

Establishment of a Cell Line (CNUH-HNSCC-1) Derived from an Advanced Laryngeal Squamous Cell Carcinoma

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Cancer cell lines are the basic material for various lines of cancer research. Diverse cancer cell lines derived from tissues of various head and neck regions are needed for biological research on head and neck cancer. However, cell lines derived from cancer of the head and neck are not common. Recently, we established and characterized a novel human squamous carcinoma cell line, CNUH-HNSCC-1. From six cases of head and neck cancer, we established one specimen that was maintained for over 50 passages. We characterized the cell line as follows: growth patterns and curve, morphology by use of phase-contrast microscopy, and tumorigenicity by implanting the cell line into nude mice and making morphological comparisons. CNUH-HNSCC-1 cells grew well *in vitro* even after passage 50. However, the cells failed to form tumors in nude mice. CNUH-HNSCC-1 cells could be used as a control cell line for studying the biology of head and neck cancer.

Key Words: Squamous cell carcinoma; Cell line; Head and neck neoplasms

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INTRODUCTION

Cancer studies using tissues are limited in that tissues are composed of a mixture of various cells, and the quantity of tissue is frequently limited. However, by using cancer cell lines, one can obtain a robust supply of cancer cells, repetitively perform experiments, conduct biological studies with live cancer cells, and perform research that is impossible or difficult *in vivo*.

Cancer cell lines are now being used very efficiently in various cancer studies, such as studies exploring the development, invasion, and metastasis of cancer cells; the expression of oncogenes; the loss or mutation of tumor suppressor genes; the development of monoclonal antibodies; the anti-cancer effects of biological response modifiers; and the promoting or inhibiting factors in cancer cells. Therefore, establishment of cell lines from malignant neoplasms can provide basic research materials for all kinds of studies.

Development of cell lines from head and neck cancer is difficult. These types of cell lines were very rare until 1980, but there are some domestic reports on head and neck squamous carcinoma cell (HNSCC) lines.¹⁻⁴

This study was designed to establish and characterize a

new squamous carcinoma cell line (CNUH-HNSCC-1) by using samples from six patients with head and neck cancer in the Department of Otolaryngology-Head and Neck Surgery, Chonnam National University Hospital.

MATERIALS AND METHODS

1. Primary culture

This study was approved by the Chonnam National University Hospital Ethics Committee. Tissues for establishing the cell line were obtained during surgery from six patients with head and neck cancer admitted between January and June 2009 to the Department of Otolaryngology-Head and Neck Surgery, Chonnam National University Hospital.

The excised tumors were handled under aseptic conditions. Tissues were placed in DPBS with antibiotics, including gentamicin, penicillin, streptomycin, and Fungizone, and were transported to the laboratory.

The tumors were rinsed two to three times with DPBS. Next, the tumors were minced into 1- to 3-mm-sized pieces with scissors and clamps in a dish containing a solution of collagenase (type I or II, 1 mg/ml), trypsin (0.05×), DPBS,

and antibiotics. After being incubated for 2 hours in a water bath, cells were selected excepting lumps, transferred to another tube, and then diluted with enough RPMI 1,640 media with 10% of fetal bovine serum to inactivate the enzyme. After centrifugation at 1,000 rpm for 5 minutes, the cell pellet was recovered and resuspended in RPMI with fetal bovine serum.

Cells and media were filtered with a cell strainer 70 and 40. This produced a population of single cells. After plating in a cell culture dish at a density of 1 to 2×10^6 cells/ml, the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% oxygen.

The medium was changed every other day, and 4 to 7 days later the cells were passaged and transferred into three new dishes when the adherent cells reached 90% confluence. Passaging continued over 30 times, and an aliquot of the cells was preserved in liquid nitrogen. No growth factors of any kind were given to the cells.

Primary cultures of cancer cells mentioned above from six different HNSCC tissues were attempted; however, only one culture was successfully passaged over 50 times. The cells were recovered from a patient with supraglottic cancer T4aN2bM0 who was treated with bilateral neck dissection, total laryngectomy, and postoperative radiation therapy and who had been without any recurrence for 16 months. Characterization of the cell line was performed to establish this cell line as an HNSCC line (CNUH-HNSCC-1).

