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Newly Established Uterine Cervical Carcinoma Cell Line with Co-amplification of Human Papillomavirus DNA and c-*myc* Gene

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A new human tumor cell line, NCC-c-CX-1 (CX-1), was established from a uterine cervical cancer xenografted in nude mice. This cell line harbored approximately 50 to 100 copies of human papillomavirus (HPV) type 18 DNA per haploid genome, and contained about 16-fold-amplified c-*myc* gene with rearrangement. These genomic alterations found in CX-1 cells were also present in both primary tumor and xenografted tumor. Histopathologically, original and xenografted tumors were poorly differentiated cancer and were characterized by neuroendocrine features such as positive neuron-specific enolase and chromogranin A by immunohistochemistry and abundant neurosecretory-type granules in the cytoplasm by electron microscopy. However, the established cell line had lost the neuroendocrine features. This cervical cancer cell line may be a useful model for studying cervical carcinogenesis, especially the interaction between HPV and c-*myc* oncogene.

Key words: Cervical cancer cell line — c-*myc* oncogene — Human papillomavirus — Neuroendocrine carcinoma

It has been suggested that human papillomavirus (HPV) infection plays an important role in the development of human uterine cervical cancers. However, HPV infection alone does not always evoke malignant change in the cervical epithelium. In cervical carcinogenesis, additional factors such as alteration of various oncogenes, herpes simplex virus infection, etc. are suspected to follow the event of HPV infection.[1)] The alteration of oncogenes including c-*myc* gene has been found so far in many human cervical cancer tissues.[2,3)] The *cis*-activation of cellular oncogenes including c-*myc* gene by HPV DNA integration nearby has been speculated to cause malignant transformation of cervical epithelium.[4)] Another report also suggested a possible correlation between the enhanced expression of the c-*myc* gene and the presence of HPV genomes in cervical cancers.[5)]

This paper describes a newly established human cervical cancer cell line in which both amplified HPV type-18 and c-*myc* genes are present. This cell line has a unique biological characteristics and may be useful for further studies of interactions between HPV and c-*myc* genome in cervical cancer.

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[5] Abbreviations: CX-1, cervical cancer cell line (NCC-c-CX-1); HPV, human papillomavirus.

MATERIALS AND METHODS

Original tumor, xenografted tumor and cultured cell line Small pieces of the original cervical cancer (Stage Ib), which had been surgically resected from a 30-year-old Japanese patient, were aseptically transplanted subcutaneously into BALB/c athymic nude mice. The xenografted tumor, grown to about 10 mm in diameter, was cut into pieces and suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum and 10^5 units of penicillin G per liter. The culture cells were incubated at 37°C under a humidified atmosphere of 5% CO_2 in air. Cultured cells were passaged every week by pipetting and, at one year after the initial cultivation, tumor cells were cloned by the limiting dilution method.

Morphological study of the tumor For light microscopy, the primary tumor, xenografted tumor and pelletted CX-1 cells were fixed in 15% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and immunohistochemically for keratin, vimentin, neuron-specific enolase (NSE), chromogranin A, serotonin, calcitonin, insulin and gastrin-releasing peptide (GRP) using the avidin-biotin peroxidase complex (ABC) method. For electron microscopy, the xenografted tumor and CX-1 cells were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, and then embedded in Epon 812. Ultra-thin sections were doubly

stained with uranyl acetate and lead citrate and observed under a Hitachi H-600 electron microscope.

Cell kinetic analysis The growth curve of CX-1 cells was constructed by seeding a single cell suspension (1.7×10^5 cells per dish) into 35 mm plastic culture dishes with 2 ml of growth medium. Cells in triplicate dishes were detached by pipetting at 12 h intervals and viable cells devoid of trypan blue dye were counted by a hemocytometer.

Southern and Northern blot analysis Genomic DNAs were extracted from the primary tumor, the xenografted tumor and CX-1 cells using the phenol/chloroform method. For Southern blot analysis, approximately 10 μg of DNA was digested with an appropriate restriction endonuclease, fractionated by 1% agarose gel electrophoresis and transferred onto nitrocellulose filters. The probes used were a 0.4 kbp *PstI/PstI* fragment of c-*myc* second exon, a 1.5 kbp *ClaI/Eco*RI fragment of c-*myc* third exon and 3′ flanking cellular sequences, a 7.9 kbp whole genome of HPV type-16, and a 7.9 kbp whole genome of HPV type-18, which were labeled with [α-^{32}P]-dCTP (Amersham, Buckinghamshire, England) by nick translation. The hybridization was performed at 42°C for 16 h in a mixture of 50% formamide, 0.65 *M* sodium chloride, 0.005 *M* EDTA, 0.1 *M* PIPES, 0.1% sodium dodecyl sulfate, 10% dextran sulfate, 5×Denhardt's solution, and 200 μg of sonicated salmon sperm DNA per ml. The filter was washed at 52°C with a solution containing 0.3 *M* sodium chloride, 0.03 *M* sodium citrate, 0.02 *M* sodium phosphate (pH 7.0), 0.06% sodium pyrophosphate and 0.05% sodium dodecyl sulfate. Then, the filter was exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 12 to 24 h.

