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Original Article

Molecular Investigation of the Human Adenovirus in Retinoblastoma Patients

Alireza Tabibzadeh [1] Ph.D., Masood Naseripour [2] M.D., Mohammad Hadi Karbalaie Niya [3] Ph.D., Davod Javanmard [1] Ph.D., Alireza Sadeghipour [4] M.D., Maryam Esghaei [1*] Ph.D.

[1] Department of Virology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2] Eye Research Center, Rasoul Akram Hospital, Iran University of Medical Sciences, Tehran, Iran
[3] Gastrointestinal and Liver Disease Research Center, Iran University of Medical Sciences, Tehran, Iran
[4] Department of Pathology, Rasoul Akram Hospital, Iran University of Medical Sciences, Tehran, Iran

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Background and Aims: Retinoblastoma tumors are the most common intraocular malignancy in childhood, leading to death after two years. The Human Adenovirus (HAdV) infection could be critical in the retinoblastoma pathogenesis due to the virus and retinoblastoma 1 interactions. The objective of the current study was to investigate the possible presence of the HAdV genome in the retinoblastoma patient's tumors.

Materials and Methods: In this study, we evaluated the HAdV infection in 96 pathological confirmed retinoblastoma samples. The DNA was extracted from formalin-fixed paraffin-embedded blocks, and the virus infection was assessed using polymerase chain reaction. SPSS version 22 was used for statistical analysis.

Results: The mean age ± SD of the retinoblastoma patients was 28.89 ± 17 months. In addition, the demographic evaluation indicated that 43 (46.7%) of patients were female. The retinoblastoma laterality assessment indicates 87 (90.4%) unilateral and 9 (9.4%) bilateral tumors. Growth pattern analysis indicates endophytic 58 (77.3%), exophytic 8 (10.7%), and 9 (12%) of tumors with mix endophytic and exophytic patterns. The polymerase chain reaction results could not found any evidence of HAdV infection in all 96 formalin-fixed paraffin-embedded samples.

Conclusions: The study results suggest that there is not any association between HAdV infection and retinoblastoma tumors in studied samples. The HAdV infection may not a concern in retinoblastoma pathogenesis. Further investigations are recommended in this field of study.

[*]**Corresponding Author:** Department of Virology, Faclty of Medicine, Iran University of Medical Sciences, Tehran, Iran. **Tel:** +982186703014, **Email:** maryam.esghaei@gmail.com

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Introduction

Retinoblastoma is the most common intraocular malignancy in childhood, critical childhood disease, and rarely diagnosed in young adults [1, 2]. The retinoblastoma prevalence in these age groups involved 4% of all malignancies [1], and their incidence is one per 15000 to 20000 population based on the geographical location [3]. The *retinoblastoma 1* gene (*RB1*) is located in the 13q14chromosome. The *RB1* gene is known as a tumor suppressor and regulator of the cell cycle and cell growth. Inactivation in both of the *RB1* genes by mutations, deletion, methylation, or single nucleotide alteration leads to tumor development [4-7]. In 1992, the Committee for the Japanese National Registry of Retinoblastoma reported a five-year survival rate of 93% in unilateral retinoblastoma and a two-year survival rate of 92% for bilateral cases [8]. Also, it was suggested that retinoblastoma could establish due to epigenetic dis-regulation [9]. The higher prevalence of retinoblastoma in low socioeconomic populations suggested a possible association with infection [10-12]. It has been reported that the human papillomavirus (HPV) infection could play a role as causative for the disease [10-14].

Human Adenovirus (HAdV) is a well-known human pathogen, especially for respiratory infections in children [15]. The HAdVs are classified into seven species named A to G and contain more than 70 serotypes [16]. The HAdV types B and D are the causative agents for the epidemic keratoconjunctivitis [17]. The etiologic role of the virus in human malignancies was rigorously studied [18]. The HAdV oncogenesis was firstly introduced by Trentin et al. in 1962 [19]. HAdVs can induce transformation in a wide range of rodents in cell culture [20]. The oncogenesis of the HAdVs in human malignancies did not report. However, there is a focus on the transformation of human cells *in vitro* [20]. In this regard, HAdV type 5 transformation in the 293 cell line and HAdV 12 transformation in the human embryo retinoblasts were reported previously [21, 22]. Also, the role of the Adenovirus E1B and E1A proteins in human embryo retinoblast transformation was illustrated [23].

Furthermore, epidemiologic investigations could show the virtual prevalence of the viral pathogens so that these studies could be beneficial in the case of retinoblastoma tumorigenesis. The current study aimed to investigate the molecular epidemiology of the HAdV infection in retinoblastoma patients.

