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# *Caspase 9* promoter polymorphisms and risk of primary lung cancer

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**Caspase-9 (CASP-9) is an initiator CASP in the apoptosome-driven apoptosis pathway and plays an important role in the development and progression of cancer. Polymorphisms in the promoter region of the *CASP-9* gene may influence the promoter activity of this gene, thereby modulating susceptibility to lung cancer. To test this hypothesis, we examined the association of four polymorphisms [−1263A>G, −905T>G, −712C>T and −293_ − 275delCGTGAGGTCAGTGCGGGGA (−293del)] in the *CASP-9* promoter with the risk of lung cancer in a Korean population. The *CASP-9* genotypes were determined in 432 lung cancer patients and 432 healthy controls that were frequency-matched for age and gender. The −1263 GG genotype was associated with a significantly decreased risk of lung cancer compared with the −1263 AA genotype or combined −1263 AA + AG genotype [adjusted odds ratio (OR) = 0.64, 95% confidence interval (95% CI) = 0.42–0.98, *P* = 0.04 and adjusted OR = 0.67, 95% CI = 0.46–0.97, *P* = 0.01, respectively]. For the −712C>T polymorphism, individuals with at least one −712T allele were at a significantly increased risk of lung cancer compared with those harboring the −712 CC genotype (adjusted OR = 1.42, 95% CI = 1.06–1.89, *P* = 0.02). Consistent with the results of genotype analyses, the −1263G/ − 712C (G-C) haplotype was associated with a significantly decreased risk of lung cancer [adjusted OR = 0.59, 95% CI = 0.47–0.75, *P* and Bonferroni corrected *P* ($P_c$) < 0.001]. Moreover, the risk of lung cancer decreased in a dose-dependent manner as the number of the G-C haplotypes increased (adjusted OR = 0.60, 95% CI = 0.45–0.81, *P* = 0.0007 and $P_c$ = 0.0014 for the G-C heterozygotes and adjusted OR = 0.34, 95% CI = 0.17–0.68, *P* = 0.0023 and $P_c$ = 0.0046 for the G-C homozygotes; $P_{trend}$ < 0.001). The promoter assay revealed the G-C haplotype to have a significantly higher promoter activity than the −1263G/ − 712T and −1263A/ − 712C haplotypes. These results suggest that *CASP-9* promoter polymorphisms affect *CASP-9* expression and contribute to genetic susceptibility to lung cancer.**

## INTRODUCTION

Although cigarette smoking is a major cause of lung cancer, only a fraction of smokers develop lung cancer during their lifetime. This suggests that the genetic constitution plays an important role in determining an individual's susceptibility to lung cancer (1,2).

Apoptosis, a genetically controlled process of programmed cell death, plays an important role in the development and maintenance of tissue homeostasis in multicellular organisms (3,4).

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Inappropriate regulation of apoptosis contributes to many human disorders, including cancer (3–6). Apoptotic cell death is orchestrated by the activation of a cascade of enzymes called caspases (CASPs), which are a family of cysteine-dependent aspartate-specific proteases (7–9). Two distinct but converging pathways for CASP activation have been delineated: the extrinsic or receptor-mediated pathway and the intrinsic or mitochondrial pathway. These two pathways have an independent group of initiator caspases but use the same group of effector caspases, primarily CASP-3, -6 and -7, that execute the final cell death program (7–11).

The extrinsic pathway is triggered by the activation of the cell surface death receptors following the binding of their specific ligand, such as the tumor necrosis factor, Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (7–11). Ligand–receptor binding recruits the adaptor molecule, FADD, resulting in the activation of the initiator CASP-8 and/or CASP-10 with formation of a death-inducing signaling complex. The activated initiator CASPs subsequently activate the downstream effector CASPs (12,13). The other principal death-signaling pathway, the intrinsic or mitochondrial pathway, is initiated by the release of cytochrome *c* from mitochondria in response to a variety of cytotoxic signals, including DNA damage, hypoxia and growth factor deprivation (6–9). Released cytochrome *c* interacts with Apaf-1, proCASP-9 and dATP to form a multiprotein complex called apoptosome. Once bound to the apoptosome, CASP-9 is activated, which subsequently triggers a cascade of effector CASPs, such as CASP-3, -6 and -7 (14–16).

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 (17,18). Recently, several candidate polymorphisms in the *CASP-9* gene have been reported in the public databases (http://www.ncbi.nlm.nih.gov/SNP). 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 CASP-9 expression or activity, thereby modulating susceptibility to lung cancer. To test this hypothesis, a case–control study was performed to evaluate the association between *CASP-9* genotypes/haplotypes and the risk of lung cancer. Among the candidate polymorphisms in the *CASP-9* gene, variants in the promoter region (1.3 kb upstream from the first exon) of the *CASP-9* gene were examined because these most probably affect gene expression. In this study, the association between the −1263A>G, −905T>G, −712C>T and −293_−275delCGTGAGGTCAGTGCGGGGA (−293del) polymorphisms (rs4645978, rs4645980, rs4645981 and rs4645982, respectively) and the risk of lung cancer was evaluated because the other five candidate polymorphisms [−1290A>T (rs2244899), −935C>G (rs4645979), −503C>G (rs28457091), −315A>C (rs10927794) and −185C>G (rs12060237)] were not detected in a preliminary study, which consisted of 27 lung cancer cases and 27 healthy controls.

