

Short Communication

CYP2D6 Genotype and the Incidence of Anal and Vulvar Cancer¹

Chu Chen,² Linda S. Cook, Xiao-Yan Li, Sarah Hallagan, Margaret M. Madeleine, Janet R. Daling, and Noel S. Weiss

Programs in Cancer Biology [C. C.] and Epidemiology [L. S. C., M. M. M., J. R. D., N. S. W.], Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024; Brigham and Women's Hospital, Boston, Massachusetts 02115 [X.-Y. L.]; and Departments of Pharmacology [S. H.] and Epidemiology [J. R. D., N. S. W.], University of Washington, Seattle, Washington 98195

Abstract

The risks of anal and vulvar cancer are strongly related to cigarette smoking. Smokers are exposed to a substantial quantity of tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is present in the mucus of the female genital tract. The enzyme debrisoquine 4-hydroxylase (CYP2D6) activates NNK and is present in foreskin keratinocytes and cervical epithelial cells. A polymorphism for the gene CYP2D6 exists, and persons who possess alleles that are associated with reduced levels of CYP2D6 activity might be expected to be at a relatively lower risk of cancers arising from NNK exposure. To test this hypothesis, we conducted a case-control study to examine the association of CYP2D6 genotype and the incidence of anal and vulvar cancer among cigarette smokers in western Washington State. We tested for 14 alleles (*1–*12, *14, and *17) among cases (25 men and 43 women with anal cancer, 64 women with vulvar cancer) and controls (30 men and 110 women). Contrary to the hypothesis, cases were not less likely than controls to have one (43.9 versus 40.7%) or two (6.8 versus 4.3%) inactivating alleles (*3, *4, *5, *6, *7, *8, *11, or *12). There was a suggestion that, if anything, the combined anal and vulvar cancer risk increased (rather than decreased) with an increasing number of CYP2D6 inactivation alleles: odds ratio = 1.2, 95% confidence interval = 0.7–2.0 with one inactivating allele; odds ratio = 1.8, 95% confidence interval = 0.6–5.4 with two inactivating alleles. These results provide no support for the hypothesis that cigarette smokers who

carry the CYP2D6 alleles that result in a low activity phenotype have a decreased risk of anogenital cancer.

Introduction

Epidemiological evidence suggests that, in addition to HPV,³ cigarette smoking predisposes to the development of anogenital cancers. (1). The large number of procarcinogenic compounds found in tobacco smoke include alkylating polynuclear hydrocarbons, arylamines, heterocyclic amines, and tobacco-specific nitrosamines. It is not clear which of these compounds, alone or as a mixture, may have a role in the etiology of anogenital cancer. However, one strong candidate is the tobacco-specific nitrosamine, NNK, for the following reasons: (a) smokers are exposed to a large quantity of NNK because a cigarette may contain 30–150 ng of NNK (2); (b) NNK is present in considerably higher levels in the mucus of the female genital tract of smokers than that of nonsmokers (3); (c) animal studies have shown that activation of NNK through α -hydroxylation leads to DNA methylation (4) and the formation of DNA adducts (5) and protein adducts (6); and (d) the cytochrome P450 CYP2D6 gene, one of those that encodes NNK-activating enzymes (7), is expressed in primary genital epithelial cells, such as foreskin keratinocytes and cervical keratinocytes, regardless of transfection with HPV16 or HPV18 (8).

The enzyme CYP2D6 catalyzes the metabolism of several dozen therapeutic compounds (9). About 5–10% of the Caucasian population are “poor metabolizers” of these compounds, due to their reduced activities of this enzyme. This reduced activity is the result of the presence of two inactivation alleles of the CYP2D6 gene. Studies of lung cancer incidence in relation to CYP2D6 phenotype or genotype have not produced consistent results (10). Although cigarette smoking is a risk factor common to lung and to anogenital cancer, the role of the CYP2D6 phenotype or genotype could differ between these cancers because of the involvement of HPV in the etiology of the latter. As part of a population-based case-control study of anal and vulvar cancer in western Washington, we sought to determine whether an association was present with the CYP2D6 polymorphism and thus, indirectly, whether tobacco-specific nitrosamines such as NNK may play a role in the development of anal and vulvar cancer.

