Infection of Human Papillomavirus (HPV) and p53 Over-Expression in Human Female Genital Tract Carcinoma

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Abstract

Inactivation of p53 gene products either by mutation or by complex formation with E6 oncoprotein encoded by high risk HPV appears to be a common event in cervical carcinogenesis. This study was designed to clarify this association in 41 primary cervical, 15 endometrial, 3 ovarian and one rectal carcinomas. Polymerase chain reaction analysis revealed presence of high risk HPV in 36 (88%) cervical, S (33%) endometrial and none of ovarian and rectal carcinomas. HPV 16 was found in 14 cervical carcinomas, HPV 18 in 19 cervical and 2 eridometrial carcinomas and HPV 33 in 28 cervical and 5 eridometrial carcinomas. Expression of tumorsuppressor protein p53 by using polyclonal antibody CM-I, was detected in 28 (68%)cervical, 7 (47%) endometrial, 2 (66%) ovarian and one (100%) rectal carcinoma. Twenty six cervical and 3 endometrial carcinoma cases were positive for both high risk HPV and p53. We conclude that beside cervical carcinoma HPV infection is not uncommon in endometrial carcinoma and in our experimental design there is no inverse correlation between HPV infection and p53 over-expression in a variety of the tumors analysed in the present study (JPMA 46:220,1996).

Introduction

HPV are associated with benign and malignant lesions of aero- digestive and ano-genital tract[1]. High risk HPV 16,18 and 33 have been consistently found in cervical, vulval and vaginal malignancies[1], beside their occasional and controversial presence in the higher female genital tract, like endometrium and ovary[2-5]. In most of the tumors HPV DNA is integrated and this phenomenon results in the deletion of large regions of the virus, but consistently leaves theE6 and E7 open reading frame intact[6]. Furthermore, E6 and E7 genes of high risk }{PV have been shown to immortalize human foreskin, cervical keratinocytes and even epithelial breast cells in tissue culture[6]. More recently it has been demonstrated that the.E6 and E7 gene products from high risk HPV bind p53 and p105-RB tumor suppressor gene products respectively[6].
Cervical cell line studies and analysis of limited number of cervical carcinoma specimens suggest that in cervical carcinoma p53 is either inactivated by complexing with high risk HPV E6 protein in HPV positive tumors or by p53 mutation in those that are HPV negative[7,8]. Previously, we have analyzed Iaiyngeal carcinomas for HPV and p539,10 and could not find the association suggested in the cervical carcinomas by other investigators[7,8] ‘ The present study analyzed a variety of cervical, endometrial and a few ovarian and one rectal carcinoma, to find HPV prevalence in these tumors and to correlate the presence or absence of HPV infection with p53 mutation which was analyzed by immuno-histochemistiy.

Material and Methods

A variety of cervical carcinoma specimens from 41 patients with mean age of 51 years (ranging from 26 to 83), endometrial carcinomas specimens from 15 patients with mean age of 58 years (ranging from 31 to 83), ovarian carcinoma specimens from 3 patients with mean age of 62 years (ranging from 60 to 69 years) and rectal carcinoma specimen from one patient of 60 year age were obtained from Pathology Laboratory of Fukui Medical School Hospital and various private hospitals scattered in Mic prefecture Japan. Staging and nistological classification were based on The General Rules for Cervical, Endometrial and Ovarian Cancer Study established by Japanese Research Society for Cervical, Endometrial and Ovarian Cancer[11]. All tissues had been fixed in 10% buffered formaline and embedded in paraffin. The paraffin blocks used in this study were stored at room temperature for 1-4 years.

Recombinant viral DNAs of HPV 16, 18 and 33 were used as target DNA for PCR amplification with their specific primers in control experiments as described previously[9]. DNA extraction was carried out according to the previously described methods for formaline-fixed, paraffln embedded tissues[9]. Briefly, the sections were deparaffiiiized twice with xylene and twice with ethanol. The residual ethanol was removed by rinsing the samples with acetone and 100-200 ul of digestion buffer [50mM Tris-HCl (pH 8.5), I mM EDTA. 0.5% Tween 20 and 200 ug/ml of protcinase K] were added. The mixture was incubated at 37°C overnight and proteinase was inactivated at 95°C for 10 min. The genomic DNA from the cell lines used as positive control was prepared according to the established procedures[12].

