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TP53, MDM2, NQO1 and susceptibility to Cervical Cancer

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Abstract

Host genetic variability modifies the risk of cervical cancer in women infected with oncogenic human papillomavirus (HPV). Studies have reported an association of the *TP53* codon 72 arginine and cervical cancer, but the results are inconsistent. We examined the association of this single nucleotide polymorphism (SNP) in women with cervical cancer and cervical intraepithelial neoplasia grade 3 (CIN3), using family-based association test. We further explored SNPs in two genes that regulate p53 stability: *MDM2* (SNP309) and *NQO1* (SNP609, SNP465). We also examined the relationship between host genotype and tumor HPV type. We genotyped 577 patients and their biological parents and/or siblings, using PCR-RFLP or TaqMan assays. HPVs were typed by sequence-based methods. The transmission/disequilibrium test was used to detect disease-susceptibility alleles. The arginine peptide of *TP53* codon 72 was overtransmitted in Caucasian families (P=0.043), and the significance of this finding was enhanced in a subgroup of women infected with HPV16- and/or 18-related HPVs (P=0.026). Allele C of *NQO1* SNP609 was also overtransmitted in all cases (P=0.026). We found no association between *MDM2* SNP309 or *NQO1* SNP465 and cervical cancer. Our results indicate that functional polymorphisms in *TP53* codon 72 and *NQO1* SNP609 associate with the risk of cervical cancer especially in women infected with type 16- and/or 18-related HPVs.

Keywords

cervical cancer; *TP53*; *MDM2*; *NQO1*; HPV; family-based association study

Introduction

Infection with oncogenic human papillomavirus (HPV), particularly type 16- and/or 18-related HPVs, is the major risk factor for invasive cervical cancer (ICC). However, disease progression is variable among infected women, because host genetics in addition to environmental, hormonal, and possibly nutritional factors influences outcomes. For example, we previously showed that the *HLA DQB1* and *CD83* variants associated with ICC in a family-based association study (1-3). A single nucleotide polymorphism (SNP) in codon 72 of *TP53* (P72R,

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rs1042522) has attracted wide attention over the past decade. The C to G base change in codon 72 replaces proline (Pro) with arginine (Arg). Storey *et al*. found that women who are homozygous for *TP53*Arg are seven times more susceptible to HPV-associated squamous carcinoma of the cervix than are heterozygous women (4). Since then, many groups have reported an effect of the *TP53* codon 72 polymorphism on cervical cancer. However, the results of those case-control studies have been inconsistent due to many factors, such as differences in the frequency of *TP*53 polymorphisms among ethnic groups, sample size, choice of control sample, and DNA sample source.

TP53 executes its tumor suppressor function by regulating DNA repair, cell cycle arrest, and apoptosis. Levels of its product, tumor protein 53 (p53), are tightly controlled, and disruption of the p53 pathway is a hallmark of most cancers. The E6 protein encoded by HPVs 16 and 18 complexes with the p53 protein, inducing its ubiquitin-dependent degradation. The resulting lack of p53 eliminates the p53-dependent control of the cell cycle (5) .

Ubiquitin-dependent degradation of p53 in the proteasome is regulated by MDM2. An alternative pathway that does not require ubiquitin is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) (6,7). Therefore, stabilization of p53 requires either MDM2 or NQO1 activity.

MDM2 binds directly to and inhibits p53 by regulating its location, stability, and ability to activate transcription (8). A SNP in the *MDM2* promoter (SNP309 T/G, rs2279744) increases affinity for Sp1 transcriptional activator, resulting in higher levels of MDM2 protein and subsequent attenuation of the p53 pathway (9). A meta-analysis of 25 published case-control studies found that *MDM2* 309GG associates with a significantly increased risk of all types of cancers (odds ratio (OR):1.17; 95% confidence interval (CI):1.04-1.33) (9).

Variants in the *NQO1* gene also associate with susceptibility to various forms of cancer. A SNP in exon 6 (*NQO1*, 609 C/T, rs1800566) replaces proline with serine at amino acid position 187. The TT genotype gives rise to an inactive enzyme, and the CT genotype produces only mild activity compared with CC (10). These substitutions have been associated with cancer susceptibility in several solid tumors (11). Another SNP, in exon 4 (*NQO1*, 465 C/T, rs4986998), shifts arginine to tryptophan at amino acid position 139. This change disrupts the consensus sequence at the 5′-splice site of intron 4, increasing alternative splicing and decreasing the expression of NQO1 protein (12). The contribution of this polymorphism to cancer has received only limited attention, however.

