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Analysis of SNP (single nucleotide polymorphism) multiplex markers related to sudden cardiac death in Brazilian families

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ABSTRACT. Sudden cardiac death (SCD) is a major public health concern worldwide, and genetic analysis may be useful in identifying the cause of death as well as in determining the possible genetic risk factors for SCD. This study analyzed eight SNPs (single nucleotide polymorphisms) highly correlated with cardiac sudden death in samples (blood and bone) from six Brazilian families with a history of cardiovascular diseases. Individuals with no family history of cardiovascular diseases were recruited as controls. Y chromosomes and mtDNA haplogroups belonging to these subjects were verified as well. We found that SNP rs16857031 showed significant differences between the group with a family history of cardiovascular diseases and the control group. Furthermore, the data obtained were compatible with known frequencies of SNPs for the haplogroups in which the samples were classified. A possible hereditary factor was identified for SNP rs4725982 in one family. These preliminary results suggest that identification of certain SNPs could be used to assess risk factors for SCD.

Key words: Cardiopathy; Sudden cardiac death; SNaPshot; SNP; Post mortem analysis

INTRODUCTION

Sudden cardiac death (SCD) is one of the most common causes of death in many countries. It is a major health issue, as sudden loss of cardiac function may lead to death in as little as a few minutes after the onset of symptoms. It is not due to trauma or violence, and can affect anyone from newborns to adults (Márban, 2002; Tester and Ackerman, 2006).

Population studies have shown that a significant number of SCDs are associated with hereditary channelopathies (Tester and Ackerman, 2006). It is recognized that changes in cardiac ion channel genes can cause sudden death without any morphological abnormalities in the heart (Márban, 2002). This can make it difficult to identify the cause of death in legal medicine reviews (Rodríguez-Calvo et al., 2008).

Post mortem genetic analyses (molecular autopsy) can potentially substantiate the pathogenic basis for the SCD, and may prevent further deaths in the same family as family history is known to be a significant risk factor (Rodríguez-Calvo et al., 2008).

In 2009, two teams of international researchers have identified several SNPs to be highly associated with QT interval syndromes (Newton-Cheh et al., 2009), a major cause of SCD. In this study, we chose eight SNPs, and examined their polymorphisms in Brazilian families. Comparisons were made between individuals with and without clinically diagnosed family history of cardiovascular diseases. In order to further complement our findings, subjects were classified into evolutionary haplogroups by polymorphisms in mitochondrial DNA and / or Y chromosome via sequencing and SNaPshot analysis, respectively. We assessed whether there may be a correlation between the occurrences of certain polymorphisms with different haplogroups. This analysis was also conducted on human remains in order to check the efficiency of this technique in deceased individuals.

MATERIAL AND METHODS

Selection of SNPs and design of primers

In the study by Newton-Chet et al. (2009), 14 SNPs were listed as having the most associations with QT interval syndromes. In this study, we selected 8 of these SNPs (Table 1) for analyses. As Brazil has a heterogeneous population, we took into account any prior knowledge in the literature on the correlation of these genes with SCD in different populations (Manta et al., 2012).

Primers for PCR and SNaPshot reactions were designed and analyzed using the programs PerlPrimer version 1.1.19 (Marshall, 2004), AutoDimer version 1 (Vallone and Butler, 2004), and the online tool PCR *in silico* on the website http://genome.ucsc.edu/ (Kent et al., 2002).

Amplification and detection of SNPs

PCR reactions were performed using 2 U Platinum Taq DNA polymerase (Invitrogen by Life Technologies); 1X Goldstar Buffer (Promega); and appropriate concentration of each primer (**Tables S1** and **S2**); 0.5 ng DNA in a final volume of 11 µL.

The following cycling parameters were performed on the Veriti thermal cycler (Applied Biosystems by Life Technologies): 95°C for 10 minutes; 35 cycles at 95°C for 30 s; 51ºC for 45 s (increasing 0.3ºC per cycle from the fourth cycle); 72°C for 30 s; holding at 4°C.

