Human papillomavirus 16 DNA in NIH3T3 cells transformed by colonic cancer cellular DNA

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Abstract

Human papillomavirus (HPV) 16 DNA is closely associated with human cancers. It has been identified as an aetiological agent in cervical cancers and, recently, in colonic neoplasms. To further understand the role of HPV 16 DNA in colorectal carcinogenesis, NIH3T3 cells were transformed with high molecular weight DNA from colonic cancer cells and the expression of HPV 16 DNA detected. Both human Alu and HPV 16 DNA sequences were found in the type II foci of CC-M2T cells by Southern blot hybridisation. Additionally, 100% tumorigenicity in nude mice was seen. This study shows the transfection of HPV DNA from colonic cancers into NIH3T3 mouse cells and suggests that HPV type 16 might be associated with the malignant transformation of colonic cells.

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Colorectal carcinoma ranks second only to lung cancer in men and to breast cancer in women in the United States.¹ The specific carcinogens, however, that cause the colorectal cancers in humans remain unknown. Human papillomavirus (HPV) have been reported to be associated with the genital tract²⁻⁵ and extragenital tumours⁶⁻⁸ in previous studies. Those works have shown that HPVs are associated with premalignant and malignant lesions of the stratified squamous epithelium. Nevertheless, reports of HPV infection in the cancer of the columnar epithelium are few. Recently, Kirgan *et al*^{9,10} have shown HPV antigen and genome in colon neoplasms. We have also found HPV DNA sequences in three cell lines of colorectal cancer from Chinese subjects.¹¹ These findings might suggest the correlation of HPV with cause of colorectal cancers.

It is uncertain whether HPV DNA acts as a causal agent or a causal passenger in colorectal carcinogenesis. Therefore, the biological activity of HPV DNA in cells has been studied to investigate this issue.¹²⁻¹⁷ We extracted the cellular DNA of human colonic cancers to transform NIH3T3 cells and investigated its possible role in colorectal carcinogenesis.

Materials and methods

CELLS

NIH3T3 cells were cultured in Dubecco modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco Laboratories, Scotland).¹³ CC-M2, CC-M3, CC-M4 cell lines were grown in RPMI (Roswell Park Memorial Institute) - 1640 (J R Scientific, Inc,

Woodland, CA, USA) medium with 10% fetal calf serum (Gibco) and cultured as previously described.¹⁸

TRANSFECTION

High molecular weight DNA was extracted from CC-M cells as in previous studies.¹¹ All DNA transfers were done by calcium phosphate precipitation.¹⁹ In brief, about 20 µg cellular DNA in 0.5 ml of 0.5 M CaCl₂ was mixed with 0.5 ml of 50 mM hydroxyethylpiperazine-ethanesulphonic acid (HEPES) (pH 7.1) and 70 mM sodium phosphate, and then agitated with a gentle stir. At room temperature, calcium phosphate was precipitated from this DNA solution in 30 minutes. Then, the solution was added to a 100 mm Petri dish containing 5 × 10⁵ NIH3T3 cells in 10 ml DMEM and 10% calf serum. Another dish, free of DNA solution, was used as a control culture. The cells were incubated for 14 hours in a humidified atmosphere with 5% CO₂ in air at 37°C. The DNA solution was then removed and 10 ml DMEM containing 10% calf serum was added. The medium was changed twice weekly. After 14 days' incubation, the number of transforming foci was obtained from the triplicate assays and the foci were harvested by the cloning cylinder procedure.

According to the description by Reznikoff *et al*,²⁰ three types of transformed cells were classified. Type I foci, which was regarded as not significant, consisted of condensed cells with little or no cellular overlap. Type II foci were formed of piled up cells that stained darkly, but had comparatively smooth edges. Type III foci had by far the most unusual morphology: highly condensed, heavily piled upon one another, and criss crossed at the edges of the focus.

DNA EXTRACTION

Total cellular DNA from each focus was extracted from these cells and from the normal epithelium of the colon, which was used as a normal control by the method previously described.^{21,22} Briefly, DNA was extracted and incubated with sodium dodecyl sulphate (SDS) and RNase at 37°C for three hours. Subsequently, proteinase K was added and incubated again overnight. Cellular DNA was then extracted with phenol/chloroform/isoamylalcohol (25:24:1), and dialysed with TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) buffer solution. After centrifugation (1000 g at 4°C for 20 minutes) the DNA in the supernatant was precipitated by the addition of ethanol. Precipitated nucleic acids were then washed with 70% ethanol and dried in the air. The absorbance of DNA at 260 nm was determined by a DU-50

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series spectrophotometer (Beckman Instruments, Inc, CA, USA) and compared with the absorbance at 280 nm. The DNA absorbance ratio ranged from 1.8 to 2.0.

