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CELL LINE

Establishment and characterization of human gingival squamous cell carcinoma cell line NOCS-1

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Abstract

The NOCS-1 cell line was established from the left gingiva tumor in an 86-year-old Japanese man. Histopathological diagnosis of the original tumor was well-differentiated squamous cell carcinoma. NOCS-1 cells were adhesive epithelial cells with neoplastic or pleomorphic features and grew without contact inhibition. It has been subcultured 70 times during the past 26 months. From passage 3, melanin-containing cells began to be observed in the NOCS-1 cell line. The plating efficiencies were 25% and 23%, doubling times were 29 and 26 h, and saturation densities were 6.9×10^4/cm^2 and 8.7×10^4/cm^2, at passage 12 and 30, respectively. When NOCS-1 cells were xenotransplanted subcutaneously into SCID mice, they produced tumors that histopathologically resembled the original tumor. In addition, NOCS-1-XG cells derived from the xenotransplanted tumor were similar to NOCS-1 cells. We believe that this cell line may be a valuable tool to develop immunotherapy and chemotherapy regimens.

Keywords Cell line · Gingival squamous cell carcinoma · G establishment · Xenograft · Anticancer drug susceptibility tests

Introduction

Squamous cell carcinoma (SCC) is the most common malignant neoplasm of the oral cavity. There are several cell lines derived from malignant tumors of the human oral cavity, such as MK-1 [1], NOS-1 [2], KOSC-2 [3], KOSK-3 [3], MSCC-1 [4], SCSC [5], H-1 [6], and Sa-3(Sa-3R) [7]. KOSK-2 and KOSK-3 cells were established through xenografted tumors in nude mice by geneticin treatment [8], which eliminated contaminating mouse fibroblasts [3]. The USC-HN1 [9] cell line was established from SCC of the upper alveolar ridge. Cell lines derived from human tumors are very useful for basic and clinical studies such as clarification of keratinization and development of chemotherapy or immunotherapy. The purpose of the present study was to establish and biologically characterize a human gingival SCC cell line.

Materials and methods

Medical history

An 86-year-old Japanese male visited the Oral and Maxillofacial Surgery Department of Niigata Hospital, Nippon Dental University (Niigata, Japan) for left side gena pain. The tumor size was 40×40 mm, oppressive pain was described. As the result of a biopsy, the diagnosis was well-differentiated SCC of the left gum. This study was approved by the Ethics Committee of the Nippon Dental University, School of Life Dentistry at Niigata, Japan (ECNG-H-120). Informed consent was obtained from participant included in the study.

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Histopathological examination

The original tumor and xenotransplanted tumor of NOCS-1 cells were fixed with a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 4 °C for about 7 days. After dehydration with graded ethanol, the tissues were immersed in xylene and then embedded in paraffin. Subsequently, 5 μm-thick sections were prepared for hematoxylin and eosin (HE) or immunohistochemical staining.

Establishment of cell lines

Small amounts of the tumor tissue extracted by surgery was rinsed several times with Hank's balanced salt solution and cut into small pieces with a razor blade. The fragments were dissociated in 0.1% trypsin (BD, MD, USA) and 0.02% EDTA/PBS (–) for 30 min at 37 °C. After vigorous pipetting, dissociated cells and small fragments were centrifuged at 430 × *g* for 5 min at room temperature. The cell pellet was resuspended with growth medium (GM) and cultured in a CO_2 incubator (4.7% CO_2 and 95.3% air) at 37 °C. The GM consisted of Dulbecco's modified Eagle's medium/F12 supplemented with 15% fetal bovine serum (Lot no. 12483), 100 μM glutaMAX, 0.1% minimum essential medium-non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml Fungizone. All materials used in GM were purchased from Life Technologies (Carlsbad, CA). The number of fibroblasts decreased gradually during culture, and epithelial cells were increased. The NOCS-1 cell line was established successfully. The GM was changed twice a week. At confluency, the culture was passaged at split ratios of 1:3–5. The cultured cells were observed under a phase contrast microscope. Aliquots of cultured cells were cryopreserved in GM containing 10% dimethyl sulfoxide (Sigma, St Louis, MO) and stored in a liquid nitrogen tank after every five passages. Cells derived from a subcutaneously xenotransplanted tumor of NOCS-1 cells in SCID mice were cultured similarly.