For Northern blot analysis, total RNA of CX-1 was prepared by guanidium thiocyanate/cesium chloride gradient centrifugation. Approximately 10 μg of total RNA was denatured, electrophoresed on 1% agarose/formaldehyde gel and transferred to nitrocellulose filters. Probes used were a 1.5 kbp *ClaI/Eco*RI fragment of c-*myc* third exon and 3′ flanking cellular sequences, and a 2.4 kbp *Bam*HI/*Eco*RI fragment of HPV type-18 early region including open reading frames E6, E7 and E1, which were labeled with isotope as described above. Then, the filter was hybridized with a probe and washed as described above for Southern blot analysis.

RESULTS

Morphological and biological characterization The xenografted tumor was established from the primary cervical cancer, which had been passaged eight times in athymic nude mice during 10 months. The xenografted tumor grew as a solid tumor with a histological appearance similar to that of the primary tumor, displaying many mitotic figures (Fig. 1). Both primary and xenografted tumors were positively stained for NSE and chromogranin A (Fig. 2), but lacked immunoreactivity to hormones investigated in this study. Electron microscopically both contained many neurosecretory-type granules of 200 to 400 nm in diameter (Fig. 3).

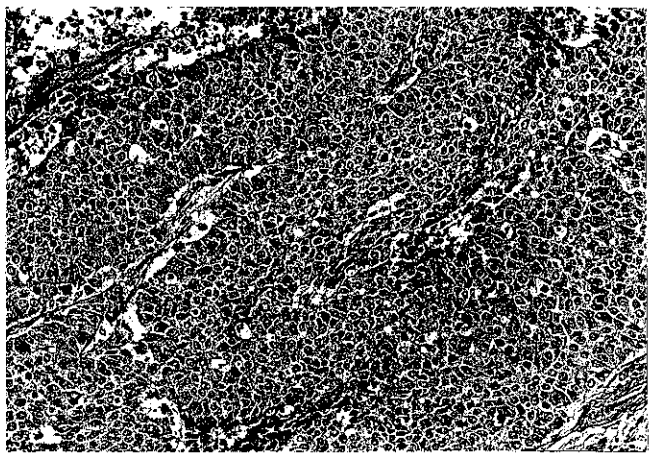

Fig. 1. Light microscopic appearance of primary tumor. Poorly differentiated tumor cells grow around small blood vessels in solid fashion. Hematoxylin and eosin stain. ×175.

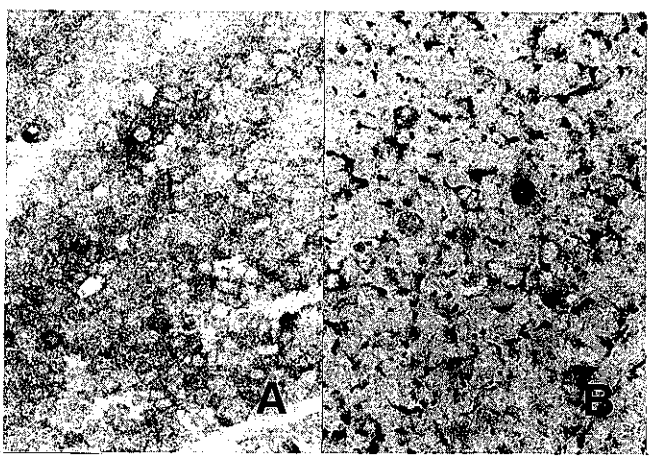

Fig. 2. Immunohistochemistry of xenografted tumor. Tumor cells are positive for neuron-specific enolase (A) and chromogranin A (B). Counterstained lightly with hematoxylin. ×350.

The CX-1 cells grew loosely adherent to the surface of the plastic culture dishes or piling up in clusters (Fig. 4). The doubling time was estimated to be 21.8 h (Fig. 5). Immunohistochemically, they were positive for keratin and vimentin, but negative for NSE and chromogranin A, in contrast with the primary and xenografted tumors.