Polymerase chain reaction (PCR) was performed as described before, with few minor modifications[9]. Briefly, 2-51.tl of the DNA solutions were used for PCR in 50 ul reaction mixture containing 10mM Tris-HCL (pH 8.8), 50mM KCI, 1.5 mM mgCl2 0.1% Triton X- 100, 80 uM of each dNTP (dATP, dCTp. dGTP and dTTP), 0.5 uM of each primer and 1.5 units of Lhe Taq DNA polymerase (Pro mega, Medison. WI). Template DNA was first denatured at 95°C for 8 min before addition of the Taq DNA polymerase at 72°C. This was followed by 35 cycles of PCR with incubation for I min at 60°C. (annealing), 1 min. at 72°C (polymerization) and 30 sec. at 95°C (denaturation). Negative controls were included in each set of reactions. As a positive amplification control, human beta globin genomic sequences were also amplified. The PCR reactions were segregated from post-PCR analysis and positive displacement pipettes and a UV-irradiated biohazard hood were used to eliminate possible contamination. Twelve ul of the PCR products were routinely checked for amplified DNA on 5%polyacrylamide gels.

Primers and probes for all HPV and beta-globin gene were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The sequences for HPV and beta-giobin gene primers and HPV probes were same as described before[11]. The oligonucleotide HPV probes were labelled by phosphoiylation with [y-32P1 ATP (specific activity >5000 Ci/mmol; 1 Ci= 37 GBq) (Amersharn, Tokyo, Japan) and T4 polynucleotide kinase (Takara Biomedicals). They were routinely purified by spun-column chro- matography through 1 ml of Sephadex 0-25 Medium (Pharmacia, LKB Biotechnology, Uppsala, Sweden). This procedure gave probes with specific activity of about 10^9 cpm/ug.

For detection of HPV, the preparation of filters and protocols forprehybridization, hybridization and washes were same as described[10]. However, the prehybridization and hybridization were extended to 3h and final stringent wash was performed at 65°C.

Alter washes, filters were dried and exposed to XAR film (Eastman Kodak Company, Rochester, NY) at -70°C for 1-12h. p53 staining was performed using a polyclonal antibody to p53 (CM 1) and a 3-layered immunoperoxidase method[10,13]. Briefly, 3-5 um thick sections from• paraffin blocks were dewaxed and after blocking endogenous petoxidase activity with 0.3% hydrogen peroxide and non-specific binding with 20% normal goat serum, these sections were incubated overnight at 4°C with the CM-i antiserum at the dilution of 1:1000. Biotinylated antirabbit imrnunoglobulins were followed by

peroxidase-labelled streptavidin The chromagen was osrnicated diaminobenzidine. The intensity and pattern of p53 iinmunostaining were tabulated according to criteria reported by Midgley et al. [13]; +++, more than 70% of tumor nuclei intensely stained; ++, between 10 and 70% tumor nuclei intensely stained; +, less than 10% of tumor nuclei intensely stained/or a variable number of tumor nuclei faintly stained; -, tumor nuclei unstained. Positive control comprised paraffin embedded specimen of a colon carcinoma previously shown to express p53 gene with activating point mutation and negative control consisted of replacement of the primaiy antibody with buffer/non-immune rabbit serum.

Results

The analysis included 41 patients with a variety of cervical carcinoma, 15 with endometrial carcinoma, 3 with ovarian carcinoma and one patient with rectal carcinoma for high risk HPV by PCR (Table I & Figure 1)

Table I. Frequency of HPV types in malignant tumors of female genital tract.

Type of tumor	Proportion of tumors positive for HPV	Frequency (%) of detection of HPV types* 16	18	33	HPV negative tumors
Cervical carcinoma	36/41	14 (34%)	19 (46%)	28 (68%)	05 (12%)
a. carcinoma in situ	11/12	07 (58%)	03 (25%)	09 (75%)	01 (08%)
b. Invasive squamous cell carcinoma	18/21	05 (24%)	10 (48%)	16 (76%)	03 (14%)
c. Adenocarcinoma	07/08	02 (25%)	06 (75%)	03 (38%)	01 (12%)
Endometrial carcinoma	05/15	0	02 (13%)	05 (33%)	10 (67%)
Ovarian carcinoma	00/03	0	0	0	03 (100%)
Rectal adenocarcinoma	00/01	0	0	0	01 (100%)

*15 cervical carcinomas and 5 endometrial carcinomas contained 2 types of HPVs and 5 cervical carcinomas harboured all three types of HPVs.

