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ORIGINAL ARTICLE

The Role of Caspase Genes Polymorphisms in Genetic Susceptibility to Philadelphia-Negative Myeloproliferative Neoplasms in a Portuguese Population

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Abstract

Our main aim was to evaluate the role of caspases' genes SNPs in Philadelphia-chromosome negative chronic myeloproliferative neoplasms (PN-MPNs) susceptibility. A case-control study in 133 Caucasian Portuguese PN-MPNs patients and 281 matched controls was carried out, studying SNPs in apoptosis related caspases: rs1045485 and rs1035142 (*CASP8*), rs1052576, rs2308950, rs1132312 and rs1052571 (*CASP9*), rs2227309 and rs2227310 (*CASP7*) and rs13006529 (*CASP10*). After stratification by pathology diagnosis for essential thrombocythemia (ET), female gender or *JAK2* positive, there is a significant increased risk for those carrying at least one variant allele for *CASP9* (C653T) polymorphism (OR 2.300 CI 95% [1.180–4.484], $P = 0.014$). However, when considered individually, none of the studied caspases polymorphisms was associated with PN-MPNs risk. Our results do not reveal a significant involvement of caspase genes polymorphisms on the individual susceptibility towards PN-MPNs as a whole. However, for essential thrombocythemia (ET), female gender or *JAK2* positive, there is a significant increased risk to those carrying at least one variant allele for *CASP9*. Although larger studies are required to confirm these results and to provide conclusive evidence of association between these and other caspases variants and PN-MPNs susceptibility, these new data may contribute to a best knowledge of the pathophysiology of these disorders and, in the future, to a more rational and efficient choice of therapeutic strategies to be adopted in PN-MPNs treatment.

Keywords Philadelphia-negative myeloproliferative neoplasms · Genetic susceptibility · Caspase genes polymorphisms · Janus kinase 2

Introduction

According to the World Health Organization (WHO-IARC) classification, myeloproliferative neoplasms (MPNs) encompass various conditions including chronic myelogenous leukemia (CML) and the most common Philadelphia-negative myeloproliferative neoplasms (PN-MPNs), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [1, 2].

Genetic insights into the pathogenesis of the PN-MPNs include the discovery of the somatic point gain-of-function mutations in the Janus kinase 2 gene (*JAK2*; exon 14 *V617F* and exon 12 mutations) [3–7], the myeloproliferative leukemia virus oncogene (*MPL*; more frequently *W515*), and recently calreticulin (*CALR*) mutations, which modified the understanding of these diseases, their diagnosis and management [3–8]. Frequencies of these mutations are approximately 95%, 0%, and 0% in PV, 60%, 3%, and 20% in ET, and 60%, 7%, and 25% in PMF, respectively [9, 10]. However, the cellular and molecular mechanisms involved in the pathophysiology of MPNs have not yet been fully clarified. These mutations cannot fully explain the phenotypic heterogeneity of PN-MPNs and further genetic alterations still await identification in around 20% of ET and PMF cases. One possible reason is

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that the outcome of a mutation can depend upon other genetic variants in the genome [11, 12]. Indeed, the possible role of silencing of *SOCS* (suppressors of cytokine signalling) by mutations or epigenomic silencing may result in the loss of negative regulators of *JAK*/STAT pathways. *SOCS* proteins bind to phosphotyrosine residues of *JAK* and may act as tumor suppressor genes, unless mutated or epigenetically silenced which may occur in PN-MPNs even if no mutation is present in the *JAK2* gene [13].

Besides modifier genes such as the *SOCS* genes whose proteins inhibit STAT phosphorylation by binding and inhibiting JAKs, polymorphic variants of several other genes together with environmental exposure/dietary exposure and immune system characteristics, may predispose to the susceptibility to these disorders [14–18]. Thus, the assessment of Single Nucleotide Polymorphisms (SNP's) at various loci may be important for individual susceptibility risk to PN-MPNs, although less specific, but prognostically relevant [9, 10, 15, 19, 20].

Apoptosis is a programmed cell death process, acting as a defense mechanism against damaged or stressed cells, to prevent accumulation of non-functional or damaged cells in the tissues. Identification of apoptotic mechanisms is critical and disturbed apoptosis pathways may lead to an accumulation of mutations that may eventually lead to cancer [21, 22].

The hematopoietic system is particularly sensitive to deregulation of the apoptotic process as these cells undergo a high turnover rate, requiring a tight balance between apoptosis and proliferation. Accordingly, apoptosis is frequently deregulated in hematologic malignancies [23].

Activation of apoptosis occurs through two major routes: the intrinsic and the extrinsic pathways. The intrinsic or mitochondrial pathway may be initiated by various apoptogenic stimuli, such as agents that cause DNA damage, rupture of microtubules, and deficiency or absence of cell growth factors. The extrinsic route is activated by death receptors of the tumor necrosis factor (TNF) family [8, 22, 24]. Caspases play a key regulatory role in both intrinsic and extrinsic pathways. Three groups of mammalian caspases exists on the basis of specific functions in different pathways, including developmental, inflammatory, and apoptotic pathways [21, 24]. The executioner caspases act in various substrates in the cytoplasm and nucleus, resulting in cellular death.