We conducted a family-based study to investigate the association between these *TP53*, *MDM2*, and *NQO1* SNPs and invasive cervical cancer and CIN3. We also examined the effect of the HPV type on the infected cervical tissue.

Material and Methods

Subjects

We genotyped a total of 577 family trios. Each trio consisted of a proband–a woman with ICC or CIN3 (CIN3 and/or adenocarcinoma in situ)–and either her biological parents or one parent and usually one or more siblings. Blood or buccal cell samples were obtained from all participants. Cervical tissue either snap-frozen in Tissue-Tek O.C.T. compound (Sakura Finetek USA Inc., Torrance CA) or formalin-fixed, paraffin-embedded blocks were used to type HPV. The characteristics of patients with ICC and CIN3 are shown in Table 1. The study was approved by Washington University's Human Research Protection Office.

SNP typing

Genomic DNA was isolated as previously described (2). The *TP53* codon 72 polymorphism (rs1042522) was determined using PCR-RFLP analysis, as described by Ara *et al*. (13). PCR was performed in a standard procedure with the following primers: forward 5′-TTGCCGTCCCAAGCAATGGATGA-3′ and reverse 5′-TCTGGGAAGGGACAGAAGATGAC-3′. The annealing temperature was 55°C. PCR products were run in a 2.0% agarose gel. After we obtained an amplified fragment of the expected size (199 bp), we digested 3 μl PCR products with 0.5 units of BstU1 at 60°C for 2 h. Then 5 μl of each PCR product was electrophoresed in a 2.5% agarose gel. The Pro allele, which is not cleaved by BstU1, yields a single band with a fragment length of 199bp. The Arg allele, which is cleaved, runs as two small fragments of 113bp and 86bp. Digesting the heterozygote samples yields three bands of 199, 113, and 86bp, respectively.

MDM2 SNP309 (rs2279744) was also genotyped by PCR-RFLP, using the primers described by Bond *et al*. (14). The primer sets were forward 5′-CGGGAGTTCAGGGTAAAGGT-3′ and reverse 5′-AGCAAGTCGGTGCTTACCTG-3′. The annealing temperature was 63°C. The PCR product was digested with 0.2 U of MspA1 I at 37°C for 3h. The GG genotype was cleaved into four fragments: 184bp, 85bp, 50bp, and 31bp. The TT genotype was cleaved into three fragments: 234bp, 85bp and 31bp. The GT genotype produced all five fragments.

PCR-RFLP results were read by three investigators with no knowledge of family structure. Inconsistent results were repeated (<1% of the samples).

SNPs rs1800566 and rs4986998 were genotyped by a TaqMan genotyping assay, using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Tumor pathology and HPV Typing

A gynecologic pathologist (PH) confirmed the histology of all the samples. HPV typing was performed on DNA extracted from cervical cancers and CIN3 lesions as previously described. (2,15). Briefly, the DNA was amplified with primers to the conserved regions of HPV L1 (L1C1/L1C2M) and E6 (E6-L/E6-R). Aliquots of PCR were run on agarose gel and dHPLC and then sequenced. If tissue was infected with multiple HPV types (indicated by multiple peaks on dHPLC), the PCR fragments were first separated by the fragment collector and then sequenced. Families were grouped according to the HPV type detected in the probands' cervical neoplasia at diagnosis. HPV16-related types are HPV16, HPV31, and HPV52. HPV18-related types include HPV18 and HPV45. HPV16, 18-related types include single infection of above HPVs and multi-infection of above HPVs plus other types.

Association Analysis

We used the family-based test of association implemented in the program TRANSMIT (16). This method is robust to population stratification, and our association study included trios of different ethnicities. The transmission/disequilibrium test (TDT) test was used to determine whether a particular focal allele was overtransmitted to the affected women and therefore be associated with disease. Since heterozygosity is needed in the parents to discern which allele has been transmitted to an affected offspring, only doubly heterozygous matings are fully informative. However, heterozygosity in one parent provides partial information, and siblings and population allele frequencies can be used internally to impute any missing genotypes under a full maximum likelihood model of transmission. Thus, while the number of families analyzed remains constant, the number of informative families varies as a function of allele frequency and the consequent degree of heterozygosity. To aid in the interpretation of our results, we

report the identities of overtransmitted alleles, the significance of the overtransmission test, and the number of informative families contributing to the result.

