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RESEARCH ARTICLE

DETERMINATION OF GENOTYPES IN CYTOMEGALOVIRUS (CMV) STRAINS OBTAINED FROM PEDIATRIC AND ADULT IMMUNOCOMPROMISED PATIENTS

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ABSTRACT

Purpose: Cytomegalovirus (CMV) causes asymptomatic disease in individuals with a normal immune system; and leads to serious complications in immunocompromised individuals and fetuses. In CMV, gB is the most studied glycoprotein in terms of genotyping. Up to now, four different gB genotypes (gB 1-4) of CMV have been identified. In this study, it was aimed to determine the genotypes of CMV strains obtained from patients with immune deficiency.

Material and Methods: Twenty children and 29 adults, 49 patients who were followed in the Department of Adult Hematology and Pediatric Hematology were included in the study. DNA isolation was performed from samples with CMV DNA levels of 1000 IU / ml and above, and 474 bp region from the gB region of the virus was amplified by nested PCR. This region was sequenced by the Sanger (ABI 3500 Prism) sequencing. Next generation sequencing (NGS) method was applied to the samples whose CMV genotype could not be determined by Sanger sequencing.

Results: Distribution of CMV genotypes of patients determined by Sanger sequencing; while it was determined as 18/49 (36.7%) type 1, 5/49 (10.2%) type 2, 5/49 (10.2%) type 3 and 1/49 (2%) type 4; 14/49 (28.5%) of them were found as mixed genotypes. CMV genotype could not be determined in 6 patients by Sanger sequencing and CMV genotype of these 6 patients was found as mixed genoype by NGS. A mixed genotype was detected in 20 (40.9%) of 49 patients, in total by Sanger sequencing and NGS.

Conclusion: Mixed genotypes were detected most commonly in our study, and it is recommended that genotypes that cannot be determined by Sanger sequencing should be studied with NGS. It can be thought that genotype determination will be a determining factor in the treatment of CMV disease and in prospective vaccine development studies.

Keywords: Cytomegalovirus, genotype, NGS, sequencing

INTRODUCTION

Cytomegalovirus (CMV) is a virus belonging to the Herpesviridae family of the Herpesvirales order. While CMV usually causes asymptomatic disease in individuals with a normal immune system, it causes serious mortality and morbidity in immunocompromised patient groups such as solid organ and bone marrow transplant patients and

patients with acquired immunodeficiency syndrome (AIDS) (1).

The CMV genome is 235 kb in size and has the largest genome among human herpesviruses. It contains more than 60 glycoproteins in its structure. Glycoprotein B (gB) (gpUL55), one of the major CMV glycoproteins, has five defined antigenic determinants (AD1-AD5), which are the target of neutralizing antibodies, enabling the cell-to-cell transmission of the virus and the fusion of infected cells. Of these, AD-1 and AD-2 are the primary targets of non-neutralizing antibodies. The gB found in the CMV envelope constitutes more than 50% of the total protein mass in the envelope and stimulates the host immune response (2).

Glycoprotein B (gB) is the most studied protein in terms of genotyping. gB is a target region for neutralizing antibodies and is a marker protein for determining transmission routes and geographic origin. In the pathogenesis, it is thought that there may be differences between genotypes in the attachment, fusion and spread of the virus from cell to cell (3). In CMV strains found in nature, four different gB genotypes (gB1-4) have been detected so far, less commonly gB 5,6, and 7 genotypes have been reported (4).

Mixed genotypes are encountered in studies on CMV genotyping. The term mixed genotype is the presence of gB1, gB2, gB3 and gB4 in pairs or triples. There are many studies in the literature in which mixed genotypes were detected. It has been reported that the risk of CMV disease is high in patients with mixed genotype, and the life span is shortened in these individuals (5,6)

DNA sequence analysis (sequencing), RFLP (Restriction Fragment Length Polymorphism), "single-strand conformation polymorphism" analysis (SSCPA), "heteroduplex motility" analysis (HMA) and multiplex PCR methods are used to determine CMV genotypes (7).

This study, it was aimed to define the CMV genotypes that provide information about the course, severity, transmission route and geographical origin of CMV disease in immunocompromised patients.

