RESEARCH ARTICLE

Interaction effects between genes involved in the AKT signaling pathway and phytoestrogens in gastric carcinogenesis: A nested case–control study from the Korean Multi-Center Cancer Cohort

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Scope: To investigate whether genes involved in AKT/nuclear factor kappa B signaling and/or gene–environment interactions between the genes and phytoestrogens may be susceptible factors for gastric cancer.

Methods and results: The representative single nucleotide polymorphisms (SNPs) identified during the primary analysis (screening a total of 622 SNPs within ± 5 kbp of the 51 target gene locations) were further investigated in 317 matched case–control sets. The summary odds ratios (ORs) and 95% confidence intervals (CIs) for gastric cancer were calculated. Interaction effects between the SNPs and phytoestrogen biomarkers (genistein, daidzein, equol, and enterolactone) were computed. *CDK1* rs4145643, *FAS* rs6586161, and *FAS* rs1468063 in the AKT signaling pathway presented significant genetic effects on gastric cancer (OR = 0.81 (95% CI: 0.66–0.99) for *CDK1* rs4145643; OR = 1.27 (95% CI: 1.03–1.58) for *FAS* rs6586161; OR = 1.29 (95% CI: 1.03–1.56) for *FAS* rs1468063; Cochran Q statistics > 0.10). Risk alleles of *FAS* rs6586161, *FAS* rs1468063, *MAP3K1* rs16886448, and *MAP3K1* rs252902 showed significant interaction effects with enterolactone ($p_{interaction} < 0.05$).

Conclusion: *CDK1* and *FAS* genes involved in AKT signaling and influenced by anti-carcinogenic property of phytoestrogens can play a role as susceptible genetic factors in gastric carcinogenesis. *FAS* and *MAP3K1* genes significantly interact with enterolactone, thereby modifying the individual's risk for gastric cancer.

Keywords:
AKT/NF-κB signaling / *CDK1* / Enterolactone / *FAS* / *MAP3K1*

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Abbreviations: BH-FDR, Benjamini–Hochberg false discovery rate; **CIs**, confidence intervals; **ERs**, estrogen receptors; **HWE**, Hardy–Weinberg equilibrium; **IRB**, Institutional Review Board; **KMCC**, Korean Multi-Center Cancer Cohort; **NF-κB**, nuclear factor kappa B; **ORs**, odds ratios; **SNPs**, single nucleotide polymorphisms

1 Introduction

Phytoestrogens are plant-derived compounds that can mimic the function of the female sex hormone, estrogen [1]. Estrogens influence the physiology of diverse target cells by interacting with two estrogen receptors (ERs; ER-α, and ER-β) [2], and phytoestrogens can mimic, modulate, and/or disrupt the actions of endogenous estrogens by binding to ERs [1]. There are two main classes of phytoestrogens, isoflavones, and lignans, that are common in the human diet: isoflavones such as genistein and daidzein are a flavonoid subclass that occurs mainly in soy products, whereas lignans such as enterolactone and enterodiol are nonflavonoid compounds that are widely distributed in vegetables, seeds, and whole-grain foods [3, 4]. A traditional Asian diet includes a rich phytoestrogen food-matrix repository, which exhibits high phytoestrogen bioavailability and results in steady-state plasma phytoestrogen concentrations [5]. Average daily intakes of phytoestrogens have been reported to be between 20 and 50 mg in Asian populations, whereas it is less than 3 mg in Western populations [6]; similarly, plasma phytoestrogen concentrations are also reported to be higher in Asians than in Europeans [7, 8].

As phytoestrogens have the capacity to function as an estrogen mimic, the putative effect of the anticarcinogenic properties of phytoestrogens on hormone-related carcinomas including breast, endometrial, and prostate cancers has been widely discussed, but the relationships remain controversial. Several epidemiologic studies have demonstrated that: (i) dietary lignan intakes were significantly associated with a reduced risk of breast cancer among pre- and postmenopausal women [9, 10]; (ii) isoflavone-rich foods and/or lignan intake have reduced the risk of endometrial cancer [11, 12]; and (iii) soy food consumption (i.e. isoflavones) has lowered the risk of both prostate and ovarian cancers [13, 14]. Additionally, emerging evidence not focused on the hormone-specific cancers has suggested that phytoestrogens might play a protective role in lung, gastric, and colorectal cancers [14–16]. On the other hand, there is doubt on the preventive effects of phytoestrogens because some studies have: (i) not shown the statistical significances of the inverse associations between those cancers and phytoestrogens [17, 18]; (ii) found significantly opposite results in studies [19–21]; and (iii) not determined whether phytoestrogen-interventions could guarantee protective effects against cancers and/or cancer-related risk factors [22–25].

