



## Article/Artigo

# Distribution of QPY and RAH haplotypes of granzyme B gene in distinct Brazilian populations

Distribuição dos haplótipos QPY e RAH do gene da granzima B em grupos populacionais brasileiros

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### ABSTRACT

**Introduction:** The cytotoxicity mediated by granules is one of the most important effector functions of cytotoxic T lymphocytes and natural killer cells. Recently, three single nucleotide polymorphisms (SNPs) were identified at exons 2, 3, and 5 of the granzyme B gene, resulting in a haplotype in which three amino acids of mature protein Q48P88Y245 are changed to R48A88H245, which leads to loss of cytotoxic activity of the protein. In this study, we evaluated the frequency of these polymorphisms in Brazilian populations. **Methods:** We evaluated the frequency of these polymorphisms in Brazilian ethnic groups (white, Afro-Brazilian, and Asian) by sequencing these regions. **Results:** The allelic and genotypic frequencies of SNP 2364A/G at exon 2 in Afro-Brazilian individuals (42.3% and 17.3%) were significantly higher when compared with those in whites and Asians ( $p < 0.0001$  and  $p = 0.0007$ , respectively). The polymorphisms 2933C/G and 4243C/T also were more frequent in Afro-Brazilians but without any significant difference regarding the other groups. The Afro-Brazilian group presented greater diversity of haplotypes, and the RAH haplotype seemed to be more frequent in this group (25%), followed by the whites (20.7%) and by the Asians (11.9%), similar to the frequency presented in the literature. **Conclusions:** There is a higher frequency of polymorphisms in Afro-Brazilians, and the RAH haplotype was more frequent in these individuals. We believe that further studies should aim to investigate the correlation of this haplotype with diseases related to immunity mediated by cytotoxic lymphocytes, and if this correlation is confirmed, novel treatment strategies might be elaborated.

**Keywords:** Granzyme B. SNP. QPY haplotype. RAH haplotype. Brazilian population. Population distribution.

### RESUMO

**Introdução:** A citólise mediada por grânulos é uma das mais importantes funções efetoras de linfócitos T citotóxicos e células natural killer. Recentemente, três polimorfismos de nucleotídeo único foram identificados nos éxons 2, 3 e 5 do gene da granzima B, resultando em um haplótipo em que três aminoácidos da proteína madura Q48P88Y245 são alterados para R48A88H245, o que leva à perda da atividade citotóxica da proteína. No presente estudo, avaliamos a frequência desses polimorfismos em populações brasileiras. **Métodos:** Avaliamos a frequência desses polimorfismos em grupos étnicos brasileiros (brancos, afro-brasileiros e asiáticos) por sequenciamento. **Resultados:** As frequências alélica e genotípica do polimorfismo 2364A/G no éxon 2 em indivíduos afro-brasileiros (42,3% e 17,3%) foram significativamente maiores ( $p < 0,0001$  e  $p = 0,0007$ ) quando comparadas a brancos e asiáticos. Os polimorfismos 2933C/G e 4243C/T também foram mais frequentes em afro-brasileiros, mas sem diferença significativa. O grupo afro-brasileiro apresentou maior diversidade de haplótipos e o haplótipo RAH foi mais frequente nesse grupo (25%), seguidos pelos brancos (20,7%) e asiáticos (11,9%), semelhante à frequência apresentada na literatura. **Conclusões:** Há uma maior frequência de polimorfismos em afro-brasileiros e o haplótipo RAH foi mais frequente nesses indivíduos. Acreditamos que novos estudos devem ter como objetivo a investigação da correlação deste haplótipo com doenças relacionadas com a imunidade mediada por linfócitos citotóxicos, e se essa correlação for confirmada, novas estratégias de tratamento poderão ser elaboradas.

**Palavras-chaves:** Granzima B. SNP. Haplótipo QPY. Haplótipo RAH. População brasileira. Distribuição populacional.

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### INTRODUCTION

Cytotoxic T lymphocytes (CTLs) and natural killer cells (NK) are effector lymphocytes that possess common cytotoxic pathways, needed for the organism's defense against cells infected with virus or neoplastic cells, and the cytotoxicity mediated by granules is one of the most important effector functions of CTLs and NK cells<sup>1</sup>. After these cells recognize their target, the cytotoxic granules move to the immunological synapse, where their membrane binds to the cellular membrane and they release their content to induce the target cell apoptosis<sup>2</sup>. The main components of these granules are the serine proteases, named granzymes and perforin.

