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Multiple HPV genotype infections in cervical cancer progression in the Study to Understand Cervical Cancer Early Endpoints and Determinants (SUCCEED)

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Abstract

Determining the causal attribution of HPV genotypes to cervical disease is important to estimate the effect of HPV vaccination and to establish a type spectrum for HPV-based screening. We analyzed the prevalence of HPV infections and their attribution to cervical disease in a population of 1670 women referred to colposcopy for abnormal cytology at the University of Oklahoma. HPV genotyping was performed from cytology specimens using the Linear Array assay that detects 37 HPV genotypes. We used different methods of type attribution to revised cervical disease categories. We found very high prevalence of multiple HPV infections with up to 14 genotypes detected in single specimens. In all disease categories except for cancers, there was a significant trend of having more infections at a younger age. We did not see type interactions in multiple genotype infections. HPV16 was the most frequent genotype at all disease categories. Based on different attribution strategies, the attribution of vaccine genotypes (6,11,16,18) ranged from 50.5% to 67.3% in cancers (n=107), from 25.6% to 74.8% in CIN3 (n=305), from 15.2% to 52.2% in CIN2 (n=427), and from 6.6% to 26.0% in <CIN2 (n=708). In the HSIL cytology group (n=651), attribution ranged from 26.1% to 64.7%. The attribution of vaccine types to HSIL was substantially higher compared to the lower cytology categories. The potential range of HPV genotype attribution is wide at the disease categories <CIN2 to CIN3. Genotyping from cervical lesions and analyzing viral oncogene expression can improve estimates of HPV genotype attribution.

Keywords

cervical cancer; HPV; SUCCEED; epidemiology; molecular

Introduction

Carcinogenic types of human papillomaviruses (HPV) are the causal agents of cervical precancer and cancer. More than 100 HPV genotypes have been identified to date. Among 30 types that infect the genitourinary mucosa, approximately 15 are carcinogenic and are highly associated with the development of cervical cancer (1). Two genotypes, HPV16 and

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HPV18, account for ~65% of cancer and its immediate tissue precursor, cervical intraepithelial neoplasia 3 (CIN 3); however, over 12 other types account for the remaining third of cases (2).

Most carcinogenic HPV infections, especially among women less than 30 years of age, are clinically occult and spontaneously regress (3). Routine cytologic screening detects a minority of such infections as equivocal (atypical squamous cells, ASC) or mildly abnormal (low-grade squamous intraepithelial lesions, LSIL) Pap tests. Referral of such women to colposcopy and biopsy may result in histologic diagnoses of CIN 1 or 2. As would be expected, data suggest that the percentage of lesions stratified by severity varies with the associated HPV type (4). Types other than HPV 16 and 18 account for a higher percentage of cytologic ASC or LSIL or histologic =<CIN2, many of which spontaneously regress.

Defining the contribution of individual HPV genotypes to each grade of severity of cervical neoplasia is important for multiple purposes. Such data are needed to estimate how much cervical disease will be prevented by current vaccination programs (5), and to determine which genotypes should be targeted by the next generation of vaccines.

Similarly, it is important to consider which genotypes should be included in future HPV screening assays. Moreover, as vaccines and type-specific HPV screening are applied, it will be important to monitor changes in disease patterns caused by other HPV genotypes, especially in the context of understanding cross-protection (i.e. prevention of infections and lesions caused by HPV genotypes closely related to those included in vaccines) and “unmasking” of putative carcinogenic types (i.e. lesions caused by non-vaccine genotypes that previously remained clinically elusive possibly because of faster progression and treatment of concurrent HPV16/18 associated lesions) (6).

However, identifying the causal genotype for each grade of cervical neoplasia is complicated because multiple HPV genotypes often co-exist within the cervical epithelium. Multiple cervical lesions within an individual patient may be caused by different genotypes and a precancerous lesion caused by a specific carcinogenic genotype can be surrounded by transient HPV infections (7).

Attribution of HPV genotypes to cervical disease is also complicated by frequent misclassification of cervical disease, related either to interpretation problems during histological examination (8) or, even more importantly, to misidentification of the worst lesion during colposcopy and biopsy (9). Based on the analysis of combinations of cytology, histology, and HPV genotyping results in the Study to Understand Cervical Cancer Early Endpoints and Determinants (SUCCEED), we recently proposed a revised disease grading algorithm that reflects the functional categories of cervical disease progression and can correct some of the misclassification errors related to biopsy placement (10).

In the present analysis, we evaluate the distribution of 37 HPV genotypes in cervical cytology specimens obtained from 1670 women at a single US institution comprising the disease continuum, from HPV infection to invasive cancer. We demonstrate the challenges of attributing HPV genotypes to disease categories and show the potential range of attribution based on different algorithms.

Methods

Study population

Participants featured in the current study were enrolled into SUCCEED starting in November 2003 and ending in September 2007. We recruited women referred to colposcopy

at the University of Oklahoma Dysplasia Clinic based at the University of Oklahoma Health Sciences Center (OUHSC), with a recent abnormal Pap smear diagnosis or a biopsy diagnosis of CIN. Details of study design and inclusion criteria have been described elsewhere (11). Briefly, exclusion criteria included women who were less than 18 years-of-age, pregnant at the time of their visit, previously treated with chemotherapy or radiation for any cancer, or women scheduled for vaginal colposcopy. Written informed consent was obtained from all women enrolled into the study and Institutional Review Board approval was provided by OUHSC and the U.S. National Cancer Institute.

