Human papillomavirus DNA in adenosquamous carcinoma of the lung

Kyoko Tsuhako, Iwao Nakazato, Tsuneo Hirayasu, Hajime Sunakawa, Teruo Iwamasa

Abstract

***Aim*—To investigate the presence of human papillomavirus (HPV) DNA in adenosquamous carcinoma of the lung—which is relatively common in Okinawa but not in mainland Japan—and examine its histological features.**

***Methods*—Of 207 cases where primary lung cancers were surgically removed between January 1995 and June 1997 in Okinawa, 23 were adenosquamous carcinoma. HPV was detected by non-isotopic in situ hybridisation (NISH) and polymerase chain reaction (PCR) amplification with primers specific for E6 and E7 regions of the HPV genome. PCR products were analysed by Southern blotting. Immunohistochemical determination of high molecular weight cytokeratin (HMC) and involucrin was also carried out.**

***Results*—18 cases were positive for HPV DNA by PCR and NISH. HPV types 6, 11, 16, and 18 were found. Seven cases were dual positive for different types of HPV. Using NISH, HPV was also found in the squamous cell components and in neighbouring enlarged adenocarcinoma cells. The HMC and involucrin were demonstrated immunohistochemically in the same areas.**

***Conclusions*—HPV DNA was found in a high proportion (78.3%) of adenosquamous carcinomas in Okinawa, a region where HPV has previously been shown to be prevalent in squamous cell carcinoma of the lung. The adenocarcinoma cells adjacent to the squamous cell carcinoma component were enlarged and positive for HPV, HMC, and involucrin. This is thought to indicate the transition from adenocarcinoma to squamous cell carcinoma.**

(*J Clin Pathol* 1998;51:741–749)

Keywords: human papillomavirus; adenosquamous carcinoma; polymerase chain reaction

Ryukyu University School of Medicine, Okinawa, Japan
Department of Pathology
K Tsuhako
I Nakazato
T Iwamasa

Department of Surgery
T Hirayasu

Department of Oral Surgery
H Sunakawa

Correspondence: Dr Kyoko Tsuhako, Department of Pathology, Ryukyu University School of Medicine, 207 Uehara, Nishihara, Okinawa 903-0215, Japan.

Accepted for publication 11 February 1998

In Japan cancer of the lung has shown a 40-fold increase in men and a 36-fold increase in women from 1950 to 1994, and in 1993 the age adjusted death rate for lung cancer surpassed that of all other carcinomas.[1] In Okinawa prefecture, a subtropical island in southern Japan, it has topped the rates for all malignant neoplasms since 1975, and is the highest in Japan. In Okinawa, squamous cell carcinoma—especially the well differentiated form—is prevalent, while this form is relatively rare in both mainland Japan[2 3] and other countries.[4] Using the polymerase chain reaction (PCR), 79% of the squamous cell carcinoma cases in Okinawa have been found to be positive for human papillomavirus (HPV) DNA, while less than 30% of cases in mainland Japan are positive.[2 5]

Vincent *et al* reported that in the 1960s squamous cell carcinoma was the most common tumour, representing about 50% of lung cancer cases.[6] However, recent reports on the incidence of the subtypes of lung carcinoma from several large studies puts adenocarcinoma ahead of squamous cell carcinoma. A relative decrease in the incidence of squamous cell carcinoma has accompanied the increased incidence of adenocarcinoma. It has long been reported that cigarette smoking is the major cause of lung cancer, and it is thus possible that the recent decrease in the frequency of squamous cell carcinoma is at least partly a reflection of changing smoking habits.[4 6 7] However, in Okinawa, squamous cell carcinoma still has a high incidence, although the prevalence of smoking in general is not particularly high, being lower than in mainland Japan.[1] Furthermore, in Okinawa, there have been significant numbers of cases with adenosquamous carcinoma. In the mainland, the frequency rate of adenosquamous carcinoma in the early 1990s was 2.6% of 2160 primary lung cancers resected in the National Cancer Centre Hospital (Tokyo, Japan).[8] Ishida *et al* also reported a similar frequency rate of 1.8% in Fukuoka, mainland Japan.[3] In the USA, Fitzgibbons and Kern[9] reported a frequency rate of 0.6%. However, the classification of adenosquamous carcinoma of the lung has been poorly defined. According to World Health Organisation (WHO) criteria,[10] adenosquamous carcinomas contain both squamous carcinomatous and adenocarcinomatous components. Colby *et al* wrote in the Armed Forces Institute of Pathology's *Atlas of Tumours of the Lower Respiratory Tract*[4] that while the proportions of each subtype required for a diagnosis are not defined in WHO criteria, a minimum of 5% for one component is reasonable, as suggested by Takamori *et al*.[8] The latter showed that there was no significant difference in prognosis among three groups with different proportions of the adenocarcinoma component (< 20%, 20–80%, and > 80%). However, according to the criteria of the Japan Lung Society,[11] to qualify as an adenosquamous carcinoma a tumour should be composed of at least 20% each of the squamous cell carcinoma component and the adenocarcinoma component. In our present report we have employed these latter criteria, but five cases of adenocarcinoma with small foci of the squamous carcinoma component (less than 20%) were also examined. The present cases also fulfilled the criteria of Fitzgibbons and

Kern[9] in that both components were at least moderately well differentiated.

We demonstrated the presence of HPV DNA in the adenosquamous carcinoma by PCR and in situ hybridisation. Detailed histological examination, including the immunohistochemical demonstration of high molecular weight keratin and involucrin, was also carried out.