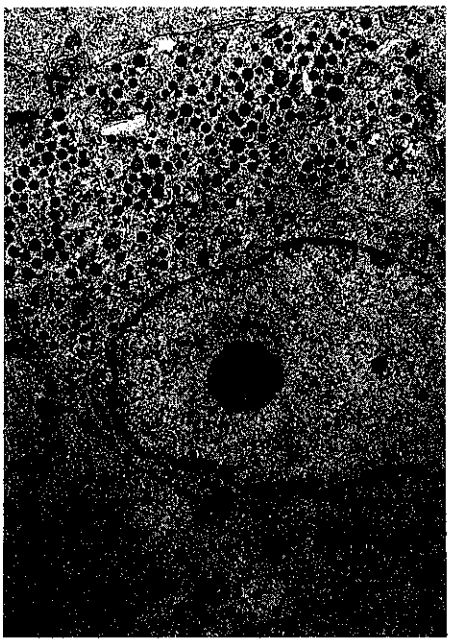

Fig. 3. Electron micrograph of xenografted tumor cell. The majority of tumor cells contain many neurosecretory-type granules in the cytoplasm. ×7250.

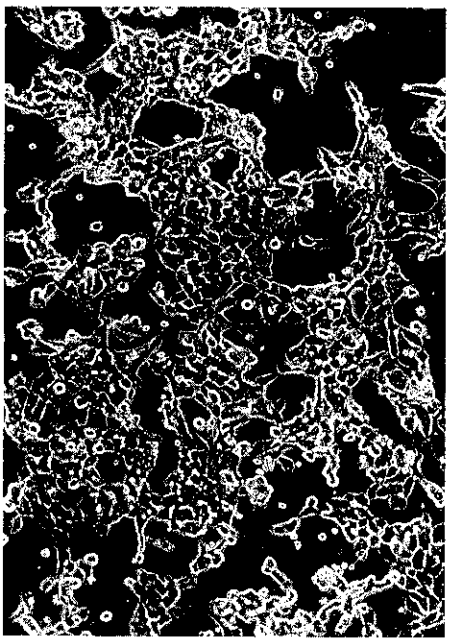

Fig. 4. Phase-contrast micrograph of CX-1 cells. Cultured tumor cells grow loosely adherent to the surface of the plastic culture dish or piling up in clusters. ×70.

Furthermore, CX-1 cells were devoid of neurosecretory-type granules electron microscopically (data not shown).

Alterations of c-*myc* gene By Southern blot hybridization using the second exon probe of the c-*myc* gene, the germ-line 12.5 kbp *Eco*RI fragments were amplified about 10-fold in the original and xenografted tumors and CX-1 cells as compared with the fragments of HeLa and NB-1 cells. However, HL-60 cells exceeded CX-1 cells in the level of c-*myc* gene amplification. Furthermore, a third exon probe detected rearranged fragments in addition to the amplified germ-line fragments (Fig. 6). Rearranged fragments were eatimated to be 1.5 kbp in size and amplified more than the germ-line fragments. Not only were these alterations of c-*myc* gene present in the primary tumor, but also they were retained in the xenografted tumor and CX-1 cells. Additionally, DNAs of CX-1 cells digested with other restriction endonucleases were hybridized with a third exon probe. Both amplified germ-line fragments and rearranged fragments of c-*myc* gene were detected in all DNAs of CX-1 cells digested with restriction endonucleases (data not shown).

No amplification of other protooncogenes (N- & L-*myc*, *myb* and H-*ras*) was observed in the original tumor of CX-1 cells.

Detection of HPV type-18 DNA sequence The primary tumor, xenografted tumor and CX-1 cells were examined for the presence of HPV DNA sequences using HPV type-16 and -18 DNA sequences as probes. They contained DNA sequences which hybridized intensely with an HPV type-18 probe (Fig. 7), but did not hybridize with an HPV type-16 probe (data not shown). Their hybridized bands showed similar patterns and intensities, and they were estimated to contain 50 to 100 copies of HPV type-18 DNA sequences per haploid genome, in contrast with HeLa cells.

Expression of c-*myc* gene and HPV type-18 genomes In order to determine the expression of c-*myc* gene and HPV type-18 genomes in CX-1 cells, total RNA was investigated by Northern blot analysis (Fig. 8). There was a high level of 2.4 kbp transcripts of c-*myc* gene in total RNA of CX-1 and HL-60 compared to that of HeLa cells. The extra bands of c-*myc* transcripts were not observed. The transcripts of HPV type-18 genomes were detected in the total RNA of CX-1 with an early region probe of HPV type-18 genome including open reading frames E6, E7 and E1.

