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DNA Viruses and Langerhans Cell Histiocytosis in Iranian Children

M. Kazemi Aghdam (MD, APCP) [1], S.A. Naji (PhD) [2], M. Khoddami (MD, APCP)* [3]

1.Pediatrics Pathology Research Center, Pediatrics Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran.
2.Virology Research Center, Institute of Tuberculosis and Pulmonary Diseases Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran.
3.Infectious Disease Research Center, Pediatrics Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran.

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ABSTRACT

BACKGROUND AND OBJECTIVE: Viruses are suggested as possible etiologic factor of Langerhans cell histiocytosis (LCH) by some investigators. Nonetheless, no report was found on this subject in Iranian children. We looked for the presence of Epstein-Barr virus (EBV), human herpesvirus-6 (HHV-6), herpes simplex virus (HSV) types 1 and 2, and Cytomegalovirus (CMV) in children with LCH.

METHODS: The investigation in this retrospective study, was for the presence of HHV-6 DNA in 48 patients and CMV, HSV types 1 and 2 and EBV DNA in 30 patients with LCH, using paraffin-embedded tissue samples and 48 and 30 (respectively) age and tissue-matched controls from the department of pediatric pathology, using nested polymerase chain reaction (nested-PCR for HHV-6 and HSV types 1 and 2), qualitative PCR method (for CMV) and qualitative TaqMan Real-time PCR (for EBV).

FINDINGS: HHV-6 was found in one (2.1%) patient and six (12.5%) control specimen (P= 0.11, OR: 0.15; 95%CI: 0.02-1.29). Two (6.66%) patients and one (3.3%) control sample had CMV, with a P value of 1.0, and OR: 2.07; 95% CI of OR: 0.18-24.15. We did not find HSV types 1 and 2 DNA in any of the patients or controls. EBV was detected in 19 (63.33%) patients and 8 (26.7%) control group. P value was 0.004 with Odds Ratio: 4.75; 95% CI of OR: 1.58-14.25.

CONCLUSION: CMV, HSV types 1 and 2, and HHV6 do not appear to have any role in the pathogenesis of LCH. However, considering the statistically significant p=0.004, our findings suggest a possible position for EBV in the pathogenesis of LCH in Iran.

KEY WORDS: *Cytomegalovirus; Epstein-Barr virus; Histiocytosis; Langerhans-Cell; Herpes Simplex Virus; Human Herpes Virus-6; Polymerase Chain reaction*.

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* **Corresponding author: M. Khoddami (MD, APCP)**
Address: Pediatric Infectious Diseases Research Center, Pediatrics Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran
Tel: +98 21 22227035
E. mail: malihehkhoddami@yahoo.com

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Introduction

Langerhans Cell Histiocytosis is a rare disease and a clonal hystiocytic proliferation with unknown etiology. This disease can affect various tissues, and it affects most of the children under the age of 5 (1) often in three forms of clinical syndromes (multiple-focal multiorgans or Letterer-Swie disease, multiple focal single-organ, or Hand Schuller Christian and single-focal disease, or the same eosinophilic granuloma). Prognostic factors include age and organs are involved, and in most cases, adults with lung involvement slowly slow down, but in children they usually need treatment (2-5). Its histological diagnostic views include granuloma structures with a mixture of cells similar to langerhans, eosinophils, multi-nucleate giant cells, macrophages and lymphocytes (1, 6, 7). Langerhans cell is a single-nucleotide non- lymphoid cell and is a member of dendritic cells family that plays the role of initiating and formulating immune responses (1, 6, 8).

Immature dendritic cells in the langerhans histocytosis are positive for langerine (CD207), CD1a and S100, and exhibit Birbec granules in the electron microscope (2, 3, 7). The association of EBV, HHV6 and CMV with LCH has been reported by some researchers and suggests a possible etiological role or their participation in the pathophysiology of the disease (9-15). But others have found that there is a link between the disease and various viruses in their studies (16-18), and thus the role of the etiologic viruses in the disease has not been clear. HHV6 was detected in 47% of LCH patients by Leahy et al. (13), and Csire et al. (11) suggested the possibility of an outbreak or progression of LCH with HHV6 infection.

