

Host Genetic Polymorphism Analysis in Cervical Cancer

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Background: The natural history of cervical cancer comprises a latency period that probably involves long-term immunologic tolerance of human papillomavirus infection. Identifying host determinants of viral persistence may help to better understand the mechanisms of tolerance and may lead to the development of tests that can allow more focused follow-up of high-risk individuals.

Methods: Genotypic frequencies of 12 polymorphic loci in four candidate genes from 127 cervical cancer patients were compared with a control group of 108 female blood donors. Genotypes were determined by PCR amplification and direct sequencing of isolated genomic DNA.

Results: The tumor necrosis factor- α (TNF α) –238 polymorphism was significantly underrepresented in cervical cancer patients [heterozygotes (HETs), odds ratio (OR) = 0.33; 95% confidence interval (CI), 0.11–0.96], as was the TNF α –376 polymorphism (P = 0.02; 0% for any variant genotype in cases vs 4.7% in controls). The NRAMP1 3' untranslated region STP+86 polymorphism also appeared to be inversely associated with cervical cancer, but this result did not reach statistical significance (HET, OR = 0.57; 95% CI, 0.32–1.02). The p53 codon 72 arginine allele showed a suggestive negative association with cervical cancer (HET, OR = 0.49; 95% CI, 0.14–1.63; homozygotes, OR = 0.35; 95% CI, 0.11–1.17). The remaining alleles tested showed no association with cervical cancer.

Conclusions: We identified host genetic polymorphisms that may be associated with cervical cancer risk, some of which have been linked to potential functional effects on cellular immune responses or

antigen processing. We failed to confirm earlier reports of increased cervical cancer susceptibility in women who harbor the p53 P72R allele. Although our findings support the general hypothesis that host immunogenetic determinants other than class II MHC may be important in the development of cervical cancer, further analysis of the HLA gene cluster comprising the implicated TNF α single-nucleotide polymorphisms will be required to determine whether their association is linkage independent.

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Cervical cancer is the third most common cancer diagnosed in women worldwide, with a disproportionate share of the mortality associated with this disease occurring in developing countries (1, 2). The impact of cervical cancer in industrialized countries, however, has been drastically reduced mainly because of the Papanicolaou smear, colposcopy, and curative excision of high-grade dysplasia (3, 4). Although most low- and many high-grade dysplastic lesions appear to resolve without intervention, women are still advised to have follow-up examinations and treatments to ensure that the significant subset of women at high risk for developing cancer are treated. Decades of research have focused on identifying molecular markers that can determine the potential for the development of invasive carcinoma. Such efforts have produced many candidates, but none more significant than the discovery that cervical cancer is predominately caused or initiated by human papillomavirus (HPV)⁴ (5, 6). Cervical tumors have been shown to harbor HPV sequences in as many as 99.7% of the cases analyzed, implying a need for the sustained presence of viral DNA during carcinogenesis (7). This finding led to the wide assumption that HPV testing would become useful for the

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⁴ Nonstandard abbreviations: HPV, human papillomavirus; TNF α , tumor necrosis factor- α ; IL-4, interleukin-4; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; HET, heterozygote; HOM, homozygote; INT, intron; UTR, untranslated region; and T_H, T-helper cell.

diagnosis and monitoring of cervical dysplasia. Unfortunately, the mere presence of viral DNA has been shown to have poor positive predictive value for cervical cancer because of high rates of transient infections in sexually active women (8, 9). Recent evidence, however, has suggested a potential role for high-risk HPV DNA testing in managing patients with atypical cytologic findings (10, 11).

Given the mounting evidence that long-term HPV infection is a prerequisite for cervical carcinogenesis (12, 13), host genetic differences that influence the primary immune response may determine those who are at highest risk for progression to invasive carcinoma. Most research efforts to date have focused on the association of cervical cancer risk with HLA, especially certain class II MHC alleles (14–17). However, with a few exceptions, strong population-independent associations with specific HLA types have been elusive, perhaps because of linkage disequilibrium with the *HLA* gene cluster (18, 19). In recent years, several polymorphic immunogenetic loci inside and outside of the MHC region have been identified that may influence the outcome of infectious processes (20–23). We investigated several polymorphic loci within the tumor necrosis factor- α (*TNF α*), interleukin-4 (*IL-4*) receptor, and *NRAMP1* genes, all of which may play important roles in the outcomes of infections of various types. Single-nucleotide polymorphisms (SNPs) in these loci alter the immune response to infectious agents by altering the balance of cellular and humoral immune responses (24–27). We also investigated several polymorphisms within the DNA damage-response gene, *p53*, based on conflicting reports of increased sensitivity of specific alleles of this protein to HPV-E6-associated ubiquitin-mediated degradation (28). Here we report the results of this investigation and discuss the potential of human genetic polymorphisms to influence the pathogenesis of cervical cancer.

