Original Articles

Immuno-histochemical expression of cytomegalovirus protein (pp65) in childhood hepatitis

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Sri Lanka Journal of Child Health, 2019; **48**(1): 7-12

Abstract

Background: Cytomegalovirus (CMV) is a known cause of both neonatal and childhood hepatitis, but its exact role is yet to be identified.

Objective: To ascertain the role played by CMV in children with chronic hepatitis who are non-hepatitis B and C.

Method: This is a retrospective study carried out on 30 liver tissue paraffin blocks of children who are non-hepatitis B and C. Diagnosis was based on the presence of CMV protein (pp65) immunohistochemically. This was correlated with available serological results for CMV, severity of liver histopathological changes, and revision of clinical manifestations of the patients. Ethical approval for this study was obtained from the College of Medicine- Al-Nahrain University.

Results: Immunohistochemistry for pp65 was negative in 10 (33.3%) cases, low positive in 4 (13.3%) cases, moderately positive in 3 (10%) cases and highly positive in 13 (43.3%) cases. Mild inflammation and severe fibrosis showed the greatest positivity for CMVpp65.

Conclusions: CMV is an important cause of chronic hepatitis in children in Iraq.

DOI: http://dx.doi.org/10.4038/sljch.v48i1.8645

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(Received on 14 March 2018: Accepted after revision on 20 April 2018)

The authors declare that there are no conflicts of interest

This research was funded by the Ministry of Higher Education on Scientific Research, Iraq.

(Keywords: Cytomegalovirus, pp65, hepatitis, immunohistochemistry, children).

Introduction

Viral hepatitis is usually caused by viruses A, B, C, D, E and G[1]. Human cytomegalovirus (HCMV) is another known cause of hepatitis in neonates and children[2]. Cytomegalovirus (CMV) is a member of the Betaherpesvirinae sub-family of Herpesviridae[3]. CMV infection is acquired perinatally or through sexual contact, organ transplantation or blood transfusion[4]. CMV sero-prevalence varies geographically ranging from 30-100%[5]. In an Iraqi study by Al-Baiati *et al* on 152 aborted women, 15 (10%) were positive for IgM CMV and 129 (85%) were positive for IgG CMV[6]. In Saudi Arabia, Redwan *et al* reported a study done on 514 of foreign manpower in Jeddah and the result was 415 (80.7%) were positive for CMV IgG[7]. From USA, Staras *et al* reported a study done on 21,639 participants aged ≥ 6 years old and the result was 12,746 (58.9%) were positive for CMV IgG[8]. CMV has a wide range of tropism into different cells[9]. It can replicate in both hepatocytes and cholangiocytes[10]. It can cause intra-hepatic cholestasis and intra- and extra- hepatic biliary atresia[11]. It might cause cirrhosis and even death[12].

Objective

To ascertain the role played by CMV in children with chronic hepatitis who are non-hepatitis B and C.

Method

A retrospective study was carried out on 30 liver tissue paraffin blocks of patients admitted to the Gastroenterology and Hepatology Teaching Hospital, Children Welfare Teaching Hospital and Baghdad Teaching Hospital (Medical City) and Central Teaching Hospital of Paediatrics in Baghdad. Specimen collection was carried out from February-August 2014 for liver biopsies performed from 2008-2014 on children with non-B, non-C hepatitis. Patient records were revised for age, sex, clinical manifestations and the serology of CMV (available for only16 patients because either the tests were not requested or because they have been lost from the file). Diagnosis was based on presence of CMV protein (pp65) immuno-histochemically.