Materials and Methods

CYP2D6 Genotyping. We sought to determine the presence of alleles *1, *2, *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, *14, and *17 (11) by analyzing point mutations at nt 212, 971, 1127, 1749, 1846, 2064, 2938, and 3023, based on the principle of an ELISA-based OLA (12); the 1–3-nt deletion (at nt 1795, 1934, 2637, and 2702) and a G-to-A substitution (at nt 1934) by

Received 8/17/98; revised 2/2/99; accepted 2/16/99.

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¹ This work was supported by National Cancer Institute (NIH) Grants R35 CA39779 and P01 CA42792 and by National Institute of Environmental Health Sciences Grant R01 ES06728. It was also supported by the Cancer Surveillance System of the Fred Hutchinson Cancer Research Center, which is funded by Contract N01-CN-05230 from the Surveillance, Epidemiology and End Results Program of the National Cancer Institute with additional support from the Fred Hutchinson Cancer Research Center.

² To whom requests for reprints should be addressed, at Program in Cancer Biology, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Mail Stop DE-320, 1100 Fairview Avenue N, Seattle, WA 98109-1024. Phone: (206) 667-6644; Fax: (206) 667-2537; E-mail: cchen@fhcrc.org.

³ The abbreviations used are: HPV, human papillomavirus; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; nt, nucleotide; OLA, oligonucleotide ligation assay; AS-PCR, allele-specific PCR; OR, odds ratio; CI, confidence interval.

AS-PCR; and the deletion of the entire gene by amplifying a 3.5-kb *2D6**5-specific fragment.

Three *CYP2D6* coding regions were first amplified to serve as OLA templates using primer pairs 5'-ACC AGG CCC CTC CAC CGG-3' and 5'-CCG GAT TCC AGC TGG GAA AT-3' (for nt 212, 971, and 1123), 5'-ATT TCC CAG CTG GAA TCC-3' and 5'-GAG ACT CCT CGG TCT CTC-3' (for nt 1749, 1846, and 2064; Ref. 13), or 5'-GCG GAG CGA GAG ACC GAG GA-3' and 5'-CCG GCC CTG ACA CTC CTT CT-3' (for nt 2938 and 3023; Ref. 13) and 100 ng of genomic DNA. Primary PCR products diluted with Triton X-100 were used for the subsequent ligation and ELISA procedures (14). Negative control PCR samples without genomic DNA were carried through the ligation and ELISA steps for background correction. Results for each polymorphism were recorded as positive or negative for the wild-type and mutant allele, respectively.

Prior to conducting AS-PCR, a primary PCR was performed that amplified the appropriate region within *CYP2D6*, specifically excluding any pseudogenes (e.g., *2D7*) or other homologous genes (e.g., *2D8*). The primary PCR products served as templates for AS-PCR. The primers and assay conditions for detecting deletion at nt 1795 (*6) were essentially according to Wang *et al.* (15) and Evert *et al.* (16), those for deletion at nt 2637 (*3) were essentially according to Kagimoto *et al.* (17), and those for deletion at nt 2071–2073 or 2072–2074 (*9) were essentially according to Tyndale *et al.* (18). The primers used for the detection of substitution at nt 1934 were 5'ATT TCC CAG CTG GAA TCC3' and 5'GAG ACT CCT CGG TCT CTC3'; the assay conditions were the same as those for nt 1795. Negative controls containing all reagents except the genomic DNA were included in both the primary PCR and AS-PCR. AS-PCR products were resolved on agarose gels along with DNA size standards, stained with ethidium bromide, and photographed over a UV transilluminator for documentation.

All samples that were identified by AS-PCR or OLA as homozygous wild-type (*2D6**1) or homozygous for mutation *2D6**2, *3, *4, *6, *7, *8, *9, *10, *11, *12, or *13 were analyzed in duplicate for the *5 allele essentially according to Steen *et al.* (19). A sample containing DNA with known *CYP2D6* deletion (previously genotyped by RFLP Southern analysis) was included in each run as a positive control; a sample without the DNA template was included as a negative control.