2. Growth characteristics

To measure the growth velocity of the tumor cells, 5×10^4 – 10^5 cells from the 50th passage were seeded in a 25-cm^2 flask. The velocity and pattern of cell growth were calcu-

lated by counting the cells every 12 hours, and the doubling time was determined.

3. Morphology

Cultured cells were examined with a phase-contrast microscope over time to characterize the cell morphologic features while a monolayer.

4. Tumorigenicity

A suspension of tumor cells (10^7 cells/0.1 ml) was transplanted into the floor of the mouth and the back of athymic nude mice to determine whether the cultured cells could

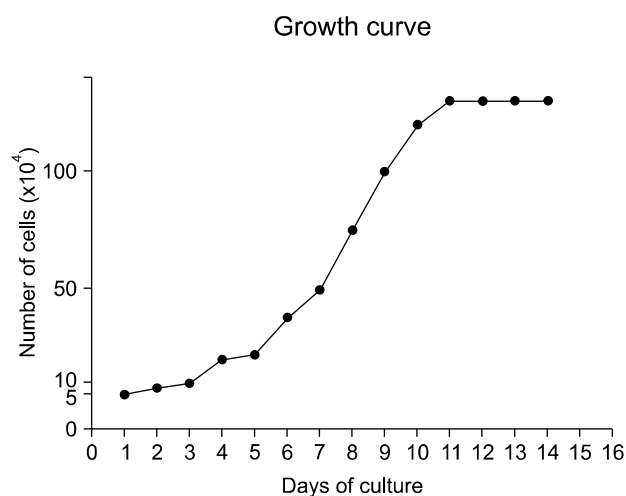


FIG. 1. Growth curve of the cultured CNUH-HNSCC-1 cells at passage 50. Each point represents the mean value of triplicate cultures.

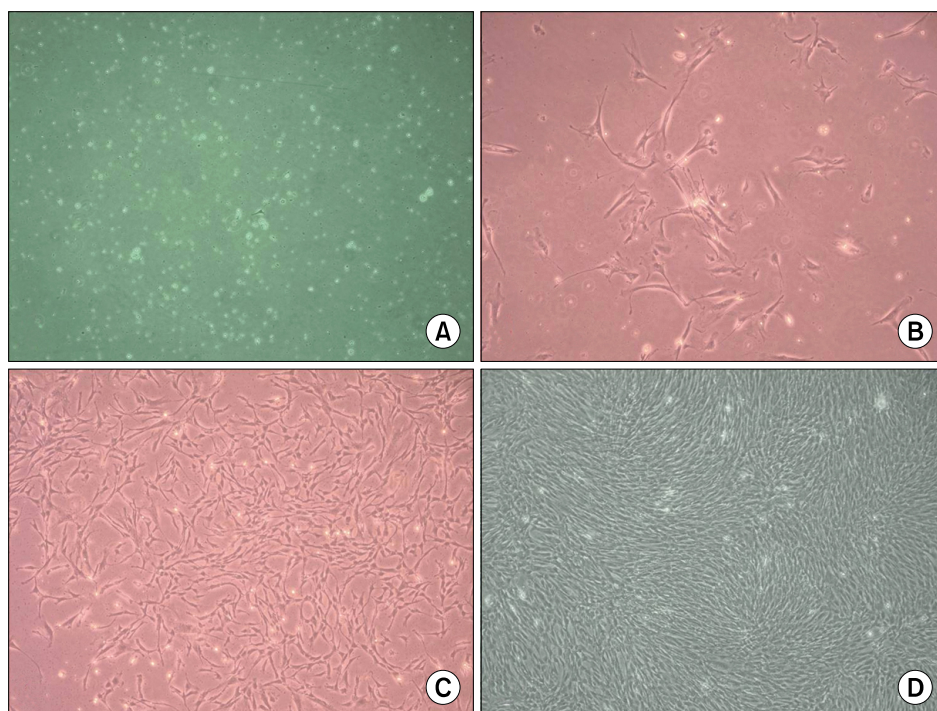


FIG. 2. Primary culture cells initially appeared as small and round single cells (A). Two days later, the cells formed branches (B), and were 60% confluent about 4 days later (C). About 7 days later, the cells were 100% confluent (D) (Magnification for all panels, $100\times$).

induce tumor mass formation and whether the primary tumor in the patient and secondary tumor in the animal model had similar pathologic and biological characteristics.