Materials and Methods

The Mayo Clinic Institutional Review Board approved this study, and informed consent was obtained from all participants. The frequency of selected polymorphisms in four candidate genes was compared between cervical cancer cases and blood donor controls. The case group consisted of 127 cases of cervical cancer seen at the Mayo Clinic from 1993 through 1996. From the original 131 patients with cervical cancer, adequate DNA was available to complete the analysis in 127. Of the 127 patients in the study group, 49 (38.6%) were from Minnesota. Of these, 16 (12.6% of the total) were from Olmsted County (location of the Mayo Clinic) or the immediately surrounding counties. Fifty-four patients (42.5%) were from the four states bordering Minnesota. Twenty-one (16.5%) were from other areas of the US, and 3 (2.4%) were from other countries. The mean (SD) age of the patients was 48.3 ± 14.4 years. The case samples were analyzed for the presence of HPV by use of the consensus L1 region

(MY09/MY11) primers, as described previously (14) and found to contain the following types: 78.7% HPV-16, 13.3% HPV-18, and 8% other types. The controls analyzed consisted of 108 randomly selected female blood donors seen at the Mayo Clinic. On the basis of historical information, most of the blood donor controls were from Olmsted County or a surrounding five-county area. Information on race is not typically collected in the Mayo Clinic health record, but on the basis of subject surnames and the usual demographics at our clinic, we estimated that >90% of the patients and controls were of white, non-Hispanic descent.

Genomic DNA was extracted from both sample sets with the Isoquick Nucleic Acid Extraction Kit (ORCA Research) according to the manufacturer's recommended protocol. Each polymorphic locus was amplified and then sequenced in both the forward and reverse directions (Table 1). Sequences were obtained by use of an ABI-377 fluorescence-based sequencing system, and base calls were determined automatically. All base calls within polymorphic sites were verified manually (Fig. 1).

Genotype frequencies for the controls were compared with Hardy-Weinberg proportions using the Fisher exact test, and none were found to exhibit excess homozygosity. The distributions of genotypes among cases and controls were compared by χ^2 tests or two-tailed Fisher exact tests when the sample size was small (*P* values reported in Table 2). Logistic regression was used to estimate the odds ratios (ORs) (7) and 95% confidence intervals (CIs) for the variant heterozygotes (HETs) and homozygotes (HOMs) relative to the wild-type HOMs. All statistical analyses were performed using the SAS statistical software (Ver. 6.12; SAS Institute).

Results

Allelic frequencies of 12 polymorphisms within 4 genes were determined by direct DNA sequence analysis for 127 cervical tumors and 108 female blood donor controls. Potential associations of individual alleles were evaluated and are reported in Table 2. Included were two *TNF α* promoter polymorphisms, -238 (A allele) and -308 (A allele), that have previously been shown to increase constitutive and inducible *TNF α* mRNA transcription and to associate with susceptibility to and severity of infections by different microbial agents (29). In our study, the frequency of G/A heterozygous individuals for the -238 polymorphism was 3.9% in the cases and 11.2% in the controls, which reached statistical significance (*P* = 0.04). Compared with the G/G genotype, there was an inverse association of the G/A genotype with risk of cervical cancer (OR = 0.33; 95% CI, 0.11–0.96). However, there was only a single A/A HOM, which occurred in the case group, so potential dosage effects of this allele could not be assessed. In contrast, for the well-studied -308 allele, the frequency of the A allele was nearly identical between cases (45 of 254; 17.7%) and controls (38 of 216; 17.6%; *P* = 0.30). Two other promoter polymorphisms (-244 and

Table 1. Oligonucleotide primer sets.

