Human Papillomavirus DNA in Invasive Cervical Carcinomas and Its Association with Patient Survival: A Nested Case-Control Study

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Abstract

This study sought to examine the association between the presence of human papillomavirus (HPV) DNA in invasive cervical cancer and prognosis. A case-control study was undertaken nested in a cohort of 208 patients with invasive cervical carcinoma in Montreal. All 40 deceased patients formed the case group. A control group of equal size was selected by matching to each case (1:1) a patient of the same age and year of admission who had survived her disease. HPV DNA was detected by the use of a PCR protocol. The odds ratio (OR) for cervical cancer death was computed by logistic regression. Detection of HPV, particularly of types 16 and 18, was negatively correlated with disease stage and histological grade. The OR for death was 0.27 [95% confidence interval (CI), 0.1–0.8] for those whose tumors were positive for HPV DNA versus those in whom HPV DNA was not detected. After adjusting for the confounding effects of stage and grade, the prognostic effect was somewhat reduced, with an OR of 0.34 (CI, 0.1–1.1), which was no longer significant. The magnitude of the HPV-survival association was not altered when the analyses were restricted to carcinomas of stages I and II. Regardless of the mechanism for the prognostic role, detection of HPV DNA in primary tumors may play an important adjunct role in assessing prognosis of patients with invasive cervical cancer.

Introduction

The overall expectation of survival for women with invasive cervical cancer is lower than that for those with breast cancer. Although early invasive disease (stage IB) follows a favorable course, with 90% survival at 5 years, most patients are diagnosed with disease extending beyond the cervix. In these cases, the prognosis is grave, with 5-year survival rates lower than 60%, and as low as 10% in patients with stage IV disease (1). Identification of prognostic factors is thus a worthwhile goal in cervical cancer research. More aggressive treatment may be given to those patients considered to be at higher risk of recurrence, whereas others with favorable prognosis may be spared the undesirable side effects of an aggressive regimen. Likewise, high-risk patients may be followed up more closely with more sensitive diagnostic tests, whereas low-risk patients may be monitored in the standard fashion.

There is substantial biological and epidemiological evidence that certain types of HPV are causally associated with the development of uterine cervical cancer (2, 3). Recent studies have also indicated that the presence of HPV in the cervical tumor may also affect the clinical prognosis (4). Interestingly, however, there are two conflicting lines of evidence relating to the putative prognostic role for HPV. Some studies have shown that the absence of HPV in the tumor confers a worse prognosis than if any HPV types were present (5–8). Other studies have shown an association between the presence of certain HPV types and poor clinical outcome, particularly HPV 18 (9, 10) and HPV 16 (11).

We describe herein a case-control study, nested within a survival cohort of cervical cancer patients, to test the hypothesis that detectability of HPV DNA in cervical tumors may affect prognosis.

Patients and Methods

Patients. We studied retrospectively by chart review all invasive squamous cell cervical cancer patients admitted between 1983 and 1990 to the Royal Victoria Hospital, a McGill University teaching institution in Montreal. A cohort of 208 patients was identified, of whom 40 had died from their disease. These deceased subjects were considered the case group. A control group of equal size was assembled by matching to each case another patient who was alive at the study closing date (October 1991). Control survivors were matched to their respective deceased cases by year of admission and age (within 5 years). Controls were chosen randomly from the lists of eligible survivors matched to each case.

HPV Testing. Specimen preparation was done as described previously (12). Multiple consecutive sections representing areas of active tumor growth were cut with a microtome from the paraffin blocks containing primary tumors. Sections were deparaffinized by sequential washes with xylene and 95% ethanol and digested with proteinase K. Extracted specimens were processed immediately or frozen at -20°C until they were tested.

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The abbreviations used are: HPV, human papillomavirus; OR, odds ratio; FIGO, International Federation of Gynecology and Obstetrics; CL, confidence limits.
Presence of HPV DNA was tested by a PCR protocol with consensus primers (GP5/GP6) flanking a 140-bp region in the L1 gene of HPV (13). Amplification products were tested by dot-blot hybridization using stringent conditions with individual random-primer-labeled cloned HPV plasmids of types 6, 11, 16, 18, and 31 (13).

As a check on the integrity of the DNA extracted from the sections, specimens were also tested by PCR for the presence of β-globin DNA. Testing was done as described previously (14) with primer pairs GH20/PC04, which amplify a 268-bp region of that gene, and PC03/PC04, which amplify a smaller 110-bp segment within the same region.

All specimens were tested blindly without knowledge of the case/control status of the patient or of any clinical information.

