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GENOTYPIC VARIATION AND PHENOTYPIC CHARACTERIZATION OF GRANZYME B GENE POLYMORPHISMS

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Abstract

Granzyme B has been associated with allograft rejection in solid organ transplantation. Single nucleotide polymorphisms (SNPs) in the Granzyme B gene might impact its expression. The aims of this study were 1) to establish the frequency of two Granzyme B SNPs (A-295G; Q-55R) in pediatric heart transplant (PHTx) recipients and 2) to determine their phenotypic expression in healthy individuals.

Methods—396 PHTx patients (245 White non-Hispanic, 49 Black non-Hispanic, 82 Hispanics, 20 others) and 52 healthy controls were screened for Q-55R and A-295G. For the control samples, we assessed the frequency of Granzyme B positive cells by ELISPOT assay following mitogen stimulation.

Results—Among the PHTx recipients, 57% percent of the population carried the Q/Q genotype, while 6 % were R/R homozygotes. Seven of 49 (14%) Black non-Hispanics (14%) were R/R homozygotes, while 13 out of 245 (5%) of White non-Hispanics and 5 out of 82 (6%) Hispanics carried the R/R genotype ($p=0.02$). The A allele frequency of Granzyme B A-295G (49.6%) was similar to that of the G allele (50.4%). However, 80% of Black non-Hispanics were A allele carriers compared to 68% of White non-Hispanics ($p < 0.0001$).

Following mitogen stimulation, the frequency of Granzyme B positive cells was higher in the Q/Q homozygotes compared to R/R carriers ($p=0.006$), while a similar frequency of Granzyme B positive cells was noticed among the genotypes of A-295G SNP.

Conclusions—These data indicate that 55 Q/Q genotype is associated with increased *in vitro* expression of Granzyme B.

Keywords

Granzyme B; gene polymorphism; transplantation; pediatric; heart

Introduction

Effector molecules released by cytotoxic T lymphocytes have been studied as non-invasive markers for acute rejection in solid organ transplantation. Increased gene expression of perforin, granzyme B and Fas/FasL molecules intra-graft and in peripheral blood has been associated with acute rejection in renal (1–3), heart (4), lung (5,6), intestinal (7–9) and hematopoietic stem cell transplantation (10).

Upon contact with target cells, activated cytotoxic T lymphocytes release cytotoxins like perforins, granzymes and death ligands into the intracellular space. Granzyme B is a pro-apoptotic serine protease with a high specificity to cleave after the aspartic acid. Classically, in the target cell, apoptosis occurs upon granzyme uptake in a perforin- dependent manner through the pores formed by this protein. However, other studies suggest that, Granzyme B may enter the cell via a cation independent mannose-6-P receptor, followed by caspase activation and apoptosis (11).

Granzyme B polymorphisms might be responsible for the impaired cytolytic effect on target cells. McIlroy and collaborators (12) have identified three single nucleotide polymorphisms (SNPs) located in exon 2 (A to G nucleotide substitution resulting in a Glutamine 48 to Arginine substitution – numbering with reference to the bovine chymotrypsinogen A sequence), exon 3 (C to G nucleotide substitution resulting in a Proline 88 to Alanine mutation) and exon 5 (T to C nucleotide substitution resulted in a Tyrosine 245 to Histidine substitution). These substitutions define an allele in which three amino acids Q⁴⁸P⁸⁸Y²⁴⁵ of the mature protein are changed to R⁴⁸A⁸⁸H²⁴⁵. The purported pro-apoptotic effect of the RAH allele is still under debate (12,13).

Recently, a Granzyme B ELISPOT technique has been demonstrated to be a superior alternative to the ⁵¹Cr-release assay to estimate cytotoxic effector cells frequency. The ELISPOT assay has significantly higher sensitivity and is able to measure the release of the cytolytic protein (14). The Granzyme B ELISPOT was applied to measure the frequency of cytotoxic T lymphocytes in transplantation (15) and in vaccine trials in cancer patients (16).