PCR gene	Region	Polymorphisms	Primer sequence	Amplicon size, bp
<i>TNFα</i>	Promoter	-238, -308, -244, -376	5'-CTCTCCCTCAACGGACTCAG-3' 5'-CTTGGTGGAGAAACCCATGAG-3'	390
<i>NRAMP1</i>	Intron 4	INT4	5'-CTTCTCTGGGTGCTGCTCTGG-3' 5'-AGACTTGGATGCCCATGG-3'	249
<i>NRAMP1</i>	3-UTR	TGTG Del, STP+86	5'-TCGGCTGGGAGTGGCATGT-3' 5'-ATGGCAAGGGTGGTGGTGTGTC-3'	223
IL-4 receptor	Cytoplasmic tail	Codon 576	5'-GAACCCGAGATGCCCTGTGTC-3' 5'-CACATTTCTCTGGGACACAGCAC-3'	284
<i>p53</i>	Intron 3-exon 4	Insertion, C/A, exon 4, codon 72	5'-AATTCATGGGACTGACTTTCTGC-3' 5'-TAGCTGCCCTGGTAGCTTTCTG-3'	374
Sequencing gene	Region	Polymorphisms	Primer sequence	Sequencing direction
<i>TNFα</i>	Promoter	-238, -308, -244, -376	5'-TGAAGCCCCTCCCAGTTCTAG-3' 5'-TCATCTGGAGGAAGCGGTAG-3'	Forward Reverse
<i>NRAMP1</i>	Intron 4	INT4	5'-TGTGGGCTTGCTGTGCCAG-3' 5'-GAAAATGGCTGTTGGGTTGG-3'	Forward Reverse
<i>NRAMP1</i>	3-UTR	TGTG Del, STP+86	5'-GGCTGGGAGTGGCATGTATG-3' 5'-GGCAACGGTGGTGGTGTCTC-3'	Forward Reverse
IL-4 receptor	Cytoplasmic tail	Codon 576	5'-CCAACCACTGTGCCCAACC-3' 5'-GCCTTGAACAGCCTCTCC-3'	Forward Reverse
<i>p53</i>	Intron 3-exon 4	Insertion, C/A, exon 4, codon 72	5'-CTGCTCTGTCTTTCAGACTTCCTG-3' 5'-TCTGGGAAGGGACAGAAGATGAC-3'	Forward Reverse

-376) have been described in the *TNF α* promoter region, but these have not yet been shown to alter transcription or to be associated with susceptibility to infection. We found only a single A (variant) allele for the -244 polymorphism, and this occurred in the control group. For the -376 polymorphism, the A (variant) allele was identified

in 0% of the cases and 4.7% (5 of 108) of the controls, or 2.3% (5 of 216) of the total alleles analyzed. The higher prevalence of *TNF α* ⁻³⁷⁶ HETs in the control group was statistically significant ($P = 0.02$).

The *Nramp* (*Bcg/Lsh/Ity*) gene was originally identified in inbred strains of mice and shown to confer increased susceptibility to infection by several infectious agents (30–32). The human *NRAMP1* gene has been shown to contain several polymorphic regions, including areas within intron 4 (INT4) and the 3'-untranslated region (UTR), that are apparently associated with increased susceptibility to infection by intracellular pathogens (22, 33, 34). However, we found that the frequency of the previously implicated INT4 C allele was similar among cases (62 of 254; 24.4%) and controls (60 of 216; 27.8%). Compared with the G/G genotype, the G/C genotype was not associated with cervical cancer risk (OR = 1.02; 95% CI, 0.59–1.76), whereas the C/C genotype was apparently inversely associated with risk (OR = 0.49), although the latter estimate was not statistical significant (95% CI, 0.17–1.43). The previously described 3'-UTR TGTG^{del} polymorphism was rare, with an allele frequency of 1.2% (3 of 254) among cases and 1.9% (4 of 216) among controls. No variant HOMs were identified.

In the course of this investigation, we discovered a previously undescribed SNP within the 3'-UTR of the *NRAMP1* gene (3'-UTR, STP+86; Fig. 1). This allele was common in both the case and control populations. The G (variant) allele was equally common in the cases (98 of 254; 38.6%) and controls (91 of 216; 42.1%). However, compared with the A/A genotype, the A/G (OR = 0.57), but not the G/G genotype (OR = 0.93), was inversely associated with cervical cancer.