MATERIAL AND METHODS

Patients and Samples

Among the patients followed in Erciyes University Health Practice and Research Center Adult Hematology and Pediatric Hematology Departments between January 2016 and September 2017, those with a CMV DNA viral load of 1000 IU/ml and above were included in the study. Plasma samples of 49 patients, 20 of whom were children and 29 of whom were adults, were included in the study.

Determination of CMV Genotypes

CMV DNA was isolated from the samples with EZ1 Advanced, Qiagen-Germany. The 474 bp region in the CMV gB gene region was amplified by "nested PCR". Amplification processes were performed on the ProFlex PCR system (Applied Biosystems).

PCR primers of CMV genotypes for g1, sense primer 5'-GATCTCCTGGGATACAGGACG-'3, antisense primer 5' for g2- GAATTGCTGATGGTTTGATCTTG-'3, sense primer 5' for g3-ACTTTCTGGGAAGCCTCGGAACG-'3, antisense primer for g4 5'-GAGTTCCTTGAAGACCTCTAGGGT-'3 was used.

First PCR protocol started with a 15 minute activation step at 95°C. The thermal cycler was programmed for 45 cycles, denaturation at 94°C for 30 sec, bonding at 55 °C for 30 sec, and at 72 °C for 30 sec. elongation stages. The final extension phase was studied at 72 °C for 10 minutes.

The amplification process was applied to the obtained amplicons by working with the g3 and g4 primers with the same protocol. PCR products were visualized by agarose gel electrophoresis. CMV genotypes were determined by Sanger sequence analysis (3500 Abi Prism). Sanger sequencing method was studied at Erciyes University Genome and Stem Cell Center (GENKÖK). Samples whose genotype could not be determined were studied by using the next generation sequencing method.

CMV genotypes were determined based on the sequences indicated in Table 1.

Ethical Approval

To carry out the study, ethical approval was obtained from the Non-Interventional Clinical Research Ethics Committee of Erciyes University School of Medicine (Date and Decision number: 22.01.2016, 2016/57).

RESULTS

Twenty of the patients were children and 29 were adults. Of all the patients, 21 (42.9%) were male and 28 (57.1%) were female. Of the 49 patients included in the study, 41 were bone marrow transplant (BMT) recipients and 8 were patients followed (not transplanted) in the hematology outpatient clinic/services. The clinical diagnoses of the patients

Table 1. CMV sequence table

gB1	A	A	A	A	G	A	A	G	T	A	C	A	G	A	T	G	G	C	A	A	C	A	A	T	G	C	A	A	C	T	C	A
gB2	A	G	A	A	G	A	A	G	T	A	C	G	A	G	T	G	A	C	A	A	T	A	A	T	A	C	A	A	C	T	C	A
gB3	A	A	G	A	G	A	A	G	T	A	C	G	-	-	-	G	G	C	A	A	T	A	C	G	A	C	C	A	C	C	-	-
gB4	A	G	A	A	G	A	A	G	T	A	C	A	G	A	T	G	G	C	A	C	C	A	A	T	G	T	A	A	C	T	C	A

Table 2. Clinical diagnosis of patients and CMV genotype distribution determined by Sanger sequencing (n = 49)

Diseases	**number**	**gB1**	**gB2**	**gB3**	**gB4**	**mixed***	**nontypeable**
Acute Myeloid Leukemia	15	6		2		6	1
Acute Lymphoblastic Leukemia	8	3	1	1		1	2
Multiple Myeloma	6	1	2	1		1	1
Non-Hodgkin Lymphoma (NHL)	2	1				1	
Hodgkin Lymphoma	1		1				
Burkit Lymphoma	1	1					
Myeloid Ciplastic Syndrome	4		1			1	2
Autoimmune Hemolytic Anemia	1				1		
Aplastic Anemia	1	1					
Acquired Aplastic Anemia	1					1	
Fanconi Aplastic Anemia	1					1	
Combined Immunodeficiency (SCİD)	4	3		1			
PNET	1					1	
Thalassemia Major	1	1					
Hemophagocytic Syndrome	2	1				1	
Total	**49**	**18**	**5**	**5**	**1**	**14**	**6**

and the CMV genotype distributions determined by the Sanger sequencing method are shown in Table 2. Of the 49 patients included in the study, 35 (71.4%) underwent allogeneic bone marrow transplantation (ABMT), 6 (12.3%) autologous bone marrow transplantation (AUBMT), and 8 (16.3%) transplantation. was not done (Table 3).