Population doubling time, saturation density, and plating efficiency

Cells at passages 12 and 30 were used to estimate the population doubling time and saturation density. A total of 2×10^4 cells/2 ml medium were seeded into each 35-mm dish and cultured for 12 days at 37 °C. After seeding, samples were obtained at 48-h intervals from three dishes, and cell counts were performed with a Turk type hemocytometer to construct growth curves for the cell line. Growth curves were drawn as the mean of three dishes. Population doubling time and saturation density were calculated from the growth curves. Plating efficiency is a number of cells that grow into colonies per 100 seeded cells, i.e., the proportion of cells that attach and grow to the number of cells originally plated expressed as a percentage.

Distribution of chromosomes

NOCS-1 cells (passage 20) were treated with 1×10^{-7} M colcemid (Grand Island Biological Co.) for 4 h at 37 °C, placed in a hypotonic solution (0.075 M KCl) for 20 min at 37 °C, and then fixed with a methanol–acetic acid (3:1) solution for 10 min at 0 °C. After fixation, the cells were stained with a 4% Giemsa solution. Cells were also treated with a 0.1% trypsin solution for 15 s at room temperature and then stained with 3% Giemsa (pH 6.8). Histograms of the chromosome number distribution were prepared based on more than 50 metaphases.

Electron microscopy

Cultured cells on 35-mm dishes were rinsed with Hank's balanced salt solution and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. The cells were rinsed with the same buffer and post-fixed with 1% osmium tetroxide. After dehydration with graded ethanol and propylene oxide, the cells were embedded in Epon. Ultrathin sections were prepared using an LKB Nova ultra-microtome, stained with uranyl acetate and lead citrate, and observed with a Hitachi HU-12A microscope.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from cultured cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). Using 1 μg of total RNA, cDNA was synthesized with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA), and amplification was performed using a PCR Supermix Platinum kit (Life Technologies) [10]. PCR amplification was performed using Veriti™ 96-well annealing for each gene, as shown in Table 1. PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide.

Transplantation

NOCS-1 cells (1×10^7 in 0.5 ml Hank's balanced salt solution/mouse) were transplanted subcutaneously into the dorsal neck skin of three SCID mice (male, 6 weeks of age; CLEA Japan Inc., Tokyo) using a 23-G needle [11]. At 5 weeks after transplantation, tumors of about 14 mm in size were observed in the three mice.

Table 1 Primers

Gene	Primer sequences, 5′ to 3′	Product size (bp)	Annealing temp. (℃)	GenBank accession number
GAPDH	S: GTCAAGGCTGAGAACGGGAA A: GCTTCACCACCTTCTTGATG	613	55	NM_001256799.1
CK17	S: GCCTGTTGTAATCGCTACGC A: GAAAAGGGGGATGTGAGCCA	741	55	Z19574.1
Ki67	S: AGTGCATCTCACGGTGTCTG A: AGCAAGTTGAACCCTCTCCG	606	55	X94762
p53	S: TACCAGGGCAGCTACGGTTT A: CCTTTCTTGCGGAGATTCTCT	572	55	X60011

Immunohistochemistry and immunocytochemistry

In each sample (original tumor tissue, NOCS-1 cell line, xenotransplanted tumor tissue of NOCS-1 cells in SCID mice, and cells derived from the xenotransplanted tumor), we performed immunohistochemistry and immunocytochemistry of CK17 (1:20, E3; Dako, Glostrup, Denmark), CK13 (1:400, DE-K13; Dako), Ki67 (1:100, ab15580; Abcam, Cambridge, UK), and p53 (1:100, PAb 240; Abcam).