At the time of the analysis, 1899 women had been enrolled; of these, we excluded 16 from the present analyses due to missing HPV results and further excluded 213 women due to unsatisfactory cytology, constituting a study population of 1670 women with a median age of 25 years (18–81 years). Of these, the following groups were excluded from the analyses of type attribution in cervical disease, because they could not be assigned to a disease category: 41 women with negative cytology, histology, and HPV result, 73 women without histology result and nine women without cytology result in the <CIN2 group, resulting in a population of 1547 women for these analyses.

Colposcopy and specimen collection

Colposcopic examination was conducted by a gynecologic oncology attending or fellow according to routine practice at OUHSC. Prior to biopsy or loop electrosurgical excision procedure (LEEP), cervical cell samples were first obtained with a Papette™ broom (Wallach Surgical, Orange, CT) and rinsed directly into PreservCyt™ solution (Cytyc Corporation, Boxborough, MA) as described previously (12). The cytology specimen was used for ThinPrep™ (Cytyc Corporation) cytology and for HPV genotyping using the Linear Array (LA) HPV Gentoyping System (Roche Molecular Diagnostics, Branchburg, NJ). Biopsy specimens were obtained for colposcopically suspected lesions. Endocervical curettage was performedin cases with glandular abnormalities in Pap cytology, with HSIL in cytology and no visible lesion, in women with unsatisfactory colposcopy and after treatment. As per standard practice, all histologically confirmed high-gradelesions diagnosed as CIN2 or above (CIN2+) were treated by LEEP of the transformation zone. Cytology and histologic diagnoses were masked to each other and to genotyping data. Further details on cytology and histology procedures are provided elsewhere (11). We used both cytology and histology results to define each distinct strata for disease progression in the SUCCEED referral population, as previously described (10): 1) normal histology, normal cytology, and HPV-negative; 2) <CIN2 histology, normal cytology, and HPV-positive; 3) <CIN2 histology, ASC (including ASC-US and ASC-H) or LSIL cytology; 4) CIN2 histology (including CIN2 histology regardless of cytology and <CIN2 histology with HSIL cytology); 5) CIN3 histology regardless of cytology; and 6) cancer regardless of cytology. Of note, the first category of histologically and cytologically normal, HPV negative women, was excluded from the analysis of HPV attribution. In addition, we present the HPV type attribution to standard histology categories without correction by cytology to allow comparison of our data with previous studies.

HPV Genotyping

Details of DNA isolation and HPV genotyping employed in SUCCEED have been previously described (13). Briefly, DNA was isolated from 1 mL aliquots of PreservCyt-fixed cells using the QIAamp DNA Blood Mini Kit (Qiagen Sciences, Germantown, MD) following a brief rinse in Hanks' Balanced Salt Solution (HBSS). Isolated DNA was stored at −70°C until PCR amplification using the Linear Array® HPV Genotyping System (Roche Molecular Diagnostics). The LA assay is capable of detecting 37 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69,

70, 71, 72, 73, 81, 82, 83, 84, IS39, CP6108). Of note, since the development of Linear Array, several type designations have changed: CP6108 is now HPV89; IS39 is now HPV82; HPV55 and HPV64 lost their type status and are now subtypes of HPV44 and HPV34 (14). To stay consistent with the literature, we use the original Linear Array designations. LA does not detect HPV52 directly, but uses a probe that simultaneously detects HPV52, 33, 35, and 58. Individual probes are present for HPV33, 35 and 58 which permits determination of HPV52 infections in the absence of HPV33, 35 and 58 but does not allow for detection of HPV52 in co-infections with any of the three other types. Two different concentrations of β-globin probes are present on each strip as internal positive controls for assuring adequate amplification of each specimen. Up to 80 patient specimens, three HPV16-positive controls and one HPV-negative control were amplified at one time using the LA Genotyping kit, following the manufacturer's instructions. Control specimens were processed through DNA isolation, amplification and detection similar to patient specimens. Hybridization of PCR products to linear arrays and subsequent signal detection were performed using the *Auto*-LiPA automated staining system (Innogenetics N.V., Belgium). Hybridization to both β-globin probes was required to report genotyping results. A hybridization signal was called "positive" when an unambiguous, continuous band was observed on the array. A single evaluator subjectively graded the intensity of each hybridization band as strong (s), moderate (m), weak (w), very weak (vw) or extremely weak (ew) as previously described in (15).

Statistical Analyses

The analytic group for our analysis of multiple infections and age consisted of those women who were HPV-positive and had genotyping and age information (n=1452). The average age is presented for five disease groups of histology and cytology (<CIN2, NILM; <CIN2, ASC/LSIL; CIN2 + <CIN2, HSIL; CIN3; Cancer) and stratified by number of infections. We used a general linear model (GLM) to calculate p-values for age trend.

We compared the observed frequencies of 2-type combinations for the 37 genotypes detected by Linear Array with expected frequencies in all women with at least 2 concurrent HPV infections (n=1060). To obtain expected frequencies for a 2-type combination, the observed genotype frequencies for both types were multiplied and the result was multiplied with the total number of subjects (n=1060). 95% confidence limits for the expected frequencies are based on the Poisson distribution. The analysis was repeated after restriction to subjects with a histology diagnosis of CIN3 and cancer (CIN3+) (n=229) to search for any combinations that were particularly common or rare in the most severe cases.

The analysis of causal attribution of HPV genotypes to disease groups was based on the following disease categories: (1) cancer, (2) CIN3, (3) CIN2, including cases with HSIL cytology and <CIN2 histology, (4) <CIN2 histology with NILM, ASC, or LSIL cytology. Fourteen HPV genotypes were considered to be carcinogenic: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 (1). In addition, we performed the analysis for the standard histologic categories CIN2 and CIN1.