**Table 1.** Characteristics of the study population

| Variable | Cases ($n = 432$) | Controls ($n = 432$) |
|---|---|---|
| Age (years) | 61.6 ± 9.0 | 60.9 ± 9.3 |
| Sex | | |
| Male | 352 (81.5)[a] | 352 (81.5) |
| Female | 80 (18.5) | 80 (18.5) |
| Smoking status* | | |
| Current | 317 (73.4) | 229 (53.0) |
| Former | 39 (9.0) | 98 (22.7) |
| Never | 76 (17.6) | 105 (24.3) |
| Pack-years[b] | 39.9 ± 17.9 | 34.4 ± 17.6 |

[a]Numbers in parentheses denote percentage.
[b]In current and former smokers, $P < 0.001$.
*$P = 0.001$.

**Table 2.** *Caspase-9* genotypes of cases and controls and their association with the risk of lung cancer

| Polymorphism | Genotype | Cases, *n* (%) | Controls, *n* (%) | Adjusted OR (95% CI)[a] |
|---|---|---|---|---|
| −1263A>G | AA | 148 (34.3) | 138 (31.9) | 1.0 |
| | AG | 225 (52.1) | 215 (49.8) | 0.94 (0.69–1.27) |
| | GG | 59 (13.7) | 79 (18.3) | 0.64 (0.42–0.98)* |
| | AA+AG | 373 (86.3) | 353 (81.7) | 1.0 |
| | GG | 29 (13.7) | 79 (18.3) | 0.67 (0.46–0.97)* |
| −905>G | TT | 143 (33.1) | 152 (35.2) | 1.0 |
| | TG | 211 (48.8) | 206 (47.7) | 1.08 (0.79–1.46) |
| | GG | 78 (18.1) | 74 (17.1) | 1.07 (0.72–1.59) |
| −712C>T[b] | CC | 261 (60.4) | 298 (69.0) | 1.0 |
| | CT | 149 (34.5) | 123 (28.5) | 1.34 (0.99–1.80) |
| | TT | 22 (5.1) | 11 (2.6) | 2.32 (1.09–4.94)** |
| | CC | 261 (60.4) | 298 (69.0) | 1.0 |
| | CT + TT | 171 (39.6) | 134 (31.0) | 1.42 (1.06–1.89)*** |
| −293del[c] | −/− | 159 (36.8) | 161 (37.3) | 1.0 |
| | −/+ | 211 (48.8) | 201 (46.5) | 1.03 (0.76–1.39) |
| | +/+ | 62 (14.4) | 70 (16.2) | 0.86 (0.57–1.30) |

[a]Adjusted for age, gender and pack-years of smoking.
[b]Comparison of genotype distribution between cases and control, $P = 0.01$.
[c]−293_−275delCGTGAGGTCAGTGCGGGGA.
*$P = 0.04$.
**$P = 0.03$.
***$P = 0.02$.

## RESULTS

Table 1 shows the demographics of the cases and controls enrolled in this study. There were no significant differences in mean age or gender distribution between cases and controls, which suggest adequate matching on the basis of these two variables. However, there were more current smokers among the cases than the controls ($P < 0.001$), and the number of pack-years in smokers was significantly higher in the cases than in the controls (39.9 ± 17.9 versus 34.4 ± 17.6 pack-years; $P < 0.001$). These differences were controlled in the later multivariate analyses.

The distributions of the *CASP-9* −1263A>G, −905T>G, −712C>T and −293del genotypes among cases and controls are shown in Table 2. The genotype distributions of the four polymorphisms among the controls were in Hardy–Weinberg

**Table 3.** LD coefficients ($|D'|$ and $r^2$) among *caspase-9* polymorphisms

| Polymorphism | | $\|D'\|$ | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| $r^2$ | −1263A>G (I) | — | 0.69 | 0.93 | 0.91 |
| | −905T>G (II) | 0.17 | — | 0.71 | 0.74 |
| | −712C>T (III) | 0.11 | 0.08 | — | 0.94 |
| | −293del[a] (IV) | 0.21 | 0.17 | 0.11 | — |

[a]−293_−275delCGTGAGGTCAGTGCGGGGA.

equilibrium. The four *CASP-9* polymorphisms were in linkage disequilibrium (LD) ($|D'|$ value: ranges from 0.69 to 0.94, Table 3). The frequency of −1263 GG genotype was borderline, significantly lower in the cases than in the controls (13.7 versus 18.3%, $P$ equals; 0.06). The distribution of the −712C>T genotypes among the cases were significantly different from that among the controls ($P = 0.01$). For the other two polymorphisms, there was no significant difference in the distributions of genotypes between cases and controls. The −1263 GG genotype was associated with a significantly decreased risk of lung cancer compared with the −1263 AA genotype or the combined −1263 AA + AG genotype [adjusted odds ratio (OR) = 0.64, 95% confidence interval (CI) = 0.42–0.98, $P = 0.04$ and adjusted OR = 0.67, 95% CI = 0.46–0.97, $P = 0.01$, respectively]. For the −712C>T polymorphism, individuals with at least one −712T allele were at a significantly increased risk of lung cancer compared with those harboring −712 CC genotype (adjusted OR = 1.42, 95% CI = 1.06–1.89, $P = 0.02$), and the risk of lung cancer increased with increasing numbers of −712T alleles (adjusted OR = 1.34, 95% CI = 0.99–1.80 for the −712 CT genotype and adjusted OR = 2.32, 95% CI = 1.09–4.94, $P = 0.03$ for the −712 TT genotype; $P_{trend} = 0.007$). The −905T>G and −293del polymorphisms were not significantly associated with the risk of lung cancer.

