FASL –844C polymorphism is associated with increased activation-induced T cell death and risk of cervical cancer

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The FAS receptor–ligand system plays a key role in regulating apoptotic cell death, and corruption of this signaling pathway has been shown to participate in tumor-immune escape and carcinogenesis. We have recently demonstrated (Sun, T., X. Miao, X. Zhang, W. Tan, P. Xiong, and D. Lin. 2004. *J. Natl. Cancer Inst.* 96:1030–1036; Zhang, X., X. Miao, T. Sun, W. Tan, S. Qu, P. Xiong, Y. Zhou, and D. Lin. 2005. *J. Med. Genet.* 42:479–484) that functional polymorphisms in FAS and FAS ligand (FASL) are associated with susceptibility to lung cancer and esophageal cancer; however, the mechanisms underlying this association have not been elucidated. We show that the FAS –1377G, FAS –670A, and FASL –844T variants are expressed more highly on ex vivo–stimulated T cells than the FAS –1377A, FAS –670G, and FASL –844C variants. Moreover, activation-induced cell death (AICD) of T cells carrying the FASL –844C allele was increased. We also found a threefold increased risk of cervical cancer among subjects with the FASL –844CC genotype compared with those with the –844TT genotype in a case-control study in Chinese women. Together, these observations suggest that genetic polymorphisms in the FAS–FASL pathway confer host susceptibility to cervical cancers, which might be caused by immune escape of tumor cells because of enhanced AICD of tumor-specific T cells.

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Abbreviations used: AICD, activation-induced cell death; CI, confidence interval; FASL, FAS ligand; HeLa, human cervical tumor; HPV, human papilloma virus; MMC, mitomycin C; OR, odds ratio; PI, propidium iodide; SNP, single nucleotide polymorphism; TIL, tumor infiltration lymphocyte.

Activation-induced cell death (AICD) refers to the induction of apoptosis of previously activated T cells on subsequent encounter with antigen (1). This mechanism plays a crucial role in both central and peripheral deletion events involved in tolerance and homeostasis of the immune system (2, 3). Accumulating evidence also demonstrates that AICD is one of the important mechanisms responsible for the increased apoptosis rate among tumor infiltration lymphocytes (TILs), which may protect transformed cells from elimination by antitumor immune responses and, therefore, contribute to carcinogenesis and cancer progression (4, 5).

AICD is a FAS ligand (FASL)–dependent process (6). AICD in peripheral T cells is often caused by the induction of expression of the death ligand, FASL. Cytotoxic T cells increase expression of both FAS and FASL on activation by antigen or other stimulation and subsequently undergo "suicide" or "fratricide" by FASL liganding to FAS (7, 8). Antigenic stimulation within the tumor microenvironment might also be involved in the enhanced expression and function of FASL on T cells, resulting in activation of autocrine or paracrine mechanisms of apoptosis (9). FASL-mediated AICD is directly regulated by the level of this death ligand (1), whereas FASL transcriptional regulation happens in both T cells and nonlymphoid cells, such as a variety of tumor cells. In a tumor microenvironment, tumor cells can also counterattack by expressing FASL and inducing the apoptosis of T cells (10, 11).

Cervical cancer is strongly associated with infection by oncogenic types of human papilloma virus (HPV) (12). However, only a portion of infected individuals develop cervical cancer during their life, indicating that genetic factors may also contribute to the formation of this cancer. A large body of epidemiological

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evidence shows that genetic predisposition to cervical cancer may exist (13, 14). It has been shown that cervical tumor cells have the ability to induce apoptosis of activated tumor cell–specific cytotoxic T lymphocytes, and this process can be blocked by an inhibitory anti-FAS antibody, suggesting that it is mediated by FAS–FASL interaction (15). Because human cervical tumors (HeLas) indeed express FASL (15–17), these tumor cells are expected to induce AICD of TILs in vivo. Thus, the expression levels of FASL and FAS might be of importance in determining individual susceptibility to cervical cancer development.

