

CASPASE-8 Gene Is Inactivated by Somatic Mutations in Gastric Carcinomas

Young Hwa Soung, Jong Woo Lee, Su Young Kim, Jin Jang, Yong Gyu Park, Won Sang Park, Suk Woo Nam, Jung Young Lee, Nam Jin Yoo, and Sug Hyung Lee

Departments of Pathology and Biostatistics, College of Medicine, Catholic University of Korea, Seoul, Korea

Abstract

Several lines of evidence indicate that deregulation of apoptosis is involved in the mechanisms of cancer development. CASPASE-8 activation plays a central role in the initiation phase of apoptosis. The aim of this study was to explore the possibility that genetic alteration of CASPASE-8 gene is involved in the development of human cancers, including gastric cancers. We have analyzed the entire coding region of human CASPASE-8 gene for the detection of somatic mutations in 162 gastric carcinomas (40 early and 122 advanced cancers), 185 non–small cell lung cancers, 93 breast carcinomas, and 88 acute leukemias by PCR-single-strand conformation polymorphism. Of the cancers analyzed, 13 cancers harbored CASPASE-8 somatic mutations. Interestingly, all of the mutations were detected in the advanced gastric cancers (10.7% of the 122 samples). We expressed the tumor-derived caspase-8 mutants in 293T, 293, and HT1080 cells and found that most of the mutants (9 of the 10 mutations tested) markedly decreased the cell death activity of caspase-8. In addition, in the cells with the inactivating caspase-8 mutants, cleavage of poly(ADP-ribose)polymerase was markedly reduced compared with that of wild-type caspase-8. The occurrence of CASPASE-8 mutation and the inactivation of cell death activity by the mutants suggest that CASPASE-8 gene mutation may affect the pathogenesis of gastric cancers, especially at the late stage of gastric carcinogenesis. (Cancer Res 2005; 65(3): 815-21)

Introduction

Apoptosis is vital to all multicellular organisms for normal development and tissue homeostasis (1). Proteases participate in protein destruction and regulation of protein activities in numerous cellular context (1-4). Among the proteases, a family of cysteine proteases that cleave the substrates at aspartate residues, known as caspases, plays a central role in the activation and propagation of death signaling (3, 4). Functionally, the mammalian caspases can be divided into either initiator caspases or effector caspases. The initiator caspases would seem to be caspase-8, -9, and -10, and the effector caspases would seem to be caspase-3, -6, and -7 (3, 4). The death signals originating from the death receptors, such as tumor necrosis factor receptors, Fas, and tumor necrosis factor–related apoptosis-inducing ligand receptors, are transduced through the recruitment of pro-caspase-8 to the death-induced signaling complex by the adaptor molecule Fas-associated death domain protein (FADD; refs. 1, 2). The local aggregation of pro-caspase-8 is sufficient to allow autoprocessing or transprocessing to produce active caspase-8, which can subsequently activate downstream executioners, such as caspase-3 and -7 (3, 4). In addition, caspase-8 plays a role in the mitochondrial apoptotic pathway by cleaving the BH3-interacting death agonist (Bid), which can then promote the release of mitochondrial factors (1).

It is now believed that clonal expansion and tumor growth are the results of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis; ref. 5). Failure of apoptosis could allow the survival of transformed cells that are prone to undergo further genetic damage and play an important role in the pathogenesis of tumors. Either inactivation of proapoptotic pathway or activation of antiapoptotic pathway results in failure of apoptosis, thereby promoting tumor cell survival. Apoptosis of cancer cells can be delayed or blocked by several ways, including somatic mutation and loss of expression of proapoptotic molecules and expression of apoptosis inhibitory molecules. Somatic mutations of apoptosis-related genes, including death receptors, Bcl-2 members, and caspases, have been reported in human cancers, and many of the mutations were proven to inactivate cell death (6-15). In human cancer tissues, somatic mutations of CASPASE-8 gene have been reported in colon cancers (15), suggesting the possibility that CASPASE-8 might be mutated widely in other human cancers. To explore the possibility, we analyzed 528 cancer tissues from various origins and found that CASPASE-8 is mutated in gastric cancers.