PCR products were purified prior to completion of the SNaPshot reaction via addition of 1 µL EXO-SAP IT (GE Healthcare) with the cycling parameter as follows: 37°C for 95 min; 78°C for

15 min; hold at 4°C.

SNaPshot reactions were performed using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems by Life Technologies) as follows: PCR Buffer II to 0.38X (Applied Biosystems by Life Technologies); 1.25 µL SNaPshot mix; and appropriate concentration of each primer (**Tables S3** and **S4**); 1 µL purified PCR products in a final volume of 6.5 µL. The cycling parameters are as follows: 30 cycles at 96°C for 3 min; 55°C for 5 s; 60°C for 30 s; hold at 4ºC.

The reaction product was purified by addition of 1µL SAP (GE Healthcare) with the following cycling protocol: 37°C for 95 min; 78°C for 15 min; hold at 4ºC.

Products of SNaPshot reactions were prepared for capillary electrophoresis (CE) by the addition of 1 µL purified SNaPshot reaction products to 10 µL HI-DI formamide and LIZ 120 (12.5 µL LIZ 120 to 1000 µL formamide HI-DI, Applied Biosystems by Life Technologies). CE was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems by Life Technologies) using the filter dye set E5 and POP-7 polymer. Analyses were performed using the GeneMapper software - IDX version 1.2 (Applied Biosystems by Life Technologies). Patent application for this methodology was filed to the INPI (National Institute of Industrial Property) under number: BR102012028699-8.

Table 1. Location and related genes on chromosomes of 8 SNPs selected for this study.

SNP	Chromosome	Gene	Ancestral allele	Derived allele
rs16857031	1q	Intron *NOS1AP*	C	G
rs2074238	11p	Intron *KCNQ1*	C	T
rs12576239	11p	Intron *KCNQ1*	C	T
rs4725982	7q	Downstrem *KCNH2*	C	T
rs1805128	21q	Missense *KCNE1*	G	A
rs12053903	3p	Intron *SCN5A*	C	T
rs2074518	17q	Intron *LIG3*	G	A
rs2968864	7q	Downstream *KCNH2*	A	G

Samples

Blood samples from individuals of five Brazilian families (N = 28) were collected on FTA cards (Whatman). Subjects were clinically diagnosed with a history of heart problems, Coronary Disease, Hypertrophic Cardiomyopathy, or had a family member who suffered a heart attack.

Samples belonging to male individuals were classified into evolutionary haplogroups by SNPs analysis of the Y chromosome (Brion et al., 2005). Blood samples from individuals who had no family history of heart diseases were used as controls (N = 30). This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences - Araraquara by Protocol: 24/2009.

DNA samples from bones and teeth were obtained from five exhumed corpses belonging to the same family, three of which with cardiovascular disorder as the cause of death. These samples were named as MPV, HV, MLV, CLV and OVB: MPV, 42 years, cardiac injury; HV, 42 years, pleuropneumonia; MLV, 79 years, myocardial infarction; CLV, 82 years, head trauma; OVB, 52 years, acute myocardial infarction/coronary insufficiency.

These samples were classified into haplogroups through evolutionary analysis of the hypervariable regions I and II of the mtDNA (Paneto et al., 2007). DNA extraction from blood samples was performed with Chelex resin (Biorad) (Singer-Sam et al., 1989).

For DNA extraction, samples of bones and teeth were subjected to demineralization, (Loreille et al., 2007) followed by centrifugation using the AMICON ULTRA filter (Millipore). Extraction procedures were carried out with the DNA IQ Kit (Promega) according to manufacturer instructions.

RESULTS AND DISCUSSION

SNPs analysis related to SCD

The amplified SNPs are shown in the electropherogram (Figure 1). Some artifacts were present, but did not have an effect on the analyses of the expected peaks for the SNPs. The primer sequences and the concentrations at which they were used are described in the supplementary material.

