Original Article

# Human papillomavirus in retinoblastoma: A tertiary eye care center study from South India

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**Purpose:** This study is aimed to investigate the presence of Human papillomavirus (HPV) DNA in tumors obtained from sporadic retinoblastoma patients. **Methods:** One hundred six tumor tissues obtained from sporadic RB patients were analyzed for HPV infection by use of both seminested PCR and real-time quantitative PCR. **Results:** Of 106 RB patients, 55 were male and 51 were female. The mean age at diagnosis was 26.77 ± 15.36 (mean ± Std. dev) months. Almost all patients presented with leukocoria. Molecular investigation by different methods revealed no HPV positivity in any tumor genome. **Conclusion:** Our study demonstrates no association between HPV and RB, postulating HPV may not be a major risk factor in the etiology of RB.

**Key words:** Human papillomavirus, nonfamilial retinoblastoma, pediatric cancer, *RB1* inactivation, real-time quantitative PCR

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Retinoblastoma (RB) is a childhood intraocular malignant tumor, occurring at an incidence of one in 15,000 live births[1] and developed upon biallelic inactivation of *RB1* gene. RB may be unilateral or bilateral and of sporadic or familial. There are two forms of the disease: hereditary and nonhereditary that account for almost 40 and 60% of RB cases, respectively. In hereditary RB, the first mutation is constitutional and the second mutation is somatic, whereas in nonhereditary, both mutations are somatic, i.e. present only in tumor cells.[2]

*RB1* gene, a tumor suppressor gene, is located on 13q14 and encodes for retinoblastoma protein (pRB). It is a negative cell cycle regulator and plays a crucial role in cell cycle arrest and apoptosis. In the $G_0$ phase of the cell cycle, pRB binds E2F inhibiting G1-S phase transition, and upon phosphorylation of pRB at multiple serine/threonine sites by different cyclin-dependent kinases in the G1 phase, E2F dissociates from pRB leading to S-phase entry. Loss of pRB results in uncontrollable cell proliferation, a neoplastic phenotype. Various mechanisms inactivating *RB1* gene or protein such as mutations, viral oncoprotein interaction and phosphorylation have been documented across multiple human cancers.[3]

*RB1* inactivation through mutation is the dogma for RB onset. Besides, multiple studies of RB have substantiated the presence of Human papillomavirus (HPV) DNA in a subset of RBs.[4-7] HPV, a double-stranded DNA virus, is a well-known causative agent of genital cancers in humans. E7 and E6 oncoproteins produced by HPV are known to induce cancer through inactivating pRB and p53, respectively.[8] Integration of the HPV genome into the host cellular genome is a critical event for malignant transformation. One case study from Mexico reported coexistence of HPV DNA and *RB1* mutation in an RB tumor, hypothesizing HPV as a cofactor in the RB pathogenesis.[9] Contradictorily, other RB studies from different regions showed no causal relationship between HPV and RB.[10,11] In India, the prevalence of HPV in RB is dynamic, ranging from 70% to no HPV.[12,13] The functional relevance of HPV in this cancer is unknown.

No RB studies from Asia have endowed the status of *RB1* gene in HPV-infected RB tumors. With an aim of investigating whether HPV is a risk factor in the etiology of RB, we performed HPV screening using seminested PCR with HPV L1 consensus primers and real-time quantitative PCR (RT-qPCR) targeted two different genes of HPV (1) L1 consensus and (2) E6 or E7 gene in RB tumor DNA samples that had already been *RB1* genotyped.

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## Methods

### Ethical consents

The study was approved by the institutional ethical committee and conducted in accordant with the Declaration of Helsinki. Informed consent from the parents of RB children were collected before sample collection.

### Study subjects and selection

We included nonfamilial RB patients whose tumor eye was enucleated as a part of treatment during January 2012 to October 2019 at a tertiary eye care center in the south zone of India. We unambiguously chose tumor samples after histopathological examination and *RB1* gene screening. After all, tumor tissues from 106 RB patients were considered for further experiments.

### Controls

HeLa cell line served as positive control, whereas DNA from 10 noncancerous retina tissue (donor eye) and genome of Herpes Simplex Virus, Cytomegalovirus, Varicella zoster virus served as negative controls.