For immunohistochemical staining of the original tumor and xenografts from SCID mice, samples were fixed in the 10% formalin neutral buffer solution. Paraffin-embedded sections were assessed using the Envision system (Dako). After blocking endogenous peroxidase by treating the sections for 10 min with Dako REAL Peroxidase-Blocking Solution, pretreatment with antigen retrieval solution (HistoVT one; Nacalai Tesque, Kyoto, Japan) was performed for 20 min at 95 °C. Sections were then incubated with primary antibodies against CK17, CK13, Ki67, and p53 overnight at 4 °C, followed by incubation with Dako Envision™+ Dual Link System-HRP for 30 min at room temperature. Color was developed with 3,3′-diaminobenzidine using an ImmPACTTM DAB Peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Sections were then observed under a light microscope (BZ-9000; KEYENCE, Osaka, Japan).

NOCS-1 cells or cells from xenografts were fixed with absolute methanol (Wako Pure Chemical Industries) for fluorescence immunocytochemistry at − 30 °C for 15 min and then incubated in Blocking One Histo (Nacalai Tesque) for 10 min at room temperature. Cultures were incubated with primary antibodies overnight at 4 °C and subsequently incubated with the following secondary antibodies for 30 min in the dark at room temperature: Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (both diluted at 1:1000; Life Technologies). Nuclei were stained with VECTASHIELD® HardSet™ Mounting Medium with DAPI (Vector Laboratories). Images were obtained under a confocal laser scanning microscope (LSM-510; Carl Zeiss, Jena, Germany).

Anticancer drug susceptibility tests of in vitro-cultured NOCS-1 and xenografted cells

Anticancer drug (CDDP, TXT, 5-FU, and a mixture of the three agents) susceptibility tests were carried out using Dox-21 (Hitachi-Aloka Medical Co., Tokyo, Japan). NOCS-1 and xenografted cell suspensions were centrifuged at 430 ×*g* for 5 min. Cell pellets were resuspended with 2 ml Hepes-GM containing each anticancer drug (final concentrations: 1 or 10 µg/ml). Cell-drug suspensions were then transferred to an oxygen electrode meter at 37 °C, and dissolved oxygen in the cell-drug medium was measured for 12 h (1×10^6 cells/tube) [12]. Analysis of susceptibility to anticancer drugs was carried out using the method of Uesu and Ishikawa [13].

Short tandem repeat analysis for authentication

To authenticate the established cell line, we analyzed short tandem repeats (STRs) of the NOCS-1 cells. The data were analyzed, and the STR profiles were compared with those recorded in public cell banks, such as the ATCC, DSMZ, RIKEN BRC Cell Bank, and JRCB, for reference matching.

Results

Histopathological diagnosis

Pathological diagnosis was well-differentiated SCC that formed a cancer pearl histopathologically, and cancer cells permeated with a restiform shape or an alveolar configuration even at a peculiar muscle layer. Tumor cells had clear nucleoli and showed neoplastic and pleomorphic features with mitosis appearing occasionally. An intermediate degree of lymphocytic infiltration was observed in interstitial tissue (Fig. 1a).