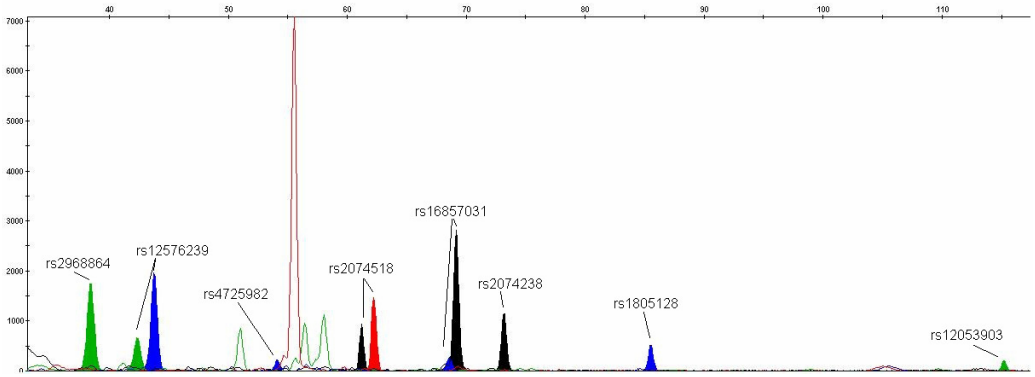

Figure 1. Electropherogram of SNaPshot reaction of eight SNPs analyzed in multiplex system.

We identified the presence of the derivative allele "T/T", which was identical for the SNP rs12053903 in all samples of from both groups. (Figure 2).

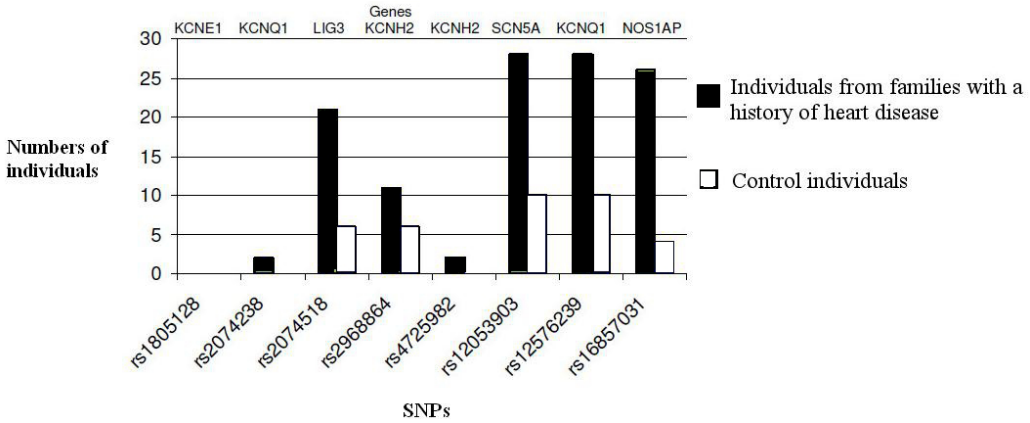

Figure 2. Occurrence of 8-SNPs with their respective frequencies which the derived alleles are present (regardless of whether homo or heterozygous) in samples of individuals from families with a history of heart disease and controls.

For the SNP rs12576239, in the group with a family history of cardiovascular diseases, 20 samples showed the alleles "C/T" and 8 samples showed the alleles "T/T". However, in the control group, we identified 14 samples with the alleles "T/T" and 16 with "C/T" (Figure 3).

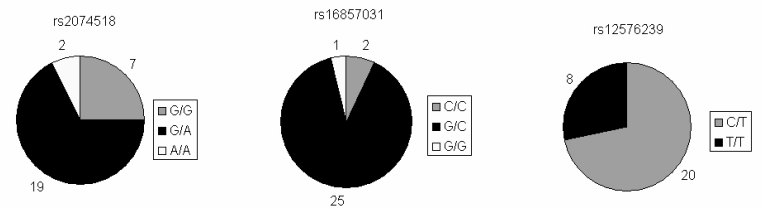

Figure 3. Profiles observed in samples of individuals with a family history of heart disease for SNPs rs2074518, rs16857031 and rs12576239.

