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ORIGINAL ARTICLE

Risk Prediction Genetic Study of Non-HLA Gene NOD2 Three Polymorphisms in Acute Graft versus Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation

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ABSTRACT

***Background**: Graft-versus-host disease (GVHD) has been associated with non- human leukocyte antigen (HLA) gene polymorphisms after allogenic Hematopoietic stem cell transplantation (aHSCT). **Objective:** This study aims to investigate the role of three polymorphisms in Nucleotide-binding Oligomerisation Domain (NOD2) gene on the occurrence / severity of acute GVHD among Egyptian population. **Methodology:** A total of 64 patients and their corresponding donors who underwent aHSCT from HLA-identical matched siblings were genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Results were confirmed by automated laser sequencer. **Results:** There was highly statistical significant association between NOD2 recipient l007fs allele and higher incidence of aGVHD p= 0.006. There was no significant association between NOD2 donor l007fs allele, G908R allele in donors and recipients, R702W in donors and recipients with incidence and severity of aGVHD. **Conclusion**: This research an original pilot study to demonstrate that the NOD2 SNP13 (Leu1007fs) is a possible risk factor for aGVHD among Egyptian population.*

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the main curative treatment for many hematological and non hematological diseases. GVHD contributes an important cause of transplant-related mortality (TRM) and morbidity >30% following aHSCT from human leukocyte antigen (HLA) identical donor siblings[1]. Therefore, polymorphisms in other genes may influence GVHD and overall survival (OS)[2]. These non-HLA-encoded genes include those involved in both the innate and adaptive immune responses, such as cytokines, chemokines and innate immune receptor genes [3]. Candidate gene studies (CGS) and genome-wide association studies (GWAS) are the two approaches commonly used to study polymorphisms in non-HLA genes associated with GVHD [4].

Nucleotide-binding Oligomerisation Domain (NOD) 2, previously known as the caspase recruitment domain 15 (CARD15) gene is a member of proteins involved in intracellular pathogen recognition and is only expressed in intestinal epithelial cells and cells of monocyte/macrophage lineage [5]. Many single nucleotide polymorphisms (SNPs) in the NOD2 gene have been reported, such as SNP8 (R702 W), SNP12 (G908R), and SNP13 (1007fs) .These polymorphisms can initiate strong GVHD responses by increasing the gastrointestinal mucosal permeability, so bacterial ligands gain access to systemic circulation [6]. In addition to recipients with NOD2 variant genotype is unable to respond efficiently to bacterial infection results in an increased level of circulating lipopolysaccharide (LPS), leading to priming of T cells[7]. According to evidence based medicine meta analysis of Zhao et al. [8] this gene was chosen in the present study. The last authors concluded that donor-recipient pairs NOD2 SNPs who received HSCT from identical siblings were associated risk of Grade III–IV aGVHD, especially in the Caucasian population. In Egypt, Mossallam and Samra, [9] studied patients and their respective HLA matched sibling donors transplanted at National Cancer Institute. They were tested for cytotoxic T-lymphocyte antigen-4 (CTLA4) gene. The authors concluded that there was no association between both polymorphisms in patients and donors and acute or chronic graft versus host disease. Another study was a PHD thesis by Abdallah Mohamed [10] conducted at National Cancer Institute investigating cytokine gene polymorphisms(CGPs): Tumor necrosis factor-alpha *(TNF-α)* (−308), transforming growth factor beta1 *(TGF-β1)* (codon10 and codon 25), interleukin *(IL)-6* (−174), *IL-10* (−1082, −819 and −592) and Interferon–gamma *(IFN-γ)* (+874) The authors concluded that there was no impact of all tested CGPs on development of GVHD or other post HSCT complications. But, no studies regarding this gene SNPs in Egyptian population.

The present study aims to highlight the importance of non-HLA gene polymorphisms on the development aGVHD following aHSCT.