Immuno-histochemical staining was done on 4µm paraffin blocks. Slides were dewaxed in xylene and rehydrated using a descending ethanol series (90%–50%). They were then placed in the antigen retrieval solution (sodium citrate buffer, pH 6.0) in a water bath at 95^0C for 5 minutes. Sections were blocked for endogenous peroxidase (3% hydrogen peroxide for 15 minutes at room temperature). Drops of protein blocks were added to slides (15 minutes at room temperature). Monoclonal antibody anti CMV pp65 (dilution 1: 150, code number: ab49214, Abcam, USA) was added to slides and incubated at 37^0C for 1 hour and then placed in 4^0C overnight. For visualisation, a horse radish peroxidase detection system was used. Finally, positive cells were visualised with the chromogen diaminobenzidine after counterstaining them with haematoxylin. Dehydration was done and sections were mounted with DPX, covered with coverslips and examined under light microscope. Results of immune-histochemical staining were classified according to the percentage of CMV-positive cells into negative, low (≤ 25%), moderate (26%–50%), or high (≥51%). Positive control slide of Newcomer supply company (USA) was positive for CMV proteins. A single experienced histopathologist revised biopsy tissue for inflammation grading and fibrosis staging. Grading of inflammation and staging of fibrosis were done according to Knodell histological activity index as follows: inflammation: nil (<3), mild (3-6), moderate (7-11) and severe (≥12) and fibrosis: nil (1), mild (2), moderate (3) and severe (≥4).

For statistical analysis SPSS 19.0 and Microsoft Excel 2013 were used. Categorical data were formulated as counts and percentages. Chi-square test was used to describe the association of these data. A level below 0.05 was considered significant.

Results

Positive immunohistochemistry for CMV pp65 was seen in 20 (66%) cases distributed as shown in figure 1.

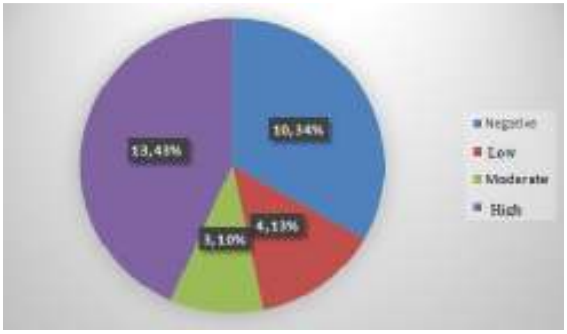

Figure 1: ***IHC scoring for CMV pp65***

There were higher positive CMV pp65 IHC in males compared to females but this was not statistically significant (Table 1). The highest positive CMV pp65 IHC results was in the age group less than 6 months 13(81.3%) as shown in Table 2.

Table 1: ***The association of CMV pp65 IHC results and gender type***

			Gender		Total
			Female	Male	
HCMV pp65 IHC	Negative	Count	6	4	10
		%	46.2%	23.5%	33.3%
	Positive	Count	7	13	20
		%	53.8%	76.5%	66.7%
Total		Count	13	17	30
		%	100%	100%	100%
p value			0.181		

Table 2: ***The association of CMV pp65 IHC results with age groups***

Age group		HCMV pp65 IHC		Total
		Negative	Positive	
< 6 months	Count	3	**13**	16
	%	18.8%	**81.3%**	100%
6-12 months	Count	2	2	4
	%	50%	50%	100%
13-18 months	Count	2	0	2
	%	100%	0%	100%
19-24 months	Count	3	2	5
	%	60%	40%	100%
>24 months	Count	0	3	3
	%	0%	100%	100%
Total	Count	10	20	30
	%	33.3%	66.7%	100%
P value		0.058		

The association between HCMV serology results and HCMV pp65 results are summarised in Table 3. Of the 16 cases where serology was available, 2 (12.5%) had negative serological tests but with positive pp65 IHC and 3 (18.8%) had at least one positive serological test but with negative pp65 IHC.

Table 3: ***Association between HCMV serology results and HCMV IHC pp65 results***

Patient number	HCMV IgM	HCMV IgG	HCMV pp65 IHC
1	+ve	+ve	+ve
2	+ve	+ve	+ve
3	-ve	-ve	-ve
4	+ve	+ve	+ve
5	+ve	-ve	+ve
6	+ve	-ve	+ve
7	+ve	-ve	-ve
8	-ve	-ve	+ve
9	-ve	-ve	-ve
10	+ve	-ve	-ve
11	-ve	+ve	+ve
12	-ve	-ve	-ve
13	+ve	-ve	+ve
14	-ve	+ve	-ve
15	+ve	+ve	+ve
16	+ve	-ve	+ve

This was available just for only 16 patients

From records, jaundice was found in 26 (86.7%) patients, hepatosplenomegaly in 12 (40%) and pale stools in 11 (36.7%) patients. In accordance with histopathology, inflammation grading and fibrosis staging are shown in tables 4 and 5.