Deregulation of pro- and anti-apoptotic genes express as cell resistance to apoptosis, culminating with the accumulation of myeloid cells and the establishing of neoplasms [8, 25–29].

A wider characterization of molecular genetic features in PN-MPNs may contribute to a better understanding of the pathogenesis of these diseases and provide new specific diagnostic, prognostic, and therapeutic tools [15, 30]. However, to date no studies have associated polymorphisms in caspases genes and risk for PN-MPNs.

Thus, the present work describes a hospital based case-control study in a Caucasian Portuguese population in order to evaluate the potential modifying role of nine apoptosis related caspases genes polymorphisms on the individual susceptibility to PN-MPNs.

Materials and Methods

Study Subjects

This case-control study involved 133 Caucasian Portuguese PN-MPNs patients (80 with ET, 39 with PV and 14 with PMF) and 281 age and sex matched controls. The patients were selected within the Portuguese population recruited in the Departments of Clinical Hematology and of Clinical Pathology, Hospital de São Francisco Xavier, Centro Hospitalar de Lisboa Ocidental (CHLO), a public general hospital that provides health care to the western population of Lisbon, where those patients were followed and treated. Diagnosis criteria for all patients were those updated by the World Health Organization. [2, 31] For all cases, at least two control individuals ($n = 281$), without neoplastic pathology, matched for age (±2 years), gender and ethnicity were recruited, with no personal or family history of PN-MPNs, no previous or current malignant disease, nor history of blood transfusions. All study subjects were Portuguese, with Portuguese ascendants. Information on demographic characteristics, family history of cancer, lifestyle habits (e.g. smoking and alcohol drinking) and exposure to ionizing radiation was collected using a questionnaire administered by trained interviewers. With respect to smoking habits, former smokers were considered as non-smokers if they gave up smoking either 2 years before PN-MPN diagnosis or 2 years before the inclusion date as control. The response rate was higher than 95% for cases and controls. A written informed consent was obtained from all those involved, prior to blood withdrawal, in agreement with the Declaration of Helsinki. The blood samples were coded to guarantee anonymity. This study was also conducted with approval by the institutional ethics' boards of the involved institutions.

General characteristics for PN-MPNs patients and control populations are summarized in Table 1.

DNA Extraction

Peripheral blood samples of all patients and controls were collected by qualified personnel into 10 ml EDTA tubes and kept thereafter at −80 °C. Genomic DNA was obtained from 250 μl of each blood sample using a commercially available kit (QIAamp® DNA mini kit; Qiagen) according to the manufacturer's instructions. All DNA samples were stored at −20 °C until analysis.

Table 1 General characteristics for the PN-MPNs cases ($n = 133$) and control population ($n = 281$) and gender distribution for the PN-MPNs cases ($n = 133$)

Characteristics	Cases, *n* (%)			Controls, *n* (%)	*P* value
Gender					
Male	61 (45.9)			133 (47.3)	0.8
Female	72 (54.1)			148 (52.7)	
Age [a,b]					
30–49	16 (12.0)			43 (15.3)	0.6
50–69	50 (37.6)			107 (38.1)	
≥ 70	67 (50.4)			131 (46.6)	
Smoking habits					
Never	104 (78.2)			213 (76.1)	0.6
Current	29 (21.8)			67 (23.9)	
Alcohol habits					
Never	103 (77.4)			191 (68.2)	**<0.0001**
Social	20 (15.0)			25 (8.9)	
Regular	10 (7.5)			64 (22.9)	
Diagnosis		*Male*	*Female*		–
ET	80	32 (40.0)	48 (60.0)		
PV	39	20 (51.3)	19 (48.7)		
PMF	14	9 (64.3)	5 (35.7		
Jak2 V617F mutation					
Yes	99 (75.0)				**0.020**
ET	58 (73.4)				
PV	34 (87.2)				
PMF	7 (50.0)				
No	33 (25.0)				

Significant *P* value: <0.05 (bold entrie)

[a] Age of diagnosis for cases

[b] Age of control population at the time of diagnosis for the matched case

SNP Selection

Publicly available on-line databases such as NCBI (http://www.ncbi.nlm.nih.gov/projects/snp/), GeneCards (http://www.genecards.org) and SNP500Cancer (http://variantgps.nci.nih.gov/cgfseq/pages/snp500.do) were used to search for SNP's reported to date on genes coding for mediators of apoptosis, to be included in this work. The eligible SNP's in the present study had to be located in a coding region giving rise to an amino acid change (non-synonymous SNP's) and exhibit a minor allele frequency (MAF) >0.1 in Caucasian populations (Table 2).

Genotyping

The polymorphisms rs2227309 and rs2227310 (*CASP7*), rs1045485 and rs1035142 (*CASP8*), rs2308950, rs1132312 and rs1052571 (*CASP9*) and rs13006529 (*CASP10*) were genotyped using real-time PCR (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology) according to manufacturer instructions and to previous reports from our group with minor modifications. Real-Time PCR genotype determinations were carried in 20% of samples in independent experiments and all the inconclusive samples were reanalyzed.