To identify possible heterogeneity of risk, we conducted follow-up tests by analyzing the subset of trios in which the proband had HPV16- and/or HPV18-related disease. Studies by us and others suggest this subgroup of high risk HPV types have increased risk and poor prognosis. (1,17,18).

Results

HPV infection status

Tumor tissues from 373 patients (64.6%) were available for HPV typing. Of those, 326 (87.4%) were positive for at least one HPV16- and/or 18-related type. The most common types detected among the cases (either alone or in co-infection with other types) were HPV16 (59.7%) and HPV18 (19.8%). Infection with multiple HPV types was detected in 20.1% of the cases.

Genotyping Results

Table 2 shows the overall analysis of codon 72 of *TP53* in all the cases. There is no significant link between the G allele and cervical cases (ICC and CIN3, P =0.213). However, the G allele of that codon was overtransmitted in Caucasian cases (P=0.043), and that association was even stronger (P=0.026) in cases with HPV16- and/or 18- related infection. The G allele frequency was 71% for the Caucasian cases and 65% for the African American cases.

Tables 3, 4, and 5 show the overall analysis of MDM2 SNP309 and NQO1 SNPs. No significant association was found between *MDM2* SNP309 (rs2279744) and the study subjects, though overtransmission of the T allele was marginally significant when all cases were analyzed (ICC and CIN3, P=0.053). This contrasts with a previous meta analysis that found the G allele associated with a slight increased risk in various tumor types (9). Significant overtransmission of the C allele (P=0.026) of *NQO1* SNP609 (rs1800566) was seen in all cervical cancer cases (Table 4). However, there was no association between *NQO1* SNP465 (rs4986998) and cervical cancer (Table 5).

Discussion

Using a family-based association study, we confirmed that the *TP53* codon 72 G (arginine) is significantly overtransmitted in Caucasian ICC and CIN3 subjects, especially in cases infected with HPV16- and/or 18-related HPVs. The stronger association with HPV16- and/or 18-related HPVs enhances the plausibility of our findings. The *NQO1* SNP609 C allele was also significantly overtransmitted in all cases. We observed no significant association between *NQO1* SNP465 or *MDM2* SNP309 and cervical cancer.

The association between the *TP53* codon 72 polymorphism and cervical cancer has been investigated in many studies and in various populations since Storey et al (4) first identified the Arg/Arg genotype as a risk marker for cervical neoplasia. However, research on this polymorphism has produced conflicting results. Two recent meta-analyses confirmed the association of homozygous Arg with invasive cervical cancer (but not with preinvasive lesions), the overall OR being 1.1 to 1.2 (19,20). One reason for this association may be that the Arg variant of the p53 protein is more vulnerable than the Pro form to being bound and degraded by the E6 oncoprotein of HPVs 16 and 18 (4,5). A third meta-analysis failed to confirm the association in most European countries, except in Italy and the United Kingdom, where it was significant for invasive cervical cancer only (21).

Several factors could be contributing to the discrepancies, such as study design, ethnicity of the subjects, DNA sample source, HPV infection status, sample size, and laboratory methods. To date, all of the *TP53* polymorphism studies have had case-control designs. Control populations have included blood donors, age-matched populations, laboratory personnel, HPV-positive individuals only, and HPV- negative subjects only. Traditional case-control designs may give rise to spurious associations because of unrecognized population substructure. Our family-based approach is robust to population stratification.

The meta-analysis by Koushik et al. identified deviation from the Hardy-Weinberg equilibrium as the principal source of divergent results (19). Studies with observed departures from the equilibrium suggest a possible issue with the control group, such as bias in control selection, ethnic admixture in the population, or misclassified genotypes due to poor laboratory techniques (such as errors in genotype calls or using tumor DNA for genotyping, which could misclassify individuals as homozygous due to loss of chromosome segment in tumors).

Also, allele frequency varies across ethnic populations with different genetic backgrounds. For example, the frequency of the arginine variant of *TP53* codon 72 changes with latitude, ranging from 31% in South Africa to 50% in India and 76% in Finland (22). Hence, the power to detect the TP53 codon 72 Arg variant with cervical cancer is related to the frequency of that variant in a population: there is generally more power to detect the effect of the variant when it is common rather than rare (22-24). A pooled analysis of 49 studies showed a significant association for arginine and cervical cancer limited to Caucasians where the frequency of arginine in the population was higher than the other ethnic groups (25). This held true in our study, which is unbiased to population stratification. The Arginine is more frequent in the Caucasian than African American population and significantly overtransmitted in the Caucasian cervical cancer patients. (Table 2). However, the sample size for African American families was smaller and no association may be due to statistical power or other biologic factors in linkage with p53 in the Caucasian population.