To analyze the attribution of HPV genotypes to the disease categories, we assumed that all infections are biologically independent. Under the assumption that productive HPV infections, precancers, and cancers are necessarily caused by HPV, the risk in unexposed women would be zero (16). With this assumption, the standard formulas of population attributable risk do not apply (17;18). We therefore subdivided cervical disease into disjoint subgroups representing the underlying type that caused the infection leading to productive infections, precancer, and cancer. Thus, applying first principles we attribute every cervical cancer carrying a HPV16 infection to HPV16, and so on for all HPV types, as well as for cervical precancers, and productive infections. In every disease category, the minimum

estimate of the % of disease caused by an HPV type was calculated as the frequency of single infections of that genotype in that category. The highest estimate was given by the frequency of that genotype assuming a causal association in every case where that genotype is present. In addition, we used "proportional" and "hierarchical" attributions of genotypes to disease categories. The proportional attribution of multiple infections was performed similar to a previously described method by Insinga et al. (19) where a case is proportionally attributed according to the frequency of that type at the respective disease category. The hierarchical attribution is based on the same frequency of that type in the respective disease category, but instead of a partial attribution to cases, only the more frequent type is attributed to the case. For example, in a multiple infection consisting of HPV16, 31, and 53 for a specific disease category where frequencies are 50%, 15%, and 20% for the respective types, using the proportional attribution, the case would be split between the three types (50/85 for HPV16, 15/85 for HPV31, and 20/85 for HPV53) while in the hierarchical attribution the case would be completely attributed to HPV16.

Finally, for cervical cancers, CIN3, and HSIL (and for the CIN2 category based on histology only), we added a category of single carcinogenic infections (Single HR), indicating the percentage of the respective carcinogenic type in the absence of other carcinogenic types while disregarding infections with non-carcinogenic types. For example, if HPV16 was detected in a multiple infection with types 6 and 61, this would be counted as a single carcinogenic infection. In contrast, co-infections comprising multiple carcinogenic HPVs, such as HPV16, 31, and 6, would not count as a single carcinogenic infection. To analyze attribution of HPV vaccine types to cytology, we added the frequencies of four types, HPV6, 11, 16, and 18, that are included in the quadrivalent vaccine.

All statistical tests were two-sided and considered to be significant at $p<0.05$. Statistical analyses were performed using SAS, version9.1 (SAS Institute, Cary, NC).

Results

Frequency of multiple genotype infections and relation to age

We analyzed the distribution of HPV genotypes and patient age in five different groups of HPV-infected women (<CIN2+NILM, <CIN2+ASC/LSIL, CIN2 including <CIN2+HSIL, CIN3, Cancer). The numbers of HPV genotypes detected in each disease and age group are shown in Table 1. Up to 14 concurrent HPV genotypes were detected in a single specimen. The highest percentage of single-HPV genotype infections was found in cancers (66.0%), while the lowest percentage was found in women with <CIN2 histology and ASC/LSIL (24.7%) (Table 1). After restricting the analysis to 14 carcinogenic types, there was a substantial reduction in the number of multiple infections, especially in the two lowest disease stages (Supplementary table 1).

Women with <CIN2 histology and ASC/LSIL cytology had the youngest average age of all disease groups (25.7 years), while women with cancers were the oldest (46.8 years). In all disease categories, we observed a relationship between younger age and multiple HPV genotype infections; there was a significant age trend in all groups except for cancers (Table 1).

Combinations of multiple genotype infections

To explore the genotype combinations in multiple infections, we compared observed versus expected numbers of 2-genotype combinations in the complete series to explore substantial deviations from the expected combinations (Supplemental Figure 1). Overall, there was a high correlation between observed and expected combinations. Three combinations of HPV16 (with HPV51, HPV39, and CP6108) were observed slightly less frequently than

expected. By contrast, two type combinations were observed substantially more frequently than expected; the combination of HPV56 and HPV66 (both alpha6 species) was predicted for 20 cases (95% CI 12–31) but was found in 52 cases, and the combination of HPV51 and HPV82 (both alpha5 species) was predicted for nine cases (95% CI 4–17) but was detected in 26 cases. In both combinations, strong signals of one type were associated with weak signals of the other type. A restricted analysis of expected and observed type combinations in CIN3+ (n=229) did not reveal any additional synergistic or antagonistic clustering of genotypes (data not shown).

Attribution of carcinogenic HPV genotypes to disease categories

We examined the association of HPV genotype with disease category (cancer, CIN3, CIN2, <CIN2) to estimate the causal attribution for each type.

Tables 2 to 5 display the attribution of HPV genotypes to each a disease category which ranged from a minimum, the frequency in single type infections (Single), to a maximum based on the inclusion of any case where the genotype is detected (Tables 2–5, Any). In the middle columns for each disease category, the proportional and hierarchical attributions of the respective types based on the frequency in the disease category are presented (Tables 2–5). In addition, for the cancer and CIN3 categories, we added the frequency of carcinogenic types in single carcinogenic infections. Likewise, we analyzed type attribution in the histologic categories CIN2 and CIN1 without considering the cytologic results (Supplemental tables 2 and 3).

Throughout all disease categories, HPV16 was the most frequent genotype in both single and multiple infections. In CIN3 and cancer, almost all single infections were caused by carcinogenic types. In cancers, the most frequent causal types (according to the hierarchical attribution) in descending frequency were HPV16, 18, 45, 33, 39, and 52. In CIN3, the most frequent genotypes were HPV16, 31, 33, 52, 18, and 35. The most frequent types in CIN2 were HPV16, 52, 51, 31, 18, and 33, in <CIN2 HPV16, 51, 53, 66, 39, and 59. Thus, the most frequent genotypes in all disease categories were carcinogenic types (except for HPV53), although the frequency and ranking of genotypes varied considerably by disease category.