As a consequence of the LD between the −1263A>G and −712C>T polymorphisms, it is difficult to determine which of the two polymorphisms was more likely to have a functional effect on the disease-associated haplotypes. In an attempt to resolve this problem, we compared three different logistic regression models (each polymorphism alone and both together) using likelihood ratio tests. The model incorporating both polymorphisms fitted significantly better than the model with either −1263A>G or −712C>T alone (both comparison, $P < 0.01$).

The association between the *CASP-9* haplotypes of the four polymorphisms examined (−1263A>G, −905T>G, −712C>T and −293del) and the risk of lung cancer was examined. Fourteen out of the possible 16 ($2^4$) haplotypes were observed. For statistical advantage, eight haplotypes with a frequency of <2% were excluded from further analysis (data not shown). The remaining six haplotypes accounted for 94.5% of the chromosomes obtained from the 864 subjects (95.5% of the cases and 93.5% of the controls). Table 4 shows the inferred haplotype distributions for the cases and controls, as well as the lung cancer risk according to haplotype. The distribution of the inferred haplotypes of the cases was significantly different from that of the controls ($P < 0.001$). The adjusted OR and 95% CI for each haplotype were determined by comparing all the other haplotypes combined. The haplotypes, G-T-C-del$^-$ and G-G-C-del$^+$, which carry the −1263G and −712C alleles, were associated with a decreased risk of lung cancer (adjusted OR = 0.36, 95% CI = 0.18–0.71, $P = 0.003$ and $P_c = 0.018$ and adjusted OR = 0.69, 95% CI = 0.54–0.90, $P = 0.006$ and $P_c = 0.036$, respectively), whereas two haplotypes, A-G-C-del$^-$ and G-T-T-del$^+$, among four haplotypes carrying one risk allele (either the −1263A allele or the −712T allele) were associated with an increased risk of lung cancer (adjusted OR = 2.61, 95% CI = 1.69–4.05, $P$ and $P_c < 0.001$ and adjusted OR = 4.44, 95% CI = 2.11–9.33, $P$ and $P_c < 0.001$, respectively).

**Table 4.** Distribution of *caspase-9* haplotypes of −1263A>G, −905T>G, −712C>T and −293del[a] polymorphisms in the cases and controls

| Haplotypes | Cases, $n$[b] (%) | Controls, $n$[b] (%) | Adjusted OR (95% CI)[c] |
|---|---|---|---|
| A-T-C-del$^-$ | 427 (51.8) | 440 (54.5) | 0.92 (0.76–1.12) |
| A-G-C-del$^-$ | 76 (9.2) | 31 (3.8) | 2.61 (1.69–4.05)* |
| G-T-C-del$^-$ | 12 (1.5) | 32 (4.0) | 0.36 (0.18–0.71)** |
| G-T-T-del$^+$ | 39 (4.7) | 9 (1.1) | 4.44 (2.11–9.33)* |
| G-G-C-del$^+$ | 130 (15.8) | 169 (20.9) | 0.69 (0.54–0.90)*** |
| G-G-T-del$^+$ | 141 (17.1) | 127(15.7) | 1.06 (0.81–1.39) |

[a]−293_ − 275delCGTGAGGTCAGTGCGGGGA.
[b]Eight haplotypes that had a frequency of <2% were excluded from analysis; cases 39 and controls 56.
[c]Adjusted (for age, gender and pack-years of smoking) OR and 95% CI for each haplotype compared with all the other haplotypes combined as shown.
*$P$ and $P_c < 0.001$.
**$P = 0.003$ and $P_c = 0.018$.
***$P = 0.006$ and $P_c = 0.036$.

We next examined the association between the haplotypes of −1263A>G and −712C>T polymorphisms and the risk of lung cancer because the haplotypes G-T-C-del$^-$ and G-G-C-del$^+$, which carry the −1263G and −712C alleles, were associated with a decreased risk of lung cancer, and logistic regression analysis for each polymorphism revealed these two polymorphisms to be associated with the risk of lung cancer. The distribution of the inferred haplotypes among the cases was significantly different from those among the controls (Table 5, $P < 0.001$). The adjusted OR and 95% CI for each haplotype were calculated by comparison with all the other haplotypes combined. Consistent with the results of genotyping analyses, the G-C haplotype, with a no risk allele at each loci, was associated with a significantly decreased risk of lung cancer (adjusted OR = 0.59, 95% CI = 0.47–0.75, $P$ and $P_c < 0.001$), whereas the G-T haplotype was associated with a significantly increased risk of lung cancer (adjusted OR = 1.38, 95% CI = 1.08–1.77, $P = 0.01$ and $P_c = 0.04$). In order to examine further the effects of the G-C haplotype on the risk of lung cancer, the remaining three haplotypes (A-C, A-T or G-T) other than the G-C haplotype were grouped as 'others', and the subjects were categorized into three groups by carrying none, one or two G-C haplotypes [i.e. 'others'/'others'; G-C heterozygotes (G-C/'others') and G-C homozygotes (G-C/G-C)]. When the 'others'/'others' was used as the reference, the risk of lung