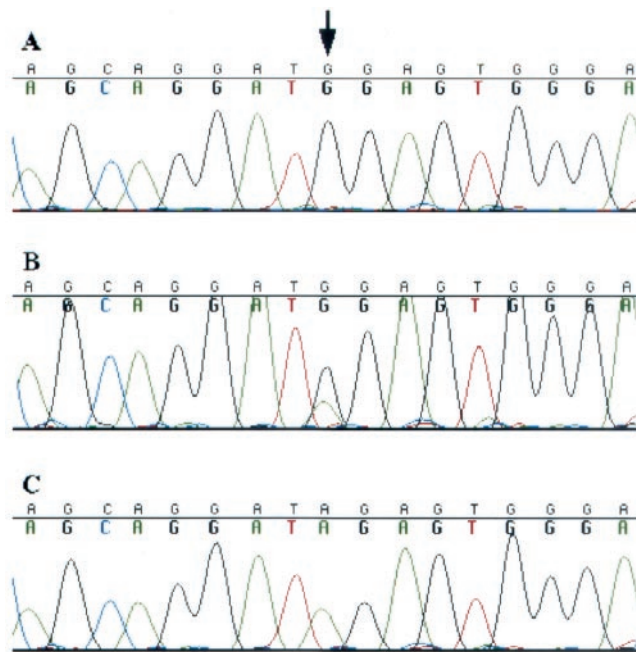


Fig. 1. *NRAMP1* STP+86 polymorphism site, depicted with the arrow, showing a homozygous G (A), a heterozygous G/A (B), and a homozygous A individual (C).

Each chromatogram is a representative example of the sequence obtained from the samples analyzed.

Table 2. Association of selected polymorphisms with risk of cervical cancer.

Gene	Polymorphism	Genotype	Cases (n = 127)		Controls (n = 108)		OR	95% CI	Global test P value ^a
			n	%	n	%			
<i>TNFα</i>	-238	G/G	121	95.3	95	88.8	1	Reference	0.04
		G/A	5	3.9	12	11.2	0.33	0.11–0.96	
		A/A	1	0.8	0	0.0			
	-308	G/G	91	71.6	73	68.2	1	Reference	
		G/A	27	21.3	30	28.0	0.72	0.40–1.32	
		A/A	9	7.1	4	3.8	1.81	0.53–6.10	
	-244	G/G	127	100.0	106	99.1			
		G/A	0	0.0	1	0.9			
		A/A	0	0.0	0	0.0			
	-376	G/G	127	100.0	102	95.3			
		G/A	0	0.0	5	4.7			
		A/A	0	0.0	0	0.0			
<i>NRAMP1</i>	Intron 4	G/G	71	55.9	58	53.7	1	Reference	
		G/C	50	39.4	40	37.0	1.02	0.59–1.76	
		C/C	6	4.7	10	9.3	0.49	0.17–1.43	
	3'-UTR TGTG Del	+/+	124	97.6	104	96.3	1	Reference	
		+ /Del	3	2.4	4	3.7	0.63	0.14–2.88	
		Del/Del	0	0.0	0	0.0			
	3'-UTR STP+86	A/A	47	37.0	29	26.9	1	Reference	
		A/G	62	48.8	67	62.0	0.57	0.32–1.02	
	IL-4 receptor	Codon 576	G/G	18	14.2	12	11.1	0.93	0.39–2.20
			A/A (Q/Q)	78	61.4	60	55.5	1	Reference
A/G (Q/R)			45	35.4	41	38.0	0.84	0.49–1.45	
<i>p53</i>	Codon 72	G/G (R/R)	4	3.2	7	6.5	0.44	0.12–1.57	
		G/G (R/R)	60	47.2	62	57.4	0.35	0.11–1.17	
		G/C (R/P)	56	44.1	42	38.9	0.49	0.14–1.63	
	Intron 3 insertion	C/C (P/P)	11	8.7	4	3.7	1	Reference	
		-/-	96	75.6	80	74.1	1	Reference	
		-/Ins	31	24.4	28	25.9	0.92	0.51–1.67	
	Intron 3 C/A	Ins/Ins	0	0.0	0	0.0			
		C/C	109	85.8	95	88.0	1	Reference	
		C/A	17	13.4	12	11.1	1.24	0.56–2.72	
	Exon 4	A/A	1	0.8	1	0.9	0.87	0.05–14.13	
G/G		125	98.4	106	98.1	1	Reference		
G/A		2	1.6	2	1.9	0.85	0.12–6.12		
	A/A	0	0.0	0	0.0			1.00	

^a P value to test whether the distribution of genotypes differs in cases and controls.

