Detection of human papilloma virus 16 and 18 DNA sequences by southern blot hybridization in oral leukoplakia and squamous cell carcinoma

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Abstract

Background The etiopathological role of human papilloma virus (HPV) in the causation of oral cancer is till a subject of speculation.

Methods We used the technique of Southern blot hybridization to detect the presence of HPV types 16 & 18 in biopsy specimens from oral cancer and leukoplakia patients as well as normal oral mucosal biopsies.

Results The prevalence of either HPV type 16 or 18 was found in 64.5% (29/45) of oral cancer, 40% (12/30) of leukoplakia and 20% (9/45) of normal oral mucosal biopsies. No association could be demonstrated between tobacco usage habits or a history of genital warts with HPV prevalence.

Conclusion A significant finding was that none of the oral cancer patients were negative for both: a history of tobacco usage as well as presence of HPV infection, on Southern blot hybridization.

Keywords Human papilloma virus · Leukoplakia · Oral cancer

Introduction

The role of several viruses in the causation of human cancers is now well accepted. Viruses strongly associated with human cancers include hepatitis B virus in hepatocellular carcinoma, papilloma virus in carcinoma of uterine cervix, Epstein – Barr virus in Burkitt's lymphoma and various retroviruses in hematopoietic and reticuloendothelial system malignancies. The role of human papilloma virus in the etiopathogenesis of oro-pharyngeal carcinoma has been the subject of much speculation but reports in the literature are conflicting. The present work was carried out to evaluate the prevalence of human papilloma virus 16 and 18 DNA in oral mucosal biopsies obtained from patients with squamous cell carcinoma of oral cavity, leukoplakia and normal healthy subjects. Southern blot DNA hybridization technique, because of its high sensitivity, was utilized to detect the viral DNA.

Patients and methods

The present work was carried out on 45 patients of oral squamous cell carcinoma, 30 patients of oral leukoplakia and 45 healthy age and sex matched controls presenting to the surgical outpatient department of University hospital, Banaras Hindu University during a 2 years period. All patients underwent detailed history and clinical examination. Biopsy from the lesion in cancer and leukoplakia patients and from the normal mucosa of controls was obtained using punch biopsy forceps. Half of the biopsy sample underwent light microscopy with conventional hematoxylin – eosin stain to confirm the diagnosis. The remaining biopsy sample was subjected to southern blot hybridization with DNA probes specific for HPV 16 and 18 genome, isolation of genomic DNA from the sample.

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The genomic DNA was extracted from the biopsy sample by the technique of phenol – chloroform digestion. The RNA present in the genome was removed by treating with DNase free RNase. The genomic DNA was collected as a pellet following centrifugation and was dissolved in TE- BUFFER. Bam H1 and Pst1 restriction endonuclease enzymes subjected ten microgram of the isolated DNA to digestion. The resulting restriction fragments were separated on agarose gel (0.7%) by electrophoresis.

Preparation of HPV 16 and 18 probes from plasmids

Bacterial cells harbouring recombinant plasmids containing HPV 16 and 18 DNA sequences were grown in LB medium containing appropriate antibiotics. The DNA was extracted by digestion with lysozyme. The recombinant plasmid DNA thus prepared was treated with BamH₁ restriction endonuclease and the fragments separated by agarose gel electrophoresis. The resultant DNA fragment was labeled with P₃₂ radioactive isotope by nick translation.

Southern blotting and hybridization

The genomic DNA of the biopsy specimens present in the agarose gel was denatured by immersion in 1.5 M NaCl, 0.5 N NaOH solutions. Blotting of the denatured DNA was carried out on nitrocellulose filter paper. Heat denatured P₃₂ labelled radioactive HPV 16 and 18 DNA probes were added to the DNA containing filter. The filter was air dried and exposed to an X-ray film to obtain autoradiographic images of the hybridized HPV 16 and 18 DNA samples.

Observations

There was a heavy male preponderance among oral cancer (37:8) and leukoplakia patients (18:12). The commonest

age group for presentation was 50–69 years for both oral cancer and leukoplakia. Most of the oral cancer patients were tobacco users (41/45, 91.2%) and only 4/45 (8.8%) had never chewed or smoked tobacco. Among leukoplakia subjects 19/30 (63.4%) were tobacco users while 11/30 (36.6%) had never used tobacco. A past history genital warts was obtained in 4/45 (8.8%) of oral cancer and 4/30 (13.4%) of leukoplakia subjects.