Single nucleotide polymorphisms (SNPs) in the promoter regions of FASL and FAS have been linked to the differential expression of these two genes. It has been shown that the T→C transition at position −844 in the promoter region of FASL is located in a binding motif for transcription factor CAAT/enhancer-binding protein β, and a considerably higher basal expression of FASL is associated with the FASL –844C allele compared with the –844T allele (18). In regards to the FAS gene, G→A transition at position –1377 and A→G transition at position −670 in the promoter region destroy stimulatory protein 1 and signal transducer and activator of transcription 1 protein binding element, respectively, and thus diminish promoter activity and decrease FAS expression (19, 20). We have recently shown that these functional polymorphisms in FASL and FAS have great impact on susceptibility to cancers of the esophagus and lung (21, 22). The effect of the FAS polymorphism on the risk of cervical cancer has been reported, but the studies were limited to the −670A→G polymorphism, and the results were controversial (23–25). To date, no study has been reported to examine the association between functional polymorphisms in FASL and the risk for the development of cervical cancer.

We wanted to determine whether the polymorphisms in FAS–FASL have effects on the expression of FAS and FASL and AICD of T cells induced by PHA and HeLa cells in vitro. We also performed a case-control study to examine the association between FAS–FASL polymorphism and the risk of cervical cancer.

RESULTS

FAS and FASL genotypes and cervical cancer risk

Baseline clinical characteristics of cases and controls are summarized in Table I. The age distribution and smoking status in patients were not significantly different from those in controls (P = 0.583 and 0.537, respectively). An absolute majority of cases (97.8%) was infected with HPV. Of 314 patients, 95.2% were classified as squamous cell carcinoma, whereas 3.5 and 1.3% were adenocarcinoma and adenosquamous carcinoma, respectively. According to the International Federation of Gynecology and Obstetrics classification, 21.3% of patients had carcinoma in situ (stage 0), whereas 21.3, 37.6, and 18.5% of patients had stage I, II, and III disease, respectively. Only 1.3% of patients had stage IV cervical cancer.

Genotype analyses of FAS and FASL polymorphisms were successful for all patients, but 13 controls failed to be

Table I. Baseline clinical characteristics of cases and controls

	Patients (n = 314)		Controls (n = 628)		P[a]
	No.	*(%)*	*No.*	*(%)*	
Age (yr)					0.583
≤40	129	(41.1)	244	(38.8)	
41–50	114	(36.3)	223	(35.6)	
>50	71	(22.6)	161	(25.6)	
Smoking status					0.537
Nonsmoker	293	(93.3)	579	(92.2)	
Smoker	21	(6.7)	49	(7.8)	
HPV infection status					
HPV+	307	(97.8)			
HPV−	7	(2.2)			
Histological type					
Squamous cell carcinoma	299	(95.2)			
Adenocarcinoma	11	(3.5)			
Adenosquamous carcinoma	4	(1.3)			
Clinical stage[b]					
Stage 0	67	(21.3)			
Stage I	67	(21.3)			
Stage II	118	(37.6)			
Stage III	58	(18.5)			
Stage IV	4	(1.3)			

[a]Two-sided χ^2 test.
[b]According to the International Federation of Gynecology and Obstetrics classification.

genotyped because of PCR amplification problems with their DNAs (Table II). The allelic frequencies for the FAS –1377A and FAS –670G alleles were 0.317 and 0.343 in controls, respectively, compared with 0.312 and 0.332 in patients. For FASL, the frequencies of the –844C alleles in controls and patients were 0.699 and 0.792, respectively. The observed genotype frequencies for both FAS and FASL in controls and patients conformed to the Hardy-Weinberg equilibrium. The two FAS polymorphisms are in almost complete linkage disequilibrium in our study population ($D' = 0.98$; $P < 0.001$). Although the distributions of the FAS –1377G→A and FAS –670A→G genotypes were not significantly different between cases and controls ($P = 0.909$ and 0.652, respectively), the frequencies for the FASL –844T→C genotypes among cases were significantly different from those among controls ($\chi^2 = 20.59$; $P < 0.0001$), and this difference was mainly caused by a higher frequency of the CC genotype among cases compared with controls (61.5 vs. 46.2%). However, no difference in the distribution of FAS and FASL genotypes among different histological types and different clinical stages of the cancer were observed (unpublished data). Multivariate regression analyses revealed that subjects carrying the FASL –844CC genotype had a threefold increased risk of developing cervical cancer (odds ratio [OR] = 3.05; 95% confidence interval [CI] = 1.43–6.52; $P = 0.004$) compared with those carrying the TT genotype. The heterozygous CT genotype also presented a higher risk for the cancer (OR = 1.68; 95% CI = 0.78–3.66), although the association was not statistically significant ($P = 0.187$). In the stratification analysis, age and smoking had no effect on the risk of cervical cancer related to FASL genotypes (unpublished data). Because of tight linkage, the haplotype of FAS −1377G→A and FAS −670A→G did not further increase the risk of the cancer (unpublished data). In view of the fact that FAS and FASL work together in apoptotic cell death, we examined whether there was a statistical interaction between the SNP in FAS and FASL that was associated with the risk of cervical cancer. However, no such interaction was evident (unpublished data).