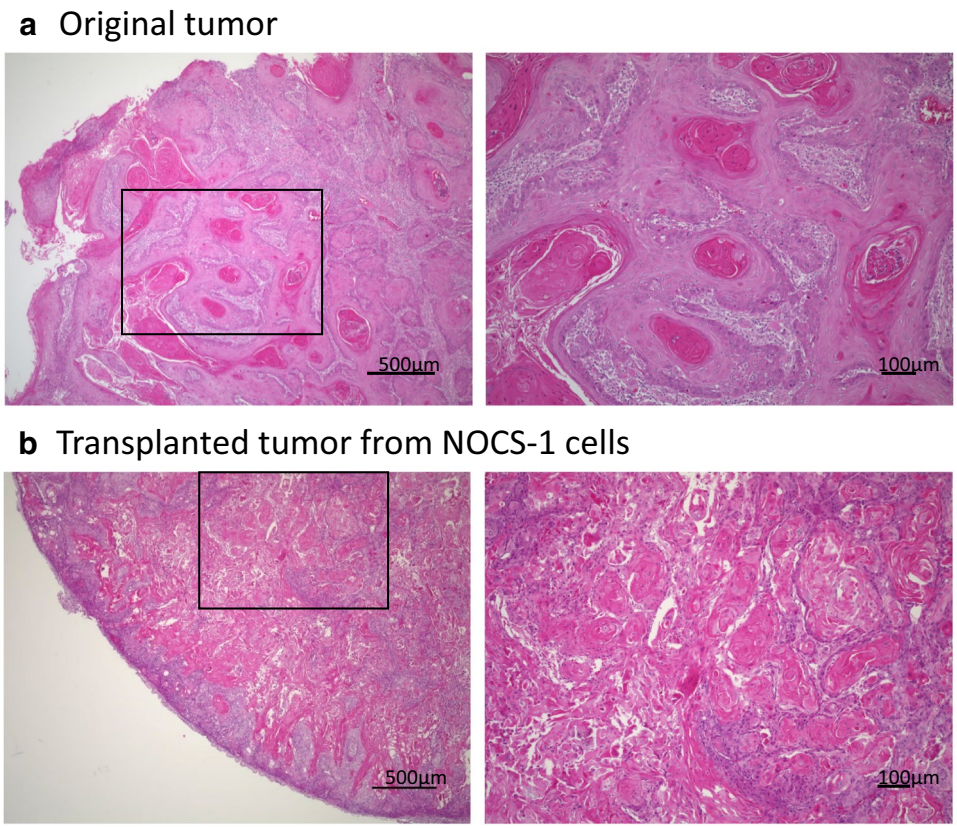

Fig. 1 HE staining. The original tumor was well-differentiated SCC (**a**). A xenotransplanted tumor of NOCS-1 cells was very similar to the original tumor (**b**)

A xenograft of about 2 cm in size was formed at 2 months after transplanting NOCS-1 cells subcutaneously into a SCID mouse. The pathological and histological types resembled those of the original tumor, and the xenograft was diagnosed as well-differentiated SCC (Fig. 1b). The original tumor and xenotransplanted tumor of NOCS-1 cells were very similar.

The xenotransplanted tumor of NOCS-1 cells was positive for CK-17, Ki67, p53, and human mitochondria, but negative for CK-13 in immunohistochemical staining (Fig. 2).

Morphological aspects of NOCS-1 cells

It was 3 months between initial culture and first passage in the culture of NOCS-1. NOCS-1 cells and the NOCS-1-XG cells derived from the xenotransplanted tumor of NOCS-1 cells were epithelial neoplastic and pleomorphic cells (Fig. 3a, b). Cell deformity was obvious, and the cell arrangement resembled a jigsaw puzzle. NOCS-1-XG cells were very similar to NOCS-1 cells.

In electron microscopic observation, the cells had a pseudomorphic nucleus and remarkable nucleoli. One of the characteristics of these cells was many microvilli on their surface. There were two kinds of intercellular attachment (Fig. 3c). One was cell attachment to each other by their microvilli, i.e., interdigitation (Fig. 3d). The other was attachment via desmosomes (Fig. 3e). Some cells had both thick cell projections and microvilli. There were two kinds of filaments. One was straight, which contacted desmosomes, while the other was waved, which existed only around the nucleus (Fig. 3c). Almost all cells had lysosomes, free ribosomes, and polysomes. Moreover, although small in number, they had secretory granule-like particles.

Biological characteristics of the NOCS-1 cell line

Population doubling time and saturation density of NOCS-1 cells at passages 12 and 30 were 29 and 26 h, and 6.9×10^4 and 8.7×10^4 cells/cm^2, respectively.

The chromosome numbers in NOCS-1 cells ranged from 31 to 110, and the modal number was 67 (Fig. 4). Plating efficiency was 25% and 23% at passages 12 and 30, respectively.