Our previous study indicated that phytoestrogens could modify the absolute risk of gastric cancer by interacting with susceptible genetic factors involved in the cytotoxin-associated gene A (CagA) transduction pathways, one of the major targets involved in gastric carcinogenesis [26]. With regard to molecular mechanisms, because they have been shown induce anti-inflammatory responses against *Helicobacter pylori* (*H. pylori*), deemed a group I human carcinogen by the International Agency for Research on Cancer (IARC), phytoestrogens appear to act as an effect modifier and/or independent protective factor in gastric carcinogenesis. Moreover, among the signal transduction pathways involved in gastric carcinogenesis, such as nuclear factor kappa B (NF-κB), extracellular signal-regulated kinase, growth factor beta, epidermal growth factor receptor, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways [27–30], phytoestrogens can inhibit AKT and NF-κB signaling, which regulates cell growth, proliferation, migration, differentiation, and apoptosis [31–34]. Through such inhibition, phytoestrogens may attenuate the growth and/or survival of cancer cells. Given that *H. pylori* can activate PI3K/AKT and NF-κB signaling in gastric epithelial cells and trigger mucosal inflammation [30], it is assumed that phytoestrogens may interfere with *H. pylori*-induced gastric carcinogenesis; thereby modifying the risk for gastric cancer. Indeed, studies have demonstrated that isoflavones and lignans can obstruct *H. pylori* proliferation and growth in vitro [35, 36]; thus, blocking an initial step toward the development of gastric cancer.

Phytoestrogens are assumed to be potent inhibitors of gastric carcinogenesis [16, 26, 37]. Moreover, interaction between phytoestrogens and the genes related to the major signal transduction systems in gastric carcinogenesis may alter an individual's susceptibility to gastric cancer. Thus, we hypothesized that: (i) genes involved in the AKT/NF-κB signaling pathways are influenced by anticarcinogenic properties of phytoestrogens, and (ii) gene–environment interactions, between genetic variants, on the AKT/NF-κB pathway and phytoestrogens may be factors for gastric cancer susceptibility. To evaluate those hypotheses, we initially conducted a two-stage genetic association study that included: (i) discovery analysis, a candidate gene approach that focused on 27 genes involved in the AKT signaling pathway and 24 genes in the NF-κB signaling pathway; and (ii) extension analysis that further examined the significant single nucleotide polymorphisms (SNPs) identified in the discovery analysis. Following those analyses, risk modification based on gene–environment interactions between serum concentrations of four phytoestrogens, consisting of three isoflavones (genistein, daidzein, and equol) and one lignan (enterolactone), and genetic polymorphisms on the AKT/NF-κB pathway was investigated.

2 Materials and methods

2.1 Study population

This study was conducted by using a two-stage approach (i.e. discovery and extension phases). In the discovery phase to identify putative genetic markers for gastric cancer, 100 matched case-controls were selected within the Korean Multi-Center Cancer Cohort (KMCC). From 1993 to 2004, a total of 19 688 healthy adults voluntarily participated in the cohort study. All participants (i) signed an informed consent

before entering the study; (ii) completed detailed standardized questionnaires by participating in a personal interview; (iii) donated blood and urine samples; and (iv) were passively followed-up through record linkages to the national death certificate and health insurance medical records databases and to the national cancer registry. A detailed description of the KMCC study protocol has been described elsewhere [38]. In December of 2005, newly diagnosed gastric cancer cases ($N = 197$) were determined according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10, C16). Excluding cases without blood samples ($N = 35$) or with a DNA level under 50 ng/μL ($N = 62$), each of the remaining 100 gastric cancer cases were matched to a cancer-free control from the KMCC by age (±5 years), sex, residential district, and enrollment year. Thus, 100 gastric cancer cases and 100-matched controls were included in the discovery phase.

In the extension phase, to evaluate further the representative genetic markers identified during the discovery analysis, 128 additional gastric cancer cases obtained from the KMCC in December 2008 and 189 gastric cancer cases obtained from two university hospitals in Korea (Chungnam University Hospital and Hanyang University GURI Hospital) were selected. Each of those 317 cases were individually matched to a cancer-free control from the KMCC by age (±5 years), sex and recruitment year.

2.2 Ethics statement

The KMCC study protocols and the current nested case–control study were approved by the Institutional Review Board (IRB) of Seoul National University Hospital and the National Cancer Center of Korea (H-0110–084-002 and C-0603–161-170, respectively). Hospital-based gastric cancer cases were recruited with IRB approval at the Hanyang University Hospital (IRB no.2003–4).