CMV in langerhans cells was found in 30% of paitients in Kawakubo et al. study (14) and the probable role of EBV in LCH pathophysiology was expressed (9, 10, 15, 19). On the other hand, Jeziorski et al. (16) and McClain et al. (17, 18) did not find a role for EBV, CMV, or HHV6 in the pathogenesis of LCH, and a positive result was not reported for HSV in LCH (13, 17, 18). Considering that viruses may play a role in the development of multiple malignancies (20-22), considering that vaccination and initial diagnosis, along with the treatment of viral infections, can play a more preventive role, more review is important. Since the presence of viruses in LCH has not been investigated in Iran, the aim of this study was to investigate the association of DNA viruses such as CMV, EBV, HHV6, and HSV 2 and 1 in this disease, which was performed using PCR techniques.

Methods

This retrospective study was conducted as case-control.

Patients and control group: Paraffin blocks of 48 patients with LCH diagnosis for HHV6 virus and 30 patients for the remaining viruses (2001-2002) were collected from the Department of Pathology of the Mofid Hospital in Tehran (one of the important centers of referral in the country). LCH diagnosis was performed by a pediatric pathologist using histological criteria in pathological reference books (granulomas consisting of langerhans cells with typical grooved nucleus and folded nucleus mixed with eosinophilsfused epidermoid cortex and eosinophilic cells and other inflammatory cells) (1,6,7).

Diagnosis was conclusive and validated using the immunohistochemistry technique for CD1a, S100, and CD68 when present. After re-examination of the slides with optical microscopy and confirmation of LCH detection, the tissues that were sufficiently sampled were selected for study, and cases with very low tissue volume were excluded. All Iranian patients were in range of 2 months to 10 years. The control group consisted of 48 tissue samples for HHV6 virus and 30 tissue samples for the remaining viruses (CMV, EBV, and HSV١ and 2) that were undergoing surgical procedures for some other reason except LHC and infectious diseases other than LHC and infectious diseases (such as pilonidal sinuses, anal fissures, eczema, hemangioma, Cystic hygroma, Emphysema, soft tissue cysts, enlarged lymph nodes, osteochondroma, etc).

These samples were also selected from files from the pediatric department of Pathology between 2002 and 2013, which matched for age and tissue location with LCH samples. The criteria for tissue placement in the control group were the lack of clinical and microscopic evidence of LCH or any other malignant tumor.

Preparation of paraffin blocks containing tissue samples and extraction of DNA: The tissue sections with5 micrometers thickness were made of paraffin blocks by Microtome and placed in the sterile tubes. Xylole and alcohol solutions were used for deparaffinization and rehydration of the tissue sections. Then, the Sliced samples were lysedby tissue buffering and K proteinzase, and the liquid tissue were used for DNA extraction. DNA was extracted from lysed tissue samples according to the company's instructions (RTP® DNA / RNA Mini Kit Procedure;

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Stratec Molecular GmbH, Berlin, Germany). The DNA was extracted and stored at -20 ° C until they were examined by PCR.

Polymerase chain reaction:

Human Herpes Virus-6: After qualitative control of extracted DNA by using the RT-PCR-Melting Curve Syber-Green for the β-globin gene, nested-PCR was used to detect the HHV6 genome in previously defined specimens. A 214 bp gene region was amplified from the main capsid protein of the virus using a Nested primer set. Primers for nested-PCR include:

HHV-1(outer):5´-CAATGCTTTTCTAGCCGCCTCTTC-3 ,´
HHV-2(outer):5´-ACATCTATAATTTTAGACGATCCC-3´
HHV-3(inner):5´-TTGTGCGGGTCCGGTTCCCATCATA-3´
HHV-4(inner):5´-TCGGGATAGAAAAACCTAATCCCT-3´

The detection limit of 50 copies of HHV6 in each reaction was determined using serial dilutions of AmpliRun® HHV6-DNA CONTROL (Vircell, S.L. Granada, Spain).