Twenty-five samples of the family history group presented tested for the SNP rs16857031 were heterozygous for that locus, namely the original or the ancestor base (C), and the derivative base which G. In addition, one sample presented with the homozygous “G/G”, while two samples alleles “C/C”. In the control group, the alleles “G/C” was found in nine samples, one sample showed the profile “G/G”, and 20 samples showed no exchange of bases, i.e., the alleles “C/C” (Figure 3).

In summary, no significant differences were found between individuals with family history of heart disease and healthy controls. However, the SNP rs16857031 was identified in a higher percentage of individuals with family history of cardiovascular complications compared to the controls. This polymorphism is located in the NOS1AP gene, which was previously described as being closely associated with heart diseases and SCD (Eijgelsheim et al., 2009). The SNP rs2968864 was identified in greater proportion of the control group, which was contrary to what was expected. However, it may be explained by the frequency of this polymorphism in the population, as will be discussed later.

While these results are still preliminary, we have demonstrated that SNP sequencing can be used efficiently in a clinical setting.

Bone and tooth samples

We were unable to analyze the eight SNPs as a multiplex with the bone and teeth samples. The SNP rs16857031 is the only polymorphism displayed on the electropherograms of the SNaPshot reactions for the five samples, and SNP rs2074238 was only amplified in one sample (MPV).

For the samples OVB, CLV, HV, and MLV, the SNP rs16857031 was identified as homozygous with the derivative allele “G/G” and for MPV, the heterozygous “G/C” was observed. It is possible that the SNP rs16857031 may be related to the cause of death for MPV, MLV and OVB, who died from heart failure.

In the MPV sample, the alleles for the rs2074238 SNP was observed to be the homozygous “C/C”, which are the ancestral alleles.

Correlation between SNPs related to SDC and mitochondrial DNA haplogroups in bones and teeth samples

The SNP rs16857031 with “G/G” alleles has a low frequency in Europeans and was identified in the samples OVB, CLV, HV, and MLV, indicating a possible hereditary factor. The alleles “C/G”, which was identified in the MPV sample, have a median frequency. As mentioned previously, the results were suggestive that these alleles may not be related to the population of origin, but are rather with cardiac irregularities. Therefore, the samples OVB, MLV, CLV, and HV

were classified by mtDNA into the “H” haplogroup, whereas the MPV sample was classified as the “T2d” haplogroup, both frequent in Europeans (Babalini et al., 2005).

SNP rs2074238 with the derived allele “T” was not identified in the MPV sample, which showed the profile “C/C”. This form was found to be very common in Europeans, which correlated with the fact that MPV was born in Portugal whereas the others were born in Brazil. Figure 4 presents the heredogram of this family.

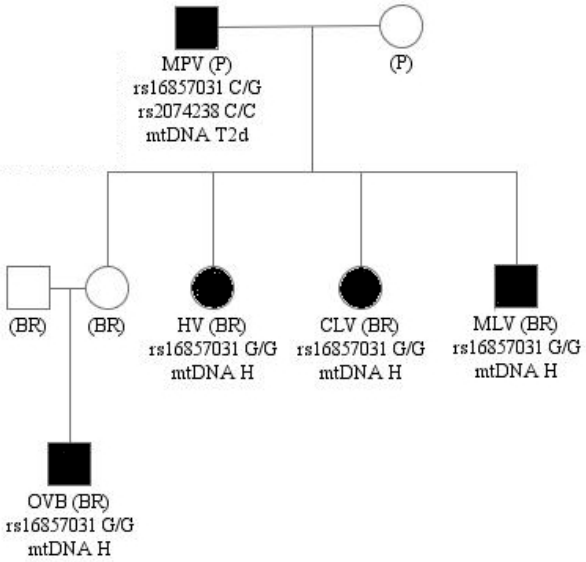

Figure 4. Ancient remains heredogram. In black, the remains analyzed in this paper showing their respective alleles for SNPs rs2074238, rs16857031 and mtDNA haplogroup. It is indicated the birth country (P = Portugal; BR = Brazil).

