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Human Papillomavirus DNA as a Possible Index of Invasiveness in Female Genital Tract Carcinomas

Maria Luisa Marcante and Aldo Venuti

Paraffin-embedded tumour sections were used for the polymerase chain reaction (PCR) with three primer sets that amplify specific regions of human papillomavirus (HPV) types 11, 16 and 18. The positive samples were confirmed by hybridisation of the amplified sequences with the specific HPV probes. In all screened metastases the same viral sequences were found as in the primary tumour. HPV 16 was the most frequently detected virus in genital tract tumours. In a metastatic ovary carcinoma with unknown primary site HPV 16 DNA was observed. Moreover, pelvic lymph nodes with no microscopic evidence of metastases contained HPV DNA of the same subtype as the primary tumour. Thus, the HPV DNA detected by PCR is a useful indicator of neoplastic cells in the earlier stages of invasiveness. The finding of specific HPVs in the metastatic lesions could also provide information about the location of the primary neoplasia.
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INTRODUCTION

HUMAN PAPILLOMARVIRUSES (HPV) have been associated with tumours of different sites. More than 60 HPV types have been defined but only a few types, mostly 16, 18 and 33, have been detected in squamous carcinoma from anogenital or oropharyngeal sites, whereas other types, such as 6 and 11 have been found in benign lesions [1–3]. The data on the presence of HPV in tumours have been obtained by Southern or dot-blot techniques and *in situ* DNA hydridisation. The first two techniques have provided evidences for an HPV role in premalignant and malignant lesions [4]. *In situ* hybridisation has revealed, in particular, the correlation between cytological morphology and the presence of HPV genome, and it has also facilitated the retrospective studies of stored samples [5–7]. All of these methods, however, have several limitations due to the low sensitivity of *in situ* techniques in high-grade lesions and to the difficulties of correlating the histological findings with Southern or dot-blot results.

The polymerase chain reaction (PCR) [8] has enhanced the detection of HPV DNA and RNA in clinical specimens and has improved the diagnostic possibilities. To evaluate the presence

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Table 1. *Oligonucleotide primers*

HPV type	Sequence (5′ → 3′) of E6 ORF	Size of amplified product (base pair)
11	AAAGATGCCTCCACGTCTGC ATTGGTTAATTTTCCCTTGC	220
16	TGCAATGTTTCAGGACCCAC CTCTATATACTATGCATAAA	170
18	ATCCCACACGGCGACCCTAC CAAATACCTCTGTAAGTTCC	120

of HPV as an indicator of neoplasia, primary and metastatic tumours were investigated by PCR adapted to paraffin-embedded tissues [9].

MATERIALS AND METHODS

After surgical removal, the specimens were immediately frozen in liquid nitrogen and stored at −80°C. Paraffin-embedded sections (5 μm) were processed essentially as described by Shibata *et al.* [9]. The section was placed in a microcentrifuge tube and deparaffinised by sequential washings with xylene, ethanol and acetone. The samples were air-dried, resuspended in 100 μl of *Taq* polymerase reaction mixture and heat-denatured.

DNA extracted from frozen biopsy samples was immobilised on a nylon membrane [10]. Probes were subgenomic region DNA of HPV 16 and 18 labelled with ^{32}P-dCTP by a random-primed DNA labelling kit (Boehringer Mannheim) (2.5 M), Bq/μg. Hybridisations and washings were carried out at high stringency (T_m −18°C).

The primers shown in Table 1 were synthesised on a DNA synthesiser at the Beatson Insitute for Cancer Research, Glasgow. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and elution of the resolved band. The PCR reaction buffer was 10 mmol/l Tris–HCl pH 9.0, 0.01% gelatin, 10 mmol/l $MgCl_2$, 0.25 mmol/l of each deoxynucleoside triphosphate and 1 μmol/l of each primer. The samples previously overlaid with paraffin oil were subjected to 30 cycles of amplification. Each cycle was 95°C for 30 s (denaturation), 49°C for 30 s (annealing) and 72°C for 2 min (extension). 2.5 U *Taq* polymerase were used in a 100 μl reaction mixture. One-tenth of each sample was processed by 2% agarose gel and dot-blot hybridisation. An example of PCR analysis is shown in Fig. 1.

