.6 E−02

GO ID : Enriched GO ID associated with this gene list, GO term : Enriched biological and functional annotation terms associated with this gene list, Count : Number of genes in the GO term, % : Term-involved genes/total genes in this gene list, *p*-value : Threshold of a modified Fisher exact *p*-value for gene-enrichment analysis ($p < 0.05$).

A, PMS1 and *RAD51* ; DNA repair (60 genes) − *DNA 2, FEN1, MSH3,6, MUTYH* and *PCNA* ; Integral component to plasma membrane (192 genes) − *SLC 29A1/ENT1* ; Regulation of metal ion transport (19 genes) − *AKT2.*

Features of frequent pathways in SP cells
Using the Functional Annotation Tool of DAVID, 23

and 10 significant KEGG pathway terms were extracted from the up- and down-regulated groups of the gene lists, respectively ($p < 0.05$) (Table 2). The representative KEGG pathway terms and the corresponding gene symbols were as follows. Up-regulation : Cytokine-cytokine receptor interaction (57 genes) − *CXCR4, IL1B, IL6, CXCL8/IL8* and *CXCR1* ; NOD-like receptor (NLR) signaling pathway

Table 2 Features of frequent pathways in SP cells

KEGG ID	KEGG pathway term	Count	%	p-value	Genes
				Up-regulation	
hsa 04060	Cytokine-cytokine receptor interaction	57	2	1.4 E−04	*CD70, BMP2, CLCF1, XCR1, CCL17, CCL18, CCL2, CCL20, CCL 25, CCL26, CCL28, CCL3L1·3L3, CCL4L1·4L2, CCL5, CCR3, CCR 4, CXCL10, CXCL14, CXCL2, CXCL3, CXCL6, CXCR4, CXCR5, CX 3CR1, GH1, INNHBA, IFNA1, IFNA17, IFNA21, IFNA5, IFNA6, IFNE, IFNK, IL1R2, IL1A, IL1B, IL2RG, IL21, IL22 RA2, IL23A, IL28 A, IFNL3/IL28B , IL6, CXCL8/IL8, CXCR1, IL9, LEP, PDGFRB, PRL, PRLR, TNF, TNFSF10, TNFSF13B, TNFSF9, TNFRSF11B, TNFRSF17, VEGFC*
hsa 04621	NOD-like receptor (NLR) signaling pathway	17	0.6	5.8 E−03	*NAIP, BIRC3, CASP8, CARD6, CARD9, CCL2, CCL5, CXCL2, IL1B, IL6, CXCL8/IL8, MAPK10, MAPK11, TAB3, NOD2, TNF, TNFAIP3*
hsa 04620	Toll-like receptor (TLR) signaling pathway	24	0.9	5.8 E−03	*CD14, CD86, CASP8, CCL5, CXCL10, IFNA1, IFNA17, IFNA21, IFNA5, IFNA6, IL1B, IL6, CXCL8/IL8, MAPK10, MAPK11, RAC1, SPP1, TLR1, TLR3, TLR4, TLR5, TLR9, TICAM1, TNF*
hsa 04622	RIG-I-like receptor (RLR) signaling pathway	17	0.6	2.2 E−02	*DDX58/RIG-I, CASP10, CASP8, CXCL10, CYLD, IFIH1/MDA5, IFNA 1, IFNA17, IFNA21, IFNA5, IFNA6, IFNE, IFNK, CXCL8/IL8, MAPK 10, MAPK11, TNF*
hsa 00982	Drug metabolism-cytochrome P450	15	0.5	3.0 E−02	*UGT2A3, UGT2B10, UGT2B11, UGT2B28, UGT2B4, ADH7, AOX1, CYP2C19, CYP2C9, FMO1, FMO4, GSTM2, GSTM5, GSTT1, MAOB*
				Down-regulation	
hsa 03030	DNA replication	18	0.6	1.3 E−06	*DNA2, FEN1, LIG1, MCM2, MCM3, MCM6, MCM7, POLA2, POLD1, POLD2, POLE, POLE2, PRIM1, PRIM2, PCNA, RFC2, RFC3, RPA4*
hsa 03430	Mismatch repair (MMR)	11	0.4	4.8 E−04	*PMS2, EXO1, LIG1, MSH3, MSH6, POLD1, POLD2, PCNA, RFC2, RFC3, RPA4*
hsa 03410	Base excision repair	13	0.4	1.7 E−03	*APEX2, FEN1, HMGB1·1L1O, LIG1, LIG3, MUTYH, NTHL1, POLD 1, POLD2, POLE, POLE2, PCNA, UNG*
hsa 00240	Pyrimidine metabolism	21	0.7	3.2 E−02	*NT5C, CTPS, DUT, DHODH, ENTPD5, POLA2, POLD1, POLD2, POLE, POLE2, POLR1A, POLR3G, POLR3GL, POLR3K, PRIM1, PRIM2, RRM1, RRM2, UMPS, UPP2, ZNRD1*
hsa 00230	Purine metabolism	30	1	4.2 E−02	*PAPSS1, NT5C, ADK, ADCY1, ADCY8, ENTPD5, ENPP3, GDA, GUCY1B3, GUCY2F, NPR1, PDE10A, PDE11A, PDE3B, PDE9A, PFAS, POLA2, POLD1, POLD2, POLE, POLE2, POLR1A, POLR3 G, POLR3GL, POLR3K, PRIM1, PRIM2, RRM1, RRM2, ZNRD1*