The experiment was designed as follows: tumor masses with an appropriate size were excised and divided into two groups. One group was fixed with formaldehyde, embedded in paraffin, and examined under a light microscope to see whether the tumors from the nude mice had a phenotype similar to the primary tumor from the patient (*i.e.*, parakeratinization, multiple keratin pearls, and intracellular keratin). The other group was minced and cultured as single cells to observe any differences between the cells from the nude mice and those from the patient.

RESULTS

1. Growth characteristics

The growth curve for cells passaged 50 times was follows: lag phase from day 0 to day 1, exponential phase from day 1 to day 9, and plateau phase after the 9th day; the doubling time was 48 hours (Fig. 1).

2. Morphology

The single cells after culturing from tumor tissue were small and round (Fig. 2A). Two days later, the round shape disappeared and the cells attached to the bottom of the dish started to form branches in many directions (Fig. 2B). Four days later, the cultures contained fusiform, spherical, or triangular cells and became 60% confluent (Fig. 2C). About 7 days later, the cultures were 100% confluent (Fig. 2D).

3. Tumorigenicity

Heterotransplantation of the cell line into nude mice was performed. Cells from the mid-exponential phase of the growth curve were obtained for tumor implantation. It took us several days to harvest enough cells for experiments. Cells in suspension (1×10^5 cells/ml) were injected into the

floor of the mouth of six 6-week-old male nude mice. However, tumors did not form after 3 weeks. Next, 1×10^7 cells/ml were injected into the floor of the mouth of another three 6-week-old male nude mice; again, no tumor formation was observed after 3 weeks. Using another three 6-week-old male nude mice obtained from a different animal company, 1×10^5 cells/ml failed to form any mass in the floor of the mouth after 3 weeks. After 1×10^7 cells/ml were injected into the back of another three 6-week-old male nude mice, a 5-mm tumor mass formed after 1 week (Fig. 3). However, this mass disappeared within 3 weeks. An injection of 2×10^6 cells/ml failed to form any mass in the floor of the mouth after 4 weeks. Three severe combined immunodeficiency (SCID) mice (males, 6 weeks old) were injected with a suspension of 1×10^9 cells/ml in the back; however, no tumor formation was observed after 6 weeks.

DISCUSSION

Cancer cell lines derived from upper aerodigestive tract tissues are important for various cancer studies. The biological characteristics of head and neck cancer are diverse; squamous carcinoma at the same stage can differ in terms of local invasion, neck metastasis, and metastasis to distant organs. Various cancer cell lines from various head and neck regions are therefore needed for biological research of head and neck cancer, and the development of new therapeutic approaches such as gene therapy requires the use of these cell lines for *in vitro* experiments.

Generally speaking, cell lines derived from head and neck cancer are not common owing to a number of difficulties. First, the tissue is infected and the infection rate can be higher in the time of culture. Second, there are typically overgrowths of fibroblasts rather than cancer cells. Third, normally developed keratinocytes may grow in these cell cultures rather than the cancer cells. Fourth, culture media can lack the growth factors necessary for the survival of the cell line, and the cancer cells may die or stop growing with repetitive passage.^{1,5-7} In total, the success rate for the establishment of HNSCC is around 30%; the success rate is lower than that for other types of malignant neoplasm, with variation from researcher to researcher.⁸⁻¹⁰

In this study, a primary cell culture was successfully produced from one out of six samples of tumor tissue taken from head and neck cancer patients. However, the cell line failed to form tumors in nude and SCID mice. The reason for this is unclear, and further study is needed to clarify this issue. There are some well-established head and neck cancer cell lines, such as PCI, SCC, FADU, and SNU-1041. One of these cell lines could be used as a control for determining *in vivo* tumorigenicity.