Cytomegalovirus: In order to evaluate the quality of the extracted genome, PCR test inhibition was also performed on all extracted nucleic acids, beta-globin PCR by using PCO3 / PCO4 primer (24).

The β-globin PCR was performed in the Syber Green RT-PCR Melting Curve format. To detect CMV, RT-PCR was performed by using the gene primer set of envelope glycoprotein B gene (gpUL 55) that 116bp gene region og virus genome (CMV-r; 5'-AAGTACCCCTATCGCGTGTG -3) and (CMV-f; 5')-ATGATGCCCTCRTCCARGTC -3) with the internal probe (CMV-p;5'-FAM-TGGCCCAGGGTACGGATCTTATTCG-BHQ1-3) amplified (25). The amplification of the gpUL55 CMV genes was carried out in the reaction volume of 20 µl under the following conditions: At first, the samples were denatured at 94 ° C for 10 minutes, followed by denaturation at 94 °C for ten seconds, after that 50 cycles extension, annealing was performed at 60 ° C for 1 minute. The RT-PCR study, the CFX-96 system (BIO-RAD, USA) was used with the HS Taq premix Taq Man reagent (GENETOBIO, Korea). The detection limit of 5 copies of the CMV gene was determined in each reaction by serial dilutions using AmpliRun® CYTOMEGALVIRUS DNA CONTROL (Vircell, Spain).

Herpes Simplex Type 1 and 2: After qualitative control of extracted DNA for the β-globin gene using Syber-Green RT-PCR-Melting Curve, two sets of nested PCR were used to identify HSV genomes 1 and 2 in samples (26).

The sequence of primers was as follows:

HSV1F1ATCRCGGTAGCCCGGCCGTGTGACA;
HSV1R1CATACCGGAAGCCACCACACAA;
HSV2F1TCAGCCCATCCTCCTTCGGCAGTA;
HSV2R1GATCTGGTACTCGAATGTCTCCG;
HSV1F2CATAYCGACCACACCGACGA;
HSV1R2GGTAGTTGGTCGTTCGCGCTGAA;
HSV2F2AGACGTGCGGGTCGTACACG;
HSV2R2CGCGGTCCCAGATCGGCA

Using the gpD primer kit for HSV1, 2, 140 parts, 1000 bp were positive for HSV1 and HSV2, respectively. Identification limit of 50 copies of HSV 1, 2 in each reaction was determined using nested PCR, which uses serial dilutions of AmpliRun® HSV1, HSV2 DNA CONTROL (Vircell, Spain).

Epstein Bar Virus: After qualitative control of extracted DNA by using the RT-PCR-Melting Curve Syber-Green for the β-globin gene, the Tag Man method was used to identify the EBV genome as a qualitative RT-PCR. To detect EBV, RT-PCR using the BamH1W EBV sequence primer set, which detects the 84bp gene region of the viral genome (ebv-f; 5'-GCAGCCGCCCAGTCTCT-3'), (ebv -r; 5'-ACAGACAGTGCACAGGAGCCT-3) with the internal probe (ebv-p; 5'-FAM-AAAAGCTGGCGCCCTTGCCTG, BHQ1-3') was used (27). The amplification of the reaction volume of 20 µl was done under the following conditions: At first, the samples were denatured at 94 ° C for 10 minutes, followed by denaturation at 94 ° C for ten seconds, after that 50 cycles extension, annealing was performed at 60 °C for 1 minute. The RT-PCR study, the CFX-96 system (BIO-RAD, USA) was used with the HS Taq premix Taq Man reagent (GENETOBIO, Korea). The detection limit of 15 copies of EBV genome was determined in each reaction by serial dilution using AmpliRun® EBV DNA CONTROL serial dilution (Vircell, S.L. Granada, Spain).