KEGG pathway ID : Enriched KEGG pathway ID associated with this gene list, KEGG pathway term : Enriched KEGG pathway term associated with this gene list, Count : Number of genes in the KEGG pathway term, % : Term-involved genes/total genes in this gene list, *p*-value : Threshold of a modified Fisher exact *p*-value for gene-enrichment analysis ($p < 0.05$), Genes : Genes involved in the term.

(17 genes) − *CARD9, CCL5, TAB3, NOD2* and *TNF ;* Toll-like receptor (TLR) signaling pathway (24 genes) − *CD14, RAC1, TLR1, TLR4* and *TICAM1 ;* RIG-I-like receptor (RLR) signaling pathway (17 genes) − *DDX58/RIG-I, IFIH 1/MDA 5, IFNA 1, IFNE* and *IFNK ;* Drug metabolism − cytochrome P450 (15 genes) − *CYP2C19, CYP2C9, GSTM2, GSTM5* and *GSTT1.* Down-regulation : DNA replication (18 genes) − *MCM2,3,6,7, POLD1,2, PRIM1,2, RFC2,3* and *RPA4 ;* Mismatch repair (MMR) (11 genes) −

PMS2, LIG1, MSH3,6, POLD1,2 and *PCNA ;* Base excision repair (13 genes) − *APEX2, FEN1, MUTYH, POLE, E2* and *UNG ;* Pyrimidine metabolism (21 genes) − *CTPS, DUT, ENTPD5, POLA2* and *RRM 1,2 ;* Purine metabolism (30 genes) − *NT5C, ADK, ADCY1,8, GUCY1B3,2F* and *PDE3B,9A,10A,11A.*

Lists of representative genes differentially expressed in SP cells

Representative genes with differences in expression

Table 3   Lists of representative genes differentially expressed in SP cells

Up-regulated Gene		
Gene Symbol	Gene Description	Fold Change
	Drug Resistance	
ABCG2/BCRP1	ATP-binding cassette, sub-family G (WHITE), member 2	1.1
ABCC2/MRP2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	1.2
	Stem Cell Marker	
CD44	CD44 molecule (Indian blood group)	1.3
SLC7A11/xCT	Solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11	1.2
PROM1/CD133	Prominin 1	1.2
CXCR4	Chemokine (C-X-C motif) receptor 4	1.4
	Self-renewal	
STAT5B	Signal transducer and activator of transcription 5B	1.1
CXCL8/IL8	Chemokine (C-X-C motif) ligand 8	1.7
	Cell-cycle Arrest at G0/1 Phase	
BTG1	B-cell translocation gene 1, anti-proliferative	1.1
FOXO1	Forkhead box O1	1.1
CDKN1A	Cyclin-dependent kinase inhibitor 1 A (p21, Cip1)	1.2

