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Adenovirus Association with Graft versus Host Disease in Paediatric Bone Marrow Transplantation Recipients

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ABSTRACT

Background: Adenovirus (Ad) infections have delayed clearance in paediatric patients. The immune suppression that occurs after hematopoietic stem cell transplantation (HSCT) can reactivate adenovirus, resulting in life-threatening disseminated disease. **Aim of the work:** to assess adenovirus infection in recipients of bone marrow transplantation and to find out if there is an association between adenovirus infection and the occurrence of graft versus host disease (GVHD) in these patients. **Patients and methods:** The study was conducted on 30 pediatric patients admitted to Nasser institute, for bone marrow transplantation. Serum and stool samples were collected one day prior to bone marrow transplantation, and every one or two weeks afterwards till the patient completed 100 days after HSCT. The adenoviral DNA was monitored in the patient's serum and stool samples by quantitative real time polymerase chain reaction (qPCR). The stool samples that tested positive for the presence of adenovirus were processed by cell culture technique for isolation of the virus. **Results:** Adenoviral DNA was detected in the fecal samples of 11 out of 30 patients (36.6% of cases); the viral load ranged from 1.2 X 10^3 copies/gram, to 8.4 X 10^7 copies per gram, two patients had 2 positive stool samples with rising titer indicating the reactivation of the adenovirus. The adenoviral DNA was detected in the serum samples of only two patients (6.6% of cases), with a low titer, one of them was only 500 copies/ml and the other one was 1.6 X 10^4 copies /ml. The remaining serum samples of the patients who shed the adenovirus in their stool were all negative for adenoviral DNA. These two patients, who had Ad DNA in their serum, had no adenoviral shedding in their stool. The adenovirus reactivation was not associated with increased risk of developing GVHD, diarrhea or CMV reactivation.

Conclusion: although the adenovirus was shed in the stool of 9 patients, the viral shedding was not associated with increased risk of developing GVHD, diarrhea or CMV reactivation.

Keywords: Adenovirus, hematopoietic stem cell transplantation (HSCT), graft versus host disease (GVHD), bone marrow transplantation.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the last treatment option for a variety of diseases, including certain hematologic malignancies, inborn errors of metabolism, immune deficiencies, and bone marrow failure syndromes [1]. Besides the risk of developing bacterial and fungal infections, there is a substantial risk of viral infection or reactivation during the immunosuppressed period after HSCT. Among all viruses; cytomegalovirus (CMV) and adenoviruses (Ad) are important [2]. Adenovirus (Ad) and cytomegalovirus infections have delayed clearance in paediatric patients. There is an immune suppression after hematopoietic stem cell transplantation (HSCT), which can reactivate these viruses, resulting in life-threatening disseminated disease [3].

Adenoviruses are nonenveloped, double-stranded deoxyribonucleic acid (dsDNA) viruses, at present, 51 serotypes and over 70 genotypes have been defined within seven species (A–G) [4]. Adenovirus species C is most frequently isolated in the general population and HCT recipients [5].

Viral reactivations, of AdV defined as viraemia with >1000 copies/mL while serum or plasma Ad DNA loads greater than 10^6 copies per mL have an increased risk for fatal complications. According to different studies [6, 7, 8] the estimated rate of Ad infection after HSCT ranges from 3–47%, with a mortality rate from 10% to 80%. In post-HSCT patients, adenoviruses are the cause of severe respiratory disease, hepatitis, and colitis or hemorrhagic cystitis [9,10]. Screening for AdV in the stool and molecular monitoring of viral load in serial stool specimens may facilitate early detection of impending viremia[11]. The frequency of Ad viremia in individuals with peak virus levels in stool specimens above 1×10^6 copies per gram was 73% vs 0% in patients with Ad levels in stool specimens below this threshold [12].

The aim of this study was to assess Adenovirus infection in recipients of bone marrow transplantation by real time polymerase chain reaction (qPCR) and to find out if there is an association between adenovirus infection and the occurrence of GVHD in these patients.

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PATIENTS AND METHODS

Patients and samples: The study was conducted on 30 pediatric patients admitted to Naser Institute for bone marrow transplantation, 13 of them were females and 17 are males, aged from 1 year to 15 years. Clinically, ten patients had β-thalassemia major, six patients had Fanconi anemia, four patients had acute lymphoblastic leukemia, one patient had chronic lymphoid leukemia, one patient had osteopetrosis, one patient had Wiskott Aldrich syndrome, one patient had Griscelli's syndrome, one patient had adrenoleukodysrtophy, one patient had severe aplastic anemia, one patient had lymphoblastic lymphoma, one patient had congenital amegakaryocytic thrombocytopenia, one patient had acute myeloblastic leukemia, and one patient had severe combined immunodeficiency

All patients were tested for the occurrence of CMV reactivation and rise in serum by qPCR which was done routinely on weekly basis. Clinical data were collected (Table 1).

