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LETTER TO THE EDITOR

Human Papillomavirus Detection Strategies in Retinoblastoma

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To editor

Epidemiological and molecular evidence has demonstrated human papillomavirus (HPV) to be the aetiological agent of both benign (warts, papillomas) and malignant tumors (anogenital and cervical carcinomas). Retinoblastoma (RB) is the most frequent childhood intraocular malignant tumour, with an incidence of 1 of 18,000–30,000 live births. Increased incidence of sporadic RB is seen in areas of low socioeconomic status (South America, Africa and Asia), raising the possibility of infection as a risk factor in its development. The development of retinoblastoma occurs as a result of the absence or inactivation of pRb protein. Earlier studies have also suggested an association of HPV with sporadic retinoblastoma, indicating that the virus may have a role in the development of these tumors [1, 2] It has been reported that vertical transmission of HPV from mothers to infants may occur through an infected birth canal. HPV DNA has also been detected in the placenta and amniotic fluid in various studies and cervical brushing from mothers of retinoblastoma [3].

Studies available on the association of HPV with retinoblastoma showed contradictory results even in a single geographical area. Studies in the South American and Mexican populations have reported HR-HPV types in 82% and 4% of retinoblastoma patients [1, 2, 4, 5]. Contrast to this findings, study from North American population showed lack of association between HPV and sporadic retinoblastoma in 40 cases [6]. Studies on Indian populations show 0%–48% of HPV positivity [7–10]. **(**Table 1**)** This variability of HPV presence in retinoblastoma cases even within the same geographical areas could be due to difference not only in the socioeconomic status, education level of patients but also due to variation in the detection methods used. Therefore, uniformity in detection methodology is recommended for comparative analysis regarding presence of HPV in retinoblastoma.

Although there are studies comparing in-situ hybridization (ISH), immunohistochemistry (IHC) and PCR of HPV in several tumours, limited data is available for retinoblastoma. In the present study, detection of HPV in 20 retinoblastoma cases and 10 controls (normal retina obtained from eye bank) was undertaken by PCR (using consensus MY09/11, PGMY09/11 primers and HPV 16 and 18 type specific primers), Immunohistochemistry (HPV L1 antigen), In situ hybridization (probes specific for HPV 6, 11, 16 and 18) and reverse hybridization (specific probes for 37 genotypes including all the major HPV subtypes/genotypes).

The male and female ratio of 20 retinoblastoma cases was 2.3:1(14 males and 6 females) with mean age of 4.04 ± 2.4 years (3 months to 8 years). Unilateral retinoblastoma cases (16) were 4 times more frequent than bilateral retinoblastoma [4]. Based on the International system of

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Table 1 Summary of published data to demonstrates association of Human papillomavirus with retinoblastoma

Study (Reference)	Nature of specimen	Detection method	Number HPV positive/total (%)	HPV type	Geographical Distribution
[1]	Fresh or fresh-frozen tumor	PCR (MY09/11 and HPV 16 and 18)	14/39 (36%)	HPV 16 and 18	North America (Mexico)
[4]	Paraffin-embedded	PCR (GP5+/GP6+) Dot blot hybridization	12/43 (27.9%)	HPV types 16 and 35	South America (Brazil)
[2]	Paraffin-embedded	PCR (CpI and CpIIG) RFLP	42/51 (82.3%)	HPV 6, 33, 16,11,31,35,51	North America (Mexico)
[6]	Fresh or fresh-frozen tumor	PCR (PGMY09/11) Reverse hybridization (Linear Array to detect 37 HPV types) Real time PCR (HPV 16 E6 and HPV 18 E7)	0/40	None	North America
[8]	Fresh or fresh-frozen tumor	PCR (HPV 16 and 18)	21/44 (47%)	HPV 16	India (South India)
[7]	Paraffin-embedded	IHC	0/30	None	India (North India)
[9]	Paraffin-embedded 64 and Fresh or fresh-frozen tumor 19	PCR (PGMY09/11) Reverse hybridization (Linear Array to detect 37 HPV types) ISH	20/83 cases (24%). Fresh 11/19 (57.9%), FFPE tissue (9/64 (14%)	HPV 16,18 45, 59, 68 52, 82 and 73	India (South India)
[5]	Paraffin-embedded	PCR (GP5+/GP6+) Hybridization assay	7/153 (4.5%)	HPV 16, 33, 45, 18, HPV 39, HPV 40, and 42	South America (Brazil)
[10]	Paraffin-embedded	PCR (MY 09/MY 11) Sequencing (HPV 16 and 18 E7) RT-PCR (HPV 16 and 18 E7) ISH	53/76 (69.7%)	HPV 16 and 18	India (Mumbai)
[15]	Paraffin-embedded	ISH	0/54	None	Korean

classification for intraocular retinoblastoma, 16 of the 19 intraocular tumours were classified as group E and 3 cases as group D. All the cases and controls were negative for HPV by PCR (multiplex and type specific PCR), IHC and ISH. (Fig. 1a-f) Results of PCR, IHC and ISH were further validated by linear array reveres hybridization. All the cases were also found to be negative for HPV by linear array reveres hybridization. (Fig. 1g).