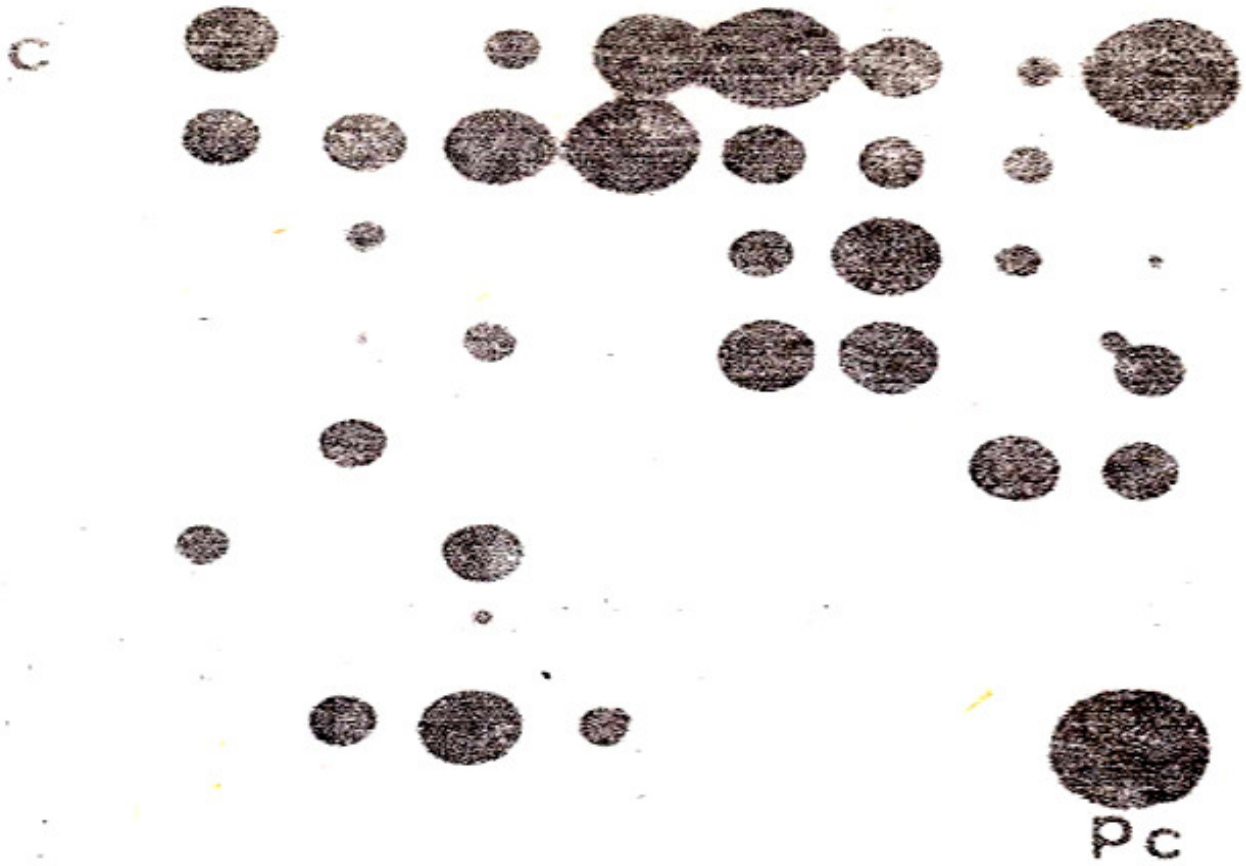

Figure 1. Detection of HPV 16, 18 and 33 sequences in 41 cervical, 15 endometrial, 3 ovarian and one rectal carcinoma specimens. The DNA from the carcinoma specimens and cloned HPV 16, 18 and 33 DNA as a positive control were amplified, spotted onto nylon filter and hybridized with P-labelled oligonucleotide probe specific for HPV 16 (a), HPV 18 (b) and HPV 33 (c)