Methods

Two hundred and seven cases of primary lung cancer (102 of which were squamous cell carcinomas and 73 adenocarcinomas) were surgically removed in the National Okinawa Hospital and Ryukyu University Hospital during the 30 months from January 1995 to June 1997. Samples from 23 cases of adenosquamous carcinoma were obtained. Eighteen of these cases have already featured in our previous review article.[12] In the present study, these 18 cases of adenosquamous carcinoma were re-examined in detail. Five cases of adenocarcinoma with small foci of squamous cell carcinoma (total squamous cell carcinoma component less than 20%, Japan Lung Cancer Society Criteria[11]) and as controls, three cases of well differentiated adenocarcinoma (papillary type) were also obtained. All these three cases were male non-smokers, and their ages were 55, 63, and 71 years. The tumours were located in the peripheral regions of the right upper lobes. We also examined 10 cases of squamous cell carcinoma (two well differentiated, four moderately differentiated, and four poorly differentiated) and three cases of adenosquamous carcinoma (adenocarcinoma component well differentiated; squamous cell carcinoma component moderately differentiated) from Kumamoto prefecture in mainland Japan (by courtesy of Dr Ohtsuka, Kumonoto Chuo General Hospital and Dr Takeya, Kumamoto University Hospital). All except one of the squamous cell carcinoma cases were male. One moderately and two poorly differentiated cases were located peripherally, but the other seven cases were in central regions. The one female case was a non-smoker, but the others were all heavy smokers. The three adenosquamous carcinomas were from two male heavy smokers and one female non-smoker.

None of the patients had been treated with radiation or chemotherapy before surgery. Most of the male patients were farmers or fishermen, and female patients were housewives. There were no miners or heavy industry workers.

HISTOLOGICAL EXAMINATION AND NON-ISOTOPIC IN SITU HYBRIDISATION

Samples were fixed in 10% phosphate buffered formalin. After fixation, the tumours were continuously sectioned at 0.5 cm intervals and all parts were subjected to routine examination. Haematoxylin and eosin, Gomori's silver impregnation, periodic acid Schiff, and alcian blue staining was performed on 4 μm sections. Antibody to involucrin (a marker of keratinocyte differentiation) was obtained from Sigma (St Louis, Missouri, USA), and antibody to high molecular cytokeratin (HMC) (Moll's No 1, 5, 10, 14)[13] from Dako (Carpinteria, California, USA).

Non-isotopic in situ hybridisation (NISH) was performed on all specimens using HPV 6/11, 16/18, and 31/33/51 biotin labelled probes from the Enzo PathoGene in situ HPV tissue hybridisation kit (Farmingdale, New York, USA). NISH was carried out according to Cooper *et al*[14] and the manufacturer's instructions. After unmasking with proteinase K (Merck, Meguro-ku, Tokyo, Japan) at a concentration of 2 mg/ml in 50 mM Tris-HCl buffer, pH 7.4, for 15 minutes at 37°C, the sections were incubated with prehybridisation solution containing 2 × SSC (sodium saline citrate), pH 7.2, 10% dextran sulphate (wt/vol), 400 mg/ml of sonicated herring sperm DNA, and 50% formamide (vol/vol). Denaturation of probe and target DNA in the sections was performed simultaneously on a hot plate (90°C) for two minutes. Hybridisation was subsequently carried out using 1 mg/ml of biotinylated HPV DNA by incubating the sections overnight in a humidified chamber at 37°C. Biotin was detected using peroxidase labelled streptavidin. H_2O_2 and DAB (3,3' diaminobenzidine) were used for the peroxidase reaction. Where there was dual positivity for different types of HPV, Dako's probe for single type HPV and a proprietary NISH detection system (also from Dako) were used according to the manufacturer's instructions.

DETECTION OF HPV TYPES 6, 11, 16, AND 18 DNA BY PCR

Within one or two weeks of obtaining samples, DNA samples from 23 cases were prepared as reported previously.[2] We also prepared DNA samples from five cases of adenocarcinoma with small foci of squamous cell carcinoma and three control cases of well differentiated adenocarcinomas, and from 10 squamous cell carcinomas and three adenosquamous carcinomas from Kumamoto prefecture.

Thirty paraffin wax sections of 10 μm thickness were placed in 15 ml tubes. The paraffin wax was removed by washing twice in 10 ml of xylene for 30 minutes and twice in 100% ethanol for 30 minutes. The specimens were then digested with proteinase K (Merck, Tokyo, Japan) in 500 mM Tris-HCl buffer, pH 7.5, containing 0.45% Tween 20 and 2.5 mM $MgCl_2$ at 37°C for 36 hours. The DNA was extracted using phenol/chloroform twice (the former equilibrated with 1 M Tris-HCl, pH 8.0, containing 0.1% quinolinol, and the latter a 24:1 (vol/vol) mixture of chloroform and isoamyl alcohol), then once more with chloroform. The DNA was precipitated with three times the volume of 100% ethanol containing 0.1 M sodium acetate at −20°C. DNA was used for PCR immediately after extraction. The 110 base pair β globin gene was detected (data not shown) in all DNA samples according to the method of Saiki *et al*,[15] using their primers (PCO_3 and PCO_4).