According to the CMV genotype distribution of 49 patients studied with the Sanger method; 18/49 gB 1 (36.7%); 5/49 gB 2 (10.2%); 5/49 of gB 3 (10.2%); GB4 (2%) was found in 1/49, and mixed genotype was found in 14/49 (28.6%). CMV genotypes could not be determined by the Sanger method in 6/49 (12.3%) of the patients. Mixed genotype was determined by next generation sequencing in 6 samples whose CMV genotype could not be determined. A mixed genotype was found in 20/49 (40.9%) of the patients with Sanger sequencing and next generation sequencing methods. The mean values of the CMV DNA viral loads of the patients included in the study according to the genotypes; gB1 42,376 IU/ml; gB2 26,099 IU/ml; gB3 32,335 IU/ml; While gB4 was 52,852 IU/ml, the average viral load of the mixed genotypes was 103,853 IU/ml.

Table 3. Distribution of CMV genotypes detected by Sanger sequencing in patients with ABMT, AUBMT and nontransplantated patients.

GENOTYPE	ABMT	AUBMT	OTHER PATİENTS	TOTAL	%
Type 1	14	-	4	18	36,7
Type 2	3	2	-	5	10,2
Type 3	3	1	1	5	10,2
Type 4	1	-	-	1	2
Mix	10	2	2	14	28,6
not typed	4	1	1	6	12,3
Total	35	6	8	49	100

ABMT: Allogeneic Bone Marrow Transplantation, AUBMT: Autologous Bone Marrow Transplantation

DISCUSSION

CMV causes serious illness in immunocompromised hosts such as solid organ or bone marrow transplant recipients, cancer patients receiving chemotherapy, and patients with AIDS. In this type of patients, virus excretion is more and longer; common infections and complications are more likely (8). Of the CMV glycoproteins, gB is the most studied protein in terms of genotyping. Four different gB genotypes (gBI-4) have been described to date in naturally occurring CMV strains. More rarely, GB 5, 6 and 7 genotypes have been reported (3). Apart from gB, genotyping studies were also carried out with gH (UL75), gN (UL73), gM (UL100), STR, UL4, and UL144 (9).

DNA sequence analysis, RFLP, SSCP, HMA and multiplex PCR methods are used to determine CMV genotypes (10).

Roubalova K. et al. in a study in which they investigated CMV genotypes, reported that CMV gB1, gB2, gB3, and gB4 genotypes were found at a rate of 30%, 17%, 26%, and 4%, respectively, in 53 patients with ACT, and atypical gB genotype was detected in one patient (11).

In another study investigating CMV genotypes in 281 patients who underwent bone marrow transplantation; researchers found gB1 48.4%, gB2 16.4%, gB3 24.6%, gB4 8.2% and mixed genotype 2.5% (12). Woo et al. investigated CMV genotypes in 33 bone marrow transplant recipients; 8 of the patients (25%), gB2 (42%), 6 (18%), 2 (6%) gB4 and 3 (9%) mixed genotype were detected (13). In a multicenter study, 26% of 239 solid organ transplant recipients with CMV infection found gB1, 10% gB2, 10% gB3 and 5% gB4 genotypes; Infection with more than one genotype has been reported with a rate of 49% (14).

Ciotti et al. reported that they found 12.8% gB1, 23.4% gB2, 4.2% gB3 and 59.6% mixed genotype in a study they conducted in 24 solid organ transplants and 23 hematopoietic stem cell recipients. gB4 was detected only in samples with mixed genotypes (5).