2.3 Gene and SNP selection

Through literature review, 27 candidate genes that may be involved in the AKT signaling pathway and 24 genes in the NF-κB signaling pathway were identified. Based on the HUGO Gene Nomenclature Committee (http://www.genenames.org), the 51 candidate genes were approved as follows: (i) AKT pathway: caspase 9, apoptosis-related cysteine peptidase (*CASP9*); caspase 1, apoptosis-related cysteine peptidase (*CASP1*); caspase 14, apoptosis-related cysteine peptidase (*CASP14*); caspase 10, apoptosis-related cysteine peptidase (*CASP10*); caspase 8, apoptosis-related cysteine peptidase (*CASP8*); caspase 7, apoptosis-related cysteine peptidase (*CASP7*); caspase 6, apoptosis-related cysteine peptidase (*CASP6*); caspase 5, apoptosis-related cysteine peptidase (*CASP5*); caspase 4, apoptosis-related cysteine peptidase (*CASP4*); caspase 3, apoptosis-related cysteine peptidase (*CASP3*); caspase 2, apoptosis-related cysteine peptidase (*CASP2*); caspase 12 (gene/pseudogene) (*CASP12*); vascular endothelial growth factor A (*VEGFA*); B-cell CLL/lymphoma 2 (*BCL2*); BCL2-like 1 (*BCL2L1*); BCL2-associated X protein (*BAX*); cyclin-dependent kinase inhibitor 1A (p21, Cip1) (*CDKN1A*); tumor protein p53 (*TP53*); cyclin-dependent kinase inhibitor 2A (*CDKN2A*); cyclin-dependent kinase 6 (*CDK6*); cyclin-dependent kinase 1 (*CDK1*); matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) (*MMP9*); phosphatase and tensin homolog (*PTEN*); Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse) (*MDM2*); forkhead box O3 (*FOXO3*); Fas (TNF receptor superfamily, member 6) (*FAS*); BCL2-antagonist/killer 1 (*BAK1*); (ii) NF-κB pathway: conserved helix-loop-helix ubiquitous kinase (*CHUK*); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (*NFKBIA*); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (*NFKBIB*); mitogen-activated protein kinase 1 (*MAPK1*); mitogen-activated protein kinase kinase 3 (*MAP2K3*); mitogen-activated protein kinase kinase 6 (*MAP2K6*); mitogen-activated protein kinase kinase kinase 3 (*MAP3K3*); mitogen-activated protein kinase kinase kinase 7 (*MAP3K7*); mitogen-activated protein kinase kinase kinase 5 (*MAP3K5*); mitogen-activated protein kinase 12 (*MAPK12*); mitogen-activated protein kinase 13 (*MAPK13*); mitogen-activated protein kinase 14 (*MAPK14*); mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase (*MAP3K1*); nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*); v-rel reticuloendotheliosis viral oncogene homolog A (avian) (*RELA*); phosphoinositide-3-kinase, catalytic, alpha polypeptide (*PIK3CA*); phosphoinositide-3-kinase, catalytic, beta polypeptide (*PIK3CB*); phosphoinositide-3-kinase, catalytic, gamma polypeptide (*PIK3CG*); phosphoinositide-3-kinase, catalytic, delta polypeptide (*PIK3CD*); phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (*PIK3R1*); phosphoinositide-3-kinase, regulatory subunit 3 (gamma) (*PIK3R3*); phosphoinositide-3-kinase, regulatory subunit 5 (*PIK3R5*); phosphoinositide-3-kinase, regulatory subunit 6 (*PIK3R6*); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (*NFKBIE*).

2.4 Genotyping

In the discovery phase, genotyping was performed using the Genome-Wide Human SNP Array 5.0 according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA) [39]. Before genotyping, genomic DNA concentrations were measured using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies). For each assay, 250 ng of genomic DNA was digested with a restriction enzyme (Nsp I or Sty I). A total of 622 SNPs (324 SNPs in the AKT pathway and 298 SNPs in the NF-κB pathway) within ± 5 kbp of the

51 target gene locations were screened; 116 polymorphisms were excluded due to the use of a SNP call rate of less than 95% ($N = 115$) or a Hardy–Weinberg equilibrium (HWE) value less than 0.0001 ($N = 1$). Among the 200 subjects, 20 cases and 14 controls were excluded due to sex discordance or poor genotyping (<90%). Finally, 506 SNPs in 51 genes (genotyping rate of 99.5%) were genotyped in 81 cases and 85 controls. Cluster images of signal intensity were reviewed for all SNPs.

Using the Illumina VeraCode GoldenGate Assay with BeadXpress according to the manufacturer's protocol (Illumina, San Diego, CA, USA) [40], 11 SNPs that had a raw p value < 0.01 or were identified as tag SNPs (*PTEN* rs11202600, *CDK1* rs4145643, *FAS* rs6586161, *FAS* rs1468063, *MAP3K7* rs150125, *MAP3K7* rs205339, *MAP3K7* rs205352, *MAPK1* rs5999749, *MAP3K1* rs16886448, *MAP3K1* rs252902, *PIK3R5* rs9895992) during the discovery phase were genotyped for the extension phase. To ensure the reliability of the two genotyping methods, the Genome-Wide Human SNP Array 5.0 and the Illumina VeraCode GoldenGate Assay, 135 samples were genotyped twice by using both methods and the concordance rate was 98.2%. Of the 11 SNPs, *MAP3K7* rs205339, *MAP3K7*rs205352, and *PIK3R5* rs9895992 were excluded due to a SNP call rate <95% or a low design score. Finally, the significant SNPs at the discovery phase (genotyping rate of 99.2% for the AKT pathway and 99.1% for the NF-κB pathway) were analyzed in 317-matched case–control sets.