**Statistical Analysis.** Survival time was considered from the date of diagnosis until the date of death or until the date of last follow-up examination. Survival distributions were computed by the Kaplan-Meier method (15) and were compared statistically by the log-rank test (16, 17). Frequencies in contingency tables were compared by Pearson’s χ² test. The Mantel-Haenszel χ² for linear trend was used to analyze the variation in HPV positivity according to ordinal variables. The association of presence of HPV with survival (i.e., with case-control status) was analyzed by multiple logistic regression using both conditional and unconditional methods (18, 19). These regression methods were used to calculate ORs and their 95% CIs as estimates of the relative risk of death from cervical cancer for HPV-positive versus HPV-negative patients.

**Results**

Table 1 shows the distributions of clinical characteristics for 39 deceased cases, their 1:1 matched survivor controls, and for the remaining 129 patients in the cervical cancer cohort. The paraffin-embedded tumor specimen from one of the deceased cases could not be obtained, which precluded its inclusion in the case-control analysis. Disease stage, disease grade, and patient age were the most important predictors of survival in the entire cohort in preliminary analyses (not shown). The matching ensured similar distributions between deceased cases and survivor controls for year of admission and age.

After eliminating tissue specimens that failed to be amplified for β-globin, the overall HPV positivity was 85.5% (59 of 69), being 82.9% among deceased cases and 88.2% among survivor controls, a non-statistically significant difference. However, when only the results from the dot-blot hybridization with HPVs 16 and 18 were considered, survivor controls were more than twice as likely to be positive as deceased cases: 55.9 versus 25.7% (P = 0.0107).

Table 1 shows the relative survival distributions of the subjects in the nested 1:1 case-control sample according to HPV positivity and subset of PCR results. Overall HPV positivity was not significantly correlated with survival (P = 0.4254; Fig. 1, top). However, the combined post-amplification dot-blot results with HPVs 16 and 18 were a significant predictor of survival (P = 0.026; Fig. 1, bottom).

