Establishment and characterization of the human SaTM-1 anal canal squamous cell carcinoma cell line derived from lymph node metastasis

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Abstract. Human anal canal squamous cell carcinoma (SCC) cell line has not yet been reported due to the rarity of this disease. Since cell lines to study this malignancy were not available, we attempted to establish and characterize anal canal SCC cell line from primary culture of lymph node metastasis. Six sublines were cloned and isolated from parental cells. They were designated as SaTM-1A, B, C, D, E and F. The features of the six sublines were characterized by reverse transcription-PCR, chemosensitivity test to 5-Fu and CDDP, immunohistochemistry, cDNA microarray analysis and tumorigenicity using immunodeficient mice. All sublines were proliferated in multiple layers at an average doubling time of 24.5 h. VEGF-A, -B, VEGFR-1, -R3 and EGFR were expressed in all sublines, whereas VEGF-D and EGF were not detected in all. SaTM-1 was proven to retain the characteristics of SCC by detection of p63 and cytokeratin 5/6. The cytotoxic effects of 5-Fu were almost similar, although those of CDDP showed different behavior, which was divided into two groups (SaTM-1A, B, E and SaTM-1C, D, F). The differences in gene expression between two groups were analyzed according to susceptibility to cytotoxic effects of CDDP. Thirty-six genes were successfully identified, which may be potentially associated with CDDP resistance. SaTM-1 cells formed tumors easily *in vivo,* therefore all subclones had tumorigenic property. This is the first report of successful establishment and characterization of a human anal canal SCC cell line, which may provide beneficial resources for investigating the biological features of human anal canal SCC.

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Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HPV, human papiloma virus; GAPDH, glyceraldehydes-3-phosphate dehydrogenase

Key words: anal canal squamous cell carcinoma, human cancer cell line, establishment, characterization

Introduction

Squamous cell carcinoma (SCC) of the anal canal is rare and accounts for 4-5% of all colorectal cancers (1). They are 20-30 times less common than colon cancer. Anal canal cancer may spread to either the inguinal or the pelvic lymph nodes. The overall incidence of clinically positive inguinal lymph node is 10-20%, in which 25% of lymph node positive patients have bilateral involvement (2). Patients with anal canal SCC are currently treated by surgery, chemo-radiotherapy or both. Abdominal perineal resection and colostomy were formerly the first choice of treatment for anal canal SCC, with 5-year survival rates achieved for 38-71% of patients and recurrence developing in 27-43% (3). The introduction of chemoradiotherapy as the primary treatment resulted in survival and recurrence rates similar to those for surgery (4), however, the prognosis of patients with inguinal lymph node involvement was poor regardless of treatment choice. At present, relatively little is known about the molecular and cellular mechanisms that modulate carcino-genesis and progression of anal canal SCC. This is due, in large part, to a lack of suitable model systems *in vitro* for advanced research because it is difficult to obtain numerous clinical specimens of anal canal SCC. Human solid tumor cell lines are important sources for studies of tumor biology including tumor cell growth, differentiation, metastases, molecular pathogenesis and susceptibility to drugs. Establishment of human cell lines that can be implanted into experimental animals is especially vital, since they are valuable for clarifying the molecular and cellular pathogenesis of drugs. To date, numerous cell lines, at least more than 50 from human colorectal cancers have been established and used worldwide. Since, to our knowledge, there have been no reports on the establishment of anal canal SCC cell lines, we

attempted to establish a permanent cell line in order to investigate the biological behavior of anal canal SCC. In the present study, a newly generated cell line and its six sublines were successfully established from anal canal SCC with inguinal lymph node metastasis, and characterized for their differentiation and biological properties. These lines were developed in our laboratory and designated as SaTM-1A, -1B, -1C, -1D, -1E and -1F.

Materials and methods

Patient and source of cell lines. A 56-year-old Japanese female was admitted to the Surgical Department of Saitama Medical University for anal bleeding and increasing abdominal pain. After a series of clinical and laboratory examinations, a diagnosis of advanced anal canal cancer involving the vagina forming micro-abscess around peri-anal tissues was made. Abdominoperineal resection combined with total vagina-hysterectomy was performed. Postoperative pathological findings indicated a well-differentiated SCC of the anal canal with vaginal invasion and multiple regional lymph node metastasis (pT4pN1M0: stage IIIB by TNM classification). Five months after surgery, bilateral inguinal lymph node swelling developed. A portion of the tumor tissues obtained at the time of lymphadenectomy was used as the source of cell culture under the approval of the patient for the collection of resected tissues. The pathological report of the removed lymph nodes confirmed a SCC recurrence. The patient died eleven months after inguinal lymphadenectomy due to intra-peritoneal hemorrhage from local recurrent tumor. Expression profile of tumor markers, CEA, CA19-9, SCC and CYFRA-21-1 was below detectable level throughout her total clinical course. Samples were collected from the patient who had given informed consent before inguinal lymphadenectomy and this study was approved by the Institutional Review Board of Saitama Medical University.