The SNP genotyping assay information for caspases genes polymorphisms is summarized in Table 2.

JAK2 V617F mutational status was determined using real-time PCR (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology), according to manufacturer instructions.

Statistical Analysis

The analysis of Hardy-Weinberg frequencies for all alleles in the control and patients' populations was carried out using exact probability tests available in SNPStat software. Differences in genotype frequency, smoking/alcohol consumption status, age class and gender distributions between PN-MPNs cancer patients and controls were evaluated by the Chi-Square (χ^2) test. The crude and adjusted odds ratio (OR) and the corresponding 95% confidence intervals (CI) were calculated using unconditional multiple logistic regression. The model for adjusted OR included terms for gender, age at diagnosis (30–49, 50–69 and ≥ 70 years), smoking habits (smokers/non-smokers), and alcohol habits (never, social and regular consumption) with male sex, lower age group and non-smokers/non-alcohol consumers being considered as the reference groups for each of these variables. For the purpose of these calculations, age at diagnosis for controls was the age at the time of diagnosis for the matched case. All analyses were performed using the Statistical Package for the Social Sciences for Windows 22.0 version (SPSS, Inc.) (Tables 3, 4 and 5). Since this is not a conclusive final study but an exploratory one on the role of apoptosis related caspases polymorphisms in PN-MPNs and the data to be obtained should be looked at as proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative.

Results

This study included 133 PN-MPNs patients and 281 age- and sex-matched controls. The baseline characteristics (sex, age alcohol consumption and smoking habits) of both case and control populations are listed in Table 1. The case group included 72 (54.1%) females and 61 (45.9%) male patients, with a mean age of 68 years, in agreement with the gender distribution usually observed in this type of pathology. No significant differences were found between the case and control groups concerning age distribution or smoking habits (see Table 1). However, alcohol consumption is significantly increased in patients when compared with the control group ($P < 0.0001$) (see Table 1).

Table 2 Selected SNP's and detailed information on the corresponding base and amino acid exchanges as well as minor allele frequency

Gene	dsSNP	Codon	Nucleotide exchange	Minor allele frequency, MAF (%)[a]
CASP7	rs2227309	249	G→A (Arg/ Lys)	28.0
	rs2227310	255	C→G (Asp/Glu)	29.0
CASP8	rs1045485	270	G→C (Asp/His)	13.0
	rs1035142	–	G→T 3'UTR	49.0
CASP9	rs2308950	173	G→A (Arg/His)	4.0
	rs1132312	136	C→T (Phe/Phe)	49.6
	rs1052571	28	C→T (Ala/Val)	49.6
CASP10	rs13006529	522	A → T (Ile/Leu)	44.0

[a] According to http://www.ncbi.nlm.nih.gov/snp/

According to diagnostic criteria patients' distribution was as follows: 80 (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF (Table 1).

The characteristics of each SNP under study are described in Table 2, while the genotype frequencies determined for all of them are shown in Table 4. All of the SNP's studied were in agreement with expectation of the Hardy-Weinberg law ($P > 0.05$, exact probability test), except for *CASP8*_rs1045485 and for *CASP9*_rs2308950 ($P = 0.006$ and $P = 0.034$ respectively, exact probability test).

The results obtained revealed that after stratification by pathology diagnosis (Table 4) a significant increased risk was observed for patients diagnosed with ET presenting at least one variant allele (T) of *CASP9*_rs1132312 polymorphism: for heterozygous individuals (OR 2.300 CI 95% [1.180–4.484], $P = 0.014$) as well as for the combination of heterozygous with homozygous variant allele (OR 2.203 CI 95% [1.163–4.176], $P = 0.015$**)**. The same effect was found, after stratification by gender, in women (OR 4.370 CI95% [1.608–11.873], $P = 0.004$) considering the presence of at least one variant allele. According to our results, the increased risk was verified in all sub-groups, as can be seen in Table 4, although when considered individually, none of the polymorphisms studied were associated with PN-MPNs risk (Table 3). No significant difference was found between the case and control groups regarding age distribution, gender, smoking habits or genotype frequencies (Table 3). As the relevance of *JAK2* mutation in PN-MPNs is well known, the population was also stratified according to the presence of *JAK2* mutation in patients, showing that there is also a significant increased risk for patients diagnosed with ET when at least one variant allele (T) for *CASP9*_rs1132312 polymorphism is present (OR 2.886 CI 95% [1.303–6.393], $P = 0.009$) (Table 4).

A key point that should be explored in studies such as this, is the effect of the combination of all genotypes since the real situation is the joint effect of the variants. That was achieved using the SNPStat software and the results yielded fifty one different combinations (data not shown). It should be noted that of all genetic variants under study, only the SNPs of *CASP8* and *CASP7* genes were in linkage disequilibrium (LD). According to the results obtained (Table 5), we could only establish a positive haplotype for *CASP9* gene correlated with a decreased risk for PN-MPN diseases in individuals.