The purpose of this study was 1) to establish in a pediatric heart transplant cohort the genotype distribution of two Granzyme B SNPs: A-55G and A-295G; and 2) to determine in a healthy control population, the phenotypic expression of these SNPs. The non-synonymous SNP A-55G (*rs 8192917*), located in exon 2, is responsible for the amino acid substitution of Q-55R (numbering with reference to the human chymotrypsinogen A sequence) (12). The Granzyme B SNP A-295G (*rs 7144366*) is a new SNPs located in the promoter of the gene.

Materials and Method

Patient population

Three hundred and ninety six pediatric heart transplant recipients from the SCCOR Program “Optimizing Outcome after Pediatric Heart Transplantation” from six medical centers (University of Pittsburgh, Stanford University, Loma Linda University Medical Center, Washington University, Columbia University, University of Alabama at Birmingham) and 52 healthy controls from the University of Pittsburgh were included in this study. The racial/ethnic background for each patient was defined as: “White non-Hispanic” for participants of white race but not Hispanic ethnicity, “Black non-Hispanic” for participants of black race but not Hispanic ethnicity and “Hispanic” if they answered yes to Hispanic ethnicity.

Anticoagulated venous blood was collected for genotyping under an Institutional Review Board approved protocol at each center. DNA was extracted using a commercially available procedure (Qiagen, Valencia, CA). Blood samples from the pediatric recipients were collected at the time of routine blood draws on a single occasion and then frozen at -80°C until DNA extraction was preformed. For the control group, we have obtained another Institutional Review Board approval, in order to draw blood, genotype various snips and perform the ELISPOT assay.

Detection of gene polymorphisms

Genomic DNA (40ng) was amplified in a total volume of 25 μl containing 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM Mg Cl₂, 200 μM each dNTP, 0.1 μM appropriate forward and reverse primers, and 1U Taq DNA polymerase (Table 1). Thermocycling conditions for all SNPs were initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, extension at 72°C for 30 seconds, with a final extension of 72°C for 8 minutes. Following amplification, PCR products were digested with the appropriate restriction enzyme following the manufacturer’s instructions (New England Biolabs, USA). Digested products were visualized on 2% agarose gels containing ethidium bromide.

Granzyme B ELISPOT assay

Peripheral blood mononuclear cells (PBMC) were isolated from fresh anticoagulated venous blood of 52 healthy controls by density centrifugation over Ficoll-Plaque (Cat# 17-1440-03, Amersham Biosciences AB). Granzyme B secretion was measured using the human Granzyme B ELISPOT kit (Cat # 3485-2HW-Plus) provided by Mabtech Inc, Sweden. Briefly, multiscreen IP 96-well plates (PDVF membrane, type ELIIP, Cat# MAIPSWU10, Millipore), pre-wetted with 50 μl 70% ethanol/well, were coated overnight at 4°C with 100 μl /well GB10 antibody (15 $\mu\text{g}/\text{ml}$). PBMCs from each healthy control were re-suspended in AIM V[®] medium (Cat # 12055-091, Invitrogen) and then, added to triplicates in 5×10^4 cells (100 μl) per well. The cells were stimulated with either 50 μl PHA (1 $\mu\text{g}/\text{ml}$, Cat# L-2769, Sigma Aldrich) or with 50 μl PMA (20ng/ml, Cat #: P8139, Sigma Aldrich) and 50 μl ionomycin calcium (1 $\mu\text{g}/\text{ml}$, Cat# I0634, Sigma-Aldrich). After 24 hours incubation, at 37°C , the plates were washed and 100 μl /well biotinylated anti-human Granzyme B antibody (clone GB11-biotin 1 $\mu\text{g}/\text{ml}$ in 1x DPBS without calcium and magnesium, Cat# 21-031-CM, Mediatech Inc. containing 0.5% human albumin serum, Cat# 100-512, Gemini-Bioproducts) was added. Plates were incubated for 2 hours at room temperature and then 100 μl /well of Streptavidin-HRP (1:1000 in 1x DPBS - 0.5% human albumin serum) was added. The reaction was developed using 100 μl /well TMB, included in the kit. The spots were counted using an ELISPOT plate reader (Cellular Technology Ltd, Cleveland, OH)

