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Investigation of INDEL variants in apoptosis: the relevance to gastric cancer



Giovanna Chaves Cavalcante¹, Milene Raiol de Moraes¹, Cristina Maria Duarte Valente¹, Caio Santos Silva¹, Antônio André Conde Modesto², Paula Baraúna de Assumpção², Paulo Pimentel de Assumpção², Sidney Santos^{1,2} and Ândrea Ribeiro-dos-Santos^{1,2*} 

Abstract

Background: Apoptosis is a type of cell death involved in different pathways inherent to the cell and the evasion from this mechanism has been related to cancer, although this process remains not very well comprehended. Gastric cancer (GC) is one of the most incident and aggressive types of cancer worldwide. In this study, we analyzed the distribution of INDEL variants in GC patients (Case) and individuals from the general population (Control) from the Amazon region, in which GC is remarkably frequent.

Methods: A panel of nine INDEL markers in apoptosis-related genes (*BCL2* rs11269260, *CASP3* rs4647655, *CASP8* rs3834129 and rs59308963, *CASP9* rs4645982 and rs61079693, *FADD* rs4197, *FAS* rs10562972 and *TP53* rs17880560) was developed and genotyped by multiplex PCR in both groups.

Results: In our analyses, only marker rs4197 (*FADD* gene) was associated to GC development as follows: INS/DEL genotype of rs4197 increasing in about 2-fold the chances of developing this type of cancer ($P = 0.046$; OR = 1.940; 95%CI = 1.011–3.725).

Conclusion: Our results suggest that rs4197 (*FADD* gene) might play a role in gastric carcinogenesis in the investigated population. More studies are needed to clarify this relation. Here, we highlight the importance of investigating INDEL variants in genes involved in apoptosis.

Keywords: Gastric Cancer, Apoptosis, INDEL, FADD

Background

Apoptosis is a type of programmed cell death (PCD) that may occur as a response to intra- or extracellular stimuli, leading to intrinsic pathway or extrinsic pathway, respectively. These are independent pathways that converge to the executor phase, the final stage of this death process [1–3]. Several genes are involved in apoptosis, out of which we highlight: *BCL2*, *CASP9* in the

intrinsic pathway; *FAS*, *FADD*, *CASP8* in the extrinsic pathway; *TP53* in both pathways; and *CASP3* in the executor phase. All of these genes are important to the apoptotic mechanism, so that variants affecting their functions might dysregulate cell homeostasis and contribute to tumor development [4]. Although the apoptosis process is still not completely comprehended, this type of cell death mechanism has been related to cancer.

Gastric cancer (GC) is one of the most frequent and aggressive types of cancer worldwide, usually presenting an unfavorable prognosis [5, 6]. In Brazil, GC is the sixth most incident and the fifth most mortal type of cancer [6]. Brazil is a country with a highly admixed population, composed mainly of European, African, Amerindian and

* Correspondence: akelyufpa@gmail.com

¹Laboratório de Genética Humana e Médica, Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Pará, Av. Augusto Correa 01, Belém, Pará 66075-110, Brazil

²Núcleo de Pesquisas em Oncologia, Programa de Pós-Graduação em Oncologia e Ciências Médicas, Universidade Federal do Pará, Belém, Pará 66073-005, Brazil



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Asian ancestry. Many studies in this country have highlighted differences between these ancestries and the Brazilian regions [7–11], including aspects of cancer development [12]. This is also notable by the higher incidence of GC in northern Brazil in comparison to the general rates of this country and the world [13].

Therefore, in this study, we aimed to analyze the distribution of nine Insertion/Deletion (INDEL) polymorphisms in apoptosis-related genes in gastric cancer patients and cancer-free individuals from the general population of the Amazon region.

Methods

Sampling

Case group was composed of 93 individuals with gastric cancer diagnosis from Pará state and control group was composed of 98 cancer-free individuals of the general population from the same state, located in the Amazon region of Brazil. From each individual, blood or buccal swab samples were collected. All participants signed an informed consent, with approval by the Committee for Research Ethics of Hospital João de Barros Barreto under Protocol Number: 64399617.7.0000.5634.

DNA extraction and quantification

DNA extraction was performed with phenol-chloroform method, based on [14]. Quantification of DNA was performed with NanoDrop 1000 and Qubit™ (Thermo Fisher Scientific, Wilmington, DE, USA).

Selection of INDEL markers

The markers were selected according to the main criteria: (i) they must be in genes involved in apoptosis

pathways; (ii) they must be INDEL-type polymorphisms, hence presenting the potential to modify the function of the protein; (iii) they must have minor allele frequency (MAF) $\geq 10\%$. Table 1 shows the technical features of the investigated markers.