Data were analyzed using Chi- Square (or Ficher's exact test) and $p < 0.05$ was considered statistically significant.

Results

Thirty patients with pathologic diagnosis of LCH (16 males and 14 females) and 30 controls without LCH were examined. DNA-HHV6 was detected in a LCH patient and 6 controls from 30 cases. The number of LCH samples and controls for the HHV6 virus increased to 48 cases (24 boys and 24 girls) based on statistician advice. All patients were Iranian and had a

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limited age of 2 months to 10 years. DNA-HHV6 was detected in Lymoph node of a 2-year-old girl with diagnosis of LCH (2.1%) from 48 cases (2.1% positive and 97.9% negative) (table 1). In our control group, HHV6 was detected in 6 cases of 48 cases (12.5% positive and 87.5% negative) with OR: 0.02- 1.29, CI 95%: 0.15 P= 0.11 that there was no significant difference in theresult of prevalence between LCH patients and the control group. DNA-CMV was detected in a 2-year-old boy with LCH in the frontal bone and a one-year-old girl with LCH of the breast skin (2 cases of 66.6%) of 30 cases (66.6% positive and 93.33% negative). In the CMV control group, in one case (3.3%), of 30 cases (3.3% positive and 96/96% negative) with 15/24-18/0, 95% CI of OR07/2, OR: p=1 which showed no statistically significant difference in CMV prevalence between LCH patients and control group. DNA-HSV was not detected in any of the 30 LCH patients nor in any of the controls. DNA-EBV was detected in 19 patients with LCH (63.33% positive and 36.67% negative). In the control group, 8 cases of 30 cases of DNA-EBV (26.7% and 73.3% negative) were detected with 95.15% -25.1: CI of OR 95%; 75.4, OR: p=0.004. LCH patients with positive results for EBV included 11 boys and 8 girls (table 1).

Table1. Age, sex, and location of patients' biopsy and their results

Age	Sex	Clinic/Biopsy Location	HHV6	HSV	CMV	EBV	Age	Sex	Clinic/Biopsy Location	HHV6	HSV	CMV	EBV
2 years	Girl	Lymph nodes	+	-	-	+	2 years	Girl	Metatarsal bone	-	-	-	-
2 years	Boy	Mediastinal mass and neck	-	-	-	+	16months	Boy	skin	-	-	-	+
10months	Boy	skin	-	-	-	-	4 months	Girl	skin	-	-	-	+
1 year	Boy	Scalp mass	-	-	-	+	1 year	Girl	Skin (multiple lesion)	-	-	-	+
2 years	Boy	Forehead bone	-	-	+	+	1.5years	Boy	Soft tissue of head	-	-	-	-
11months	Boy	Mastoid	-	-	-	+	7 years	Boy	Soft tissue around the anus	-	-	-	-
1 year	Girl	Chest wall skin	-	-	+	+	5 years	Girl	skin	-			
3 years	Boy	Iliac bone	-	-	-	+	6 years	Boy	skin	-			
6 years	Girl	The upper part of the tibia	-	-	-	+	1 year	Girl	bone	-			
10 years	Boy	Soft skull tissue	-	-	-	-	2 years	Girl	Soft tissue	-			
2 years	Girl	parietal bone	-	-	-	-	17months	Boy	skin	-			
2 years	Boy	skin	-	-	-	+	4 years	Girl	Lymph node	-			
8 years	Boy	Soft tissue of head	-	-	-	+	1 year	Girl	skin	-			
2 years	Girl	Soft tissue under the chin	-	-	-	-	2 years	Girl	skin	-			
2 years	Boy	vertebrae bone	-	-	-	+	22months	Boy	bone	-			
7 years	Boy	Clavicle and sft tissue	-	-	-	+	2 years	Girl	skin	-			
15months	Boy	Abdominal mass	-	-	-	-	1 year	Boy	Soft tissue	-			
4 years	Girl	Skull	-	-	-	+	18months	Boy	skin	-			
2.5years	Girl	Right lung lobe	-	-	-	+	2 years	Girl	skin	-			
8 years	Girl	Abdomen skin	-	-	-	-	1 year	Boy	skin	-			
2 months	Girl	Soft tissue	-	-	-	+	1 year	Boy	Soft tissue	-			
2 years	Boy	Soft tissue of head	-	-	-	+	2 years	Girl	skin	-			
3 years	Girl	Bone of the forehead and soft tissue	-	-	-	-	5 years	Boy	skin	-			
3 years	Girl	Soft tissue of head	-	-	-	-	3 years	Girl	skin	-			