We found the range of potential attributions to be wide, particularly in <CIN2 to CIN3. For example, the combined percentage for the two carcinogenic vaccine types HPV16 and HPV18 ranged from 5.4% (sum of single percentages) to 24.0% (sum of hierarchical percentages) in <CIN2, from 15.0% to 51.2% in CIN2, from 25.6% to 74.9% in CIN3 and from 50.4% to 67.2% in cancer. For cancers and CIN3 we can define the lower end of the potential attribution for a specific type by its frequency in single carcinogenic infections, assuming that precancers and cancers are almost exclusively caused by carcinogenic types.

The range of attribution of the HPV types included in the quadrivalent vaccine (6, 11, 16, and 18) was from 50.5% (sum of single percentages) to 67.3% (sum of hierarchical percentages) in cancers, from 25.6% to 74.8% in CIN3, from 15.2% to 52.2% in CIN2, and from 6.6% to 26.0% in <CIN2 (Tables 2–5). When the lowest estimate of attribution was defined by single carcinogenic infections, the potential range of attribution was narrowed from 56.1% to 67.3% for vaccine types in cancers and from 37.0% to 74.8% in CIN3. Of note, the differences in type attribution ranges between the classic histologic categories CIN2 and CIN1 (Supplemental tables 2 and 3) and the revised disease categories <CIN2 and CIN2+HSIL (Tables 4 and 5) were only minor.

Attribution of quadrivalent HPV vaccine genotypes to cytology groups

To estimate the impact of vaccination on current screening procedures, it is important to know the HPV genotype distributions in cytology categories. Similar to our analysis of type attribution to the revised disease categories that mainly emphasizes histology, we computed single HPV genotype frequencies, proportional and hierarchical attributions, and the overall percentage of cases positive by HPV genotypes for the four major cytology categories (HSIL, LSIL, ASC, and NILM). We computed the frequency of single carcinogenic genotypes for the HSIL cytology group (Supplemental tables 4–7). To estimate the potential impact of the quadrivalent vaccine on cytology categories, we added the percent attribution of HPV6, 11, 16, and 18 (Table 6). The range of vaccine type attribution to HSIL was from 26.1% in single infections, 35.9% in single carcinogenic infections to 58.2% and 64.7% for the proportional and hierarchical attributions, respectively. Notably, none of the HSIL cases showed a single type infection with the non-carcinogenic vaccine types 6 and 11. The lowest estimate of attribution (based on single type infections) to LSIL and ASC was 9.0% and 10.4% while the highest attribution (hierarchical attribution) was 35.9% and 35.1%, respectively.

Discussion

Twenty years ago, cross-sectional and cases-control studies established the etiological link between major carcinogenic HPV types and cervical cancer (20–22). Although the most frequent carcinogenic types have been refined for cervical cancers since then, the carcinogenicity of rare HPV genotypes is still being studied (1). Vaccination against HPV 16 and 18 will likely change the associations between different grades of cytologically or histologically defined lesions, the causative HPV types, and the biological potential and clinical threat posed by such disease states (23;24). Accordingly, estimating the attribution of carcinogenic HPV genotypes to cancer and CIN 3 is an important step in considering the application of HPV genotyping in screening and management and in developing multivalent prophylactic vaccines. However, developing these estimates has been challenging because of the frequent occurrence of multiple type infections, imprecision in defining disease states, differences in detection between cytologic and histologic specimens and other factors.

We present a comprehensive analysis of HPV genotypes in SUCCEED, a large cross-sectional study of women referred to colposcopy due to recently abnormal cytology or histology in the US. The current analysis includes 1670 women spanning all categories of cervical squamous neoplasia, with a high proportion of cervical cancers and precancers. Since we analyzed a population referred mainly due to abnormal Pap results, women with transient HPV infections that do not cause cytological abnormalities are underrepresented in our population. In contrast, due to the large catchment area of the OUHSC dysplasia clinic, we have very good representation of women with productive HPV infections, precancers, and cancers from an urban population from Oklahoma City and a rural population from the state of Oklahoma.

We demonstrate a strong association between patient age and number of HPV genotypes for all disease categories from <CIN2 to CIN3, however, disease category itself showed no association with number of genotypes. Prevalence and natural history studies on HPV infection have demonstrated that the peak age of HPV infections is between 20 and 25 years, related to the highest level of sexual activity and associated risk of exposure to HPV infections (25–30). In agreement with that, the average age of women with 2 or more HPV infections in our study was 30 or younger in the respective disease categories (except for women with cancer).

We note that our analysis of observed and expected type combinations is exploratory. Since stratification into double, triple, and quadruple combinations of the 37 genotypes led to large numbers of potential combinations with extremely few expected and observed events, we combined all cases with 2 or more infections. Previous studies have used different approaches, e.g. including cases with single infections, limiting the analysis to the most frequent types, or analyzing combinations of clades rather than single types (31–34). While some previous studies have shown specific clustering of different genotypes (31;34), we only observed two more frequent than expected combinations of closely related types that suggest cross-hybridization of the genotyping probes rather than biologic clustering.

While HPV genotyping data from cancers are the most important parameters for public health purposes, currently, the attribution of the vaccine types HPV16 and HPV18 (and, to a lesser extent, that of HPV6 and HPV11) to the overall disease burden is being used extensively to make assumptions on HPV vaccine efficiency (5;35). Similarly, when designing HPV screening assays, ultimate clinical sensitivity for the detection of precancers by inclusion of types rarely associated with cancer has to be weighed against the potentially dramatic loss of specificity when the respective type is frequent in low-grade disease (e.g., HPV53) (36).