Establishment of a cell line. Immediately following surgery, fresh metastatic tumor tissues from the inguinal lymph nodes were rinsed three times with phosphate-buffered saline (PBS) after the surrounding connective tissues were removed. The tumor tissues were minced finely by sterile scissors to approximately 1 mm^3 pieces and dissociated into small aggregates by repeated pipetting under aseptic conditions. Neither enzymatic nor mechanical dissociation of the tumor cells was performed. The dispersed tumor cells were suspended in a medium on gelatin-coated 100-mm plastic dishes (BD Biosciences, San Jose, CA). The medium for the primary culture was EGM-2MV (Clonetics, San Diego, CA) containing 20% fetal bovine serum (FBS) (Invitrogen). When cell growth stability was obtained after five to six passages, the culture medium was changed to Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) (Nissui, Tokyo, Japan), and the concentration of FBS was decreased from 20 to 10%. Cells were refed with fresh medium every 3-4 days and were passaged on reaching confluency by trypsinization. Six sublines were able to be cloned and isolated from SaTM-1 parental cells by the limiting dilution method *in vitro*. Sublines were developed by seeding 60 single cells of parental cells in a 96-well culture plate containing the culture medium in a 5% CO_2 atmosphere at 37˚C for 17 days. Individual clones were picked up and seeded in a 24-well plate as SaTM-1A, B, C, D, E and F, which were cultured and harvested separately in T-25 cm^2 flasks. All procedures of the present study were performed in compliance with regulations administered by Saitama Medical University.

Cell culture condition and morphological analysis. All established subclones were cultured in 1:1 mixture of DMEM and Ham's F-12 supplemented with 100 U/ml penicillin, 100 μl/ml streptomycin and 10% heat-inactivated FBS in a 5% CO_2 atmosphere at 37˚C. For morphological analysis, $1.5x10^6$ cell were cultured in 75 cm^2 flask for 3 days and images were obtained using a Axiovert 200M phase contrast microscope (Zeiss, Germany). All experiments were performed in our laboratory in 50 and more passages.

Cell proliferation assays. Cell proliferation assays were performed using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's instructions (Promega). Briefly, each subline was plated in a 96-well plate at $3.5x10^3$ cells/well. After 24, 48, 72 and 96 h, CellTiter 96 AQueous One solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium was added to each well at a ratio of 20/100 μl culture media followed by incubating the plates in the dark at 37˚C for 3 h, after which the absorbance at 490 nm was read using 1420 Multilabel Counter ARVO MX (PerkinElmer). The experiment was performed in triplicate and repeated three times. The doubling time was estimated with Cell Doubling Time Calculator program (http://pozharov.com/Dtime.html).

Chemosensitivity assay. In order to investigate susceptibility to anti-tumor drugs, $3.5x10^3$ cells were cultured in a 96-well plate for 24 h followed by treatment with several concentration of Cisplatin (CDDP) (2.5, 5, 7.5, 10, 15, 20 and 50 μg/ml) and 5-fluorouracil (5-Fu) (1, 5, 10, 50, 100, 500 and 1,000 μg/ml) for another 24 and 48 h, respectively. The relative number of viable cells was assessed using the CellTiter 96 AQueous cell proliferation kit according to the manufacturer's instructions. Each experiment was performed in triplicate and repeated three times.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA were extracted from six established subclones using TRIzol reagent according to the manufacturer's instructions (Invitrogen). First strand cDNA was synthesized from 5 μg of total RNA using SuperScript III RNase H-reverse transcriptase (Invitrogen) in a total volume of 20 μl reaction mixture containing 0.5 mM dNTP mixture and 0.025 μg/(l oligo$(dT)_{12-18}$ primer. The PCR reactions contained 1 μl cDNA, 1X PCR buffer (Takara), 200 μM dNTP each, 0.5 unit of Ex Taq Polymerase (Takara), and 0.2 μM of each primer and were subjected to the following amplification scheme. For VEGFR-3, 1 cycle of 94˚C for 2 min, 35 cycles of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 2 min, and final extension at 72˚C for 5 min after the last cycle. For the other genes, 1 cycle of 95˚C for 2 min, 30-35 cycles of 95˚C for 30 sec, 56˚C (VEGF-C, VEGF-D, and

