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Functional polymorphism in manganese superoxide dismutase and antioxidant status: Their interactions on the risk of cervical intraepithelial neoplasia and cervical cancer

Seo-Yun Tong [a], Jong-Min Lee [a,*], Eun-Seop Song [b], Kwang-Beom Lee [c], Mi-Kyung Kim [d], Jae-Kwan Lee [e], Sung-Kyong Son [f], Jung-Pil Lee [g], Jae-Hoon Kim [h], Yong-Il Kwon [i]

[a] Department of Obstetrics and Gynecology, East-West Neo Medical Center, Kyung Hee University, #149 Sangil-Dong, Gangdong-Gu, Seoul 134-890, Republic of Korea
[b] Department of Obstetrics and Gynecology, Inha University School of Medicine, Inchon, Republic of Korea
[c] Department of Obstetrics and Gynecology, Gil Medical Center, Gachon University of Medicine and Science, Inchon, Republic of Korea
[d] Carcinogenesis Branch, Division of Basic Sciences, National Cancer Center, Goyang, Republic of Korea
[e] Department of Obstetrics and Gynecology, Korea University College of Medicine, Seoul, Republic of Korea
[f] Department of Obstetrics and Gynecology, Chungnam National University College of Medicine, Daejon, Republic of Korea
[g] Department of Obstetrics and Gynecology, Ajou University School of Medicine, Suwon, Republic of Korea
[h] Department of Obstetrics and Gynecology, Yongdong Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea
[i] Department of Obstetrics and Gynecology, Hallym University College of Medicine, Seoul, Republic of Korea

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ABSTRACT

Objective. Manganese superoxide dismutase (MnSOD), the primary antioxidant enzyme in mitochondria, plays a key role in protecting cells from oxidative stress. Furthermore, the *MnSOD rs4880* polymorphism is associated with enzyme activity. The authors evaluated the interaction between *MnSOD* genotypes and cervical carcinogenesis risk and the modulating effects of serum antioxidant nutrient status (β-carotene, lycopene, zeaxanthin/lutein, retinol, α-tocopherol and γ-tocopherol).

Methods. Cases and controls for this study were recruited between June 2006 and July 2007 (263 controls, 84 cervical intraepithelial neoplasia (CIN), 94 CIN 2/3, and 99 cases of cervical cancer). The *MnSOD* polymorphism at rs4880T/C was examined using SNaPshot assays. Serum antioxidant vitamin concentrations were measured by reverse-phase gradient high-pressure liquid chromatography. Odds ratios (OR) and 95% confidence intervals (95%CI) were estimated after adjusting for age, menopause, parity, oral contraceptive use, smoking and alcohol consumption.

Results. No association was found between the *MnSOD rs4880* polymorphism and cervical cancer. However, genotypes significantly modified the risk of cervical cancer in association with the serum statuses of micronutrients ($P_{interaction}<0.05$ for β-carotene, lycopene, zeaxanthin/lutein, α-tocopherol, and γ-tocopherol). Decreased CIN1 risk in association with the MnSOD rs4880 variant genotype was also observed particularly for subjects with higher β-carotene and γ-tocopherol levels. Similar results were observed for lycopene and α-tocopherol in relation to the risk of CIN2/3.

Conclusion. Our findings suggest that a higher antioxidant micronutrients status may decrease the risk of CIN and cervical cancer and modify the effect of the *MnSOD* polymorphism on disease risk.

Introduction

Cervical cancer is the second most commonly diagnosed cancer and the third most common cause of cancer mortality in women worldwide [1]. In Korea, although incidence and mortality rates have been decreasing, cervical cancer remains the sixth most common form of cancer [2].

Virologic, molecular, clinical and epidemiological studies support the conclusion that persistent high-risk human papillomavirus (HPV) infection is a primary cause of cervical cancer [3]. However, most HPV infections are transient and rarely progress to a significant cervical lesion [4]. Cofactors that modulate the risk of chronic HPV infection and progression to cervical intraepithelial neoplasia (CIN) include environmental and lifestyle factors, such as, smoking, long-term oral contraceptive (OC) use, high parity, nutritional status, coinfection with another sexually transmitted disease, and host immunity [5–7].

Chronic inflammation has long been recognized to be a risk factor of various cancers, such as, gastric cancer, hepatocellular cancer, and cervical cancer [8]. Although the mechanisms underlying these associations are uncertain, chronic inflammation is known to induce oxidant-generating enzymes and the subsequent production of

* Corresponding author. Fax: +82 2 440 7894.
E-mail address: kgo02@hanmail.net (J.-M. Lee).