**Table 5.** Distribution of the *caspase-9* haplotypes (−1263A>G and −712C > T) in the cases and controls

| Haplotypes | Cases, *n* (%) | Controls, *n* (%) | Adjusted OR (95% CI)[a] |
|---|---|---|---|
| Haplotype | | | |
| A-C | 511 (59.1) | 488 (56.5) | 1.16 (0.95–1.40) |
| A-T | 8 (0.9) | 3 (0.4) | 2.77 (0.72–10.58) |
| G-C | 158 (18.3) | 231 (26.7) | 0.59 (0.47–0.75)* |
| G-T | 187 (21.6) | 142 (16.4) | 1.38 (1.08–1.77)** |
| G-C haplotype | | | |
| G-C/G-C | 13 (3.0) | 29 (6.7) | 0.34 (0.17–0.68)***,**** |
| G-C/Others[b] | 132 (30.6) | 172 (39.8) | 0.60 (0.45–0.81)****,***** |
| Others/Others | 287 (66.4) | 231 (53.5) | 1.0**** |

[a]Adjusted for age, gender and pack-years of smoking.
[b]Haplotype A-C, A-T or G-T.
*$P$ and $P_c$) < 0.0001.
**$P = 0.01$ and $P_c = 0.04$.
***$P = 0.002$ and $P_c = 0.005$.
****$P_{trend} < 0.001$.
*****$P = 0.0007$ and $P_c = 0.0014$.

cancer decreased in a dose-dependent manner as the number of the G-C haplotypes increased (adjusted OR = 0.60, 95% CI = 0.45–0.81, $P = 0.0007$ and $P_c = 0.0014$ for the G-C heterozygotes and adjusted OR = 0.34, 95% CI = 0.17–0.68, $P = 0.0023$ and $P_c = 0.0046$ for the G-C homozygotes; $P_{trend} < 0.001$).

The association between the *CASP-9* haplotypes of −1263A>G and −712C>T polymorphisms and the risk of lung cancer was examined further after stratifying the subjects according to age, gender, smoking status and tumor histology (Table 6). For this analysis, the remaining three haplotypes other than the G-C haplotype were also grouped as 'others' and was used as the reference. The protective effect of the G-C haplotype on the risk of lung cancer was similar in younger and older individuals, as well as in males and females. When stratified according to the smoking status, the protective effect of the G-C haplotype was significant in the smokers (adjusted OR = 0.57, 95% CI = 0.45–0.74, $P < 0.001$) but not in never-smokers. When the ever-smokers were dichotomized by the pack-years of smoking, the protective effect of the G-C haplotype was significant in the light smokers (adjusted OR = 0.46, 95% CI = 0.32–0.68, $P < 0.001$), whereas there was no significant association in the heavy smokers. The protective effect of the G-C haplotype on the lung cancer risk was observed for the non-small cell lung cancer and small cell lung cancer, although this was only statistically significant for the non-small cell lung cancer (adjusted OR equals; 0.56, 95% CI equals; 0.44–0.72, $P < 0.0001$).

In addition to the stratification analyses, the joint effects of the *CASP-9* haplotypes of −1263A>G and −712C>T polymorphisms and smoking status on the risk of lung cancer were also investigated (Table 7). When the group of never-smokers with the G-C haplotype was used as the reference group, the group of heavy smokers with the 'others' carried the highest risk (adjusted OR = 5.30, 95% CI = 2.86–9.81, $P < 0.0001$). The group of light smokers with the 'others' had a significantly increased risk of lung cancer compared with the reference group (adjusted OR = 2.36, 95% CI = 1.30–4.30, $P = 0.005$), whereas the group of light smokers with the G-C haplotype had a similar risk to the reference group (adjusted OR = 1.08, 95% CI = 0.55–2.12). Nevertheless, we did not observe statistically significant evidence for interactions between the caspase-9 haplotypes and smoking in the multivariate logistic regression analysis ($P$ equals; 0.26 for the interaction term).

The effects of the −1263A>G and −712C>T polymorphisms on the promoter activity of *CASP-9* were investigated using a luciferase assay. The promoter activity of the three common haplotypes (i.e. A-C, G-C and G-T) was compared because these two polymorphisms were in LD. In A549 cells, the G-C haplotype significantly increased promoter activity compared with the G-T haplotype and the A-C haplotype ($P < 0.001$ and $P < 0.01$, respectively). Similarly, in 1299 cells, the G-C haplotype significantly increased promoter activity compared with the G-T haplotype ($P < 0.01$, Fig. 1).