The final immunogenetic candidate locus investigated in this study was the human IL-4 receptor. Hershey et al. (25) in 1997 described a single C-terminal nucleotide polymorphism (codon 576 G allele) that introduces a gain-of-function mutation leading to increased IL-4 signaling, presumably producing type 2 T-helper cell (T_HII) immune bias. We reasoned that such a biological environment could make such individuals susceptible to HPV infection, which instead may require a type 1 T-helper (T_HI; cytotoxic) immune response for clearance of primary infection (35, 36). However, we failed to observe significant differences in allele frequencies between cases (53 of 254; 20.9%) or controls (55 of 216; 25.5%), and although the G/G genotype was inversely associated with cervical

cancer (OR = 0.44), this was not statistically significant (95% CI, 0.12–1.57).

Finally, in addition to immunogenetic analyses, we determined the allele frequency of another candidate susceptibility locus, the *p53* codon 72 polymorphism. This SNP produces a missense mutation (Arg/Pro) at codon 72 within the *p53* coding region, which has been implicated in some studies of HPV pathogenesis. Recent studies of the candidate susceptibility genotype (*p53*^{72Arg/Arg}) have reported conflicting results (28, 37–42). In our studies, which included larger numbers of cases and controls than most previous studies, the Arg/Arg genotype was actually more common in controls (62 of 108; 57.4%) than in cases (60 of 127; 47.2%). Consistent with overall allele

frequency data, both HETs and HOMs had a possible decreased risk for developing cervical cancer (HET, OR = 0.49; 95% CI, 0.14–1.63; HOM, OR = 0.35; 95% CI, 0.11–1.17). We also determined the frequency of three other *p53* polymorphisms (INT3 insertion, INT3 C→A, and Exon 4G→A). No association was found with these polymorphisms in our datasets.

Discussion

In this study we found that four polymorphic alleles, *TNF α* ⁻²³⁸ and *TNF α* ⁻³⁷⁶, *NRAMP1* 3'-UTR STP+86, and *p53* codon 72^{Arg}, were associated with cervical cancer. However, only two of these associations (*TNF α* ⁻²³⁸ and *TNF α* ⁻³⁷⁶) were statistically significantly associated (*P* < 0.05). The *TNF α* ⁻²³⁸ promoter polymorphism leads to high inducible concentrations of TNF α and the potential for severe inflammatory complications of *P. falciparum* infection (43). Increased susceptibility to chronic active hepatitis B and C has been suggested, which may also be attributable to the proinflammatory effects of TNF α after establishment of infection (44, 45). We found a statistically significant underrepresentation of the *TNF α* ⁻²³⁸ polymorphism, which may suggest a role for this polymorphism, or another in linkage disequilibrium, in enhancing the resolution of primary HPV infection or of cervical dysplasia. Jang et al. (46) recently reported similar findings in a South Korean population. Indeed, if overexpression of TNF α was an isolated protective factor, we would have expected the *TNF α* ⁻³⁰⁸ polymorphism to also be significantly underrepresented in cervical cancer cases relative to controls. We did not observe this, nor was a significant association with the *TNF α* ⁻³⁰⁸ allele noted in a recent study of Swedish cervical cancer patients (19). A recent analysis of the effects in vitro of TNF polymorphisms suggested that the *TNF α* ⁻²³⁸ SNP, and not the better-studied *TNF α* ⁻³⁰⁸ SNP, may have a greater impact on tissue-specific gene expression (47). An equally plausible explanation is that the *TNF α* ⁻²³⁸ allele, by virtue of its map location within the *HLA* gene cluster, is contained within an extended haplotype comprising other more closely disease-linked susceptibility loci.

We also evaluated two other polymorphisms in the TNF α promoter not previously reported to be functionally relevant in controlling gene expression. Although there was no association with the *TNF α* -244 polymorphism, we found an inverse association with the -376 polymorphism for which the biological significance is not yet known. Regardless, given the relative rarity of the variant allele, this polymorphism is unlikely to be an important practical determinant of cervical cancer risk, at least within similar study populations.