With regard to the number of SNPs of the different genes under study, and grouping the initiator caspase genes as a whole, we stablished a new haplogroup (Table 6). The result obtained substantiate the decreased risk for PN-MPN in our population, as observed for the *CASP9* haplotype. To our knowledge, this is the first study where this association is described.

Discussion

Polymorphisms in apoptosis related genes may contribute to individual susceptibility to MPNs and, hence, modify disease risk. However, to the best of our knowledge, no clinical association studies have been performed thus far to evaluate the role of caspases genes polymorphisms on PN-MPNs susceptibility.

The present study revealed an increased incidence of the *JAK2* V617F mutation in ET patients and a decreased incidence in PV patients, compared with the literature [9, 10], probably due to the small population studied. Moreover, the cases included were incident cases diagnosed in a hospital hematological consultation.

This study was intended to ascertain the possible role of genetic polymorphisms *CASP8* (Asp302His and 3'UTR_G/T), *CASP9* (Arg221Gln, Arg173His, Phe136Phe and Ala28Val), *CASP7* (Lys249Arg and Asp255Glu) and *CASP10* (Ile522Leu), on the individual susceptibility for PN-MPNs. Caspases are main components of the apoptotic pathway. This specific group of cysteine aspartate proteases are a family of intracellular proteins responsible for the dismantling and destruction of the cell components [24, 32]. These proteins are produced as proenzymes (zymogens) and can be activated by proteolytic cleavage in response to various apoptotic stimuli. Each caspase is cleaved to produce a large and a small subunits, forming an active tetrameric form from two molecules of pro-enzyme [33].

Table 3 Genotype distribution and myeloproliferative risk for the *CASP7*Lys249Arg, *CASP7*Asp255Glu, *CASP8*Asp302His, *CASP8*Tyr12STOP, *CASP9*Arg173His, *CASP9*Phe136Phe, *CASP9*Val28Ala, and *CASP10*Ile522Leu polymorphisms in the MPNs case ($n = 133$) and control ($n = 281$) populations

Genetic polymorphism	Controls, *n* (%)	Cases, *n* (%)	*P* value[a]	OR crude (95% CI)	OR adjusted (95% CI)[b]
CASP7 (Lys249Arg; rs2227309)					
G/G	154 (55.2)	77 (57.9)	0.811	1 (Reference)	1 (Reference)
G/A	109 (39.1)	50 (37.6)		0.917 (0.595–1.414)	0.899 (0.578–1.398)
A/A	16 (5.7)	6 (4.5)		0.750 (0.282–1.993)	0.855 (0.309–2.364)
G/A + A/A	125 (44.8)	56 (42.1)		0.896 (0.590–1.360)	0.894 (0.583–1.370)
CASP7 (Asp255Glu; rs2227310)					
C/C	154 (55.8)	73 (55.3)	0.968	1 (Reference)	1 (Reference)
C/G	106 (38.4)	52 (39.4)		1.035 (0.671–1.596)	1.032 (0.662–1.608)
G/G	16 (5.8)	7 (5.3)		0.923 (0.364–2.341)	1.014 (0.386–2.669)
C/G + G/G	122 (44.2)	59 (44,7)		1.020 (0.672–1.549)	1.030 (0.671–1.580)
CASP8 (Asp270His; rs1045485)					
G/G	220 (78.9)	101 (76.5)	0.819	1 (Reference)	1 (Reference)
G/C	51 (18.3)	26 (19.7)		1.110 (0.655–1.882)	1.092 (0.634–1.881)
C/C	8 (2.9)	5 (3.8)		1.361 (0.435–4.265)	1.194 (0.372–3.829)
G/C + C/C	59 (21.1)	31 (23.5)		1.144 (0.698–1.877)	1.083 (0.643–1.823)
CASP8 (3'UTR; rs1035142)					
G/G	97 (34.8)	50 (37.6)	0.816	1 (Reference)	1 (Reference)
G/T	137 (49.1)	61 (45.9)		0.864 (0.548–1.362)	0.861 (0.540–1.375)
T/T	45 (16.1)	22 (16.5)		0.948 (0.514–1.752)	1.072 (0.568–2.025
G/T + T/T	182 (65.2)	83 (62.4)		0.885 (0.576–1.358)	0.912 (0.581–1.432)
CASP9 (Arg173His; rs2308950)					
G/G	276 (98.9)	129 (97.0)	0.224	1 (Reference)	1 (Reference)
G/A	3 (1.1)	3 (2.3)		2.140 (0.426–10.746)	1.704 (0.335–8.661)
A/A	0 (0.0)	1 (0.8)		ND	ND
G/A + A/A	3 (1.1)	4 (3.0)		2.853 (0.629–12.932)	2.170 (0.472–9.983)
CASP9 (Phe136Phe; rs1132312)					
C/C	87 (31.2)	31 (23.3)	0.146	1 (Reference)	1 (Reference)
C/T	128 (45.9)	74 (55.6)		1.622 (0.984–2.675)	1.669 (1.000–2.783)
T/T	64 (22.9)	28 (21.1)		1.228 (0.671–2.247)	1.299 (0.699–2.413)
C//T + T/T	192 (68.8)	102 (76.7)		1.491 (0.927–2.398)	1.548 (0.952–2.517)
CASP9 (Ala28Val; rs1052571)					
C/C	70 (25.1)	25 (18.8)	0.176	1 (Reference)	1 (Reference)
C/T	129 (46.2)	74 (55.6)		1.606 (0.937–2.753)	1.580 (0.911–2.743)
T/T	80 (28.7)	34 (25.6)		1.190 (0.648–2.186)	1.150 (0.618–2.142)
C/T + T/T	209 (74.9)	108 (81.2)		1.447 (0.861–2.415)	1.415 (0.838–2.390)
CASP10 (Ile522Leu; rs13006529)					
A/A	82 (29.4)	36 (29.8)	0.647	1 (Reference)	1 (Reference)
A/T	123 (44.1)	58 (47.9)		1.074 (0.651–1.773)	1.113 (0.675–1.834)
T/T	74 (26.5)	27 (22.3)		0.831 (0.461–1.499)	0.893 (0.496–1.608)
A/T + T/T	197 (70.6)	85 (70.2)		0.983 (0.616–1.568)	0.973 (0.602–1.572)