Statistics

Chi-square statistics were used to compare the gene polymorphisms and allele frequencies for Granzyme B A-295G and Granzyme B Q-55R among race/ethnicity groups within the pediatric heart transplant patients and between the transplant group and the healthy control group. For each type of stimulation, the distribution of the change in spot frequency, based on the ELISPOT assay, was examined by genotype, and medians and interquartile ranges (IQR) are reported. Kruskal-Wallis nonparametric tests (with 2 d.f.) were used to statistically compare the median change in spot frequency among the 3 genotype groups for Granzyme B Q-55R and among the 3 genotype groups for Granzyme B A-295G.

Results

Genotypic distribution of Granzyme B SNPs in pediatric heart transplant patients

In order to characterize the frequency of the studied Granzyme B SNPs, we report on 396 pediatric heart recipients. The racial/ethnic distribution in the pediatric heart recipients was as follows: 245 were White non-Hispanics, 49 were Black non-Hispanics, 82 were Hispanics, while 20 carried other racial backgrounds (Asian, American- Indian etc).

The majority of the pediatric heart transplant recipients expressed the Q allele (75%) of the Granzyme B Q-55R SNP. Fifty-seven percent of the total pediatric population carried the Q/Q genotype, while only 6 % were homozygotes for the R/R genotype. The racial/ethnic genotype distribution is depicted in table 2. Seven of 49 (14%) Black non-Hispanic recipients were R/R homozygotes, while 13 out of 245 (5%) White non-Hispanics and 5 out of 82 (6%) Hispanic recipients carried the R/R genotype ($p=0.02$).

The distribution of A and G alleles of Granzyme B A-295G SNP was balanced in our population (49.6% vs 50.4%, respectively). However, allele distribution varied significantly by race/ethnicity. Eighty percent of Black non-Hispanics and 84% of Hispanic patients were A allele carriers compared to 68% of White non-Hispanics ($p < 0.0001$).

The majority of the healthy volunteers were White- non Hispanics (47), 4 of them were Asians and only one was Black-non Hispanic. The control group was screened for two additional SNPs (A94P; Y247H) (12) that, as previously shown, were found in complete linkage disequilibrium with Q-55R. No differences in genotype distribution of Q-55R were noticed in healthy controls compared to pediatric heart transplant recipients.

Granzyme B polymorphisms showed altered phenotypic expression in vitro

The frequency of Granzyme B producing cells was determined by ELISPOT assay in 52 healthy controls that exhibited various genotypes of the studied Granzyme B SNPs. We have found significant differences among Q-55R genotype variants following mitogen stimulation. As depicted in the example in Figure 1, the Q/Q homozygotes had increased number of Granzyme B positive cells as compared to the R/R variant carriers.

Following PHA stimulation the Q/Q carriers exhibited a median change of 280 (IQR 111 to 432) as compared to 38 (IQR 36 to 54) Granzyme B positive cells/well in R/R variant homozygotes ($p=0.0068$) (Figure 2a). The same pattern was observed following PMA and ionomycin calcium stimulation; the Q/Q carriers exhibited significantly higher number of Granzyme B positive cells as compared to R/R genotype (median change of 705 (IQR 512 to 930) versus 267 (IQR 247 to 363), respectively) ($p = 0.0062$) (Figure 2b).

In contrast, the frequency of Granzyme B producing cells was similar among the three genotypes of A-295G SNP. Thus, following PHA stimulation a median change of 184 (IQR

101 to 356) Granzyme B positive cells/well were noticed in A/A carriers, compared to 164 (IQR 54 to 405) in G/G homozygotes ($p = 0.33$) (Figure 3a). The results remained consistent following the stimulation with PMA and ionomycin calcium (636 ± 64 in A/A carriers compared to 614 ± 79 Granzyme B positive cells/well in G/G genotype carriers $p = 0.65$) (Figure 3b).