Identities of the NOCS-1 cell line and NOCS-1-XG cells

NOCS-1 cells were positive for Ki67 and p53, but CK17 and CK13 were stained weakly in immunocytochemistry

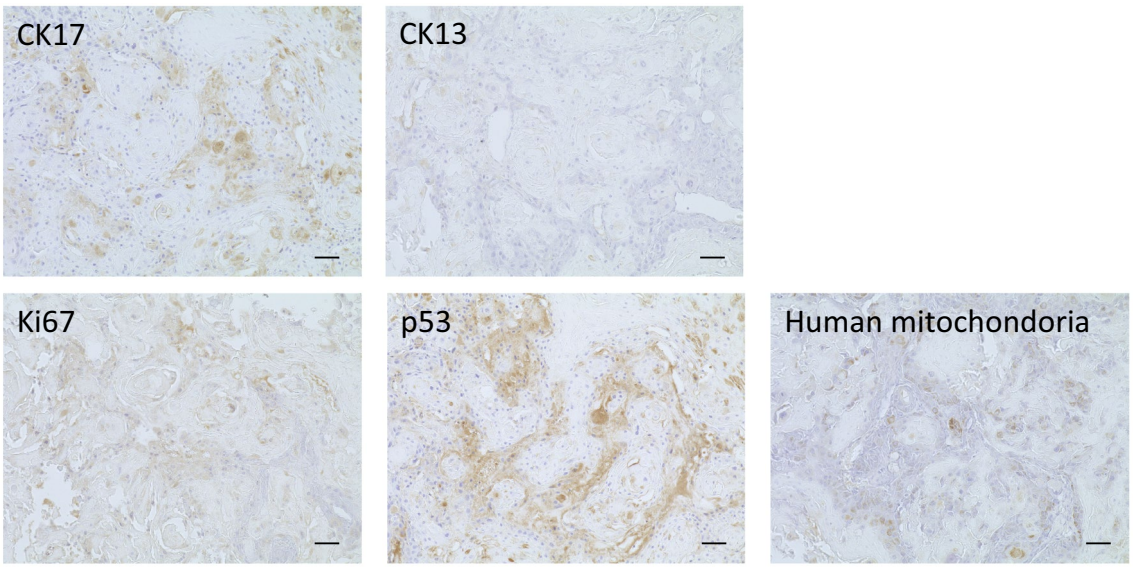

Fig. 2 Immunohistochemical staining. The xenotransplanted tumor of NOCS-1 cells was positive for CK17, Ki67, p53, and human mitochondria, but negative for CK13