ND – Non Determined

[a] P-value determined by $\chi 2$ test

[b] ORs were adjusted for age (30–49, 50–69, >70 years), smoking status (never and former, and current) and alcohol consumption (never, social and regular drinkers)

There are 14 different caspases that can be classified as initiator, effector and cytokine activators [22]. The initiator caspases (caspase-2, 8, 9, 10) activate the effector caspases (caspase 3, 6, 7 and 14), which are capable of degrading direct multiple substrates leading to deregulation of vital cellular processes and cellular death [22, 32, 34–36], and also the cytokine activator caspases (caspase 1, 4, 5, 11, 12 and 13).

While initiator caspases are self-activated, effector caspases activation is dependent of initiator caspases via internal cleavages. Furthermore, most of caspase family members are functional in cellular proliferation, survival, and inflammation, whereas some of them are essential for apoptosis [22].

All stimuli that lead to apoptosis appear to initiate a sequence of events that culminate in the activation of caspases,

Table 4 ORs (95% CI) for *CASP9* (Phe136Phe) polymorphisms and PN-MPNs association

Pathology stratification	N	*CASP9* (Phe136Phe; rs1132312)	*P* value[a]	OR crude (95% CI)	OR Adjusted (95% CI)[b]
ET	80	C/C	**0.050**	1 (Reference)	1 (Reference)
		C/T		**2.233 (1.157–4.309)**⅄	**2.300 (1.180–4.484)**⅄⅄
		T/T		1.942 (0.912–4.133)	2.009 (0.932–4.330)
		C//T + T/T		**2.136 (1.138–4.010)**◆	**2.203 (1.163–4.176)**◆◆
ET, females	48	C/C	**0.009**	1 (Reference)	1 (Reference)
		C/T		**4.403 (1.599–12.126)**†	**4.663 (1.667–13.045)**††
		T/T		**3.920 (1.256–12.231)**×	**3.777 (1.198–11.911)**××
		C//T + T/T		**4.257 (1.586–11.425)***	**4.370 (1.608–11.873)****
ET, *JAK2* positive	58	C/C	**0.021**	1 (Reference)	1 (Reference)
		C/T		**3.059 (1.356–6.896)**‡	**3.104 (1.366–7.052)**‡‡
		T/T		2.379 (0.942–6.009)	2.447 (0.960–6.239)
		C//T + T/T		**2.832 (1.288–6.229)**+	**2.886 (1.303–6.393)**++

Significant *P* value: <0.05 (bold entrie)

⅄PCrude = 0.017; ⅄⅄PAdjusted = 0.014 (*P*-values are adjusted by unconditional multiplicative logistic regression).

◆PCrude = 0.018; ◆◆PAdjusted = 0.015 (P-values are adjusted by unconditional multiplicative logistic regression).

†PCrude = 0.004; ††PAdjusted = 0.003 (P-values are adjusted by unconditional multiplicative logistic regression).

×PCrude = 0.019; × × PAdjusted = 0.023 (P-values are adjusted by unconditional multiplicative logistic regression).

*PCrude = 0.004; **PAdjusted = 0.004 (P-values are adjusted by unconditional multiplicative logistic regression).