Discussion

The current study investigated the correlation between various genotypes of two Granzyme B SNPs (Q-55R and A-295G) and the frequency of Granzyme B precursor cells in PBMC of normal healthy controls. In order to establish the prevalence of these Granzyme B SNPs, we have examined a large and racially diverse pediatric heart transplant cohort.

Recently, McIlroy et al. (12) identified a mutated Granzyme B allele responsible for the substitution of three amino acids ($R^{48}A^{88}H^{245}$) that encodes for a stable protein. A prevalence of 25–30% of RAH allele was reported in European, African and Asian populations (12,13). Concordant with previous reports, in this large cohort of pediatric heart recipients, we also found a similar prevalence of the R allele. Furthermore, we report a significant difference in the distribution of -55 R/R genotype among various racial/ethnic groups. In addition, the pediatric group was genotyped for Granzyme B A-295G SNP and the racial/ethnic distribution of this SNP was reported. Black non-Hispanics were identified as being more likely to be homozygous for the A allele compared to White non-Hispanic and Hispanic recipients.

The impact of Q-55R SNP on cytolytic function of Granzyme B is still controversial. One report showed that $R^{48}A^{88}H^{245}$ allele was incapable of apoptosis *in vivo* (12), while other authors showed that this allele retained the biochemical and cytotoxic function of the wild type variant (13,17).

Our investigation focused on establishing a correlation between the Granzyme B SNPs and protein expression following non-specific mitogen stimulation of fresh, unmanipulated cells from normal individuals. Granzyme B ELISPOT assay was performed to evaluate the frequency of Granzyme B precursor cells. We did not have the opportunity to measure the frequency of Granzyme B precursor cells in the pediatric heart transplant group. Furthermore, donor blood and/or tissue was not available for cytotoxic assays. However, these results were likely to be impaired by different immunosuppression regimens used across the various centers.

Our results on normal controls clearly showed a significant difference among Q-55R genotypes: carriers of Q/Q genotype exhibited a higher frequency of Granzyme B precursor cells as compared to R/R genotype. In contrast to our findings, other investigators have reported similar expression of Granzyme B among the two genotypes. These discrepancies might be explained by the source of the cells, different cohorts and the method of detection. We examined the frequency of Granzyme B precursor cells in freshly isolated PBMC from normal healthy controls, as Granzyme B might be produced by several types of cells in PBMC including NK cells, CD8+ and CD4+ T cells (11). In contrast, the other investigators limited their observations to a subset of cells, CD8 positive T cells, from frozen samples of HIV positive donors. Furthermore, we analyzed the frequency of Granzyme B by release of protein and capture at single cell level with the Granzyme B ELISPOT assay, whereas previous investigators applied a less sensitive assay of intracellular staining for Granzyme B in CD8 positive T cells. Although PMA and ionomycin calcium induced a stronger stimulation than PHA, following both modalities of activation we detected a similar pattern. In contrast, for the other Granzyme B SNP A-295G we did not find a functional correlation as assessed by ELISPOT assay.

Using ELISPOT assay, immune cell frequencies can be measured at the single cell level without elaborate expansion or manipulation of cell populations (20). ELISPOT assay may be preferable to assess the functional expression of certain molecules due to its capacity to detect low-level responses, while flow cytometry allows more for phenotypic discrimination of responding cells upon stimulation (21). Furthermore, Granzyme B ELISPOT assay measures the release of a cytolytic protein (14) and was documented to correlate with the frequency of cytotoxic T lymphocytes after HLA-identical living-related kidney transplantation (15) and to measure ex vivo antigen-specific cytotoxicity of PBMC in clinical trials for cancer vaccines (16). Moreover, ex vivo ELISPOT measurements of Granzyme B within 24 hours after antigen challenge also allowed for discrimination of active memory CD8⁺ cells from resting memory cells (22).