Table 4: Distribution of CMV PP65 IHC according to inflammation

Inflammation							
Total	**Severe**	**Moderate**	**Mild**	**Nil**			
30	05	09	13	03	**Total**		**CMV pp65 IHC score**
10	03	01	04	02	**No.**	**Negative**	
33.3	60.0	11.1	30.8	66.7	**%**		
20	02	08	**09**	01	**No.**	**Positive**	
66.7	40.0	88.9	**69.2**	33.3	**%**		
0.162					***p* value**		
10	03	01	04	02	**No.**	**Negative**	**CMV pp65 IHC score**
33.3	60.0	11.1	30.8	66.7	**%**		
04	0	01	**02**	01	**No.**	**Low**	
13.3	0	11.1	**15.4**	33.3	**%**		
03	0	01	**02**	0	**No.**	**Moderate**	
10	0	11.1	**15.4**	0	**%**		
13	02	06	**05**	0	**No.**	**High**	
43.3	40.0	66.7	**38.5**	0	**%**		
0.434					***p* value**		

The nil, mild, moderate and severe is the grading of inflammation and every one of them contain cases with positive or negative reacting for pp65, the positive results were classified into low, moderate and high according to the percentage of staining cells by pp65

Table 5: Distribution of CMV PP65 IHC according to fibrosis

Fibrosis							
Total	**Severe**	**Moderate**	**Mild**	**Nil**			
30	12	10	05	03	**Total**		**CMV pp65 IHC score**
10	02	04	02	02	**No.**	**Negative**	
33.3	16.7	40.0	40.0	66.7	**%**		
20	**10**	06	03	01	**No.**	**Positive**	
66.7	**83.3**	60.0	60.0	33.3	**%**		
0.348					***p* value**		
10	02	04	02	02	**No.**	**Negative**	**CMV pp65 IHC score**
33.3	16.7	40.0	40.0	66.7	**%**		
04	**03**	0	0	01	**No.**	**Low**	
13.3	**25.0**	0	0	33.3	**%**		
03	**0**	03	0	0	**No.**	**Moderate**	
10	**0**	30	0	0	**%**		
13	**07**	03	03	0	**No.**	**High**	
43.3	**58.3**	30.0	60.0	0	**%**		
0.231					***p* value**		

The nil, mild, moderate and severe is the staging of fibrosis and every one of them contain cases with positive or negative reacting for pp65, the positive results were classified into low, moderate and high according to the percentage of staining cells by pp65

Nine (69.2%) of 13 cases with mild histological inflammation were positive for CMV pp65 IHC with the distribution with immune-histochemical scoring of 2 (15.4%), 2(15.4%), 5(38.5%) in low, moderate and high respectively, while 2 of 5 (40%) of those with severe histological inflammation were positive for CMV pp65 IHC, both with high IHC reaction. The highest positivity rate of immune-histochemical reaction was in cases with severe fibrosis 10 of 12 (83.3%) with the distribution of 3 (25%), 0 (0%), 7 (58.3%) in low, moderate and high IHC reaction respectively. Positive and negative results of CMV pp65 immunohistochemistry are shown in Figures 2 and 3.