Materials and Methods

Participants, demographic and clinical data

In this cross-sectional study, enrolled patient samples were obtained from 96 patients with confirmed retinoblastoma based on the clinical and pathological assessments referred to affiliated hospitals to Iran University of Medical Sciences, Tehran, Iran, from 2015 and 2018. All included patients were primary retinoblastoma cases, and an expert pathologist confirmed the retinoblastoma. The Ethics Committee of Iran University of Medical Sciences (code IR.IUMS.REC.1397.333) approved the study procedure. Written

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informed consent was obtained from all the included patients. The patients without written informed consent were excluded from the study. The demographic and clinical data were obtained from the laboratory data repository.

Sample preparation and DNA extraction

The pathologically confirmed retinoblastoma formalin-fixed paraffin-embedded (FFPE) tissue blocks were sectioned and prepared for the DNA extraction. Sectioning and extraction were macroscopically performed in the tumor site of the tissue blocks. The DNA extraction was performed by the QIAamp DNA FFPE Tissue Kit (Qiagen, Dusseldorf, Germany) based on the manufacturer's protocols. The NanoDrop ND-1000® spectrophotometry assessed the extraction (Thermo Fisher Scientific Inc., Waltham, MA, USA).

HAdV detection

The human genome in the extracted samples was confirmed using polymerase chain reaction (PCR) for the Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. GAPDH was amplified using 5'-CGACCACTTTGTCAAGCTCA-3' forward and 5'-AGGGGTCTACATGGCAACTG-3' reverse primers by 228 base pair PCR product in size. The HAdV detection performed by a conventional PCR method using 5'-GCTTCGGAGTACCTGAGYCC-3' forward and 5'-GGCCATRTCCAGCACTCKGT-3' reverse degenerated primers that can amplify a 234 base pair from the HAdV hexone gene. The thermal program for HAdV detection was provided by the Bio-Rad thermal cycler (T100™ Thermal Cycler) and included one step for 5 minutes at 95˚C (pre-denaturation), 40 cycles of 30 seconds at 95˚C (denaturation), 30 seconds at 60˚C (annealing) and 20 seconds at 72˚C (extension) and a final step of 10 minutes at 72˚C (final extension). The same protocol was used for GAPDH, but the annealing was performed at 55˚C for 30 seconds. The PCR products were run in 1% agarose gel after electrophoresis and visualized by ultraviolet light. In all the procedures, careful handling of the samples for minimalizing possible cross-contamination was considered.

Statistical analysis

The statistical evaluation was performed by SPSS version 22 (SPSS Inc., Chicago, IL, USA) using chi-square and Mann–Whitney U test based on the variables. The statistically significant results in the current study considered as $p < 0.05$.

Results

Patients demographical and pathological features

By assessing 96 included patients, the mean age ± SD of the patients was 28.89 ± 17 months. 49 (53.3%) of patients were male, and 43 (46.7%) were female. The retinoblastoma laterality assessment indicates 87 (90.4%) unilateral and nine (9.4%) bilateral retinoblastomas. The differentiation and growth patterns of tumors are summarized in Table 1. Growth pattern analysis indicates 9 (12%) of tumors with mix endophytic and exophytic patterns. The dominant growth pattern was endophytic 58 (77.3%) and followed by exophytic 8 (10.7%). Tumor involvement of the optic nerve margin was seen in 7 (8.4%) patients. Statistical assessment of the pathological features

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indicates a statistically significant association between laterality and tumor growth patterns (p = 0.03). In addition, a statistically significant association was seen in optic nerve margin involvement and tumor growth pattern (p = 0.04).

HAdV infection

The retinoblastoma tumor tissue sections were assessed for the HAdV genome using PCR amplification. It was found that there was no HAdV positive sample by the PCR method. Three different concentrations of positive control were used to ensure the primer efficacy for detecting HAdV in RB tumors (Figure 1). The GAPDH gene was used to confirm the presence of the genome in the studied samples. However, all were negative for the virus genome.

Table 1. The differentiation, laterality and growth pattern of tumors

Differentiation Pattern	Growth Pattern				Laterality		
	Endophytic (%)	Exophytic (%)	Mix (%)	P-value	Bilateral (%)	Unilateral (%)	P-value
Well differentiated	22 (37.9)	2 (25)	1 (12.5)	0.152	1 (16.7)	24 (31.6)	0.182
differentiated	15 (25.9)	1 (12.5)	1 (12.5)		1 (16.7)	19 (25)	
Poorly differentiated	1 (1.7)	0 (0)	1 (12.5)		0 (0)	3 (3.9)	
Undifferentiated	11 (19)	4 (50)	3 (37.5)		3 (50)	17 (22.4)	
Moderate differentiated	3 (5.2)	1 (12.5)	2 (25)		0 (0)	6 (7.9)	
Undetermined	6 (10.3)	0 (0)	0 (0)		1 (16.7)	7 (9.2)	