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reactive oxygen and nitrogen species (ROS and RNS) [9]. Furthermore, endogenous antioxidant enzymes, such as, manganese superoxide dismutase (MnSOD), and antioxidants, such as, carotenoids and tocopherols are known to play significantly roles in cellular defense against oxidative stress [10].

MnSOD is a major antioxidant enzyme in mitochondria and catalyzes the conversion of superoxide radicals to hydrogen peroxide (H_2O_2), which is subsequently converted into water by catalase and glutathione peroxidase [11]. The most common functional polymorphism in *MnSOD* is a T-to-C transition in its −9 position that results in a valine (GTT) to alanine (GCT) exchange and affects localization and transport of the enzyme into mitochondria [12,13]. Furthermore, the alanine-containing protein has 30–40% greater activity than the valine form [13].

Thus, in this study, we investigated whether the *MnSOD rs4880* (V16A) polymorphism is associated with CIN and cervical cancer risk. In addition, we evaluated whether serum antioxidant nutrients modify relationships between *MnSOD* genotypes and CIN/cervical cancer risk.

Materials and methods

Subjects

The subjects enrolled were patients with a histologically proven diagnosis of CIN or cervical cancer who registered at six tertiary medical centers in Korea between June 2006 and July 2007. Participants in this study were recruited from among women that underwent Papanicolaou (Pap) smears and Hybrid Capture® II (Digene Corporation, Gaithersburg, MD) tests for cervical cancer screening. All women with abnormal Pap smear results also underwent biopsy. CIN lesions were subdivided as CIN1 or CIN2/3 based on the American Society for Colposcopy and Cervical Pathology 2006 guidelines [14]. Histopathological diagnoses included 84 cases of CIN1, 94 cases of CIN2/3, and 99 cases of cervical cancer. Control subjects ($n=263$), who had a normal Pap smear on the day of recruitment and no history of an abnormal Pap smear, were randomly selected from health promotion center at the same hospitals during the study period.

Epidemiological data and sample collection

After informed consent had been obtained, subjects were asked to complete questionnaires regarding lifestyle and to provide 20 ml of peripheral venous blood before the initiation of any treatment. The research protocol was approved by the institutional review boards at each institute. All blood samples were centrifuged within 1 h of collection. After separating plasma, samples were stored at −80 °C until assayed.

Subjects were interviewed by trained interviewers who were unaware of subjects' disease statuses. A wide range of information was collected on socio-demographic characteristics, body size, reproductive and menstrual history, exogenous hormone use, medical history and family history of cervical and other cancers at study enrollment or before the onset of disease for controls and case subjects, respectively. Socio-demographic characteristics included education, occupation, cigarette smoking, alcohol consumption, and habitual exercise, and detailed information was obtained on exposure times.

Genotyping of MnSOD rs4880

Genomic DNA was prepared from peripheral blood samples using QIAamp DNA Blood mini kits (QIAGEN, Valencia, CA). MnSOD *rs4880* genotyping was performed using a single-base primer extension assay using SNaPShot assay kits (Applied Biosystems Inc., Foster City, CA). Briefly, genomic DNA flanking the SNP was amplified by PCR using forward (5-GGCTGTGCTTTCTCGTCTT) and reverse (5-TAGTCGTA-GGGCAGGTCG) primer pairs and standard PCR reagents in 10 μm reaction volumes, containing 10 ng of genomic DNA, 0.5 pM of each oligonucleotide primer, 1 μm of 10×PCR Gold buffer, 250 μM dNTP, 3 mM $MgCl_2$ and 0.25 U of i-StarTaq DNA Polymerase (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea). PCR reactions were carried out as follows: 10 min at 95 °C for 1 cycle, 30 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, followed by 1 cycle of 72 °C for 7 min. One microliter aliquots of the purified amplification products were added to a SNaPshot Multiplex Ready reaction mixture containing 0.15 pmol of genotyping primer (5-CTGCCTGGAGCCCAGATACCCCAAA). Primer extension reactions were carried out over 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Reaction products were treated with 1 U of SAP at 37 °C for 1 h and 72 °C for 15 min to remove excess fluorescent dye terminators. One microliter aliquots of final reaction samples that contained the extension products were then added to 9 μm of Hi-Di formamide (ABI, Foster City, CA). Mixtures were incubated at 95 °C for 5 min, followed by 5 min on ice, and analyzed by electrophoresis using an ABI Prism 3730×l DNA analyzer. Results were analyzed using Gene Scan analysis software (ABI).