Currently, PCR amplification is considered as the most sensitive method for detection of HR-HPV DNA. Consensus L1 primers are frequently used owing to their ability to detect a wide spectrum of HPV types. However, PCR techniques have certain drawbacks as they may give false negative results when there are multiple HPV type infections and also when the virus is integrated into host genome. PCR primers targeting L1 can be considered less reliable than PCRs directed against the E6 or E7 genes, which encode oncogenic products and always remain intact [11]. HPV 16 and 18 have been reported to be most predominant genotypes in various malignancies including retinoblastoma. Therefore, in this study we have used type specific primers for HPV 16 and HPV 18 specific for E6 region to rule out the possibility of false negative results of consensus primer (PGMY09/11). Immunohistochemical detection of HPV viral protein has been described in cervical biopsies as well as in smears and has proved to be useful in HPV detection [12]. ISH techniques

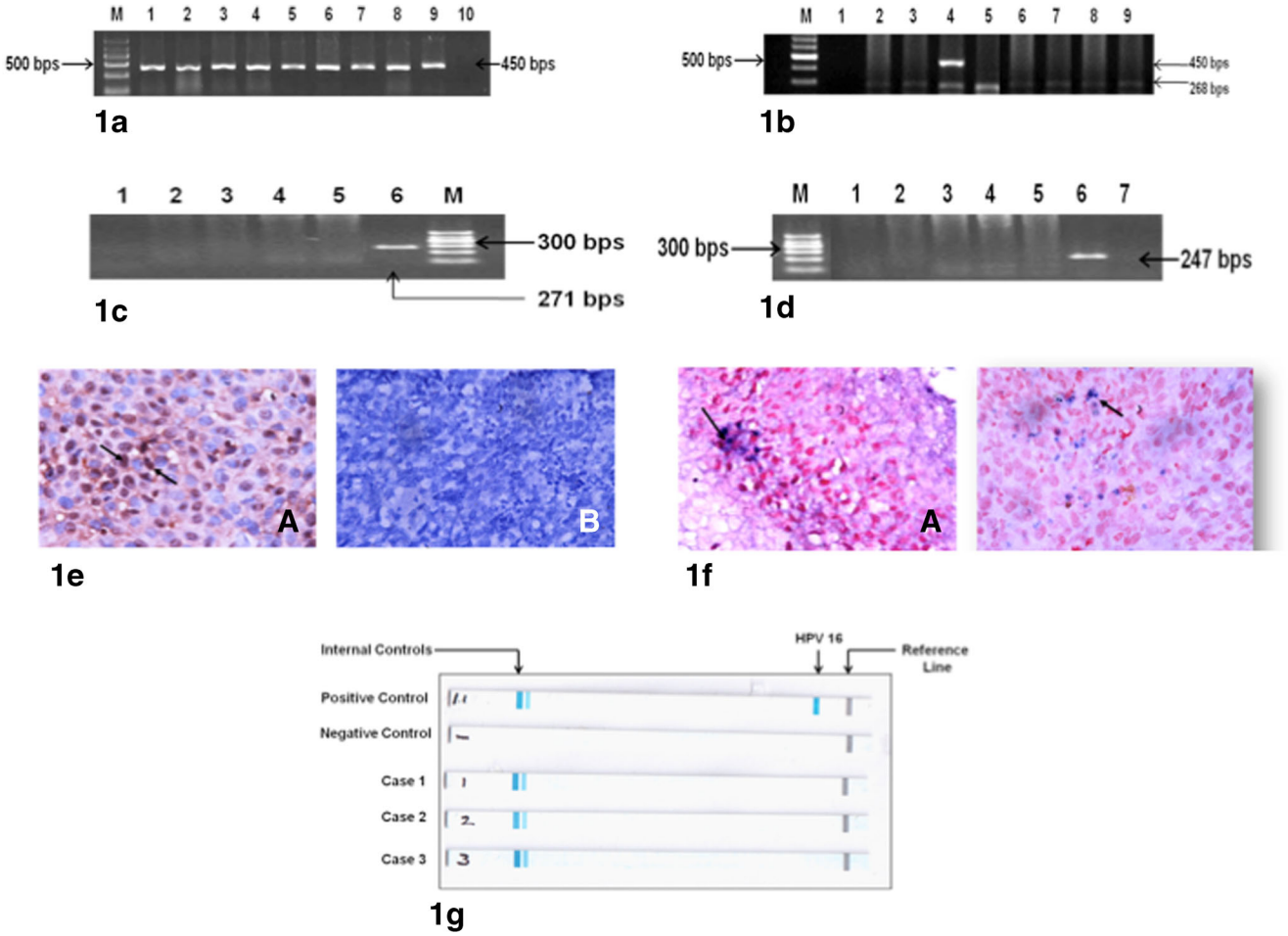

Fig 1. (**1a**) Analysis of the β-globin PCR products (to check the quality of DNA) on 1.2% agarose gel stained with ethidium bromide (450 bps product). Lane M: 100 bp ladder, Lane 1–9: Retinoblastoma samples, Lane 10- PCR negative control. (1**b**) PCR amplification of HPV L1 region using PGMY09/11 consensus showing the amplimer of 450 bp and internal control (β-globin) of 268 bp. Lane 1: Negative control (dH_2O), Lane 2: Control retinal tissue, Lane 3: HPV negative cell line (C33a DNA), Lane 4: HPV positive cell line (Hela DNA), Lane 5–9: Retinoblastoma samples, Lane M: 100 bp DNA molecular weight marker. (I**c**) PCR amplification of HPV16 E6 gene showing amplimer of 271 bp. Lane 1: Negative control (dH2O), Lane 2–5: Retinoblastoma samples, Lane 6: HPV positive cell line (CasKi DNA), Lane M: 100 bp DNA molecular weight marker. (1**d**) PCR amplification of HPV18 E6 gene showing amplimer of 247 bp. Lane 1–5: Retinoblastoma samples, Lane 6: HPV positive cell line (CasKi DNA), Lane 7: Negative control (dH_2O), Lane M: 100 bp DNA molecular weight marker. (1**e**) HPV immunostaining (IHC) to show strong nuclear and cytoplasmic positivity of HPV in (A) positive control (carcinoma cervix) and (B) retinoblastoma case negative for immunoexpression. (1**f**) Non-isotopic in situ hybridization (ISH) using digoxigenin-labelled human papillomavirus (HPV) generic probe (HPV 6, 11, 16 and 18). Nuclear positivity of HPV in (A) positive control and (B) High power view to show localization of HPV in positive control (carcinoma cervix). (1**g**) Identification of HPV genotypes using linear array. Representative retinoblastoma cases (Case 1 to 3) negative for HPV with β-globin internal control and negative and positive (HPV type 16) assay controls