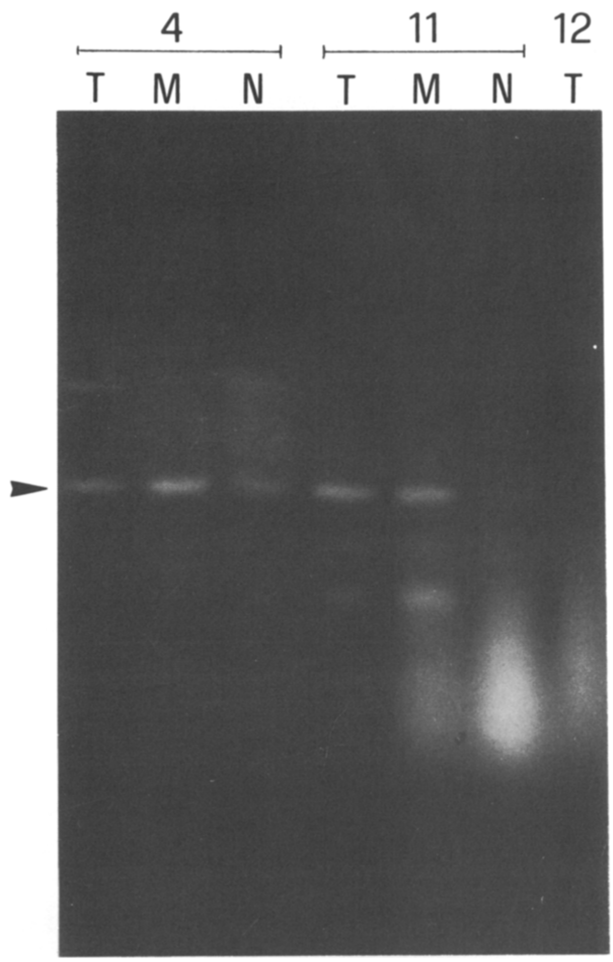

Fig. 1. PCR analysis for HPV sequences on primary and metastatic tumour DNAs. Numbers over lanes are patients as in Table 3. Arrow indicates position of amplified DNA sequences of HPV 16. T = tumour, M = metasatic lymph nodes and N = histologically normal lymph nodes.

RESULTS

Selection of primers

Analysis of several frozen biopsy samples from genital tract tumours [11] revealed that HPV genomic sequences were present in the neoplastic tissue, mostly integrated in the cell genome. Interestingly, the sequences corresponding to the E6–E7 regions were always detected, even in the occurrence of deletions in the virus genome as shown by the lack of hybridisation with E2/L1 subgenomic probes (Table 2). Thus we selected a series of primers corresponding to the E6 region, which amplify sequences of different length for each HPV tested (Table 1).

Detection of HPV by PCR in primary and metastatic tumours

PCR analysis of tumours from different paraffin-embedded tissues (Fig. 1) revealed that HPV 16 and 18 DNA sequences were present in over 90% (14/15) of the genital tract carcinomas. Type 16 was the most frequently found (Table 3.)

Positivity of the primary tumour corresponded in the metastatic locations in almost all the cases (Table 3). The only metastatic lymph node that was negative in PCR was case 12, in which we failed to demonstrate HPV sequences even in the primary location. A metastatic ovary carcinoma (case 2) with unknown primary location was positive for HPV 16.

In the extragenital tumours, the presence of HPV 16 was limited to tongue (1/2), breast (1/3) and finger (2/2) (Table 4).

HPV 16 sequences as index of earlier stages of invasiveness

Pelvic lymph nodes from 5 patients, clinically suspected and thereafter found microscopically negative, have also been

Table 2. *HPV subgenomic sequences in genital neoplasms*

		HPV			Integration	
		None	E6-E7	E2-L1	Yes	No
Histology						
Squamous cell carcinoma	(n = 22)	2	20	3	19	1
Paget's carcinoma	(n = 1)	—	1	—	1	—
Bowenoid lesion	(n = 1)	—	1	1	1	—

Table 3. Detection of HPV in primary and metastatic genital carcinomas

Patient	Metastatic location	HPV 11	HPV 16	HPV 18	Primary location	HPV 11	HPV 16	HPV 18
1	Pelvic LN	—	+	—	Vulva	—	+	—
2	Ovary	—	+	—	Unknown	ND	ND	ND
3	Pelvic LN	—	—	+	Vulva	—	—	+
4	Pelvic LN	—	+	—	Vulva	—	+	—
5	Pelvic LN	—	+	—	Vulva	—	+	—
6	Pelvic LN	—	+	—	Vulva	—	+	—
7	Pelvic LN	—	+	—	Vulva	—	+	—
8	Pelvic LN	—	+	—	Vulva	—	+	—
9	Pelvic LN	—	+	—	Vulva	—	+	—
10	Pelvic LN	—	+	—	Cervix	—	+	—
11	Retroperit-oneal LN	—	+	—	Cervix	—	+	—
12	Pelvic LN	—	—	—	Vulva	—	—	—
13	Pelvic LN	—	+	—	Vulva	—	+	—
14	ND	ND	ND	ND	Penis	—	+	—
15	Pelvic LN	—	—	+	Cervix	—	—	+

LN = lymph-node, ND = not done.

analysed. In 2 metastases-free lymph nodes, HPV 16 sequences corresponding to the transforming E6 region were found. The same sequences were also present in the primary tumour (vulva and cervix, respectively). The other primary tumours (all vulval) were HPV 16 positive. None of the lymph nodes or primary specimens were positive for HPV 11 or 18.