FAS and FASL genotypes and their expression on T cells

To determine the effects of the SNPs in FAS and FASL on expression of the genes, PBMCs isolated from 40 healthy donors carrying different genotypes were incubated with PHA or mitomycin C (MMC)–treated HeLa cells. The proportion of $CD25^+/CD4^+$ cells to $CD3^+$ cells was determined as an index of T cell activation. In addition, the levels of IL-2 in the supernatants of culture media of PBMCs were also determined as an indicator of T cell activation. It was found that the proportion of $CD25^+/CD4^+$ cells (mean ± SD, $n = 40$) in the PBMC cultures with PHA was 50.83 ± 14.08%, which was significantly higher than that in the cultures without PHA (2.27 ± 0.88%; $P < 0.001$). In parallel, the IL-2 level (mean ± SD, $n = 40$) in the PBMC culture media with PHA was significantly higher than that in the PBMC culture media without PHA (191.25 ± 66. 41 pg/ml vs. 28.82 ± 15.39 pg/ml; $P < 0.001$). Similarly, the proportion of $CD25^+/CD4^+$ cells was significantly elevated when PBMCs were incubated with MMC-treated HeLa cells compared with those incubated without MMC-treated HeLa cells (9.98 ± 3.32% vs. 6.83 ± 2.61%; $P < 0.001$). The activation of T cells by HeLa antigen was also evidenced by the fact that the mean IL-2 level in the PBMC culture media incubated with HeLa cells was significantly higher than that in the PBMC culture media without HeLa cells (150.63 ± 48.05 pg/ml vs. 98.93 ± 53.47 pg/ml; $P < 0.001$). These results demonstrated that both PHA and HeLa cell antigens were able to activate PBMCs under our study

Table II. Allele and genotype frequencies of FAS and FASL among controls and cases and their association with cervical cancer

Genotype	Controls ($n = 615$)	Patients ($n = 314$)	OR[a] (95% CI)	P
	No. (%)	*No. (%)*		
FAS –1377G→A				
GG	282 (45.8)	144 (45.9)	reference	
AG	277 (45.1)	144 (45.9)	1.01 (0.75–1.37)	0.934
AA	56 (9.1)	26 (8.2)	0.87 (0.51–1.49)	0.617
A allele frequency	0.317	0.312		
FAS –670A→G				
AA	268 (43.6)	138 (43.9)	reference	
AG	272 (44.2)	144 (45.9)	1.09 (0.81–1.47)	0.850
GG	75 (12.2)	32 (10.2)	0.85 (0.53–1.36)	0.425
G allele frequency	0.343	0.332		
FASL –844T→C				
TT	40 (6.5)	10 (3.2)	reference	
CT	291 (47.3)	111 (35.3)	1.68 (0.78–3.66)	0.187
CC	284 (46.2)	193 (61.5)	3.05 (1.43–6.52)	0.004
C allele frequency	0.699	0.792		

[a]Data were calculated by unconditional logistic regression, adjusting for age and smoking status.