DISCUSSION

The cervical cancer cell line, CX-1, is a newly established cell line with co-amplification of integrated HPV type-18 DNA and c-*myc* genes. The c-*myc* gene was rearranged with amplification. Up to the present, many

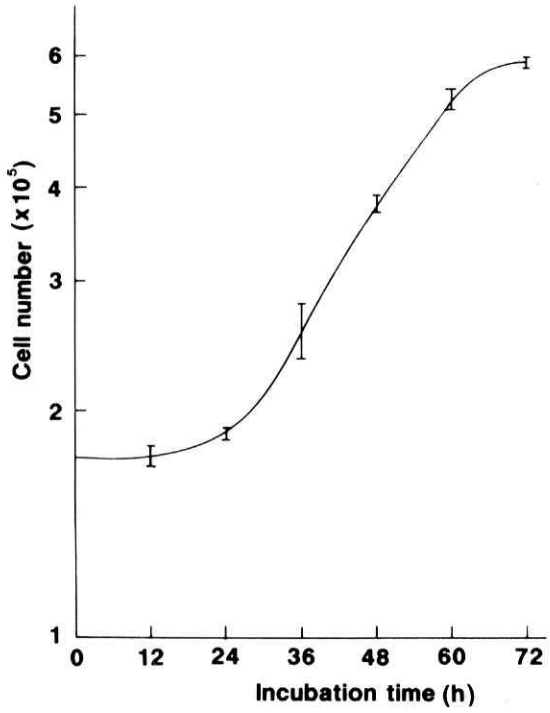

Fig. 5. Growth curve of CX-1 cells.

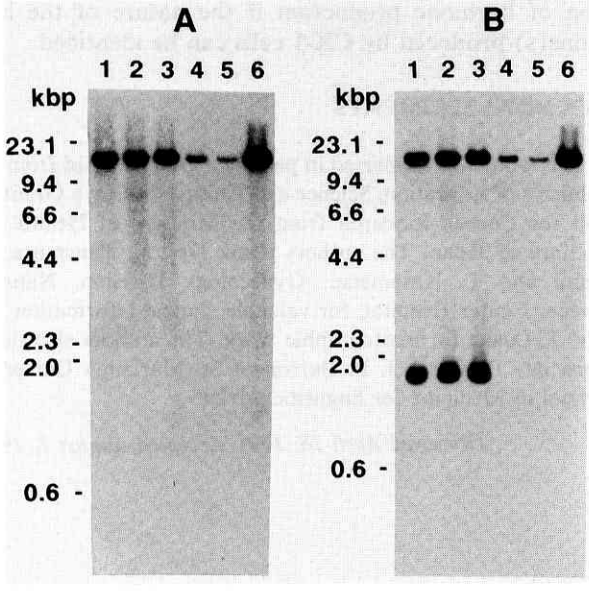

Fig. 6. Southern blot analysis of c-*myc* gene. Extracted DNAs (lanes from 1 to 6) digested with *EcoR*I were hybridized with c-*myc* probes (A: second exon probe, B: third exon probe). Lane 1: original tumor, lane 2: xenografted tumor, lane 3: CX-1 cells, lane 4: HeLa cells, lane 5: NB-1 cells, lane 6: HL-60 cells.

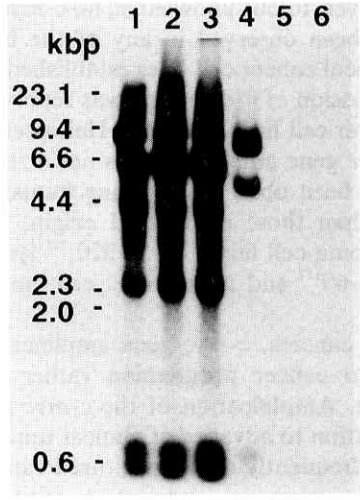

Fig. 7. Southern blot analysis of HPV type-18 DNA sequence. Extracted DNAs (lanes from 1 to 6) digested with *Pst*I were hybridized with HPV type 18 DNA probe. lane 1: original tumor, lane 2: xenografted tumor, lane 3: CX-1 cells, lane 4: HeLa cells, lane 5: NB-1 cells, lane 6: HL-60 cells.