METHODOLOGY

Subjects

Sixty four HLA-matched aHSCT donor-recipient pairs from Naser Institute, Ministry of Health. Cairo, Egypt were enrolled in this study along October 2016 to march 2018. Peripheral blood samples were collected from all participants prior to the transplant into EDTA tubes**.** An informed consent was obtained from all participants. This study was approved by the ethics Committee of Faculty of Medicine for Girls, Al Azhar University. The Mean±SD age of the patients and donors was 25, 25±10 years. Indications for aHSCT included severe aplastic anaemia 42.2%, acute myeloid leukemia (17.2%%), myeilodysplastic syndrome (14.1%), Fanconi anaemia (10.9%), chronic myeloid leukemia (7.8%), acute lymphoblastic leukemia (6.2%), and paroxysmal nocturnal hemoglobinuria (1.6%) (Table 1).

Table 1: Characteristics of HSCT patients & donors

Characteristics	**N (%)**
Gender mismatch	23(35.9)
Diagnosis	
Severe aplastic anaemia	27(42.1)
Acute myeloid leukemia	11(17.1)
Fanconi anaemia	7(10.9)
Chronic myeloid leukemia	5(7.8)
MDS	9(14.1)
Acute lymphoblastic leukemia	4(6.3)
PNH	1(1.7)
Type of transplant	
Myeloablative	64(100)
Stem cell source	
Peripheral blood	64(100)
Acute GVHD	14(21.9%)
Grade 0–1	9 (14.1%)
Grade II–IV	5(7. 8%)
Chronic GVHD	
Yes	8(12.5%)
Survival status	
Alive	56(87.5%)

MDS: Myelodysplastic syndrome; PNH: Paroxyismal nocturnal hemoglobinuria; GVHD: Graft-versus-host disease

Conditioning regimen used for the myeloablative transplant consisted of busulfan/cyclophosphamide. All patients had peripheral blood as their stem cell source (100%). Acute GVHD was graded according to standard criterion. Patients were followed up 12 months after transplantation. Routine conditioning regimens included Bu/ cy:Busulphan 4 mg/Kg p.o. daily for four days (D-7 to D-4) and Cyclophosphamide 60 mg/Kg/d IV for two days (D-3 and D-2) given with 2-mercaptoethane sulfonate sodium (MESNA). GVHD prophylaxis was given by Cyclosporin-A (CSA) 3 mg/kg IV from day one to be changed to oral form (3 mg/kg) once the patient can swallow for 9–12 months post-transplant.

Genomic DNA extraction from blood

Genomic DNAs were extracted from HLA-matched aHSCT donor-recipient pairs according protocol of Iranpur & Esmailizadeh [11]. 2 volumes of red blood cell lysis buffer were added to the EDTA-whole blood. Then, 400 µl of nucleic lysis buffer, 100 µl of saturated NaCl (5M) and 600 µl of chloroform were added for lysing white blood cells. After centrifugation, the purified DNA was collected by ethanol precipitation. Finally, gel electrophoresis and UV spectrophotometer were used to determine the DNA concentration and purity. The sample DNA was diluted with TE buffer to make a final concentration of 100 µg/ml working solution.

PCR amplification

- NOD2 gene polymorphism primers: Three primer pairs (Table 2) were chosen in the present study – manufactured (Biosearch.USA).
- Internal control primer pair: Single primer pair were utilized for B- actine for all specimens.

PCR amplification was performed using purified DNA in a 50 µl reaction at 95°C for 2 min, 95°C for 30 s, 60°C for 30 s, followed by 35 cycles at 72°C for 55 s and a final extension at 72°C for 10 min [12].PCR reactions were carried out using thermal cycle (Biometra 2000, Germany).

