Int J Colorectal Dis (2011) 26:1113–1118
DOI 10.1007/s00384-011-1217-5

ORIGINAL ARTICLE

Polymorphisms of *Caspase 8* and *Caspase 9* gene and colorectal cancer susceptibility and prognosis

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Accepted: 14 April 2011 / Published online: 3 May 2011

Abstract
Purpose 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 between *CASP8* and *CASP9* gene polymorphisms and colorectal cancer (CRC) susceptibility.
Methods A case–control study at 402 CRC patients and 480 healthy controls was undertaken in order to investigate the association between the genotype and allelic frequencies of *CASP8* −652 6N ins/del and *CASP9* −1263 A>G polymorphisms and the CRC susceptibility. The polymerase chain reaction (PCR) restriction fragment length polymorphism method was used and the incidence of polymorphisms on messenger RNA (mRNA) expression levels was detected by quantitative reverse-transcriptase PCR in CRC tissues.
Results No statistical significant association was observed between *CASP8* −652 6N ins/del polymorphism frequencies and CRC susceptibility. *CASP9* −1263 G allele was observed to be significant associated with reduced risk of CRC. Homozygotes for the −1263 GG *CASP9* genotype, and hetrozygotes for the −1263 AG genotype expressed 6.64- and 3.69-fold higher mRNA levels of Caspase-9, respectively compared to the −1263 AA genotype cases. No significant association was observed between CASP9 −1263 A>G polymorphism and tumor characteristics. The *CASP9* −1263 GG genotype was associated with increased overall survival in CRC patients.
Conclusion The *CASP9* −1263 A>G polymorphism was observed to play a protective role in CRC predisposition, while the *CASP9* −1263 GG genotype may confer a better prognosis at CRC patients.

Keywords Caspase-9 · Caspase-8 · Colorectal cancer · SNPs

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Introduction

Colorectal cancer (CRC) is the third most common cancer with a 5-year survival rate of 30–65% [1]. A portion of the inter-patient variability in its clinical outcome is attributed to inherited and somatic genetic factors. The development and progression of CRC involves unregulated epithelial cell proliferation associated with a series of accumulated genetic alterations [2]. There is evidence that prolonged survival of such genetically unstable colorectal epithelial cells, leading eventually to their ultimate malignant transformation, is associated with progressive inhibition of apoptosis. Genetic polymorphisms for genes controlling cell cycle or apoptosis have been found to increase the risk for a number of human

malignancies [3, 4]. However, such studies on CRC risk are scarce.

The two main apoptotic pathways 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 lead to cell death by nuclear membrane breakdown, DNA fragmentation, chromatin condensation and the formation of apoptotic bodies [5, 6].

CASP8 is a key regulator of apoptosis, an essential defense mechanism against hyperproliferation and tumorigenesis. Polymorphisms in *CASP8* gene have been reported to influence cancer risk. The variant D302H (rs1045485) has been associated with risk of breast cancer in the European population [7]. Additionally, the −652 6N ins/del promoter variant (rs3834129) has been associated with the risk of developing multiple cancer types, including CRC in the Chinese population [8]. It has been postulated that this deletion polymorphism has direct functional effects on cancer risk on the basis that the deletion destroy a stimulatory protein 1 binding site and decreases *CASP8* transcription [8]. On the contrary, Haiman et al. [9] reported that the −652 6N ins/del promoter polymorphism in the *CASP8* gene is not associated with cancer risk, and, more recently, Gangwar et al. [10] have confirmed this finding on bladder cancer.

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 APAF-1 and forms the apoptosome with procaspase-9 and activates the *CASP9* cascade. *CASP3* is then activated, leading to cell death [11]. Single-nucleotide polymorphisms are the most common human genetic variation and may contribute to an individuals' 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 the cancer risk. Several candidate polymorphisms in the *CASP9* gene have been recently reported in the public databases (http://www.ncbi.nlm.nih.gov/SNP) [12]. 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.

Hence, in order to confirm or refute the purported association between *CASP8* −652 6N ins/del and *CASP9* −1263 A>G polymorphisms and CRC risk, we have performed a hospital-based case–control study on Greek population.

