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ORIGINAL ARTICLE

Caspase 8 and caspase 9 gene polymorphisms and susceptibility to gastric cancer

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Abstract
Background Caspase-8 (CASP8) and caspase-9 (CASP9) play crucial roles in regulating apoptosis, and their functional polymorphisms may alter cancer risk. Our aim was to investigate the association of CASP8 and CASP9 gene polymorphisms with gastric cancer (GC) susceptibility.
Methods We undertook a case–control study of 88 GC cases and 480 controls to investigate the association between CASP8 −652 6N ins/del and CASP9 −1263 A>G polymorphisms and GC susceptibility by a polymerase chain reaction (PCR)-restriction fragment length polymorphism method.
Results CASP8 −652 6N ins/del polymorphism and CASP9 −1263 GG genotype were observed to be significantly associated with a reduced risk of GC. No significant association was observed between CASP8 −652 6N ins/del and CASP9 −1263 A>G polymorphisms and tumor characteristics. However, both CASP8 del/del and CASP9 −1263 GG genotypes were associated with increased overall survival in GC patients.
Conclusions The CASP8 −652 6N ins/del and the CASP9 −1263 A>G polymorphisms were observed to play a protective role in GC predisposition.

Keywords Caspase 8 · Caspase 9 · Gastric cancer · Gene polymorphisms

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Introduction

Gastric cancer (GC) is still a major health problem worldwide due to its frequency, poor prognosis, and limited treatment options [1]. Currently available therapeutic approaches for GC are not highly effective, and thus the prognosis is poor. The selective targeting of cancer cells by the induction of apoptosis is a major goal of current cancer research. Genetic polymorphisms for genes controlling the cell cycle or apoptosis have been found to increase the risk of a number of human malignancies [2, 3]. However, such studies on GC risk are limited.

Apoptosis, is a highly organized cell programmed death process. It is an evolutionarily conserved process and is essential for organ development, tissue remodeling, immune response, and tumor suppression. Aberrant apoptosis is believed to contribute to cancer initiation and progression, and treatment failure. In addition, as apoptosis usually does not elicit the host inflammatory or immune response, this type of cell death is the preferred way of cancer cell killing by various treatments. Accordingly, selectively inducing apoptosis in cancer cells has been increasingly recognized as a promising therapeutic approach for many cancers. The broad apoptotic pathways

that occur in humans–the extrinsic or receptor-mediated pathway and the intrinsic or mitochondrial pathway– both utilize the caspase enzyme cascade. The extrinsic pathway utilizes caspase-8 (CASP8) and caspase-10 (CASP10) as initiator caspases, while the intrinsic pathway employs caspase-9 (CASP9). Both pathways converge to use caspase-3 (CASP3), caspase-6 (CASP6), and caspase-7 (CASP7) as the effector caspases, which leads to typical apoptotic features such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing [4, 5].

CASP8 is a key regulator of apoptosis, an essential defense mechanism against hyperproliferation and tumorigenesis. Polymorphisms in the CASP8 gene have been reported to influence cancer risk. The variant D302H (rs1045485) has been associated with the risk of breast cancer in the European population [6]. Additionally, the −652 6N ins/del promoter variant (rs3834129) has been associated with the risk of developing multiple cancer types [7]. It has been postulated that this deletion polymorphism has direct functional effects on cancer risk, on the basis that the deletion destroys a stimulatory protein 1 binding site and decreases CASP8 transcription [7]. In contrast, Haiman et al. [8] reported that the −652 6 N ins/del promoter polymorphism in the CASP8 gene was not associated with cancer risk, and more recently Gangwar et al. [9] confirmed this finding in bladder cancer. According to our knowledge, limited data exist in the literature with regard to CASP8 polymorphisms and GC. Soung et al. [10] reported the occurrence of CASP8 mutations mainly in advanced GC. These authors also reported that CASP8 mutations decreased the cell death activity of CASP8 [10].