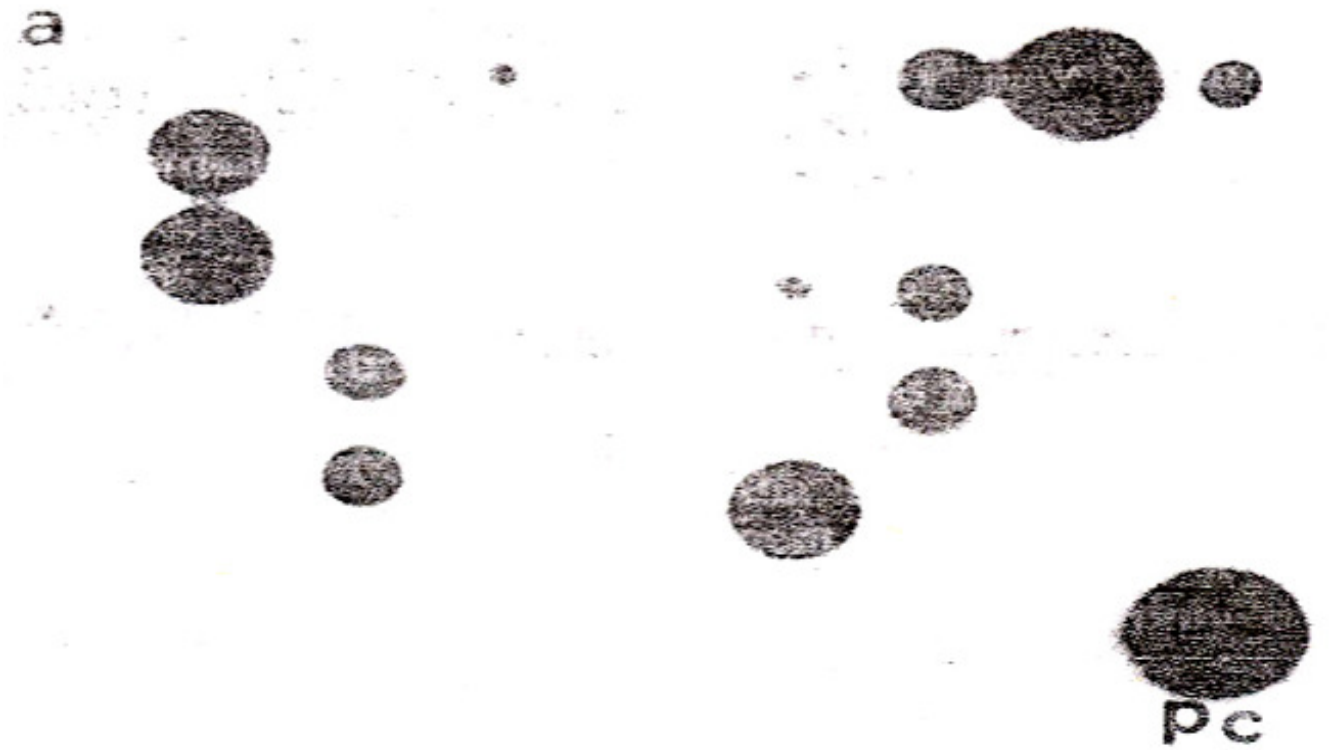

Figure 1 (a)

and p53 by immunohistochemistzy (Table II. Figure 2 and 3).

Table II. Over-Expression of p53 in malignant tumors of female genital tract.

Type of tumor	Total number of cases	Number of cases in relation to intensity and pattern of CM-1 staining*			
		-	+	++	++++
Cervical carcinoma	41	13 (32%)	14 (34%)	10 (24%)	04 (10%)
a. Carcinoma in situ	12	06 (50%)	04 (33%)	01 (08%)	01 (08%)
b. Invasive squamous cell carcinoma	21	04 (19%)	09 (43%)	07 (33%)	01 (05%)
c. Adenocarcinoma	08	03 (38%)	01 (13%)	02 (25%)	02 (25%)
Endometrial carcinoma	15	08 (53%)	01 (07%)	04 (27%)	02 (13%)
Ovarian carcinoma	03	01 (33%)	01 (33%)	01 (33%)	0
Rectal adenocarcinoma	01	0	01 (100%)	0	0

*The intensity and pattern of p53 immunostaining were graded as follows: +++, more than 70% of tumor nuclei intensely stained; ++, between 10 and 70% of tumor nuclei intensely stained; +, less than 10% of tumor nuclei intensely stained and/or a variable number of tumor nuclei faintly stained; -, tumor nuclei unstained.

Figure 2. Immunohistochemical analysis of a squamous cell carcinoma of cervix. (a) Section stained with H & E:;(b) the same section stained with CM-1 polyclonal anti-serum, demonstrating intense nuclear staining in less than 10% of neoplastic cells (+), for p 53 protein. (Original magnificationx300).