## DISCUSSION

This study investigated the potential association between *CASP-9* polymorphisms (−1263A>G, −905T>G, −712C>T and −293del) and the risk of lung cancer. In addition, the *CASP-9* haplotypes of four polymorphisms were estimated, and their frequency distributions in lung cancer cases and controls were compared. Among the four polymorphisms examined, the −1263A>G and −712C>T polymorphisms and their haplotypes were associated with risk of lung cancer. This finding suggests that polymorphisms in the *CASP-9* gene might be useful markers for determining genetic susceptibility to lung cancer. Moreover, the results suggest that the *CASP-9* gene might be involved in the development of lung cancer. Several studies have demonstrated that polymorphisms in the cell death pathway genes *Fas* and *FasL* contribute to the genetic susceptibility to various human cancers including lung cancer (19–22). This study is an important addition to previously published work investigating polymorphisms in the genes involved in the apoptotic pathways as markers for genetic susceptibility to cancer.

In this study, the G-C haplotype with a no-risk allele at both −1263A>G and −712C>T loci was associated with a significantly decreased risk of lung cancer compared with either the A-C or the G-T haplotypes carrying one risk allele at either the −1263A>G locus or the −712C>T locus. To determine if the association between the *CASP-9* haplotypes and the risk of lung cancer is due to differences in the transcriptional activity of the *CASP-9* promoter, we compared the promoter activity of these three haplotypes using a luciferase assay. The *in vitro* promoter assay revealed the G-C haplotype to have a significantly higher transcriptional activity than the G-T haplotype and the A-C haplotype. This suggests that the haplotypes of the −1263A>G and −712C>T polymorphisms influence *CASP-9* expression, thus contributing to the genetic susceptibility to lung cancer. The mechanism by which the *CASP-9* G-C haplotype leads to a higher promoter activity is unknown. An analysis of the potential transcription factor-binding sites using the Alibaba2 program (23) showed that the −1263 A to G change leads to the

**Table 6.** Analysis of *caspase-9* haplotype (−1263A>G/−712C>T) frequencies, ORs and 95% CIs for lung cancer by selected variables

| | Cases ($n$ =864) | | Controls ($n$ = 864) | | Adjusted OR (95% CI) for the haplotype G-C versus others | $P$-value |
|---|---|---|---|---|---|---|
| | G-C | Others[a] | G-C | Others[a] | | |
| All subjects | 158 (18.3) | 706 (81.7) | 231 (26.7) | 633 (73.3) | 0.59 (0.47–0.75)[b] | <0.0001 |
| Age (years) | | | | | | |
| <62 | 59 (17.0) | 289 (83.0) | 123 (28.7) | 305 (71.3) | 0.49 (0.35–0.70)[c] | <0.0001 |
| ≥62 | 99 (19.2) | 417 (80.8) | 108 (24.8) | 328 (75.2) | 0.70 (0.51–0.96)[d] | 0.03 |
| Gender | | | | | | |
| Male | 135 (19.2) | 569 (80.8) | 192 (27.3) | 512 (72.7) | 0.61 (0.47–0.78)[e] | 0.001 |
| Female | 23 (14.4) | 137 (85.6) | 39 (24.4) | 121 (75.6) | 0.54 (0.30–0.96)[e] | 0.04 |
| Smoking status | | | | | | |
| Never | 26 (17.1) | 126 (82.9) | 42 (20.0) | 168 (80.0) | 0.76 (0.44–1.32)[d] | 0.33 |
| Ever | 132 (18.5) | 580 (81.5) | 189 (28.9) | 465 (71.1) | 0.57 (0.45–0.74)[d] | <0.0001 |
| Smoking level[f] | | | | | | |
| ≤39 pys[g] | 45 (16.1) | 235 (83.9) | 120 (30.3) | 276 (69.7) | 0.46 (0.32–0.68)[d] | <0.0001 |
| >39 pys | 87 (20.1) | 345 (79.9) | 69 (26.7) | 189 (73.3) | 0.69 (0.48–1.00)[d] | 0.05 |
| Histologic type | | | | | | |
| NSCLC[h] | 126 (17.6) | 592 (82.4) | 231 (26.7) | 633 (73.3) | 0.56 (0.44–0.72)[b] | <0.0001 |
| SCLC[i] | 32 (21.9) | 114 (78.1) | 231 (26.7) | 633 (73.3) | 0.74 (0.48–1.14)[b] | 0.18 |

[a]Haplotype A-C, A-T or G-T.
[b]Adjusted for age, gender and pack-years of smoking.
[c]Adjusted for gender and pack-years of smoking.
[d]Adjusted for age and gender.
[e]Adjusted for age and pack-years of smoking.
[f]In ever-smokers (current and former).
[g]Pack-years of smoking.
[h]Non-small cell lung cancer.
[i]Small cell lung cancer.