VEGFR-2) or 60˚C (VEGF-A, VEGF-B, VEGFR-1, EGF and EGFR) for 30 sec, and 72˚C for 1 min, and final extension at 72˚C for 5 min after the last cycle. For GAPDH, 1 cycle of 95˚C for 2 min, 25 cycles of 95˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 1 min, and final extension at 72˚C for 5 min after the last cycle. Detection of human papilloma viruses (HPVs) associated with anal canal carcinoma progression was performed to evaluate the infection of HPV-16 and HPV-18, whose amplification scheme was 1 cycle of 94˚C for 3 min, 35 cycles of 94˚C for 30 sec, 55˚C for 1 min and 72˚C for 30 sec, and final extension at 72˚C for 5 min after the last cycle. Amplified fragments were visualized by ethidium bromide staining of the 2% agarose gel and photographed under UV light. Primer sequences used in these experiments were as followed: forward primer and reverse primer, respectively. VEGF-A, 5'-GCAGAATCATC ACGAAG TGG-3' and 5'-G CATGGTGATGTTGGACTCC-3'; VEGF-B, 5'-CCTTGACTGTGGAGCTCATG-3' and 5'-TGTCTGGCTTCACAGCACTG-3'; VEGF-C, 5'-AGACTC AATGCATGCCACG-3' and 5'-TTGAGTCATCTCCAG CATCC-3'; VEGF-D, 5'-GCTGTTGCAATGAAGAGAGC-3' and 5'-TCTTCTGTTCCAGCAAGTGG-3'; VEGFR-1, 5'-AAGAGAGCTTCCGTAAGGCG-3' and 5'-G CATCCTCTT CAGTTACGTCC-3'; VEGFR-2, 5'-CCTTCTTCGAAGC ATCAGC-3' and 5'-AGAGATTCCATGCCACTTCC-3'; VEGFR-3, 5'-CAGGATGAAGACATTTGA-3' and 5'-AAG AAAATGCTGACGTATGC-3'; EGF, 5'-TGCAACTGTG TTGTTGGCTACATC-3' and 5'-TGGTTGACCCCCATT CTTGAG-3'; EGFR, 5'-CAGCGCTACCTTGTCATTCAG-3' and 5'-TCATACTATCCTCCGTGGTCA-3'; HPV-16, 5'-AAGGGCGTAACCGAAATCGGT-3' and 5'-GTTTGCA GCTCTGTGCATA-3'; HPV-18, 5'-AAGGGAGTAACC GAAAACGGT-3' and 5'-GTGTTCAGTTCCGTGCACA-3'; GAPDH, 5'-ATGTTCGTCATGGGTGTGAA-3' and 5'-TGTGGTCATGAGTCCTTCCA-3'.

Immunohistochemistry. In order to prove that the newly established SaTM-1 itself still characterizes squamous cell carcinoma *in vitro*, p63 and cytokeratin 5/6 expressions were investigated by immunohistochemistry using subcutaneously implanted tumor to immunodeficient mice of each SaTM-1 subline. Immunohistochemical staining was performed using an ENVISION+ kit according to the manufacturer's instructions. Sections were deparaffinized in xylene, rehydrated through graded alcohols, and immersed in 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. Antigen retrieval was carried out by autoclave for 5 min at 121˚C in 0.01 M citrate buffer (pH 6.0). Primary monoclonal antibodies used for immunohistochemistry were as follows: human anti-p63 (clone 4A4, Dako Cytomation, Denmark, dilution 1:50) and human anti-cytokeratin 5/6 (clone D5/16B4, DakoCytomation, dilution 1:100).

cDNA microarray experiment. Total RNA was extracted from SaTM-1 six sublines using RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instruction. Gene expression analysis was performed using Affymetrix GeneChip® according to the manufacturer's instruction. Briefly, 5 μg of total RNA was reversely transcribed to obtain a second strand cDNA, after which the complementary RNA (cRNA) was synthesized by *in vitro* transcription (IVT), incorporating biotin-labeled nucleotide. Fragmented cDNA was hybridized on Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA) and stained by streptavidin phycoerythrin using Fluidics Station 450 (Affymetrix). The fluorescent intensity was assessed by scanning using Chip Scanner 3000 (Affymetrix). After scaning, signal values detection and scaling were performed using GeneChip Operating software (Affymetrix), and quantified data were analyzed by MAS5 normalization and visualized by Spotfire Decision Site for Functional Genomics (Spotfire Inc, MA, USA). Records were visualized in a dendrogram (a tree graph) based on the similarity between them. Clustering method, Ward's method; Similarity measure, Half square euclidean distance; Ordering function, Average value.

Microarray data analysis. A hierarchical clustering using the Ward's method was applied to clarify the six SaTM-1 sublines in gene expression patterns from the output data of the PRMI program. As the result produced two major clusters, next a pattern matching program was used to identify genes that discriminate the two major clusters. The p-values of the identified genes were evaluated by the difference in signal values between high and low IC_{50} score groups for CDDP. Of these, genes having $p<0.0001$ were selected, and, furthermore, with those of lower sensitivity genes for CDDP were selected. The final option was to obtain several genes with > signal values of 3000 from the high chemosensitivity group.

Tumorigenicity in immunodeficient mice. Subconfluent SaTM-1 cells were harvested with trypsin-EDTA and resuspended to a final concentration of $1x10^6$ and $1x10^7$ cells/ml in Hank's balanced salt solution (Invitrogen). Five female immunodeficient mice (5 weeks old, BALB/c-*nu*/*nu*) purchased from CLEA Japan were inoculated subcutaneously at the left and right flanks with $1x10^5$ and $1x10^6$ cells/ 0.1 ml using a 26-gauge needle attached to a 1 ml syringe, respectively. Each tumor size was measured with a caliper at 20 days post-inoculation, and tumor volumes were determined using the following formula: volume = 0.5 x (shorter diameter)^2 x longer diameter. All animal work was carried out at the animal facility of Saitama Medical University under approved animal protocols and in accordance with institutional guidelines.

