Human papillomavirus DNA in adenosquamous carcinoma of the lung

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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.

Keywords: human papillomavirus; adenosquamous carcinoma; polymerase chain reaction

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. 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 and other countries. 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.

Vincent et al reported that in the 1960s squamous cell carcinoma was the most common tumour, representing about 50% of lung cancer cases. 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. 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. 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). Ishida et al also reported a similar frequency rate of 1.8% in Fukuoka, mainland Japan. In the USA, Fitzgibbons and Kern 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, 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 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. 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, 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 adenosquamous carcinoma with small foci of the squamous carcinoma component (less than 20%) were also examined. The present cases also fulfilled the criteria of Fitzgibbons and...
Tsuhako, Nakazato, Hirayasu, et al were no miners or heavy industry workers. Radiation or chemotherapy before surgery. Most non-smoker. From two male heavy smokers and one female smoker, but the others were all heavy smokers. The three adenosquamous carcinomas were located centrally, but the other seven cases were in peripheral regions. The one female case was a non-smoker, but the others were all heavy smokers. All except one of the squamous cell carcinoma cases were male. One moderately differentiated, four poorly differentiated, and four moderately differentiated, and four poorly differentiated) and three cases of adenosquamous carcinoma (adenocarcinoma component well differentiated; squamous cell carcinoma component moderately differentiated) were 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, Kumamoto 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.

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. 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) 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, Kumamoto 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.

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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) 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 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. H2O2 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. 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 MgCl2, 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 parts of 2 mg/ml in 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.4 mM each of 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 MgCl2, 0.01% gelatin, 0.4 mM each of primers of 110 base pair (bp) long.
Table 1 Primers and probes

<table>
<thead>
<tr>
<th>HPV 6 E6</th>
<th>Primer</th>
<th>Sense: 5'-GCTGGGATCTGACACGTG-3'</th>
<th>Antisense: 5'-CATGGCTGGTCAAGAGATG-3'</th>
<th>Probe: 5'-GCTACCGTGTCTGCAGACCGG-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16 E6</td>
<td>Primer</td>
<td>Sense: 5'-GCTGGGATCTGACACGTG-3'</td>
<td>Antisense: 5'-CATGGCTGGTCAAGAGATG-3'</td>
<td>Probe: 5'-GCCACTGCTGGTCAGACCGG-3'</td>
</tr>
<tr>
<td>HPV 18 E6</td>
<td>Primer</td>
<td>Sense: 5'-GCTGGGATCTGACACGTG-3'</td>
<td>Antisense: 5'-CATGGCTGGTCAAGAGATG-3'</td>
<td>Probe: 5'-CCACTGCTGGTCAGACCGG-3'</td>
</tr>
</tbody>
</table>

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

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 unresctable 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 28) was of tubular type. The squamous cell carcinoma component was well or moderately differentiated, showing cellular keratinisation and intercellular bridges. In the
Table 4 Sequence of the PCR amplified DNA (HPV 18 E7 region)

<table>
<thead>
<tr>
<th>Case</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
<th>Predominant component</th>
<th>Detection of HPV by PCR†</th>
<th>NISH‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>Well</td>
<td>Moderate</td>
<td>Equal mixture</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Well</td>
<td>Well</td>
<td>SCC</td>
<td>11,16</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Moderate</td>
<td>Well</td>
<td>Equal mixture</td>
<td>16,18</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Moderate</td>
<td>Well</td>
<td>Equal mixture</td>
<td>16,18</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>11,16</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>16,18</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Equal mixture</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>Well</td>
<td>Well</td>
<td>SCC</td>
<td>11,16</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Well</td>
<td>Well</td>
<td>Adenocarcinoma</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Well</td>
<td>Well</td>
<td>Adenocaricnom</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>22</td>
<td>Well</td>
<td>Moderate</td>
<td>Adenocarcinoma</td>
<td>6,16</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>Moderate</td>
<td>Well</td>
<td>Equal mixture</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Cases are numbered in order of the surgical procedures.

†HPV types detected.

‡Episomal and integrated forms of HPV were demonstrated on the squamous cell carcinoma component, as were enlarged adenoacanthoma cells adjacent to the squamous cell carcinoma component.

As reported previously, 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, lower limits of 85, 740, 55, and 7700 viral copies, respectively, were detected. 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 mm² of the tumour tissue, while the second type was detected at a much lower level (less than 200 copies/10 mm²). 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 adenoacanthomas from Okinawa, HPV DNA was not detected by PCR (data not shown). Four of five Okinawan adenoacanthomas with small focus of squamous cell carcinoma components, and two of 10 squamous cell carcinoma components from Kumamoto, were positive for HPV DNA by PCR and NISH (data not shown).

Sequence variants were 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.
Discussion
In this study we employed the General Rules for Clinical and Pathological Records of Lung Cancer published by the Japan Lung Cancer Society. 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
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-
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, 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...
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). In addition, using the reverse transcription polymerase chain reaction (RT-PCR), 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. We therefore postulate that adenocarcinoma cells are infected 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, 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, the near-est neighbour to Okinawa. HPV DNA has often been shown in oesophageal squamous cell carcinoma in China, South Africa, and Portugal, but rarely in mainland Japan or the USA. 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. 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. On the other hand, Suzuki et al recently reported that there was no correlation between the histological features of the tumour and the presence of viral sequences. 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. Nevertheless, such superimposed infection might influence the histological differentiation of the tumours, as previously reported. 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. and Colby et al. 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.