**Table 7.** Interaction of *caspase-9* haplotypes (−1263A>G/−712C>T) and tobacco smoking on risk of lung cancer

| Smoking status | *Caspase-9* haplotype | | | |
|---|---|---|---|---|
| | G-C | OR[a] (95% CI) | Others[b] | OR[a] (95% CI) |
| Never smoker | 26/42[c] | 1.0 (reference) | 126/168 | 1.32 (0.76–2.30) |
| Smoker | | | | |
| ≤39 pack-years | 45/120 | 1.08 (0.55–2.12) | 235/276 | 2.36 (1.30–4.30)* |
| >39 pack-years | 87/69 | 3.67 (1.88–7.15)** | 345/189 | 5.30 (2.86–9.81)*** |

$P = 0.26$ for the interaction term between haplotype and smoking in the multivariate model.
[a]Data were calculated by logistic regression, with haplotype G-C in never-smokers as reference group and adjusted for age and gender.
[b]Haplotype A-C, A-T or G-T.
[c]Number of cases/controls.
*$P = 0.005$.
**$P = 0.0001$.
***$P < 0.0001$.

creation of an additional simian virus-40 protein 1-binding site, whereas the −712 C to T change eliminates Krox-20, NF-1 and ETF-binding sites. Therefore, it is possible that the predicted changes in the putative transcription factor-binding sites owing to the −1263A>G and −712C>T polymorphisms might lead to enhanced promoter activity. However, this hypothesis has to be verified in future studies.

In this study, the G-C haplotype with a higher *CASP-9* promoter activity was associated with a significantly decreased risk of lung cancer. This suggests that the 'higher production' haplotype for CASP-9 may offer protection against the development of lung cancer. This is biologically plausible because CASP-9, as an initiator CASP, plays an important role in the apoptosome-driven apoptosis pathway, which is essential for eliminating mutated or transformed cells from the body (5,6,24,25).

Recent studies have demonstrated that an analysis of the haplotypes might be superior in predicting the disease associations compared with an analysis of a single polymorphism (21,22,26,27). Therefore, our investigation was extended to analyzing the haplotypes of −1263A>G and −712C>T polymorphisms. Although logistic regression analysis of each polymorphism showed that both polymorphisms were associated with the risk of lung cancer, these two polymorphisms within a haplotype had an additive effect on the risk of lung cancer when considering (i) the model incorporating both polymorphisms fitted significantly better than the model with −1263A>G or −712C>T alone; and (ii) the effect of the G-C haplotype on the risk of lung cancer was greater than either −1263G or −712T alone.

Another interesting finding of this study was that *CASP-9* polymorphisms and their haplotypes interacted with tobacco smoking. *CASP-9* polymorphisms were significantly associated with the risk of lung cancer in the smokers but not in the never-smokers, which reflects a gene–environment interaction. Such an interaction is biologically plausible because smoking is a major risk factor for lung cancer. It was also found that the association between *CASP-9* polymorphisms and the risk of lung cancer was statistically significant in the light smokers but not in the heavy smokers. This might be

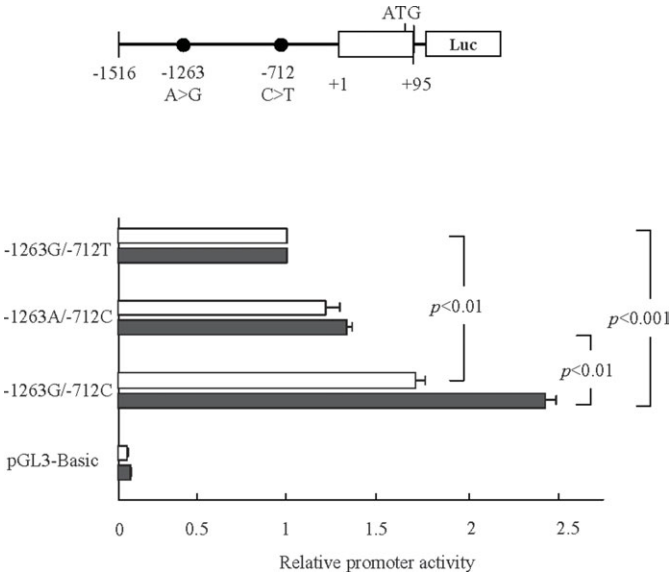


**Figure 1.** Transcription activity analysis of *CASP-9* haplotypes of the −1263A>G and −712C>T polymorphisms. The transcription activity was measured using the Dual-Luciferase Reporter Assay System in H1299 cells (white columns) and A549 cells (black columns). Columns, means from four independent experiments done in triplicate; bars, SD. In A549 cells, the −1263G/−712C haplotype significantly increased promoter activity compared with the −1263G/−712T haplotype and the −1263A/−712C haplotype ($P < 0.001$ and $P < 0.01$, respectively). Similarly, in 1299 cells, the G-C haplotype significantly increased promoter activity compared with the G-T haplotype ($P < 0.01$). Luc, luciferase.

due to that the effect of genetic differences on the risk may be smaller at higher dose of carcinogen when environmental influences may overpower any genetic predisposition (21,28,29). However, the failure to observe a significant effect in both never-smokers and heavy smokers might be due to the relatively small number of subjects in these groups. Therefore, additional studies with a greater number of subjects are needed to confirm these findings.