Statistical analysis. Statistics were performed using one-way ANOVA for cell proliferation assay and susceptibility to anti-tumor drugs. Results are expressed as mean ± SE based on at least three independent experiments that were carried out in quadruplicate. A $p<0.05$ was considered to be statistically significant. A probability level of <0.0001 as a p-value of genes which have a difference in signal values between high and low IC_{50} score groups for CDDP was adopted throughout to determine statistical significance.

Results

Morphology of cells. Six sublines having different forms of growth were proliferated in multiple layers and observed for their adhesive and floating properties. Major populations

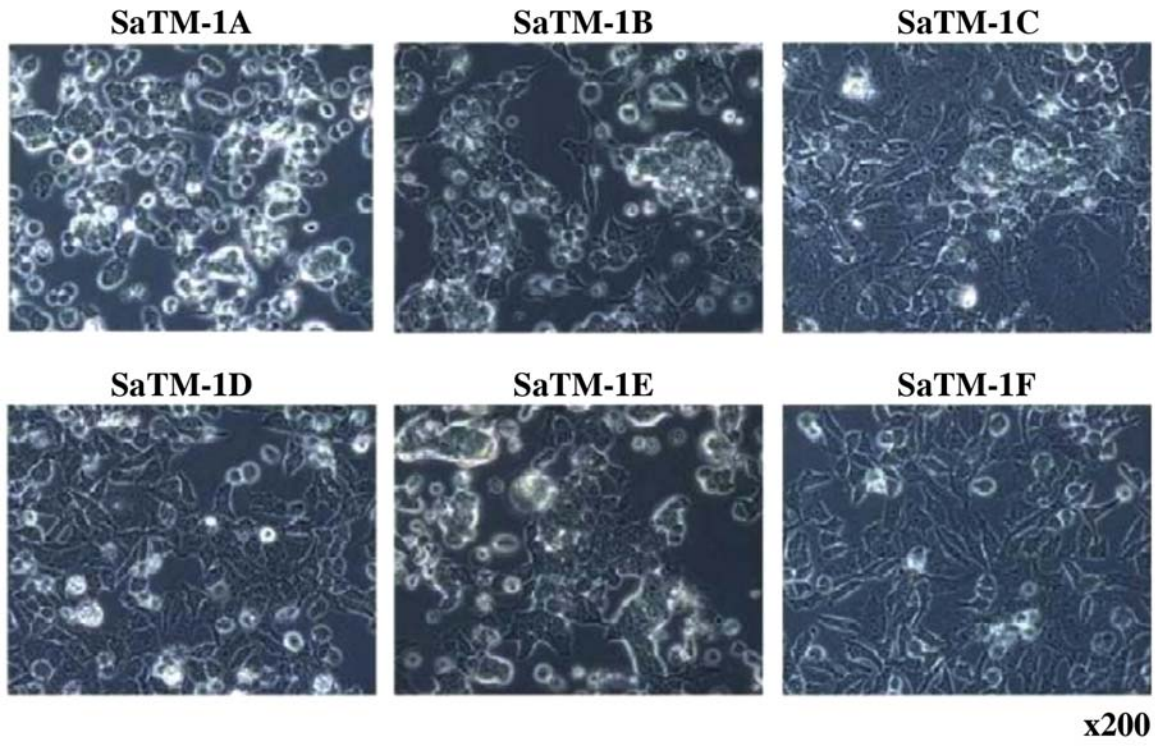

Figure 1. Six established sublines of SaTM-1 were cultured in 1:1 mixture of DMEM and Ham's F-12 with 10% heat-inactivated FBS in a 5% CO_2 atmosphere at 37˚C. For morphological analysis, $1.5x10^6$ cells were cultured in 75 cm^6 flask for 3 days and images were obtained using Axiovert 200M phase contrast microscope.

observed in SaTM-1A, B and E were floating cells with spherical shape, whereas flat-shaped adhesive cells were dominant in SaTM-1C, D and F (Fig. 1). The growth of the cell line was stable. To data, it has been passaged more than 50 times in the past 5 years. Cells that were kept frozen and then cultured showed the same morphologic characteristics and proliferation. Contamination by mycoplasma and bacteria was excluded by routine assays.

Cell proliferation assay. In order to determine characteristics of six sublines, cell proliferation assay was performed. The doubling times of SaTM-1A, B, C, D, E and F were estimated at 23.2, 24.7, 26.7, 24.6, 23.6 and 24.1 h, respectively. The average doubling time was 24.5 h. The results showed that all sublines grew at similar rates in conventional condition (data not shown).

Susceptibility to CDDP and 5-Fu. To assess susceptibility to anti-tumor drugs, six sublines were treated with CDDP and 5-Fu for 24 and 48 h, respecitively (Fig. 2A and B). Figures show the relative number of viable cells after drug-treatment. The cytotoxic effects of CDDP and 5-Fu were both dose-dependent and time-dependent. Interestingly, in contrast to observation of similar cytotoxic effects of 5-Fu against all sublines, CDDP showed different cytotoxic activity. The chemosensitivity for CDDP of SaTM-1A, B and E were two-fold lower than these of SaTM-1C, D and F.