Materials and methods

Subjects

A hospital-based case–control study was undertaken. The study population consisted of a well-documented series of 402 patients (203 men and 199 women; mean age, 66.8 years; range, 33–93 years), who underwent surgery for CRC at our institutions between January 2004 and December 2009. Patients with hereditary CRC and inflammatory bowel disease-related cancers were excluded from the study. None of the patients underwent preoperative chemoradiotherapy. The tumor was located in the right colon in 91 cases (22.64%), in the left colon in 177 cases (44.03%), and in the rectum in 134 cases (33.33%). The histological grade was assessed according to WHO criteria [13]: 52 tumors (12.93%) were well differentiated, 284 (70.65%) moderately differentiated, and 64 (15.92%)

Table 1 Histopathological characteristics of Greek colorectal cancer samples at diagnosis

Characteristics	Colorectal cancer patients, *n*
Tumor location	
Rectum	134
Left colon	177
Right colon	91
Tumor size	
≤ 4 cm	226
> 4 cm	176
Growth pattern	
Ulcerative	223
Protruding	179
Differentiation	
Good	52
Moderate	286
Poor	64
T stage	
T1	16
T2	68
T3	290
T4	28
N stage	
N0	179
N1	152
N2	71
TNM stage	
I	39
II	141
III	195
IV	27

poorly differentiated. According to the International Union Against Cancer classification and TNM staging system [14], 39 of the tumors (9.70%) were stage I, 141 (35.07%) stage II, 195 (48.51%) stage III, and 27 (6.72%) stage IV (Table 1). Family history and microsatellite instability (MSI) status had been evaluated in three young (<50 years of age) patients (all microsatellite-stable, MSS) and no known inherited or familial predisposition pattern had been identified. In 40 stage II patients (all T3N0M0) MSI status had also been assessed by their oncologist in order to make appropriate decisions on adjuvant treatment.

The age and gender matched 480 healthy controls used were randomly selected from a pool of healthy volunteers who visited the hospital during the same period. The study was approved by the hospital review board, and written informed consent was obtained from each participant.

Genotyping

DNA was isolated from peripheral blood using the Nucleospin blood kit (Macherey-Nagel, Germany). To confirm the integrity of DNAs, initially, a 430-bp sequence of the human glyceraldehydes 3-phosphate dehydrogenase gene (GAPDH) was amplified.

Polymorphisms in *CASP8* (−652 6N ins/del) and *CASP9* (−1263 A>G) were analyzed by PCR restriction fragment length polymorphism. Details of primers and PCR conditions were described earlier [8, 12].

Reverse-transcriptase PCR

Total RNA was extracted from 46 fresh biopsies from the pathologic area of CRC patients using the NucleoSpin RNA isolation kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Hereafter, reverse transcription was performed by incubating 1 μg total RNA for 1 h at 42°C in the presence of 500 μg/mL of Oligo dT 12–18, 10 mM deoxyribonucleotide triphosphates, 5× first-strand buffer, 0.1 M dithiothreitol, and 200 U/ml MMLV reverse transcriptase (Invitrogen). Prior to RT-PCR analysis, all of the RNA samples used had been DNase-treated to eliminate the risk of DNA contamination. Assessment of the *CASP9* mRNA levels was performed by employing the GAPDH expression levels as a reference gene. Real-time quantitative RT-PCR was conducted on an ABI Prism 7000 apparatus (Applied Biosystems, Foster City, CA, USA). Each cDNA sample was mixed with specific primer sets and PCR master mix (Applied Biosystems, No. 4312704). The levels of genes expression were normalized after subtracting the Ct value of the GAPDH RNA internal control from that of the *CASP9* Ct value for samples ($\Delta Ct = -|Ct_{CASP9}$ (samples) $- Ct_{GAPDH}|$). In order to compare the levels of *CASP9* expression between the samples tested, the ΔΔCt value was determined using the formula ($\Delta\Delta Ct = \Delta Ct_{CASP9(\text{sample A})} - \Delta Ct_{GAPDH(\text{sample B})}$). Then, the relative level of *CASP9* in cancer samples was compared to normal samples by setting the *CASP9* expression in normal samples value to 1 and determining the fold change in expression against this value using the following formula $2^{\Delta\Delta Ct}$.

Statistical analysis

Genotype and allele frequencies were compared with the chi-square with Yate's correction using S-Plus (v. 6.2, Insightful, Seattle, WA). Odds ratios (ORs) and 95 confidence intervals (CIs) were obtained with GraphPad (v. 3.00, GraphPad Software, San Diego, CA). The *P* values

Table 2 *Caspase* 8–6 N ins/del and *Caspase* 9–1263 A>G polymorphisms and susceptibility to colorectal cancer

Genotype	Cases, *n* (%) (*n*=402)	Controls, *n* (%) (*n*=480)	*P* value; OR (95%CI)
Caspase 8 6 N			
ins/ins	103 (25.62)	120 (25)	Reference
del/ins	201 (50)	254 (52.92)	0.62; 0.92 (0.67–1.27)
del/del	98 (24.38)	106 (22.08)	0.77; 1.07 (0.74–1.58)
(del/ins) + (del/del)	299 (74.38)	360 (75)	0.88; 0.97 (0.71–1.31)
ins allele	407 (50.62)	494 (51.46)	Reference
del allele	397 (49.38)	466 (48.54)	0.74; 1.03 (0.86–1.25)
Caspase 9–1263 A>G			
AA	155 (38.55)	116 (24.16)	Reference
AG	181 (45.02)	239 (49.79)	0.0003; 0.57 (0.42–0.77)
GG	66 (16.45)	125 (26.04)	<0.0001; 0.39 (0.27–0.58)
AG + GG	247 (61.44)	364 (75.83)	<0.0001; 0.51 (0.38–0.68)
A allele	491 (61.22)	471 (49.06)	Reference
G allele	313 (39.03)	489 (50.94)	<0.0001; 0.61(0.51–.74)