Correlation between SNPs related to SDC and Y chromosome haplogroups

The genetic characteristics that affect the QT interval may be inherited in approximately 35% of the general population (Eijgelsheim et al., 2009). The genetic inheritance can be illustrated in the analysis of the SNP rs4725982 in two male individuals from family eight. Both the father and son were presented with the polymorphic base “T” for this SNP. Following analyses of the Y chromosome, these individuals were classified into the rare T haplogroup, which is found in low frequencies in Europe and parts of the Middle East, North and West Africa (King et al., 2007).

The frequency of the alleles “T/T” in Europeans and African is also relatively low (http://www.ncbi.nlm.nih.gov/snp/) which may indicate some correlation between the onset of heart disease or SCD in male individuals belonging to this particular haplogroup. Confirmation of this hypothesis requires larger sample of individuals with the syndrome.

The profile “C/C” in the same locus found in samples belonging to the other families have a high frequency in both Europeans and Africans, which correlates with the populations included in this study. Almost all subjects were classified into the European haplogroups once the frequency of haplogroup J2 was identified in Spain and Germany, and absent in China and Japan (Brión et al., 2005); R1b1 is highly common in Europe; haplogroup B is almost entirely restricted to sub-Saharan Africa (Karafet et al, 2008), C is quite found in Asia (Hammer et al., 2001), as haplogroup D, and

it is not found anywhere outside of Asia (Karafet et al., 2001) and haplogroup E is found in high frequency in Africa and moderate frequencies in Europe (Jobling and Tyler-Smith, 2000).

The results presented in this study are in agreement with the literature as it recognized the predominance of European lineages for in the Brazilian populations (Carvalho-Silva et al., 2001).

The finding that the SNP rs1805128 was not identified with the derived allele "A" in any of the samples and rs2074238 with the derived allele "T" in only two samples can be explained by the high frequency of "G/G" and "C/C" alleles in Europeans.

On the other hand, the rs12576239 SNP with allele derivative "T" was identified in all samples and has a median frequency for the profile "C/T" and almost zero for the profile "T/T" in Europeans. The rs16857031 SNP, which was also identified in all samples with the allele "G", has low to medium frequency for the profiles "C/G" and "G /G", respectively. These evidences suggest that these alleles are not population-specific, but rather, are associated with a family history of cardiac irregularities.

Frequency of the profiles "A/A" and "A/G" in SNP rs2968864 is high in Europeans. This agrees with the results from our study, which showed high percentage of the "A/G" alleles in the individuals classified into the European haplogroups.

The frequency of the allele "A" in SNP rs2074518 was found to be slightly higher in the group with a family history of cardiac diseases as compared to the controls. In addition, Europeans show high occurrences of profile "A/G", and median occurrences for the alleles "A/A"and "G/G" (http://www.ncbi.nlm.nih.gov/snp/).

The possibility that ancestral origin may influence the development of cardiovascular diseases is an interesting notion (Fridman et al., 2011). In this preliminary study, we have established a reliable method for SNP analysis related to SCD, which can be used in a larger number of samples to confirm the association between ancestral origin and disease.

CONCLUSIONS

The data are consistent with the known frequencies of the SNPs; SNP rs16857031 presented the polymorphic base with greater frequency in individuals with a family history of heart failure compared with the control group.

Lastly, we recommend the use of *monoplex* for analysis of SNP samples from partially degraded DNA obtained from ancient remains (exhumed bones and/ or teeth).