Down-regulated Gene		
Gene Symbol	Gene Description	Fold Change
	Drug Resistance	
SLC29A1/ENT1	Solute carrier family 29 (nucleoside transporters), member 1	0.9
	DNA Replication	
POLA2	Polymerase (DNA directed), alpha 2, accessory subunit	0.9
PRIM1,2	Primase, DNA, polypeptide 1 (49 kDa), 2 (58 kDa)	0.9
POLD1,2	Polymerase (DNA directed), delta 1, catalytic subunit, delta 2, accessory subunit	0.9
POLE,2	Polymerase (DNA directed), epsilon, catalytic subunit, epsilon 2, accessory subunit	0.8∼0.9
MCM2,3,6–9	Minichromosome maintenance complex component 2,3,6–10	0.8∼0.9
RPA4	Replication protein A4, 30 kDa	0.8
RFC2,3	Replication factor C (activator 1) 2, 40 kDa, 3, 38 kDa	0.9
DNA2	DNA replication helicase 2 homolog (yeast)	0.8
	DNA Repair	
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	0.9
FEN1	Flap structure-specific endonuclease 1	0.9
MSH3,6	MutS homolog 3, 6 (E. coli)	0.8∼0.9
MUTYH	MutY homolog (E. coli)	0.8

between SP cells versus MP cells on the basis of results from GO and KEGG pathway enrichment analyses are presented in Table 3. *ABCC2/MRP2* and *ABCG2/BCRP1* were up-regulated and *SLC29A1/ ENT1* was down-regulated (Table 3). Using significantly enriched KEGG pathway maps from DAVID analysis as reference, up-regulated genes were classified as drug resistance, stem cell marker, self-renewal or cell-cycle arrest at G0/1 phase. The down-regulated genes were classified as drug resistance, DNA replication or DNA repair.

Close-up specific pathways identified using KEGG Mapper Search & Color Pathway analysis

NF-κB signaling pathway (Fig. 3 A), DNA replication pathway (Fig. 3 B), MMR pathway (Fig. 3 C), and ABC transporter pathway (Fig. 3 D) were extracted by KEGG Mapper Search & Color Pathway analysis. In particular, inflammatory cytokines such as *IL1B* and *TNF,* transmembrane receptors such as *CD14* and *TLR4,* and intracellular receptors such as *DDX58/ RIG-I* were enhanced in the canonical (classical) NF-κB signaling pathway and these signals were terminated to enhancement of genes encoding survival, activation of non-canonical pathway, cytokines as positive feedback, and inflammation (Fig. 3 A). There were no up-regulated genes except for one gene in the DNA replication pathway (Fig. 3 B). Seven genes were markedly decreased in the 12 genes encoding the DNA polymerase family (α, δ, and ε). The MMR pathway also had no up-regulated genes (Fig. 3 C). Moreover, 10 genes, including the *PMS2, MSH3* and *MSH6* genes, were down-regulated among the 22 genes in this pathway. The ABC transporter pathway was shown for an overview of the ABC transporter genes (Fig. 3 D). The *ABCA2/ABC2, ABCC2/MRP2* and *ABCG2/BCRP1* genes were up-regulated.

Prospects of activated signaling pathways associated with anti-cancer drug resistance in SP cells

The results of GO and KEGG pathway enrichment analyses and KEGG Mapper Search & Color Pathway analysis predicted the specific biological functions and activated pathways in SP cells (Fig. 4). The NF-κB signals through PRR (TLR, NLR and RLR) path-

ways induce cancer progression relating to anti-apoptosis, invasion and angiogenesis. The STAT5 and CXCL8/IL8 signals contribute to self-renewal of stem cells. The FOXO1 and BTG1 signals contribute to cell-cycle arrest at the G0/1 phase. In SP cells, these signals were enhanced. DNA replication and pyrimidine/purine metabolism were suppressed. The mismatch repair system may be decreased in SP cells, suggesting that the depression of mismatch repair may induce insensitivity to cisplatin. *SLC29A1/ ENT1,* which is involved in the uptake of fluorouracil, was also suppressed.