The genetic effect of polymorphisms on the lung cancer risk often depends on age and gender (30–33). In addition, it is possible that the *CASP-9* genotypes/haplotypes have different effects on the lung cancer risk according to the histological type because the different histological types of lung cancer are distinct diseases in terms of their etiologies and carcinogenesis pathways (34,35). Therefore, stratification analysis was performed to determine if the *CASP-9* genotypes/haplotypes might have differential effects on the risk of lung cancer according to age, gender and tumor histology. However, in this study, there was no clear evidence that gender, age or tumor histology modified the effect of the *CASP-9* polymorphisms on the risk of lung cancer.

The other five candidate polymorphisms (−1290A>T, −935C>G, −503C>G, −315A>C and −185C>G) were not detected in the preliminary study including 27 healthy controls. These samples included 54 chromosomes, which provided at least a 95% confidence level in detecting the alleles with frequencies >5%. Therefore, it is very likely that if this polymorphism exists, it may not play a major role in the genetic susceptibility to lung cancer in the Korean population (36,37).

Genetic polymorphisms often show ethnic variation. In this study, the frequency of the variant alleles of the −1263A>G, −905T>G, −712C>T and −293del polymorphisms among healthy controls were 0.432, 0.410, 0.168 and 0.395, respectively; these were different from the frequencies (0.523, 0.525, 0.013 and 0.464, respectively) reported in the NIH Database (http://www.ncbi.nlm.nih.gov/SNP). Ethnic variation in the *CASP-9* polymorphisms warrants additional study to clarify the association of the *CASP-9* polymorphism with the risk of lung cancer in diverse ethnic populations.

In conclusion, *CASP-9* promoter polymorphisms and their haplotypes have an influence on the *CASP-9* promoter activity and are significantly associated with the risk of lung cancer. The association between *CASP-9* polymorphisms and the risk of lung cancer appeared to be influenced by tobacco smoking. These results suggest that the *CASP-9* gene may be involved in the development of lung cancer. Because genetic polymorphisms often show ethnic variation, further studies are needed to clarify the association between *CASP-9* polymorphisms and lung cancer in diverse ethnic populations.

## MATERIALS AND METHODS

### Study population

This case–control study included 432 lung cancer patients and 432 age and gender matched healthy controls. The details of the study population are described elsewhere (38–41). Briefly, eligible cases included all patients who were newly diagnosed with primary lung cancer at the Kyungpook National University Hospital, Daegu, Korea from January 2001 to February 2002. There were no age, gender, histological or stage restrictions; however, patients with a prior history of cancer were excluded. The cases included 210 (48.6%) squamous cell carcinomas, 141 (32.6%) adenocarcinomas, 73 (16.9%) small cell carcinomas and 8 (1.9%) large cell carcinomas. The control subjects were randomly selected from a pool of healthy volunteers who visited the general health check-up center at Kyungpook National University Hospital during the same period. The control subjects were frequency-matched (1:1) to cancer cases on the basis of gender and age (±5 years). All cases and controls were ethnic Koreans who resided in Daegu City or in the surrounding regions. A trained interviewer completed a detailed questionnaire for each patient and control. The questionnaire included information on the average number of cigarettes smoked per day and the number of years the subjects had been smoking. For the smoking status of the subjects, a person who had smoked at least once a day for more than 1 year during the lifetime was regarded as a smoker. A former smoker was defined as one who had stopped smoking at least 1 year before either the diagnosis of lung cancer (cases) or the date the informed consent form had been signed (controls). The cumulative cigarette dose (pack-years) was calculated using the formula: pack-years = packs per day × years smoked. This study was approved by the institutional review board of the Kyungpook National University Hospital, and written informed consent was obtained from each participant.

**Table 8.** Primer sequences, annealing temperatures and restriction enzymes for genotyping of *caspase-9* polymorphisms

| Polymorphism[a] | rs number[b] | Primer | T (°C)[c] | Restriction enzyme |
|---|---|---|---|---|
| −1263A>G | 4645978 | F:5′-GGGAATACTTTCCTGGCAGG-3′<br>R: 5′-GTCTTCCATTCCCTCTTCCG(C → G)TC-3′ | 59 | *Bsm*AI |
| −905T>G | 4645980 | F:5′-GAAGAGGGAATGGAAGACTGTG-3′<br>R: 5′-GCCCCCGGGGTCAATCCTCA-3′ | 56 | *Bse*YI |
| −712C>T | 4645981 | F:5′-AGTCGCGGAGGTGCCGCCTT-3′<br>R: 5′-AGGGCTAGCCTCGTGCCAG(C → G)C-3′ | 59 | *Hae*II |
| 293del[d] | 4645982 | F:5′-CGTTGGAGATGCGTCCTGCG-3′<br>R: 5′-CGCCCTCAGGACGCACCTCT-3′ | 60 | — |

[a]Calculated from transcription start site of exon 1 (reference genomic sequence, AY2141168).
[b]Accessible at http://www.ncbi.nlm.nih.gov/SNP.
[c]Annealing temperature for PCR amplification.
[d]−293_−275delCGTGAGGTCAGTGCGGGGA.

### Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol/chloroform extraction. The *CASP-9* −1263A>G, −905T>G and −712C>T genotypes were determined using a PCR-RFLP assay, and the −293del genotypes ($-293del^{-/-}$, $del^{-/+}$, $del^{+/+}$) were tested using a PCR assay. The PCR primers were designed on the basis of the GenBank reference sequence (accession no. AY214168). Table 8 shows the primer sequences and annealing temperatures used in PCRs. The PCR reactions were performed in a total volume of 20 μl containing 100 ng of the genomic DNA, 10 pM of each primer, 0.2 mM dNTPs, 1 unit of *Taq* polymerase (New England BioLabs, Beverly, MA, USA) and 1× reaction buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100]. For the −1263A>G, −905T>G and −712C>T genotyping, the PCR products were digested overnight with the appropriate restriction enzymes (New England BioLabs, Table 8) at 37°C. The digested PCR products were resolved on 6% acrylamide gel (−1263A>G and −712C>T) or 2% agarose gel (−905T>G) and stained with ethidium bromide for visualization under UV light. For the −293del genotyping, the PCR products were resolved on 8% acrylamide gel. Genotyping analysis was performed 'blind' with respect to the case/control status in order to ensure quality control. Approximately 10% of samples were randomly selected to be genotyped again by a different investigator, and the results showed 100% concordance. The genotyping results were confirmed by examining selected PCR-amplified DNA samples ($n = 2$, respectively, for each genotype) by DNA sequencing. The results were also 100% concordant.

### Promoter–luciferase constructs

To examine the potential effects of the −1263A>G and −712C>T polymorphisms on the *CASP-9* transcription activity, we compared the promoter activity of the three common haplotypes [i.e. −1263A/−712C (A-C), −1263G/−712C (G-C) and −1263G/−712T (G-T)]. The fragments of the *CASP-9* promoter region (from −1516 to +95, transcription start site of exon 1 counted as +1) were synthesized by PCR using genomic DNA from donors carrying each haplotype. The PCR primers for the *CASP-9* promoter were 5′-CGGGGTACCCCCTGAACCCTAGGGTCTAC-3′ (forward, *Kpn*I restriction site) and 5′-CCCAAGC TTGTCCATGGCGAGTAGCCAA-3′ (reverse, *Hin*dIII restriction site). The PCR products were inserted upstream of the luciferase gene in the pGL3-basic plasmid (Promega, Madison, WI, USA). The correct sequence of all the clones was verified by DNA sequencing.

### Transient transfection and luciferase assay

The promoter activity was measured using the Dual Luciferase Reporter Assay System (Promega) in A549 and H1299 lung cancer cells. The A549 and H1299 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Cells ($1 \times 10^5$) were plated on a six-well plate the day before transfection in order for the cells to be ~60% confluent by the next day. The pRL-SV40 plasmid and the pGL3-basic plasmid with the synthesized fragments of the *CASP-9* promoter region were cotransfected using Lipofectine reagent (Invitrogen, Carlsbad, CA, USA). The pRL-SV40 vector that provided the constitutive expression of *Renilla* luciferase was used as an internal control to correct for the differences in transfection and harvesting efficiency. The cells were collected 48 h after transfection, and the cell lysates were prepared according to Promega's instruction manual. Luciferase activity was measured using a Lumat LB953 luminometer (EG & G Berthhold, Bad Wildbad, Germany), and the results were normalized using the activity of *Renilla* luciferase. Independent triplicate experiments were performed four times, and the results were reported as mean ± standard deviation.

## STATISTICAL ANALYSIS

The cases and controls were compared using the Student's *t*-test for continuous variables and the $\chi^2$ test for categorical variables. Hardy–Weinberg equilibrium was tested for using the goodness-of-fit $\chi^2$ test with one degree of freedom to compare observed genotype frequencies with expected genotype frequencies among the subjects. The LD among the polymorphisms was examined using Lewontin's standardized

coefficient $D'$ ($|D'|$) (42) and LD coefficient $r^2$. The haplotypes and their frequencies were estimated on the basis of a Bayesian algorithm using the Phase program (43). In order to confirm the inferred haplotypes by the Phase program, we further used the Haplo.stats program (http://www.mayo.edu/hsr/Sfunc.html) developed by Schaid *et al*. (44). The two methods yielded similar results (data not shown). As a result, the Phase program was used for subsequent analyses. The cancer risk associated with the genotypes and haplotypes was estimated as an OR and 95% CI using unconditional logistic regression. Crude ORs and ORs adjusted for possible confounders (gender as a nominal variable; age and pack-years smoked as continuous variables) were calculated. When multiple comparisons are made, the $P_c$ values were also calculated for multiple testing using Bonferroni's inequality method. For the gene–smoking interaction analyses, we used three approaches to evaluate consistency of results: (i) stratified analyses in specific categories of cumulative smoking exposure, (ii) genotype/haplotype–smoking joint effects and (iii) logistic regression model including the interaction term between genotype/haplotype and smoking. For these analyses, the subjects were categorized into three groups according to the level of smoking exposure: never-smokers, ever-smokers $\leq 39$ pack-years (light smokers) and ever-smokers $> 39$ pack-years (heavy smokers, by median pack-year value of the ever-smokers). All the analyses were performed using Statistical Analysis Software for Windows, version 8.12 (SAS institute, Gary, NC, USA).

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*Conflict of Interest statement*. None declared.

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