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Discussion

In this study, DNA-EBV was found in 19 of 30 cases of LCH (63.33%), which suggests the possibility of its etiologic involvement in the disease. HSV1, HSV2, CMV, or HHV6 did not show significant correlation with LCH. There is no report on the association of viruses in Iranian children with LCH. CMV can infect dendritic cells (DC) and langerhans (LC) cells (28- 30), EBV infect monocytes and LC during infectious period (31, 32). In addition, EBV and CMV appear to be involved in hemophagocytic syndromes, in patients with congenital immunodeficiency (28-30).

The findings of Jeziorski et al. (16) were against the hypothesis of the role of EBV, CMV, or HHV6 in the pathogenesis of LCH. McClain et al. (17, 18) failed to detect adenovirus, CMV, EBV, HSV, HHV6, Human Immune Deficiency Virus (HIV), Human Type I and II Tumor Cells (HTLV I, II, and parvovirus genome in 56 LCH cases using the Institute Hybridization (ISH) and PCR techniques. There are different opinions about the etiologic role of HHV6 in LCH (11-13, 16-18). HHV6 was detected in 47% of LCH patients by using PCR technique by Leahy et al. (13). Glotzbecker et al. (12) reported 71% positive results for HHV6 with IHC and ISH methods, but by using quantitative and qualitative RT-PCR, there was no significant difference between the 13 patients with LCH compared to the control group. Csire et al. (11) introduced a patient with LCH, in whom HHV6 was continuously positive over the course of 17 years and expressed the possible association of HHV6 infection with the flare or progression of LCH. Identification of CMV in LCs in 30% of 29 patients by Kawakubo et al. (14) by using ISH, IHC, and PCR may be due to the fact that the release of cytokines can stimulate proliferation of LCs and cause LCH. No positive results have been reported in articles about the association of HSV with LCH (13, 17, 18). EBV is known to affect many malignancies and identified herpesviruses are involved in continuous infections (20- 22) and possible etiologic role or participation of EBV is expressed in the pathophysiology of LCH (9, 10, 15, 19). These contradictory results in studies can be due to different sensitivities of techniques or regional differences. Previous studies using serology, IHC, ISH, and PCR techniques have not been reported any limitation (8- 13, 15- 18).

The IHC, ISH techniques were not available to us, and serologic testing for these infections has not been performed for patients. In addition to studies done by other researchers who achieved positive results (9, 10, 15), we also detected DNA-EBV in 19 of the 30 cases of LCH (63.33%) with p=0.004, 1.25-55.25: CI of OR 95%; 75.4 Odds Ratio. Positive results in 8 cases (26.7%) can be random. In the recent study, as in other negative reports (16-18), there was no significant difference in the identification of HHV6 in patients with LCH and in the control group. CMV-DNA was found in two patients with LCH (66.6%) and P-value is equal to 1, in support of the negative results of other studies (16-18).

Like previous studies (13, 17, 18), we also failed to detect HSV1 and HSV2 DNA in any of the LCH patients. Other techniques to confirm the presence of EBV in langerhans cells did not exist in our study and the etiologic role of EBV is not conclusively verified, but the positive results reported in other studies (9, 10, 15) in addition to 63.33% positive in our study, strongly suggest an effective role of EBV in LCH induction. These results are important because vaccination or early diagnosis and the treatment of EBV infection can possibly prevent the occurrence of LCH in the future.

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