These drug metabolic enzymatic signals may contribute to suppression of cyclophosphamide activity. Moreover, *ABCC2/MRP2* and *ABCG2/BCRP1,* which are involved in export of anti-cancer drugs, were up-regulated.

Expression of genes encoding transporter proteins associated with anti-cancer drug resistance

The expression of genes encoding proteins that confer drug resistance was determined using RT-PCR analysis. *ABCC2/MRP2* and *ABCG2/BCRP1* were up-regulated ; however, the expression of *SLC29A1/ ENT1* was down-regulated in SP cells (Fig. 5). Data analyzed using ImageJ are shown in the graph. The values were normalized to that of an internal control (*RPS18*).

DISCUSSION

The population of SP cells present in human cancer cell lines varies markedly (0.2%–10%).[5, 17] In the present study, the SP and MP cell populations in SCC-4 were 1.48% and 35.4%, respectively (Fig. 1 A). In the presence of the ABC transporter inhibitor reserpine, the ratio of the SP cell fraction decreased to 0.150% and that of the MP cell fraction was almost unchanged (Fig. 1 B), suggesting that SP cells with a strong ability to excrete Hoechst 33342 existed in the SCC-4.[4, 5, 17, 18] Because the uptake of Hoechst 33342 mediated by ABCG2/BCRP1 is inhibited by reserpine, expression of *ABCG2/BCRP1* is an important determinant of the SP phenotype.[18] Although it is shown that only 44 probes were differentially expressed by 2-fold in SP cells, the expression of *ABCG2/BCRP1* was in-
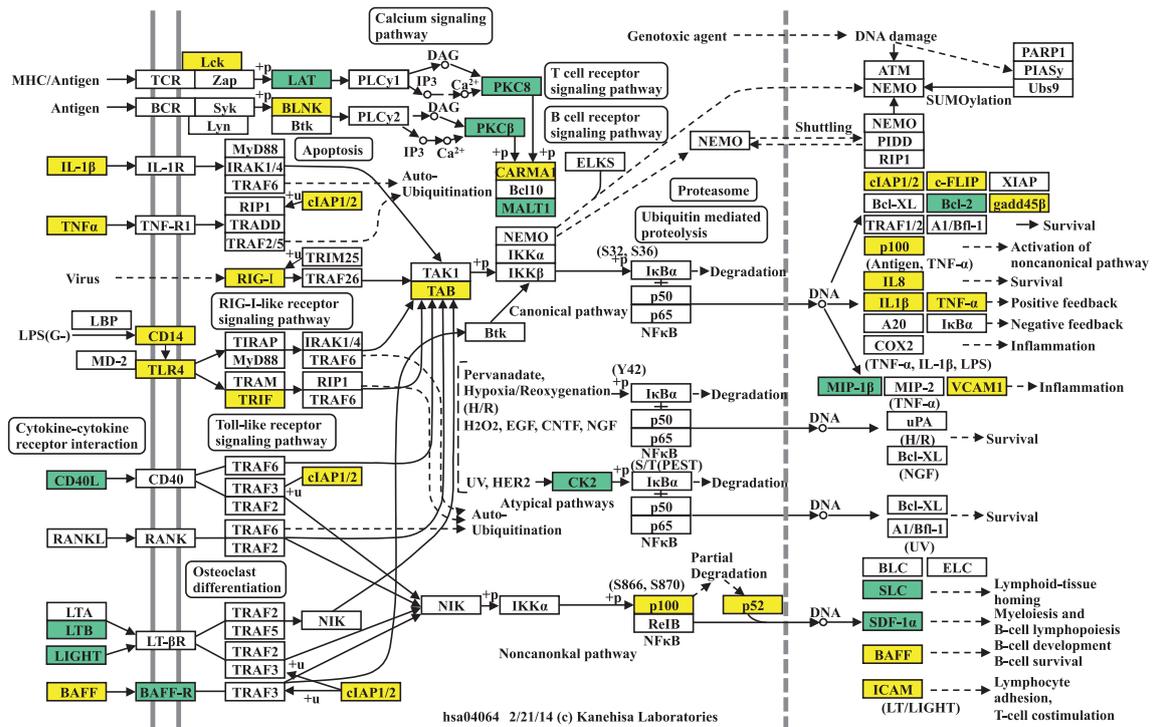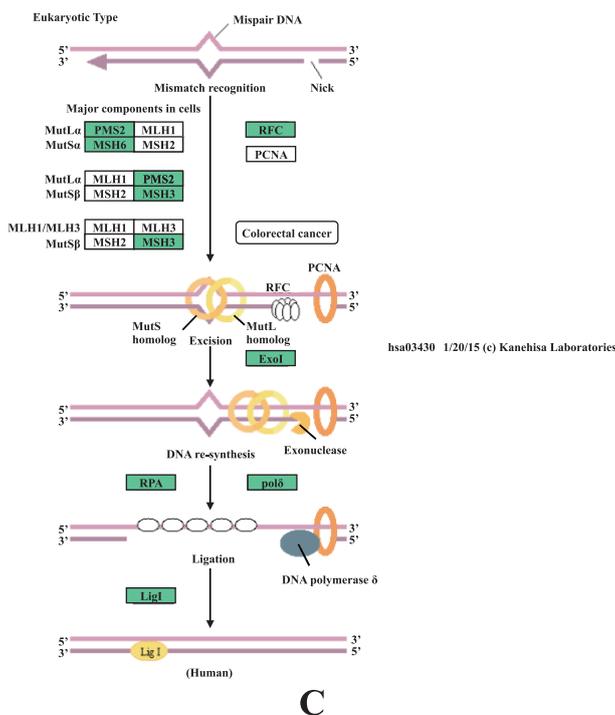
A
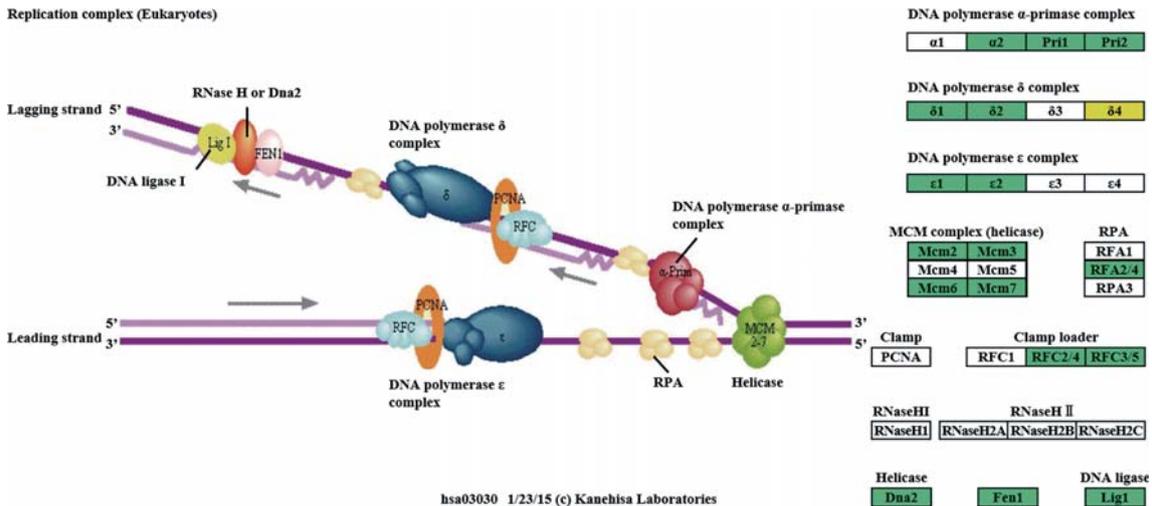
B