In our study, only about a third of the CIN2 and CIN3 cases had infections with single HPV genotypes. Even after restriction to carcinogenic types, only 50% of CIN2 and CIN3 lesions could be attributed to single carcinogenic type infections (Supplementary Table 1). In the remaining cases, assumptions underlying attribution matter, but there are no accepted rules on how to attribute causative HPV genotypes without further functional data.

We present a range of potential attributions for each genotype, based on the frequency in single infections and counting all lesions containing the respective genotype. Due to the high proportion of multiple infections observed, this range can be very wide. In CIN3, HPV16 was found in 25% of the cases as a single infection, but was present in 73% of the overall cases. We can provide more precise estimates of type-specific causality for cancers since most cancers contain only one carcinogenic HPV type. The percentage of cancers caused by each genotype is very similar for single-type, all-type, proportional, or hierarchical attribution (e.g., the range of potential attribution of HPV16 varied only from 41% to 55%). A notable difference in HPV genotype attribution to cancers in our study was an underrepresentation of HPV31 compared to previous studies from North America (2).

We have used two different approaches to attribute HPV genotypes to cervical disease categories: In proportional attribution, a fraction of each case is attributed to every genotype in a multiple infection, while in hierarchical attribution a case is completely attributed to the most frequent type. Thus, the hierarchical attribution favors the more frequent types, especially HPV16, while the proportional attribution is more likely to attribute some cases to types without carcinogenic potential. Ideally, to use data on truly causal HPV infections, the frequency of types in single infections should constitute the underlying hierarchy/ proportions (as previously described by (19)). However, the low number of single genotype infections in our study prohibited us from using the single type frequencies. The high number of multiple infections observed in our study can be attributed to the use of a sensitive assay that is capable of detecting 37 HPV genotypes simultaneously. In most previous studies, HPV typing covered fewer types and consequently, these studies have described lower frequencies of multiple infections. We also acknowledge that there may be minor errors in our genotyping results leading to additional variation in HPV attribution.

Within the constraints of our analysis, we observe that the percentage of vaccine types in <CIN2 is substantially lower (range from 6.6% to 26.0%) than in CIN3 (range from 25.6%

to 74.8%). While at least 26.1%, but up to 64.7% of HSIL cytology might be eliminated by the quadrivalent vaccine, probably much less than a third of ASCUS and LSIL will be prevented. This confirms the notion that cytological screening will suffer substantially in a vaccinated population, since the relative excess of low-grade lesions will increase further (23;24). In our attribution of vaccine types to disease categories, we have not accounted for potential partial cross-protection against closely related types, especially HPV45 and HPV31, that has been previously demonstrated in vaccine trials (37). Based on our data, cross-protection would not reduce cases by more than 4 to 6% in the CIN3 and HSIL categories, and even less in cancers and the lower disease categories.

Our HPV genotyping data were derived from sampling the complete cervical surface which could involve multiple lesions each with their own independent causative HPV genotype. In theory, it is possible to determine the causal type in multiple infections by analyzing DNA from isolated lesions, HPV RNA expression patterns, oncoprotein expression, and viral integration. However, it has been demonstrated that it is challenging to obtain lesion-specific genotypes from histological specimens (7;38), and other more specific assays are currently not available in reliable high-throughput formats..

In our molecular studies in SUCCEED, further determination of causal HPV genotype attribution will require analyzing additional markers such as viral oncogene expression and integration. Only based on these additional data can we move forward with improved estimates of genotype attribution, validate the attribution models described here, and gain a better understanding of the potential effects of HPV vaccination on cervical disease, and which genotypes to include in new generation HPV detection assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Number of HPV infections and average patient age by disease groups

	<CIN2 NILM			<CIN2 ASC/LSIL			CIN2 + <CIN2 HSIL			CIN3			CA		
types	**n**	**%**	**mean age**	**n**	**%**	**mean age**	**n**	**%**	**mean age**	**n**	**%**	**mean age**	**n**	**%**	**mean age**
All	167	100%	27.7	481	100%	25.7	418	100%	27.0	303	100%	29.5	97	100%	46.8
1	61	36.5%	29.7	119	24.7%	28.7	110	26.3%	30.3	99	32.7%	30.8	64	66.0%	46.3
2	43	25.7%	27.1	117	24.3%	26.1	109	26.1%	27.3	81	26.7%	30.1	17	17.5%	52.4
3	30	18.0%	27.9	97	20.2%	25.0	70	16.7%	24.4	58	19.1%	27.9	11	11.3%	46.1
4	13	7.8%	28.2	65	13.5%	23.8	51	12.2%	25.3	37	12.2%	29.4	3	3.1%	37.7
5+	20	12.0%	22.6	83	17.3%	23.0	78	18.7	25.4	28	9.2%	26.7	2	2.1%	33.5
p-trend			0.01			<0.001			<0.001			0.03			0.3

NILM= negative for intraepithelial lesion and malignancy; ASC= atypical squamous cells; LSIL= low grade squamous intraepithelial lesion; HSIL= high grade squamous intraepithelial lesion; CIN= cervical intraepithelial neoplasia; CA= carcinoma. Only HPV-positive women with histology/cytology results and age information are included (n=1452). Up to 14 concurrent infections were detected.