The *Nramp* (*Bcg/Lsh/Ity*) gene was originally identified among inbred strains of mice that were susceptible to infection with several different intracellular pathogens, including *Mycobacterium bovis*, *Leishmania donovani*, *Salmonella typhimurium*, and *M. lepraemurium* (31, 32, 48–50). Soon after its identification in mice, the human homolog,

named *NRAMP1*, was cloned, localized to chromosome 2q35, and found to be a lysosomal membrane protein with a putative divalent cation transporter function (24, 51, 52). *NRAMP1* has been shown to contain several polymorphic regions, including areas within INT4 (the G→C transition) and the 3'-UTR (the TGTG deletion), which are associated with an increased susceptibility to infection by intracellular pathogens (24, 33, 34). We found little evidence for a significant association for either of these polymorphisms. During the course of this investigation, however, we identified an A→G 3'-UTR polymorphism 86 nucleotides downstream of the termination codon (3'-UTR STP+86) in *NRAMP1* that has not previously been described. The variant G allele was relatively common, and we found that the HETs appeared to be at lower risk of cervical cancer, although this was not statistically significant. Moreover, the variant HOMs had a risk similar to the wild-type HOMs, further raising the issue of a chance finding, because variant HOMs would presumably have a risk similar to or greater than that of the HETs based on dosage effects.

The IL-4 receptor polymorphism we evaluated is reported to increase the signaling activity for this receptor and lead to T_HII immune bias (25). Consequently, patients with this polymorphism are at risk for development of atopic disorders such as hyper IgE syndrome and atopic dermatitis. Various immunosuppressive states associated with a reduction in cellular immune responses are associated with increasingly severe cervical dysplasia and increased viral shedding, suggesting that a T_H bias that opposes T_HI responses (i.e., T_HII bias) might predispose to chronic infection (36). Our data, contrary to our own prediction, suggested a nonsignificant inverse trend with this relatively common gain-of-function polymorphism. It is possible that the impact this polymorphism has on the immune response is overestimated or that the functional effects of this SNP do not extend to, or operate differently in, the context of mucosal immunity.

The *p53* codon 72 polymorphism has received considerable attention over the last few years after the initial report by Storey et al. (28), which described a polymorphism-dependent increase in sensitivity of *p53* to HPV-E6-mediated ubiquitin-dependent proteolysis, as well as increased risk of cervical cancer in carriers of the putative susceptibility allele. Subsequent studies attempted to reproduce this study, with mixed results (37–42). Our inability to confirm the association of the *p53*^{Arg/Arg} codon 72 genotype in a much larger population of cervical cancer patients is thus consistent with other studies of sufficiently large size. Differences among reports may be the result of variation in allele frequency among different ethnic populations, interlaboratory variation in the methods used to determine allele frequencies (39), or differences in the frequencies of HPV types represented in the cervical tumors tested [e.g., HPV-16 was 10% more common in our samples than in the population studied by Storey et al. (28)]. Although we cannot rule out the

possibility that this polymorphism may be an important determinant of cervical cancer risk in certain ethnic populations that are not well represented in our studies, the preponderance of evidence seems to weigh against the impact of this allele as a significant risk factor.

This exploratory study had some limitations that require comment. We had minimal demographic data on our control group of blood donors, although it should be broadly representative of the predominately upper-Midwestern population from which the cases were derived (>95% Caucasian). Likewise, we did not know the HPV infection status of our controls at the time of analysis, but based on the frequency of cervical dysplasia among healthy women in Rochester, MN and the surrounding area, this rate would be expected to be <5%. Overall, we found that the frequency of several alleles in our control group were consistent with other published data for Caucasians, who comprised >95% of the blood donor population in our study (25, 44, 53). Additional bias can be introduced by virtue of analyzing a large number of candidate SNPs in either the case or control population. We evaluated 12 polymorphisms in four candidate genes, raising a concern about false positives attributable to multiple statistical comparisons. Therefore, we did not choose to use the *P* value in isolation to determine the importance of our results, but also used the strength of the ORs and the precision of the 95% CIs to guide the interpretation of our results.

In conclusion, our findings are generally supportive of a role for host genetic susceptibility in the etiology of cervical cancer. The discovery of candidate susceptibility alleles provides a theoretical framework for further studies of the functional effects of these polymorphisms and facilitates the discovery of extended haplotypes that may confer even greater risk for cervical cancer susceptibility (18, 19, 54). In the context of managing cervical cancer risk, it is conceivable that some day a combination of HPV testing and host genetic testing will provide better positive predictive value than is currently afforded by HPV testing alone.

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