There are some possibilities for the lack of tumor formation. First, implantation of cancer cells into an immunodeficient mouse does not guarantee a 100% success rate of tumor formation. This is a result of tumor biology. Baker¹¹ heterotransplanted 21 human SCC lines into 112 mice. In that study, 95 of the mice developed viable persistent sub-

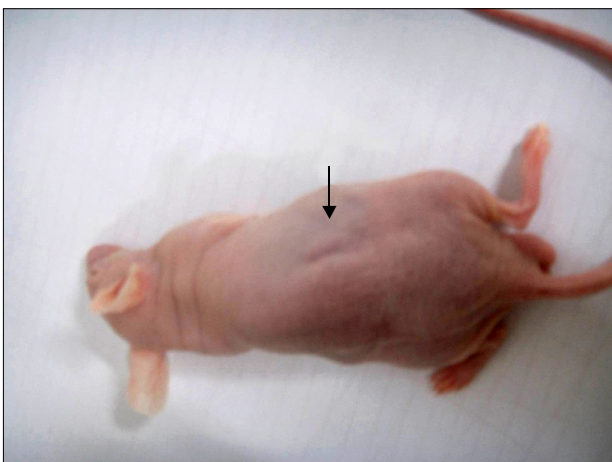


FIG. 3. A small round nodule (arrow) about 5×3 mm developed in the back of the nude mice 1 week after the injection with CNUH-HNSCC-1 cells.

cutaneous nodules and 56% of the mice developed progressively enlarging tumors. However, tumors did not grow in 14 (12.5%) of the mice, 5 (24%) of the 21 SCC cell lines regressed until no discernable tumor could be observed, and 7 SCC lines (33%) formed stable non-enlarging subcutaneous tumors. These findings were also observed in non-SCC lines for which the regression rate was greater; 13 of the 44 (29.5%) inoculated mice developed tumors that regressed. Interestingly, the cell lines derived from primary cancer and biopsy specimens were found to not grow in nude mice. However, the cell lines derived from metastatic or residual tumors that were later resected demonstrated progressive growth. This finding suggests that the tumor biology may have changed sufficiently over the intervening intervals to alter the physiologic properties of the second group of SCC lines. This emphasizes the continual changes in tumor physiology as tumors persist and progress in the human host.

Second, it is possible that a longer period might have been needed for tumor formation in our study. In a study by Baker,¹¹ the lag phase between inoculation and the beginning of tumor growth varied considerably among cell lines, ranging from 7 to 188 days. Baker also reported that SCC lines varied widely in the rate of growth and the size of the tumor produced in nude mice.¹¹

Third, it is possible that SCC lines vary in their ability to induce a stromal proliferative response in the host to the extent that some fail to infiltrate the dermal connective tissue.¹² For example, Rheinwald and Beckett¹² reported that tumorigenic keratinocyte lines cultured from human squamous cell carcinomas require anchorage and fibroblast support.

Fourth, tumors may not be formed due to a host immune response. Heo et al¹³ reported that successful tumor growth was obtained by injecting cells into nude mice except for PCI-13 cells, which were injected into nude mice that were not pretreated with cyclophosphamide (an immunosuppressant).

There are several specific markers for HNSCC. The epidermal growth factor receptor (EGFR) has emerged as one of the most promising therapeutic targets in the treatment of solid malignancies, including HNSCC.^{14,15} The squamous cell carcinoma antigen (SCCA) is a useful tumor marker for predicting disease-free and overall survival in HNSCC.^{16,17} Variant isoforms of CD44 are expressed in HNSCC.^{18,19} It was recently reported that KITENIN is highly expressed in tumors and metastatic lymph nodes compared with adjacent mucosa and non-metastatic lymph nodes in head and neck cancer,²⁰ that it is highly expressed in advanced laryngeal cancer compared with early laryngeal cancer,²¹ and that it represents a more aggressive phenotype in an HNSCC model.²² It is possible that we could characterize the CNUH-HNSCC-1 cell line by use of these markers and apply them to *in vivo* experiments.

In summary, we attempted to establish and characterize a new HNSCC line (CNUH-HNSCC-1). Primary cell cultures were derived from supraglottic SCC that were able to un-

dergo repetitive passage. However, the tumor formation in an animal model failed, and the use of this cell line may therefore be limited. Further attempts to establish a stronger HNSCC line are needed to obtain cells with more tumorigenicity potential through studies with longer periods of tumor growth observation and in animal models pretreated with immunosuppressants.