Table 2

Attribution of HPV types to cancer cases

Cancer, n=107										
Type	**Single type infection**	**%**	**Single carcinogenic type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
16	44	41.1%	50	46.7%	57.17	53.4%	59	55.1%	59	55.1%
18	10	9.3%	10	9.3%	12.5	11.7%	13	12.1%	15	14.0%
45	2	1.9%	2	1.9%	3.9	3.6%	5	4.7%	8	7.5%
52	1	0.9%	2	1.9%	3.1	2.9%	3	2.8%	6	5.6%
59	1	0.9%	2	1.9%	2.52	2.4%	2	1.9%	6	5.6%
33	4	3.7%	4	3.7%	4.08	3.8%	4	3.7%	5	4.7%
35	1	0.9%	2	1.9%	1.69	1.6%	2	1.9%	4	3.7%
39	2	1.9%	3	2.8%	2.89	2.7%	3	2.8%	4	3.7%
53	0	0.0%	NA		0.84	0.8%	1	0.9%	4	3.7%
84	0	0.0%	NA		0.4	0.4%	0	0.0%	4	3.7%
66	0	0.0%	1	0.9%	0.72	0.7%	0	0.0%	3	2.8%
72	1	0.9%	NA		1.09	1.0%	1	0.9%	3	2.8%
83	0	0.0%	NA		0.14	0.1%	0	0.0%	3	2.8%
31	0	0.0%	0		0.32	0.3%	0	0.0%	2	1.9%
62	0	0.0%	NA		0.28	0.3%	0	0.0%	2	1.9%
70	0	0.0%	NA		0.42	0.4%	0	0.0%	2	1.9%
73	0	0.0%	NA		0.95	0.9%	1	0.9%	2	1.9%
81	0	0.0%	NA		0.23	0.2%	0	0.0%	2	1.9%
56	0	0.0%	0		0.08	0.1%	0	0.0%	1	0.9%
58	1	0.9%	1	0.9%	1	0.9%	1	0.9%	1	0.9%
6	0	0.0%	NA		0.04	0.0%	0	0.0%	1	0.9%
54	0	0.0%	NA		0.14	0.1%	0	0.0%	1	0.9%
55	0	0.0%	NA		0.01	0.0%	0	0.0%	1	0.9%
61	0	0.0%	NA		0.33	0.3%	0	0.0%	1	0.9%
67	1	0.9%	NA		1	0.9%	1	0.9%	1	0.9%
69	1	0.9%	NA		1	0.9%	1	0.9%	1	0.9%

Cancer, n=107										
Type	**Single type infection**	**%**	**Single carcinogenic type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
82	0	0.0%	NA		0.14	0.1%	0	0.0%	1	0.9%
89	0	0.0%	NA		0.02	0.0%	0	0.0%	1	0.9%
Vaccine types	54	50.5%	60	56.1%	70	65.4%	72	67.3%		
All 37 types	69	64.5%	77	72,0%	97	90.7%	97	90.7%		

Types 51, 68, 11, 26, 40, 42, 64, 71, and 82v were not detected in cancers and are not displayed. Single type infection: minimum attribution; Single carcinogenic type infection: frequency of single carcinogenic infections irrespective of additional non-carcinogenic type infections; Proportional attribution: each type is proportionally attributed to a case according to its frequency in the respective disease category; Hierarchical attribution: the most frequent type (according to its frequency in the respective disease category) is attributed to the case; Any: inclusion of any case where type is detected (maximum attribution).

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Table 3

Attribution of HPV types to CIN3 cases

CIN3, n=305										
Type	**Single type infection**	**%**	**Single carcinogenic type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
16	75	24.6%	109	35.7%	198.75	65.2%	221	72.5%	221	72.5%
31	10	3.3%	12	3.9%	20.68	6.8%	24	7.9%	42	13.8%
52	4	1.3%	5	1.6%	10.31	3.4%	8	2.6%	35	11.5%
51	1	0.3%	1	0.3%	6.28	2.1%	6	2.0%	27	8.9%
66	0	0.0%	0	0.0%	4.7	1.5%	5	1.6%	27	8.9%
18	3	1.0%	4	1.3%	7.58	2.5%	7	2.3%	25	8.2%
33	5	1.6%	6	2.0%	8.94	2.9%	9	3.0%	20	6.6%
45	4	1.3%	6	2.0%	7.06	2.3%	6	2.0%	20	6.6%
61	0	0.0%	NA		2.42	0.8%	1	0.3%	20	6.6%
35	4	1.3%	6	2.0%	8.5	2.8%	7	2.3%	19	6.2%
59	1	0.3%	1	0.3%	3.12	1.0%	2	0.7%	18	5.9%
83	0	0.0%	NA		2.09	0.7%	0	0.0%	18	5.9%
56	0	0.0%	0	0.0%	1.6	0.5%	0	0.0%	16	5.2%
58	6	2.0%	6	2.0%	7.32	2.4%	6	2.0%	16	5.2%
53	1	0.3%	NA		2.2	0.7%	1	0.3%	16	5.2%
39	0	0.0%	1	0.3%	2.66	0.9%	0	0.0%	15	4.9%
89	0	0.0%	NA		1.49	0.5%	0	0.0%	15	4.9%
6	0	0.0%	NA		0.71	0.2%	0	0.0%	14	4.6%
84	0	0.0%	NA		1.09	0.4%	0	0.0%	14	4.6%
54	0	0.0%	NA		1.06	0.3%	0	0.0%	13	4.3%
62	0	0.0%	NA		0.96	0.3%	0	0.0%	12	3.9%
70	0	0.0%	NA		1.04	0.3%	0	0.0%	12	3.9%
42	0	0.0%	NA		0.5	0.2%	0	0.0%	10	3.3%
55	0	0.0%	NA		0.59	0.2%	0	0.0%	10	3.3%
68	0	0.0%	0		0.39	0.1%	0	0.0%	9	3.0%
82	1	0.3%	NA		1.21	0.4%	1	0.3%	7	2.3%