The primers and probes used are listed in table 1. The PCR reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.4 mM each of

Table 1 Primers and probes

HPV 6 E6	**HPV 11 E6**
Primer	*Primer*
Sense: 5'-GCTGGATATGCAACAACAGTTG-3'	Sense: 5'-GCAGCGTGTGCCTGTTGCTTA-3'
Antisense: 5'-CATGCATGTTGTCCAGCAGTG-3'	Antisense: 5'AAGCAACGACCCTTCCACTGG-3'
Probe: 5'-GCTACCTGTGTCACAAACCG-3'	*Probe*: 5'-CCTGTGTCACAAGCCGTTGTG-3'
189 bp PCR product is obtained	230 bp PCR product is obtained
HPV 16 E6	**HPV 16 E7**
Primer	*Primer*
Sense: 5'-GATGGGAATCCATATGCTGTA-3'	Sense: 5'-CCAGAGACAACTGATCTCTAC-3'
Antisense: 5'-TCGACCGGTCCACCGACCCCT-3'	Antisense: 5'-GTGTGTGCTTTGTACGCACAAC-3'
Probe: 5'-GCCACTGTGTCCTGAAGAAAAGC-3'	*Probe*: 5'-TAACCTTTTGTTGCAAGTGTGACTCTACGCTTCG-3'
240 bp PCR product is obtained	171 bp PCR product is obtained
HPV 18 E6	**HPV 18 E7**
Primer	*Primer*
Sense: 5'-CAGTATACCCCATGCTGCATGCC-3'	Sense: 5'-GAGCCGAACCACAACGTCAC-3'
Antisense: 5'-CGGTTTCTGGCACCGCAGGCACC-3'	Antisense: 5'-GGATGCACACCACGGACACA-3'
Probe: 5'-CAGACTCTGTGTATGGAGACAC-3'	*Probe*: 5'-TCCAGCAGCTGTTTCTGAACACCCTG-3'
160 bp PCR product is obtained	152 bp PCR product is obtained

The primers for E6 regions of HPV 6, 11, 16, and 18 and probes for Southern blot analysis were the same as those reported by McNicol *et al.*[16]

four dNTPs, 0.32 μm each primer, and 2.4 units of Taq DNA polymerase (Cetus-Takara, Seta, Otsu, Japan). Reaction conditions were the same as reported previously[2]: annealing at 62°C for three minutes, denaturation at 94°C for two minutes, and extension at 74°C for four minutes for 40 cycles. The amplified DNA was transferred on to nylon membranes (Hybond-N^+, Amersham Life Science, Buckinghamshire, UK) after routine polyacrylamide gel (10%) electrophoresis, and Southern blot analysis was carried out using a probe and the Amersham system ECL 3'-oligolabelling and detection system, according to the instructions provided by the manufacturer (Amersham Life Science). The sensitivity of the PCR method was tested using various concentrations of HPV 11, 16, and 18 DNA in plasmid pBR 322, and HPV 6 in plasmid pML, which were obtained from the Japanese Cancer Research Resource Bank, with permission from Dr Zur Hausen.

Table 2 Adenosquamous carcinoma of the lung

Case	*Sex*	*Age*	*Stage†*	*Location‡*	*Smoking (cigs/d×years)*	*Occupation*
1	M	70	IIIa	C	20×28	Shopkeeper
2	M	67	IIIa	P	30×40	Fisherman
3	F	58	I	P	30×39	Housewife
4	F	58	I	P	20×52	Housewife
5	M	61	IIIa	P	20×25	Fisherman
6	F	51	I	P	0	Housewife
7	M	71	I	P	20×52	Farmer
8	M	76	I	P	?	Farmer
9	M	79	IIIb	P	?	Farmer
10	F	77	IIIa	P	30×43	Housewife
11	M	70	I	P	30×40	Engineer
12	M	68	I	P	20×39	Farmer
13	M	51	II	P	20×27	Farmer
14	F	73	I	P	0	Shopkeeper
15	M	79	II	P	?	Government employee
16	F	50	I	P	0	Housewife
17	F	52	IIIa	P	?	Housewife
18	M	65	I	P	10×54	Farmer
19	M	74	II	P	30×37	Farmer
20	M	76	I	C	20×49	Fisherman
21	M	77	II	P	20×48	Government employee
22*	F	84	IIIb	P	0	Housewife
23	M	55	I	P	20×30	Shopkeeper

Cases were numbered in order of the surgical procedures.
Cig, cigarettes; F, female; M, male.
†Stage: International staging system.
‡Location: C, central; P, peripheral.
*Dead (April, 1997).

The PCR products of particular interest in dual positive cases for different types of HPV were extracted from the agarose gel. The extracted DNA was cloned into the pGEM-T vector (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The sequence analysis was carried out using Hitachi SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan).

Results

Twenty three of 207 primary lung cancers (11.1%) were adenosquamous carcinomas. In addition, 4.1% of biopsy specimens from unresected or unresectable tumours over the same period (6/147 biopsied cases) had two components, adenocarcinoma and squamous cell carcinoma, though the diagnosis of adenosquamous carcinoma in such cases should only be made after examining all parts of the tumour. The incidence of this tumour in Okinawa was considered high, though the true incidence is uncertain. Table 2 shows that the mean (SD) age of the patients was 67.6 (10.4) years (range 50 to 80); 15 were male and eight female. Twenty one cases were peripherally located and two centrally. Twelve cases (52.1%) were stage I, four (17.4%) were stage II, and seven (30.4%) were stage III.

HISTOLOGICAL OBSERVATIONS AND NISH

Adenosquamous carcinoma of the lung is composed of admixed adenocarcinoma and squamous cell carcinoma. The grades of differentiation of the two components are shown in table 3. Most of the cases showed well differentiated regions of both components (fig 1A and B). The adenocarcinoma component was predominant in 16 cases, but five cases consisted of an equal mixture of the two components, and in two the squamous cell carcinoma component was predominant. The adenocarcinoma component of 22 cases was of papillary type, and only one case (No 23) was of tubular type. The squamous cell carcinoma component was well or moderately differentiated, showing cellular keratinisation and intercellular bridges. In the

Table 3 Adenosquamous carcinoma of the lung and detection of human papillomavirus (HPV)