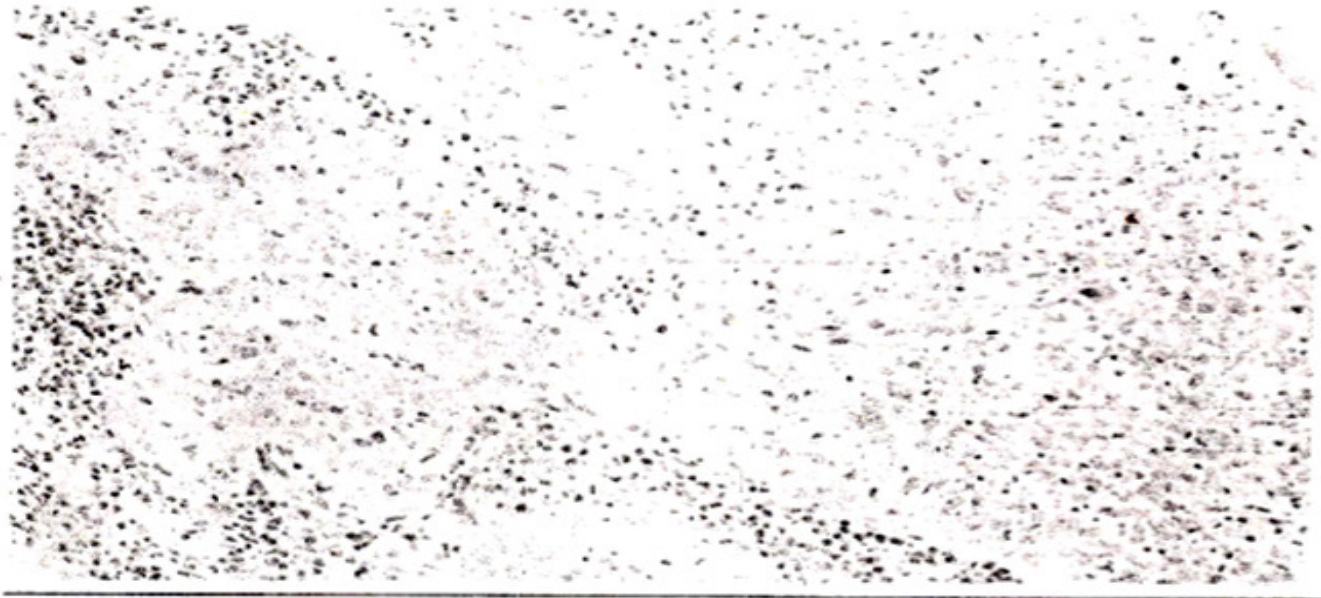

Figure 2 (a)