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REFERENCES

1. Kim KH, Chung PS, Park HM, Rhee CS, Park JG. Establishment and characterization of cell lines derived from squamous cell carcinoma of the head and neck. *Korean J Otolaryngol* 1996;12: 181-7.
2. Jun HK, Kim SY, Gong GY. Histologic similarity between the primary tumor and xenograft of AMC-HN cell lines in nude mice. *Korean J Otolaryngol-Head Neck Surg* 1997;40:1546-53.
3. Kim SY, Chu KC, Lee HR, Lee KS, Carey TE. Establishment and characterization of nine new head and neck cancer cell lines. *Acta Otolaryngol* 1997;117:775-84.
4. Roh HJ, Goh EK, Wang SG, Chon KM, Kim YS, Han JY. Establishment and characterization of a novel cell line (PNUH-12) derived from a human squamous cell carcinoma of the hypopharynx. *Korean J Otolaryngol-Head Neck Surg* 1999;42:72-81.
5. Krause CJ, Carey TE, Ott RW, Hurbis C, McClatchey KD, Regezi JA. Human squamous cell carcinoma. Establishment and characterization of new permanent cell lines. *Arch Otolaryngol* 1981; 107:703-10.
6. Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science* 1955;122:501-14.
7. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, et al. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* 1973;51:1417-23.
8. Easty DM, Easty GC, Carter RL, Monaghan P, Butler LJ. Ten human carcinoma cell lines derived from squamous carcinomas of the head and neck. *Br J Cancer* 1981;43:772-85.
9. Rupniak HT, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B, et al. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *J Natl Cancer Inst* 1985;75:621-35.
10. Carey TE. Head and neck tumor lines. In: Hay R, Gazdar A, Park JG, eds. *Atlas of Human Tumor Cell Lines*. Orlando, FL: Academic Press, 1994;79-120.
11. Baker SR. An *in vivo* model for squamous cell carcinoma of the head and neck. *Laryngoscope* 1985;95:43-56.
12. Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. *Cancer Res* 1981;41:1657-63.
13. Heo DS, Snyderman C, Gollin SM, Pan S, Walker E, Deka R, et al. Biology, cytogenetics, and sensitivity to immunological effec-

- tor cells of new head and neck squamous cell carcinoma lines. *Cancer Res* 1989;49:5167-75.
14. Ang KK, Berkey BA, Tu X, Zhang HZ, Katz R, Hammond EH, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 2002;62:7350-6.
 15. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res* 1993;53:3579-84.
 16. Lara PC, Cuyás JM. The role of squamous cell carcinoma antigen in the management of laryngeal and hypopharyngeal cancer. *Cancer* 1995;76:758-64.
 17. Snyderman CH, D'Amico F, Wagner R, Eibling DE. A reappraisal of the squamous cell carcinoma antigen as a tumor marker in head and neck cancer. *Arch Otolaryngol Head Neck Surg* 1995;121:1294-7.
 18. Sandström K, Nestor M, Ekberg T, Engström M, Anniko M, Lundqvist H. Targeting CD44v6 expressed in head and neck squamous cell carcinoma: preclinical characterization of an 111In-labeled monoclonal antibody. *Tumour Biol* 2008;29:137-44.
 19. Reategui EP, de Mayolo AA, Das PM, Astor FC, Singal R, Hamilton KL, et al. Characterization of CD44v3-containing isoforms in head and neck cancer. *Cancer Biol Ther* 2006;5:1163-8.
 20. Lee JK, Bae JA, Sun EG, Kim HD, Yoon TM, Kim K, et al. KITENIN increases invasion and migration of mouse squamous cancer cells and promotes pulmonary metastasis in a mouse squamous tumor model. *FEBS Lett* 2009;583:711-7.
 21. Lee JK, Yoon TM, Seo DJ, Sun EG, Bae JA, Lim SC, et al. KAI1 COOH-terminal interacting tetraspanin (KITENIN) expression in early and advanced laryngeal cancer. *Laryngoscope* 2010;120:953-8.
 22. Lee JK, Lim SC, Kim HD, Yoon TM, Kim K, Nam JH, et al. KITENIN represents a more aggressive phenotype in a murine model of oral cavity squamous carcinoma. *Otolaryngol Head Neck Surg* 2010;142:747-52.