Table 2 shows ORs for cervical cancer death by HPV positivity according to HPV types probed in the amplification products. Apart from a gain in precision, OR estimates obtained by unconditional logistic regression did not differ materially from those obtained in conditional (matched) analysis. They
from the loss in precision resulting from the presence of apart and was no longer significant. When the analysis was restricted slightly decreased the magnitude of the association with the only when viral DNA detection was limited to probing with 5 only overall HPV positivity was considered because of the small confirming the findings from some of the previous studies detection of HPV DNA in the primary tumor is negatively The results from the present investigation suggest that the Discussion of amplified products with HPVs 6, 11, 16, 18, and 31; bottom, HPV positivity with HPV status. In agreement with the results from the uni- were chosen, therefore, to represent the prognostic relations with HPV status. In agreement with the results from the univariate survival analysis, the ORs in models adjusted for age and survival time end points, such as death or recurrence (5-8). In mediates end points, such as stage, nodal status, or unfavor- were based on correlating the presence of HPV with inter- were hypothesized to presumptively identify specimens most likely to determine the HPV type. Our use of genomic probes was not permit direct probing of the amplified products for determining the HPV type. Our choice of PCR method was based on this fact. The GP5/6 protocol amplifies a genome sequence smaller than 200 bp. On the other hand, the main disadvantage of the GP5/6 protocol is that it does not permit direct probing of the amplified products for determining the HPV type. Our use of genomic probes was intended to presumptively identify specimens most likely to have high copy numbers of HPVs 16 and 18, the two most common oncogenic types, and not to determine type-specific HPV prevalence in the tumors. Most studies of the association of HPV and prognosis were based on correlating the presence of HPV with intermediate end points, such as stage, nodal status, or unfavorable histology (9, 11, 21). Because these end points are predictors of cervical cancer survival, these studies assumed that if a correlation with HPV was identified, then the presence of HPV might eventually affect survival as well. Although useful to identify correlations among prognostic factors, such studies do not necessarily yield the same results as those focusing on survival time itself. It is noteworthy that all studies identifying a negative association between HPV positivity and cervical cancer prognosis were based on survival time end points, such as death or recurrence (5-8). In these studies, tumor specimens from all cohort members were tested for the presence of HPV, which incurred substantial costs related to specimen processing and testing. In the present study, we used a nested case-control study design to minimize the costs associated with HPV testing. We tested only those patients who had died from cervical cancer and a sample of those who had survived the disease after having been followed up for a comparable clear association with survival was observed when viral DNA detection was defined as presumptive positivity with only HPVs 16 and 18. This prognostic relation was only partially explained by a secondary relation between presence of HPV and stage because advanced-disease patients were less likely to have HPV detected in their tumors than those with early stage disease. In consequence, the negative association between presence of HPV and risk of death tends to become weaker after controlling statistically for the prognostic effects of stage. On the other hand, the prognostic relation with HPV seemed to be negatively confounded by histological grade because there was a trend for increased HPV DNA detection as the degree of differentiation decreased. Apart from the loss in precision, the magnitude of the association with survival did not change materially by restricting the analysis to smaller tumors (e.g., stages I and II). As a further restriction to even smaller tumors, patients with stage Ib carcinomas (those with parametrial invasion) were also eliminated resulting in 11 cases and 24 controls. The ORs for HPV 16/18 presumed positivity were 0.19 (P = 0.049), adjusted for age and year of diagnosis, and 0.04 (P = 0.0245), adjusted additionally for grade and stage, which indicates an independent prognostic effect in early disease patients. Before considering the implications of the present study, one must first consider its limitations. First, because of the retrospective nature of the investigation, we used archival pathology specimens to analyze the presence of HPV DNA. A substantial level of DNA degradation may have occurred in many of these samples as a consequence of the process of formol fixation and inclusion in paraffin. In such specimens, protocols based on primers flanking relatively large HPV gene segments are more likely to yield false-negative results than those designed to amplify smaller (i.e., less than 200 bp) sequences (20). Our choice of PCR method was based on this fact. The GP5/6 protocol amplifies a genome sequence smaller than 200 bp. On the other hand, the main disadvantage of the GP5/6 protocol is that it does not permit direct probing of the amplified products for determining the HPV type. Our use of genomic probes was intended to presumptively identify specimens most likely to have high copy numbers of HPVs 16 and 18, the two most common oncogenic types, and not to determine type-specific HPV prevalence in the tumors.
length of time. Instead of selecting control patients randomly from the survivors in the cohort, we matched controls to deceased cases on variables that are potential confounders of the prognostic association of HPV and survival (i.e., age and years since admission to the cohort). Previous studies found a negative correlation between the patient’s age and the likelihood of the tumor being positive for HPV (6, 10). Because age itself may be correlated with disease stage and directly influences length of survival, it is important to eliminate the confounding effect of age when studying the prognostic value of the presence of HPV. Matching on year of admission helps to ensure comparable conditions with respect to specimen quality, thus minimizing the possibility of a differential loss in HPV detectability over time in archived specimens of cases and controls. By using this strategy, we restricted HPV testing to the most informative patients, thus ensuring an efficient statistical analysis allowing for adequate control of other potential confounders. We did not choose to further match on disease stage because this would have prevented us from assessing a prognostic effect mediated by stage. Moreover, this maneuver would have caused the sample of controls to be considerably nonrepresentative of the base cohort of cervical cancer survivors.

What is the mechanism for the differing biological behavior of cervical tumors on the basis of detectability of HPV DNA? Recent evidence from an international study indicates that meticulous testing of nonfixed cervical carcinomas by PCR results in positivity rates greater than 95% (22). Combined testing by more than one PCR protocol and systematic testing of multiple specimens of the same primary tumor tend to increase the proportion of HPV positivity to nearly 100%. If HPV is a necessary cause of cervical cancer and eventually all cervical tumors will be shown to contain HPV DNA, then our findings and those of previous studies (5–8) indicate a prognostic value related to the decreased detectability of the virus rather than to its absence.

The lower detection rate in archival specimens may indicate that only in tumors with a high viral load were there enough target genome regions remaining nonfragmented after formal fixation and further processing. Had we worked with fresh tumor tissue, the detectability would have been higher than the overall 85% rate we obtained. It could be hypothesized that tumors with a more aggressive behavior may have lower viral copy numbers. They would be considered HPV positive if testing were done with fresh tissue, but the deleterious effect of fixation and embedding on the quality of the DNA could lead to false negative HPV results with the corresponding archival specimens.

Regardless of its mechanism, the prognostic relation with HPV detection, be it real or artifactual, is an intriguing finding. The magnitude of the ORs seen in the present study (in the range of 0.25–0.35) can be equated to risks of death approximately 3–4 times higher among those with the unfavourable trait (undetectability or absence of HPV DNA in the tumor). These levels of relative risk are comparable to those seen with features, such as stage of disease or nodal status, in predicting survival. Prospective studies involving fresh or frozen tissues and measuring viral burden should help to solve questions related to specimen quality and HPV detectability, thus shedding light on the association with prognosis.

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