Common genetic variations (genetic polymorphisms) in transplant recipients (and possible donors) may influence post-transplant outcomes (23–25). In our prior studies, we have focused on the impact of various cytokine and growth factor gene polymorphisms on post-transplant outcomes of PHTx recipients (26,27). We have also suggested that the racial/ethnic distribution of certain genetic polymorphisms might explain some of the observed variation in post-transplant outcomes among different racial groups (28). In the current study, we turn our attention to effector molecules, which are key components of the host response to the allograft and mediate cell death (11). Although Black recipients exhibited a higher frequency of the Granzyme B R/R genotype in comparison with Whites, the overall frequency of this genotype/phenotype is low and we do not expect a significant change in their rejection profile based only on this parameter. In order to evaluate the risk of rejection for a certain race/ethnic group, multiple parameters (e.g. age at the time of transplant, donor/recipient race, HLA compatibility) need to be considered, including additional cytokine and/or growth factor genetic polymorphisms that may also affect the clinical outcome (26). Furthermore, the information that we have obtained on the association between specific polymorphisms and the function/phenotype of a given mediator is very useful for designing future studies that correlate clinical outcomes with various genetic variations.

We have shown that the prevalence of two Granzyme B SNPs was also influenced by race/ethnicity. In addition, we determined a significant correlation between the frequency of Granzyme B precursor cells and certain genetic polymorphisms. Further analysis will focus on the clinical impact of Granzyme B polymorphisms in pediatric heart transplant recipients.

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Abbreviations

SNP	Single nucleotide polymorphism
PHTx	Pediatric heart transplant
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PMA	

Phorbol 12-myristate 13-acetate

IQR

Interquartile ranges

dNTP

Deoxyribonucleotide triphosphate (dNTP)

Streptavidin-HRP

Streptavidin -Horseradish Peroxidase

DPBS

Dulbecco's Phosphate Buffered Saline

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Figure 1.

Figure 2.

Figure 3.

Table 1

PCR Primer Sequences and Restriction Enzymes used to genotype the Granzyme B polymorphisms

Granzyme B SNP	PCR Primers	Restriction Enzyme
Granzyme B A-295G <i>rs 7144366</i>	5'-GCGGTTCTATCACTGGGAACT -3' 5'-GCAGGAACCCAAGCCTATG -3'	Sfcl
Granzyme B Q-55R <i>rs 8192917</i>	5'-TGTCCCTAAGACAGGTATGCTC -3' 5'-GTGTTTCCAGGAGGGTGT -3'	BsmA I

Genotype distribution of Granzyme B SNPs in different racial/ethnic groups in pediatric heart transplant recipients and healthy controls.

Table 2

Gene Polymorphism	Pediatric Heart Transplant Patients N=396				Healthy Controls N=52
	White NH N=245	Black NH N=49	Hispanic N=82	Other N=20	
Granzyme B A-295G *					
A/A	43 (18%)	16 (33%)	37 (45%)	7 (35%)	13 (25%)
G/A	123 (50%)	23 (47%)	32 (39%)	9 (45%)	24 (46%)
G/G	79 (32%)	10 (20%)	13 (16%)	4 (20%)	15 (29%)
Granzyme B Q-55R **					
Q/Q	141 (58%)	21 (43%)	55 (67%)	9 (45%)	30 (58%)
Q/R	91 (37%)	21 (43%)	22 (27%)	11 (55%)	17 (33%)
R/R	13 (5%)	7 (14%)	5 (6%)	0 (0%)	5 (10%)

* p<0.001 for race/ethnicity comparison within pediatric heart transplant cohort; p=0.95 for pediatric heart transplant recipients vs Controls

** p=0.022 for race/ethnicity comparison within pediatric heart transplant cohort; p=0.62 for pediatric heart transplant recipients vs Controls

White NH = White non-Hispanic; Black-NH = Black non-Hispanic