Because of its strong proapoptotic activity, granzyme B is the most well-characterized protein from the granzyme family. Granzyme B is a serine protease of approximately 32kDa, which has the specificity of cleaving aspartate residues<sup>3</sup>. The gene that codifies granzyme B in humans is located in chromosome 14 and possesses five exons and 4 introns.

Functionally, it is known that granzyme B is responsible for inducing a rapid deoxyribonucleic acid (DNA) fragmentation of target cells by activating the apoptosis by the caspase pathway. This was demonstrated in knockout mice for granzyme B whose CTLs are incapable to induce a fast DNA fragmentation of the target cells *in vitro*<sup>4</sup>. It also was demonstrated that the granzyme B plays an important role *in vivo*, promoting protection against viral infections in rats<sup>5</sup>. Although a complete loss of perforin expression is associated with a fatal immune disorder, familial hemophagocytic lymphohistiocytosis<sup>6</sup>, any deficiency of the granzyme B expression in this condition was not described<sup>7</sup>.

Three single nucleotide polymorphisms (SNPs) were recently identified, located at 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 resulting in a tyrosine 245 to histidine substitution). These substitutions define a haplotype in which the three amino acids Q48P88Y245 of the mature protein are altered to R48A88H245. These polymorphisms seem to lead to a loss of cytotoxic activity of the protein against tumor target cells<sup>8</sup>.

The frequency of these polymorphisms was evaluated in racial groups, and the haplotype RAH was found in a high frequency-25% to 30% in Europeans, Africans, and Asians<sup>8,9</sup>.

Because of the importance of this protein in the immune response mediated by CTLs and NK cells and because of the findings related to the haplotype RAH, which demonstrated a reduction of the cytolytic capacity against transformed cells, we decided to evaluate the frequency of these polymorphisms in Brazilian ethnic groups to investigate whether these groups have the same genetic profile regarding the presence of these SNPs.

## METHODS

### Population samples

Three Brazilian urban groups from Ribeirão Preto, a city in the northwestern region of the State of Sao Paulo, were analyzed. DNA samples were obtained from 55 white, 52 Afro-Brazilian, and 50 Asian subjects. Individuals were assigned to each group based on phenotypic characteristics and only if they reported the absence of any other ethnic group in their two preceding generations.

Blood cells obtained from 5mL of blood collected from each individual into tubes containing ethylenediaminetetraacetic acid as an anticoagulant were separated from plasma under aseptic conditions by centrifugation at 900 × g for 10min at 4°C.

### DNA extraction, amplification, and genotyping

Red blood cells were lysed using a 0.144M NH<sub>4</sub>Cl and 0.01M NH<sub>4</sub>HCO<sub>3</sub> solution. Next, DNA was extracted from the buffy coat using the Super Quick Gene DNA isolation kit (Analytical Genetic Testing Center [AGTC], Denver, CO, USA) according to the manufacturer's instructions. Primer sequences were designed using Primer3 software version 0.4.0<sup>10</sup> based on the GenBank reference sequence (accession no. M28879) and were as follows: Exon 2 (forward): 5'-TCT GGA GCC ACT AAG CAG GT- 3' and Exon 2 (reverse): 5'-AAC CAA CCT TCC CCT CTC TC- 3'; Exon 3 (forward): 5'-GTT TCT AGG GGG AAC CGA AC- 3' and Exon 3 (reverse): 5'-GCT GCA CAA TCT TCC CTC TC- 3'; Exon 5 (forward): 5'-TCT AAA AGG GGG CTT GAG ATG- 3' and Exon 5 (reverse): 5'-CTC TCC CAG TGT AAA TCT GGA C- 3'.

DNA (100-200ng) was amplified in a 25-μL polymerase chain reaction (PCR) mixture containing: 5pmol of forward and reverse primers, 1X PCR buffer (Invitrogen Life Technologies, Brazil), 2.5mM dNTPs, and 1 U *Taq* polymerase (Invitrogen Life Technologies, Brazil). The PCR conditions were as follows: 94°C for 5min, 35 cycles of 94°C for 40s; 57-61°C for 40s; 72°C for 1min, and 72°C for 10min. The amplified products were visualized on 1% agarose gel stained with 0.5μg/mL ethidium bromide. Fragments of 385, 459, and 299bp were visualized and matched with exons 2, 3, and 5 pairs of primers, respectively.