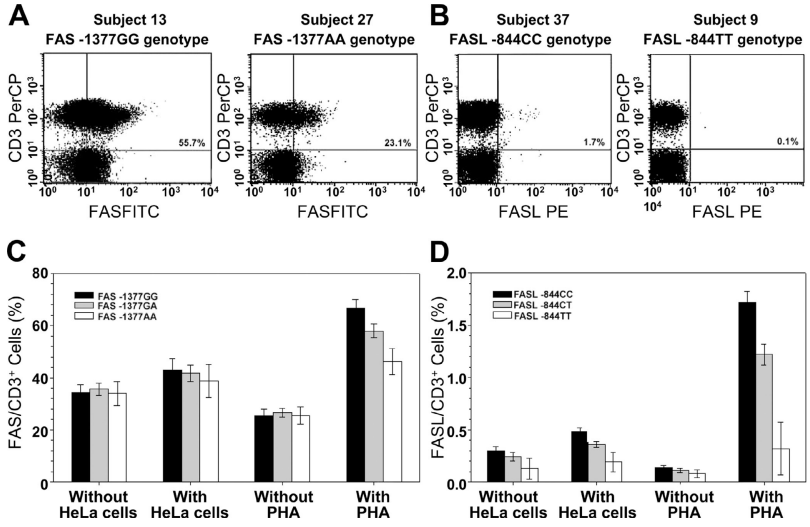

Figure 1. Differential expression of FAS and FASL on CD3$^+$ cells in PHA- or HeLa cell–stimulated PBMCs from individuals carrying different FAS or FASL genotypes. (A and B) Representative flow cytometry pictures showing differential FAS or FASL expression in different genotypes. Values indicate the percentage of FAS/CD3$^+$ and FASL/CD3$^+$ cells, respectively. (C and D) FAS or FASL expression levels on T cells as a function of genotypes. PBMCs from individuals were cultured with or without HeLa cell antigen and MMC-treated HeLa cells for 72 h or cultured with or without PHA for 6 h. FAS and FASL expression levels were adjusted for CD25 and IL-2 levels. FAS expression levels (C) on T cells with PHA but not HeLa cells were significantly different among three FAS –1377 genotypes ($P < 0.05$). FASL expression levels on T cells with both PHA and HeLa cells were significantly different among three FASL –844 genotypes ($P < 0.05$).

conditions. Moreover, neither the elevated population of CD25$^+$/CD4$^+$ cells nor IL-2 concentration in the media was correlated to FAS –1377, FAS –670, or FASL –844 genotypes (unpublished data).

Next, we examined whether the FAS –1377 or FAS –670 and FASL –844 SNPs had effects on the expression of FAS and FASL on T cells in PHA- or HeLa cell–stimulated PBMCs. The representative flow cytometry determination of FAS and FASL expression is shown in Fig. 1, A and B. We observed a significant difference ($P < 0.05$) in the FAS expression on T cells after incubation with PHA as a function of FAS genotype (Fig. 1 C). The FAS –1377GG or GA genotype had a significantly heightened FAS expression levels (mean ± SD) compared with the AA genotype (66.63 ± 3.53% [$n = 12$] and 57.92 ± 2.61% [$n = 22$] vs. 46.31 ± 5.02% [$n = 6$]; $P = 0.002$ and 0.048, respectively), whereas the levels were not significantly different ($P = 0.355$) between the GG and GA genotype. Similar results were obtained for the FAS –670 polymorphism, which is in almost complete linkage disequilibrium with the FAS –1377 polymorphism. However, the expression of FAS in PBMCs incubated with HeLa cells among three genotypes was not statistically significant ($P > 0.05$; Fig. 1 C). For FASL expression after stimulation by PHA, the FASL –844CC genotype had significantly higher levels than the CT or TT genotype (1.72 ± 0.10% [$n = 18$] vs. 1.22 ± 0.10% [$n = 18$] and 0.32 ± 0.25% [$n = 4$]; $P = 0.002$ and $P < 0.0001$, respectively), and the difference between the heterozygous CT genotype and the variant homozygous TT genotype was also statistically significant ($P = 0.002$). After stimulation by HeLa cells, the FASL expression in subjects with the CC genotype was 0.48 ± 0.04%, which was significantly higher than that in subjects with the CT (0.36 ± 0.03%; $P = 0.018$) or TT (0.19 ± 0.09%; $P = 0.004$) genotypes, although the levels between the CT and TT genotypes were not statistically different, possibly because of limited statistical power (Fig. 1 D). These findings clearly demonstrated that the investigated polymorphisms in the promoter region of FAS and FASL have a substantial impact on the activation-induced expression of FAS and FASL on T cells.

FAS and FASL genotypes and AICD of T cells

To examine the effects of FAS and FASL genotypes on AICD of T cells, PBMCs incubated with PHA or HeLa cells were labeled with FITC–Annexin V, propidium iodide (PI), and PerCP-Cy5.5-CD3, and the proportion of Annexin V$^+$ cells in the CD3$^+$ gate were determined by flow cytometry (Fig. 2 A). We found that neither FAS nor FASL genotypes had an effect on the spontaneous apoptosis of T cells during culture. However, when T cells were incubated with either PHA or HeLa cells, the differences in AICD among three different FASL –844 genotypes were striking. As shown in Fig. 2 B, after incubation with HeLa cells, the mean proportion (±SD) of Annexin V$^+$ cells was significantly higher in T cells carrying the FASL –844CC genotype (11.59 ± 5.39) compared with that in T cells carrying the –844CT (7.36 ± 3.45; $P = 0.006$) or –844TT (6.76 ± 0.34; $P = 0.068$) genotype. Similarly, after incubation with PHA, the differences in AICD of T cells among three FASL genotypes were also significant, with the proportion of Annexin V$^+$ cells being higher in T