Case	Grade of differentiation: Adeno-carcinoma	Grade of differentiation: Squamous cell carcinoma	Predominant component	Detection of HPV by PCR†	NISH‡
1	Well	Moderate	Adenocarcinoma	-	-
2	Well	Moderate	Equal mixture	18	+
3	Well	Well	Adenocarcinoma	16	+
4	Well	Moderate	Adenocarcinoma	18	+
5	Well	Well	Adenocarcinoma	16	+
6	Well	Well	Adenocarcinoma	16	+
7	Well	Well	SCC	11,16	+
8	Moderate	Well	Equal mixture	16,18	+
9	Moderate	Well	Equal mixture	16,18	+
10	Well	Well	Adenocarcinoma	16	+
11	Well	Well	Adenocarcinoma	11,16	+
12	Well	Moderate	Adenocarcinoma	16,18	+
13	Moderate	Moderate	Equal mixture	-	-
14	Well	Well	Adenocarcinoma	-	-
15	Well	Well	SCC	11,16	+
16	Well	Well	Adenocarcinoma	6	+
17	Well	Well	Adenocarcinoma	16	+
18	Well	Moderate	Adenocarcinoma	18	+
19	Well	Well	Adenocarcinoma	6	+
20	Well	Moderate	Adenocarcinoma	18	+
21	Well	Moderate	Adenocarcinoma	-	-
22	Well	Moderate	Adenocarcinoma	6,16	+
23	Moderate	Well	Equal mixture	-	-

Cases are numbered in order of the surgical procedures.
NISH, non-isotopic in situ hybridisation; PCR, polymerase chain reaction; SCC, squamous cell carcinoma.
†HPV types detected.
‡Episomal and integrated forms of HPV were demonstrated on the squamous cell carcinoma component, as were enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component.

well differentiated cases, pearl formation was observed. In areas where both components were intermingled, adenocarcinoma cells adjacent to the squamous cell carcinoma component were enlarged with wide cytoplasms and had an irregular tubulopapillary structure (fig 1C). Small nodules of the squamous carcinomatous component often protruded into tubulopapillary structures of the adenocarcinoma component (fig 1D). This is a feature of the transition of adenocarcinoma to squamous cell carcinoma.

Immunohistochemically, HMC and involucrin, a marker of keratinocyte differentiation,[17] were strongly stained in the squamous carcinoma component (fig 2A, B, and C), and in the enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component (fig 2D). In 18 cases the squamous cell components were found to be positive for HPV DNA by the use of NISH (fig 3A, table 3). The presence of HPV was confirmed in the nuclei in these cases. We demonstrated episomal and integrated HPV using Cooper's criteria.[14] Furthermore, positive reactions for both episomal and integrated HPV were clearly obtained on the enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component (fig 3B). However, HPV DNA was not demonstrated by NISH in either the surrounding non-tumour regions or in the bronchial epithelium (fig 3C). In the five cases of adenocarcinoma with small foci of squamous cell components, the adenocarcinomatous components were all well differentiated, while the squamous cell regions were moderately differentiated. The HPV DNA positive reaction by NISH was also demonstrated in the squamous cell carcinoma components of four of these five cases: two were positive for HPV 6, one each for HPV 16 and 18. The reactions were for both episomal and integrated forms. Only one case was negative for HPV DNA by NISH. Again, enlarged adenocarcinoma cells were observed adjacent to the squamous cell carcinoma components and were positive for HPV DNA, but in one of the five cases in which the squamous cell carcinoma component was negative for HPV DNA, the enlarged adenocarcinoma cells were also negative for HPV DNA by NISH. All three control adenocarcinoma cases were negative for HPV by NISH (fig 3D). Of the 10 cases of squamous cell carcinoma from Kumamoto prefecture, one well differentiated case was positive for HPV 16, and one moderately differentiated case was positive for HPV 18. However, none were dual positive for two types of HPV. Three adenosquamous carcinomas from Kumamoto prefecture were negative for HPV.

Table 4 Sequence of the PCR amplified DNA (HPV 18 E7 region)

	764
(A)	CACACAATGTTGTGTATGTGTTGTAAGTGTGAAGCCAGAATTGAGCTA
(B)	CACACAATGTTGTGTATGTGTTGTAAGTGTGAAGCCAGAATTGAGCTA
(A)	GTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAGCAGCTGTTT
(B)	GTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAGCAGCTGTTT
	898
(A)	CTGAACACCCTGTCCTTTGTGTGTCCGTGGTGTGCATCC
(B)	CTGAACACCCTGTCCTTTGTGTGTCCGTGGTGTGCATCC

(A) published sequence (Cole and Danos[18]); (B) PCR amplified DNA (Okinawan case No 9).

DETECTION OF HPV DNA BY PCR

As reported previously,[2] using Southern blot analysis with chemiluminescence probes (Amersham Life Science), lower limits of 55 and 77 viral copies of the HPV 16 and 18 E7 regions, respectively, were detected. In the cases of HPV 6, 11, 16, and 18 E6, using McNicol's primer and probes,[16] lower limits of 85, 740, 55, and 7700 viral copies, respectively, were detected.[2] The sensitivities of PCR using various primers and probes varied. Cases where either one or both of the E6 and E7 regions are detected are counted as positive for HPV DNA. Eighteen cases with positive NISH reaction were also positive for HPV DNA by PCR (table 3, fig 4A, B, C, and D). Seven cases were dual positive for two types of HPV DNA. In these dual positive cases, one type of HPV DNA was detected at a level greater than 1000 copies per approximately 10 mm^3 of the tumour tissue, while the second type was detected at a much lower level (less than 200 copies/10 mm^3). HPV DNA was not detected from any surrounding non-tumour parts of the lung by PCR (data not shown).

In three adenosquamous carcinomas from Kumamoto prefecture and three adenocarcinomas from Okinawa, HPV DNA was not detected by PCR (data not shown). Four of five Okinawan adenocarcinomas with small foci of squamous cell carcinoma components, and two of 10 squamous cell carcinomas from Kumamoto, were positive for HPV DNA by PCR and NISH (data not shown).