Most studies to date have analyzed relatively small samples and have provided little or no information on tumor histology or HPV type/variants. Furthermore, only a few studies have evaluated the HPV status of cervical lesions. Lack of data on HPV status weakens a study's power, given that the association of the *TP53* polymorphism with the risk of cervical cancer is HPV-correlated. In our study, the tumor histology was confirmed and HPV was typed. The association was stronger in subjects affected with HPV16- and/or 18-related HPVs. However, family trios are unable to provide estimates of population parameters, such as the genotype relative risk.

The DNA source is yet another factor attributed to the inconsistency of the association. The sources used in the *TP*53 polymorphism assays varied from white blood cells, cervical cells, tumor biopsies, paraffin-embedded tissue, or remains unreported. Using tumor cells may lead to spurious results because cancer DNA often loses one allele at the *TP53* locus (26). Also, poor-quality DNA, such as that derived from formalin-fixed tissue, can inhibit PCR amplification. One review demonstrated the overall OR that Arg/Arg women would develop cervical cancer varied by DNA source. The OR appeared to increase when the DNA came from formalin-fixed tissues (20). In the present study, we used genomic DNA from blood or buccal cells for cases and their parents.

Another possible reason for discrepancies is that the significant SNPs identified in this study may predispose to rapid-onset cervical cancer since the probands in this study are predominantly young women. If age of onset is a factor studies of women with older age may dilute that effect.

Finally, one study showed that inter-laboratory differences may impair or destroy the ability to detect an association between p53 arginine and cervical cancer. The proportions of the Arg/Arg, Pro/Pro, Arg/Pro genotypes varied substantially among laboratories, with Kappa coefficients ranging from 0.49 to 0.63 (27). The OR for the Arg/Arg genotype in cervical cancer increased from 1.5 to 8.0 when discordant genotypes were excluded. By using stringent allele typing and restricting the comparison to HPV-positive controls, these investigators increased the OR to 21.5 (95%CI 3.4, 137.8) (27). To reduce errors in our study, the genotypes were read by three different investigators with no knowledge of family structure. Inconsistent results were less than 1% and all were repeated.

The combination of high levels of *MDM2* with the G-allele of SNP309 can severely weaken the p53 tumor suppressor pathway, resulting in a higher mutation rate, less efficient DNA repair, and reduced apoptosis. All of these deficits lead to faster and more frequent tumor formation (28). This SNP has been evaluated in several tumor types in diverse populations. A meta-analysis of 25 population case-control studies representing more than 2900 individuals found that the homozygous 309GG variant associated with a significantly increased risk of all types of cancers (OR 1.17) (9). A subsequent case-control study from Brazil, which found no association of *MDM2* with cervical cancer, was very small (72 cervical cancer cases (65.5% CIN3) and 100 healthy controls) (29). Our study may also have lacked the power to identify an association at such a small OR.

A meta-analysis suggested that the *NQO1* SNP609 TT genotype, with null enzyme activity, may affect individual susceptibility to lung, bladder, and colorectal cancer (11). In a Japanese study, TT genotype was a risk factor for cervical squamous cell carcinoma but not for adenocarcinoma or adenosquamous carcinoma (30). Also, a study of head and neck squamous cell carcinomas found no association with *NQO1* SNP609 or SNP465 (31). Our study showed that the C allele of SNP609 was significantly overtransmitted in ICC and CIN3, the opposite allele from these previous reported studies. This conflicting result may be attributable to differences in sample size and population characteristics. Niwa et al. studied a relatively small sample (total 131 cervical cancers), and the frequency of the T allele is known to differ among ethnic groups, being higher in Japanese people and lower in Caucasians (0.398 vs. 0.217) (30). Additional studies are needed to clarify this association and determine the reason for the variability.

Our study is the first to use a family-based sample to evaluate the risk of variants in *TP53*, *MDM2*, and *NQO1* genes in women with CIN3 and ICC. However, the number of informative family trios might have been too small to detect relatively small odds ratios, as identified in the meta-analyses for *MDM2* and *TP53*. For example, we may not have had sufficient power in our subgroup analyses.