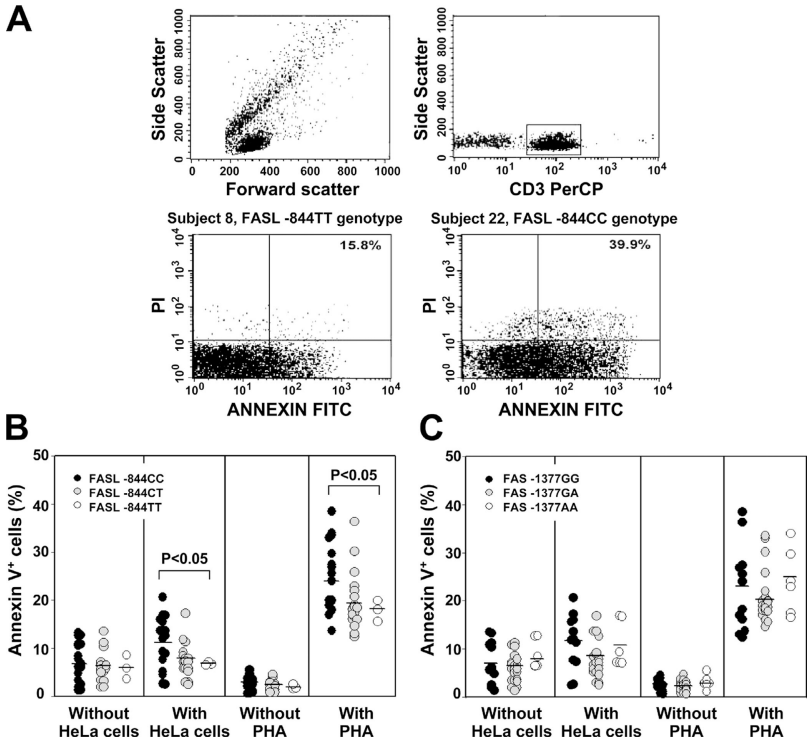

Figure 2. AICD of T cells in PBMCs from healthy individuals carrying different FAS or FASL genotypes determined by flow cytometry. (A, top) Annexin V assay was performed to detect the apoptosis index by flow cytometry in $CD3^+$ cells within a lymphocyte gate. Apoptosis index was defined as the ratio of Annexin $V^+/CD3^+$ cells. (A, bottom) Representative flow cytometry showing apoptotic cells in PBMCs from two individuals with different FASL genotypes after simulation by PHA. The percentages of Annexin V^+ cells are shown. (B and C) The relationship between genotypes of FAS and FASL rates of AICD of T cells. The FASL −844 genotype but not the FAS −1377 genotype was associated with AICD. The FASL −844CC genotype had a significantly higher rate ($P < 0.05$) of AICD compared with the FASL −844CT or TT genotype when T cells were incubated with HeLa cells or PHA.

cells carrying the −844CC genotype than that in T cells carrying the −844TC or −844TT genotype ($P < 0.05$ for both). However, the differences in AICD of T cells stimulated either with HeLa cells or with PHA were not significant ($P > 0.05$) among different FAS −1377 genotypes (Fig. 2 C).

DISCUSSION

Nearly every cell in the body has the potential to threaten the life of an individual if it transforms into cancer. Therefore, the immune surveillance of T cells plays an essential role in maintaining homeostasis (1–3). The FAS–FASL system regulates the AICD process of T cells, and genetic variations in these cell death pathway genes may thus influence susceptibility to cancers. Functional germline and somatic mutations in the FAS gene, and perhaps also in the FASL gene, that impair apoptotic signal transduction are associated with susceptibility to cancer (26–30). We have recently reported that functional SNPs in FAS and FASL increase the risk of esophageal cancer and lung cancer in a Chinese population (21, 22). Cervical cancer is a unique tumor that tightly links to infection of oncogenic types of HPV. So far, no specific oncogenes or tumor suppressor genes have been defined in this cancer (12). Therefore, we performed a case-control study to investigate whether genetic polymorphisms in FAS and FASL confer susceptibility to cervical cancer, a typical viral infection–associated cancer, in a Chinese population. Based on the analysis of 314 patients and 615 frequency-matched controls, we found that subjects carrying the FASL −844CC genotype had a threefold increased risk for cervical cancer compared with those carrying the FASL −844TT genotype, whereas the FAS polymorphisms seemed not to be associated with the risk of this cancer. In addition, we observed no associations between genotypes and histological types or clinical stages of cervical cancer. However, this might be the result of a limited sample size in each stratum, and more studies with stronger statistical power would be helpful to clarify the associations.