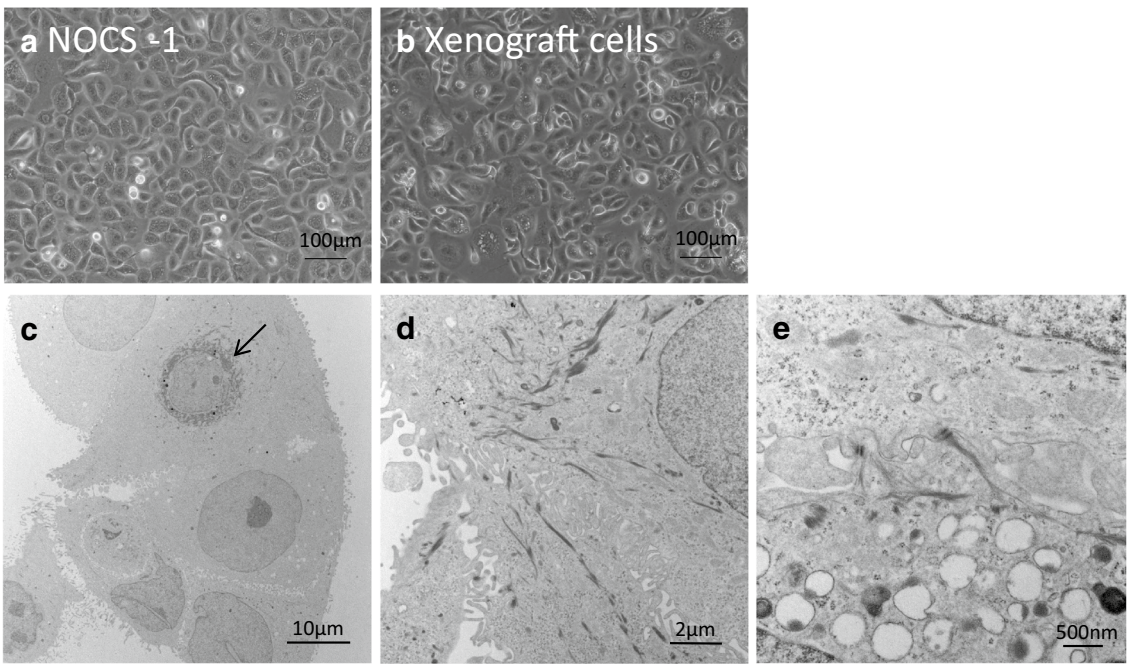

Fig. 3 Morphological aspects. NOCS-1 and xenografted cells (NOCS-1-XG) were epithelial cells with neoplastic and pleomorphic features in phase-contrast microscopy (**a, b**). In electron microscopic observation, there were two kinds of intercellular attachments. Thick waved filaments (arrow) were observed only around the nucleus (**c**). Cells attached to each other by microvilli, i.e., interdigitation (**d**), or desmosomes (**e**)

(Fig. 5a). NOCS-1-XG cells were positive for CK17, Ki67, and p53, but CK13 was stained weakly (Fig. 5b).

Expression of CK17, CK13, Ki67, and p53 genes was confirmed in both cells by RT–PCR analysis (Fig. 6).

Cross contamination test

It was confirmed that NOCS-1 cells did not correspond to any cells in the database of the JCRB Cell Bank (a

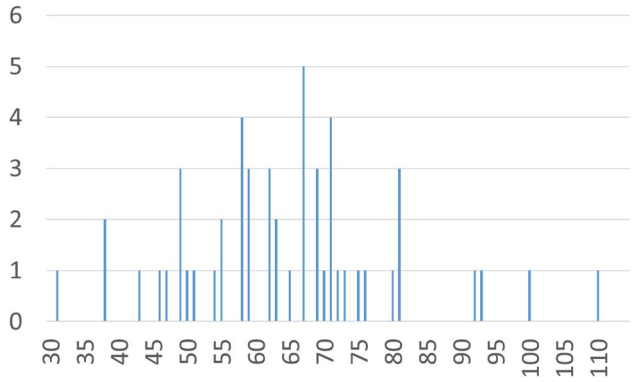

Fig. 4 Chromosome numbers. Chromosome numbers in NOCS-1 cells ranged from 31 to 110, and the modal number was 57–72

database of data from the ATCC, DSMZ, RIKEN BRC Cell Bank, and JCRB Cell Bank) (Table 2).

Anticancer drug susceptibility test of NOCS-1 cells

Susceptibility testing for anticancer drugs revealed that NOCS-1 cells were sensitive to a mixture of the three agents at 10 µg/ml each, but were not sensitive to 1 or 10 µg/ml TXT, 1 or 10 µg/ml CDDP, 1 or 10 µg/ml 5-FU, or the mixture of the agents at 1 µg/ml each (Fig. 7).

Discussion

To authenticate the NOCS-1 cell line, we examined cross-contamination with other cell lines by STR analysis. As a result, it was shown that NOCS-1 is new cell line.