CASP9 is a member of the intrinsic pathway, which is activated as a result of mitochondrial damage and cytochrome c release. After cytochrome c is released into the cytoplasm, it complexes with apoptosis protease activating factor-1 (APAF-1) and forms an apoptosome with procaspase-9 and activates the CASP9 cascade. Single-nucleotide polymorphisms are the most common human genetic variation and may contribute to an individual's susceptibility to cancer. Many studies have demonstrated that some variants affect either the expression or the activities of various enzymes and are therefore associated with cancer risk. Several candidate polymorphisms in the CASP9 gene have been reported recently in public databases (http://www.ncbi.nlm.nih.gov/SNP) [11]. Although the functional effects of these polymorphisms have not been elucidated, it has been hypothesized that some of these variants, particularly their haplotypes, can influence CASP9 expression or activity, thereby modulating susceptibility to cancer. Limited data exist on the specific association between CASP9 polymorphisms and GC pathogenesis. Soung et al. [12] have suggested that the caspase-9 gene is rarely mutated in GC patients.

In the present study, in order to examine the possible association between CASP8 −652 6N ins/del and CASP9 −1263 A>G polymorphisms and GC risk we performed a hospital-based case–control study in a Greek population.

Patients, materials, and methods

Patient information

The subjects in this hospital-based case–control were 88 unrelated GC patients together with 480 ethnically and sex- and age-matched healthy individuals (controls). All patients and controls were born and have been living in Greece. All patients gave their informed consent and the hospital review board approved the study. The patients were followed up until August 2010 or death. The median time (±SD) of follow-up was 36.27 (±19.5) months. Seventeen patients were lost to follow-up. The characteristics of the GC patients at diagnosis are presented in Table 1.

Genotyping

DNA was isolated from peripheral blood using the Nucleospin blood kit (Macherey-Nagel, Düren, Germany). To confirm the integrity of DNAs, initially a 430 bp

Table 1 Histopathological characteristics of the gastric cancer (GC) patients at diagnosis

Characteristics	Gastric cancer patients, *n*
Age at diagnosis (years), ±SD	63.33 ± 12.13
Male/female	62/26
Lymph node metastasis	
Negative	26
Positive	62
Other metastasis	
Negative	66
Positive	22
TNM stage at diagnosis	
I	24
II	16
III	27
IV	21
Tumor size (cm)	
≤5	40
>5	48
Lauren classification	
Intestinal	39
Diffuse	49

sequence of the human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was amplified. Polymorphisms in CASP8 (−652 6N ins/del) and CASP9 (−1263 A>G) were analyzed by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP). Details of primers and PCR conditions were described earlier [7, 11]. The mutations were confirmed by sequencing analysis using a Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI 377 automated sequencer. As a negative control of the PCR amplifications, we used distilled water instead of genomic DNA and confirmed the fidelity of the reactions.

Statistical analysis

Genotype and allele frequencies were compared with the χ^2 test with Yates' correction using S-Plus (v. 6.2; Insightful, Seattle, WA, USA). Odds ratios (ORs) and 95% confidence intervals (CIs) were obtained with GraphPad (v. 3.00; GraphPad Software, San Diego, CA, USA). The *P* values are all two-sided. Hardy–Weinberg equilibrium was verified by calculation of the expected frequencies and numbers, and significance testing was based on the 1 *df* χ^2. Strong association (significance) was assumed at $P < 0.001$. A weak but still significant association, meriting attention, was considered for values ranging between 0.01 and 0.05.

Results

A total of 480 controls and 88 GC cases were recruited for this study. The histological characteristics of the patients are presented in Table 1. The genotypic distributions of the two gene polymorphisms were in Hardy–Weinberg equilibrium. The genotype and allele frequencies of the *CASP8* and *CASP9* gene polymorphisms in healthy controls and GC patients are shown in Table 2. The variant −652 6N del/del genotype was 22.08% prevalent in the controls compared with 12.5% in the cases ($P = 0.005$). The del −652 6N allele was significantly higher in controls as compared with cases (48.54 vs. 36.36%, $P = 0.004$). Carriers of the −652 6N del/ins or del/del genotypes were at lower risk for GC than carriers of other genotypes. Concerning the *CASP9* −1263 A>G polymorphism, the G allele was overrepresented in controls as compared with cases (50.94 vs. 30.68%, $P < 0.0001$). Individuals with the *CASP9* −1263 AG or GG genotype were at lower risk for GC than those with other genotypes.