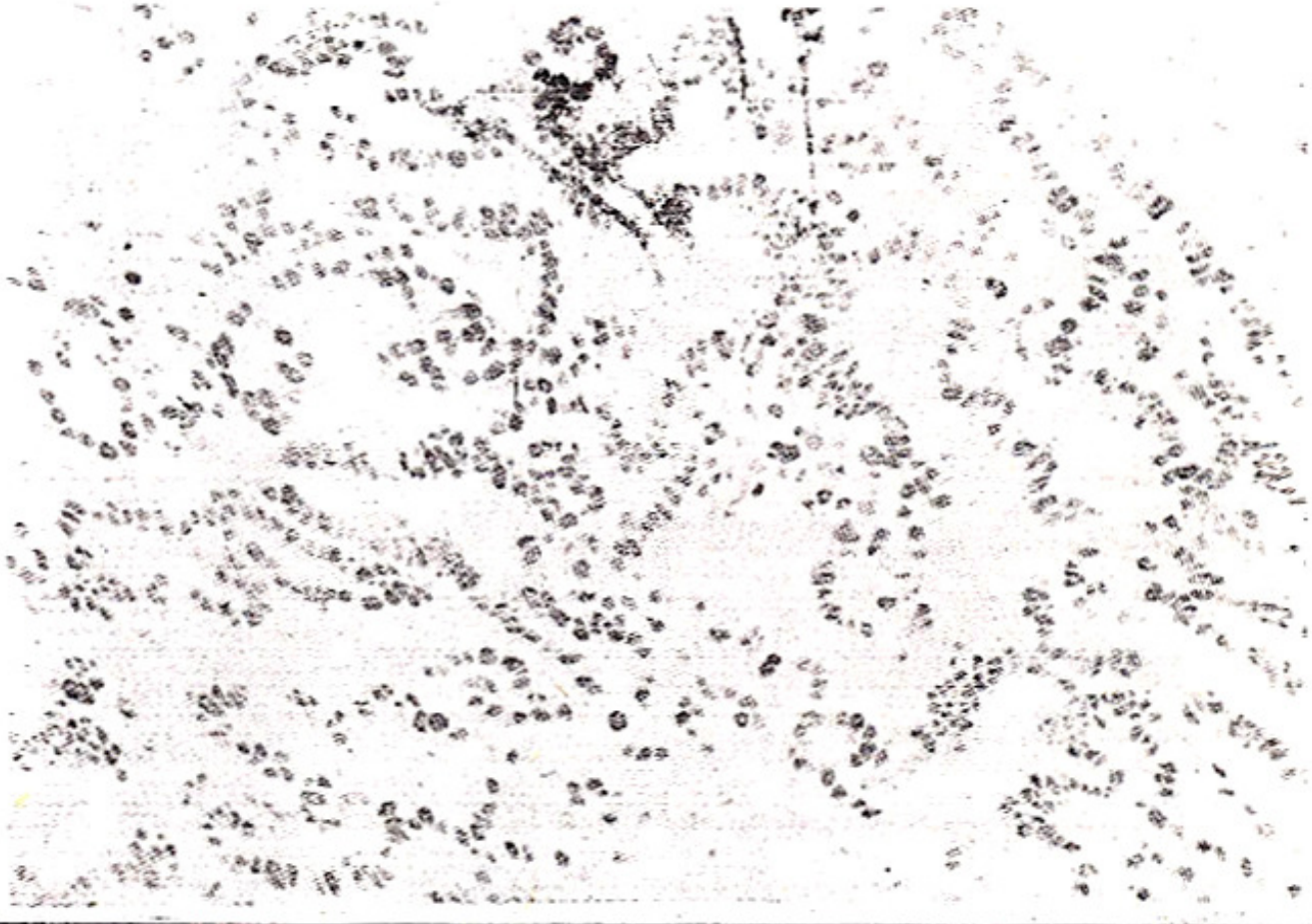

Figure 3: Immunohistochemical analysis of a endometrial adenocarcinoma (a) Section stained with H&E; (b) the same section stained with CM-1 polyclonal anti-serum, demonstrating intense nuclear staining in more than 70% of neoplastic cells (+++), for p53 protein. (Original magnificationx300).

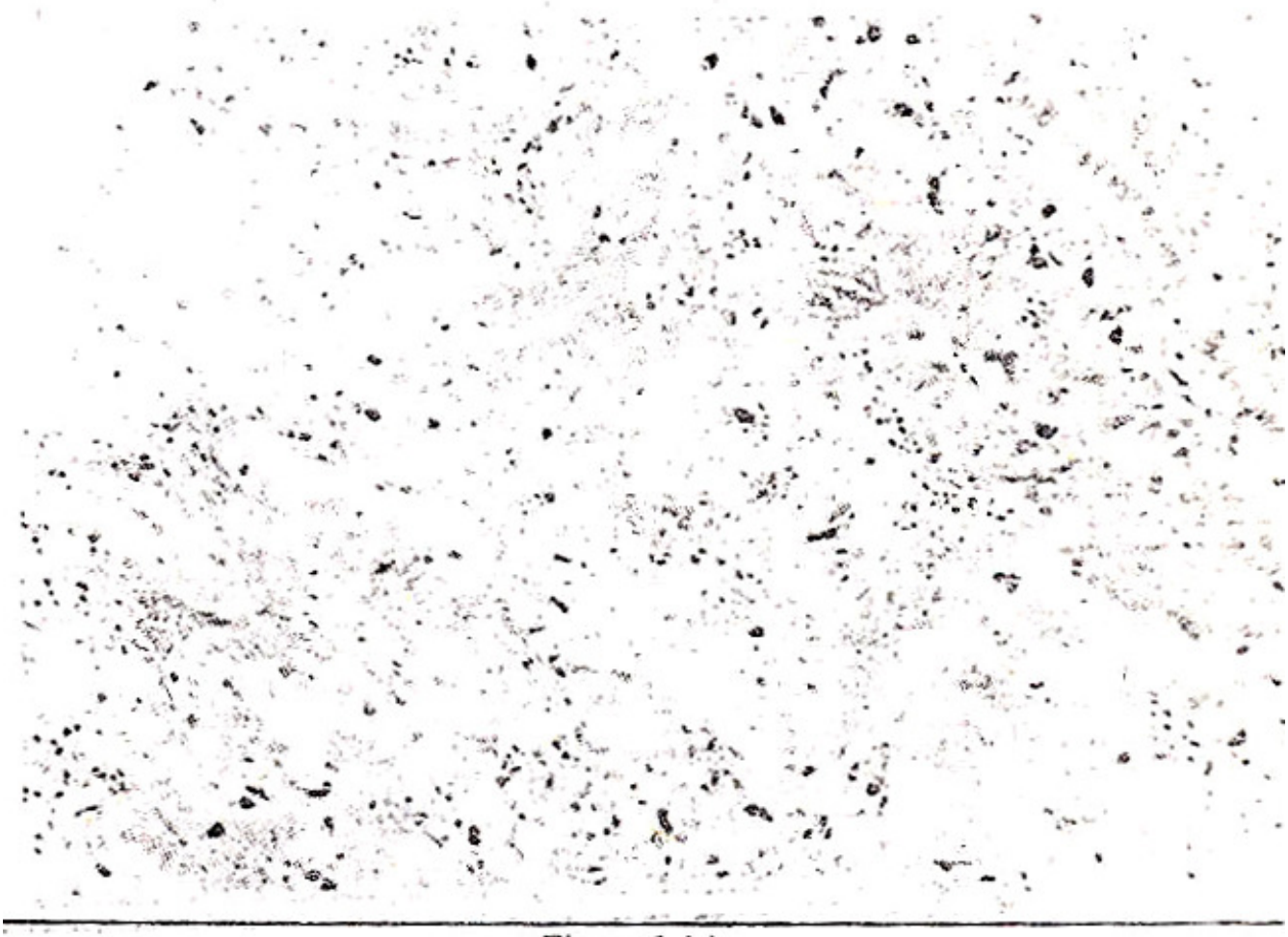

Figure 3 (a)