DISCUSSION

An interesting point about the association of HPV with carcinoma is the integration of viral genome into the host DNA [12–14]. The analysis of our frozen biopsy samples has confirmed these data but also stressed the necessity of using specific probes

Table 4. Detection of HPV in primary and metastatic extragenital tumours

Patient	Metastatic location	HPV 11	HPV 16	HPV 18	Primary location	HPV 11	HPV 16	HPV 18
16	Retroperit-oneal LN	—	—	—	Kidney	—	—	—
17	Cervical LN	—	—	—	Thyroid	—	—	—
18	Cervical LN	—	+	—	Tongue	—	+	—
19	ND	ND	ND	ND	Brain	—	—	—
20	Cervical LN	—	—	—	Tongue	—	—	—
21	Retroperit-oneal LN	—	—	—	Kidney	—	—	—
22	Lung	—	—	—	Liver	—	—	—
23	Axillary LN	—	—	—	Breast	—	+	—
24	Cervical LN	—	—	—	Vocal chords	—	—	—
25	ND	ND	ND	ND	Breast	—	—	—
26	Cervical LN	—	—	—	Oesophagus	—	—	—
27	ND	ND	ND	ND	Finger	—	+	—
28	Cervical LN	—	—	—	Thyroid	—	—	—
29	ND	ND	ND	ND	Breast	—	—	—
30	Cervical LN	—	—	—	Larynx	—	—	—
31	ND	ND	ND	ND	Finger	—	+	—

of a conserved region. The integration of viral genome could occur in a downstream region of the E6/E7 regions and could cause deletion and rearrangement. In our samples these deletions seem to involve mostly the E2/L1 regions, but the E6/E7 regions are always retained in the host genome [12,15,16] . For this reason we used oligomers of the E6 region designed specifically to amplify and detect HPV 11, 16 and 18 in tumours with different locations and tissue origin by agarose gel or dot-blot hybridisation with radiolabelled HPV sequences.

The sensitivity of our procedure was determined by the analysis of serial dilutions of HeLa and SiHa cells (data not shown). In our conditions, PCR detected 10–20 copies of HPV, as already reported by Shibata *et al.* [9].

HPV occurrence, at least for the screened HPV types, was limited only to tumours of stratified epithelia located mostly, but not exclusively, in the genitalia. The presence of HPV 16 in finger and oropharngeal carcinoma has been reported [17–19]. So far, positivity in breast carcinoma has not been reported, although keratinocytes isolated from mammary tissue could be efficiently immortalised by HPV 16 [20]; our findings agree with these experimental results.

Our data indicate that HPV sequences are a reliable pointer for neoplastic cells because over 90% of metastases were positive by PCR and, in 1 case, the same integration was found in the primary and metastatic location (data not shown). The high concordance between tumours and metastases for HPV DNA could reflect some contamination during surgical manipulations; but, if this were so, all the tissues removed during operation would be positive. On the contrary, normal tissues surrounding the tumours (cases 5 and 13) were negative in PCR (data not shown).

Even a metastasis with unknown primary location was scored positive and therefore HPV DNA could also be useful in the study of cases in which it is hard to define the site of the primary lesion.

Thus, the presence of sequences of particular types of HPV in the neoplastic cells could permit the definition of an epithelial origin even in poorly differentiated tumours, and eventually the location of the primary lesion, because over 90% of genital tumours were positive to HPV 16 and 18.

Others [21] have revealed HPV sequences by PCR in a limited number of metastatic lymph nodes but positivity in metastasis-free lymph nodes has not been reported. Our data indicate that PCR can detect carcinoma cells that have just crossed regional lymph nodes.

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Diagnostic Accuracy of Combination of Assays for Immunosuppressive Acidic Protein and Carcinoembryonic Antigen in Detection of Recurrence of Gastric Cancer

Norio Shimizu, Hiroshi Yamashiro, Atsunobu Murakami, Ryuichi Hamazoe and Michio Maeta

Two tumour markers, immunosuppressive acidic protein (IAP) and carcinoembryonic antigen (CEA), were assayed in gastric cancer patients. Levels of IAP and CEA were measured simultaneously in the preoperative and postoperative periods. The usefulness of the combined assay of these markers for detection of recurrence of cancer was investigated in terms of sensitivity, specificity and diagnostic accuracy. Sensitivity was not high (69.2%), but specificity and diagnostic accuracy were 96.7% and 86.9%, respectively. In cases with metastases in the liver, sensitivity (100.0%), specificity (100.0%) and diagnostic accuracy were high. In cases of peritoneal dissemination, these indices were low. The combination assay of IAP and CEA appears to be useful for detection of recurrence of gastric cancer, especially in patients with liver metastases.
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INTRODUCTION

Many tumour markers have been used to detect malignancies, to predict staging or prognosis, to estimate the effects of treatment and to detect recurrence [1–5]. Carcinoembryonic antigen (CEA) has generally been used to predict the stage or prognosis of colorectal cancer and to detect recurrence. We have used CEA as a marker for gastric cancer [6]. Immunosuppressive acidic protein (IAP) was first found by Tamura *et al.* [7]. It is an α-1 acid glycoprotein and has been used as a marker for various malignancies, (e.g. gynaecological [8], testicular [9], colorectal [10], pancreatic and choledochal [10], and gastric cancers [11]). We have measured plasma levels of CEA and IAP in patients with gastric cancer and investigated the usefulness of these tumour markers for the detection of gastric cancer and of recurrence.

PATIENTS AND METHODS

Plasma levels of IAP and CEA were measured simultaneously in 349 patients with gastric cancer admitted to the Hospital of