No sequence variation was noted in the HPV 16 and 18 DNA of the PCR products from either region E6 or E7 (table 4) compared with the published sequences.[18]

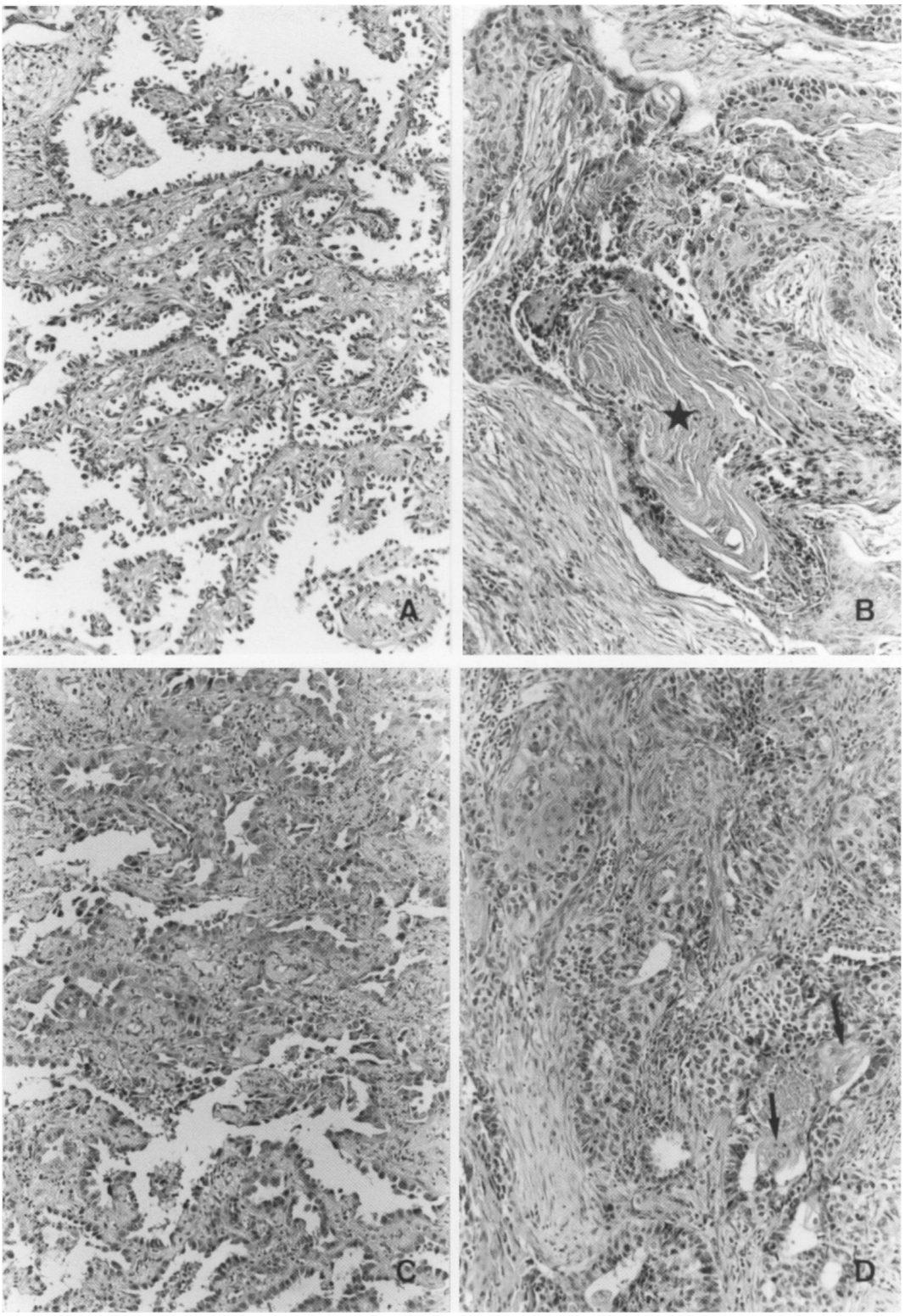

*Figure 1 (A) The well differentiated adenocarcinoma component of adenosquamous carcinoma (case No 1). The papillary structure can be seen. (H&E stain, ×123.) (B) The well differentiated squamous cell carcinoma component of adenosquamous carcinoma (case No 3). *keratin pearls. (H&E stain, ×123.) (C) Enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component (case No 1). Enlarged adenocarcinoma cells with relatively wide cytoplasm show the tubulopapillary structure in the upper part of the figure. (H&E stain, ×123.) (D) Small nodules of squamous cell carcinoma. Small nodules of squamous cell carcinoma protruded into the lumens of adenocarcinomatous components in areas where the two components are intermingled. Arrows indicate the nodule of squamous cell carcinoma components. (H&E stain, ×123.)*

Discussion

In this study we employed the *General Rules for Clinical and Pathological Records of Lung Cancer* published by the Japan Lung Cancer Society.[11] Adenosquamous carcinoma is defined as tumour which is composed of at least 20% each of the squamous cell carcinoma component and the adenocarcinoma component, so we only classified as adenosquamous carcinoma those tumours showing obvious areas of squamous cell carcinoma and obvious areas of adenocarcinoma. Although adenosquamous carcinoma of the lung is reported to be a relatively rare tumour and its biological behaviour is still unclear, in Okinawa there was a high incidence of adenosquamous carcinoma in the current