2.5 Evaluation of *H. pylori* status and CagA seropositivity

Using immunoblot assay, Helico Blot 2.1™ (MP Biomedicals Asia Pacific, Singapore), all study subjects were evaluated their *H. pylori* infection status and CagA seropositivity. Helico Blot 2.1™ kits have reported high sensitivity (99% for both *H. pylori* and CagA seropositivity) and specificity (98% for *H. pylori* and 90% for CagA seropositivity) among Korean population [41].

2.6 Measurement of phytoestrogen biomarkers: genistein, daidzein, equol, and enterolactone

Plasma concentrations of four phytoestrogen biomarkers, three isoflavones (genistein, daidzein, and equol), and one lignan (enterolactone), were measured with time-resolved fluoroimmunoassay (TR-FIA) kits according to the manufacturer's instructions (Labmaster, Turku, Finland). Detailed methods for measurement of the biomarkers have been described elsewhere [16]. Briefly, after the free phytoestrogen biomarkers were extracted from 200 μL of each subject's plasma sample, the extracts' time-resolved fluorescence was measured by using a VICTOR3™ 1420 Multilabel Counter (Perkin-Elmer, Waltham, MA, USA). Standard curves produced by diluted standards (final concentrations: 300 nmol/L for genistein, 240 nmol/L for daidzein, 330 nmol/L for equol, and 300 nmol/L for enterolactone) were run with each assay. Plasma concentrations were calculated including analytic recovery and dilution information based on the formula: Final result = concentration (a reading value) × 0.8 (recovery, %) × dilution factor [42]. Measurements were obtained from extension phase subjects with sufficient plasma volume (>200 μL), i.e. from 191 gastric cancer cases and their matched controls.

2.7 Statistical analysis

Using Fisher's exact test, with a HWE cut-off level of <0.0001, the HWE within the control group was evaluated before genetic analyses.

In the discovery phase, an individual SNP effect on the risk for gastric cancer was evaluated based on both raw and permutated p values using the likelihood-ratio test with 1 degree of freedom in the additive model. Permutated p values were estimated by using 100 000 permutation tests. Absolute risk for gastric cancer was estimated as odds ratios (ORs) and 95% confidence intervals (CIs) using an unconditional logistic regression model and adjusting for the risk factors of age, smoking status (ever versus never), *H. pylori* infection (positive versus negative), and CagA seropositivity (positive versus negative). To avoid spurious association with false positive results, Benjamini–Hochberg false discovery rate (BH-FDR) corrected p values were computed.

In the extension phase, the most significant SNPs that had raw p value <0.01 or were identified as tag SNPs in the discovery phase were reevaluated. Using a conditional logistic regression model and adjusting for risk factors, including smoking status (ever versus never), *H. pylori* infection (positive versus negative), and CagA seropositivity (positive versus negative), ORs and 95% CIs for gastric cancer were calculated. To summarize the results from each phase, data pooling and meta-analysis were also conducted. The summary ORs and 95% CIs were calculated based on a fixed-effect model and heterogeneity was evaluated using Cochran Q statistics [43].

Based on the results of spline analysis, the best cut-off point of each phytoestrogen biomarker was detected (genistein 76 nmol/L, daidzein 37 nmol/L, equol 8.8 nmol/L, and enterolactone 24 nmol/L). Analysis of data stratified by high and low levels of phytoestrogen biomarkers was conducted using a conditional logistic regression model. Interaction effects between the representative SNPs and phytoestrogen biomarkers were computed as ORs and 95% CIs, adjusted for the covariate risk factors listed in the preceding paragraph.

Statistical analyses were performed using SAS software version 9.2 (SAS Institute, Cary, NC), and PLINK software version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink) [44]. Meta-analyses were conducted using STATA version 10 (Stata, College Station, TX).

Table 1. Characteristics of gastric cancer cases and controls included in the genetic analyses: discovery, extension, and pooled analyses