### PCR product sequencing

PCR products (1.0-1.5μL) were sequenced using PCR primers. Sequencing reactions were carried out using the DYEnamic™ ET dye terminator kit (Amersham Biosciences, Uppsala, Sweden) according

to the manufacturer's instructions. The cycling conditions were as follows: 95°C for 1min, 25 cycles of 95°C for 20s, 50°C for 20s, and 60°C for 1min. Next, the products were electrophoresed directly with the automated apparatus MegaBace DNA sequencing system 1,000 (Amersham Biosciences, Uppsala, Sweden). The electropherograms were analyzed with Sequence Analysis Software version 2.4 (Amersham Biosciences, Uppsala, Sweden). Chromatograms were processed using the base program Phred<sup>11,12</sup>. The SNPs were detected in the highly similar regions of multiple alignments using the PolyPhred program<sup>13</sup>.

### Statistical analysis

Allelic frequencies and heterozygosity observed were computed using the direct counting method. Adherence of genotypic proportions to expectations under Hardy-Weinberg equilibrium was tested by the exact test of Guo and Thompson<sup>14</sup>, and the presence of a significant association between loci was determined using a likelihood ratio test of linkage disequilibrium<sup>15</sup>, both using Arlequin 3.1 software<sup>16</sup>.

The GenePop 3.4 program was used to perform the pairwise exact test of population differentiation based on allele and genotype frequencies<sup>17</sup>. Haplotypes defined by the three SNPs were constructed using Arlequin 3.1 software<sup>16</sup>. The allele, genotype, and haplotype frequencies were compared using Fisher exact test and  $\chi^2$  square test implemented in the GraphPad-Instat, version 3.05 (GraphPad Software, Inc., San Diego, CA), to determine the significance of the deviation, with the level of significance set at  $p < 0.05$ .

### Ethical considerations

The study protocol was approved by the institutional ethics committee of the University Hospital, Medical School of Ribeirão Preto (process n° 8705/2000). All subjects were informed about the procedure and gave written informed consent to participate in this study.

## RESULTS

### Polymorphism detection

Three coding mutations were detected: 2364A/G at exon 2, 2933C/G at exon 3, and 4243C/T at exon 5. These coding SNPs define an allele of the *Gzmb* gene in which three amino acids of the mature protein Q48P88Y245 are mutated to R48A88H245. Two noncoding SNPs at exon 3 were also detected: 2893A/G (74 K < K) and 2973C/T (101N < N). Genotypic frequencies of SNPs 2364A/G and 2933A/G in all groups and of SNP 4243C/T in whites and Afro-Brazilians were in agreement with that expected by Hardy-Weinberg equilibrium. However, the genotypic distribution of 4243C/T in Asians differed significantly from expected ( $p < 0.0297$ ). The presence of a significant association between SNPs was estimated using a likelihood ratio test of linkage disequilibrium considering all of the samples from the control admixed and ethnic groups. A positive association was detected between all loci (exact  $p = 0.00000 \pm 0.00000$  in all analyses).

### Genotype and allele distribution among ethnic groups

To investigate the ethnic distribution of *Gzmb* alleles, white, Afro-Brazilian, and Asian individuals were genotyped for the 2364A/G, 2933C/G, and 4243C/T SNPs. Both allele and genotype

frequencies were significantly higher among Afro-Brazilians to SNP 2364A/G at exon 2 ( $p < 0.0001$  and  $p = 0.0007$ , respectively). The 2364G allele was present in 42.3% of the Afro-Brazilians, 25% of the whites, and 14.9% of the Asians (Table 1).