On Southern blot hybridization evidence of either HPV 16 or 18 infection was found in 29/45 (64.5%) of oral cancer specimens, 12/30 (40%) of leukoplakia and 9/45 (20%) of controls. No association could be demonstrated between either tobacco usage or a history of genital warts with HPV positivity in either group.

Discussion

The human papilloma virus (HPVs) is a large family of related DNA viruses. More than 60 HPV types are currently known and the typing is based on DNA homology rather than on serotype [1]. HPVs are specific for squamous epithelium for their replication although they are able to infect respiratory epithelium and the columnar cells of the endocervix. Different types of HPVs show distinct tissue specificity with a major sub division between mucosal and epidermal viruses [2].

The commonest outcome of HPV infection in humans is latent infection, which can exist for years. In this state viral DNA is present without any clinical or histological evidence of disease. The second possible outcome of HPV infection the formation of a benign papilloma or wart. These lesions consist of tissue hyperplasia with thickening of the spinous layer and increased capillary proliferation [3].

The third outcome of HPV infection is conversion of a benign papilloma to a malignant carcinoma. This is an extremely rare event even with high-risk HPV types such as

Table 1 Demographic profile of oral cancer patients, leukoplakia patients and controls

Characteristics	Oral Cancer (n = 45)		Leukoplakia (n = 30)		Controls (n = 45)	
Age	n	%	n	%	n	%
20–49	16	35.5	14	46.6	14	31.1
50–69	26	57.7	15	50.0	27	60.0
≥ 70	3	6.6	1	3.3	4	8.9
Mean age	52 years		47 years		53 years	
Sex						
Male	37	82.2	18	60.0	35	77.8
Female	8	17.8	12	40.0	10	22.2
Marital status						
Married	35	77.5	18	60.0	38	84.5
Unmarried	4	8.8	1	3.3	2	4.5
Widowed	6	13.4	11	36.7	5	11.0

Table 2 Risk factors among oral cancer patients, leukoplakia patients and controls

Risk Factor	Oral Cancer (n = 45)		Leukoplakia (n = 30)		Controls (n = 45)	
	n	%	n	%	n	%
Tobacco chewing						
Never	8	17.8	14	46.6	26	57.8
Former	6	13.4	9	30.0	7	15.5
Current	31	68.8	7	23.4	12	26.7
Tobacco smoking						
Never	14	31.2	16	53.3	28	62.2
Former	9	20.0	8	26.7	11	24.4
Former	22	48.8	6	20.0	6	13.4
Never chewed or smoked tobacco or consumed alcohol	4	8.8	11	36.6	22	48.8
Past diagnosis of genital warts						
No	41	91.1	25	86.4	38	84.4
Yes	4	8.9	4	13.4	7	15.6

16 and 18. Environmental factors such as X-ray therapy in laryngeal papillomas, tobacco smoking in cervical cancer among women and ultraviolet light exposure in the malignant conversion of skin warts are an important cofactor besides HPV infection in such patients [4]. Combination of HPV plus a carcinogen is said to increase the probability of malignant conversion of a pre existing benign lesion.

The etiological role of human papilloma virus in the causation of invasive carcinoma of the uterine cervix is well proven [4]. HPV types 16 and 18 predominate in cervical cancer tissue. The E6 and E7 genes of HPVs that are always expressed in HPV associated cervical cancer have been found to code for proteins that inactivate tumour suppressor proteins P53 and Rb and lead to genetic instability and cellular changes leading to carcinogenesis [5].

HPV infections also seem to be related to cancers at other ano-genital sites such as vulva, vagina anal canal and penis. However the contribution of HPV infections to cancers at nongenital sites remains uncertain. Carcinoma of the oropharynx shares a great deal of histological similarity with that of the uterine cervix. Speculation on the role of human papilloma virus in the etiopathogenesis of oro-pharyngeal carcinoma has been voiced frequently. The HPV prevalence rates in oral cancers have been reported to vary from 31% to 74% indifferent studies [6]. The rate of detection of HPV also depends on the diagnostic technique being used with polymerase chain reaction being most sensitive followed by southern blot hybridization.