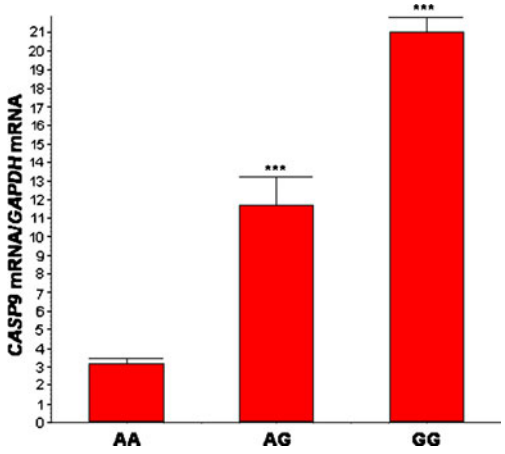

Fig. 1 Comparison of relative expression levels of *CASP9* mRNA as determined by real-time RT-PCR among the different *CASP9* −1263 A>G genotypes

are all two-sided. Hardy–Weinberg equilibrium was verified by calculation of expected frequencies and numbers, and significance testing was based on the 1 *df* χ^2. The survival curves were made using the Kaplan–Meier method and comparison was with the log-rank test. Inference for the OR was aided by GraphPad InStat (version 3.00, GraphPad Software, Inc., San Diego, CA). The effect of various variables on outcome was investigated by multivariate analysis using the Cox proportional hazards model. 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 402 CRC cases were recruited for this study. The histological characteristics of the patients are presented in Table 1. Six patients were MSI-H. No association was found with the examined polymorphisms in the limited subgroup of patients with known MSI status. The genotypic distributions of the two gene polymorphisms were in Hardy–Weinberg equilibrium. The genotype and allele frequencies of *CASP8* and *CASP9* gene polymorphism in healthy controls and CRC patients are shown in Table 2. No statistically significant association was observed in *CASP8*. The variant −652 6N del/del genotype was 22.08% prevalent in controls compared with 24.38 in CRC cases ($P=0.88$). The G allele of *CASP9* −1263 A>G polymorphism was higher in controls as compared with CRC cases (50.94% versus 39.03%, $P<0.0001$). Individuals with *CASP9* −1263 GG genotype were at lower risk of CRC ($P<0.0001$; OR=0.39, 95%CI: 0.27–0.58).

Concerning the tumor characteristics, no statistically significant association was observed in case of either *CASP8* or *CASP9* allelic and genotypic frequencies with any of the examined parameters (i.e., stage, differentiation status, etc.). To analyze the combined effect of these polymorphisms, we conducted gene–gene interaction analysis; however, we did not observe any statistically significant result.

Since the *CASP9* −1263 G allele showed the higher association with CRC risk, we further evaluated its effect on *CASP9* mRNA expression levels. As illustrated in Fig. 1, in CRC cases, homozygous carriers of *CASP9* −1263 GG genotype and heterozygotes for the −1263 AG genotype expressed 6.64- and 3.69-fold higher mRNA levels of *CASP9*, respectively, compared to cases with the −1263 AA genotype.

Follow-up information regarding survival was available for all the patients (Table 3). The median duration of the follow-up was 36 months (range, 6–60 months). A total of 118 patients suffered cancer-related death during the follow-up period. The hazard ratios of the *CASP9* genotypes of the patients for overall specific survival are presented in Table 3. The −1263 AA genotype was significantly associated with poor survival ($P=0.04$; Fig. 2). At multivariate analysis, the disease stage and −1263AA genotype emerge as independent variables of adverse prognostic significance (Table 4). *CASP8* −652 6N ins/del polymorphism was not associated with survival.

Discussion

Recently, genetic variants in caspase-mediated apoptosis and their role in human cancer susceptibility have been attracting increasing attention. The present study investigated the potential association between *CASP8* (−652 6N ins/del) and *CASP9* (−1263 A>G) polymorphisms and the risk for CRC.