Study Subjects and Analysis. In-person interviews and peripheral blood specimens were obtained as part of case-control studies of anal and vulvar cancer. The subjects were 18–79-year-old residents of King, Pierce, and Snohomish Counties in western Washington. Vulvar and anal cancer cases diagnosed between April 1991 and December 1994 were identified from a population-based cancer registry. Controls were identified by random digit telephone dialing (20) and were frequency matched to cases in 5-year age groups. Previous reports from this study describe the methods in more detail (1, 21–23).

For the purposes of this analysis, only cases with squamous (or cloacogenic) tumors were included because cigarette smoking appears to be associated only with these histological subgroups (1). We further restricted the study population (both cases and controls) to current or former smokers. Among the 29 interviewed cases with invasive vulvar cancer, the 15 who gave blood at the time of interview and had a history of smoking were tested for *GSTMI* and *CYP2D6*. Among the 160 women with *in situ* vulvar cancer who were interviewed, a random

sample of 43 were chosen from those who had ever smoked and had a blood sample available. For the cases with anal cancer, there were 22 men and 42 women who gave a blood specimen and had smoked cigarettes, and they were tested for *GSTMI* and *CYP2D6* status. Among controls, blood samples were available for genotype analysis from 31 of 41 men who had ever smoked cigarettes and a random sample ($n = 113$) of the 190 women who had smoked.

The presumed phenotype for each *CYP2D6* genotype was based on previous reports (11, 24). The genotype for two women with anal cancer, two female controls, and one male control could not be determined due to a small or poor-quality blood sample. Additionally, one female control had results that were not consistent with any previously reported allelic polymorphism (11, 24). Thus, there were 58 vulvar cancer cases, 62 anal cancer cases, 110 female controls, and 30 male controls available for the final analysis. Subjects were grouped into three categories for analysis according to the number of inactivation alleles they carried (24).

Logistic regression (EGRET Version 26.6; Statistics and Epidemiology Research Corporation, Seattle, WA) was used to determine the relative ORs (as an estimates of the relative risk) of cancer associated with having one or two *CYP2D6* inactivation alleles and 95% CIs (25). ORs were determined separately and combined for vulvar and anal cancers and also stratified by *GSTMI* status (null/positive), recency of smoking (former/current), and the presence of antibodies to HPV16 capsids in sera (negative/positive), as determined by ELISA (26). The referent group in all comparisons was comprised of individuals with no *CYP2D6* inactivation alleles. All relative risk estimates were adjusted for age (continuous). Separate analyses of men and women diagnosed with anal cancer were limited by the small number of subjects with two inactivation alleles but produced a similar pattern of results as that for the two sexes combined.

Results

Selected characteristics of the cases and controls are presented in Table 1. In general, persons with cancer were more likely than controls to have a history of genital warts, to have a positive *GSTMI* genotype, to be HPV16 seropositive, and to be a current smoker at the reference date.

The genotyping assays did not allow us to distinguish genotype *1/*4 from *4/*10 for 12 cases and 16 controls and genotype *2/*4 from *4/*17 for 14 cases and 18 controls; however, each of these subjects were classified in the same analysis category (i.e., those with one inactivation allele). The frequency of *CYP2D6* wild-type *1 (0.254–0.305 for cases and 0.300–0.357 for controls), *2 (0.283–0.343 for cases and 0.271–0.336 for controls), and *4 (0.208 for cases and 0.207 for controls) were the most common, with a combined allelic frequency of >0.700 in both cases and controls. None of the cases or controls carried alleles *7, *8, *11, *12, and *14, all of which are inactivation alleles (11). The frequency of all inactivating alleles combined (*3, *4, *5, *6) was slightly greater among cases (0.288) than controls (0.247).