SOUTHERN BLOT HYBRIDISATION

Filter hybridisation was carried out as described by Southern.²³ Briefly, each DNA was cleaved with the restriction enzyme EcoR I and then electrophoresed in 1% agarose gel. After staining with ethidium bromide, the DNA was denatured in situ and transferred to nitrocellulose filter paper (Bio-Rad Laboratories, Richmond, CA, USA) with an electroblot apparatus (Bio-Rad). To show the presence of human genes in mouse cells, the filters were hybridised with a human DNA marker, Alu family sequences,²⁴ with specific activity ranging 2×10^8 to 2×10^9 cpm/ μ g in high stringency conditions, then washed several times at 55°C, and finally exposed to an x ray film by an autoradiographic method at -70°C. In this assay, normal NIH3T3 cells and human leucocytes were used, as negative and positive controls, respectively. After removal of Alu probes, the filters were also hybridised to nick translated ³²P-labelled HPV DNA (2×10^8 cpm/ μ g) under stringent conditions (50% formamide, 10% dextran sulphate, 5× Denhardt's solution, 1% SDS-1 M NaCl, 50 mM TRIS-HCl, pH 7.4, 500 μ g/ml depurinated salmon sperm DNA) at 42°C for 24 hours. Filters were washed twice in 2×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% SDS for 30 minutes at room temperature and then in 0.1×SSC containing 0.1% SDS at 50°C for 30 minutes before autoradiography. Filters were dried and exposed to XAR 2 film (Eastman Kodak Co, Rochester, NY, USA) with an enhancing screen at -70°C.²⁵ DNA molecular weight marker was obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA).

TUMORIGENECITY

Transformed cells were subcutaneously injected into bilateral flanks of six adult female BALB/c nude mice. About 5×10^6 cells were injected per site. Within one to two months, the newly grown tumours were removed and their representative sections were stained with haematoxylin and eosin.

Results

TRANSFORMATION OF NIH3T3 CELLS

The transforming activity of DNAs, which induced morphological transformation of

Transforming efficiency and tumorigenicity of NIH-3T3 cells transformed by CC-M cellular DNAs

Human donor DNA	Foci/ μ g DNA			Tumorigenicity (no of tumours/no of injections)
	Type I	Type II	Type III	
CC-M2	0.017 (1/60)	0.25 (15/60)	0	6/6
CC-M3	0.033 (2/60)	0	0	0/6
CC-M4	0.017 (1/60)	0	0	0/6
None	0	0	0	0/6

Figure 1: Phase contrast of micrographs of (A) morphological appearance of NIH-3T3 cells transformed with the high molecular weight DNA of CC-M2 cells, original magnification $\times 100$ and (B) longterm culture of CC-M2T, original magnification $\times 200$ (C) typical uniformly flat morphology of NIH-3T3 cells, original magnification $\times 200$.

NIH3T3 fibroblasts in the cancer cells were estimated. The Table shows that only high molecular DNA isolated from CC-M2 cells was able to induce type II transforming foci of which the transforming efficiency was 0.25 focus per μ g of DNA. In contrast with type II focus, the type I ranged from 0.017 to 0.033. These results indicate that CC-M2 cells contain activated transforming genes. The morphology of type II focus was comprised of tightly packed cells, which were extensively piled upon one another and had comparatively smooth edges (Fig 1A). These cells, however, showed a criss cross appearance after longterm cultivation (Fig 1B). They were significantly different from non-transformed NIH3T3 cells, which showed contact inhibition of cell growth, strong cell to substrate adhesion, and uniformly flat morphology (Fig 1C).

HPV DNA IN TRANSFORMED CELLS

Cell lines, derived from transformed foci that were induced by CC-M2, CC-M3, CC-M4, were designated as CC-M2T, CC-M3T, CC-M4T respectively. All transformed cell lines were examined by Southern blot hybridisation. The

Figure 2: Southern blot analysis of human Alu family sequences in CC-M2T transfected by CC-M2 cellular DNA and control cells. Non-transfected and positive controls: 3T3-C and Alu-C.

results showed that all type II foci of CC-M2T cells were the only positive foci in the DNA hybridisation assay of human Alu family sequences (Fig 2). These results show that human DNAs integrate only into the type II foci of CC-M2T. CC-M2T that contained Alu sequences was also positive in the hybridisation with ^{32}P -labelled HPV-16 DNA sequences (Fig 3). Under highly stringent conditions, two important HPV-16 related DNA bands of 7.9 and 2.2 Kb were found in CC-M2T cells. Other cell lines, however, in this assay were all negative.