Materials and Methods

Tissue Samples and Microdissection. Methacarn-fixed tissues of 162 gastric carcinomas, 93 breast carcinomas, 185 non–small cell lung cancers, and nonfixed fresh tissues of 88 acute leukemias (subgroup details available on request) were randomly selected for PCR-single-strand conformation polymorphism (SSCP) of CASPASE-8 gene. Approval was obtained from the Catholic University of Korea, College of Medicine institutional review board for this study. Informed consent was provided according to the Declaration of Helsinki. The gastric carcinoma samples consisted of 70 diffuse-type, 55 intestinal-type, and 37 mixed-type gastric carcinomas by Lauren’s classification and 40 early and 122 advanced gastric carcinomas according to the depth of invasion. The tumor-node-metastasis stages of the gastric cancers were 72 stage I, 40 stage II, 35 stage III, and 15 stage IV.

Malignant cells and normal cells were selectively procured from H&E-stained slides using a 30.5-gauge hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator as described previously (16). In this study, primary lesions, but not metastatic lesions, were analyzed for the mutation detection. DNA extraction was done by a modified single-step DNA extraction method as described previously (16).
PCR-SSCP Analysis. Genomic DNA from tumor cells and normal cells from the same patients were amplified with 14 primer pairs covering the entire coding region (8 exons) of human \textit{CASPASE-8} gene. Numbering of cDNA of \textit{CASPASE-8} was done in respect of the ATG start codon (NM_033355). Radioisotope ([32P]dCTP) was incorporated into the PCR products for detection by autoradiogram. The procedures of PCR and SSCP analyses were done as described previously (8–11). After SSCP, bands showing mobility shifts were cut out from the dried gel and reamplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA).

To analyze the allelic status of \textit{CASPASE-8} gene in the tumors, we have used three intragenic polymorphisms (960 A/G, IVS9-19 A/G, and 789 A/G). SSCP analysis at these polymorphic sites was used for the detection of loss of heterozygosity (LOH) as well as for the detection of mutations. Complete or nearly complete absence of one allele in tumor DNA of informative cases, as defined by direct visualization, was considered as LOH.

Site-Directed Mutagenesis. The plasmid constructs encoding FADD and caspase-8 were described previously (15). Site-directed mutagenesis was done using a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. To change a base, a plasmid that contained the \textit{CASPASE-8} gene in pcDNA3.1 with Flag tag at the NH2 terminus was used as a template. The nucleotide sequences of the mutagenized plasmids were confirmed by DNA sequencing.

Transfection and Cell Death Assay. 293T (human embryonic kidney cells with SV40), 293 (human embryonic kidney with adenovirus 5), and HT1080 (human fibrosarcoma) cell lines in log phase were transfected in six-well plates with expression vectors together with 0.2 \( \mu \)g of green fluorescence protein marker plasmid pEGFP N2 (Clontech, Palo Alto, CA) using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after the transfection, the adherent and attached cells were pooled, fixed in 10% methanol for 15 minutes, and stained with 1 \( \mu \)g/mL 4',6-diamidino-2-phenylindole for 15 minutes. The percentage of green fluorescence protein–positive cells with nuclear apoptotic morphology was counted under

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**Figure 1.** Mutations of \textit{CASPASE-8} gene in gastric cancers. SSCP (A) and sequencing analysis (B) of \textit{CASPASE-8} DNA from tumors (lane \( T \)) and normal tissues (lane \( N \)). A, arrows (lane \( T \)), aberrant bands compared with SSCP from normal tissue (lane \( N \)). B, arrows, nucleotide changes in tumor tissue compared with normal tissue.
fluorescent microscope (mean ± SD; n = 3). For the statistical analysis of the cell death data, we used ANOVA and Duncan’s multiple range tests.