Table 2 Primer sequences and restriction digestion predictions

Polymorphism	Primer sequences (5′→3)	Restriction enzyme	Amplified products length (bp)	Restriction products length (bp)
1007fs	F: GGC AGA AGC CCT CCT GCA GGG CC R: CCT CAA AAT TCT GCC ATT CC	ApaI	151	Leu1007Leu homozygotes ;151 Leu1007Pro heterozygotes: 151,130, 21 Pro1007Pro homozygotes: 130, 21
G908R	F: TCT TTT GGC CTT TTC AGA TTCTG R: CAG CTC CTC CCT CTT CAC CT	HhaI	163	G908G homozygotes : 163 G908R heterozygotes 27, 136, and 163 R908R homozygotes : 27, 136
R702W	F: AGA TCA CAG CAG CCT TCCTG R: CAC GCT CTT GGC CTC ACC	MspI	185	R702R homozygotes : 20, 35, 54, 76 R702W heterozygotes 20, 35, 54, 76, 130 W702W homozygotes allele: 20, 35, 130
B-actin	F CTT CGA GCA AGA GAT GGC CAC R: TTG CTG ATC CAC ATC TGC TGG AAG	-	350	-

Restriction endonuclease analysis:

- Target variants were detected using PCR-RFLP method. Restriction enzymes MspI, HhaI and ApaI were chosen to detect the specific SNPs. The digestion reaction and restriction enzyme conditions included to kit instructions included with the restriction enzymes MspI, HhaI (New England BioLab-USA), FastDigest ApaI (Thermo Scientific, Fermentas)[12]. Then 20 µl of digestion products were loaded on 4% agarose gel containing ethidium bromide electrophoresed and imaged using the gel imager Figure 1.
- Optimization of gel electrophoresis: concentration of agarose, voltage and duration were checked in repeated runs until optimized.
- Digestion products were loaded on submarine electrophoresis through 4% agarose gel and visualized by UV transiluminator after staining with ethidium bromide Figure 2.

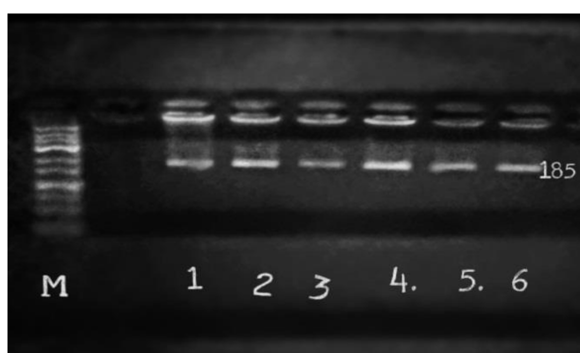

Fig. 1: Agarose electrophoresis of NOD2 SNP R702W PCR products.

This figure shows agarose electrophoresis of NOD2 PCR products; M :(25-750bp) molecular marker, 1-6: amplified PCR products of R702W gene.

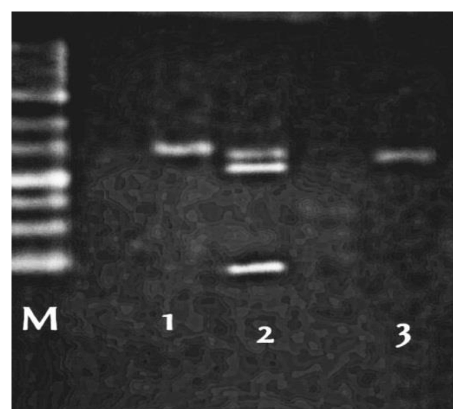

Fig. 2: Agarose electrophoresis of digested NOD2 SNP 1007fs, G908R PCR products.

This figure shows agarose electrophoresis of digested NOD2 PCR products; M: (25-750bp) molecular marker, 1: GG genotype of G908R gene (163bp), 2: LP genotype of 1007fs gene (151, 130, 21 bp), 3: LL genotype of 1007fs gene (151).

DNA sequencing

- The PCR amplicons were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed on an ABI 3500XL (Applied Biosystems, USA) sequencer
- Confirmation of DNA sequence results; by alignment with Gene Bank data and compared Gene Bank data which confirm the mutation sites Figure 3.