are more sensitive and are currently more often used in routine diagnostics. It has been demonstrated that ISH can be used for the detection of very low copy number of HPV DNA sequences in paraffin-embedded tissue sections. Although more sensitive methods such as PCR can be performed in formalin-fixed and paraffin-embedded tissues, DNA damage and DNA extraction in these tissues can reduce the sensitivity of PCR. Thus, ISH can detect HPV in cases that may not be identified by PCR. However, a potential limitation of this technique is the failure to detect HPV subtypes other than the genotypes tested with the targeted probes [13].

In the present study, results of PCR, IHC and ISH were further validated by reverse hybridization based Linear array (Roche diagnostic), which is highly accurate and sensitive assay for HPV genotype detection in clinical samples and, unlike DNA sequencing, it is able to identify multiple infections [14].

In the present study there was a lack of association between HPV and retinoblastoma from North Indian population by PCR,IHC, ISH and linear array. Absence of HPV has also been reported from Asian population including North India. However, in these studies only IHC or ISH has been used [7, 15]. Earliar Studies from South India have shown HPV positivity in 24–70% retinoblastoma cases by PCR. HPV 16 and 18 were the most predominant genotypes [8–10]. Recently presence of other HPV genotypes (45, 59, 68 52, 82 and 73) has also been reported in retinoblastoma cases using reverse hybridization linear array [4].

Studies from North America also have found no association between HPV and retinoblastoma using PGMY09/11 primers and reverse hybridization linear array [6]. In contradiction, Orjuela et al. reported 36% HPV positivity by PCR (MY09/11) in retinoblastoma cases [1]. Studies from South America have reported the incidence of HPV between 4% to 28% with degenerate PCR technique followed by dot blot analysis [4, 5]. (Table 1) To conclude, the results from the present study indicate that HPV may not beassociated with retinoblastoma in Indian population.

Author's Contribution SC: Perform all experimental work, data collection, analysis, interpretation and writing. SS,NS AS,: Conception and design. BC,SK,: Acquisition of clinical data.

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Compliance with Ethical Standards

Conflict of Interest None.

Patient Consent Obtained.

Ethics Approval This study was conducted after approval from the Institute Ethics Committee, AIIMS, New Delhi and carried out in accordance with Declaration of Helsinki principles. Informed consent was obtained from all patients participating in this study.

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