**Immunoprecipitations and Immunoblotting Assay.** After transfection, cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-Flag M2 antibodies (Sigma, St. Louis, MO). Immunocomplexes were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The resulting blots were incubated with anti-hemagglutinin (HA; 1:1,000 v/v, Roche Molecular Biochemicals, Mannheim, Germany) followed by horseradish peroxidase–conjugated secondary antibodies and detection by an enhanced chemiluminescence method (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Alternatively, lysates were analyzed directly by immunoblotting after normalization for total protein content.

**In vitro Translation.** In vitro translation was done using a TNT Quick Coupled Transcription/Translation system (Promega, Madison, WI) according to the instructions of the manufacturer. The resulting products were analyzed by immunoblotting using anti-caspase-8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Results**

**Mutations and Allelic Status of CASPASE-8 Gene.** Genomic DNA isolated through microdissection were analyzed for the detection of mutations in the coding region and the exon-intron junctions of CASPASE-8 gene by PCR-SSCP analysis. Enrichment and DNA sequencing analysis of aberrantly migrating bands on

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<th>LOH analysis</th>
<th>Mutation type</th>
<th>Mutation site (domain)*</th>
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**Abbreviations:** NI, not informative (homozygosity); HET, retention of heterozygosity.
*p20, large subunit; p10, small subunit.
*Polymorphisms at nucleotides 789 and 960 of CASPASE-8 cDNA.
*The polymorphism at the intron 7 of CASPASE-8.
the SSCP led to the identification of 15 mutations (Fig. 1; Table 1). None of the normal samples from the same patients showed evidence of mutations by SSCP (Fig. 1), indicating the mutations had risen somatically. Of note, the CASPASE-8 mutations were detected in gastric cancers but not in other cancer types. The incidence of CASPASE-8 mutation in gastric cancer is statistically higher than those of non–small cell lung cancer, breast cancer, and acute leukemias (Fisher’s exact test, P < 0.01). In addition, interestingly, all of the 15 mutations were detected in advanced gastric cancers but not in early gastric cancers. Significantly, incidence of CASPASE-8 mutation in the advanced gastric cancers is higher than that in the early gastric cancers (Fisher’s exact test, P < 0.05). Of the 122 advanced gastric cancers analyzed, 13 (10.1%) cancer samples harbored CASPASE-8 mutations (Fig. 2A; Table 1). In addition, among the 15 mutations detected, 2 cancers (cases 24 and 224) harbored 2 mutations each. The mutations consisted of 3 missense, 1 in-frame deletion, and 5 frameshift mutations in the coding sequences; 2 mutations in the initiation codon; 3 mutations in the introns; and 1 mutation in the 3’ untranslated region. Regarding the histologic subtypes and staging of the gastric cancers, there was no significant correlation with CASPASE-8 mutations (Fisher’s exact test, P > 0.05). We repeated the experiments twice, including PCR, SSCP, and sequencing analysis, to ensure the specificity of the results and found that the data were consistent (data not shown). In addition, we repeated the mutational screening by a direct sequencing to exclude the possibility that there would be missed CASPASE-8 mutations, but we could not find any more mutations by the direct sequencing.

The missense mutations detected in the present study would result in the substitution of amino acids in the death effector domains (DED) and the p10 small protease subunit. The frameshift mutations would result in premature terminations of amino acid synthesis in caspase-8 protein (Table 1). Using in vitro translation and subsequent immunoblotting, we observed that the caspase-8 mutants were expressed well and the sizes of the detected mutants were matched with the predicted amino acid changes (Fig. 2B; Table 1). In the two mutations at the initiation codon, we used caspase-8 expression vector without any tag for in vitro translation. These two mutants (cases 252 and 220) were detected by immunoblotting with caspase-8 antibody (Fig. 2B).

The sizes of the two mutant proteins on the immunoblot seemed to be equal and were apparently smaller than that of wild-type caspase-8 (Fig. 2B).

We examined the allelic status of CASPASE-8 in the tumors by using the three intragenic polymorphisms. Among the 13 cases with the CASPASE-8 mutations, 7 were heterozygous for one or more polymorphism(s) and 1 (case 108) of them (14%) showed evidence of allelic loss (Table 1).