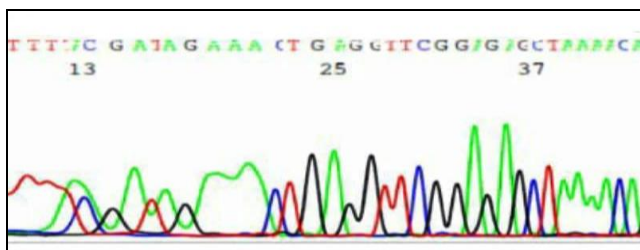

Fig. 3: Gene sequencing analysis of 1007fs NOD2 polymerase chain reaction products.

The reverse sequencing map of the polymerase chain reaction (PCR) product of heterozygote 1007fs NOD2 (LP).

Statistical Analysis

Data were collected, coded, revised and entered to the Statistical Package for Social Science (IBM SPSS) version 20. The data were presented as number and percentages for the qualitative data, mean, standard deviations and ranges for the quantitative data with parametric distribution and median with inter quartile range (IQR) for the quantitative data with non parametric distribution.

Chi-square test was used in the comparison between two groups with qualitative data and *Fisher exact test* was used instead of the Chi-square test when the expected count in any cell found less than 5.

Independent t-test was used in the comparison between two groups with quantitative data and parametric distribution and *Mann-Whitney test* was used in the comparison between two groups with quantitative data and non parametric distribution. A p-value < 0.05 was considered significant.

Validation and Quality control:

B-actin gene as internal control was included in each specimen. B-actin PCR amplicon was detected internal positive control ampilicon was detected at 350bp.

RESULTS

Distribution of NOD2 gene polymorphisms of recipients and donors

- Range of follow-up post aHSCT was (6-12 months). Incidence of aGVHD in the whole cohort was 21.9%. 9 (14.1%) were grade 0-I aGVHD and 5 (7. 8%) were II-IV aGVHD while that of chronic GVHD (cGVHD) was 12.5%. 12.5% died during study period (Table 1)
- Sixty four pairs of recipients and their corresponding matched sibling donors were genotyped for three NOD2 polymorphisms **R702W, G908R** and **1007fs**. The genotype distributions of the study group are demonstrated in Table 3.

Table 3: Genotype frequencies of the NOD2 polymorphisms in patients and donors

NOD2 SNP	Genotype	Patients N (%)	Donors N (%)
1007 FS	**LL**	38(59.4)	46(71.9)
	LP	25(39)	18(28.1)
	PP	1(1.6)	0
G908R	**GG**	62(96.9)	63(98.4)
	GR	2(3.1)	1(1.6)
	RR	0	0
R702W	**RR**	62(96.9)	64(100)
	RW	2(3.1)	0
	WW	0	0

LL, GG, RR are wild genetic alleles (no mutation)
LP, GR, RW are heterozygous mutation
PP, RR, WW are homozygous mutation

Association of NOD2 gene polymorphisms and GVHD

There was significant association between recipient 1007fs SNP and occurrence of aGVHD p=0.006. There was no significant association between donor NOD2 1007fs SNP and aGVHD p=0.1. The association between donors/recipients NOD2 G908, R702 SNPs regarding occurrence/severity of aGVHD was not applicable as 100% of cases were GG and RR genotypes respectively (Table 4, 5).

Table 4: Influence of recipient NOD2 genotypes (1007fs, R702 and G908) on aGVHD

NOD2 SNPs	genotypes	aGVHD (NO.=14)		Chi square test	
		No.	%	X^2	P value
1007fs	LL	4	28.6	7.476	**0,006**
	LP	10	71.4		
G908	GG	14	100%	NA	NA
R702	RR	14	100,0%	NA	NA

P > 0.05: Non significant, P < 0.05: Significant, P < 0.01: highly significant, NA: not applicable

Table 4 shows that there was highly statistically significant difference in NOD2 (1007fs) in recipients regarding aGVHD *p* value = 0.006, NOD2 (R702 and G908) in recipients regarding aGVHD are not applicable because all data were RR and GG.