**TABLE 1 - Allele and genotype distribution of SNPs 2364A/G, 2933C/G, and 4243T/C from the granzyme B gene among Brazilian ethnic groups (whites, Afro-Brazilians, and Asians).**

| SNPs        | Ethnic groups |      |                |                   |         |      |
|-------------|---------------|------|----------------|-------------------|---------|------|
|             | White         |      | Afro-Brazilian |                   | Asian   |      |
|             | n             | %    | n              | %                 | n       | %    |
| <b>2364</b> |               |      |                |                   |         |      |
| A           | 81            | 75.0 | 60             | 57.7              | 80      | 85.1 |
| G           | 27            | 25.0 | 44             | 42.3 <sup>a</sup> | 14      | 14.9 |
| AA          | 30            | 55.6 | 17             | 32.7              | 35      | 74.5 |
| AG          | 21            | 38.9 | 26             | 50.0              | 10      | 21.3 |
| GG          | 3             | 5.5  | 9              | 17.3 <sup>b</sup> | 2       | 4.2  |
| pHW         | 1.00000       |      | 1.00000        |                   | 0.19933 |      |
| <b>2933</b> |               |      |                |                   |         |      |
| C           | 83            | 75.5 | 71             | 68.3              | 82      | 82.0 |
| G           | 27            | 24.5 | 33             | 31.7              | 18      | 18.0 |
| CC          | 31            | 56.4 | 25             | 48.1              | 34      | 68.0 |
| CG          | 21            | 38.2 | 21             | 40.4              | 14      | 28.0 |
| GG          | 3             | 5.4  | 6              | 11.5              | 2       | 4.0  |
| pHW         | 1.00000       |      | 0.49819        |                   | 0.60100 |      |
| <b>4243</b> |               |      |                |                   |         |      |
| T           | 82            | 77.4 | 68             | 70.8              | 83      | 83.0 |
| C           | 24            | 22.6 | 28             | 29.2              | 17      | 17.0 |
| TT          | 33            | 62.3 | 25             | 52.1              | 37      | 74.0 |
| TC          | 16            | 30.2 | 18             | 37.5              | 9       | 18.0 |
| CC          | 4             | 7.5  | 5              | 10.4              | 4       | 8.0  |
| pHW         | 0.42754       |      | 0.49837        |                   | 0.02977 |      |

SNPs: single nucleotide polymorphisms; pHW: probability of adherence to the Hardy-Weinberg equilibrium exact test; <sup>a</sup> $p = 0.0007$  and <sup>b</sup> $p < 0.0001$  (Fisher exact test).

Allele and genotype frequencies of the polymorphisms 2933C/G and 4243C/T also were higher in Afro-Brazilians but without statistical significance. The frequency of 2933G allele in Afro-Brazilians was 31.7%, 24.5% in whites, and 18% in Asians. The same trend was observed for 4243T allele, found in higher frequency in Afro-Brazilians (29.2%), followed by whites (22.6%) and Asians (17%).

The number of heterozygous individuals for the three SNPs also was higher in Afro-Brazilians. The 2364AA, 2933CC, and 4243CC genotypes were more frequent in Asians (74.5%, 68%, and 74%, respectively), which appears to be the least polymorphic group.

#### Haplotype distribution among ethnic groups

The association of the polymorphisms gave origin to six haplotypes with a frequency of more than 1% among the ethnic groups. The haplotype RPH was found only in Afro-Brazilians in a frequency lower than 1%. The haplotypes QPY, QPH, RAY, and RAH were shared by whites, Afro-Brazilians, and Asians. The haplotype QAH was observed in Afro-Brazilians (1%) and Asians (3%), and the RPY haplotype occurred only in Afro-Brazilians individuals (12%).

The haplotype QPY was the most frequent in all groups (79%, 73%, and 53% in the Asian, white, and Afro-Brazilian groups, respectively). The RAH allele was more frequent in the Afro-Brazilians group (25%), followed by whites (21%) and Asians (12%). The frequency of the remaining haplotypes is presented in Table 2.

**TABLE 2 - Frequencies of haplotypes identified in granzyme B gene in Brazilian ethnic groups (whites, Afro-Brazilians, and Asians).**

| Haplotypes | SNPs |      |      | Ethnic groups |                |       |
|------------|------|------|------|---------------|----------------|-------|
|            | 2364 | 2933 | 4243 | White         | Afro-Brazilian | Asian |
| QPY        | A    | C    | T    | 0.73          | 0.53           | 0.79  |
| RAH        | G    | G    | C    | 0.21          | 0.25           | 0.12  |
| RAY        | G    | G    | T    | 0.04          | 0.05           | 0.02  |
| QPH        | A    | C    | C    | 0.02          | 0.03           | 0.04  |
| QAH        | A    | G    | C    | -             | 0.01           | 0.03  |
| RPY        | G    | C    | T    | -             | 0.12           | -     |

SNPs: single nucleotide polymorphisms.