High-pressure liquid chromatographic analysis of serum antioxidants

Serum carotenoids and tocopherols levels were determined by high-performance liquid chromatography (HPLC). The methods used to extract analytes from serum and the quality control parameters and the reverse-phase gradient HPLC system used have been described previously [15]. Serum samples from study subjects were randomized and analyzed together under subdued light. All HPLC solvents were obtained from J.T. Baker (Phillipsburg, NJ) and were filtered through a 0.2-μm membrane filter (Waters, Millipore, MA) before use. Briefly, serum carotenoids, retinol, and tocopherols were extracted using $CHCl_3$-CH_3OH (2:1, v/v). Retinol acetate and tocopherol acetate were added as internal standards for the analysis of retinol, carotenoids, and tocopherols. All sample processing was performed under red light. To separate micronutrients simultaneously, a mobile phase gradient was used. The detector was set at 292 nm for tocopherols, 340 nm for retinol, and at 450 nm for carotenoids. Handling losses were corrected for by measuring internal standard recoveries. Because zeaxanthin and lutein isomers could not be separated, they eluted together, and thus, we refer to them as zeaxanthin plus lutein. The mean coefficient of variation was 8% for retinol acetate and tocopherol acetate, and the variation between duplicate serum samples was <1%. The coefficients of variation were <8% for both tocopherols. The relative standard deviations of pooled controls ranged from 4% to 10%. The accurate concentration of the standard solution was determined spectrophotometrically using an extinction coefficient E at 275 nm. Results were calculated by dividing the peak area of a compound by that of the internal standard.

Statistical analysis

The χ^2 test was used to compare differences in the proportions of categorical covariates between cases and controls. Mean values and standard deviations were calculated for continuous demographic variables, and differences between means were tested by ANOVA. Unconditional logistic regression models were used to estimate crude and multivariate odds ratios (ORs) and corresponding 95% confidence intervals (CIs) and to determine whether the *MnSOD rs4880* polymorphism was associated with the risk of CIN or cervical cancer [16]. Risk estimates were computed after making multivariate adjustments for age, menopausal status (premenopause versus postmenopause), parity (one versus two versus >three), oral contraceptive use, smoking status, alcohol consumption status (ever versus never), and HPV infection status. Risk estimates were calculated with

the wild-type as the reference category. Tests for Hardy–Weinberg equilibrium among controls were conducted using observed genotype frequencies and the χ^2 test with 1 *df*.

Gene–nutrient interactions were evaluated using unconditional logistic regression models by joint categories of polymorphisms of *MnSOD rs4880* and serum micronutrients. We evaluated whether these association between micronutrients and the risk of CIN or cervical cancer were modified by genotypes of the *MnSOD rs4880* polymorphism. Variables for joint effects were coded using *MnSOD rs4880 TT* genotypes using low categories of serum micronutrients as referent groups. Low and high nutrient levels were defined based on their being ≤ or > the median levels of controls, respectively. Interaction tests were performed by entering into the model multiplicative interaction terms of the ordinal score for each genotype and serum measurements. Interactions were tested using the log likelihood ratio test, whereby the model that included the interaction term was compared with that without the term. All *P* values were two-sided, and the analysis was performed using SAS 8.0 software. ([17]: SAS Institute, Inc., Cary, NC).

Results

The characteristics of cases and controls are shown in Table 1. Significant differences were found in terms of age, alcohol drinking status, menopausal status, and parity between cases and controls.

The genotypes and allele frequencies of the rs4880 polymorphism in cases and controls are presented in Table 2. The observed genotype frequency in the control subjects was in agreement with the Hardy–Weinberg equilibrium ($P>0.05$).

The frequency of the rs4880 CT/CC genotype (OR = 0.427, 95% CI = 0.203–0.9) was significantly lower in CIN1 cases compared to that of controls. Our results showed that women with the rs4880 CT/CC genotype had a 57.3% lower risk of developing CIN1 than women with the rs4880 TT genotype.