Table 5 Influence of donor NOD2 genotypes (1007fs, R702 and G908) on aGVHD

NOD2 SNPs	genotypes	aGVHD (NO.=14)		Chi square test	
		No.	%	X^2	P value
1007fs	LL	8	57.1	1.912	0.1
	LP	6	42.9		
G908	GG	14	100,0%	NA	NA
R702	RR	14	100,0%	NA	NA

P > 0.05: Non significant, P < 0.05: Significant, P < 0.01: highly significant, NA: not applicable

This table shows that there was no significant difference in NOD2 (1007fs) in recipients regarding aGVHD *p* value = 0.1, NOD2 (R702 and G908) in donors regarding aGVHD are not applicable because all data were RR and GG.

There was no significant association between donors/recipients NOD2 1007fs and severity of aGVHD p=0.5, p=0.3 respectively (Table 6, 7).

Table 6: Influence of donor NOD2 genotypes (1007fs, R702 and G908) on severity of aGVHD

		grade 0-1 (N0.=9)		grade II-IV (NO.=5)		Chi square test	
		No.	%	No.	%	X^2	P value
1007fs	LL	5	55,6%	3	60,0%	2,246	0,523
	LP	4	44,4%	2	40,0%		
R702	RR	9	100,0%	5	100,0%	NA	NA
G908	GG	9	100,0%	5	100,0%	NA	NA

P > 0.05: Non significant, P < 0.05: Significant, P < 0.01: highly significant, NA: not applicable

This table shows that there was no statistically significant difference in NOD2 (1007fs) in donor regarding aGVHD severity **p=0.5**. NOD2 (R702 and G908) are not applicable because all data were RR and GG.

Table 7 Influence of recipient NOD2 genotypes (1007fs, R702 and G908) on severity of aGVHD

		grade 0-1 (N0.=9)		grade II-IV (NO.=5)		Chi square test	
		No.	%	No.	%	X^2	P value
1007fs	LL	2	22.2%	2	40,0%	1.014	0.314
	LP	7	77.8%	3	60,0%		
R702	RR	9	100,0%	5	100,0%	NA	NA
G908	GG	9	100,0%	5	100,0%	NA	NA

P > 0.05: Non significant, P < 0.05: Significant, P < 0.01: highly significant, NA: not applicable

This table shows that there was no statistically significant difference in NOD2 (1007fs) in donor regarding aGVHD severity p=0.3. NOD2 (R702 and G908) are not applicable because all data were RR and GG.

There was no significant association between donors/recipients NOD2 1007fs SNP and cGVHD p=0.5, p=0.3 respectively, however trending is observed in recipients (62.5% versus 37.5%). There was no significant association between donors / recipients G908 SNP and cGVHD p=0.7, p=0.5 respectively. There was no significant association between donors / recipients R702 SNP and cGVHD p=0.5.

Association of NOD2 gene polymorphisms and TRM

There was no significant association between donors/ recipients NOD2 1007fs SNP and TRM, however trending is observed in recipients p= 0.1, p=0.3. There was no significant association between donors and recipients G908 SNP and TRM p=0.5, p=0.7 respectively. There was no significant association between donors and recipients R702 SNP and TRM p=0.5.

DISCUSSION

GVHD constitutes a major complication following HSCT transplants from HLA identical siblings >30%, revealing that non-HLA immune genes are also implicated in the process[13]. Non-HLA genes involve cytokine, cytokine receptor and innate immune receptor genes [14]. Polymorphisms in TNF and IL-10 genes were the first non-HLA genes reported by Middeleton et al. [15], showing their potential role in aGVHD prediction.

The effect of polymorphisms in innate immune receptor genes on HSCT complication has been reported. NOD2 protein regulates the infection by recognition of pathogen associated molecular patterns, mainly muramyl dipeptide (MDP), a derivative of peptidoglycan, which is a component of both Gram-positive and –negative bacterial cell walls. NOD2 responds by initiating inflammatory responses via a number of pathways[16]. *In vitro* studies have shown that NOD2 can also respond to viral single-stranded (ss) RNA ligand [17].