Table (1): incidence of clinical complications that occurred after bone marrow transplantation in patients included in the study:

Clinical data		Frequency	Percent
CMV reactivation	negative	27	90
	Positive	3	10
Diarrhea	negative	23	76.7
	Positive	7	23.3
GVHD	negative	24	80
	Positive	6	20

GVHD = graft versus host disease, CMV =Cytomegalovirus

The inclusion criteria were:

Age is less than 18 years, and undergoing allogenic hematopoietic stem cell transplantation.

Sample collection:

The serum and stool samples were taken one day prior to bone marrow transplantation, and every one or two weeks afterwards till the patient completes 100 days after BMT. Total number of samples was 100 stools and 234 serum samples. The stool samples were markedly lower in number than serum samples because patients were discharged from hospital after 3 weeks of HSCT, they were asked to attend in the outpatient clinic on weekly basis for follow up and blood sampling for routine investigations, most of them found some burden to carry stool samples from their distant homes to the outpatient clinic. All samples were stored at -20 °C till DNA extraction and sample inoculation into tissue culture.

Ethical consideration: to participate in the study, a written consent was obtained from each patient's guardian following the indications of the ethical committee of the faculty of medicine for girls, Al Azhar University, Egypt. All study participants approved the storage of their frozen specimens for the study research purposes, in our laboratory. **The study was approved by the Ethics Board of Ain Shams University.**

Materials required for cell culture:

- Hep -2 cells (human epithelial carcinoma strain 2) were purchased from and propagated in the virology lab in VACSERA (the holding company for biological products and vaccines) Cairo, Egypt.
- Minimum Essential Medium (MEM) with Earle's Balanced Salts containing 10% fetal bovine serum, penicillin, streptomycin, and gentamicin was used.
- Reference adenovirus strains:
- Adenovirus serotype 1, Accession number (2010904126) ~1ml, and
- Adenovirus serotype 5, Accession number (2010907500) ~1ml
- They were kindly obtained from the viral and zoonotic diseases research program at NAMRU-3.
- Adenovirus specific primer were adopted from Hiem[13] and the sequence of them were as follows:
- 5'-GCC-ACG-GTG-GGG-TTT-CTA-AAC-TT-3' Adeno-quant 1 (AQ1) and
- 5'-GCC-CCA-GTG-GTC-TTA-CAT-GCA-CAT-C-3', Adenoquant 2 (AQ2).
- The sequence of the probe was 5'-TGC-ACC-AGA-CCC-GGG-CTC-AGG-TAC-TCC-GA-3' (Adenoprobe, AP) with FAM labelled as a fluorescent dye on the 5' end and TAMRA as a fluorescence quencher dye labelled to the 3' end. Reaction conditions were 5 minutes 35ºC for Uracil-DNA-Glycosylase incubation followed by 95ºC for 10 minutes to activate the ''hot start'' Taq-polymerase. Forty-five cycles that consisted of denaturation at 95ºC for 3 seconds, annealing at 55ºC for 10 seconds, and extension at 65ºC for 60 seconds were performed with a temperature increase of 0.5ºC/ seconds between the annealing and extension step.

METHODS

The procedure of Sub culturing, maintaining Hep-2 cells, maintaining the virus stock and isolation of the adenoviral causative agent from stool samples was

performed as recommended by ATCC (American type culture collection).

DNA was extracted from serum samples by using QIAamp MinElute Virus Spin Kit (Qiagen, Dusseldorf, Germany) and the procedure was done as recommended by manufacturer. DNA was extracted from stool samples by using QIAamp DNA Stool Mini Kit (Qiagen, Dusseldorf, Germany) and the procedure was done as recommended by manufacturer.

Setting the standard real time PCR curve:

In order to draw the standard curve for real time PCR, the exact number of the copies of the virus had to be known, this was achieved through the following four main steps:

First: running a conventional PCR. The primers were adopted from Hiem[13] these primers were designed to achieve DNA amplification of all 51 types of the genus human AdV.

Second: purification of the amplicon by using QIAquick PCR purification kit (Qiagen, Dusseldorf, Germany).

Third: measuring the amount of DNA in the amplicon by using Qubit DNA high sensitivity assay kit (Invitrogen, California, USA) and using the Qubit device.