		Discovery scan			Extended reanalysis			Pooled analysis (total study population)		
		Case ($N = 81$)	Control ($N = 85$)	$p^{c)}$	Case ($N = 317$)	Control ($N = 317$)	$p^{c)}$	Case ($N = 398$)	Control ($N = 402$)	$p^{c)}$
Age	Mean (SD)	64.2 (±7.9)	63.3 (±8.0)	0.44	62.8 (±8.9)	63.0 (±8.5)	0.78	63.1 (± 8.8)	63.1 (±8.4)	0.96
Sex	Female	26 (32.1)	26 (30.6)	0.83	94 (30.0)	94 (30.0)	1.00	120 (30.2)	120 (29.9)	0.93
H. pylori infection	Positive (+)	72 (88.9)	68 (80.0)	0.12	280 (88.3)	270 (85.2)	0.24	352 (88.4)	338 (84.1)	0.07
CagA	Positive (+)	78 (96.3)	74 (87.1)	**0.03**	293 (92.4)	277 (87.4)	**0.03**	371 (93.2)	351 (87.3)	**0.01**
VacA	Positive (+)	50 (61.7)	46 (54.1)	0.32	219 (69.0)	213 (67.2)	0.61	269 (67.6)	259 (64.4)	0.35
Smoking status	Ever smokers[a)]	52 (64.2)	47 (55.3)	0.24	203 (64.0)	178 (56.2)	**0.04**	255 (64.1)	225 (55.9)	**0.02**
Drinking status	Ever drinkers[b)]	46 (56.8)	50 (58.8)	0.79	196 (62.0)	190 (59.9)	0.59	242 (61.0)	240 (59.7)	0.72
Gastric ulcer history	Positive (+)	14 (17.3)	9 (10.6)	0.43	49 (17.9)	47 (19.2)	0.70	63 (18.6)	56 (18.1)	0.87

a) Defined as former and current smokers.
b) Defined as former and current drinkers.
c) Statistically significant values indicated by bold font.

3 Results

Several characteristics of the participants in each study phase are summarized in Table 1. Gastric cancer cases showed significantly higher rates of CagA seropositivity than the controls ($p = 0.03$, discovery phase; $p = 0.03$, extension phase; and $p = 0.01$ pooled phases). Smokers, including former and current smokers, were significantly more common among gastric cancer cases than among the controls in the extension and pooled-phase analyses ($p = 0.04$, $p = 0.02$, respectively; Table 1).

In the primary scan of the discovery phase, *CDK1* rs4145643 and *CDK1* rs2127356 in the AKT pathway and *MAP3K1* rs16886448 and *PIK3R5* rs9895992 in the NF-κB pathway were significantly associated with a reduced risk of gastric cancer (OR = 0.60 (95% CI: 0.37–0.97), OR = 0.61 (95% CI: 0.39–0.98), OR = 0.30 (95% CI: 0.14–0.63), and OR = 0.49 (95% CI: 0.30–0.81), respectively). However, the BH-FDR p values in all genetic models were not significant ($p > 0.05$; Table 2). All associations between the significant SNPs in the discovery scan and gastric cancer risk were relatively weaker in the extension phase. Of the eight significant SNPs in six genes, *PTEN* rs11202600, *MAPK1* rs5999749, and *MAP3K1* rs252902 did not show statistical significance in the extension phase's reevaluation (data not shown).

In the combined analysis, which included the discovery and extension analyses, *CDK1* rs4145643, *FAS* rs6586161, and *FAS* rs1468063 in the AKT signaling pathway presented significant genetic effects on gastric cancer without heterogeneity (meta OR = 0.81 (95% CI: 0.66–0.99), meta OR = 1.27 (95% CI: 1.03–1.58), and meta OR = 1.29 (95% CI: 1.03–1.56), respectively; Cochran Q statistics > 0.10). For the NF-κB signaling pathway, the risk estimates for *MAP3K7* rs150125 and *MAP3K1* rs16886448 were significantly associated with gastric cancer in both the pooled and meta-analysis results (meta OR = 1.27 (95% CI: 1.03–1.57) and meta OR = 0.63 (95% CI: 0.46–0.86), respectively), whereas heterogeneity across the analyses was founded (Cochran Q statistics < 0.05; Table 3).

Among the four phytoestrogen biomarkers, a significant gene–environment interaction with the representative SNPs was only observed in enterolactone. Risk alleles of *FAS* rs6586161, *FAS* rs1468063, *MAP3K1* rs16886448, and *MAP3K1* rs252902 showed significant interaction effects with enterolactone: the A allele of *FAS* rs6586161 and the T allele of *FAS* rs1468063 represented significantly increased risks for gastric cancer at a low level of enterolactone (OR = 2.31 (95% CI: 1.20–4.43), $p_{interaction} = 0.01$; and OR = 2.04 (95% CI: 1.04–3.98), $p_{interaction} = 0.05$; respectively). The C allele of *MAP3K1* rs16886448 also showed a significantly increased risk for gastric cancer at a low level of enterolactone (OR = 3.07 (95% CI: 1.21–7.79), $p_{interaction} = 0.003$). The A allele of *MAP3K1* rs252902 was associated with a reduced risk of gastric cancer at a high enterolactone level, indicating marginal significance for a gene–environment interaction (OR = 0.65 (95% CI: 0.44–0.98), $p_{interaction} = 0.06$; Table 4).

4 Discussion

CDK1 rs4145643, *FAS* rs6586161, and *FAS* rs1468063 involved in the AKT signaling pathway presented strong associations with the development of gastric cancer. Moreover, a gene–environment interaction between the *FAS* genetic polymorphisms and enterolactone appeared to be a significant risk modifier in gastric carcinogenesis. Regardless of the unclear genetic effects on the NF-κB pathway, it appears that genetic polymorphisms of *MAP3K1* might actively interact with enterolactone.