‡PCrude = 0.007; ‡‡PAdjusted = 0.007 (P-values are adjusted by unconditional multiplicative logistic regression)

+PCrude = 0.010; ++PAdjusted = 0.009 (*P*-values are adjusted by unconditional multiplicative logistic regression)

[a] P-value determined by $\chi 2$ test

[b] ORs were adjusted for age (30–49, 50–69, >70 years), smoking status (never and former) and alcohol consumption (never, social and regular drinkers)

Table 5 Haplotype Association response for SNPs of *CASP9* gene

Haplotype association response				
CASP9			OR (95% CI)	*P* Value
rs2308950	rs1132312	rs1052571		
G	C	T	1.00 (Reference)	
G	T	C	1.15 (0.79–1.68)	0.46
G	**C**	**C**	**0.24 (0.11–0.52)**	**$3e^{-4}$**
G	**T**	**T**	**0.41 (0.25–0.70)**	**0.001**

Significant *P* value: <0.05 (bold entrie)

but they do it in different ways. Three pathways are associated with activation of caspases: (1) the intrinsic or mitochondrial pathway, which is initiated by cellular stress signals such as DNA damage (induced by genotoxic agents or defects in DNA repair), endoplasmic reticulum (ER) stress (induced by the accumulation of unfolded proteins), rupture of microtubules; this pathway converges on mitochondria, resulting in permeabilization of the outer membrane and subsequent cytochrome c release [37, 38]; (2) the extrinsic pathway, which is initiated by the activation of death membrane receptors of the tumor necrosis factor (TNF) family, induced by ligands; and (3) the pathway involving granzyme B [8, 22, 24, 39–41].

The ratio of the pro-and anti-apoptotic proteins plays an important role in the regulation of cell death, and disruption in the balance between these proteins has been established to contribute to carcinogenesis by reducing apoptosis in malignant cells [21, 29, 42]. In particular in MPNs, the deregulation of apoptosis is involved in the pathophysiology of these diseases [8, 43, 44].

The present study involved four different genes and nine polymorphisms from the caspase cascade, and our results didn't confirm the correlation between different SNPs in PN-MPN diseases as a whole. However, the results reveal that for ET patients alone, or after stratification by female gender or when applied to *JAK2* positive cases, there is a significant increased risk when cases of these sub-groups carried at least one variant allele for *CASP9*_rs1132312 (C653T) polymorphism. Previous studies have described the involvement of *CASP9* gene polymorphisms in the pathogenesis of various types of cancer, such as non-Hodgkin's lymphoma [45, 46], lung cancer [47, 48], breast cancer [49], and gastric cancer [50].

By definition, MPNs are a group of clonal disorders derived from the proliferation of one or more myeloid lineages, in which megakaryocytes are the 'key-cells' for diagnostic histological features [44, 51]. Previous studies have correlated the uncontrolled proliferation of megakaryocytes to dysregulation of pro-apoptotic and anti-apoptotic mechanisms [44, 51].

Caspase-9 is important in regulating megakaryocyte turnover in MPNs. Malherbe and colleagues [44] showed that disruptions targeting the intrinsic apoptotic cascade (caspase-9 action) apparently promote megakaryocyte accumulation and thrombocytosis in MPNs. Considering that our results revealed an increased risk for ET in patients who present at least one variant allele, we might anticipate that polymorphisms in *CASP9* gene might be responsible for a high proliferation leading to increased risk for ET.

With regard to the analyses of haplogroups' association response, our results didn't establish a global haplogroup. However, the correlation for SNPs of *CASP9* gene showed a decreased risk for two haplotypes (GCC and GTT), as shown in Table 5. Interestingly, a similar effect was obtained when initiator caspases where grouped. However, the mechanism by which the studied haplogroups lead to a decreased risk for MPNs remains unknown. These results suggest that polymorphisms may exert independent or interactive effects on the development of MPNs.

With regard to smoking habits, although some published studies refer smoking as a contributing factor for PN-MPNs [52, 53], this study did not reveal an important association, probably due to the small number of smoking individuals included.

Additional studies involving larger populations should be pursued to further clarify the potential value of the different apoptosis related caspase genotypes as predictive biomarkers of susceptibility to PN-MPNs and also allow for the study of gene-environment and gene-gene interactions as well as stratified analysis according to histological subtype and disease stage.

Table 6 Haplogroup Association response for SNPs present in all initiator caspases studied

Haplogroup association response							
CASP8		*CASP9*			*CASP10*	*OR (95% CI)*	*P Value*
rs1045485	rs1035142	rs2308950	rs1132312	rs1052571	rs13006529		
G	G	G	C	T	A	1 (Reference)	
G	**G**	**G**	**T**	**T**	**A**	**0.18 (0.03–0.96)**	**0.046**
G	**T**	**G**	**C**	**C**	**T**	**0.08 (0.01–0.82)**	**0.034**

Significant *P* value: <0.05 (bold entrie)

A better understanding of the pathophysiological mechanisms will allow the development of more directly and specifically targeted drugs, with high efficacy, fewer adverse effects, contributing to compliance of the patients with treatments.

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Compliance with Ethical Standards

Conflict of Interest The authors claim no competing financial or intellectual conflicts of interest in the preparation and submission of this manuscript.