### DNA extraction

Total genomic DNA (gDNA) was extracted from RB and control samples using Qiagen DNA mini Kit (Qiagen, USA), as per the manufacturer's instructions. The purity and yield of the extracted gDNA were determined using Nano spectrophotometer (NanoDrop Technologies Inc, Wilmington).

### HPV quantification targeting HPV L1 consensus

SYBR green chemistry-based RT-qPCR was employed to quantify HPV DNA. HPV L1 consensus (MY11-GP06) primers were retrieved from previous study[4] [Table 1]. The reaction mixture contained 1X SYBR Green Master mix (Takara, Japan), 400 nM each primer, and 2 µL (~ 50–100 ng) of gDNA. Thermal cycle conditions were initial denaturation of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C.

The specificity of these primers was evaluated through their ability of discriminating against other viral genomes. Then, detection limit of these primers was determined with 10-fold serial dilution of HPV L1 fragment, ranging from $10^6$ to 1 copy. The integrity of extracted DNA was checked by amplifying the *beta-2-microglobulin* gene (*B2M*) [Table 1]. Standard curve, between Ct verses HPV DNA copy number, was constructed with known copies of HPV DNA ranging from $10^6$ to 1 per reaction. The standards, RB DNA samples along with both positive and negative controls, were parallelly inspected in triplicate for every run. The reactions with amplification efficiency of above 90% and $R^2$ value of ≥0.98 were only considered. True Ct value was obtained based on melt curve analysis as it aids to differentiate nonspecific amplification. The viral load (copies/µg) in RB samples was determined through interpolating corresponding Ct value in the standard curve.

### Seminested PCR

This method efficiently detects even low copy number, and was conducted as described previously[14] with little modification. In the first round PCR, MY11-MY09 primers were used [Table 1]. The reaction was conducted in 10 µL volume containing 50–100 ng of gDNA, 1X PCR buffer, 50 µM each dNTPs, 0.5 U *Taq* DNA polymerase (Sigma-Aldrich, USA), and 4 µM each primer. Thermal cycles were initial denaturation of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C followed by final extension of 7 min at 72°C. Appropriate positive and negative controls were included in each run. Then, the PCR reaction mixture was purified using ExoSAP-IT™ (Thermo scientific, USA), according to manufacturer's protocol.

In the second round PCR, the purified PCR mix was reamplified by MY11-GP06 primers [Table 1] with the reaction composition as described above. Thermal cycles were initial denaturation of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C followed by final extension of 7 min at 72°C. The resultant PCR mixture was resolved on 1.5% ethidium bromide stained agarose gel. Then, amplification positive reaction mix was further purified and subjected to Sanger sequencing. The HPV genotype was identified using nucleotide- Basic Local Alignment Search Tool/n-BLAST (National Centre for Biotechnology Information).

### HPV quantification targeting HPV early genes

TaqMan chemistry-based RT-qPCR was performed using HPV 14 high-risk viruses real-time PCR kit (Helini Biomolecules, Chennai, India), according to manufacturer's protocol. This kit contained TaqMan probe targeting E6/E7 gene of 14 different HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and an endogenous control to assess reaction performance and DNA integrity. The standard curve was plotted to quantify the viral loads (copies/µg) in clinical samples using HPV 16 standards provided in this kit.

## Results

The clinical characteristics and *RB1* mutation status of each study patient are given in Supplemental Table S1. Of 106 RB patients, 83% had ($n$ = 88) unilateral and 17% had ($n$ = 18) bilateral disease. The male ($n$ = 55) to female ($n$ = 51) ratio was 1.07:1, with mean age at diagnosis

**Table 1: Primer pairs used in this study**

| S. No | Primer sequence | Product length, bp |
|---|---|---|
| 1 | B2M-1: 5'-GCTGGGTAGCTCTAAACAATGTATTCA-3'<br>B2M-2: 5'-CCATGTACTAACAAATGTCTAAAATGGT-3' | 95 |
| 2† | MY11: 5'-GCMCAGGGWCATAAYAATGG-3'<br>MY09: 5'-CGTCCMARRGGAWACTGATC-3' | 452 |
| 3† | MY11: 5'-GCMCAGGGWCATAAYAATGG-3'<br>GP06: 5'-GAAAAATAAACTGTAAATCA-3' | 190 |

†M=A + C; W=A + T; Y=C + T; R=A + G.