Genotyping

The panel of markers described above was genotyped by Multiplex PCR, allowing the amplification of all of these markers in a single reaction, followed by capillary electrophoresis and fragment analysis. Protocol preparation of PCR samples was: 5.0 μL of QIAGEN Multiplex PCR Master Mix, 1.0 μL of Q-solution, 2.0 μL of water, 1.0 μL of primer mix and 1.0 μL of DNA per sample. Amplification reaction was done using Veriti thermal cycler (Thermo Fisher Scientific) with the following protocol: 95 °C for 15 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 90 s and 72 °C for 1 min, with a final extension at 70 °C for 30 min. The protocol for capillary electrophoresis was: 1.0 μL of PCR product, 8.5 μL of HI-DI deionized formamide and 0.5 μL of GeneScan 500 LIZ pattern size standard (reagents by Thermo Fisher Scientific). DNA fragments were separated and analyzed using ABI PRISM 3130 genetic analyzer and GeneMapper ID v.3.2 software (both by Thermo Fisher Scientific).

In addition to the panel of apoptosis markers, a panel of 61 ancestry informative markers (AIM) previously developed and expanded by our research group was also employed in this study, following the established protocols [15, 16]. This was done considering that the Brazilian population is highly admixed in terms of genetic contributions from different parental populations, mainly European, African and Native American. In order

Table 1 Technical characterization of the markers included in the panel

Gene	ID	Region	Alleles	MAF	Primers	Amplicon
<i>BCL2</i>	rs11269260	Intron	TCTATCACCGATCATT/–	0.37	F5'GCTTCCAGTTCATCCATGT3' R5'CTCAGCGTGGTAGTGTGGGA3'	189–205
<i>CASP3</i>	rs4647655	Intron	–/AAATCCTGAA	0.28	F5'AGGAGTATCCCCTCGTGAC3' R5'CAAGAGTCAGGCAAAAACAGG3'	379–389
<i>CASP8</i>	rs3834129	Promoter	AGTAAG/–	0.39	F5'CTCTTCAATGCTTCCTTGAGGT3' R5'CTGCATGCCAGGAGCTAAGTAT3'	249–255
<i>CASP8</i>	rs59308963	Intron	ATTCTGTC/–	0.26	F5'TTTTTGTCTCCAAGCTTCC3' R5'GAACAAGAGAGAGGGCAGGA3'	261–269
<i>CASP9</i>	rs4645982	Intron	–/TCCCCGCACTGACCTCAGC	0.42	F5'GGTGACCCCAAGATTGACCT3' R5'GCCCTCAGGACGCACTCTG3'	336–353
<i>CASP9</i>	rs61079693	Intron	AAAA/–	0.32	F5'CATGCACAGCTATCCAGGAG3' R5'TTGTTCTGTCCGATAGATGC3'	458–462
<i>FADD</i>	rs4197	3' UTR	–/TGT	0.47	F5'TGCCCCACTTAGCAGTCTCA3' R5'GAGAGGTGGAGAACTGGGATT3'	278–281
<i>FAS</i>	rs10562972	Intron	TTC/–	0.12	F5'GCATCAGGACGCTGAACATA3' R5'AATGCAACTTGCTCCAGAGG3'	368–371
<i>TP53</i>	rs17880560	3'-Flanking	–/GCCGTG	0.21	F5'CTGTGTGTCTGAGGGGTGAA3' R5'ATCCTGCCACTTCTGATGG3'	400–406

to control the possible influence of these ancestries in the genotypic distribution, leading to misinterpretation, we have used this AIM panel, composed of ancestry-specific markers that are able to estimate individual and population genetic contribution.

Statistical analysis

Inference of genetic ancestry based on the AIM panel was done with Structure software v.2.3.4 [17]. R language [18] was used to assess Hardy-Weinberg Equilibrium (HWE) of genotype distribution. JASP software v. 0.9.2.0 [19] was used to perform all other statistical analyses (Student's *t* test, chi-squared test, logistic regression). *P*-values ≤ 0.05 were considered statistically significant.

Results

Firstly, we assessed HWE for genotypic distribution and found that only two markers, rs4645982 (*CASP9*) and rs4197 (*FADD*), deviated from HWE in case group ($P = 0.0035$ and $P = 0.0026$, respectively).

Then, we compared sex, age and controlled genetic ancestry of case and control groups in order to verify possible confounding factors. In addition, staging (I-IV) of patients was assessed. These data are described in Table 2.

In these analyses, only age presented a statistically significant difference and it was included for correction in the panel analysis. It is noteworthy that 53% of the patients included in case group presented diffuse-type adenocarcinoma, while 47% presented intestinal-type adenocarcinoma, and the observed mean age (mean age \pm SE) for these subtypes was 39.20 ± 1.397 and 44.34 ± 1.526 , respectively, so that the mean age for case group as a whole was lower than it is usually observed for patients with GC in the world.