References

1. Swerdlow CESH, Harris NL et al (2008) WHO classification of Tumours of Haematopoieticand lymphoid tissues., 4 edition (October 27, 2008) ed. World Health Organization, Lyon
2. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW (2016) The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127:2391–2405
3. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, Garçon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 434:1144–1148
4. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352:1779–1790
5. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Fröhling S, Döhner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 7:387–397
6. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR, Project CG (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 365:1054–1061
7. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, Warren AJ, Gilliland DG, Lodish HF, Green AR (2007) JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 356:459–468
8. Tognon R, Nunes NeS, Castro FA (2013) Apoptosis deregulation in myeloproliferative neoplasms. Einstein (Sao Paulo) 11:540–544
9. Tefferi A, Pardanani A (2015) Myeloproliferative neoplasms: a contemporary review. JAMA Oncol 1:97–105
10. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, Avezov E, Li J, Kollmann K, Kent DG, Aziz A, Godfrey AL, Hinton J, Martincorena I, Van Loo P, Jones AV, Guglielmelli P, Tarpey P, Harding HP, Fitzpatrick JD, Goudie CT, Ortmann CA, Loughran SJ, Raine K, Jones DR, Butler AP, Teague JW, O'Meara S, McLaren S, Bianchi M, Silber Y, Dimitropoulou D, Bloxham D, Mudie L, Maddison M, Robinson B, Keohane C, Maclean C, Hill K, Orchard K, Tauro S, Du MQ, Greaves M, Bowen D, Huntly BJ, Harrison CN, Cross NC, Ron D, Vannucchi AM, Papaemmanuil E, Campbell PJ, Green AR (2013) Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 369:2391–2405
11. Rueff J, Rodrigues AS (2016) Cancer drug resistance: a brief overview from a genetic viewpoint. Methods Mol Biol 1395:1–18
12. Rice KL, Lin X, Wolniak K, Ebert BL, Berkofsky-Fessler W, Buzzai M, Sun Y, Xi C, Elkin P, Levine R, Golub T, Gilliland DG, Crispino JD, Licht JD, Zhang W (2011) Analysis of genomic aberrations and gene expression profiling identifies novel lesions and pathways in myeloproliferative neoplasms. Blood Cancer J 1:e40
13. Valentino L, Pierre J (2006) JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem Pharmacol 71:713–721
14. Bolufer P, Barragan E, Collado M, Cervera J, López JA, Sanz MA (2006) Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. Leuk Res 30:1471–1491
15. Delhommeau F, Jeziorowska D, Marzac C, Casadevall N (2010) Molecular aspects of myeloproliferative neoplasms. Int J Hematol 91:165–173
16. Beer PA, Delhommeau F, LeCouédic JP, Dawson MA, Chen E, Bareford D, Kusec R, McMullin MF, Harrison CN, Vannucchi AM, Vainchenker W, Green AR (2010) Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. Blood 115:2891–2900
17. Kilpivaara O, Levine RL (2008) JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. Leukemia 22:1813–1817
18. Björkholm M, Hultcrantz M, Derolf Å (2014) Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? Best Pract Res Clin Haematol 27:141–153
19. Levine RL (2009) Mechanisms of mutations in myeloproliferative neoplasms. Best Pract Res Clin Haematol 22:489–494
20. Campregher PV, Santos FP, Perini GF, Hamerschlak N (2012) Molecular biology of Philadelphia-negative myeloproliferative neoplasms. Rev Bras Hematol Hemoter 34:150–155
21. Goldar S, Khaniani MS, Derakhshan SM, Baradaran B (2015) Molecular mechanisms of apoptosis and roles in cancer development and treatment. Asian Pac J Cancer Prev 16:2129–2144
22. Kiraz Y, Adan A, Kartal Yandim M, Baran Y (2016) Major apoptotic mechanisms and genes involved in apoptosis. Tumour Biol 37:8471–8486
23. Zaman S, Wang R, Gandhi V (2014) Targeting the apoptosis pathway in hematologic malignancies. Leuk Lymphoma 55:1980–1992
24. Green DR, Llambi F (2015) Cell death signaling. Cold Spring Harb Perspect Biol 7
25. Nunes NS, Tognon R, Moura LG, Kashima S, Covas DT, Santana M, Souto EX, Zanichelli MA, Simões BP, Souza AM, Castro FA (2013) Differential expression of apoptomiRs in myeloproliferative neoplasms. Leuk Lymphoma 54:2047–2051
26. Tognon R, Gasparotto EP, Leroy JM, Oliveira GL, Neves RP, Carrara ReC, Kashima S, Covas DT, Santana M, Souto EX, Zanichelli MA, Velano CE, Simões BP, Alberto FL, Miyashiro K, de Souza AM, Amarante-Mendes GP, de Castro FA (2011) Differential expression of apoptosis-related genes from death