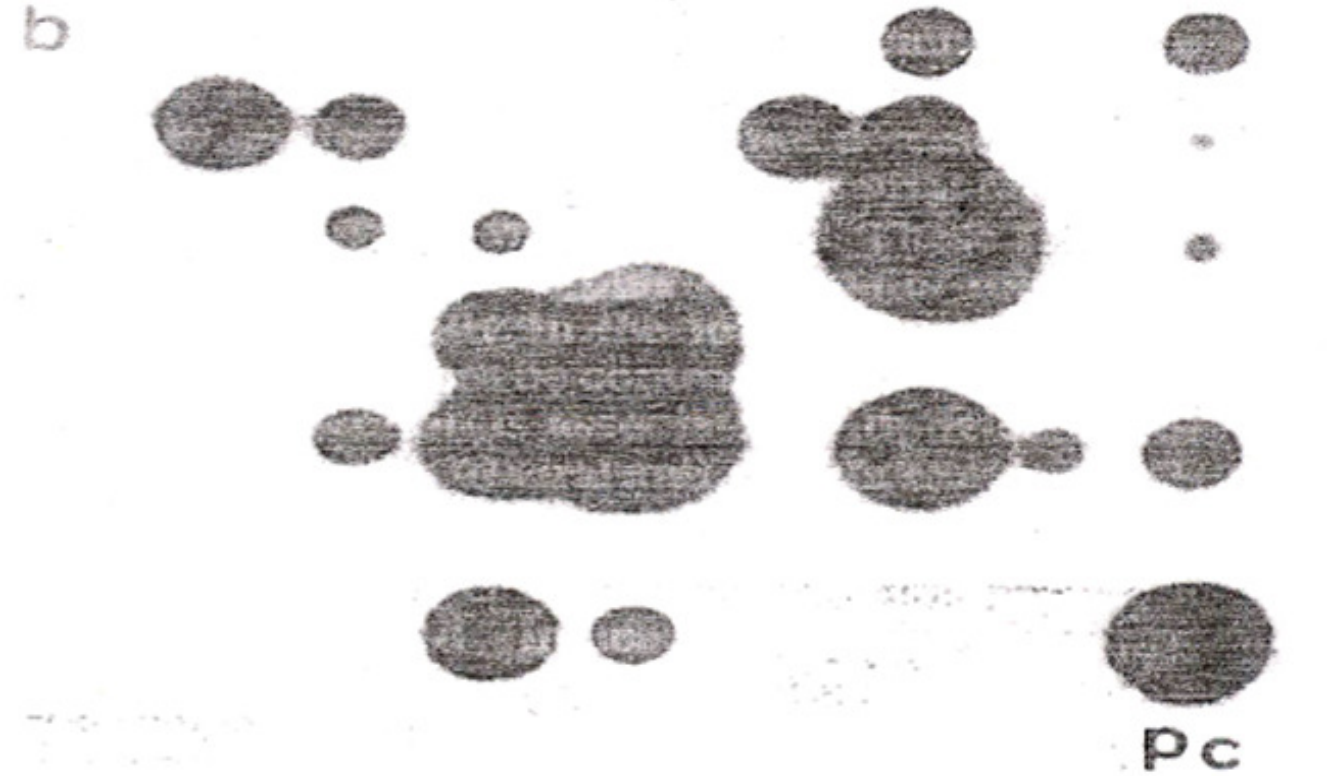

Figure a (b).

The high risk HPVs were found in 36 (88%) of 41 cervical 5 (33%) of 15 endometrial and none of ovarian (n3) and rectal (n= 1) carer omas I IPV 33 was predominant both in cervical (28 cases) and endometrial careinomas (5 cases). HPV 18 was found in 19 cervical and 2 endometrial carcinomas, while HPV 16 was found only in 14 cervical carcinomas. Single HPV infection was detected in 16 cervical arid 3 endometrial carcinomas. On the other hand, 20 cervical and 2 endometrial carcinomas harboured more than two types of HP V. p53 over-expression was found in 28 (68%) of 41 cervical carcinomas and 7 (47%) of 15 endometrial carcino mas, 2 (66%) of 3 ovarian carcinomas and one rectal carcinoma. Fourteen cervical and 6 endometrial carcinomas found positive for p53 alteration contained more than 10% of cells stained strongly with CM-1 polyclonal antibody. The correlation between presence or absence of HPV and p53 mutation detected by irnmunohistochemistiy is shown in Table III.