Fourth: the exact virus copy number was calculated by using online software called "copy number calculator for real time PCR".

The exact number of virus copies present in the amplicon was 2.88×10^{10} copy/ µL, Then a serial tenfold dilution was done and they were put in real time PCR to set up the standard curve. Finally a real time PCR reaction was done for the DNA extracted from all serum and stool samples to detect the presence and the quantity of adenoviral DNA.

RESULTS

The Adenoviral DNA was detected in the fecal samples of 11 patients; the viral load ranged from 1.2×10^3 copies/gram, to 8.4×10^7 copies per gram, two patients had 2 positive stool samples with rising titer indicating the progression of the disease.

The serum samples of the patients who shed the adenovirus in the stool were all negative for adenoviral DNA. The adenoviral DNA was detected in only two serum samples, with a low titer, one of them was only 500 copies/ml and the other one was 1.6×10^4 copies /ml.

These two patients, who had Adv DNA in their serum, had no adenoviral shedding in their stool.

All stool samples which were taken prior to bone marrow transplantation tested negative for adenoviral DNA, which indicates that the adenovirus reactivation was due to immune suppression by immune-suppressive medications. The stool samples that tested positive for the presence of adenoviral DNA were processed by cell culture and the viruses were successfully isolated.

The relation between the presence of adenoviral DNA in serum and stool samples and age of the patients was calculated using Wilcoxon sum rank test (Table 2). The relationship between the presence of adenoviral DNA in serum and stool samples were studied against source of graft, gender, CMV, diarrhea, GVHD & the underlying illness (malignant vs. benign) using chi square & fisher exact test as appropriate (Table 3, 4). None were significant.

Table (2): The relation between the presence of adenoviral DNA in serum and stool samples and age of the patients.

Descriptive characteristics			**Wilcoxon sum rank test**		
Fecal samples	**Neg. Fecal**	**Pos. Fecal**	**Z**	**P**	**Significance**
N	19	11			
Median	7	6			
Min.	1	3			
Max.	14	15			
			-0.347	0.729	NS
Serum samples	**Neg. Serum**	**Pos. Serum**	**Z**	**P**	**Significance**
N	28	2			
Median	7	12.5			
Min.	1	11			
Max.	15	14			
			-2.053	0.04	Significant

N= number, NS= not significant

Table (3): Relation between the underlying disease, source of the graft, GVHD, CMV, gender and adenoviral DNA presence in fecal samples:

			Presence of adenoviral DNA in stool samples			P value
			Negative	Positive	Total	
GVHD	Negative	Count	15	9	24	0.36 *
		% within GVHD	62.5%	37.5%	100.0%	
	Positive	Count	2	4	6	
		% within GVHD	33.3%	66.7%	100.0%	
Gender	Female	Count	9	4	13	0.225 @
		% within Gender	69.2%	30.8%	100.0%	
	Male	Count	8	9	17	
		% within Gender	47.1%	52.9%	100.0%	
CMV	negative	Count	15	12	27	1 *
		% within CMV	55.6%	44.4%	100.0%	
	Positive	Count	2	1	3	
		% within CMV	66.7%	33.3%	100.0%	
Diarrhea	negative	Count	15	8	23	0.19 *
		% within Diarrhea	65.2%	34.8%	100.0%	
	Positive	Count	2	5	7	
		% within Diarrhea	28.6%	71.4%	100.0%	
diagnosis	benign	Count	11	12	23	0.427 *
		% within diagnosis	47.8%	52.2%	100%	
	malignant	Count	2	5	7	
		% within diagnosis	28.6%	71.4%	100%	
Source of graft	1st degree	Count	1	1	2	1*
		% within Source of graft	50.0%	50.0%	100.0%	
	2nd degree	Count	16	12	28	
		% within Source of graft	57.1%	42.9%	100.0%	
Total number of patients whose stool samples tested positive for the presence of adenoviral DNA		Count	19	11	30	
		%	56.7%	43.3%	100.0%	