FASL −844T→C polymorphism has been shown to have a substantial impact on promoter activity of the FASL genes in an in vitro assay system because it is located in a binding motif for transcription factor CAAT/enhancer-binding protein β (18). However, little or nothing is known so far about genotype and phenotype association; i.e., the effect of this polymorphism on the expression of FASL in vivo in cells. Our data in the present study provide evidence for the first time that the −844T→C polymorphism in FASL strongly in-

fluences the expression of FASL ex vivo on T cells stimulated by either cervical cancer cells or PHA. More importantly, our study demonstrates that the FASL –844C allele, which had higher expression of FASL on T cells, was associated with an enhanced rate of AICD of T cells. This genotype and phenotype correlation between FASL –844T→C polymorphism and AICD of T cells was consistent with molecular epidemiological findings, demonstrating the FASL –844C allele as an at-risk allele for cervical cancer. Because FASL–FAS–mediated AICD is considered to be one of the important mechanisms responsible for the TIL apoptosis occurring in the tumor microenvironment (4–7), individuals carrying the FASL –844C allele and, thus, with higher FASL expression on T cells on tumor antigenic stimulation would be anticipated to be susceptible to cancer development. On the other hand, it has been shown that various types of tumors express FASL, and aberrant FASL expression has been linked to cervical carcinogenesis and tumor progression (15–17). Because of the functional consequence of the FASL –844T→C polymorphism, it would be expected that individuals carrying the FASL –844C allele may also have higher FASL expression on tumor cells compared with those carrying the FASL –844T allele. Heightened expression of FASL may facilitate transformed cells to counterattack the FAS-expressing TILs (4, 10, 11, 15, 31), resulting in immune evasion of these malignant cells. This mechanism may also contribute to an increased risk for developing cervical cancer among subjects carrying the risk FASL allele.

We did not observe any substantial association between the investigated functional polymorphisms in FAS and the rate of AICD of T cells stimulated by PHA or HeLa cells, although the polymorphisms did somewhat influence the expression of the FAS gene ex vivo on T cells. This finding was consistent with the population data in the present study but was inconsistent with our previous results from large molecular epidemiological studies on esophageal and lung cancer showing a positive association between the FAS polymorphisms and risk of these cancers (21, 22). This discrepancy may reflect the essential difference in carcinogenesis between cervical cancer and lung or esophageal cancer. However, these results may also suggest that, unlike FASL, a subtle change of widely expressed FAS might not be sensitive enough to detect a small change of AICD of T cells and the risk of the cancer. More studies with a larger sample size and stronger statistical power are needed to get conclusive results.

The data from the genotype–phenotype analysis in the present study are reliable because our experimental model using ex vivo PBMCs treated with HeLa cells mimicked the situation in vivo. HeLa cells are integrated with HPV-18 (32), and the antigen extracted from these cancer cells contains HPV oncoproteins, which are suitable for testing the response of cytotoxic T lymphocytes to both HPV infection and cervical tumor cells. In addition, it has been shown that HeLa cells persistently express FASL (15), which allows tumor cells in our experimental model to counterattack the Fas-expressing T lymphocytes using the T cell's own principal mechanism of cytotoxicity. Finally, all PBMC samples we selected for testing were of the HLA-A2 genotype, which are matched to HeLa cells (33). Therefore, the T cells sensitized by HeLa cell antigen could be activated by the following treatment with HeLa cells. Our results showing increased AICD caused by interaction of T cells with HeLa cells or PHA clearly demonstrate that our experimental system is valid.