Fig. 3   Pathways identified using KEGG Mapper Search & Color Pathway analysis. The pathways extracted using this analysis were (A) the NF-κB signaling pathway, (B) the DNA replication pathway, (C) the MMR pathway, and (D) the ABC transporter pathway. Genes that were up-regulated by more than 10% are displayed in yellow and those down-regulated by more than 10% are shown in green. Abbreviations (Fig. 3 A) CD14 : CD14 molecule ; DDX58/RIG-I : DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 /Retinoic acid-inducible gene 1 ; IL1B : Interleukin 1, beta ; TLR4 : Toll-like receptor 4 ; and TNF : Tumor necrosis factor ; (Fig. 3 B) DNA polymerase α complex : Polymerase (DNA directed), alpha (POLA) 1～2 and Primase, DNA, polypeptide (PRIM) 1～2 ; DNA polymerase δ complex : Polymerase (DNA directed), delta (POLD) 1～4 ; and DNA polymerase ε complex : Polymerase (DNA directed), epsilon (POLE) 1～4 ;

C

D

(Fig. 3 C) PMS2 : Postmeiotic segregation increased 2 ; MSH3 : MutS homolog 3 ; and MSH6 : MutS homolog 6 ; (Fig. 3 D) ABCA2/ABC2 : ATP-binding cassette, sub-family A (ABC1), member 2/ATP-binding cassette 2 ; ABCC2/MRP2 : ATP-binding cassette, sub-family C (CFTR/MRP), member 2/Multidrug resistance-associated protein 2 ; and ABCG2/BCRP1 : ATP-binding cassette, sub-family G (WHITE), member 2/ Breast cancer resistance protein 1.
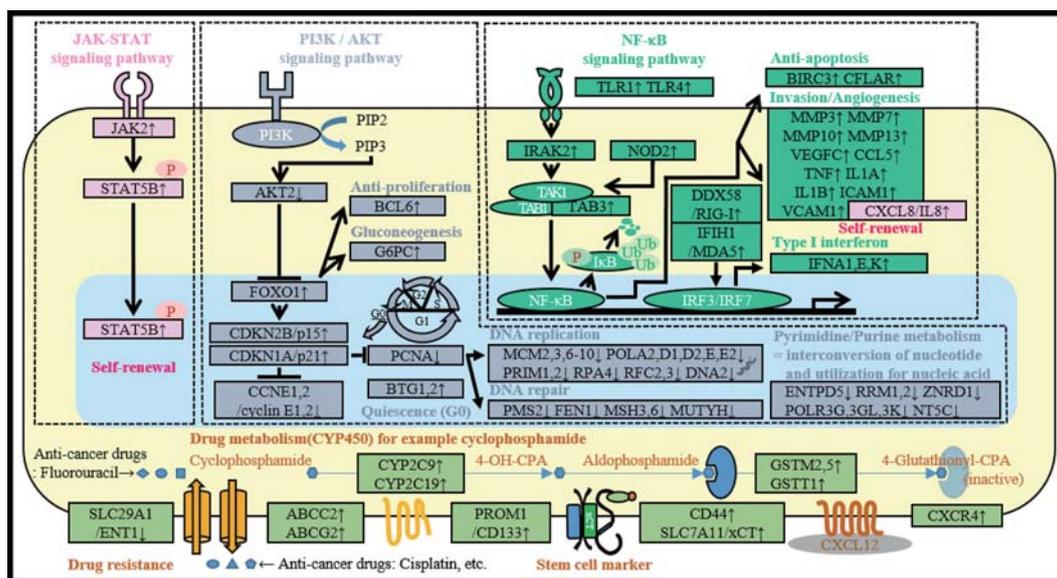
Fig. 4   Proposal of signaling pathways activated in SP cells. The activation of signaling pathways based on the comparative gene expression profile in SP cells are shown here. The NF-κB signals through the PRR (TLR, NLR and RLR) pathways induce cancer progression related to anti-apoptosis, invasion and angiogenesis. The STAT5 and CXCL8/IL8 signals contribute to self-renewal of stem cells. The FOXO1 and BTG1 signals contribute to cell-cycle arrest at the G0/1 phase. These signals were enhanced in the SP cells. DNA replication and pyrimidine/purine metabolism were suppressed. The mismatch repair system may be decreased in SP cells, suggesting that the depression of mismatch repair may induce insensitivity to cisplatin. *SLC29A1/ENT1* involved in the uptake of fluorouracil was also suppressed. These drug metabolic enzymatic signals may contribute to suppression of cyclophosphamide activity. *ABCC2/MRP2* and *ABCG2/BCRP1,* which are involved in the excretion of anti-cancer drugs, were upregulated. This shows the identities and functions of the pathways related to drug-resistance.
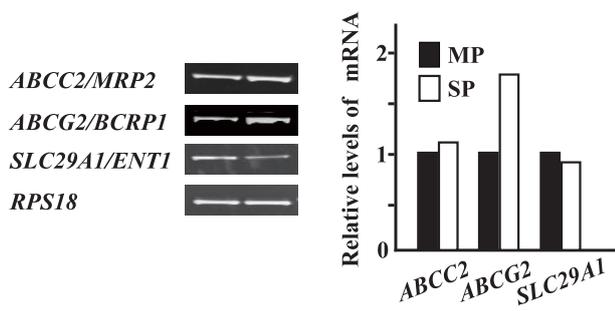