Gene expression of growth factors and their receptors. RT-PCR showed expression of VEGF-A, VEGF-B, VEGFR-1, VEGFR-3 and EGFR in all sublines, whereas expression of VEGF-D and EGF were not observed in all lines. Interestingly, VEGF-C was expressed only in SaTM-1C and VEGFR-2 was not detected only in SaTM-1F (Fig. 3). GAPDH expression showed equal loading of all growth factors and their receptor expressions.

Detection of human papilloma viruses. RT-PCR analysis could not detect genomic DNAs of HPV-16 and HPV-18, most typical cancer-associate HPV types, in all six sublines, indicating that HPV-16 and HPV-18 may not be the causative agents of these lines (data not shown).

p63 and cytokeratin 5/6 expression. Cytokeratin 5/6 was expressed mainly in the cytoplasm and p63 expressed in the nucleus of the tumor cells. Immunohistochemical analysis of xenograft tumors used on immunodeficient mice showed that p63 was diffusely expressed in SaTM-1B, C and E, but focally expressed in SaTM-1A, D and F. Cytokeratin 5/6 expression varied from few cells (SaTM-1D, E and F) to almost diffusely stained cells (SaTM-1A and C). SaTM-1B demonstrated negative staining for cytokeratin 5/6 (Fig. 4). From these findings, our newly established SaTM-1 cell line has been demonstrated to have the characteristics of SCC.

Gene expression profile associated with susceptibility to cytotoxic effects of CDDP. The differences in gene expression among the six SaTM-1 sublines were analyzed according to susceptibility to cytotoxic effects of CDDP using Affimetrix GeneChip^R system. Normalized expression data are shown as a dendrogram (a tree graph) based on the similarities between them. Hierarchical clustering produced two major clusters

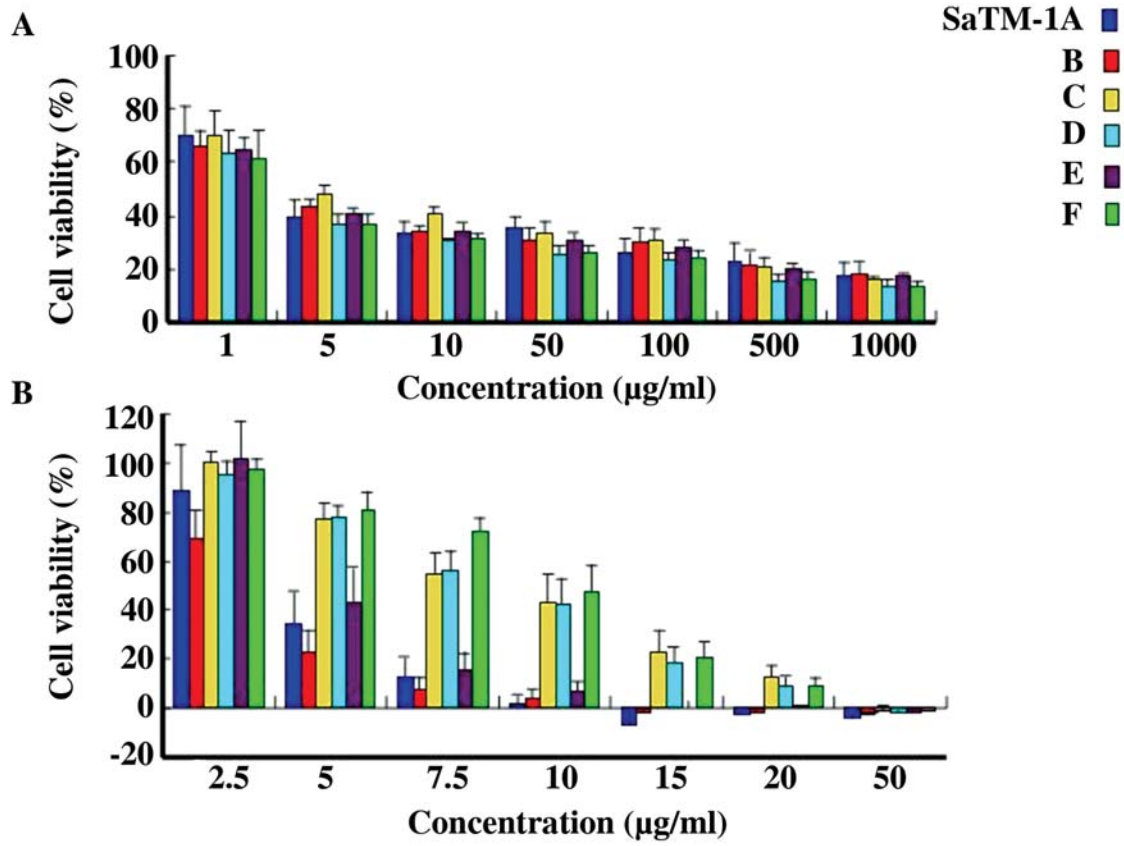

Figure 2. To investigate susceptibility to anti-tumor drugs, $3.5x10^3$ cells were cultured in a 96-well plate for 24 h followed by treated with several concentration of cisplatin (CDDP) (2.5, 5, 7.5, 10, 15, 20 and 50 μg/ml) and 5-fluorouracil (5-Fu) (1, 5, 10, 50, 100, 500 and 1,000 μg/ml) for another 24 and 48 h, respectively. Each experiment was performed in triplicate and repeated three times. Fig. 2A showed susceptibility to 5-Fu (48 h) and Fig. 2B to CDDP (24 h).