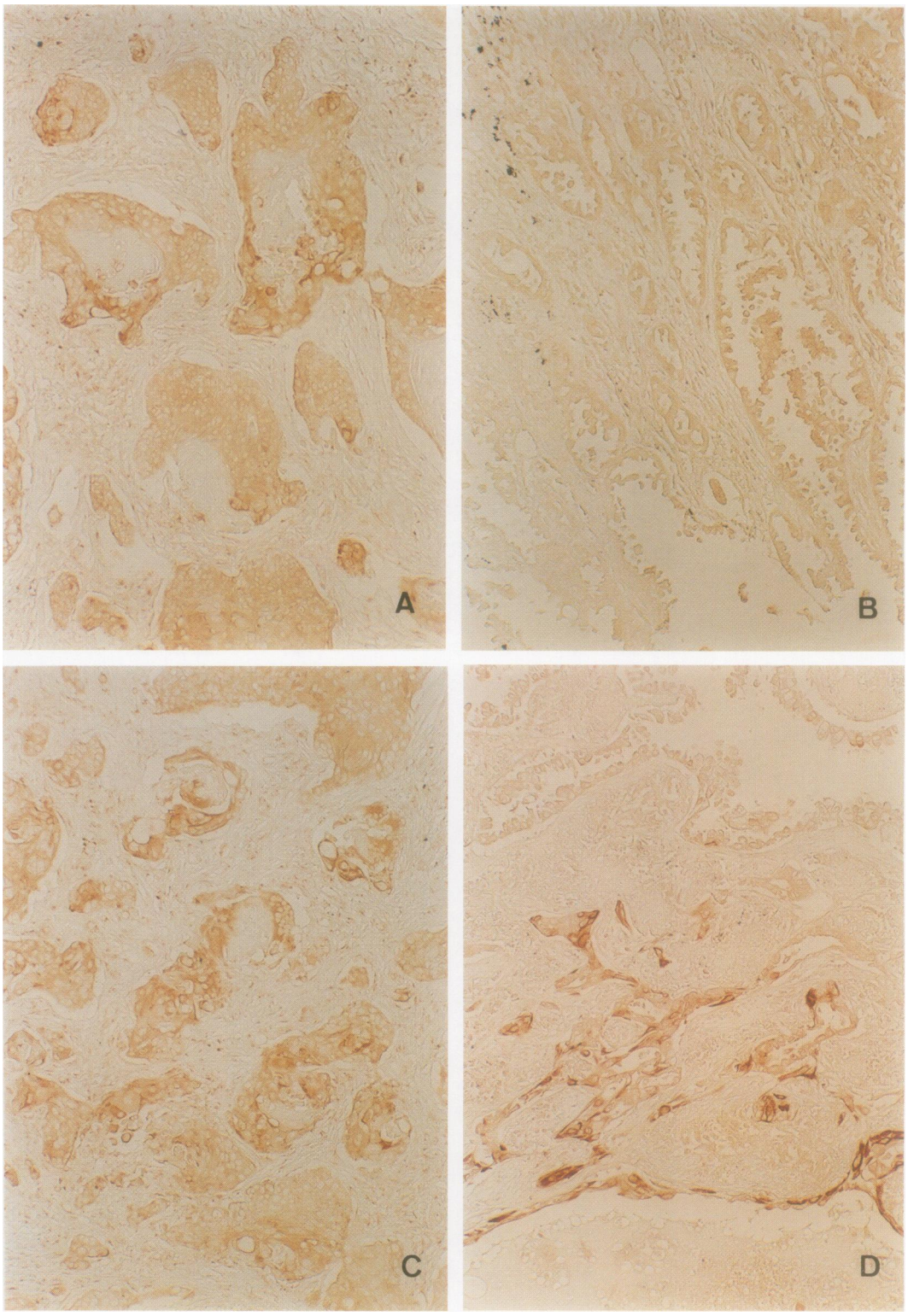

Figure 2 (A) Immunohistochemical demonstration of high molecular weight cytokeratin. Staining for high molecular weight cytokeratin is strongly positive in the squamous cell carcinoma component. (×123.) (B) Immunohistochemical demonstration of high molecular weight cytokeratin. The adenocarcinoma component shows negative reaction. (×123.) (C) Immunohistochemical demonstration of involucrin. The squamous cell carcinoma component shows a strongly positive reaction. (×123.) (D) Immunohistochemical demonstration of involucrin on the enlarged adenocarcinoma cells. These cells showed a strongly positive reaction (lower part), which was absent in the ordinary adenocarcinoma component (upper part). (×123.)

series of surgically resected cases. Eighteen (78.3%) of the present 23 cases were positive for HPV DNA by both PCR and NISH. There were no sequence variations in the HPV amplified by PCR. The squamous cell carcinoma component showed a strongly positive reaction to HMC and involucrin antibodies, while pearl formations were also demonstrated in well differentiated cases. The adenocarcinoma cells adjacent to the squamous cell carcinoma components had an increase in cytoplasm. These enlarged adenocarcinoma cells also stained positively for HMC and involucrin antibodies and contained HPV DNA. The small foci of squamous cell carcinoma in adenocarcinoma cases were positive for HPV DNA by NISH. Enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component in these cases were also posi-