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

REFERENCES

Babalini C, Martínez-Labarga C, Tolk HV, Kivisild T, et al. (2005). The population history of the Croatian linguistic minority of Molise (southern Italy): a maternal view. *Eur. J. Hum. Genet.* 13: 902-912.

Brion M, Sobrino B, Blanco-Verea A, Lareu MV, et al. (2005). Hierarchical analysis of 30 Y-chromosome SNPs in European populations. *Int. J. Legal Med.* 119: 10-15.

Carvalho-Silva DR, Santos FR, Rocha J and Pena SDJ (2001). The Phylogeography of Brazilian Y-Chromosome Lineages. *Am. J. Hum. Genet*. 68: 281-286.

Eijgelsheim M, Aarnoudse AL, Rivadeneira F, Kors JA, et al. (2009) Identification of a common variant at the *NOS1AP* locus strongly associated to QT-interval duration. *Hum. Mol. Genet.* 18: 347-357.

Fridman C, Cardena M, Krieger JM and Pereira AC (2011). Evaluation of the relationship between mitochondrial haplogroup and development of heart failure in Brazilian sample. *Forensic Sci. Int. Genet.* 3: 77-78.

Hammer MF, Karafet TM, Redd AJ, Jarjanazi H, et al. (2001). Hierarchical patterns of global human Y-chromosome diversity. *Mol. Biol. Evol.* 18: 1189-1203.

Jobling MA and Tyler-Smith C (2000). New uses for new haplotypes: the human Y chromosome, disease, and selection. *Trends Genet.* 16: 356-362.

Karafet T, Mendez FL, Meilerman MB, Underhill PA, et al. (2008). New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. *Genome Res.* 18: 830-838.

Karafet T, Xu L, Du R, Wang W, et al. (2001). Paternal population history of East Asia: sources, patterns, and microevolutionary processes. *Am. J. Hum. Genet.* 69: 615-628.

Kent WJ, Sugnet CW, Furey TS, Roskin KM, et al. (2002). The human genome browser at UCSC. *Genome Res.* 6: 996-1006.

King TE, Bowden GR, Balaresque PL, Adams SM, et al. (2007). Thomas Jefferson's Y chromosome belongs to a rare European lineage. *Am. J. Phys. Anthropol.* 132: 583-589.

Loreille OM, Diegoli TM, Irwin JA, Coble MD, et al. (2007). High efficiency DNA extraction from bone by total demineralization. *Forensic Sci. Int.* 1: 191-195.

Manta F, Caiafa A, Pereira R, Silva D, et al. (2012). Indel markers: Genetic diversity of 38 polymorphisms in Brazilian populations and application in a paternity investigation with post mortem material. *Forensic Sci. Int. Genet.* 6: 658-661.

Marbán E (2002). Cardiac channelopathies. *Nature* 415: 213-218.

Marshall OJ (2004). PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* 20: 2471-2472.

Newton-Cheh C, Eijgelsheim M, Rice KM, de Bakker PI, et al. (2009). Common variants at ten loci influence QT interval duration in the QTGEN Study. *Nat. Genet.* 41: 399-406.

Paneto GG, Martins JA, Longo LV, Pereira GA, et al. (2007). Heteroplasmy in hair: Differences among hair and blood from the same individuals are still a matter of debate. *Forensic Sci. Int.* 173: 117-121.

Rodríguez-Calvo MS, Brion M, Allegue C, Concheiro L, et al. (2008). Molecular genetics of sudden cardiac death. *Forensic Sci. Int.* 182: 1-12.

Singer-sam J, Tanguay R and Riggs AD (1989). Use of Chelex to improve the PCR signal from a small number of cells. *Amplifications* 3: 11.

Tester DJ and Ackerman J (2006). The role of molecular autopsy in unexplained sudden cardiac death. *Curr. Opin. Cardiol.* 21: 166-172.

Vallone PM and Butler JM (2004). AutoDimer: a screening tool for primer-dimer and hairpin structures. *Biotechniques* 37: 226-231.