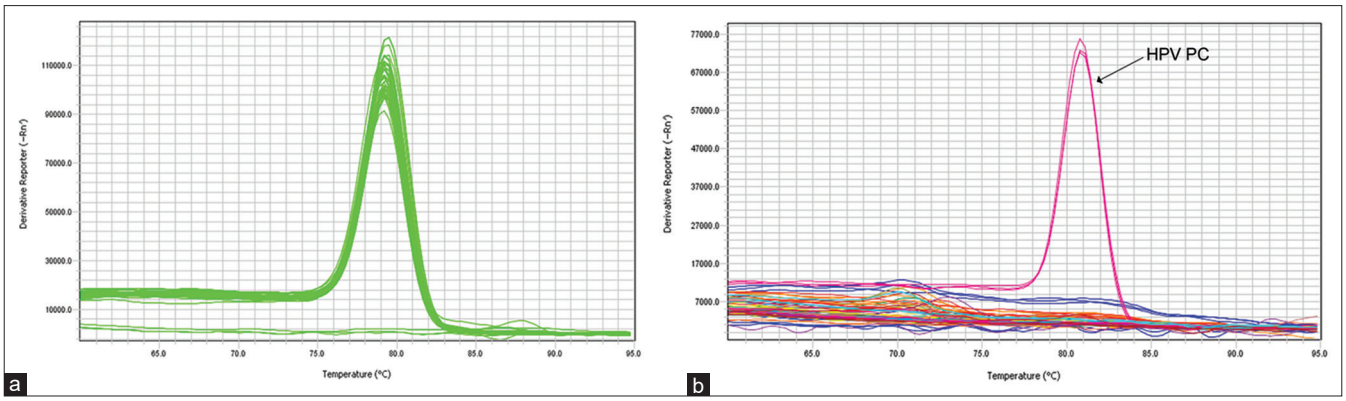


**Figure 1:** HPV quantification by HPV L1 consensus targeted RT-qPCR. (a) Melt curve plot for *B2M* gene. (b) Melt curve plot for HPV L1 consensus shows amplification only in positive control (HPV PC, black arrow)

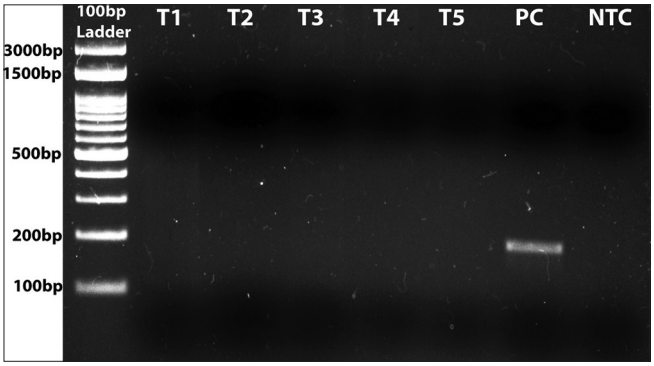


**Figure 2:** HPV detection by seminested PCR. Lane 1: 100 bp ladder (GeneDireX®), Lane T2–T5: RB DNA samples, Lane PC: HeLa DNA (HPV 18 positive), and NTC: nontemplate control

was 26.77 ± 15.36 (mean ± Std. dev) months (ranges from 2 to 72 months). Most of the patients presented with leukocoria ($n$ = 104) as their first clinical sign and other presentations included squint ($n$ = 1) and defective vision ($n$ = 1) are infrequent. Furthermore, *RB1* screening by Sanger sequencing and Multiplex ligation-dependent probe amplification identified 98 tumors with *RB1* mutations and 8 tumors with no detectable *RB1* mutation (Data not shown).

The specificity test of MY11-GP06 primers showed amplification only in HPV positive control, which is depicted in Supplemental Fig. S1. The detection limit of MY11/GP06 primers was found to be 10 copies of HPV. The HPV investigation by use of real-time PCR targeted HPV L1 gene showed none of DNA samples extracted from RB tissue and cell lines (Y79 and Weri-RB1) were positive, which is shown in Fig. 1. Similarly, seminested PCR targeted HPV L1 region did not observe HPV positivity in any tumor DNA [Fig. 2].