We then evaluated the combined effect of the *MnSOD rs4880* genotype and serum micronutrient levels (Table 3), in women with high and low levels of analytes. In terms of the risk of CIN1, among cases with high β-carotene levels, the rs4880 CT/CC alleles were found to be associated with a significantly lower risk of CIN1 (OR = 0.286, 95% CI = 0.086–0.953), whereas the rs4880 TT genotype had an OR = 0.533 (95% CI = 0.259–1.099) versus cases with a low β-carotene level and the rs4880 TT genotype. Furthermore, a significant interaction was observed between the rs4880 polymorphism and serum β-carotene levels ($P_{interaction}=0.002$). A significant interaction was also observed between γ-tocopherol and the rs4880 genotype in relation to the risk of CIN1 ($P_{interaction}=0.033$). Moreover, women in high retinol level with the rs4880 TT genotype had an ~2-fold increased risk of CIN1 (OR = 2.13, 95% CI 1.003–4.523).

In terms of the risk of CIN2/3 and the variant (CT/CC) allele, a high lycopene level was found to be associated with a decreased risk (OR = 0.126, 95% CI = 0.025–0.644), and a significant interaction was found between serum lycopene level and rs4880 genotype ($P_{interaction}=0.013$). Similarly, a high α-tocopherol serum level, clearly reduced the risk for both the wild allele (OR = 0.439, 95% CI = 0.224–0.863) and the variant allele (OR = 0.296, 95% CI = 0.095–0.921), as compared with a low serum α-tocopherol in wild allele carriers. In addition, a significant interaction was observed between the rs4880 genotype and serum α-tocopherol level in terms of the risk of CIN2/3 ($P_{interaction}=0.001$).

Regarding the risk of cervical cancer, a significant reduction in risk was observed for those with a high β-carotene, zeaxanthin/lutein, α-tocopherol, and γ-tocopherol levels, regardless of the rs4880 T/C genotype. A significant interaction was found between serum β-carotene ($P_{interaction}<0.001$), lycopene ($P_{interaction}=0.011$), zeaxanthin/lutein ($P_{interaction}=0.017$), α-tocopherol ($P_{interaction}<0.001$), and γ-tocopherol ($P_{interaction}<0.001$) levels and the rs4880 genotype in relation to the risk of cervical cancer, such that the presence of the variant alleles (CT/CC) was related to a lower risk when micronutrients levels were high and to an elevated risk when micronutrient levels were low.

Table 1
Clinical characteristics and the mean serum levels of micronutrients of study subjects.

Variables	Control ($n=263$)	CIN1 ($n=84$)	CIN2/3 ($n=94$)	Cervical cancer ($n=99$)
Age, median (range), y	47(41–53)	39(32–48)***	39(33–46)***	50(43–61)***
Ever smoker, %	9.5	15.9**	14.5*	12.3
Ever drinker, %	45.4	66.9***	64.7***	47.2
Oral contraceptive use, %	15.1	21.4	19.1	20.3
Post menopause, %	44	22***	17***	68***
Number of parity, means ± SD	2.28 ± 0.92	2.28 ± 0.85	2.15 ± 0.81	2.66 ± 1.31**

* *P*-value<0.05.
** *P*-value<0.01.
*** *P*-value<0.001.

Table 2
The association between the MnSOD rs4880 polymorphism and the risk of cervical carcinogenesis.

Genotype	Control		Case		OR (95% CI)*
	N	%	*N*	%	
CIN1					
TT	194	74	73	87	1 (ref.)
CT/CC	69	26	11	13	0.427 (0.203–0.9)
CIN2, 3					
TT	194	74	77	82	1 (ref.)
CT/CC	69	26	17	18	0.699 (0.376–1.298)
Cervical cancer					
TT	194	74	72	73	1 (ref.)
CT/CC	69	26	27	27	0.950 (0.548–1.647)

OR indicates odd ratio; 95% CI, 95% confidence interval; *MnSOD*, manganese superoxide dismutase; T, thymine; C, cytosine; CIN, cervical intraepithelial neoplasia.
* ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause versus postmenopause), number of parity (one versus two versus over three), oral contraceptive use, smoking habit (ever versus never), alcohol consumption status (ever versus never), HPV infection status.

Discussion

In this study, we observed an inverse association between the risk of cervical cancer and serum concentrations of β-carotene, lycopene, zeaxanthin plus lutein, retinol, α-tocopherol, and γ-tocopherol, regardless of rs4880 genotype. No overall association was observed between *MnSOD rs4880* genotypes and cervical cancer risk. However, an interaction was noted between *MnSOD rs4880* genotypes and serum micronutrient levels. We found that the *MnSOD rs4880* CT/CC genotype was associated with a reduced risk of CIN1 particularly in subjects with higher β-carotene and γ-tocopherol levels. Furthermore, similar results were observed for lycopene and α-tocopherol in relation to the risk of CIN2/3.