Fig. 5   The expression of genes associated with anti-cancer drug resistance. The electrophoresis bands represent the expression levels of each gene by RT-PCR. *ABCC2/MRP2* and *ABCG 2/BCRP1* were up-regulated, while *SLC29A1/ENT1* was down-regulated in the SP cells. Data analyzed using ImageJ are shown in the graph. The relative level is presented as the fold changes in SP versus MP cells. The values were corrected by an internal control (RPS18).

creased by more than 10% in SP compared with MP cells. For this reason, we defined a change of greater than 10% as the critical threshold. While 15.8% of the up-regulated genes showed this critical degree of differential expression (Fig. 2 A), 16.6% of the down-regulated genes did (Fig. 2 B).

The significant enrichment GO terms revealed by DAVID analysis represented by differentially expressed genes are presented in Table 1 ($p < 0.05$). Among the up-regulated genes, the associated GO terms include immune and inflammatory responses, positive regulation of NF-κB transcription factor activity, and PRR signaling pathway. The GO terms are related to cell migration, negative regulation of cell growth, and angiogenesis. GO terms related to DNA replication, DNA metabolic processes, and DNA repair were the most highly enriched with genes that were down-regulated.

The significant enrichment KEGG pathway terms revealed by DAVID analysis are presented in Table 2 ($p < 0.05$). Enriched KEGG terms are related to the cytokine − cytokine receptor interaction pathway, the NLR signaling pathway, the TLR signaling pathway, the RLR signaling pathway, and the drug metabolism -cytochrome P450-pathway in the up-regulated genes. NLR, TLR, and RLR signaling pathways de-

rived from KEGG pathway analysis correlate with PI3 K-AKT, NF-κB, JAK-STAT, and MAPK-signaling pathways. KEGG terms related to DNA replication, MMR, base excision repair, pyrimidine metabolism, and purine metabolism (synthesis of nucleotides) were enriched for genes that were down-regulated. Representative genes associated with sufficient enrichment GO terms as well as KEGG pathway terms revealed by DAVID analyses are shown in Table 3.

Although DAVID analyses are advantageous for identifying specific GO and KEGG pathway terms, which include genes that are significantly enriched (p ＜0.05), they do not distinguish between an increase or decrease in gene expression. To compensate for this, KEGG Mapper Search & Color Pathway analysis was performed by classifying changes of gene expression according to the numerical size of the genes. From specific GO and KEGG pathway terms associated with SP cells, the representative examples implicate the following pathways : the NF-κB signaling pathway (Fig. 3 A), the DNA replication pathway (Fig. 3 B), the MMR pathway (Fig. 3 C), and the ABC transporter pathway (Fig. 3 D). The NF-κB signals that mediated *CD14, DDX58/RIG-I, IL1B, TLR4* and *TNF* were especially enhanced downstream in the canonical (classical) NF-κB signaling pathway (Fig. 3 A). The DNA replication and MMR pathways did not include all the up-regulated genes except *POLD4* (Figs. 3 B and 3 C). Seven of the 12 genes encoding the main DNA polymerase family (α, δ and ε) for human DNA replication were decreased in the DNA replication pathway. Ten of the entire 22 genes including *PMS2, MSH3* and *MSH6,* were suppressed in the MMR pathway. Although the ABC transporter pathway was not extracted by KEGG pathway enrichment analysis, the results in Fig. 3 D showed that the *ABCA2/ABC2, ABCC2/MRP2* and *ABCG2/BCRP1* genes were up-regulated in 11 ABC transporter genes (*ABCA2/ABC 2, ABCB1/MDR1, ABCC1～6/MRP1～6, ABCC10/ MRP7, ABCC11/MRP8* and *ABCG2/BCRP1*) associated with export of anti-cancer drugs.