CIN3, n=305										
Type	**Single type infection**	**%**	**Single carcinogenic type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
73	0	0.0%	NA		0.22	0.1%	0	0.0%	6	2.0%
81	0	0.0%	NA		0.08	0.0%	0	0.0%	5	1.6%
40	0	0.0%	NA		0.22	0.1%	0	0.0%	4	1.3%
67	0	0.0%	NA		0.03	0.0%	0	0.0%	3	1.0%
72	0	0.0%	NA		0.04	0.0%	0	0.0%	3	1.0%
11	0	0.0%	NA		0.01	0.0%	0	0.0%	2	0.7%
26	0	0.0%	NA		0.1	0.0%	0	0.0%	2	0.7%
82v	0	0.0%	NA		0.04	0.0%	0	0.0%	2	0.7%
69	0	0.0%	NA		0	0.0%	0	0.0%	1	0.3%
71	0	0.0%	NA		0	0.0%	0	0.0%	1	0.3%
Vaccine types	78	25.6%	113	37.0%	207	67.9%	228	74.8%		
All 37 types	115	37.7%	157	51.5%	303.99	99.7%	304	99.7%		

HPV64 was not detected in CIN3 and is not displayed. Single type infection: minimum attribution; Single carcinogenic type infection: frequency of single carcinogenic infections irrespective of additional non-carcinogenic type infections; Proportional attribution: each type is proportionally attributed to a case according to its frequency in the respective disease category; Hierarchical attribution: the most frequent type (according to its frequency in the respective disease category) is attributed to the case; Any: inclusion of any case where type is detected (maximum attribution).

Table 4

Attribution of HPV types to CIN2 cases

CIN2, n=427								
Type	**Single type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
16	52	12.2%	155.84	36.5%	200	46.8%	200	46.8%
52	11	2.6%	29.61	6.9%	37	8.7%	64	15.0%
51	6	1.4%	26.48	6.2%	37	8.7%	61	14.3%
31	8	1.9%	23.23	5.4%	29	6.8%	52	12.2%
66	1	0.2%	12.22	2.9%	12	2.8%	46	10.8%
18	12	2.8%	19.38	4.5%	19	4.4%	43	10.1%
53	0	0.0%	8.99	2.1%	7	1.6%	42	9.8%
84	0	0.0%	9.07	2.1%	8	1.9%	41	9.6%
35	6	1.4%	14.46	3.4%	14	3.3%	39	9.1%
62	1	0.2%	8.37	2.0%	4	0.9%	39	9.1%
39	2	0.5%	9.73	2.3%	3	0.7%	38	8.9%
33	9	2.1%	18.01	4.2%	17	4.0%	37	8.7%
59	1	0.2%	8.72	2.0%	4	0.9%	36	8.4%
6	1	0.2%	8.39	2.0%	4	0.9%	35	8.2%
56	1	0.2%	6.56	1.5%	2	0.5%	34	8.0%
58	3	0.7%	10.55	2.5%	5	1.2%	34	8.0%
89	0	0.0%	6.39	1.5%	0	0.0%	33	7.7%
45	3	0.7%	9.09	2.1%	5	1.2%	31	7.3%
42	0	0.0%	4.26	1.0%	0	0.0%	26	6.1%
61	1	0.2%	4.47	1.0%	2	0.5%	26	6.1%
73	2	0.5%	4.26	1.0%	2	0.5%	23	5.4%
54	0	0.0%	2.21	0.5%	0	0.0%	21	4.9%
68	0	0.0%	2.86	0.7%	0	0.0%	19	4.4%
81	0	0.0%	1.97	0.5%	0	0.0%	17	4.0%
55	0	0.0%	1.94	0.5%	0	0.0%	16	3.7%
67	4	0.9%	5.18	1.2%	4	0.9%	15	3.5%

CIN2, n=427								
Type	**Single type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
40	1	0.2%	1.55	0.4%	1	0.2%	12	2.8%
82	0	0.0%	0.71	0.2%	0	0.0%	10	2.3%
70	2	0.5%	2.41	0.6%	2	0.5%	9	2.1%
83	0	0.0%	0.36	0.1%	0	0.0%	9	2.1%
82v	0	0.0%	0.36	0.1%	0	0.0%	7	1.6%
72	0	0.0%	0.32	0.1%	0	0.0%	6	1.4%
11	0	0.0%	0.07	0.0%	0	0.0%	4	0.9%
26	1	0.2%	1	0.2%	1	0.2%	1	0.2%
Vaccine types	65	15.2%	184	43.1%	223	52.2%		
All 37 types	128	30.0%	419.02	98.1%	419	98.1%		

Types 64, 69, and 71 were not detected in cancers and are not displayed. Single type infection: minimum attribution; Proportional attribution: each type is proportionally attributed to a case according to its frequency in the respective disease category; Hierarchical attribution: the most frequent type (according to its frequency in the respective disease category) is attributed to the case; Any: inclusion of any case where type is detected (maximum attribution).