**Reduction of Apoptotic Activities by the CASPASE-8 Mutations.** To determine whether the mutant forms of caspase-8 are functionally defective, we generated caspase-8 expression vectors containing the mutations found in the coding region of CASPASE-8. On transfection into 293T, 293, and HT1080 cells, we found that all of the mutants, except the mutant case 183 (1427T > C), showed significant defects in apoptosis induction compared with the wild-type caspase-8 (Fig. 3A; ANOVA and Duncan’s multiple range test, P < 0.001). We used a dominant-negative caspase-8 (an artificial caspase-8 C360A mutant that has a mutation at the activation site) as a negative control for the caspase-8 activation. We also compared the apoptotic activities of the CASPASE-8 mutants of the gastric cancers with the three inactivating CASPASE-8 mutants of the colorectal cancers that had been reported in our previous study. The mutants of colorectal cancers had similar apoptotic activities with the 492_493delTG, 698delE, and 969_972delCTAT mutants of gastric cancers (Fig. 3A). Figure 3B shows that the mutants were expressed well and the levels of the expression were similar among them.

In addition, to see whether the reduced cell death by the CASPASE-8 mutations was accompanied with the decreased cleavage of key proteins destructed in the cell death, we transfected the caspase-8 mutants or wild-type caspase-8 in 293T cells and compared poly(ADP-ribose)/polymerase (PARP) cleavage in the lysates by immunoblotting with anti-PARP antibody. All of the mutants, except two (cases 133 and 183), showed decrease or absence of PARP cleavages compared with the discernable cleavage of PARP of the wild-type caspase-8 (Fig. 4).
Binding of Caspase-8 Mutants with FADD. The DED of caspase-8 is important in transmitting death signals from FADD. Because two missense mutations (cases 24 and 133) and an in-frame deletion (case 224) would change amino acids in the DED without any changes in other domains, we tested whether the reduced cell death activities by these mutations (Fig. 3) arose from diminished binding of these mutants with FADD. To see this, we coexpressed FADD with each caspase-8 mutant (cases...

Figure 3. Defective apoptotic activities of tumor-derived caspase-8 mutants. A, 293T, 293, and HT1080 cells were transfected with 1.3 μg of wild-type caspase-8 or each tumor-derived caspase-8 mutant together with 0.2 μg of pEGF. 1238G > A, 1237C > T, and 1381insG were the mutants detected in the previous studies (15). Twenty-four hours after transfection, cell death was examined under fluorescence microscopy. Columns, mean (n = 3); bars, SD. B, mutants were transfected in 293T cells, and cell lysates were immunoblotted with anti-caspase-8 antibody or anti-FLAG antibody.

Figure 4. Impaired PARP cleavage by CASPASE-8 mutations. 293T cells were transfected with 1.3 μg of wild-type caspase-8 or each tumor-derived caspase-8 mutant. One day after transfection, cell lysates were normalized for total protein content and then employed by direct immunoblot analysis by anti-PARP antibodies (Pharmingen, San Diego, CA).
24, 133, and 224). The coimmunoprecipitation analysis shows that FADD coimmunoprecipitates well with the mutants (Fig. 5), suggesting that these mutants are recruited in the death-induced signaling complex. As a positive control, Fig. 5 also shows that FADD coimmunoprecipitates with the dominant-negative caspase-8. In contrast, FADD did not coimmunoprecipitate with caspase-7 (data not shown), thus demonstrating the specificity of these results.

Discussion

Our previous observation (15) that CASPASE-8 gene is mutated in colon cancers led us to analyze other human cancers besides colon cancer. We found that only gastric carcinomas harbored CASPASE-8 mutation among the cancers analyzed. Furthermore, we observed that the incidence of CASPASE-8 mutation in advanced gastric cancer was higher than that in early gastric cancer. We compared the apoptotic activities of the previously detected CASPASE-8 mutants of the colorectal cancers with those of the mutants of the gastric cancers and found that they were similar. These data suggest that CASPASE-8 mutation may contribute to the pathogenesis of both colorectal and gastric cancers.