Accordingly, the prediction of activated pathway maps based on the profile in this study is also shown in Fig. 4. Inflammation induces activation of NF-κB and its downstream target genes that encode IL-6 and CXCL8/IL-8, which mediate angiogenesis, tumor growth and metastasis.[24] IL-6 and CXCL8/IL-8, in turn, function as positive feedback regulators by activating the NF-κB/STAT3 pathways to stimulate further cytokine production.[24] Moreover, IL-6 and CXCL 8/IL-8 regulate the self-renewal of breast cancer stem cells.[25, 26] SP cells expressed higher levels of *IL-6* and *CXCL8/IL-8* mRNAs compared with MP cells, suggesting that they may mediate inflammation and self-renewal.

AKT inhibition and FOXO activation maintain leukemia stem cell homeostasis.[27] CDKN1A/P21 maintains the quiescence of stem cells and contributes to long-term remodeling of bone marrow.[28] Consistent with these findings, we found that transcription of *AKT2* decreased and those of *FOXO1, CDKN1A/P21* and *CDKN2B/P15* increased in SP cells. Moreover, the expression of genes related to the DNA replication pathway mediated by *CDKN1A/P21* and *PCNA,* such as *MCM2, 3, 6–10 ; POLD1 ;* and *POLD2* was decreased, suggesting that the SP cells of SCC-4 might not exist at the S phase.[29]

BTG1 is expressed at high levels during G0/G1 and is down-regulated during the transition to G1.[30] In the present study, the high level of *BTG1* expression suggests that SP cells may arrest in G0/G1. The expression levels of *FOXO1* and *CDKN1A/P21* are consistent with this possibility. Cell-cycle arrest impedes therapy using conventional anti-cancer drugs that inhibit dividing cells. In particular, it is known that MMR inactive cells developed high resistance to multi-anti-cancer agents such as monovalent alkylating agents, 6-thioguanine and 6-mercaptopurine,[31, 32] or cisplatin.[33] These findings indicate that SP cells may be relatively insensitive to these drugs.

Taken together, the profiles obtained in this study indicate that SP cells may have multi-resistance for anti-cancer drugs. The SP phenotype is determined by the expression of the ABC transporter genes such as *ABCG2/BCRP1*.[18] The ABC transporters are classified as "excretion type" and SLC transporters as "uptake type." RT-PCR analysis (Fig. 5) detected up-regulation of the expression of *ABCG2/BCRP1* and *ABCC2/MPR2* in SP cells by 1.8- and 1.1-fold, respectively. On the other hands, the expression of *SLC*

29A1/ENT1 was down-regulated by 0.9-fold.

ABCC2/MRP2 and *ABCG2/BCRP1* involved in export of anti-cancer drugs, such as cisplatin and gefitinib respectively, were up-regulated.[34] The drugs VX 710 (biricodar) and GF120918 (elacridar) were developed to overcome resistance to chemotherapeutic drugs by targeting ABC transporters.[35] Moreover, *SLC 29A1/ENT1* involved in uptake of anti-cancer drugs such as the nucleoside derivative fluorouracil was also suppressed.[36] The drug sulfasalazine is administered as an SLC transporter inhibitor to prevent cysteine uptake by the xCT subunit of system xc (−), resulting in suppression of CD44 function.[7, 37] The differential expression of genes related to these transporters demonstrated here suggests that they might mediate excretion or uptake of the anti-cancer drugs in SP cells of SCC-4.

The pattern of gene expression of SP cells isolated from cultures of the SCC-4 might be similar to that of stem cells that show the self-renewal and cell-cycle arrest in G0/G1. Furthermore, these data predict that SP cells are resistant to anti-cancer drugs. We propose that a promising pharmacological approach for the treatment targeting the cancer stem-like cells is to develop specific inhibitors of the drug resistance associated pathway. Based on this study, we need further research focused on the sensitivity of SP cells to drugs used for oral cancer therapy.

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