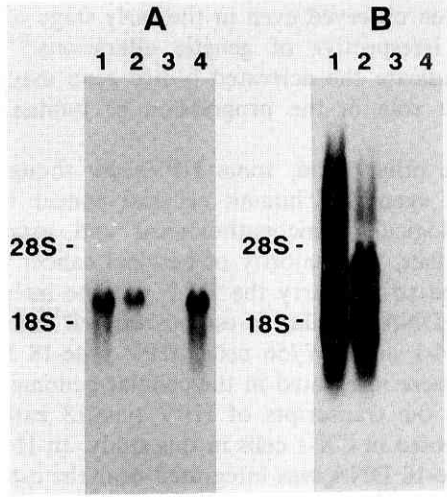

Fig. 8. Northern blot analysis of c-*myc* (A) and HPV type 18 (B) transcripts. Total RNAs extracted from CX-1 cells (lane 1), HeLa cells (lane 2), NB-1 cells (lane 3) and HL-60 cells (lane 4) were employed. The level of transcript of β-actin was used as a control.

huan cancer cell lines of cervical origin have been established and used for the study of cervical carcinogenesis by HPV infection.[6,7] In particular, HeLa cells played an important role in molecular biological studies to elucidate the transcription of HPV integrated in human

cells.[8] However, to our knowledge, no c-*myc* gene amplification has been observed in any of the human HPV-positive cervical cancer cell lines established so far. Only the overexpression of c-*myc* gene was reported in several cervical cancer cell lines including HeLa cells.[4, 9] Nevertheless, c-*myc* gene amplification is not a rare phenomenon and has been observed in many human cancer cell lines other than those of cervical origin, for example, colon carcinoma cell line, COLO 320,[10] lymphoblastoid cell line, HL-60[11] and many small cell lung cancer cell lines.[12]

In human cancers, c-*myc* gene amplification seem to be related to cancer progression rather than cancer developmemt. Amplification of the c-*myc* gene was observed in relation to advance of clinical tumor stage, and it was more frequently seen in cultured cancer cells and xenografted tumors in nude mice than in primary tumors, suggesting its relation to the proliferative activity of cancers. In human cervical cancers, alterations of c-*myc* gene including gene amplification, overexpression and rearrangement have been reported.[2, 3, 13] The genetic alterations of c-*myc* gene in cervical cancer were not frequent in the early stage of tumors, but increased in more advanced stage cases, as in other cancers.[14] In addition, the overexpression of the c-*myc* gene has commonly been observed even in the early stage of cervical cancers, irrespective of genetic alterations.[5, 13] These results indicate the activated c-*myc* gene may play an important role in the progression of human cervical cancers.

On the other hand, some HPVs are thought to be causative agents of human cervical cancer based on epidemiological, clinicopathological and experimental data. In fact, the majority of cervical cancer cell lines established so far carry the HPV genome integrated in host cell DNA. Similar to established cell lines such as HeLa, C4-1 and SW756 cells, HPV type-18 DNA sequences were integrated in the cellular genome of CX-1 cells and the transcripts of HPV type-18 early region were detected in CX-1 cells in this study. In HeLa cells, HPV type-18 DNA was integrated near the c-*myc* gene, suggesting that malignant transformation might have occurred by *cis*-activation of a cellular oncogene by HPV.[4] Other authors speculated that the presence of a high copy number of HPV DNA sequences might be correlated to the increase of c-*myc* gene alterations.[2, 5] Therefore, CX-1 cells appear to be a useful cell line to elucidate the interaction between c-*myc* gene and HPV-18, which might play a key role in maintaining the malignant phenotype of cervical cells.

Histologically, the original tumor of CX-1 cells was poorly differentiated carcinoma with neuroendocrine features, although the majority of cervical cancer cell lines have been derived from squamous cell carcinoma or adenocarcinoma. In general, HPV type-16 and -18 infections were frequently observed in squamous cell carcinoma and adenocarcinoma of the cervix, respectively.[15, 16] Recently, both HPV type-16 and -18 DNA was demostrated in small cell carcinoma of the cervix which was classified as a neuroendocrine carcinoma.[17, 18] HPV type-18 was suggested to be a viral type specifically associated with cervical small cell neuroendocrine carcinomas.[18] The phenomenon that morphological neuroendocrine features disappeared in the process of establishing the CX-1 cell line was occasionally experienced in the culture of small cell carcinoma of the lung.[19] These phenomena may be attributed to the difference of the expression level of the gene controlling morphological neuroendocrine features, because the genomic patterns of c-*myc* gene and HPV were well conserved from the original tumor to CX-1 cells. Though this study failed to elucidate which hormones CX-1 cells secrete, this cell line might be utilized for the study of the genetic regulation of hormone production if the nature of the hormone(s) produced by CX-1 cells can be identified.

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