Cases were somewhat more likely than controls to carry one (44.1 versus 40.7%) or two (6.8 versus 4.3%) inactivating alleles; there was a suggestion that the combined anal and vulvar cancer risk increased with an increasing number of *CYP2D6* inactivation alleles (with one inactivation allele, OR = 1.2, 95% CI = 0.7, 2.0; with two inactivating alleles, OR = 1.8, 95% CI = 0.6, 5.4; Table 2). This association was entirely due to the one between *CYP2D6* genotype and anal

Table 1 Selected characteristics of anal and vulvar cancer cases and controls (King, Pierce, and Snohomish Counties, Washington, 1991–1994)

	Cases (%)			Controls (%)	
	Anal, male (n = 22)	Anal, female (n = 40)	Vulvar (n = 58)	Males (n = 30)	Females (n = 110)
Age (yr)					
<30	13.6	2.5	13.8	10.0	7.3
30–39	36.4	20.0	31.0	13.3	28.2
40–49	18.2	17.5	29.3	30.0	30.9
50–59		17.5	13.8	20.0	14.6
60–69	27.3	30.0	6.9	13.3	11.8
70–79	4.6	12.5	5.2	13.3	7.3
Race					
White	86.4	97.5	93.1	93.3	93.6
African-American	9.1		3.4		0.9
Native-American				3.3	0.9
Asian-American			1.7		1.8
Other	4.6	2.5	1.7	6.7	2.7
Lifetime no. of sexual partners ^a					
1–4	9.1	42.5	39.7	40.0	53.6
≥5	90.9	57.5	60.3	60.0	45.5
History of genital warts					
No	72.7	77.5	58.6	96.7	86.4
Yes	27.3	22.5	41.4	3.3	13.6
Cigarette smoking					
Recency					
Former	18.2	35.0	19.0	60.0	57.3
Current	81.8	65.0	81.0	40.0	42.7
Pack-years smoked					
≤10	13.6	20.0	22.4	33.3	37.3
10–29	40.9	32.5	53.4	33.3	38.2
≥30	45.5	47.5	24.1	33.3	24.6
HPV16 antibody status ^b					
Negative	36.4	65.0	43.1	73.3	76.4
Positive	63.6	30.0	55.2	26.7	23.6
GSTM1 status					
Null	31.8	40.0	41.4	60.0	54.5
Positive	68.2	60.0	58.6	40.0	45.5

^a Unknown for one vulvar case; one female control reported no male partners.

^b Unknown for two female anal cases and one vulvar case.

cancer (OR = 1.4, 95% CI = 0.8, 2.6 and OR = 2.6, 95% CI = 0.7, 9.2 for one and two inactivation alleles, respectively). Although there were slight suggestions of an increased cancer risk associated with one or with two inactivating alleles in some subgroups based on the presence or absence of other risk factors (e.g., persons who had at least one active *GSTM1* allele or had serological evidence of HPV infection), the relatively small number of subjects within the subgroups argues for a cautious interpretation.

Discussion

Contrary to our initial hypothesis, the results of this study suggest that the presence of one or two inactivation alleles at the *CYP2D6* locus is not associated with a decreased incidence of anal and vulvar cancer among smokers and may even be related to an increased risk of anal cancer. However, these results should be viewed as preliminary due to a number of limitations. (a) Our study was based on a relatively small number of subjects, particularly those with two inactivation mutations. (b) We had a fairly low level of participation. Although this potentially could skew the results, it seems unlikely that *CYP2D6* status would differ appreciably between participating and nonparticipating cases and controls with a history of smoking. (c) Potential misclassification of genotypes among a few individuals in our study does exist. For example,

because our study participants are predominantly Caucasians, we did not test for the “ultrametabolizer” allele, *2XN, which was found in 1% of a Swedish population studied (27). Also, we have no information on alleles *13, *15, and *16, which have been previously identified by others, and our methods did not allow us to distinguish *1/*4 from *4/*10 and *2/*4 from *4/*17.

Is it possible that our observations are valid, i.e., there is no decreased risk among persons with one or more *CYP2D6* inactivation mutations, and that, nonetheless, tobacco-specific nitrosamines play a role in the etiology of anal and vulvar cancers? We believe the answer to this question is “yes.” (a) Enzymes other than *CYP2D6*, such as *CYP2A6* and *CYP2E1*, are capable of metabolizing nitrosamines (28–31). Perhaps only an individual who has an inactivation mutation at all of these loci is at decreased risk. (b) There is evidence to indicate that the reactive intermediate produced from NNK by hydroxylation, methyl diazohydroxide, has an extremely short half-life (32). Thus, any effect that NNK might have on cancer risk would largely be due to activation of NNK at the target tissue. Whether the *CYP2D6* enzyme is produced in the anal and vulvar epithelia is not clear.