receptor pathway in chronic myeloproliferative diseases. J Clin Pathol 64:75–82
27. Tognon R, Gasparotto EP, Neves RP, Nunes NS, Ferreira AF, Palma PV, Kashima S, Covas DT, Santana M, Souto EX, Zanichelli MA, Simões BP, de Souza AM, Castro FA (2012) Deregulation of apoptosis-related genes is associated with PRV1 overexpression and JAK2 V617F allele burden in essential thrombocythemia and myelofibrosis. J Hematol Oncol 5:2
28. Tognon R, Nunes NS, Ambrosio L, Souto EX, Perobelli L, Simões BP, Souza MC, Chauffaille MeL, Attié de Castro F (2016) Apoptosis- and cell cycle-related genes methylation profile in myeloproliferative neoplasms. Leuk Lymphoma 57:1201–1204
29. Olsson M, Zhivotovsky B (2011) Caspases and cancer. Cell Death Differ 18:1441–1449
30. Mambet C, Matei L, Necula LG, Diaconu CC (2016) A link between the driver mutations and dysregulated apoptosis in BCR-ABL1 negative myeloproliferative neoplasms. J Immunoassay Immunochem 37:331–345
31. Tefferi A, Vardiman JW (2008) Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. Leukemia 22:14–22
32. Riedl SJ, Salvesen GS (2007) The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 8:405–413
33. Cho SG, Choi EJ (2002) Apoptotic signaling pathways: caspases and stress-activated protein kinases. J Biochem Mol Biol 35:24–27
34. Philchenkov A, Zavelevich M, Kroczak TJ, Los M (2004) Caspases and cancer: mechanisms of inactivation and new treatment modalities. Exp Oncol 26:82–97
35. Ng PW, Porter AG, Jänicke RU (1999) Molecular cloning and characterization of two novel pro-apoptotic isoforms of caspase-10. J Biol Chem 274:10301–10308
36. Oliver L, Vallette FM (2005) The role of caspases in cell death and differentiation. Drug Resist Updat 8:163–170
37. Petit E, Oliver L, Vallette FM (2009) The mitochondrial outer membrane protein import machinery: a new player in apoptosis? Front Biosci (Landmark Ed) 14:3563–3570
38. Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629
39. Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15:2922–2933
40. Peter ME, Krammer PH (2003) The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 10:26–35
41. Creagh EM, Conroy H, Martin SJ (2003) Caspase-activation pathways in apoptosis and immunity. Immunol Rev 193:10–21
42. Ding HF, Lin YL, McGill G, Juo P, Zhu H, Blenis J, Yuan J, Fisher DE (2000) Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. J Biol Chem 275:38905–38911
43. Testa U (2004) Apoptotic mechanisms in the control of erythropoiesis. Leukemia 18:1176–1199
44. Malherbe JA, Fuller KA, Mirzai B, Kavanagh S, So CC, Ip HW, Guo BB, Forsyth C, Howman R, Erber WN (2016) Dysregulation of the intrinsic apoptotic pathway mediates megakaryocytic hyperplasia in myeloproliferative neoplasms. J Clin Pathol 69(11):1017-1024
45. Lan Q, Morton LM, Armstrong B, Hartge P, Menashe I, Zheng T, Purdue MP, Cerhan JR, Zhang Y, Grulich A, Cozen W, Yeager M, Holford TR, Vajdic CM, Davis S, Leaderer B, Kricker A, Schenk M, Zahm SH, Chatterjee N, Chanock SJ, Rothman N, Wang SS (2009) Genetic variation in caspase genes and risk of non-Hodgkin lymphoma: a pooled analysis of 3 population-based case-control studies. Blood 114:264–267
46. Kelly JL, Novak AJ, Fredericksen ZS, Liebow M, Ansell SM, Dogan A, Wang AH, Witzig TE, Call TG, Kay NE, Habermann TM, Slager SL, Cerhan JR (2010) Germline variation in apoptosis pathway genes and risk of non-Hodgkin's lymphoma. Cancer Epidemiol Biomark Prev 19:2847–2858
47. Park JY, Park JM, Jang JS, Choi JE, Kim KM, Cha SI, Kim CH, Kang YM, Lee WK, Kam S, Park RW, Kim IS, Lee JT, Jung TH (2006) Caspase 9 promoter polymorphisms and risk of primary lung cancer. Hum Mol Genet 15:1963–1971
48. Lin J, Lu C, Stewart DJ, Gu J, Huang M, Chang DW, Lippman SM, Wu X (2012) Systematic evaluation of apoptotic pathway gene polymorphisms and lung cancer risk. Carcinogenesis 33: 1699–1706
49. Theodoropoulos GE, Michalopoulos NV, Pantou MP, Kontogianni P, Gazouli M, Karantanos T, Lymperi M, Zografos GC (2012) Caspase 9 promoter polymorphisms confer increased susceptibility to breast cancer. Cancer Genet 205:508–512
50. Liamarkopoulos E, Gazouli M, Aravantinos G, Tzanakis N, Theodoropoulos G, Rizos S, Nikiteas N (2011) Caspase 8 and caspase 9 gene polymorphisms and susceptibility to gastric cancer. Gastric Cancer 14:317–321
51. Florena AM, Tripodo C, Di Bernardo A, Iannitto E, Guarnotta C, Porcasi R, Ingrao S, Abbadessa V, Franco V (2009) Different immunophenotypical apoptotic profiles characterise megakaryocytes of essential thrombocythaemia and primary myelofibrosis. J Clin Pathol 62:331–338
52. Lindholm Sørensen A, Hasselbalch HC (2015) Smoking and Philadelphia-negative chronic myeloproliferative neoplasms. Eur J Haematol 97(1):63-69
53. Hasselbalch HC (2015) Smoking as a contributing factor for development of polycythemia vera and related neoplasms. Leuk Res 39: 1137–1145