The standard curve constructed between Ct versus log known quantity of HPV in real-time TaqMan probe-based PCR assay had slope of -3.566 with a correlation coefficient of > 0.99. The standard curve and the endogenous amplification plot obtained from this assay are shown in Fig. 3. A total of 40 blinded RB DNA samples were subjected to this assay and found positivity in none of these samples for oncogenic HPV types investigated. The Ct value of each sample for HPV 16, 18 and other 12 types is given in in Supplemental Table S2.

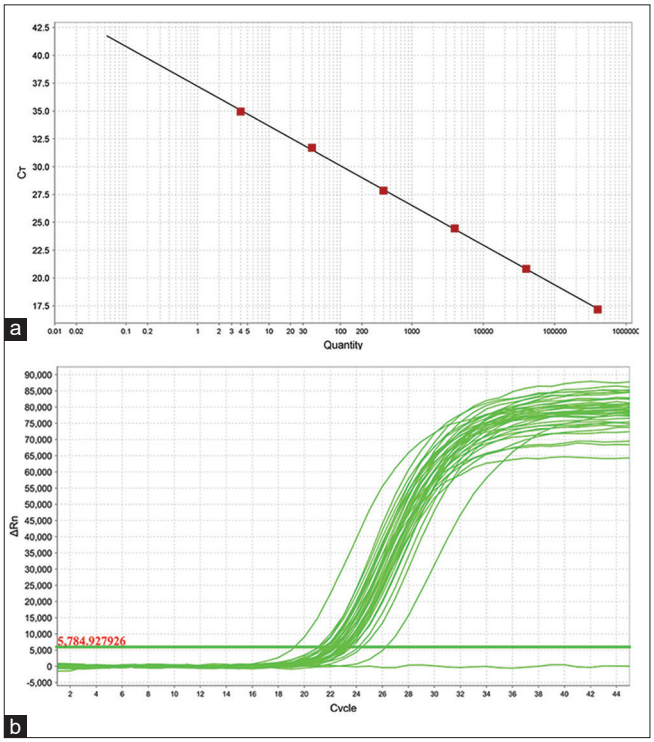


**Figure 3:** HPV quantification by TaqMan probe RT-qPCR. (a) Standard curve. (b) Endogenous control amplification plot

## Discussion

The present study was aimed to investigate the presence of HPV DNA in sporadic RB tumors that had previously been *RB1* genotyped. Interestingly, our analyses identified that no tumor DNA was positive for HPV, indicating that HPV has no relevance in the pathogenesis of RB with or without *RB1* biallelic mutations. Over the past two decades, several reports regarding the role of HPV in RB have been published and showed their prevalence in this pediatric cancer, ranging from 0 to 82%.[11,15] Our finding is in concordant with study by Gillison and coworkers where clear evidence of no association between HPV and RB, regardless of *RB1* genotype, was demonstrated.[11] A study by Ryoo and coworkers also reported that none of RB tumors were positive for HPV analysis by *in-situ* hybridization (ISH).[10] In parallel, Saktanasate and coworkers

by use of real-time PCR also did not identify HPV DNA in RB tumors.[16]

However, the HPV infection in RB is still open to debate as many earlier studies of RB have reinforced positive correlation between HPV infection and RB pathogenesis. Orjuela and coworkers identified HPV DNA, particularly HPV 16 ($n = 4$) and HPV 18 ($n = 11$), in 14 of 39 tumor genome and observed intact RB1 protein by immunohistochemistry (IHC) in 3 of 14 HPV positive tumor sections.[7] Another Mexico study from different group showed 42 out of 51 RBs, both familial and nonfamilial, positive for different HPV genotypes 6, 11, 31, 33, 35, and 51.[15] Palazzi and coworkers[17] detected oncogenic HPV genotypes 16 and 35 in 12 of 43 sporadic RBs, which was in contradiction to another Brazilian study by Antoneli and coworker, where only 7 of 153 tumors were shown positive for HPV, indicated low prevalence of HPV in RB children from Brazil.[6]