In a study by Soleimani et al. in Iran, the distribution of CMV genotypes in kidney transplant recipients was investigated and gB1 was the most common genotype with 35.3%. following gB1; gB3 (17.6%) and gB4 (17.6%) were found equally. As gB2 and mixed genotypes (gB 1+ gB3) and (gB1 +gB 2), 14.7% were detected (15).

Barrado et al. stated that viral load was found to be higher in samples with mixed genotype, but genotype was not a distinguishing factor in the course of the disease (16).

Parkan et al. studied different gene regions in 33 samples of 12 patients diagnosed with congenital CMV using the next generation sequencing technique (17). Dominant genotypes for UL6, gN, gO, UL139, UL146, gB and gH, respectively; genotypes 2 (58%), genotypes 1, and 4c (33.3% each), genotypes 1a and

1c (33.3% each), genotype 5 (50%), genotypes 8, and 12 (25% each) , genotype 1 (75%) and genotype 2 (83.3%) were determined.

In a study investigating CMV gB genotypes in different patient groups in our country; The most common genotypes were gB1 (32.5%) in kidney transplant recipients, gB1 (34.3%) in stem cell transplant recipients, gB3 (57.1%) in heart transplant recipients, and gB1 (38.4%) in newborns (4).

In a study conducted by Kılınç et al. in our region, 33 of 74 samples taken from 53 patients were gB1 (45%), 7 (9%) gB2, 14 (19%) gB4, 2 (% 4), mixed genotype was detected in 18 (24%) (5).

In our study, gB1 was found in 18 (36.7%) of 49 patients, most of whom underwent bone marrow transplantation using the Sanger method, gB2 in 5 (10.2%), gB3 in 5 (10.2%) and gB4 in 1 (2%) of the patients. Mixed genotype was found in 20 (40.8%) of them. In the study of Kılınç et al., the most common genotype was gB1; the most common mixed genotype was found in this study (18).

In the study performed by Kılınç et al. (17) in our region, the viral loads of the samples with mixed genotype were determined as 103-105 copies/ml; found the viral loads of the samples with a single genotype to be 102-103 copies/ml.

In the study of Vinuesa et al., using next-generation sequencing technique in 25 ACT patients with CMV viremia; They detected more viral load in samples with mixed variants (19).

In our study, the viral loads of the samples with mixed genotypes were found to be higher than those with a single genotype. The results of all three studies were similar.

Coaquette et al. state that infection with more than one genotype progresses to CMV disease in immunocompromised patients, and that graft rejection, viral load, and coinfection with other herpesviruses are higher in these patients (20).

In another study investigating the response of CMV gB genotypes to antiviral treatment; It has been shown that although the basal viral load level is higher in infections with more than one genotype, it decreases more quickly in the first days of treatment compared to other genotypes, and the gB1 genotype responds to treatment later in the first days (21). Despite all these studies, it remains unclear whether different gB genotypes are at risk in the development and prognosis of CMV disease in humans.

PCR techniques, which are widely used in genotyping of CMV, have been replaced by sequence analysis techniques, and typing of mixed genotypes in the Sanger technique, which is accepted as classical sequencing, is not possible in some isolates, as seen in our study. For this reason, it is considered appropriate to use next-generation sequencing for samples that cannot be determined by the Sanger method for CMV genotype determination. In Arav's research, it is stated that next-generation sequencing techniques are superior to other methods in determining mixed CMV genotypes (22). It is known that CMV disease caused by CMV mixed genotypes detected in immunocompromised patients is more severe than in patients with a single genotype. Therefore, mixed genotype determination should be investigated with sensitive methods in these patient groups (3).

In our study, 18 (36.7%) of the patients found gB1, 5 (10.2%) gB2, 5 (10.2%) gB3, 1 (2%) gB4, 20 (20.9%) mixed genotype.

CONCLUSION

As a result; It is noteworthy that the highest rate of mixed genotype was detected in our patients. It was concluded that the next generation sequencing method is appropriate to study in patients whose CMV genotype could not be determined by the Sanger method. With CMV genotype determination, parameters such as the severity of the CMV disease, the route of transmission, the geographical region where the disease was first seen are illuminated, and it is obvious that it will also shed light on treatment, prevention and vaccine development studies.

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