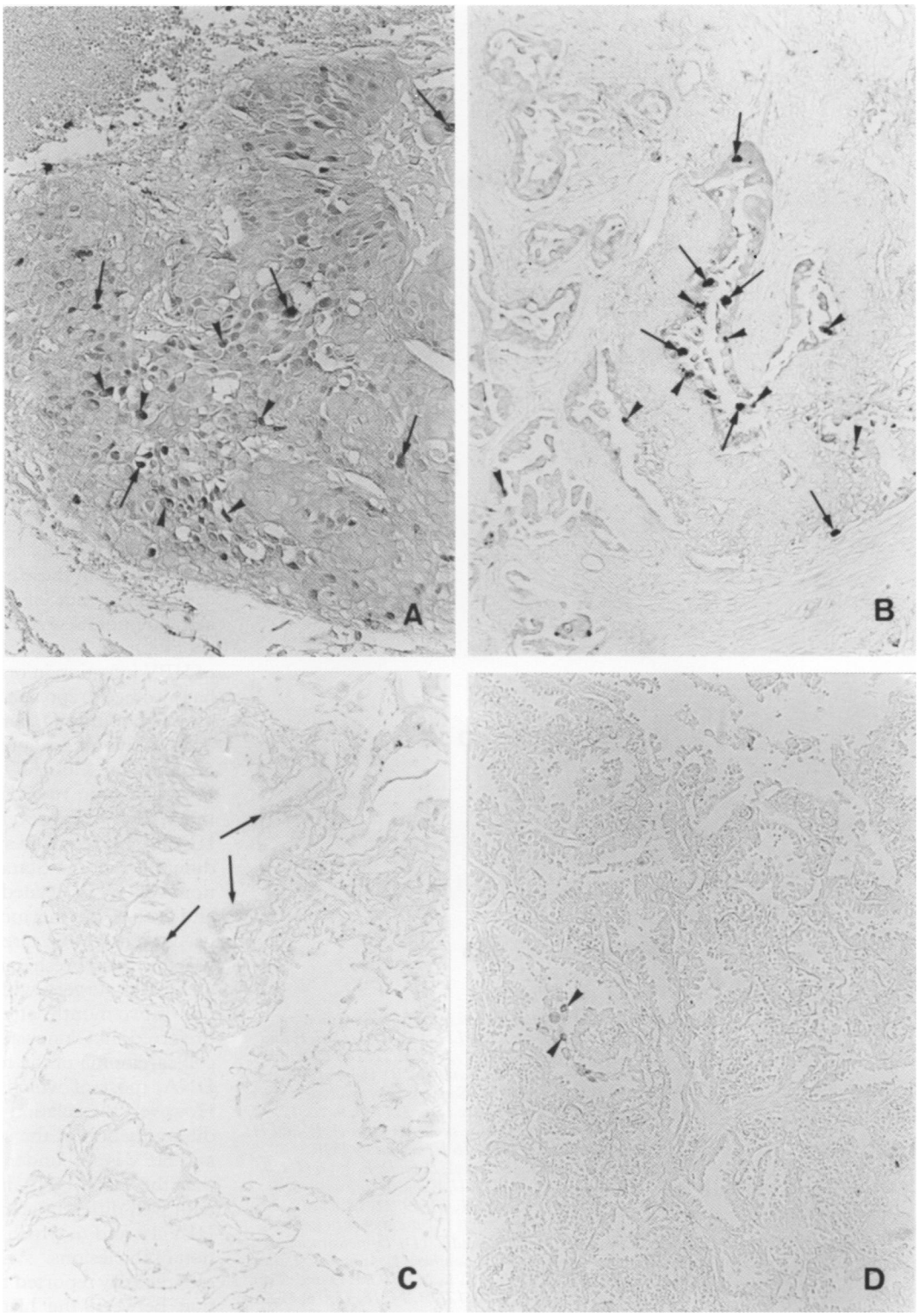

Figure 3 (A) Demonstration of human papillomavirus (HPV) DNA in the squamous cell carcinoma component by non-isotopic in situ hybridisation (NISH). Signals of HPV 16 DNA are found on the nuclei of squamous cell carcinoma components. Arrows: episomal forms; arrowheads: integrated form. (Case No 3, ×123.) (B) Demonstration of HPV DNA in the enlarged adenocarcinoma cells by NISH. Signals of HPV 16 are found on the nuclei. Arrows: episomal forms of HPV DNA; arrowheads: integrated forms of HPV DNA. (Case No 3, ×123.) (C) Demonstration of HPV DNA by NISH. HPV DNA was not demonstrated on either the surrounding non-tumour regions or in bronchial epithelium (arrows) (Case No 2, ×123.) (D) Demonstration of HPV DNA by NISH. No positive signal was found on the control adenocarcinoma cases. This case was a 55 year old non-smoking male. The tumour was 1.2 × 2.8 cm in diameter and located at right upper lobe (S3). Arrowheads: carbon laden macrophages. (×123.)

tive for HPV DNA by NISH, and for high molecular weight cytokeratin and involucrin antibodies.

The enlarged adenocarcinoma cells adjacent to the squamous cell carcinoma component showed an irregular papillotubular structure, into which squamous cell carcinoma components often protruded. This is considered to be an event marking the transition from adenocarcinoma to squamous cell carcinoma. Many scenarios for the development of adenosquamous carcinoma have been reported,[8] including the possibility of adenocarcinoma with squamous metaplasia, high grade mucoepidermoid carcinoma, and a bipotential undifferentiated cell origin. On the basis of the present results, we