Correspondingly, the prevalence of HPV in Indian RB is shown to be varying between 0 and 70% owing to differences in study population and sensitivity of different detection methods. Mohan and coworkers employed nested or seminested PCR and found HPV positivity in 47% of unilateral nonfamilial RBs from South India.[4] Using Southern blot technique, Shetty and coworkers identified HPV genotypes 16 and 18 in 40 and 30% of RB tumors, respectively.[13] Anand and coworkers found 24% of unilateral RB tumors positive for different high-risk and intermediate-risk HPV types and reported HPV genotypes 45, 52, 59, 68, 73, and 82 first time in association with RB, due to the employment of linear array HPV genotyping method.[18] The same group also conducted HPV analysis in 21 RB tumors and 15 of 21 corresponding mothers' cervical brushing samples and identified 3 of 12 HPV positive tumors had the same HPV genotypes in their mother's cervical brushing samples, referring the maternal transmission as possible route of HPV infection in children with RB.[19]

Moreover, a case-control study by Naru and coworkers revealed a causal relationship between HPV 16 and RB in 25% of cases.[5] Another study by these authors compared the proteome between HPV infected and uninfected RBs using 2D-DIGE-coupled MALDI TOF/TOF mass spectrometry and identified 11 differentially expressed proteins. Eight upregulated genes included *ENO2*, *CKB*, *LDHB*, *VIM*, dodecanoyl-CoA isomerase, ubiquitin carboxyl terminal hydrolase isozyme L1, *TPM3*, and *YWHAE*, whereas three downregulated genes included *TUBB2A*, *APOA1*, and *P4HB*.[20] Contradictorily, study by Chauhan and coworkers found no causal relationship between HPV and RB.[12]

Certain studies have also enunciated the role of HPV in RB genesis or progression via analyzing the expression of pRB and/or HPV L1 protein. Montoya and coworkers furthermore observed the expression of HPV L1 protein in 7 of 10 HPV positive RB sections using IHC.[15] All of these studies were investigating HPV as an independent risk factor other than *RB1* biallelic mutations. Ironically, a case study demonstrated coexistence of *RB1* deletion and HPV 6 in a sporadic bilateral tumor and postulated HPV could be a cofactor in RB pathogenesis.[9] This had pushed us forward to investigate whether HPV is a cofactor or an independent factor in the pathogenesis of RB, and there are no reports from India evaluating the HPV DNA in RB tumors that had previously been *RB1* genotyped.

In the present study, we first utilized RT-qPCR targeted HPV L1 consensus and found no tumors were positive. Seminested PCR with HPV L1 consensus primers are shown to be more sensitive in the detection of broad spectrum of HPV infections than single-step PCR with either primer set.[14] Since there is a chance of cross-contamination in seminested PCR,[21] following precautions were taken to avoid such cross-contamination (1) inclusion of appropriate negative controls (non-HPV viral DNA), (2) usage of fresh aliquoted PCR reagents for each run, and (3) preparation of PCR master mix in PCR work station and addition of samples in different laminar flow chambers. With all these precautions, we found no positivity for HPV in any RB samples by seminested PCR as well. These negative results might be of having mutations in HPV L1 consensus primer binding site or disruption of L1 gene resulting from integration. HPV-specific PCR was reported to be more sensitive than degenerate PCR due to degeneracy nature of HPV general primers.[22] As a confirmatory analysis, TaqMan chemistry-based RT-qPCR targeted E6 or E7 gene was alternatively utilized for 40 blinded RB tumor DNA samples and also revealed HPV positivity in no tumor DNA.

Only two coherent studies that contradict each other had systematically analyzed the biallelic status of *RB1* gene followed by HPV DNA.[9,11] Similarly, we conducted HPV analysis in RB tumors, which had already been screened for mutations or methylation in *RB1*. Moreover, no aforementioned studies conveying positive relationship between HPV and RB have correlated the disease pathogenesis with either the viral copy number or expression of active viral oncogene and/or proteins included E6 and E7.

The lacuna of few studies evidencing no relationship between HPV and RB included (i) targeted only HPV L1 gene, (ii) use of a single technique, and (iii) analysis on DNA from the FFPE section. An ideal way to investigate HPV in clinical samples include targeting at least two different genes of HPV and using more than one technique to confirm. Gillison and coworkers employed multiplex PCR-coupled line blot hybridization detecting HPV L1 gene and RT-qPCR detecting HPV E6 or E7 gene and strongly concluded no relationship between HPV and RB.[11] Equivalently, we also adapted different detection methods, each targeted HPV L1 or E6/7 genes and did not find HPV DNA in any tumor genome.