TUMORIGENECITY

The Table shows the results of tumour induction. Tumours developed only from the CC-M2T cell line within six weeks. The tumorigenicity of CC-M2T in nude mice was 100% (6/6). The tumours were then removed to establish another cell line for future studies. The histological examination of these tumours showed a fibrosarcoma like picture.

Discussion

Human papillomavirus has been shown to play a causal part in cervical neoplasia.²⁻⁵ It has also been found closely associated with human extra-genital cancers.⁶⁻⁸ Few reports, however, have shown the relation between HPV and colonic cancers. Kirgan *et al*^{9,10} found that colonic neoplasms contain both HPV antigen and viral genome. Nevertheless, Southern blot is a well known standard technique in DNA hybridisation. Therefore, we have shown the expression of HPV-16 and HPV-18 DNA in colonic cancer

Figure 3: Southern blot analysis of HPV 16 DNA sequences in the same nitrocellulose filter as Figure 2.

(CC-M) cell lines by Southern blot hybridisation.¹¹ From this study, we believe that HPV may play an important part in colorectal carcinogenesis. Although a number of studies have described the HPV transforming activity in the transformation of normal cells,¹²⁻¹⁶ the carcinogenic mechanisms of HPV is not yet completely understood. Also, activated transforming genes in some human tumours have been detected by DNA transfection.²⁶⁻²⁸ There is still not one study concerning the transmissible condition of HPV DNA in colonic cancers. To identify the HPV transfection of human tumour cell lines to normal mouse cells, we have shown the Alu sequences of human DNA marker in the genome of NIH3T3 transformants, although CC-M2 was the only cell line that transfected successfully. Our transfection rate was higher compared with previous reports^{26,27} Perucho *et al*²⁶ have shown that only five of 21 human tumour cell lines contain a gene or genes capable of transforming mouse cells. In Krontiris and Cooper's²⁷ series, only two of eight human tumour cell lines and none (0/13) of primary tumours can transform NIH3T3 cells. The low efficiency of transformation with human tumour DNA may be attributed to several reasons. Firstly, the molecular heterogeneity of tumours with the same origin, histological type, and differentiated level.^{27,29} In other words, cell lines derived from tumours of the same tissue may contain two different transforming genes.²⁶ Secondly, the long latency in the transformation of rodent cell lines by HPV.¹³ This suggests that longer than 14 days' incubation is required to obtain transforming foci. Thirdly, the lack of cooperative activated oncogenes. Matlashewski¹⁴ has described two groups of genes that are required in the morphological transformation of normal

primary cells in vitro. One group is the establishing genes such as *myc*, *Ela* and the other is the transforming genes including *ras* and polyoma middle T genes. He has also shown that activated *ras* gene is required in the morphological transformation of HPV-16 DNA. In other words, negative cell lines may lack some genes capable of transforming NIH/3T3 cells or contain a set of genes that could transfer 3T3 cells, but at an efficiency of focus induction too low for detection.²⁶ Fourthly, the size of transforming genes is too large at transfer. Peruchio²⁶ and Wigler *et al*³⁰ have shown that the efficiency of transformation may be subject to the size and nature of the transforming fragment.

Dürst³¹ has reported that the sequences of papillomavirus may be integrated near the cellular oncogenes in some cervical carcinomas. These activated cellular oncogenes by HPV DNA may participate in the malignant transformation of cervical cells. Interestingly, in our study, all type II foci of CC-M2T cells contained HPV DNA sequences and showed a 100% (6/6) tumorigenicity in nude mice. The tumour cells grown from nude mice were also found to have HPV DNA sequences (unpublished data). This suggests that HPV may be an active gene in carcinogenesis and thus does not lose in vivo passage. HPV-16 DNA has been detected in all CC-M colonic cancer cell lines.¹¹ Moreover, HPV genomes was found in 43% of colonic carcinomas and none in normal mucosa (unpublished data). In this study, we have exclusively shown the transfection of HPV-16 DNA from colonic cancer cells to NIH3T3 cells. Because HPV-16 DNA is known to activate cellular oncogene³¹ and is closely associated with malignant transformation,^{13,14} these findings suggest that an association of HPV-16 DNA with the multistep development of colorectal cancers may exist. As in the report by Reznikoff *et al*,²⁰ all the type I foci in this study had a non-tumorigenic character in vivo and were considered to have no transforming response.

In conclusion, the HPV DNA of colonic cancer cells can integrate and induce neogrowth in mouse cells. These data suggest that HPV-16 may play an active part in colorectal carcinogenesis and may not be simply a casual DNA in transformed cells. This study provides valuable insight into the association of HPV DNA with colonic cancers. Further investigations into the mapping of HPV integration sites and nearby activated oncogene will be very helpful in better understanding colorectal carcinogenesis.

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