Concerning the tumor characteristics, overall no statistically significant association was observed for *CASP8* and *CASP9* and any parameter investigated (e.g. stage, differentiation status). To analyze the combined effect of these polymorphisms we conducted gene–gene interaction analysis; however, we did not observe any statistically significant result.

Follow-up information regarding survival was available for all the patients (Table 3). The median duration of the follow up was 36.27 months (range 1–60 months). A total of 17 patients suffered cancer-related death during the follow-up period. The hazard ratios of the *CASP8* and *CASP9* genotypes of the patients for overall specific survival are presented in Table 3. The −652 ins/ins and the −1263 AA genotypes were associated with poor survival (Fig. 1).

Table 2 Caspase 8 −6N ins/del and caspase 9 −1263 A>G polymorphisms and susceptibility to gastric cancer

Genotype	Cases, *n* (%) (*n* = 88)	Controls, *n* (%) (*n* = 480)	*P* value; odds ratio (OR) (95% confidence interval [CI])
Caspase 8 6N			
ins/ins	35 (39.77)	120 (25)	Reference
del/ins	42 (42.73)	254 (52.92)	0.03; 0.57 (0.34–0.93)
del/del	11 (12.5)	106 (22.08)	0.005; 0.35 (0.17–0.73)
(del/ins) + (del/del)	53 (60.23)	360 (75)	0.006; 0.50 (0.31–0.81)
ins allele	112 (63.64)	494 (51.46)	Reference
del allele	64 (36.36)	466 (48.54)	0.004; 0.61 (0.44–0.85)
Caspase 9 −1263 A>G			
AA	44 (50)	116 (24.16)	Reference
AG	34 (38.64)	239 (49.79)	0.0001; 0.37 (0.23–0.62)
GG	10 (11.36)	125 (26.04)	<0.0001; 0.21 (0.10–0.44)
AG + GG	44 (50)	364 (75.83)	<0.0001; 0.32 (0.19–0.51)
A allele	122 (69.32)	471 (49.06)	Reference
G allele	54 (30.68)	489 (50.94)	<0.0001; 0.43 (0.30–0.60)

Table 3 Caspase 8 −6N ins/del and caspase 9 −1263 A>G genotypes in association with GC overall survival

Genotypes	Overall survival			*P* value
	Events (*n*)	5-Year survival (%)[a]	Hazard ratio (95% CI)	
Caspase 8 −6N ins/del				
ins/ins (35)	14	71.90	1.00 (reference)	
del/ins (42)	3	97.16	5.9 (2.09–21.07)	0.0008
del/del (11)	0	100	3.74 (0.83–16.86)	0.086
Caspase 9 −1263 A>G				
AA (44)	15	71.80	1.00 (reference)	
AG (34)	2	95.34	5.87 (2.18–15.80)	0.0004
GG (10)	0	100	6.27 (1.97–19.99)	0.0019

[a] Survival proportion derived from Kaplan–Meier analysis

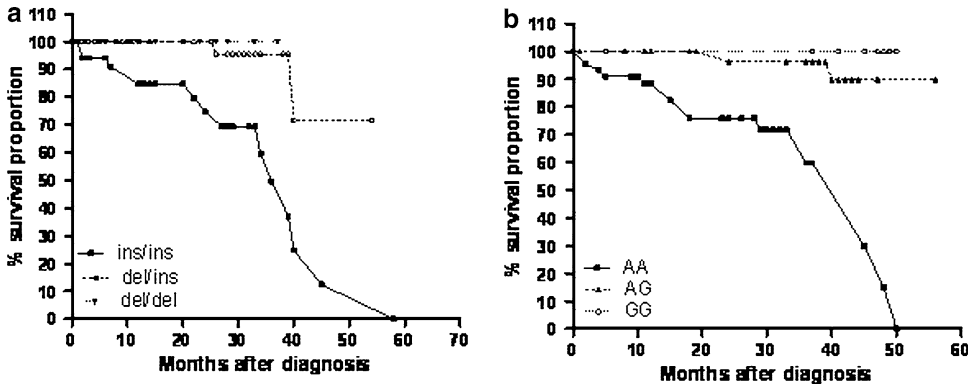

Fig. 1 Kaplan–Meier survival curves for gastric cancer (GC) patients stratified by *CASP8* −6N ins/del (**a**) and *CASP9* −1263 A>G genotypes (**b**)