In summary, although there likely are some genetic characteristics that influence the ability of the carcinogens in cigarette smoke to give rise to anal or vulvar tumors, our data argue that *CYP2D6* status is not one of them.

Table 2 Risk of vulvar and anal cancer by number of CYP2D6 inactivation alleles stratified by GSTM1 genotype, recency of smoking, and the presence of HPV-16 antibodies (King, Pierce, and Snohomish Counties, Washington, 1991–1994)

No. of CYP2D6 inactivation alleles	Anal				Vulvar				Combined anal and vulvar			
	Cases ^a (n)	Controls (n)	Age-adjusted OR ^b	(95% CI)	Cases ^a (n)	Controls (n)	Age-adjusted OR ^b	(95% CI)	Cases ^a (n)	Controls (n)	Age-adjusted OR ^b	(95% CI)
Overall												
0	27	77	1.0	(Referent)	31	61	1.0	(Referent)	58	77	1.0	(Referent)
1	30	57	1.4	(0.8–2.7)	24	43	1.1	(0.6–2.1)	52	57	1.2	(0.7–2.0)
2	5	6	2.6	(0.7–9.2)	3	6	0.8	(0.2–3.6)	8	6	1.8	(0.6–5.4)
By GSTM1 status												
GSTM1 null												
0	11	40	1.0	(Referent)	15	30	1.0	(Referent)	26	40	1.0	(Referent)
1	11	34	1.1	(0.4–2.8)	8	26	0.6	(0.2–1.7)	19	34	0.9	(0.4–1.8)
2	1	4	0.9	(0.1–9.4)	1	4	0.5	(0.1–4.6)	2	4	0.8	(0.1–4.5)
GSTM1 positive												
0	16	37	1.0	(Referent)	16	31	1.0	(Referent)	32	37	1.0	(Referent)
1	19	23	1.9	(0.8–4.5)	16	17	1.8	(0.7–4.6)	33	23	1.7	(0.8–3.4)
2	4	2	5.1	(0.8–31.8)	2	2	1.5	(0.2–12.0)	6	2	3.5	(0.7–18.6)
By recency of smoking												
Former smoker												
0	8	41	1.0	(Referent)	6	34	1.0	(Referent)	14	41	1.0	(Referent)
1	8	37	1.0	(0.3–3.1)	3	26	0.7	(0.2–2.9)	11	37	0.8	(0.3–2.1)
2	2	3	3.4	(0.5–25.1)	2	3	3.7	(0.5–27.1)	4	3	4.1	(0.8–20.6)
Current smoker												
0	19	36	1.0	(Referent)	25	27	1.0	(Referent)	44	36	1.0	(Referent)
1	22	20	2.0	(0.9–4.6)	21	17	1.3	(0.6–3.1)	41	20	1.7	(0.8–3.3)
2	3	3	2.2	(0.4–12.5)	1	3	0.3	(0.1–3.0)	4	3	1.1	(0.2–5.4)
By HPV16 AB ^c status												
HPV16 AB–												
0	15	58	1.0	(Referent)	16	46	1.0	(Referent)	31	58	1.0	(Referent)
1	16	43	1.3	(0.6–3.1)	8	33	0.7	(0.3–1.8)	23	43	1.0	(0.5–1.9)
2	3	5	2.4	(0.5–11.7)	1	5	0.5	(0.1–4.8)	4	5	1.5	(0.4–6.0)
HPV16 AB+												
0	12	19	1.0	(Referent)	14	15	1.0	(Referent)	26	19	1.0	(Referent)
1	12	14	1.4	(0.5–4.0)	16	10	1.8	(0.6–5.2)	27	14	1.5	(0.6–3.7)
2	2	1	3.0	(0.2–39.0)	2	1	1.7	(0.1–22.1)	4	1	2.4	(0.2–23.8)

^a Two females with a vulvar and an anal cancer diagnosis are included in each of the site-specific analyses (*i.e.*, they are included as cases in the vulvar-specific analysis and as cases in the anal-specific analysis) but are included only once in the combined analysis. Thus, the total number of vulvar cases plus anal cases will exceed the number of combined cases by 2.

^b Adjusted for age (continuous).

^c AB, antibody.

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