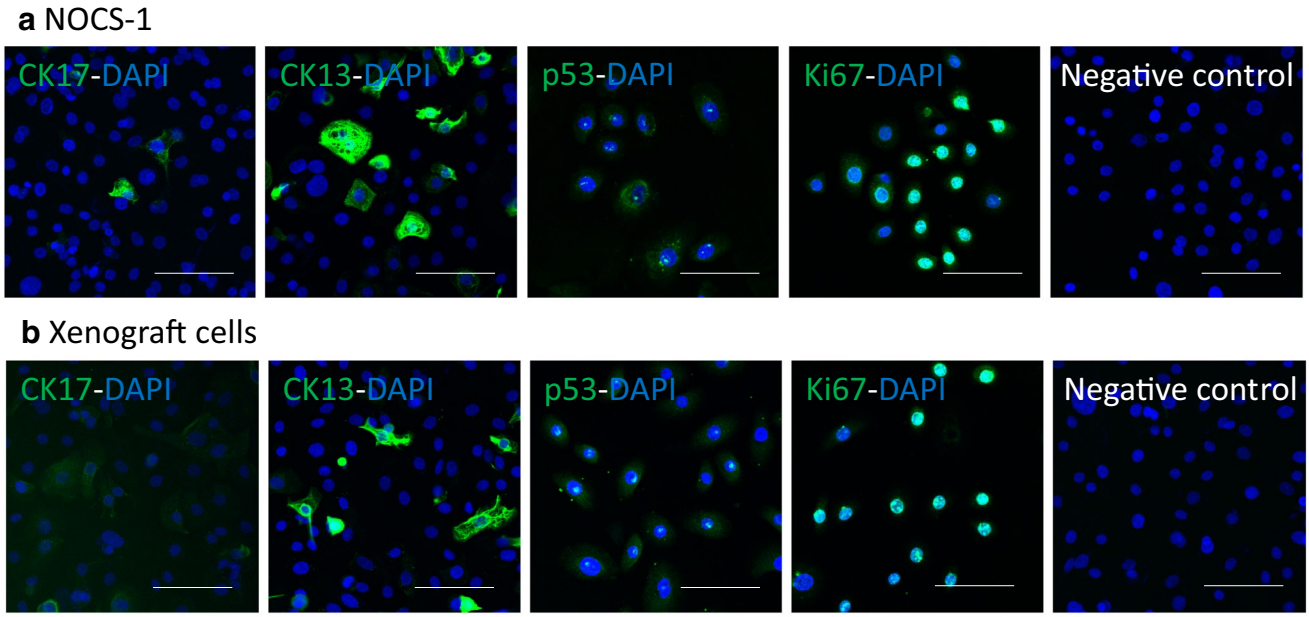

Fig. 5 Immunocytochemical staining. NOCS-1 cells were positive for Ki67 and p53, but CK17 and CK13 were stained weakly (**a**). Xenografted cells were positive for CK17, Ki67, and p53, but CK13 was stained weakly (**b**)

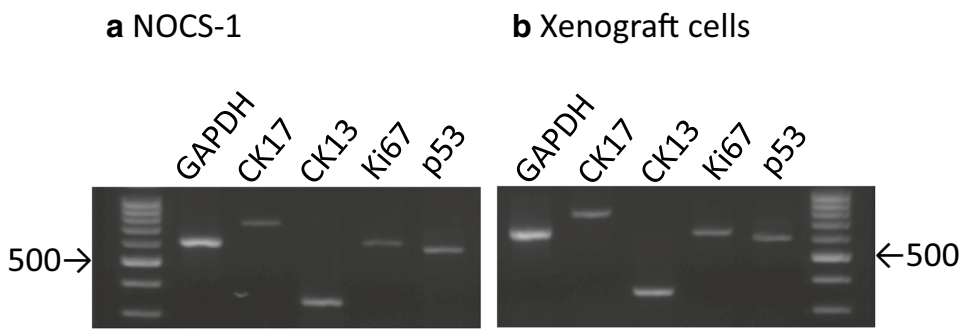

Fig. 6 RT-PCR analysis. Expression of CK17, CK13, Ki67, and p53 genes was confirmed in NOCS-1 and xenografted cells

Table 2 Results of STR analysis

Microsatellite (chromosome)	NOCS-1
D3S1358	15
TH01	9
D21S11	30
D18S51	14,27
Penta_E	14,15
D5S818	11
D13S317	11,13
D7S820	8,11
D16S539	11
CSF1PO	10
Penta_D	8,11
AM	X,Y
vWA	16,17
D8S1179	10,13
TPOX	8
FGA	22,26