CDK1, which is synonymous with *CDC2* (cell division control protein 2 homolog), is in charge of cell-cycle progression. CDK activity can regulate cell growth, DNA replication, and mitotic distribution of chromosomes to daughter cells and some CDKs have been implicated in oncogenic progression [45]. Human genome sequencing results have identified dozens of CDKs, but CDK1 is the only indispensable cell-cycle CDK that can interact with all other cyclins and compensate

Table 2. Selected SNPs in the AKT/NF-κB pathway genes associated with gastric cancer risk as detected in the discovery phase

Pathway	GENE	CHR[a)]	#SNPs[b)]	db SNP ID	CHR position	Minor allele	Global p[c)]	$p_{permutated}$[d)]	$p_{corrected}$[e),f)]	OR (95% CI)[g)]
AKT	*PTEN*	10	5	rs11202600[h)]	89672813	C	0.006341	0.00766	0.4740	3.99 (1.37–11.6)**
				rs17107001	89676489	T	0.035550	0.05849	0.9813	0.22 (0.04–1.08)*
	CDK1	10	10	rs4145643[h)]	62232861	C	0.008677	0.00863	0.5865	0.60 (0.37–0.97)**
				rs2127356	62231701	A	0.010070	0.01196	0.6462	0.61 (0.39–0.98)**
	FAS	10	13	rs6586161[h)]	90731239	A	0.011140	0.01371	0.6750	1.77 (1.11–2.82)**
				rs1468063[h)]	90765271	T	0.017310	0.02296	0.8291	1.84 (1.14–2.98)**
				rs2296601	90757620	T	0.028750	0.03474	0.9587	1.75 (1.08–2.84)**
	TP53	17	3	rs2909430	7519370	C	0.028110	0.02643	0.9561	7.29 (0.81–65.40)*
	CASP2	7	5	rs10500136	142724366	C	0.033970	0.04958	0.9761	0.15 (0.02–1.11)*
	CASP14	19	2	rs33933508	15015126	G	0.039120	0.04917	0.9886	1.77 (1.11–2.81)**
NF-kB	*MAP3K7*	6	17	rs150125[h)]	91295724	T	0.001546	0.00191	0.1326	2.38 (1.44–3.95)**
				rs205339[h),i)]	91288700	A	0.001546	0.00191	0.1326	2.38 (1.44–3.95)**
				rs205352[h),i)]	91305939	A	0.003573	0.00384	0.2923	2.26 (1.35–3.79)**
	MAPK1	22	16	rs5999749[h)]	20517660	C	0.001647	0.00189	0.1432	2.83 (1.42–5.65)**
	MAP3K1	5	28	rs16886448[h)]	56206570	G	0.001961	0.00249	0.1698	0.30 (0.14–0.63)**
				rs252902[h),j)]	56151842	A	0.004284	0.00544	0.3399	1.92 (1.20–3.08)**
				rs252904	56154490	A	0.004284	0.00544	0.3399	1.92 (1.20–3.08)**
				rs252915	56157511	A	0.004284	0.00544	0.3399	1.92 (1.20–3.08)**
				rs2113079	56193708	T	0.008453	0.01036	0.5682	1.81 (1.13–2.89)**
				rs832584	56214969	C	0.008453	0.01036	0.5682	1.81 (1.13–2.89)**
				rs860580	56185186	T	0.009932	0.01093	0.6226	1.79 (1.11–2.87)**
				rs1423621	56136792	C	0.011110	0.01102	0.6670	1.77 (1.09–2.85)**
	PIK3R5	17	16	rs9895992[h),i)]	8764493	A	0.004341	0.00466	0.3439	0.49 (0.30–0.81)**
	MAPK14	6	12	rs7761118	36176281	A	0.012140	0.01251	0.6962	2.83 (1.37–5.84)**
				rs16884628	36165946	A	0.024820	0.02178	0.9221	2.60 (1.30–5.21)**
				rs3761980	36101884	G	0.031280	0.03347	0.9588	2.56 (1.28–5.12)**
				rs16883860	36110440	G	0.031280	0.03347	0.9588	2.56 (1.28–5.12)**
				rs16884919	36179495	A	0.031280	0.03347	0.9588	2.56 (1.28–5.12)**
	PIK3R1	5	16	rs706713	67558478	C	0.027330	0.02686	0.9398	1.84 (1.03–3.28)**
				rs706714	67558607	A	0.040220	0.04858	0.9838	1.77 (1.02–3.07)**
	MAPK12	22	1	rs1555048	49030475	A	0.044810	0.06067	0.9910	2.13 (0.94–4.80)*
	PIK3R6	17	16	rs196449	8649052	A	0.048320	0.06390	0.9976	0.60 (0.34–1.02)*

a) Chromosome number.
b) Total number of SNPs selected within each candidate gene.
c) Raw p value calculated in the trend model with a cut-off level ≤ 0.05.
d) 100 000 permutations for single SNP in the trend model.
e) Corrected p values for multiple comparison with 100 000 permutations in trend model.
f) All BH-FDR p values were not significant ($p > 0.05$).
g) Adjusted for age, smoking (never versus ever), *H. pylori* infection (positive versus negative), and CagA seropositivity (positive versus negative).
h) Representative SNPs selected for the extension phase analysis.
i) Excluded due to a SNP call rate <95% or a low design score.
j) Tag SNPs.
** $p < 0.05$, * $0.05 \leq p < 0.10$