Although little is known about polymorphisms in FASL and susceptibility to cervical cancer, at least three studies have been published regarding the FAS –670 polymorphism and the risk of cervical cancer. Lai et al. (23) conducted a study in a Chinese population in Taiwan and reported that the FAS –670A allele was associated with an increased risk of squamous intraepithelial lesions and squamous cell carcinoma. However, Dybikowska et al. (24) and Engelmark et al. (25) have recently shown that the FAS –670 genotype is not associated with a risk of the cancer in Caucasian populations. In this study, we analyzed both FAS –670 and –1377 polymorphisms and found that these two tightly linked polymorphisms were not associated with an increased risk of cervical cancer. The reasons for the inconsistent results among these studies are not clear, but geographic or ethnic differences in genotype frequencies should be considered, as have been extensively discussed in our previous reports (21, 34). In this study with 615 healthy controls, we observed a frequency of 12.2% for the variant FAS –670GG genotype, which is not significantly different from that reported by Dybikowska et al. (18.5%; $P = 0.06$) but significantly lower than that reported by Lai et al. (20.5%; $P < 0.001$). The genotype frequencies are not available in the report of Engelmark et al. because of the sib-pair design in their study (25). These geographic or ethnic differences in genotype frequencies might partially explain the dissimilar results.

Both FAS −670A→G and FAS −1377G→A SNPs were shown to have a biological impact on the transcriptional activity of the FAS gene, although the effect of −670A→G is still ambiguous (20, 35). We found a heightened expression of FAS on T cells stimulated by PHA that was associated with either the FAS −1377AA or FAS −670GG genotype. However, the role which SNP actually plays in influencing the gene expression remains unclear, because these two SNPs are in almost completely linkage disequilibrium in our donor population. It would be interesting and important to distinguish between an effect at −1377 and the effect at −670, and studies are underway to explore this issue. Another limitation of our study is that the HPV status of controls is unknown, and this did not allow us to explore whether the findings reflect the association of the genotypes with control of HPV infection (i.e., the ability to prevent persistent HPV infection) or the ability to eliminate infected cells before they undergo the transformation process.

In conclusion, our study demonstrates an association between the genotype and phenotype of FASL polymorphism

and suggests that heightened AICD of T lymphocytes and/or counterattack of tumor cells against TILs resulting from functional polymorphisms in the FAS–FASL pathway may be involved in the mechanism underlying an individual's susceptibility to the development of cervical cancer.

MATERIALS AND METHODS

Study subjects. The case-control study consisted of 314 patients with cervical cancer and 628 controls. All subjects were ethnic Han Chinese and residents of Beijing and its surrounding regions. Patients were recruited between June 2001 and March 2002 at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). All eligible patients diagnosed with cervical carcinoma at the hospital during the study period were recruited, with a response rate of 94%. Healthy controls were from a nutritional survey conducted in the same region during the period of case collection. The control subjects were randomly selected from a database consisting of 2,500 individuals based on a physical examination, including chest radiography and abdominal ultrasonography. Gynecological and cervical cytological examinations were also performed. The selection criteria included a history of no cancer or previous operations on the uterine cervix and free of cervical intraepithelial neoplasia. Controls were frequency matched to cervical cancer patients by age (±5 yr). Most control subjects (n = 520) were characterized in a breast case control study as reported previously (36). We added 108 more controls selected from the same database to make a 2:1 matching to the cases and to increase the statistical power. For FAS/FASL expression and AICD assays, blood samples were obtained from a group of 40 healthy volunteers (22 males and 18 females) working in our institution aged 19–49 yr (mean ± SD = 31.8 ± 8.6). All these volunteers were HLA-A2 positive with no history of malignant disease and clinically evident HPV infection. At recruitment, informed consent was obtained from each subject, and this study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

Genotype analysis. Genotypes of FAS −1377G→A, FAS −670A→G, and FASL −844T→C polymorphisms were determined by PCR-based restriction fragment length polymorphism assays as described previously (21). All subjects were successfully genotyped except for 13 controls whose DNA samples failed to be amplified. To ensure quality control, genotyping was performed without knowledge of case/control status of the subjects, and a 15% random sample of cases and controls was genotyped twice by different persons; the reproducibility was 100%.

Antibodies and reagents. PE-labeled mouse anti–human FASL (NOK-1, IgG1), FITC-labeled mouse anti–human FAS (DX2, IgG1), FITC-labeled mouse anti–human CD25 (2A3, IgG1), PerCP-Cy5.5–labeled anti–human CD4 (RPA-T4, IgG1), and PerCP-Cy5.5–labeled mouse anti–human CD3 (OKT3, IgG1) were purchased from BD Biosciences. The Annexin V–FITC apoptosis detection kit was purchased from BD Biosciences. MMC and PHA were obtained from Sigma-Aldrich.