Table III. Correlation between presence of high risk HPV DNA and over-expression of p53 in malignant tumors of female genital tract.

Type of tumor	Total number of cases	Number of cases positive for HPV DNA	Number of cases Positive for p53	Number of cases positive for both HPV DNA and p53
Cervical carcinoma	41	36 (88%)	28 (68%)	26 (63%)
a. Carcinoma in situ	12	11 (92%)	06 (50%)	05 (42%)
b. Invasive squamous cell carcinoma	21	18 (86%)	17 (81%)	16 (76%)
c. Adenocarcinoma	08	07 (88%)	05 (63%)	05 (63%)
Endometrial carcinoma	15	05 (33%)	07 (47%)	03 (20%)
Ovarian carcinoma	03	0	02 (66%)	0
Rectal adenocarcinoma	01	0	01 (100%)	0

There were 26 cases of cervical and 3 cases of endometrial carcinomas which were simultaneously positive for both HPV DNA and p53 alteration. There was no significant association between the presence of specific type of HPV and the detectable level of p53, however, out of six cervical and endometrial malignant tumor revealing highest expression of p53 (-H-+), three harboured only HPV 33 and one was HPV negative.

Discussion

Recent epidemiological and experimental data suggest that specific high risk HPV are causally involved in the pathogenesis of anogenital cancer, particularly in cancer of cervix[14]. The frequencies of HPV positive specimen from patients with cervical cancer have ranged from 15-92%[14]. In this study we found presence of high risk HPV in 88% of cervical cancer specimens in Japanese patients, which confirms our previous fmdings in which by using in situ hybridization techniques, HPV 16 and 18 were detected in 68% of cases[15]. Beside cervical carcinoma, 33% of endometrial carcinomas, also harboured high risk HPV, while nçne of ovarian and rectal carcinomas were found positive for HPV DNA. There are conflicting reports about the presence of HPV DNA in ovarian and endometrial carcinoma[2-5]. However; in a most recent report high risk HPV were not only found in 27% of malignant ovarian tumors and 38% of endOmetrial carcinomas, but also more than 50% of benign ovarian and endometriai specimens were positive for HPV DNA[2]. Although biological significance of the presence of HPV in benign and malignant endometrial specimens is not known, the HPV may play the same role in this site as has been suggested for its presence in other genital sites like vulva, vagina and cervix.
We could detect overexpression of p53 in 68% of cervical carcinoma, 47% of endometrial carcinoma, 2 (of 3) malignant epithelial ovarian tumors and I (of 1) rectal adenocarcinoma. These figures are in accordance with the recent studies indicating presence of p53 alterations in 62% of invasive squamous cell carcinoma of cervix and 11% of cervical adenocarcinoma[16]. 21% of endometrial carcinoma[17] and more than 50% of ovarian carcinoma[18]. . Previous studies have shown that there is inverse relation between the presence of high risk HPV DNA sequences and p53 mutation in a series of primary cervical carcinoma[8] and cervical cell lines[7]. However, in our study this correlation could not be substantiated. On the contrary, 26 cervical and 3 endometrial carcinoma cases were found positive for these two parameters. These results are similar to one recent study indicating presence of p53 overexpression in 59% of HPV positive cervical carcinomas. Similar results were observed in one of our previous study, in which variable p53 over-expression was detected in 69% of HPV positive laryngeal carcinomas[10]. These findings suggest the possibility of secondary HPV infection in these carcinoma cases which already have p53 mutations or there may be heterozygosity of the tumor cells for harbouring HPV and p53 mutation. Likewise, there can be stabilization of p53 in these HPV infected cases by preferential complex formation with unknown protein specifically expressed by the tumor cells or there may be failure of ubiquitin mediated proteolysis. Crook and Vousen[19] have reported low frequency of p53 formation in primary cervical carcinomas with metastasis, but in our present study this was not the case. All of the cervical and endometrial carcinomas positive for both HPV DNA and p53 over-expression were non-metastatic. Lo et al[20] have reported presence of p53 mutation in cervical carcinomas infected with HPV33, but in this series the presence of p53 over-xpression was equally distributed in cases infected with HPV 16, 18 and 33. However, out of six cervical and endornetrial malignant tumors revealing highest expression of p53 (+++), only three harboured HPV 33 and one was HPV negative. Ourfindings suggest that HPV infection is also common in endometrial carcinomas beside cervical malignancy. More over, there is no inverse correlation between HPV infection and presence of p53 mutation detected by immunohistochemistry.

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