Table 3. Pooled analysis for significant SNPs in the AKT/NF-κB pathway genes associated with gastric cancer risk as detected in the extension phase

Pathway	Gene	db SNP ID	MAF[a)] (%)	OR (95% CI)[b),c)]	OR (95% CI)[b),d)]	p heterogeneity
AKT	*CDK1*	rs4145643	C (45.5)	0.80 (0.65–0.98)	0.81 (0.66–0.99)	0.184
	FAS	rs6586161	A (44.7)	1.27 (1.04–1.56)	1.27 (1.03–1.58)	0.118
		rs1468063	T (45.5)	1.29 (1.05–1.59)	1.29 (1.03–1.56)	0.113
NF-kB	*MAP3K7*	rs150125	T (41.0)	1.28 (1.04–1.57)	1.27 (1.03–1.57)	0.007
	MAP3K1	rs16886448	G (17.1)	0.63 (0.47–0.85)	0.63 (0.46–0.86)	0.034

a) Minor allele frequency among controls.
b) All ORs were adjusted for age, smoking (never versus ever), *H. pylori* infection (positive versus negative), and CagA seropositivity (positive versus negative).
c) Pooled analysis including all gastric cases and controls from both study data sets (discovery and extension).
d) Metaanalysis using fixed effect model for combined analysis.

Table 4. Gastric cancer risk associated with the interaction between SNPs[a] in the AKT/NF-κB pathway genes and phytoestrogens[b]

				Enterolactone[c]		*p* interaction
				Low level	High level	
Pathway	Gene	db SNP ID	Risk allele	**OR (95% CI)**[d]	**OR (95% CI)**[d]	
AKT	*FAS*	rs6586161	A	**2.31 (1.20–4.43)**	0.89 (0.61–1.30)	0.01
		rs1468063	T	**2.04 (1.04–3.98)**	0.96 (0.66–1.40)	0.05
NF-kB	*MAP3K1*	rs16886448	C	**3.07 (1.21–7.79)**	0.69 (0.41–1.19)	0.003
		rs252902	A	1.26 (0.70–2.27)	**0.65 (0.44–0.98)**	0.06

a) Additive effects of allele dosage estimated in the additive model.
b) Cut-off level of each biomarker was decided using spline analysis.
c) Mean of biomarkers among low and high concentration groups: 4.0 nmol/L versus 77.8 nmol/L, respectively.
d) Adjusted for age, smoking (never versus ever), *H. pylori* infection (positive versus negative), and CagA seropositivity (positive versus negative).

for the absence of interphase CDKs [46, 47]. Moreover, aberrant activity of CDK1 can be directly connected to tumor-associated cell-cycle defects; thus, CDK1 is in the spotlight as an anti-cancer therapeutic target. Though a single gene effect of *CDK1* has not been fully elucidated and an exact biological function remains to be explored, given CDK1's key role throughout the entire cell-cycle sequence, *CDK1* genetic polymorphisms that alter CDK1 activity and/or cellular capacity may be involved in human carcinogenesis, especially that of gastric cancer. Our findings suggest a potential genetic effect of *CDK1* rs4145643 on the development of gastric cancer. Future investigations should assess whether the SNP could be explained by a biological function related to CDK activity and/or cellular capacity and should determine the functional *CDK1* SNPs.

FAS, also known as tumor necrosis factor receptor superfamily member 6, cluster of differentiation 95, and apoptosis antigen 1, appears to interfere with the development and/or progression of gastric cancer. FAS is a representative death receptor that modulates programed cell death, apoptosis, homeostasis in immune systems, and carcinogenic activity [48, 49]. Although previous studies have reported conflicting results on whether *FAS* genetic variants such as 1377G>A (rs2234767) and 670A>G (rs1800682) could be prime determinants of human cancers [50, 51], the potent connection between the *FAS* gene and the development of gastric cancer is still supported by the observation that *FAS* polymorphisms are significantly associated with gastric atrophy and intestinal metaplasia among *H. pylori*-infected persons [52]. Furthermore, the minor allele of rs1468063 (T allele), identified as a susceptible genetic marker in the present study, has been significantly associated with Alzheimer's disease, implicating FAS-mediated apoptosis in neuronal cells [53]. Given that FAS-mediated apoptosis in gastric epithelial cells may lead to gastric disorders, *FAS* rs1468063 can be a potent trigger for the development of gastric cancer and may determine individual susceptibility to oncogenesis. Additionally, in spite of a lack of experimental evidence confirming a functional effect of the SNP in a molecular mechanism, but considering that *FAS* rs1468063 is located in 3′ untranslated regions containing regulatory elements that control the spatial and temporal expression of messenger RNA [54], it is speculated that the SNP may be a regulatory factor and may induce diverse variation in messenger RNA expression. Further studies focused on functional genomics are needed to elucidate the molecular basis of this SNP's effects.