In the present study the prevalence rates of HPV types 16 and / or 18 among oral cancer patients, leukoplakia patients and control subjects was 64.5, 40 and 20% respectively as detected by Southern blot hybridization. (Table 3). Tobacco chewing, smoking and alcohol drinking are well known risk

factors for oral cancer. We found that 8.8% of oral cancer patients (4/45), 36.6% of leukoplakia patients (11/30) and 48.8% of control subjects (22/45) had never chewed or smoked tobacco or consumed alcohol.

Genital infection with human papilloma virus is extremely common in the population and a oro-genital sexual contact has been postulated to be mode of transmission of HPVs to the oral cavity [7]. Schwartz et al. [8] found that risk of squamous cell carcinoma of oro-pharynx in males increased with decreasing age at first intercourse, increasing number of sex partners and a history of genital warts. In their series 26% of tumours in case subjects contained HPV DNA of which 16.5% were type 16 HPV. They concluded that HPV type 16 infections might contribute to the development of a small percentage of oral squamous cell carcinoma most likely in association with cigarette smoking.

Our data shows that among oral cancer patients, there were 16 (35%) who were HPV negative but used tobacco, while there were 4 patients (9%) who were HPV infected but did not use tobacco. Thus, among oral cancer patients 25 (55%) were both HPV infected and tobacco users. The corresponding figures of positivity for both HPV and tobacco among leukoplakia patients and controls were respectively 24% and 9%. Significantly none of oral cancer patients was HPV negative as well as non-tobacco user while 20% of leukoplakia patients and 37% of controls were negative for both.

The individual role of HPVs in the causation of oral cancer is difficult to evaluate due to the strong confounding influence of tobacco usage, which is a well-recognized risk factors. Significantly HPVs were found at a higher rate among oral cancer patients (64.5%) than healthy controls (20%) ($p < 0.05$). Also none of cancer patients was negative for both HPV infection and tobacco usage while 4 patients

Table 3 Prevalence of HPV type 16 and 18 DNA on Southern blot hybridization in biopsies from oral cancer patients, leukoplakia patients and controls

HPV DNA	Oral Cancer (n = 45)		Leukoplakia (n = 30)		Controls (n = 45)	
	n	%	n	%	n	%
Absent	15	33.3	18	60.0	36	80.0
HPV type 16	24	53.3	10	33.3	7	15.5
HPV type 18	6	13.3	2	6.6	2	4.4
Either or both present (HPV 16 and 18)	29	64.5	12	40.0	9	20.0

(9%) had HPV infection only and no history of tobacco use. This clearly suggests that the increased risk of oral cancer due to HPV infection is independent of tobacco use. The malignant transformation rate of leukoplakia is 5 to 7% and we found that 40% of these patients ($p < 0.05$) had HPV infections. Whether HPV was responsible as a cofactor in the malignant transformation or an innocent bystander requires the attention of future researchers.

Smith et al. [9] report that among those who are infected with HPV in the oral cavity the relative risk of developing cancer is higher (Odd's Ratio = 3.7) than in those who are exposed to tobacco or alcohol (Odd's Ratio = 2.6). They suggest that HPV positive individuals who are non-smokers and drinkers are potentially at high risk of developing cancer. Cox et al. [10] have reported the presence of HPV type 16 in normal, premalignant and malignant oral tissue but were unable to support the hypothesis that HPV 16 infection represents a clear risk factor for oral carcinogenesis. They postulate that other oncogenic HPV types might also be responsible. In their work human simplex virus sequences were detected more frequently in potentially malignant lesions than carcinomas.

Min et al. [11] found aberrant expression of P53 gene and altered level of P53 protein to be extremely frequent in oral cancer cell lines. They speculate that these changes may result from either mutation or infection with high risk HPV. Yeudall et al. [12] point out that unlike in cervical cancer, papilloma virus DNA integration is not a common event in oral cancer cells and loss of viral sequences may occur more readily. They demonstrated dual presence of HPV sequence and mutant P53 in the keratinocyte culture, which could represent polyclonal tumors. Additionally, P53 mutations were detected in cells derived from patients with no positive tobacco history.

Conclusion

On Southern blot hybridization evidence of either HPV 16 or 18 infection was found in 29/45 (64.5%) of oral cancer specimens, 12/30 (40%) of leukoplakia and 9/45 (20%) of controls. No association could be demonstrated between either tobacco usage or a history of genital warts with HPV positivity in either group. A significant finding was that none of the oral cancer patients was negative for both a history of tobacco usage as well as presence of HPV infection on Southern blot hybridization.

Conflict of interest The authors do not have any disclosable interest

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