Table 5

Attribution of HPV types to <CIN2 cases

Less than CIN2, n=708								
Type	**Single type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
16	28	4.0%	85.92	12.1%	147	20.8%	147	20.8%
51	20	2.8%	64.23	9.1%	94	13.3%	121	17.1%
53	15	2.1%	45.6	6.4%	64	9.0%	98	13.8%
66	12	1.7%	40.55	5.7%	56	7.9%	94	13.3%
39	10	1.4%	41.8	5.9%	42	5.9%	92	13.0%
62	5	0.7%	33.37	4.7%	29	4.1%	88	12.4%
59	9	1.3%	32.38	4.6%	30	4.2%	81	11.4%
56	5	0.7%	26.93	3.8%	22	3.1%	73	10.3%
52	8	1.1%	24.94	3.5%	19	2.7%	68	9.6%
18	10	1.4%	28.2	4.0%	23	3.2%	67	9.5%
42	3	0.4%	17.59	2.5%	11	1.6%	63	8.9%
31	8	1.1%	23.95	3.4%	17	2.4%	62	8.8%
89	2	0.3%	17.32	2.4%	9	1.3%	57	8.1%
54	4	0.6%	16.2	2.3%	7	1.0%	55	7.8%
6	9	1.3%	20.63	2.9%	13	1.8%	54	7.6%
84	3	0.4%	13.54	1.9%	4	0.6%	52	7.3%
35	10	1.4%	20.2	2.9%	14	2.0%	49	6.9%
58	4	0.6%	12.77	1.8%	7	1.0%	46	6.5%
61	2	0.3%	10.07	1.4%	4	0.6%	41	5.8%
45	6	0.8%	12.89	1.8%	8	1.1%	39	5.5%
73	3	0.4%	7.79	1.1%	5	0.7%	32	4.5%
68	2	0.3%	7.83	1.1%	4	0.6%	31	4.4%
83	2	0.3%	6.23	0.9%	2	0.3%	30	4.2%
55	5	0.7%	8.07	1.1%	6	0.8%	27	3.8%
70	5	0.7%	8.36	1.2%	5	0.7%	26	3.7%
82	1	0.1%	3.9	0.6%	1	0.1%	26	3.7%

Less than CIN2, n=708								
Type	**Single type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
81	2	0.3%	5.16	0.7%	2	0.3%	25	3.5%
67	2	0.3%	4.58	0.6%	2	0.3%	24	3.4%
40	0	0.0%	2.64	0.4%	0	0.0%	19	2.7%
33	3	0.4%	4.39	0.6%	3	0.4%	18	2.5%
72	1	0.1%	2.05	0.3%	1	0.1%	15	2.1%
11	0	0.0%	1.19	0.2%	1	0.1%	10	1.4%
26	0	0.0%	0.37	0.1%	0	0.0%	4	0.6%
82v	0	0.0%	0.11	0.0%	0	0.0%	4	0.6%
64	0	0.0%	0.13	0.0%	0	0.0%	3	0.4%
69	1	0.1%	1.03	0.1%	1	0.1%	3	0.4%
71	0	0.0%	0.07	0.0%	0	0.0%	3	0.4%
Vaccine types	47	6.6%	136	19.2%	184	26.0%		
All 37 types	200	28.2%	652.98	92.2%	653	92.2%		

Single type infection: minimum attribution; Proportional attribution: each type is proportionally attributed to a case according to its frequency in the respective disease category; Hierarchical attribution: the most frequent type (according to its frequency in the respective disease category) is attributed to the case; Any: inclusion of any case where type is detected (maximum attribution).

Table 6

Attribution of vaccine types to cytology categories

HSIL, n=651										
Type	**Single type infection**	**%**	**Single carcinogenic type infection**	**%**	**Proportional attribution**	**%**	**Hierarchical attribution**	**%**	**Any**	**%**
16	144	22.1%	206	31.6%	333	51.2%	376	57.8%	376	57.8%
18	26	4.0%	28	4.3%	40	6.1%	45	6.9%	72	11.1%
6	0	0.0%	NA		6	0.9%	0	0.0%	35	5.4%
11	0	0.0%	NA		0	0.0%	0	0.0%	3	0.5%
Vaccine types	170	26.1%	234	35.9%	379	58.2%	421	64.7%		
All 37 types	267	41.0%	369	56.7%	640.96	98.5%	641	98.5%		
LSIL, n=312										
Type	Single type infection	%			Proportional attribution	%	Hierarchical attribution	%	Any	%
16	16	5.1%			55	17.7%	93	29.8%	93	29.8%
6	8	2.6%			16	5.2%	13	4.2%	39	12.5%
18	4	1.3%			9	3.0%	6	1.9%	27	8.7%
11	0	0.0%			0	0.1%	0	0.0%	6	1.9%
Vaccine types	28	9.0%			81	26.0%	112	35.9%		
All 37 types	70	22.4%			308	98.7%	308	98.7%		
ASC, n=425										
Type	Single type infection	%	Type		Proportional attribution	%	Hierarchical attribution	%	Any	%
16	34	8.0%			94	22.1%	132	31.1%	132	31.1%
18	8	1.9%			15	3.6%	13	3.1%	34	8.0%
6	2	0.5%			8	1.8%	4	0.9%	25	5.9%
11	0	0.0%			0	0.0%	0	0.0%	4	0.9%
Vaccine types	44	10.4%			117	27.5%	149	35.1%		
All 37 types	121	28.5%			381	89.6%	381	89.6%		

HSIL= high grade squamous intraepithelial lesion; LSIL= low grade squamous intraepithelial lesion; ASC= atypical squamous cells; Single type infection: minimum attribution; Single carcinogenic type infection: frequency of single carcinogenic infections irrespective of additional non-carcinogenic type infections; Proportional attribution: each type is proportionally attributed to a case according to its frequency in the respective disease category; Hierarchical attribution: the most frequent type (according to its frequency in the respective disease category) is attributed to the case; Any: inclusion of any case where type is detected (maximum attribution).