Few epidemiologic studies have explored the possible significance of micronutrients on the clearance of HPV or on the risk of CIN or cancer [18–23]. In a recent review study, it was reported that α-carotene, β-carotene, lycopene, zeaxanthin plus lutein, and cryptoxanthin had protective effects on cervical neoplasia [21]. However, to the best of our knowledge, no investigation has previously been undertaken on the association between cervical cancer risk and *the MnSOD rs4880* polymorphism. Several studies have evaluated associations between *MnSOD rs4880* genotypes and other cancers, but results have been inconsistent with respect to cancer sites and the combinatorial effects of other risk factors. Recently, Bergman et al. [24] reported that the wild-type genotype (rs4880 TT) is associated with a 2.7-fold (95% CI, 2.2–5.5) increased

Table 3
Odds ratios (ORs) of CIN and cervical cancer according to *MnSOD* rs4880 T/C genotypes and serum micronutrient levels.

	MnSOD rs4880	CIN 1 ($n=84$)		CIN 2,3 ($n=94$)		Cervical cancer ($n=99$)	
		TT	CT/CC	TT	CT/CC	TT	CT/CC
β-carotene (μg/ml)[a]							
Low (≤0.205)	*N* (control/case)	100/48	31/7	100/42	31/11	100/59	31/22
	OR (95% CI)[b]	1.0[c]	0.424 (0.128–1.399)	1.0[c]	1.123 (0.435–2.897)	1.0[c]	1.162 (0.56–2.412)
High (>0.205)	*N* (control/case)	94/25	38/4	94/35	38/6	94/13	38/5
	OR (95% CI)[b]	0.533 (0.259–1.099)	0.286 (0.086–0.953)	0.993 (0.53–1.861)	0.429 (0.148–1.243)	0.243 (0.116–0.508)	0.223 (0.079–0.63)
	$P_{interaction}$[d]	0.002		0.237		<0.001	
Lycopene (μg/ml)[a]							
Low (≤0.006)	*N* (control/case)	96/39	32/7	96/47	32/15	96/43	32/18
	OR (95% CI)[b]	1.0[c]	0.335 (0.097–1.158)	1.0[c]	1.112 (0.483–2.558)	1.0[c]	1.083 (0.503–2.333)
High (>0.006)	*N* (control/case)	95/29	37/4	95/29	37/2	95/21	37/6
	OR (95% CI)[b]	0.552 (0.263–1.16)	0.415 (0.125–1.376)	0.606 (0.322–1.142)	0.126 (0.025–0.644)	0.565 (0.294–1.086)	0.435 (0.162–1.173)
	$P_{interaction}$[d]	0.082		0.013		0.011	
Zeaxanthin/Lutein (μg/ml)[a]							
Low (≤0.49)	*N* (control/case)	99/30	32/5	99/38	32/7	99/45	32/21
	OR (95% CI)[b]	1.0[c]	0.453 (0.093–2.195)	1.0[c]	0.598 (0.202–1.767)	1.0[c]	1.12 (0.527–2.38)
High (>0.49)	*N* (control/case)	95/43	37/6	95/39	37/10	95/27	37/6
	OR (95% CI)[b]	1.773 (0.86–3.656)	0.738 (0.246–2.211)	0.936 (0.5–1.753)	0.738 (0.298–1.827)	0.501 (0.269–0.932)	0.346 (0.128–0.936)
	$P_{interaction}$[d]	0.043		0.447		0.017	
Retinol (μg/ml)[a]							
Low (≤0.64)	*N* (control/case)	98/26	33/7	98/42	33/9	98/35	33/16
	OR (95% CI)[b]	1.0[c]	0.668 (0.183–2.438)	1.0[c]	0.638 (0.238–1.708)	1.0[c]	1.049 (0.456–2.411)
High (>0.64)	*N* (control/case)	96/47	36/4	96/35	36/8	96/37	36/11
	OR (95% CI)[b]	2.13 (1.003–4.523)	0.761 (0.223–2.605)	0.892 (0.479–1.659)	0.686 (0.264–1.78)	0.71 (0.383–1.318)	0.648 (0.276–1.516)
	$P_{interaction}$[d]	0.011		0.397		0.767	
α-tocopherol (μg/ml)[a]							
Low (≤1.20)	*N* (control/case)	101/42	30/7	101/57	30/13	101/56	30/22
	OR (95% CI)[b]	1.0[c]	0.419 (0.12–1.465)	1.0[c]	0.747 (0.313–1.783)	1.0[c]	1.181 (0.563–2.479)
High (>1.20)	*N* (control/case)	93/31	39/4	93/20	39/4	93/16	39/5
	OR (95% CI)[b]	0.833 (0.414–1.675)	0.405 (0.126–1.307)	0.439 (0.224–0.863)	0.296 (0.095–0.921)	0.193 (0.093–0.401)	0.147 (0.047–0.461)
	$P_{interaction}$[d]	0.066		0.001		<0.0001	
γ-tocopherol (μg/ml)[a]							
Low (≤0.30)	*N* (control/case)	90/42	41/6	90/42	41/13	90/55	41/21
	OR (95% CI)[b]	1.0[c]	0.395 (0.121–1.284)	1.0[c]	0.778 (0.329–1.838)	1.0[c]	0.814 (0.394–1.681)
High (>0.30)	*N* (control/case)	104/31	28/5	104/35	28–4	104/17	28/6
	OR (95% CI)[b]	0.521 (0.256–1.06)	0.272 (0.079–0.944)	0.665 (0.355–1.243)	0.327 (0.097–1.103)	0.224 (0.111–0.453)	0.309 (0.114–0.837)
	$P_{interaction}$[d]	0.033		0.159		0.0001	