In 2013, Data base SNP national centre for biotechnology information (NCBI) reported 854 SNPs within NOD2 gene [18]. In the present study, we chose NOD2 polymorphisms associated with Crohn's disease located in or near the leucine-rich region (LRR) of the NOD2 gene: Leu1007fsinsC, Gly908Arg and Arg702Trp [19].

In the present study PCR-RFLP was chosen for genotyping of NOD2 gene, as it is a cost-effective method and releasing accurate and précised results comparable to RT-PCR and sequencing [20].our results were validated by sequencing of some alleles to confirm the mutation sites.

The frequencies of NOD2 1007 FS were 39%, 28.1% in recipients and donors and for Gly908Arg were 3.1%, 1.6% in recipients and donors while that of Arg702Trp genotype were 3.1%, 0% in recipients and donors which is comparable to the study of Kassem et al. [12] who investigated NOD2 among Egyptian Crohn's disease patients and its relation to disease severity.

The present study shows highly statistical significant association between recipient 1007fs SNP and risk of aGVHD **p=0.006.**These results were consistent with other studies[8, 21-23] .Interestingly, Jakusla et al. [20] & Grube et al. [24] reported that NOD2 SNP13 more susceptible to septic complication which coordinate with our results as in both cases due to bacterial improper membrane localization of NOD2 leading to access of bacteria to enter the intestinal wall so increasing both sepsis and aGVHD.

The detection of SNP13 mutations in the recipient as a prognostic factor can be explained by complete loss of function of the NOD2 receptor variant.

In this study no significant association between donor NOD2 1007fs SNP and aGVHD p=0.5.These results are consistent with other studies [25,26]. In contrast, some studies show significant association between donor NOD2 1007fs SNP and aGVHD [8,23]. This conflict may be attributed to ethnic variation / narrow scale study population.

Moreover the finding that polymorphism in NOD2 gene locus of the recipient, not donor can negatively influence HSCT outcome coordinates with other the study of Penack et al[27] who reported that NOD2 deficiency in donor murine model has no impact of GVHD while recipient NOD2 deficiency increase risk of GVHD.

In the present study, no significant association between donors/ recipients G908 SNP and aGVHD p=0.8. As well as donors / recipients R702W SNP and aGVHD p=0.8.Our results are consistent with Nguyen et al.[26] & Tanabe et al. [28].Controversly, Van der et al.[29] & Zhao et al.[23] reported significant association. These conflicting results regarding G908R and R702W SNPs with our results may be due to first, the number of NOD2 genotypes analyzed were limited 3.1%, 1.6% compared to 7%, 8%, and may, therefore, ultimately reduce the power of analysis. Second, due to ethnic variation. Third, overall incidence of GVHD, and type of conditioning regimen, donor source.

In the present study, no significant association between donor/recipients NOD2 1007fs SNP and cGVHD p=0.5, p=0.3 respectively, however trending is observed in recipients SNP (62.5% versus 37.5%). As well as no significant association between donors / recipients G908, R702 SNPs and cGVHD. These results were consistent with Gruhn et al.[30] who reported no significant association between donor / recipients NOD2 SNPs and cGVHD. Zhao et al. [23] also reported no significant association between cGVHD and NOD2 genotype in donor and recipient.

In the present study, no significant association between donor NOD2 1007fs SNP and TRM p=0.9. There was no significant association between recipient

1007fs SNP and occurrence of TRM, however trending is observed. There was no significant association between donors / recipients G908 SNP and TRM. As well as no significant association between donors / recipients G908, R702 SNPs and TRM. In contrary Van der et al[29] who reported significant association between donor/ recipients NOD2 SNPs and TRM p=0.02.The difference may be attributed to smaller overall incidence of TRM 8% in our study versus 16% the other study.

CONCLUSIONS & RECOMMENDATIONS

Based upon the results 1007fs NOD2 genotyping could predict outcome in patients post aHSCT while G908R, R702W NOD2 polymorphisms couldn't predict outcome in patients and donors post aHSCT. A limitation to this study is the narrow scale population; further studies on a larger cohort are needed.

Conflict of interest

The authors have declared no Conflict of interest.

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