Table 2 Demographic data for case (gastric cancer patients) and control groups

Variable	Case	Control	<i>P</i> -value
N	91	95	
Age, years ^a	42.15 \pm 1.66	32.18 \pm 1.17	< 0.001
Sex, % of male/female ^b	48.9/51.1	50.0/50.0	1.000
European ancestry ^c	0.541 \pm 0.028	0.587 \pm 0.021	0.251
African ancestry ^c	0.152 \pm 0.018	0.124 \pm 0.013	0.966
Native American ancestry ^c	0.307 \pm 0.022	0.289 \pm 0.019	0.649
Stage I, %	3.3	NA	0.241
Stage II, %	20.7	NA	
Stage III, %	26.0	NA	
Stage IV, %	50.0	NA	

^aValues are expressed as mean \pm SE (Standard Error of Mean), Student's *t*-test;

^bValues are expressed as distribution percentages, chi-squared test; ^cValues are expressed as mean \pm SE, Mann-Whitney test. NA Not Applicable

Nevertheless, in the analysis performed for each genotype (carriers of a genotype vs. non-carriers of such genotype as a reference group), only INS/DEL genotype of rs4197 ($P = 0.046$; OR = 1.940; 95%CI = 1.011–3.725) presented significant association to risk of developing GC in the investigated population (Table 3).

Discussion

In the last decade, it has been established that tumors display different abilities in order to grow, survive and proliferate, of which we highlight here resistance to cell death, including apoptosis [20]. However, there is still a lot to discover about these mechanisms.

Apoptosis is a complex process that involves the protein action of several different genes, such as *BCL2*, *CASP3*, *CASP8*, *CASP9*, *FADD*, *FAS* and *TP53*. Dysfunction in these genes may lead to deregulation of cell death and, thus, tumor development. Therefore, in this study, we investigated whether nine INDEL variants in the mentioned genes might influence gastric carcinogenesis, by comparing their distribution in GC patients and cancer-free individuals.

Regarding the observed deviation from HWE in the distribution of rs4197 and rs4645982 in case group, no previous studies were found with these markers, but their genotype distribution varies greatly between different populations in 1000 Genomes Project database [21]. Curiously, their distribution also presented a deviation from HWE in most populations in that database, suggesting that there could be a selective advantage leading to this pattern and/or that the deviation observed here could be due to population substructure, especially considering the relatively recent admixture process in Brazil, as previously observed for other markers in this population [7, 22]. As such, it is an expected process in admixed populations, and it could even highlight the potential of these markers. In fact, it has been suggested that HWE deviation only in patient's group could support possible locus-disease associations [23].

Then, we carried on to the analyses performed for each genotype (carriers of a genotype vs. non-carriers of such genotype as a reference group) in cases and controls. In these analyses, our results suggest that individuals carrying INS/DEL of rs4197 have about 2-fold chances of developing GC than non-carriers ($P = 0.046$; OR = 1.940; 95%CI = 1.011–3.725).

To the best of our knowledge, this is the first study investigating variant rs4197. It is a 3-bp INDEL in the 3' UTR of *FADD* gene, a key adaptor molecule that transmits death signals from death receptors during extrinsic apoptosis, thus being crucial to a variety of processes [24]. In fact, it seems that it could affect protein features due to the importance of 3'-UTR [25]. Depletion of *FADD* protein action may lead to failure of apoptosis and, thus, to tumor development. Absence of *FADD*

Table 3 Genotypic distribution of the investigated variants for patients with GC in comparison to control group. *P*-value, OR and 95%CI were obtained with logistic regression adjusted for age, which was done for the genotype as reference in each line (DEL/DEL vs. the other genotypes of that marker, and the same for INS/DEL and INS/INS)