The central aim of cancer research has been to identify the mutated genes that are causally implicated in tumorigenesis. Mutations in cancer could be categorized either as functional alterations affecting key genes underlying the neoplastic process or as nonfunctional "passenger" changes. However, unfortunately, the prevalence of passenger mutations in the cancer genomes is not known. In general, high incidence and functional derangements related to the characteristics of cancers may favor that mutations related to the characteristics of cancers may favor that mutations are conserved in these species.

Among the 13 types of CASPASE-8 mutations, the frameshift mutations would change the amino acids after the DED (cases 17 and 240) or the p20 subunit (cases 108 and 207) or the p10 subunit (case 24) and hence resemble typical loss-of-function mutations. Because the active caspase-8 consists of a (p20/p10)2 tetramer (17, 18), the frameshift mutations may also lead to abnormal construction of the tetramer. Mutations at the position +1 of the consensus sequence of the donor site of an intron have been shown to cause cryptic splice site utilization and exon skipping in various human disease genes (19). Thus, although functional studies could not be done, it could be conceived that the splice-site mutation (IVS2 + 1G > A) found in case 224 may result in abnormal splicing of CASPASE-8 mRNA. The missense mutation 1427T > C would result in the substitution of Phe767 with Ser767 at the COOH terminus (Table 1). The Phe767 residue is located within the last 10 amino acids from the COOH terminus where no -helices or β-strands or loops exist (20), which play an important role in the cell death activity, suggesting that this amino acid alteration may not be related to cell death function. In addition, by analyzing the Genbank database for CASPASE-8 genes of human, mouse, rat, and chicken, we found that the amino acids, which were changed from wild-type in cases of 24 (C164), 183 (F476), and 224 (E84), are conserved in these species.

We have explored the possibility that the reduced cell death activities of the two missense mutations (cases 24 and 133) in the DED could arise from impaired binding of the mutants with FADD. However, we found that there was no discernable decrease of binding of the mutants with FADD (Fig. 5) compared with the wild-type, suggesting that there may be other mechanisms to reduce the cell death function of the mutants. Several examples of mutations in Met (ATG) translational initiation codon have been reported, with a preponderance of Met-to-Val substitution (19). In the present study, two mutations (cases 220 and 252) affected the initiation codon (Table 1). In vitro translation products by the mutations were smaller than the wild-type caspase-8 (Fig. 2), suggesting that the consequence by the initiation codon mutations may be the initiation of translation at the next available site downstream. In addition, the immunoreactivity of the mutant products to caspase-8 antibody indicates that the newly created initiation codon may be in-frame with the wild-type CASPASE-8 cDNA.

Chromosome 2q33, where CASPASE-8 gene resides, has been reported as a common deleted region in gastric cancers, and the LOHs were observed in ~30% of the informative cases in earlier studies (21, 22). However, we showed that the second allele of CASPASE-8 was only one (case 108) of the seven informative cases with the CASPASE-8 mutations, indicating that CASPASE-8 mutation may not be responsible for the LOH at 2q33-34 in gastric cancers.

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Figure 5. Binding of caspase-8 mutants to FADD. 293T cells were cotransfected with expression constructs for human HA-FADD either with or without the Flag-caspase-8 mutant. Transfected cells were harvested, lysed 1 day later, immunoprecipitated with anti-Flag antibodies, and sequentially immunoblotted with anti-HA antibody. Aliquots of the same lysates (normalized for total protein content) were also analyzed directly by SDS-PAGE/immunoblotting. IP, immunoprecipitation; WB, Western blotting.
cancers. Thus, the hemizygously mutated caspase-8 protein might bind with other normal caspase-8 protein(s), construct a structurally abnormal caspase-8 tetramer, and could therefore block the death signal transduction. In contrast, in the gastric cancer cells of case 108, mutated caspase-8 proteins might bind each other and form a mutant tetramer.

All gastric cancers presumably begin as early gastric cancers, which develop over time into advanced lesions (23). However, the whole mechanism of this process is still poorly understood. The significant difference in CASPASE-8 mutation frequency between early and advanced gastric carcinomas suggests that CASPASE-8 mutation may be involved in the late event of gastric carcinogenesis but not in the initiating event of it.

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