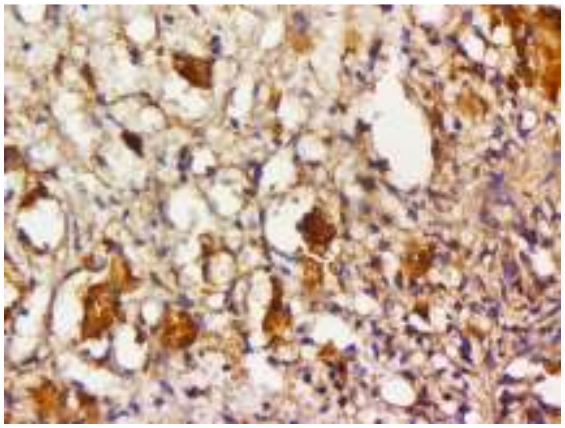

Figure 2: ***Positive result of CMV pp65 immunohistochemistry showing brown cytoplasmic and perinuclear stain (IHC stain, 40x)***

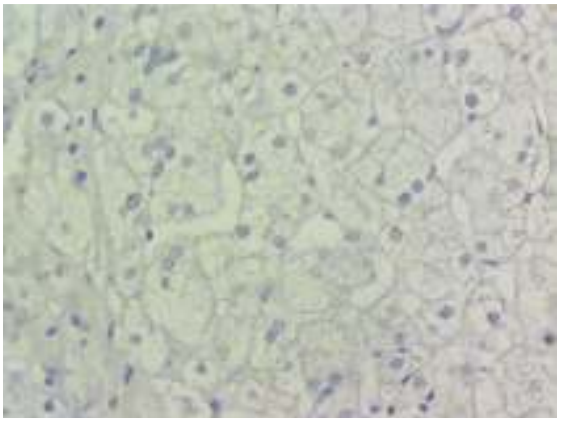

Figure 3: ***Negative result of CMV pp65 immunohistochemistry showing absence of brown cytoplasmic and perinuclear stain (IHC stain, 40x)***

Discussion

This study detects CMV in liver biopsy in infants and children with different diseases related to the liver. The idea came from the fact that CMV serology is difficult to interpret because HCMV virus serology may be related to the maternal immunity during pregnancy. The virus can replicate in hepatocytes and cholangiocytes during infection. These cells are one of the many cells that the virus can infect[9,10]. In the current study the detection of CMV was based on the presence of pp65. Expression of pp65 coincides with viral lytic replication[13]. Thus, the presence of pp65 represents the virus in lytic status and not in latent status.

The positive IHC expression of CMV pp65 was 66% of the studied samples. The pp65 expression reflects active viral infection[14]. This means that CMV is a frequent cause of hepatitis in neonates and children. The higher positive CMV pp65 IHC results in the

age group less than 6 months is probably due to congenital infection, perinatal infection and immaturity of the immune system in this age group[15,16]. Similar age related prevalence was reported by Shibata *et al.* in 2005 and Na SY in 2012[17,18].

From the patient records, only 16 patients had serological investigations for CMV. After comparing the results of serology and CMV pp65 IHC, it was noted there were mismatches in the results of these investigations in some cases. The serological diagnosis is an indirect method to detect CMV infection; the diagnosis depends on the presence of antibodies against the virus (IgM, IgG). Some cases were serologically negative (IgM, IgG) but positive for CMV pp65 IHC. This may be due to lower sensitivity and specificity of serological tests in comparison to IHC. Finally, the presence of a previous infection, or the passive transmission from the mother transplacentally, may be the cause of positive IgG antibodies with negative staining reaction, thus nullifying the possible role of HCMV as the cause of the hepatitis. The sensitivity and specificity of available HCMV serological test was 81% and 66% respectively. Similar findings were reported by Shibata *et al* in 2005, Na SY in 2012 and Hasosah *et al* in 2012[17-19].

Cases with mild histological inflammation 13 (43.3%) were the highest among the positive staining cases, and cases with severe histological fibrosis 12 (40%) were also the highest, probably pointing to the tendency of the virus to induce rapid tissue damage in the infected liver and reflects the extent of persistent or progressive hepatic injury[20].

Conclusions

CMV virus is an important cause of chronic hepatitis in children in Iraq who are negative for hepatitis B and C. CMV protein pp65 immunohistochemistry is a reliable method to diagnose CMV hepatitis. Most of the positive reacting cases to CMV pp65 IHC were in the high score, reflecting heavier viral load in the liver tissue, with tendency for rapid induction of tissue damage.

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