HeLa cell culture, antigen preparation, and MMC treatment. HeLa cells (HLA-A2.1) were cultured in RPMI 1640 medium with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For production of HeLa cell antigen, portions of logarithmic growth HeLa cells were collected and washed with cold PBS. These cells were then shattered by freezing in liquid nitrogen and melted in a 37°C water bath alternately five times, followed by centrifugation at 2,200 *g* for 15 min at 4°C. The supernatants were collected and the total amount of protein was determined. Another portion of 10^6 HeLa cells/ml in RPMI 1640 medium was treated with 100 mg/ml MMC for 1 h at 37°C with occasional shaking to inhibit proliferation. These cells were then washed four times with the medium before use in the activation of PBMCs as described below.

PBMC isolation and culture. 5×10^6 PBMC/ml were isolated by Ficoll-Paque (Sigma-Aldrich) density-gradient centrifugation and suspended in RPMI 1640 medium, as used for HeLa cells. PBMCs were distributed into 24-well tissue culture plates at 1 ml/well and cultured in the presence of 25 μg/ml PHA for 6 h or cultured with 50 μg/ml HeLa cell antigen for the first 4 h, followed by the addition of MMC-treated HeLa cells (1:10 PBMCs) and cultured for the next 68 h. PBMCs cultured without PHA or HeLa antigen/cells served as respective controls. Cultured PBMCs were used to assay expression of FAS, FASL, and CD25 and the apoptotic index among $CD3^+$ or $CD4^+$ cells, and the cell culture supernatants were collected to determine IL-2 production as described below.

Flow cytometry analysis. A flow cytometer (FACSCalibur; BD Biosciences) was used to determine the cell surface expression of FAS, FASL, CD25, CD3, and CD4 and cell apoptosis index indicated by Annexin V and PI staining. Cultured PBMCs, as described above, were washed twice in cold PBS and incubated in PBS plus 3% FCS with 5 μl PE-labeled anti-FASL, 5 μl FITC-labeled anti-FAS, and 5 μl PerCP-Cy5.5–labeled anti-CD3 (tube 1); 5 μl FITC-labeled anti-CD25, 5 μl PE-labeled anti-CD3, and 5 μl PerCP-Cy5.5–labeled mouse anti-CD4 (RPA-T4, IgG1; tube 2); or 5 μl FITC-labeled Annexin V, 5 μl PI staining solution, and 5 μl PerCP-Cy5.5–labeled anti-CD3 (tube 3) in a 100-μl volume for 15 min in the dark at room temperature (25°C). The samples were washed twice with PBS-FCS, followed by the addition of 500 μl PBS with 1% paraformaldehyde to tubes 1 and 2 or 400 μl 1× BD Annexin V binding buffer to tube 3. The Annexin V–PI was analyzed by flow cytometry within 1 h, and other samples were stored at 4°C in the dark and analyzed within 24 h. A total of 50,000 events were counted within the lymphocyte gate for each samples.

IL-2 assay. PBMC culture media were collected, and IL-2 concentrations were determined by a human IL-2 ELISA kit (R&D Systems), according to the manufacturer's protocol. ODs were measured at 450 nm with a multilabel counter (Wallac Victor^2 1420; PerkinElmer Life and Analytical Sciences).

Statistical analysis. Results of cell surface molecule and expression, apoptosis index, and secreted IL-2 were expressed as means ± SD or as median. Statistical analysis of these results was done by the one-way analysis of variance test. Because the expression levels of FAS and FASL on T cells might be influenced by the activation status of T cells (37), an analysis of covariance was performed when analyzing FAS/FASL expression by using the expression level of $CD25^+/CD4^+$ cells and the concentration of IL-2 in cell culture media as covariates. A p-value of <0.05 was used as the criterion of statistical significance, and all statistical tests were two-sided tests. The association between FAS and FASL polymorphisms and the risk of cervical carcinoma was estimated using ORs and their 95% CIs, which were calculated by unconditional logistic regression. All the ORs were adjusted for age and smoking status. We tested the null hypotheses of additive and multiplicative gene–gene interactions between the FAS and FASL polymorphisms and evaluated departures from additive and multiplicative interaction models (38) by including main effect variables and their product terms in the logistic regression model. All analyses were performed with Statistical Analysis System software (version 6.12; SAS Institute). Haplotype frequencies and linkage disequilibrium coefficient were estimated using PHASE (39) and 2LD software (40).

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