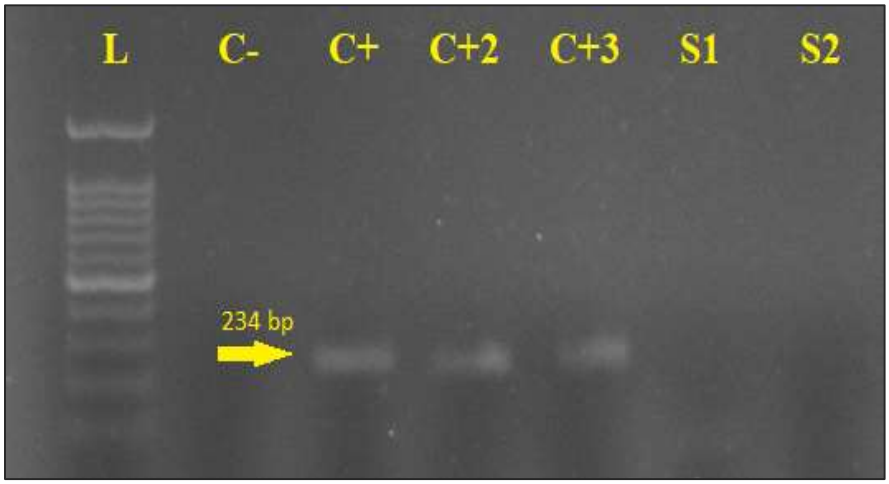

Fig. 1. There was no evidence of the HAdV genome in retinoblastoma patient's tumors. The gel electrophoresis illustration for HAdVs Hexon gene amplicon in three concentration from the positive control (C+ as no diluted, C+2 ½ diluted, C+3 ¼ diluted), in compare with retinoblastoma patient's tumors samples (S1 and S2).

Discussion

The most important etiology of retinoblastoma progression was related to the changes in the *RB1* gene as a tumor suppressor. For tumor induction, both alleles of *RB1* dysfunction seem to be necessary. In some familial and inherited cases, dysfunction in one allele could be sufficient for tumor induction [24, 25]. As mentioned, the HAdV proteins (E1B and E1A)

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can transform human embryo retinoblast cell lines [23]. The objective of the current study was to investigate the molecular epidemiology of the HAdV infection in FFPE tissue DNA extracts of retinoblastoma patients by molecular testing. In the present study, 96 patients were included by the mean age ± SD, 28.89 ± 17 months and, 49 (53.3%) of patients were male. The pathological finding for the current study seems to be in accordance with other studies [14, 26]. In the conducted study by Surakiatchanukul et al. [27], the assessment of 82 RB tumors indicates exophytic and endophytic growth patterns in 51 (62%) and 31 (38%) of the patients, respectively. The growth pattern and age were statistically associated with Surakiatchanukul's study. A statistically significant association between laterality and optic nerve margin involvement with tumor growth pattern was observed in our current study. In addition, growth pattern analysis in our current study indicates nine (12%) tumors with mix endophytic and exophytic patterns, endophytic 58 (77.3%), and exophytic 8 (10.7%). These differences between our study and the conducted study by Surakiatchanukul et al. could be due to the difference in sample size or geographical locations. The conducted study suggested that HAdV 12 can induce transformation in human embryo retinoblasts cells [21]. Gillison et al. [28] suggested that there are no clues about the presence of HAdVs in retinoblastoma by assessing 40 RB tumor samples. Besides, the result of the current study indicates that there was no evidence of the HAdV genome in retinoblastoma samples. The presence of human DNA viruses in RB was suggested by Palazzi and colleagues [29], assessing 43 Brazilian retinoblastoma patients. Palazzi's study indicates the presence of the human papilloma virus (HPV) genome in 27% of the RB tumors. Also, the HAdV in retinoblastoma tumors in the Palazzi was not found. This absence of the HAdV genome in retinoblastoma was either suggested by Ueno et al. [30]. Meanwhile, Javanmard et al. [14] reveal that 9.8% of the assessed retinoblastoma samples show evidence of the HPV genome. The presence of HPV in retinoblastoma samples was reviewed by Chauhan et al. [10]. Furthermore, recent investigations in the field of oncolytic tumors by Pascual-Pasto et al. [31] suggest that the VCN-01 (clinical-grade oncolytic adenovirus) can acts as a therapeutic agent by targeting the *RB1* gene in mice model by retinoblastoma xenografts. The major limitations in the current study could include sample size and limitation in the patient's clinical and pathological data, including a date for retinoblastoma diagnosis, records of HAdV infection before cancer diagnosis, tumor type, and tumor staging. Furthermore, the quantitate assessment of virus and the absence of expression level for some important genes in this field could be mentioned as the limitation of this study.

Conclusion

The current study could not show any HAdV genome infection in the assessed retinoblastoma samples. The study results suggest no association between HAdV infection and retinoblastoma tumors in the studied samples. The HAdV infection may not a concern in retinoblastoma

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pathogenesis. Furthermore, further studies might use full for a clear conclusion about the role of the HAdV or other DNA viruses oncogenesis in retinoblastoma.

Conflict of Interest

There is no conflict of interest.

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