## DISCUSSION

In contrast with the molecular and cellular findings, we notice that little progress has been achieved regarding the definition of the granzyme B functional significance *in vivo*, especially in humans. In mice, the granzyme B deficiency is associated with the extreme susceptibility to ectromelia poxvirus, which is worsened by granzyme A simultaneous deficiency<sup>5</sup>. Although a complete lack of perforin expression is associated with a deregulation and fatal insufficiency of the immune system in children with autosomal recessive disorder-familial lymphohistiocytosis hemophagocytic<sup>6</sup>, the deficiency of granzyme B expression was not identified in this condition<sup>7</sup>. The description of one haplotype with three mutations in the granzyme B gene, whose product presents normal proteolytic activity, with reduced apoptotic capacity<sup>8</sup>, offers a unique opportunity to define the functions of granzyme B in human beings. These substitutions define a haplotype in which three amino acids Q48P88Y245 of the mature protein are altered to R48A88H245.

In our study, the research on the frequency of these polymorphisms was conducted in Brazilian population groups. The distribution of the SNPs genotypes evaluated was in agreement with that expected from the Hardy-Weinberg equilibrium, except for the polymorphism 4243T/C in Asians. We could consider, from among the factors that interfere in this equilibrium, the gene flux resulting from migrations because such event inflates the diversity of the population and simultaneously leads to a 4243TT heterozygous deficiency, or it is possible that occurrence might have been merely casual.

By analyzing 2364A/G SNP at exon 2, we verified that the allelic and the genotypic frequencies were significantly higher ( $p < 0.0001$  and  $p = 0.0007$ , respectively) in Afro-Brazilian individuals but without any statistical significance.

Consequently, the frequency of haplotype RAH was higher in the Afro-Brazilian individuals (25%). In this group, we found the largest number of different haplotypes, which is in accordance with the results by McIlroy and collaborators<sup>8</sup>, who identified the RPY and RAY haplotypes only in West African individuals. In our study, we did not find RAY haplotype, and RPY and RPH haplotypes were found only in Afro-Brazilians, the last one presenting a frequency lower than 1%. A hypothesis for these findings is that RAH haplotype might have an African ancestor origin, in which multiple mutations transformed the sequence QPY in RPY and then in RAY and finally in RAH. However, it also may be possible that the haplotype RAH is the ancestor form and the QPY haplotype that present wider distribution appeared more recently; in this case, the H245Y mutation might have been the first to appear, followed by A88P and then by R48Q<sup>8</sup>.

In our research, we identified QPY, QPH, QAH, RPY, RPH, RAY, and RAH haplotypes. The RPH haplotype, found only in Afro-Brazilian individuals in a frequency of 0.005%, was not described in the studies of McIlroy and collaborators<sup>8</sup>, probably because they have only described the haplotypes with a frequency higher than 1%. In the same study, the authors investigated the presence of RAH haplotype in various ethnic groups and found an allelic frequency varying from 25 to 30%. The wild QPY haplotype was found in a frequency of 54 to 71%. Sun and collaborators found frequencies similar to the ones by McIlroy et al.<sup>8,9</sup>. We verified that the frequencies of RAH, RAY, and QPH haplotypes in ethnic groups did not differ significantly from the ones found in previous studies, but the frequency of wild QPY haplotype differed from McIlroy and collaborators' findings, especially in relation to Asian individuals (79.3% vs. 65.7%, respectively), with  $p = 0.0294$ . The QAH haplotype had been found before in white, black, and Asian subjects<sup>8</sup>, but in our investigation, we did not find this haplotype in white individuals. However, the RPY haplotype had not been described in black and Asian subjects, and it was identified only in Afro-Brazilian individuals.

The frequency of RAH haplotype among whites, blacks, and Asians was 20.7%, 24.7%, and 11.9%. According to McIlroy and collaborators<sup>8</sup>, this haplotype frequency was higher in Asians, whereas in our study, it was higher in Afro-Brazilians. This difference could be caused by the different origin of individuals included in the investigation. In our study, most Asian individuals came from Japan or China, and black individuals from our country have their origin in several countries from Sub-Saharan Africa<sup>18</sup>, whereas in the work previously cited, Asian individuals were Vietnamese and black individuals from West Africa.