Gene	Genotype	Case (%)*	Control (%)*	<i>P</i> -value	OR (95%CI)
<i>BCL2</i>	rs11269260	93	98		
	DEL/DEL	13 (14)	19 (19.4)	0.844	1.090 (0.463–2.567)
	INS/DEL	51 (54.8)	42 (42.9)	0.149	1.589 (0.848–2.977)
	INS/INS	29 (31.2)	37 (37.8)	0.092	0.556 (0.281–1.101)
<i>CASP3</i>	rs4647655	93	96		
	DEL/DEL	47 (50.5)	48 (50.0)	0.898	0.959 (0.511–1.802)
	INS/DEL	39 (41.9)	39 (40.6)	0.995	1.002 (0.528–1.904)
<i>CASP8</i>	rs3834129	93	98		
	DEL/DEL	27 (29.0)	19 (19.4)	0.098	1.851 (0.893–3.835)
	INS/DEL	38 (40.9)	42 (42.9)	0.987	0.995 (0.526–1.880)
<i>CASP9</i>	rs59308963	89	93		
	DEL/DEL	30 (33.7)	29 (31.2)	0.749	1.116 (0.568–2.195)
	INS/DEL	41 (46.1)	44 (47.3)	0.727	0.893 (0.471–1.690)
	INS/INS	18 (20.2)	20 (21.5)	0.953	1.024 (0.467–2.244)
<i>FADD</i>	rs4645982	81	58		
	DEL/DEL	16 (19.8)	13 (22.4)	0.897	0.943 (0.392–2.272)
	INS/DEL	24 (29.6)	20 (34.5)	0.583	0.808 (0.377–1.732)
	INS/INS	41 (50.6)	25 (43.1)	0.535	1.254 (0.613–2.567)
	rs61079693	89	94		
	DEL/DEL	22 (24.7)	26 (27.7)	0.418	0.742 (0.360–1.529)
<i>FAS</i>	rs4197	93	97		
	DEL/DEL	31 (33.3)	41 (42.3)	0.099	0.573 (0.295–1.111)
	INS/DEL	57 (61.3)	48 (49.5)	0.046	1.940 (1.011–3.725)
<i>TP53</i>	rs10562972	89	93		
	DEL/DEL	1 (1.1)	0 (0.0)	0.988	991,452.554 (0.000–∞)
	INS/DEL	10 (11.2)	21 (22.6)	0.260	0.603 (0.250–1.454)
<i>FAS</i>	rs17880560	87	93		
	DEL/DEL	57 (65.5)	58 (62.4)	0.738	1.119 (0.579–2.166)
	INS/DEL	26 (29.9)	31 (33.3)	0.714	0.880 (0.445–1.741)
<i>FAS</i>	INS/INS	4 (4.6)	4 (4.3)	0.964	1.035 (0.232–4.623)

*Sample number; *P*-value: *P*-value adjusted for age, OR Odds Ratio, CI Confidence Interval

expression has been suggested as marker of tumor development in mice and cancer prognosis in humans, due to the involvement of this protein with cell apoptosis, survival and proliferation [26]. Therefore, investigation of *FADD* gene should help the understanding of cancer development, including gastric carcinogenesis.

As for the variants that did not present a statistical association here, this may suggest that they either do not play a role in gastric carcinogenesis or it is a population-specific trait. Indeed, some of these variants have been associated cancer development in a few studies, but not in others. For instance, one of these variants (rs3834129

in *CASP8*) has been previously studied by our research group in other sampling of the same region: we have associated *INS/INS* genotype of this variant to reduced chances of developing B-cell acute lymphoblastic leukemia [27], but have not found any association of this variant with GC or colorectal cancer [22], so that our study corroborates the findings in the latter. Thus, the association of such variants to GC and other types of cancer is still a matter of great discussion.

Conclusions

Despite a few limitations (sample number and average age of both groups), this study contributed to an increased knowledge on variants in apoptosis-related genes in regard to GC development. As future perspectives, we recommend studies in the same population with a greater sample number and in different populations for comparison, as well as functional studies focused on the proteins to possibly reinforce the involvement of *FADD* gene in gastric carcinogenesis. Although such future studies are recommended to strengthen our results, this work contributes to a better understanding of these genes and INDEL variants in regard to gastric cancer.

Abbreviations

AIM: Ancestry Informative Marker; BCL2: BCL2 apoptosis regulator; CASP3: Caspase 3; CASP8: Caspase 8; CASP9: Caspase 9; CI: Confidence Interval; DEL: Deletion; DNA: Deoxyribonucleic Acid; FADD: Fas associated via death domain; FAS: Fas cell surface death receptor; GC: Gastric Cancer; HWE: Hardy-Weinberg Equilibrium; ID: Identification; INDEL: Insertion/Deletion; INS: Insertion; MAF: Minor Allele Frequency; OR: Odds Ratio; PCD: Programmed Cell Death; PCR: Polymerase chain reaction; TP53: Tumor protein p53; UTR: Untranslated Region

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Authors' contributions

GCC designed the study, performed part of the experiments, performed data analysis and wrote the manuscript; MRM, CMDV, CSS and AACM performed part of the experiments; PBA and PPA participated in the acquisition of data and gave contributions to the manuscript; SS gave major contributions to the study design and to the manuscript; ARS designed and coordinated the study and gave major contributions to the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Figshare repository (<https://doi.org/10.6084/m9.figshare.13040078>).

Ethics approval and consent to participate

All participants signed an informed consent, with approval by the Committee for Research Ethics of Hospital João de Barros Barreto under Protocol Number: 64399617.7.0000.5634.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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