Majority of the studies utilized qualitative methods. PCR was the most commonly employed techniques followed by dot-blot hybridization[5] to detect HPV DNA in RB tumors. Other techniques included RFLP,[15] Southern blot,[13] ISH,[10] and real-time PCR.[11,16] We used both qualitative and two different chemistries-based quantitative PCR assays in order to minimize the possibility of false-negative outcomes.

## Conclusion

Our molecular analyses clearly show the HPV may not be a major risk factor in the RB development or pathogenesis and also ensure that genetic inactivation of *RB1* gene is of great consequences in RB tumorigenesis.

### Ethical approval and consent to participate

This study was approved by the Institutional Ethics Review Board, Aravind Medical Research Foundation, India (Ethical approval no: IRB2016017BAS) and conducted in

accordant with 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written consent was collected from the parents of RB children enrolled in the study.

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### Conflicts of interest

There are no conflicts of interest.

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**Table S1: Clinical and genetic characteristics of retinoblastoma patients from this study**

| Sample | Age of diagnosis (months) | Sex | RB Stages | Laterality | Clinical presentation | *RB1* mutation |
|---|---|---|---|---|---|---|
| O1 | 18 | F | E | Uni | Leucokoria | Present |
| O2 | 24 | F | A, E | Bi | Leucokoria | Present |
| O4 | 9 | M | E | Uni | Leucokoria | Present |
| O5 | 24 | F | E | Uni | Leucokoria | Present |
| O6 | 17 | F | E | Uni | Leucokoria | Present |
| O7 | 24 | F | E | Uni | Leucokoria | Present |
| O8 | 24 | F | E | Uni | Leucokoria | Present |
| O9 | 36 | M | E | Uni | Leucokoria | Present |
| O12 | 36 | F | E, E | Bi | Leucokoria | Present |
| O13 | 24 | M | E | Uni | Leucokoria | Present |
| O14 | 12 | F | E | Uni | Leucokoria | No mutation |
| O15 | 3 | M | E | Uni | Leucokoria | Present |
| O16 | 24 | M | E | Uni | Leucokoria | Present |
| O18 | 30 | M | E, A | Bi | Leucokoria | Present |
| O19 | 18 | M | B, E | Bi | Leucokoria | Present |
| O20 | 36 | M | E | Uni | Leucokoria | Present |
| O22 | 12 | M | E | Uni | Leucokoria | Present |
| O24 | 12 | M | E | Uni | Leukocoria | Present |
| O25 | 24 | M | E | Uni | Leukocoria | Present |
| O26 | 24 | M | D | Uni | Leukocoria | Present |
| O27 | 12 | F | E, D | Bi | Leukocoria | Present |
| O28 | 72 | F | E | Uni | Leukocoria | No mutation |
| O29 | 36 | M | A, E | Bi | Leukocoria | Present |
| O30 | 24 | F | A, E | Bi | Leukocoria | Present |
| O31 | 48 | F | E | Uni | Leukocoria | Present |
| O32 | 9 | M | E | Uni | Leukocoria | Present |
| O33 | 36 | M | D | Uni | Leukocoria | Present |
| O34 | 18 | M | E | Uni | Leukocoria | Present |
| O35 | 3 | M | B, E | Bi | Leukocoria | Present |
| O36 | 72 | M | D | Uni | Leukocoria | Present |
| O37 | 36 | M | E | Uni | Leukocoria | Present |
| O38 | 30 | M | E | Uni | Leukocoria | Present |
| O39 | 34 | M | E | Uni | Squint | Present |
| O40 | 18 | F | E | Uni | Leukocoria | Present |
| O41 | 24 | F | E | Uni | Leukocoria | Present |
| O42 | 15 | M | E | Uni | Leukocoria | Present |
| O44 | 4 | M | E | Uni | Leukocoria | Present |
| O45 | 36 | F | E | Uni | Leucokoria and squint | Present |
| O46 | 26 | M | E, A | Bi | Leucokoria | Present |
| O47 | 48 | F | E | Uni | Leucokoria | Present |
| O48 | 21 | M | E | Uni | Leucokoria | Present |
| O49 | 24 | M | A, E | Bi | Leucokoria | Present |
| O50 | 24 | M | E | Uni | Leucokoria | Present |
| O51 | 28 | M | E | Uni | Leucokoria | Present |
| O52 | 28 | M | D, A | Bi | Leucokoria | Present |
| O53 | 24 | F | E | Uni | Leucokoria | Present |
| O54 | 41 | F | E | Uni | Leucokoria | Present |
| O55 | 8.5 | F | D, E | Bi | Leucokoria | Present |
| O56 | 5 | M | E | Uni | Leucokoria | Present |
| O57 | 32 | F | E | Uni | Leucokoria | Present |