* P value using fisher exact test, @ p value using chi square test

Table (4): Relation between the underlying disease, source of the graft, GVHD, CMV, gender and adenoviral rise in serum samples:

			resence of adenoviral NA in serum samples			P value
			Positive	Negative	Total	
GVHD	negative	Count	2	22	24	1 *
		% within GVHD	8.3%	91.7%	100.0%	
	Positive	Count	0	6	6	
		% within GVHD	0.0%	100.0%	100.0%	
Gender	Female	Count	2	11	13	0.179 *
		% within Gender	15.4 %	84.6%	100 %	
	Male	Count	0	17	17	
		% within Gender	0 %	100 %	100 %	
CMV	negative	Count	1	26	27	0.193 *
		% within CMV	3.7%	96.3%	100.0%	
	Positive	Count	1	2	3	
		% within CMV	33.3%	66.7%	100.0%	
Diarrhea	negative	Count	2	21	23	1 *
		% within Diarrhea	8.7%	91.3%	100.0%	
	Positive	Count	0	7	7	
		% within Diarrhea	0.0%	100.0%	100.0%	
diagnosis	benign	Count	1	22	23	0.418 *
		% within diagnosis	4.3%	95.7%	100.0%	
	malignant	Count	1	6	7	
		% within diagnosis	14.3%	85.7%	100.0%	
urce of graft	1st degree	Count	0	2	2	1*
		% within Source of graft	0.0%	100.0%	100.0%	
	2nd degree	Count	2	26	28	
		% within Source of graft	7.1%	92.9%	100.0%	
otal number of patients ose serum samples tested sitive for the presence of adenoviral DNA		Count	2	28	30	
		%	6.7%	93.3%	100.0%	

* P value using fisher exact test

DISCUSSION

This study was conducted on paediatric patients only because other studies confirmed that the adenoviral infection rate in adult BMT recipients is extremely low [14]. Other similar studies utilized multiplex PCR by using 5 sets of primers, this was due to the high genetic diversity of the all human AdV types which made it difficult to design a single set of primers to detect all Ad serotypes [12]. However, Hiem managed to design a single set of primers to detect all known HAd serotypes. This was achieved by a strategy of constructing consensus sequences that balanced mismatches with various so far sequenced human AdV types achieving similar melting temperatures [13.]

In this study, adenoviral DNA was detected in the fecal samples of 11 patients out of thirty patients (36.6%), the virus load ranged from 1.2 X 10^3 copies/gram, to 8.4 X 10^7 copies per gram, two patients

had 2 positive stool samples with rising titer indicating the progression of the disease. This result is very close to other similar studies [11, 12, 15]. All stool samples which were taken prior to bone marrow transplantation tested negative for adenoviral DNA, which indicates that the adenovirus infection was due to immune suppression by immune-suppressive medications. Conversely with other studies denoting higher frequency of adenoviral DNA rise in the serum of BMT patients that may reach (44.8%) [5,9,10], our study showed very low frequency (6.6%). For explanation, we point out that all patients were given prophylactic dose of ganciclovir to guard against CMV infection, which probably has limited the adenoviral replication and rise in the serum of patients and limited the replication to intermediate viral load (maximum copy number in stool samples was 8.4 X 10^7 copy/ gram). In addition, the rise of AdV DNA in the serum of these two patients can be also explained by the fact that AdV DNA can be detected with low level (1.7 X 10^3 copies/ml) in blood samples of any person; this is related to a latent or persistent infection of lymphoid cells [13].

Consistent with Bil-Lula, there's a higher frequency of adenoviral reactivation in male patients [16]. In contrast with Mynarek that point out that the frequency of adenoviral infection is higher in patients younger than 5 years [8], our study showed no relation between the age of the patients and adenoviral shedding in stool. This may be due to small number of patients included in this study compared with other studies.

Van Montfrans showed that there was a higher frequency of adenoviral reactivation in HSCT patients who experienced moderate to severe GVHD, however, our study showed no relation between adenoviral reactivation and development of GVHD [17].This is because most of the patients in our study experienced a very mild GVHD, which showed gradual improvement after a short period of time.

In accordance with Matthes-Martin study, there was no relation between the adenoviral reactivation and the occurrence of diarrhea[18].

Mynarek reported that adenoviral infection rises in HSCT patients who received the graft from unrelated donor [8]. In our study, patients received grafts from first and second degree relatives, none of them received graft from unrelated donor.

In accordance with Rustia study, there's no relation between CMV rise in the serum of the patients and adenoviral shedding in stool. On the other hand, one of the two patients in this study who had a rise in adenoviral DNA in their serum, showed concomitant rise of CMV also, which is consistent with the same study that denote that adenoviral-CMV co-reactivation and rise in serum in BMT patients may reach 14.3% [6].

CONCLUSION

Eleven out of thirty patients experienced adenoviral reactivation and shedding in the stool, this shedding did not lead to rise of adenoviral DNA in their serum, and didn't increase the frequency of occurrence of diarrhea, GVHD or related to CMV rise in serum.

Therefore, monitoring of adenoviral reactivation in pediatric bone marrow transplantation recipients is recommended by detection of the adenoviral DNA load in stool samples by real time PCR.

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