The importance of the research on this haplotype frequency was evidenced by the work that verified that RAH haplotype is incapable of inducing apoptosis in tumor cell lines<sup>8</sup>. According to this study, granzyme B is expressed in similar amounts in individuals who possess wild QPY haplotype and in those with RAH; however, the cells that presented RAH haplotype seemed to be incapable of inducing apoptosis in primary glioblastoma cell lines, at least for the first time interval. Moreover, the RAH haplotype was associated with a predisposition to develop breast cancer. People presenting genotype RAH/RAH were 16 times more likely to have breast cancer as compared with those with genotype QPY/QPY, and individuals with the wild-type QPY/QPY displayed higher cytotoxicity against tumor cell lines, in comparison to donors with the mutated type RAH/RAH<sup>19</sup>.

In conclusion, we verified in our ethnic groups that there was a higher allelic frequency of polymorphisms in Afro-Brazilian individuals, with a significantly higher frequency of SNP 2364A/G at exon 2 in this group compared with the others, besides presenting a larger number of different haplotypes. The frequency of wild QPY haplotype differed among our findings and the ones described in the literature, especially regarding Asian individuals. The RAH haplotype was more frequent in Afro-Brazilian individuals (25%), followed by the whites (20.7%) and then the Asians (11.9%), similar to the frequencies previously described. We believe that further studies should investigate this haplotype correlation with the prognosis and the response to treatment for diseases related to immunity mediated by CTLs and NK cells. Therefore, if this correlation is confirmed, novel strategies for treatment might be elaborated, aiming to help the individuals' immune response in the defense against these diseases.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## REFERENCES

1. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2002; 2:735-747.
2. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2002; 2:401-409.
3. Poe M, Blake JT, Boulton DA, Gammon M, Sigal NH, Wu JK, et al. Human cytotoxic lymphocyte granzyme B. Its purification from granules and the characterization of substrate and inhibitor specificity. *J Biol Chem* 1991; 266:98-103.
4. Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* 1994; 76:977-987.
5. Mullbacher A, Waring P, Tha Hla R, Tran T, Chin S, Stehle T, et al. Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. *Proc Natl Acad Sci USA* 1999; 96:13950-13955.
6. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999; 286:1957-1959.
7. Ericson KG, Fadeel B, Andersson M, Gudmundsson GH, Gurgey A, Yalman N, et al. Sequence analysis of the granzysin and granzyme B genes in familial hemophagocytic lymphohistiocytosis. *Hum Genet* 2003; 112:98-99.
8. McIlroy D, Cartron PF, Tuffery P, Dudoit Y, Samri A, Autran B, et al. A triple-mutated allele of granzyme B incapable of inducing apoptosis. *Proc Natl Acad Sci USA* 2003; 100:2562-2567.
9. Sun J, Bird CH, Thia KY, Matthews AY, Trapani JA, Bird PI. Granzyme B encoded by the commonly-occurring human RAH allele retains pro-apoptotic activity. *J Biol Chem* 2004; 279:16907-16911.
10. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; 132:365-386.
11. Ewing B, Green P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 1998; 8:186-194.
12. Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 1998; 8:175-185.
13. Nickerson DA, Tobe VO, Taylor SL. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res* 1997; 25:2745-2751.
14. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 1992; 48:361-372.
15. Excoffier L, Slatkin M. Incorporating genotypes of relatives into a test of linkage disequilibrium. *Am J Hum Genet* 1998; 62:171-180.
16. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online* 2005; 1:47-50.
17. Raymond M, Rousset F. GenePop (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered* 1995; 86:248-249.
18. Abe-Sandes K, Silva Jr WA, Zago MA. Heterogeneity of the Y chromosome in Afro-Brazilian populations. *Hum Biol* 2004; 76:77-86.
19. Gaafar A, Aljurf MD, Al-Sulaiman A, Iqniebi A, Manogaran PS, Mohamed GE, et al. Defective gamma delta T-cell function and granzyme B gene polymorphism in a cohort of newly diagnosed breast cancer patients. *Exp Hematol* 2009; 37:838-848.