among SaTM-1 sublines. Cluster 1 consisted of low chemosensitivity for CDDP group (SaTM-1A, B and E). Cluster 2 was composed of high chemosensitivity group (SaTM-1C, D and F). Genes showing remarkably differential expressions between Clusters 1 and 2 were selected. The significant p-value was set at lesser than 0.0001 and defined 1463 genes below the p-value ($p<0.0001$). A total of 209 genes were selected which have greater two-fold average signal values between high and low IC_{50} score groups. Of the 209 genes, the selected 36 genes with >3000 of average signal values in high chemosensitivity group are shown in Table I. Each gene is denoted by the gene symbol, average signal values in SaTM-1A, B, E and SaTM-1C, D, F and the ratio between two groups. Some of the genes we identified here may be potentially associated with the chemotherapeutic targets for CDDP.

Tumorigenecity in immunodeficient mice. After 20 days, no tumor development was observed in the left flank that was injected with $1x10^5$ cells of each line. In contrast, injections of $1x10^6$ cells at the right flank produced tumors from all sublines. Tumor incidences for SaTM-1A, B, C, D, E and F cells were 40, 20, 80, 40, 40 and 80% at 20 days post-inoculation, respectively. Mean tumor volumes (mm^3) were as follows: 2245±2149 (SaTM-1A), 992 (B), 1050±450 (C), 297±100 (D), 1025±762 (E) and 485±562 (F). These results indicate that all sublines had tumorigenic property by subcutaneous injection in immunodeficent mice (Table II).

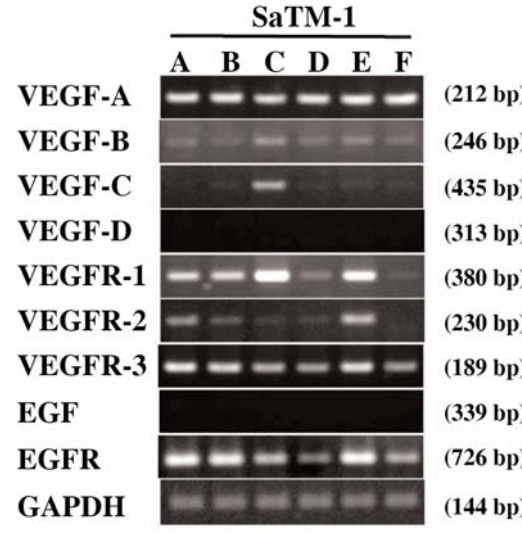

Figure 3. Gene expression of growth factors and their receptors were evaluated using the conventional RT-PCR methods. GAPDH expression showed the equal loading of all growth factors and their receptor expression.