*Contd...*

**Table S1: Contd...**

| Sample | Age of diagnosis (months) | Sex | RB Stages | Laterality | Clinical presentation | *RB1* mutation |
|---|---|---|---|---|---|---|
| O58 | 36 | M | D | Uni | Leucokoria | Present |
| O59 | 18 | M | D | Uni | Leucokoria | Present |
| O60 | 24 | F | E | Uni | Leucokoria | Present |
| O61 | 2 | F | E | Uni | Leucokoria | Present |
| O62 | 48 | F | E | Uni | Defective vision | Present |
| O64 | 18 | M | E | Uni | Leucokoria | Present |
| O65 | 28 | F | E | Uni | Leucokoria | Present |
| O66 | 66 | M | D | Uni | Leucokoria | Present |
| O67 | 12 | M | D | Uni | Leucokoria | Present |
| O68 | 24 | F | D, A | Bi | Leucokoria | Present |
| O69 | 18 | M | D | Uni | Leucokoria | Present |
| O70 | 16 | F | B, E | Bi | Leucokoria | Present |
| O71 | 36 | F | D, E | Bi | Leucokoria | Present |
| O72 | 25 | F | E | Uni | Leucokoria | Present |
| O73 | 24 | F | E | Uni | Leucokoria | No mutation |
| O75 | 30 | F | E, A | Bi | Leucokoria | Present |
| T1 | 7 | M | E | Uni | Leukocoria | Present |
| T2 | 9 | F | D | Uni | Leukocoria | Present |
| T3 | 27 | M | E | Uni | Leukocoria | Present |
| T4 | 29 | F | E | Uni | Leukocoria | Present |
| T5 | 17 | F | E | Uni | Leukocoria | Present |
| T6 | 36 | M | E | Uni | Leukocoria | No mutation |
| T7 | 24 | F | D | Uni | Leukocoria | Present |
| T8 | 4 | M | E | Uni | Leukocoria | No mutation |
| T9 | 36 | M | E | Uni | Leukocoria | Present |
| T10 | 36 | M | E | Uni | Leukocoria | Present |
| T11 | 48 | F | E | Uni | Leukocoria | Present |
| T12 | 36 | F | E | Uni | Leukocoria | No mutation |
| T13 | 48 | F | E | Uni | Leukocoria | Present |
| T14 | 48 | F | E | Uni | Leukocoria | Present |
| T15 | 6 | M | E | Uni | Leukocoria | Present |
| T16 | 72 | M | E | Uni | Leukocoria | Present |
| T17 | 36 | F | E | Uni | Leukocoria | Present |
| T18 | 39 | M | E | Uni | Leukocoria | Present |
| T19 | 20 | M | E | Uni | Leukocoria | Present |
| T20 | 24 | F | E | Uni | Leukocoria | Present |
| T21 | 36 | M | E | Uni | Leukocoria | Present |
| T22 | 24 | F | E | Uni | Leukocoria | Present |
| T23 | 2 | M | E | Uni | Leukocoria | Present |
| T24 | 24 | F | E | Uni | Leukocoria | Present |
| T25 | 24 | F | A, E | Bi | Leukocoria | Present |
| T26 | 24 | F | E | Uni | Leukocoria | Present |
| T27 | 19 | F | E | Uni | Leukocoria | Present |
| T28 | 24 | M | D | Uni | Leukocoria | Present |
| T29 | 12 | M | D | Uni | Leukocoria | Present |
| T30 | 60 | F | E | Uni | Leukocoria | Present |
| T31 | 9 | F | E | Uni | Leukocoria | Present |
| T32 | 29 | M | D | Uni | Leukocoria | Present |
| T33 | 10 | F | E | Uni | Leukocoria | Present |
| T34 | 36 | M | E | Uni | Leukocoria | No mutation |

*Contd...*

**Table S1: Contd...**

| Sample | Age of diagnosis (months) | Sex | RB Stages | Laterality | Clinical presentation | *RB1* mutation |
|---|---|---|---|---|---|---|
| T35 | 19 | M | E | Uni | Leukocoria | No mutation |
| T36 | 72 | F | E | Bi | Leukocoria | Present |
| T37 | 24 | M | E | Uni | Leukocoria | Present |
| T38 | 24 | M | D | Uni | Leukocoria | Present |
| T39 | 36 | F | E | Uni | Leukocoria | Present |
| T40 | 36 | F | E | Uni | Leukocoria | Present |

Uni – Unilateral; Bi – Bilateral.