In conclusion, this family-based association study indicated a significant association between *TP53* codon 72 and cervical cancer in a Caucasian population, and that association was even stronger in subjects infected with HPV16- and/or HPV18-related types. These results provide evidence at the molecular level for heritability of cervical cancer risk. The study suggests that further work on interactions among genetic variants in the p53 pathway, HPV, and other environmental stresses are warranted.

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Table 1

Characteristics of the study subjects

	Number of subjects
Total	577
Race	
Caucasian	514
African American	62
Asian American	1
Age	
Mean age (y) ± SD	33.97 ± 8.62
<40	425
≥40	144
Unknown	8
Stage	
0 (CIN3, adenocarcinoma in situ)	223
I	290
II	39
III	15
IV	1
Unknown	9
Histology (Stage I-IV)	
Squamous cell	220
Adenocarcinoma	89
Adenosquamous	15
Other	21
Unknown	9
HPV infections	
Overall HPV infection rate (%)	87.4% (326/373)
HPV16	59.7%
HPV18	19.8%
Multiple HPVs	20.1%

Table 2

Analysis of *TP53* codon 72 (rs1042522) by subgroups

Number of informative families	MAF*	Overtransmitted allele	*P* value	Race	HPV**	Stage
554	0.319	G	0.213	all	all	ICC/ CIN3
268	0.301	G	0.243	all	high	ICC/ CIN3
309	0.319	G	0.526	all	all	ICC
195	0.301	G	0.154	all	high	ICC
182	0.319	G	0.291	all	all	CIN3
70	0.301	G	0.882	all	high	CIN3
469	0.287	G	0.043	Caucasian	all	ICC/ CIN3
241	0.269	G	0.026	Caucasian	high	ICC/ CIN3
272	0.287	G	0.160	Caucasian	all	ICC
173	0.269	G	0.040	Caucasian	high	ICC
148	0.287	G	0.161	Caucasian	all	CIN3
65	0.269	G	0.593	Caucasian	high	CIN3

*MAF = Minor allele frequency

**High = HPV 16 and/or 18-related HPV

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Table 3

Analysis of *MDM2* (rs2279744) by subgroups

Number of informative families	MAF*	Overtransmitted allele	*P* value	Race	HPV**	Stage
577	0.328	T	0.053	all	all	ICC/ CIN3
353	0.328	T	0.278	all	high	ICC/ CIN3
344	0.328	T	0.051	all	all	ICC
238	0.328	T	0.257	all	high	ICC
221	0.328	T	0.156	all	all	CIN3
112	0.328	T	0.651	all	high	CIN3
513	0.348	T	0.131	Caucasian	all	ICC/ CIN3
325	0.348	T	0.363	Caucasian	high	ICC/ CIN3
310	0.348	T	0.076	Caucasian	all	ICC
218	0.348	T	0.304	Caucasian	high	ICC
193	0.348	T	0.242	Caucasian	all	CIN3
104	0.348	T	0.767	Caucasian	high	CIN3

*MAF = Minor allele frequency

**High = HPV 16 and/or 18-related HPV

Table 4

Analysis of *NQO1* (rs1800566) by subgroups

Number of informative families	MAF*	Overtransmitted allele	*P* value	Race	HPV**	Stage
524	0.209	C	0.026	all	all	ICC/ CIN3
251	0.203	C	0.166	all	high	ICC/ CIN3
284	0.209	C	0.092	all	all	ICC
182	0.203	C	0.548	all	high	ICC
176	0.209	C	0.263	all	all	CIN3
66	0.203	C	0.147	all	high	CIN3
441	0.211	C	0.195	Caucasian	all	ICC/ CIN3
224	0.205	C	0.516	Caucasian	high	ICC/ CIN3
248	0.211	C	0.314	Caucasian	all	ICC
160	0.205	C	0.826	Caucasian	high	ICC
143	0.211	C	0.239	Caucasian	all	CIN3
61	0.205	C	0.228	Caucasian	high	CIN3

*MAF = Minor allele frequency

**High = HPV 16 and/or 18-related HPV

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Table 5

Analysis of *NQO1* (rs4986998) by subgroups

Number of informative families	MAF*	Overtransmitted allele	*P* value	Race	HPV**	Stage
349	0.034	C	0.509	all	all	ICC/ CIN3
245	0.032	C	0.564	all	high	ICC/ CIN3
232	0.034	C	0.420	all	all	ICC
178	0.032	C	0.220	all	high	ICC
117	0.034	C	0.960	all	all	CIN3
67	0.032	T	0.426	all	high	CIN3

*MAF = Minor allele frequency

**High = HPV 16 and/or 18-related HPV