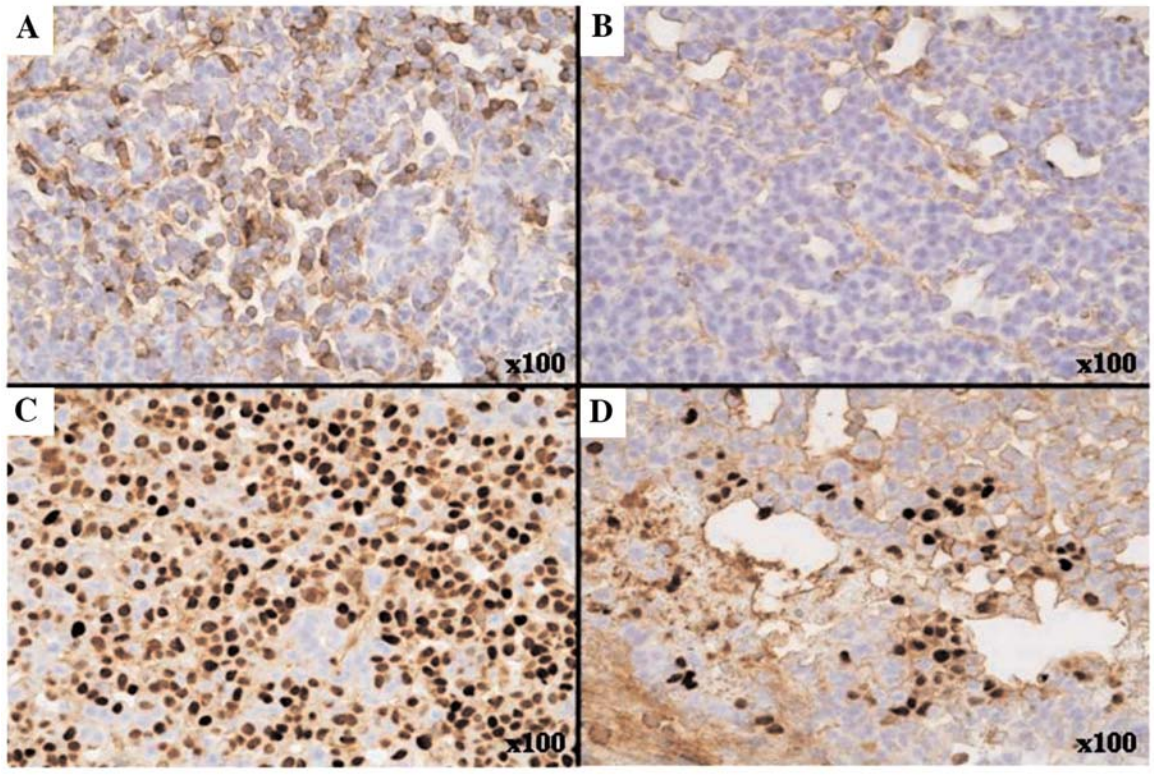

Figure 4. Immunohistochemical analysis of cytokeratin 5/6 and p63 using SaTM-1 xenograft tumors to immunodeficient mice. Cytokeratin 5/6 revealed almost diffuse expression in SaTM-1C (A) and only a few cells detected in SaTM-1E (B). The p63 was diffusely expressed in SaTM-1B (C) and focally in SaTM-1A (D). We showed the typical histological pictures (magnification, x100) for cytokeratin 5/6 and p63 expression in all SaTM-1 sublines.

Discussion

The development of tumor cell lines has been vital for studying the biological properties of various tumors. Here, we generated a stable cell line SaTM-1 and its six cloned sublines from human anal canal SCC. A large number of colorectal cancer cell lines are available for cancer research, though, no reports or collections are known of anal canal SCC by ATCC (American Type Culture Collection) or Riken Cell Bank (Japan). This is probably due to the difficulty of obtaining specimens because the incidence of this disease is uncommon. Indeed, our new cell line, SaTM-1 is thought to be the first cell line developed from human anal canal SCC. We have presented here details of the development and initial characterization by a variety of biological means. HPV-16 and -18 of most typical cancer-associated types of anal canal SCC were not detectable in all the sublines, which demonstrated the possibility of infection of other types of HPVs should not be excluded. The average cell doubling times of all sublines were similar at approximately 24.5 h, which does not demonstrate an extremely rapid growth compared to other SCC lines (5). In six sublines, the results of chemosensitivity assay against 5-Fu were similar in values, but CDDP showed different cytotoxic activity among the six lines, which fell into two groups (SaTM-1A, B, E vs. SaTM-1C, D, F). As shown in the susceptibility study to CDDP, two groups have clear differences of cytotoxic effects against CDDP (Fig. 2B). To identify genes that are associated with chemosensitivity to CDDP, gene expression profiles of the SaTM-1 six sublines were investigated by cDNA microarray. As shown in Table I, the number of genes identified by our selection method described previously was 36. Among them, 34 genes were already known genes and 2 genes were predictive genes demonstrating only coding sequences without gene symbol. Of the 36 genes, 9 genes (GJA1, KLF-1, EMP-1, DNM3, PAM, FAP, SOCS2, ETV1 and TMSB10) show some involvement in cancer. Four genes were already reported to play an important role in CDDP sensitivity in some types of cancers: GJA1 (6) and SOCS2 (7) for ovarian cancer, KLF6 (8) for colon cancer, TMSB10 (9) for breast cancer. The remaining genes showed no apparent relations to CDDP treatment. There have been numerous reports on analysis of gene expression profiles associated with drug resistance using clinical samples (10); however, our data were based on serial analysis of gene expression using sublines derived from the parental cell line we established, which would bring about significant results and reasonable explanations. Further studies using the genes we identified may lead us to confirm the clinically useful genes affecting CDDP resistance, possible to apply them to personalization of chemotherapy in the future. We investigated whether the six sublines also possess some characteristics of established carcinoma cell line in nature as evidenced by the expression of growth factors and their receptors, such as VEGF family genes and their tyrosine kinase receptors. As shown in Fig. 3, both VEGF-A and VEGF-B were expressed in all sublines, but VEGF-D and EGF were undetectable. VEGF-C was expressed in SaTM-1C alone. For receptor analysis, VEGFR-1, VEGFR-3 and EGRF were detected in all sublines and VEGFR-2 was expressed in all lines except

Table I. Selected genes associated with susceptibility to CDDP.