Discussion

Defects in apoptosis signaling play an important role in the pathogenesis of cancers. Cancer cell survival can be induced by the inactivation of pro-apoptotic signaling or the activation of anti-apoptotic pathways. There are two main ways that can down-regulate cancer cell apoptosis: (a) somatic and non-somatic mutation and loss of expression of pro-apoptotic molecules; and (b) overexpression of apoptosis inhibitory molecules [10, 13]. Mutations within caspase family proteases are not uncommon in cancers [14]. The present study investigated the potential association between *CASP8* (−652 6N ins/del) and *CASP9* (−1263 A>G) polymorphisms and the risk of GC.

Several reports show that *CASP8* is mutated in different types of cancers. Soung et al. [10] examined gastric, breast, non-small cell lung cancers, and acute leukemia for mutations within the *CASP8* gene using single-strand conformation polymorphism (SSCP) methodology. Interestingly, the *CASP8* mutations were mostly detected in GC cases but not in other cancers.

The *CASP8* −652 6N del allele has been found to destroy a binding element for stimulatory protein 1 and to reduce the expression of CASP8, thus resulting in a reduction in the apoptosis reactivity of T lymphocytes upon stimulation by cancer cells or phytohemagglutinin in an ex vivo model [7]. This deletion variant was found to be associated with a reduced risk of lung, esophageal, stomach, breast, and cervical cancers in a Chinese population [7]. However, these findings failed to be replicated in subsequent larger studies [8, 9, 15]. In agreement with Sun et al. [7], we observed that the *CASP8* −652 6N del allele was associated with a significantly decreased risk of GC. Additionally, the data of our study provided evidence for the association of the *CASP8* −652 6N ins/del polymorphism and overall survival. The Kaplan–Meier curve showed a potential association of the presence of the del allele with increased survival.

CASP9 is involved in activation through the apoptosome-driven intrinsic pathway [16]. The release of apoptogenic proteins, caused by perturbation of the mitochondria, results in the activation of caspases which are responsible for most of the biochemical and morphological changes observed during apoptosis. APAF-1 in the presence of cytochrome c and dATP oligomerizes to form a very large apoptosome complex. The apoptosome recruits and processes caspase-9 to form a holoenzyme complex, which, in turn, recruits and activates the effector caspases. The influence of the CASP9 −1263 A>G polymorphism on the development and progression of gastric, colorectal, or other cancers remains to be determined, as its functional importance in the activation of CASP9 by the apoptosome complex and in the efficiency

of effector caspase activation by CASP9 is largely obscure for both these actions. One can only speculate that the CASP9 −1263 A>G polymorphism may affect either the binding affinity and stability of the apoptosome or the CASP9-induced activation of other caspases, in order to influence positively or adversely the intrinsic apoptotic pathway. Taking into consideration the biological role of CASP9, such functional alterations, either towards the activating or the repressing side, may also be expressed in an inherently variable, promoting or prohibitive, tumor microenvironment.

No data exist regarding *CASP9* gene polymorphisms and the risk of GC. However, studies of different cancer types have shown that the *CASP9* −1263 A>G polymorphism was associated with a significantly decreased risk of lung and bladder cancer [9, 17]. In the present study we found that the carriers of the −1263 G allele had a reduced risk of GC. Furthermore, the data of our study provided evidence for the association of the *CASP9* −1263 A>G polymorphism and overall survival. The Kaplan–Meier curve showed a potential association of the presence of the G allele with increased survival.

In conclusion, *CASP8* −652 6N ins/del and *CASP9* −1263 A>G promoter polymorphisms are significantly associated with the risk of GC. Because genetic polymorphisms often show ethnic differences, further studies are needed in diverse ethnic populations to clarify the association between caspase gene polymorphisms and GC.

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