**Table S2: Ct value obtained from TaqMan assay for each blinded sample**

| Ct value for HPV 16 | Ct value for HPV 18 | Ct value for other 12 HPV types | Ct value for endogenous control |
|---|---|---|---|
| Undetermined | 41.953 | Undetermined | 21.549 |
| Undetermined | Undetermined | Undetermined | 22.857 |
| Undetermined | 44.405 | Undetermined | 21.082 |
| Undetermined | Undetermined | Undetermined | 21.296 |
| Undetermined | Undetermined | Undetermined | 23.422 |
| Undetermined | Undetermined | 42.91 | 19.201 |
| Undetermined | Undetermined | Undetermined | 24.064 |
| Undetermined | Undetermined | Undetermined | 22.006 |
| Undetermined | Undetermined | Undetermined | 22.983 |
| Undetermined | Undetermined | 41.306 | 22.892 |
| Undetermined | Undetermined | Undetermined | 21.465 |
| Undetermined | Undetermined | Undetermined | 23.590 |
| Undetermined | Undetermined | Undetermined | 21.607 |
| Undetermined | Undetermined | Undetermined | 26.218 |
| Undetermined | Undetermined | Undetermined | 21.828 |
| 41.733 | Undetermined | Undetermined | 22.055 |
| Undetermined | Undetermined | Undetermined | 21.563 |
| Undetermined | Undetermined | Undetermined | 22.934 |
| Undetermined | Undetermined | Undetermined | 22.792 |
| Undetermined | Undetermined | Undetermined | 22.158 |
| Undetermined | Undetermined | Undetermined | 23.133 |
| Undetermined | Undetermined | Undetermined | 22.305 |
| Undetermined | Undetermined | Undetermined | 21.685 |
| Undetermined | Undetermined | Undetermined | 23.156 |
| Undetermined | Undetermined | Undetermined | 21.935 |
| Undetermined | Undetermined | Undetermined | 22.360 |
| Undetermined | Undetermined | Undetermined | 22.670 |
| Undetermined | Undetermined | Undetermined | 22.387 |
| Undetermined | Undetermined | Undetermined | 23.281 |
| Undetermined | Undetermined | Undetermined | 23.394 |
| Undetermined | 34.481 | Undetermined | 22.057 |
| Undetermined | Undetermined | Undetermined | 22.335 |
| Undetermined | Undetermined | Undetermined | 22.715 |
| Undetermined | Undetermined | Undetermined | 20.982 |
| Undetermined | Undetermined | Undetermined | 24.440 |
| Undetermined | Undetermined | Undetermined | 22.557 |
| Undetermined | Undetermined | Undetermined | 22.212 |
| Undetermined | Undetermined | Undetermined | 21.803 |
| Undetermined | Undetermined | Undetermined | 23.057 |
| Undetermined | Undetermined | Undetermined | 22.174 |

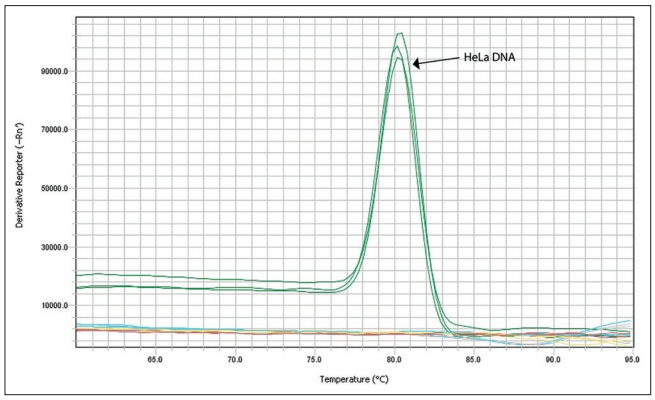


**Figure S1:** Melt curve plot of MY11-GP06 specificity test showing amplification in HPV positive control (HeLa cell DNA)