Table 3 *CASP9* −1263 A>G genotypes in association with CRC overall survival

Genotypes	Overall survival			*P* value
	Events (*n*)	5-year survival (%)[a]	Hazard ratio (95%CI)	
AA (155)	58	79.82	1.00 (reference)	
AG (181)	50	79.93	1.07 (0.72–1.59)	0.72
GG (66)	10	85.31	1.92 (1.01–3.14)	0.04

[a] Proportion of survival derived from Kaplan–Meier analysis

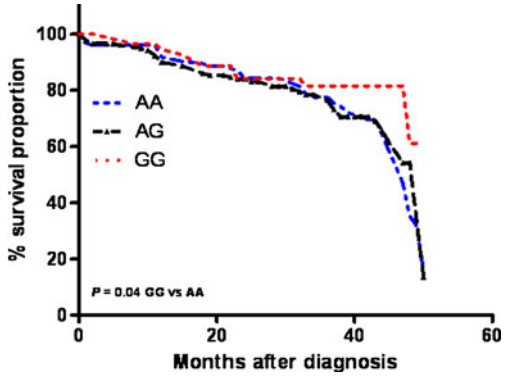

Fig. 2 Kaplan–Meier survival curves for CRC patients stratified by *CASP9* −1263 A>G genotypes (AA, AG, and GG)

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*, resulting in reduction of the apoptosis reactivity of T lymphocytes upon stimulation by cancer cells or phytohemagglutinin in an ex vivo model [8]. This deletion variant was found to be associated with a reduced risk of lung, esophageal, stomach, breast, and cervical cancer in a Chinese population [8]. However, these finding failed to be replicated in subsequent larger studies [9, 10, 15]. Our data are in agreement with Pittman et al. [15] and Liu et al. [16] who also supported that *CASP8* may not contribute to the risk of CRC.

CASP9 is involved in activation through apoptosome-driven intrinsic pathway [17]. The homozygous −1263 GG genotype in our study was observed to be significantly associated with reduced risk of CRC ($P<0.0001$, OR=0.39). Our observations were compatible with a Korean study and an Indian study where *CASP9* −1263 A>G polymorphism was observed to be associated with significantly decreased risk of lung and bladder cancer, respectively [10, 18]. Up to now, no related study dealing with the effect of this polymorphism on CRC exist in the international literature.

To investigate if the association between *CASP9* −1263 A>G polymorphism and the risk of CRC is due to differences in the transcriptional activity of the *CASP9* promoter, we compared the mRNA levels in carriers of the three different genotypes (AA, AG, and GG). The carriers of the G allele exhibited higher mRNA levels than the carriers of the AA genotype. These findings are partially in agreement with Park et al. [18] who have shown that the G-C haplotype of the −1263 A>G and −712 C>T polymorphisms, have significantly higher transcriptional activity than the A–C haplotype. The mechanism by which these polymorphisms lead to a higher promoter activity is unknown. Park et al. [18] suggested that the −1263 A to G transition leads to the creation of an additional simian virus-40 protein 1-binding site. Therefore, it is possible that this change in the putative transcription factor-binding sites owing to the −1263 A>G polymorphism might lead to enhanced transcription levels. Our results suggest that the presence of G allele resulted in a higher *CASP9* "production" and may offer protection against the development of CRC.

It is noteworthy that our study provided evidence for the association between *CASP9* −1263 A>G polymorphism and overall survival, on the grounds of lack of any association of the *CASP9* polymorphism with any of the conventional prognostic parameters, i.e., stage, that were taken into account. The potential association of GG genotype with increased survival merits further evaluation, since its oncologic significance may have important prognostic and therapeutic implications.

Excluding studies focusing on the predictive role of gene polymorphisms to the response to chemotherapy, where MSI may have a principal role, MSI has not been generally included in CRC risk studies analyzing the role of specific polymorphisms [19]. Due to the relatively small number of patients with known MSI status in the current study, no definite conclusions can be derived for a potential relationship with the examined polymorphisms. Nevertheless, the degree of influence of the major CRC molecular pathways (chromosomal instability, MSI) on genetic polymorphisms remains to be further elucidated.

In conclusion, in the current study that comprised a homogeneous Mediterranean population, the *CASP9* −1263 A>G promoter polymorphism was significantly associated with the susceptibility to CRC. Since genetic polymorphisms often show ethnic differences, further studies are needed in populations with diverse backgrounds, in order to further elucidate the association between caspase gene polymorphisms and CRC.

Table 4 Cox proportional hazard estimation of overall survival

Covariant	B coefficient	SE	*P*	Correlation coefficient (*r*)	95% CI for relative risk
Overall survival					
TNM stage	0.378	0.038	0.02	0.129	0.28–0.47
−1263 AA CASP9	0.321	0.033	0.04	0.191	0.23–0.42

The analysis for disease status was performed by grouping patients in two groups (TNM stage I+II and III+IV).

Acknowledgments The study was supported by National Resources "KAPODISTRIAS" 70/4/9923 to GE Theodoropoulos.

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