Gene title	Symbol	Ave. values		Ratio
		SaTM-1A,B,E	SaTM-1C,D,F	C,D,F/A,B,E
mRNA;cDNA DKFZp564E143 (from clone DKFZp564E143)	---	31.4	8160	259.6
Neuron navigator 3	NAV3	176.4	11310.2	64.12
Gap junction protein, α1	GJA1	1303.7	27035.1	20.74
Kruppel-like factor 6	KLF6	299	4383.4	14.66
Potassium inwardly-rectifying channel	KCNJ2	1856.2	14715.8	7.93
Germ cell associated 1	GSG1	594.8	4204.8	7.07
RAB31, member RAS oncogene family	RAB31	1467.6	7810.7	5.32
Family with sequence similarity 62, member B	FAM62B	1285.3	6702.8	5.22
Epithelial membrane protein1	EMP1	961.4	4895.5	5.09
Pleckstrin homology-like domain, family A, member 2	PHLDA2	945.9	4763.8	5.04
Dynamin 3	DNM3	804.7	3195.2	3.97
Peptidylglycine α-amidating monooxygenase	PAM	1698.5	6549.2	3.86
Fibroblast activation protein	FAP	862.2	3283.1	3.81
EF-hand domain family, member A2	EFHA2	2334.6	8623.9	3.69
Coiled-coil domain containing 46	CCDC46	4607.7	16322.8	3.54
Mdm4, transformed 3T3 cell double minute 1, p53 binding protein (mouse)	MDM1	2868	9963.4	3.47
Sorting nexin 9	SNX9	1193.3	3692.1	3.09
Roundabout, axon guidance receptor, homolog 2	ROBO2	1276.6	3799.9	2.98
Urotensin 2 domain containing	UTS2D	4899.3	12824.2	2.62
Solute carrier family 25, member 24	SLC25A24	1350.3	3476.5	2.57
Multimerin 1	MMRN1	3175.1	8084.5	2.55
G protein-coupled receptor 126	GPR126	3196.9	7808.4	2.44
Coiled-coil domain containing 50	CCDC50	1748.5	4251.9	2.43
Myosin regulatory light chain	MRCL3	2881	6680.6	2.32
Suppressor of cytokine signaling 2	SOCS2	2264.5	5226.5	2.31
Cytochrome b-245, α polypeptide	CYBA	1621	3730.7	2.3
FLB7348 PRO1953 mRNA	---	1966.9	4444.4	2.26
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	3141.8	7089.5	2.26
Amyloid ß (A4) precursor-like protein 2	APLP2	2163.5	4817.2	2.23
Ets variant gene 1	ETV1	1859.7	4125.5	2.22
Tubulin, ß6	TUBB6	3760	8274.1	2.2
CD9 molecule	CD9	3262.2	7165.8	2.2
Thymosin, ß10	TMSB10	16097.4	34737.3	2.16
Proteolipid protein 2 (colonic epithelium-enriched)	PLP2	3056.6	6588.9	2.16
Translocator protein (18 kDa)	TSPO	2240.4	4689.2	2.09
Serine incorporator 1	SERINC	2969.7	6093.2	2.05

Table II. *In vivo* tumorigenecity.

Cell line	Tumor incidence		Mean of tumor volume (mm^3)[a]
	$1x10^5$ cells	$1x10^6$ cells	
SaTM-1A	0/5	2/5	2245±2149[b]
SaTM-1B	0/5	1/5	992
SaTM-1C	0/5	4/5	1050±450
SaTM-1D	0/5	2/5	297±100
SaTM-1E	0/5	2/5	1025±762
SaTM-1F	0/5	4/5	485±562

[a]Tumor volume = (longer diameter) x (shorter diameter)^2 x0.5. [b]Mean ± S.D.

SaTM-1F. These results indicated that each line was genetically different, although all cloned sublines were established from the same parental cells. VEGF family is well known to be angiogenic and lymphangiogenic factors, and their receptors are generally expressed by endothelial cells. We additionally confirmed that SaTM-1 cells could not express endothelial markers, such as CD31, LYVE-1 using immunohistochemistry to omit the possibility of contamination of endothelial cells. Furthermore, SaTM-1 cells formed tumors in nude mice at subcutaneous injection of $1x10^6$ cells for each subline (Table II). Heterotransplantation of SaTM-1 cells into immunodeficent mice will provide a useful model for the study of human anal canal SCC whose incidence was extremely low. Microscopically, all SaTM-1 xenografts proved to be typical well-differentiated SCC with abundant keratinocytosis. p63 is a nuclear protein homologue of the tumor suppressor gene p53 that appears to be important in the development of epithelial tissues in many organs. p63 nuclear expression is seen most frequently in SCC (11,12). Cytokeratin 5/6 expression is also observed as diffuse cytoplasmic distribution with perinuclear enhancement in SCC, although sometimes observed in adenocarcinoma (13). p63 is an important marker for carcinomas with squamous differentiation, while it is negative for adenocarcinomas. Positive staining in nucleus for p63 was found in almost all sublines of SaTM-1 cells. We are convinced that SaTM-1 cell line itself retains the characteristics of SCC *in vitro*. We are going to deposit the parental cell lines at a public cell line bank to make them available to suitable requests by outside investigators. This report presents the SaTM-1 cell line derived from lymph node metastasis as the first established cell line developed from human anal canal SCC, which has also been cloned into six sublines with unique biological properties. This cell line may therefore serve as a useful tool to understand the molecular mechanism of tumor differentiation and proliferation as well as to investigate new chemotherapeutic targets in the treatment of anal canal SCC.

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