OR indicates odd ratio; 95% CI, 95% confidence interval; *MnSOD*, manganese superoxide dismutase; T, thymine; C, cytosine; CIN, cervical intraepithelial neoplasia.
[a] High and low levels of serum micronutrients are based on median values in the control group.
[b] ORs and 95% CIs calculated by unconditional logistic regression, adjusted for age, menopausal status (premenopause versus postmenopause), number of parity (one versus two versus over three), oral contraceptive use, smoking habit (ever versus never), alcohol consumption status (ever versus never), HPV infection status.
[c] Reference category.
[d] Based on the likelihood ratio test when models were compared with and without an interaction term between serum micronutrients and MnSOD genotype.

risk of breast cancer, whereas in stark contrast, Ambrosone et al. [25] and Cai et al. [26] reported a positive association between the *MnSOD* C allele and breast cancer in premenopausal women. Furthermore, the *MnSOD rs4880* polymorphism was not found to be associated with prostate cancer risk. [27,28] However, interestingly, Choi et al. [27] reported that the risk of prostate cancer increased with iron intake among men homozygous for the TT genotype (P for trend = 0.02). Mikhak et al. [28] also reported that the CC genotype plus a low lycopene level is associated with a higher risk of aggressive prostate cancer.

Although the functional effects of the *MnSOD* polymorphism are not completely understood, it has been reported that the CC genotype has higher enzyme activity [13,29], and that the effect of the *MnSOD rs4880* polymorphism on cancer risk is dependent on antioxidant status. [25,30] In mitochondria, higher *MnSOD* expression may result in elevated H_2O_2 levels, which are produced by the dismutated superoxide anion, and these may induce toxicity if glutathione peroxidase activity or antioxidant levels are low [30]. However, when antioxidant levels are adequate, the increased rate of superoxide anion quenching afforded by the C genotype may be beneficial [28]. In this study, we observed that women with the C genotype and a higher antioxidant status appeared to be protected from cervical carcinogenesis.

Our study is limited in that blood measurements might not be a reliable index of long-term dietary intake, given the duration of cancer development. Furthermore, although we enrolled a large number of individuals, the study is still limited because our subgroup analysis lacked statistical power. Third, because the age of CIN cases are younger than that of cancer cases, the variables, such as age, smoking, alcohol drinking and menopausal status, are also statistically significantly different between cases and controls. Generally, the average age of women with CIN is 15 years younger than that of women with invasive carcinoma [31]. To compensate for this, we estimated the odds ratio after making multivariate adjustments for age, menopausal status, parity, smoking status, and alcohol consumption status.

Nevertheless, the study has strengths that should also be borne in mind. First, it produced the statistically significant result that women with the *MnSOD* rs4880 C allele with a low antioxidant status have a higher risk of cervical carcinogenesis than individuals with the T allele. Second, this study had a case-control design and extensive information was collected on lifestyle factors, which allowed us to adjust for possible confounders. Third, this study was free of the possible confounding effect of ethnicity, because of the homogenous nature of the Korean population.

In conclusion, our results provide strong plausible evidence that the association between antioxidant status and the risk of CIN/cervical cancer is dependent on *MnSOD* rs4880 genotype, and that endogenous and exogenous antioxidants both have important effects on cervical carcinogenesis.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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