Interaction between the *FAS* gene involved in the AKT signaling pathway and phytoestrogens such as enterolactone appears to play a regulatory role in gastric carcinogenesis. Enterolactone was found to inhibit: (i) AKT phosphorylation, which is one of the most frequent conversions observed in human cancers, intensifying in a dose- and time-dependent manner [31]; and (ii) not only the AKT signal transduction pathway but also its upstream targets such as insulin-like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) signaling [55]. Studies have demonstrated that enterolactone is an independent beneficial marker of human health such as cancers [16, 56, 57], with a high enterolactone concentration indicative of a healthy diet and healthy body. Our findings indicated that *FAS* genetic variants such as rs6586161 and rs1468063 could interfere in enterolactone activities, and may control the absolute risk for gastric cancer. Focused on the AKT signal transduction pathway, *FAS* gene and enterolactone may be interconnected and control AKT signaling in both dependent and/or independent manners, thus playing a regulatory role in gastric carcinogenesis. Given the results of an animal study in which some lignans induced inhibitory effects on *H. pylori* proliferation in vitro and reduced the numbers of *H. pylori* colonies [36], the potential regulatory effects of enterolactone on *H. pylori*-induced gastric carcinoma is supported by our results.

Despite the inconclusive results of our genetic analysis not overcoming heterogeneity, the interactions between *MAP3K1* genetic variants (rs16886448 and rs252902) related to the NF-κB signaling and enterolactone should be treated as putative risk modifiers in gastric carcinogenesis. NF-κB signaling controls various cellular activities such as those related to immune systems, inflammatory responses, cytokine/chemokine production, and apoptosis [58], and thus it plays a crucial role in the development of gastric cancer.

With regard to the *MAP3K1* gene, some of its genetic variants (rs889312 and rs16886165) have been demonstrated to be genetic susceptibility markers for hormone-related tumors such as breast cancer [59, 60]. The hormone-dependent effect of *MAP3K1* on gastric carcinogenesis is noteworthy because: (i) this gene is perhaps a clue to explaining a sex-related difference in gastric cancer incidence (i.e. incidence differences may be related hormonal differences); and (ii) gene–environment interactions between *MAP3K1* and phytoestrogens may modify an individual's susceptibility to gastric cancer. Further studies with a greater number of gastric cancer cases, along with in-depth in vitro experiments, will help elucidate such unproven mechanisms in the etiology of gastric cancer.

Although the present study is a two-phase genetic and gene–environment interaction study based on the use of nested case–control methodology, several study limitations remain. First, we did not confirm the sufficiency of the statistical power in our genetic analyses. Also, in the extension phase, hospital-based gastric cancer cases were matched to community-based controls; thus, selection bias may be induced. However, to diminish that potential bias, we established strict methodological criteria as follows: all cases were matched to controls according to the major covariates in the initial study design stage and multivariable models were used to control the effects of confounding factors. Given that genetic traits are innate and not easily transformable, the bias might not affect the study results. Finally, stratified analyses concerning independent risk factors for gastric cancer such as histological subtypes (i.e. cardiac versus noncardiac; diffuse type versus intestinal type), age (young versus old), and sex (male versus female) could not be conducted due to the small sample size and the relatively homogeneous nature of the study population (i.e. most patients were noncardiac types; most participants were over 60 years of age; and most women were assumed to be menopausal, so estrogen effects were diminished). Therefore, the results should be interpreted with caution. Nevertheless, the present study provides integrated evidence of the role of phytoestrogens and of related genes in gastric carcinogenesis, thereby elucidating the etiology of gastric cancer. Through our primary candidate analysis, putative genetic modifiers were screened and subsequently intensively reanalyzed. Furthermore, gene–environment interactions between phytoestrogens and the genetic modifiers were evaluated as individual susceptibility factors.

In summary, the present study demonstrates that *CDK1* and *FAS* genes involved in the AKT signaling pathway and influenced by anti-carcinogenic property of phytoestrogens can play a role as genetic factors in the susceptibility to gastric carcinogenesis. In terms of gene–environment interactions, *FAS* and *MAP3K1* genes involved in the AKT/NF-κB pathway significantly interact with enterolactone and can modify the individual's risk for gastric cancer. Larger replication studies will further clarify the biological and causal mechanisms involved in gastric carcinogenesis.

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