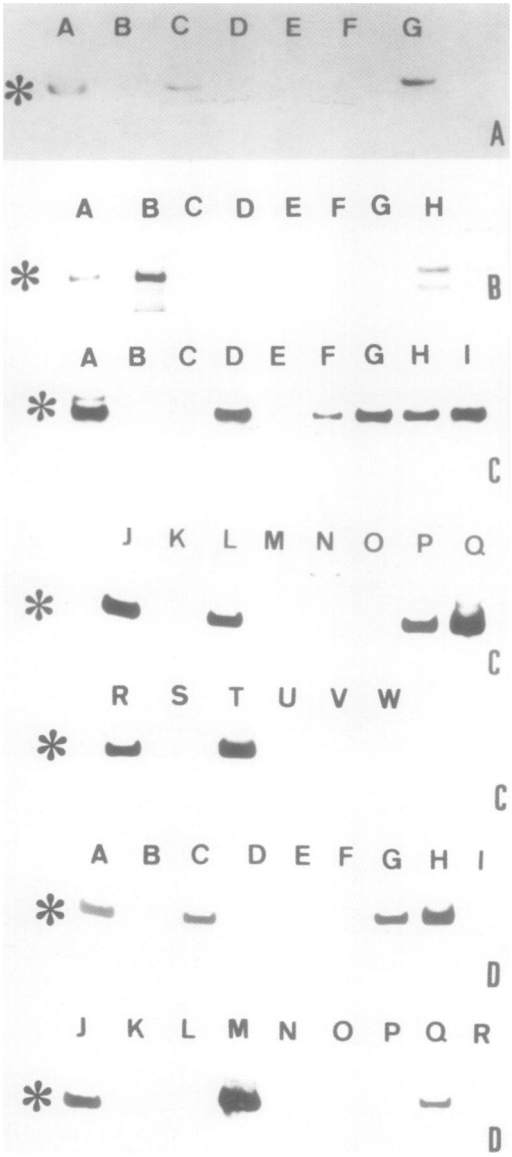

Figure 4 (A) Demonstration of HPV 6 E6 region by PCR, Southern blot analysis. Lanes A, B, C, D, E, F, and G are cases 16, 17, 19, 20, 21, 23, and 22; 189 bp bands (*) are shown in lanes A, C, and G. (B) Demonstration of HPV 11 E6 region by PCR, Southern blot analysis. Lanes A, B, C, D, E, F, G, and H are cases 7, 11, 8, 9, 10, 12, 13, and 15; 230 bp bands (*) are shown in lanes A, B, and H. (C) Demonstration of HPV 16 E7 region by PCR, Southern blot analysis. Lanes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, and W are cases 3, 4, 15, 5, 16, 6, 7, 8, 9, 10, 18, 11, 13, 19, 21, 12, 15, 17, 20, 22, 23, 1, and 2; 171 bp bands (*) are shown in lanes A, D, F, G, H, I, J, L, P, Q, R, and T. (D) Demonstration of HPV 18 E7 region by PCR Southern blot analysis. Lanes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q and R are cases 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 18, 15, 16, 17, 20 and 21; 152 bp bands (*) are shown in lanes A, C, G, H, J, M, and Q.

postulate that adenocarcinoma cells are infected with HPV, transform to enlarged cells expressing high molecular cytokeratin and involucrin, and progress to squamous metaplasia. However, it remains to be clarified whether HPV infection occurs before or after the squamous cell component develops. Small foci of squamous components in adenocarcinomas were positive for HPV. The enlarged adenocarcinoma cells adjacent to squamous cell carcinoma components were also positive for HPV by NISH. Furthermore, squamous metaplasia is induced by transfection of HPV into cultured adenocarcinoma cell lines (DLD-1, moderately differentiated adenocarcinoma of the colon, and PC-14, poorly differentiated adenocarcinoma of the lung).[12] In addition, using the reverse transcription polymerase chain reaction (RT-PCR),[12] HPV mRNA (E6 and E7) was detected from fresh samples of cases 2 and 8, from which adenocarcinoma and squamous cell carcinoma components were demonstrated at biopsy (data not shown). The remaining 21 cases were not examined by RT-PCR because fresh samples were not obtainable. However, HPV is considered to be play a role in the development of the tumours. Sun *et al* also reported squamous metaplasia caused by HPV 16 in normal uterine endocervical cells.[19] We therefore postulate that adenosquamous carcinoma might be induced by HPV infection. On the other hand, 15 cases in the present study were heavy smokers, of whom three were negative for HPV DNA and three from Kumamoto prefecture were also negative for HPV. It has been reported that smoking causes squamous metaplasia of the bronchial epithelium[20 21]; thus smoking needs to be taken into account in these cases.

The incidence of HPV DNA in squamous cell carcinoma varies significantly in different geographical regions. A high prevalence (76%) of HPV infection in oral epidermoid carcinoma has also been reported from Taiwan,[22] the nearest neighbour to Okinawa. HPV DNA has often been shown in oesophageal squamous cell carcinoma in China,[23] South Africa,[24 25] and Portugal,[26] but rarely in mainland Japan[27 28] or the USA.[29 30] However, the sensitivities of the HPV DNA detection systems have varied in the different reports. Standardisation of the detection system is needed, including the primers and probes. Furthermore, when old samples are used the detection rate of HPV DNA decreases year on year. Fresh samples obtained immediately after surgery without fixation, or at most one or two months after surgery, are much to be preferred. In Okinawa, many cases of squamous cell carcinoma of the lung are positive for HPV DNA, most of which are well differentiated.[2] However, the relation between the histological differentiation of the squamous cell carcinoma and HPV is still under discussion. It is reported that the HPV DNA is significantly associated with well differentiated carcinoma, particularly HPV 16 and 6, which results in the keratinisation of the lesions.[2 31] On the other hand, Suzuk *et al* recently reported that there was no correlation between the histological features of the tumour and the presence of viral sequences.[30] These contradictory results might in part be caused by the different detection systems and condition of the samples used.

Seven cases in our study were positive for two types of HPV DNA. The copy number of one type is high and of the other low. The second type of HPV detected might be a superimposed infection. In HPV DNA transfection experiments, the copy number of superimposed HPV transfection in cells which are already transfected with another type of HPV is indeed usually very low.[12] Nevertheless, such superimposed infection might influence the histological differentiation of the tumours, as previously reported.[2] Furthermore, adenocarcinoma with

small foci of squamous cell carcinoma showed similar immunohistochemical characteristics and HPV DNA detection rate to the adenosquamous carcinoma. Based on these results we believe that the histological criteria for adenosquamous carcinoma proposed by Takamori *et al*[8] and Colby *et al*[4] seem reasonable. However, the clinical course of the present cases has only been observed over a short period (January 1995 to June 1997), and 22 cases are still alive. The prognosis of the tumour needs to be examined further.

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