Establishment of an oral cancer cell line from the primary site is particularly difficult, and few successful derivations have been reported [14]. One of the reasons for the difficulty is that bacterial and/or fungal contaminations occur frequently in the primary oral tumor. Most cell lines reported previously were established from lymph node metastatic foci or transplanted tumors in nude mice [15, 16]. There are currently not enough cell lines to allow research of gingival cancer. One of the major problems with successful culture is overgrowth of fibroblasts. Inagaki et al., established KOSC-2 and KOSK-3 cell lines [3] through xenografted tumors in nude mice. Then, they used geneticin for selective elimination of host fibroblasts during primary culture [3]. It is a very useful method to establish tumor cell lines, because mouse fibroblasts inhibit the growth of tumor fibroblasts. In xenografted tumor tissues, it is thought that host fibroblasts replace fibroblasts constituting the grafted tumor. By geneticin controlling the proliferation of host fibroblasts, establishment of a cell line becomes easy. The mechanism of selective elimination probably depends on the difference in chemosensitivities of fibroblasts and cancer cells. Fortunately, our tumor had good proliferation, and we established a cell line without xenografting.

Wavy thick filaments were observed only around the nucleus. These filaments are also observed in Nialym cells established from cancer of the tongue [17].

In SCC of the oral cavity, actions of both oncogenes and tumor suppressor genes have been identified during the disease course. It has been reported that *c-myc* overexpression appears to correlate with a poor prognosis [18], and that the tumor suppressor gene *p53* is commonly involved in the history of heavy smokers and drinkers with oral cancer [19].

When NOCS-1 cells were xenotransplanted into a SCID mouse, they generated the same histopathological tumor as the original tumor. Thus, the NOCS-1 cell line appears to have inherited the nature of the original tumor.

This cell cline appears to be very useful for research on the causation of cancer and development of innovative drugs or immunotherapies for gingival cancer.

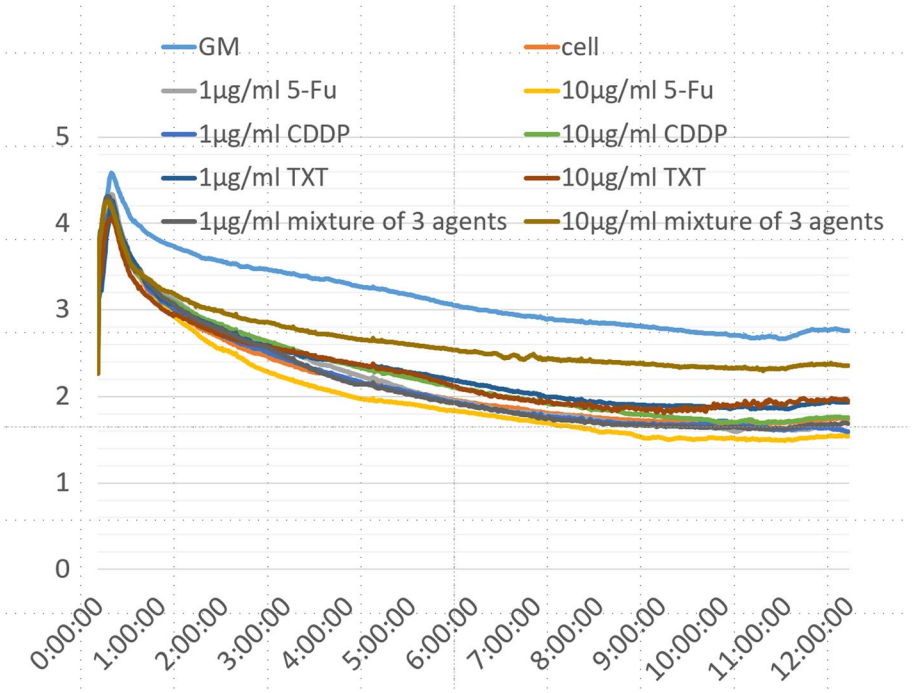

Fig. 7 Anticancer drug susceptibility test. NOCS-1 cells were sensitive to a mixture of the three agents at 10 μg/ml each, but not sensitive to 1 or 10 μg/ml TXT, 1 or 10 μg/ml CDDP, 1 or 10 μg/ml 5-FU, or a mixture of the three agents at 1 μg/ml each

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Compliance with ethical standards

Conflict of interest No competing financial interests exist. The authors declare that there are no conflicts of interest related to the publication of this study.

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