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Neutralizing antibodies against oncogenic human papillomavirus as a possible determinant of the fate of low-grade cervical intraepithelial neoplasia

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Abstract

To determine whether neutralizing antibodies (NAs) against HPV16 is responsible for a higher regression rate of low-grade cervical intraepithelial neoplasia (CIN1), we investigated an association between the presence of the NAs and the fate of the HPV16-related CIN1. All the women examined in this study had HPV16 positive cervix. The women were allocated into four groups by their cervical pathology, i.e., non-pathological (n:7), CIN1 (n:37), CIN2/3 (n:19), and cervical cancer (n:13). Their sera were tested for the presence of NAs against HPV16 by an in vitro assay using HPV16-pseudovirions. As for the CIN1 cases, clinical regression of the lesions were compared between NA-positive and NA-negative groups. Copy number of HPV16-DNA in smear samples was measured by quantitative PCR. The incidence of the presence of the NAs in the women with a non-pathological cervix (85.7%) was significantly higher than in the CIN1 cases (21.5%), the CIN2/3 cases (15.7%), and the cervical cancer cases (0%) ($p < 0.0001$). The regression of the CIN1 lesion was closely associated with the presence of the N As ($p = 0.0002$). The presence of the NAs was associated with low-level copy number of the viral DNA relative to the NA-negative group ($p = 0.05$). The presence of the NAs against HPV16 was associated with a higher regression rate of HPV-related CIN1 lesions. The NAs seem to have a role in deterring HPV-related cervical lesions from progressing to CIN2/3 by inhibiting the infection with de novo replicated HPV. This study further suggests that HPV vaccine to induce the NAs may be effective in eliminating CIN lesions, especially in the NA-negative cases.

Keywords: Human papillomavirus; Neutralizing antibodies; Pseudovirion; Low-grade cervical intraepithelial neoplasia; Regression

Genital human papillomavirus (HPV) is a major risk factor for the development of cervical cancer, the annual mortality being in the order of 200,000 per year [1]. Among women infected with oncogenic HPV, a small percentage of them later develop low-grade cervical intraepithelial neoplasia (CIN1), the precursor lesion of cervical cancer [2]. CIN1 is thought to regress spontaneously in about 60% of the patient cohort [6,7]. On the other hand, persistent HPV infection is associated with the progression to high-grade CIN (CIN2/3) or cervical cancer [3–5]. To know what determines the fate of CIN1 goes some way to developing the strategy for the prevention of its progression.

To address this, we focused our attention to neutralizing antibodies (NAs) against HPV, antibodies known to prevent its infection, in view of the fact that chronic production of oncogenic HPV can occur in CIN1 which might lead to the persistent infection [8].

It was shown that sera from the majority of cases with HPV11-positive condyloma acuminata have the ability to neutralize the HPV11 authentic virions using an in vitro assay system to monitor the viral infection [9,10]. We have already established the system for HPV

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using HPV pseudovirions, which enabled us to detect the NAs present in sera or vaginal washes from immunized mice with capsid proteins of HPV16 [11–13]. Using this system, we examined the presence of the NAs against HPV16 in HPV16-positive women without cervical pathology, CIN and cervical cancer. Furthermore, we observed natural courses of HPV16-positive CIN1 cases in a cohort study as related to the NAs with a view to seeing a possible association between the presence of the NAs and the fate of the disease.

Materials and methods

Patients and samples. We sampled sera from seven women with HPV16-positive non-pathological cervix (non-CIN cases), 37 cases with HPV16-positive CIN1, 19 cases with HPV16-positive CIN2/3, and 13 cases with HPV16-positive cervical cancer. We followed up the CIN1 cases by using colposcopy and Pap smear at four-month intervals with no intervention, except for cases progressing to CIN3. Upon enrollment we sampled serum for serologic study and exfoliated cells of the cervical lesion for HPV-DNA detection with informed consent to participate in this study. An expert pathologist panel confirmed CIN lesions based on the review of cytologic and histologic materials.

HPV-DNA detection, typing, and quantitative PCR. HPV-DNA was detected in exfoliated cervical cells collected from HPV-infected women by the PCR-based method as described previously [14]. In brief, total cellular DNA was extracted from each specimen by the standard procedure. HPV-DNA was amplified by PCR using the consensus-primers for the HPV L1 region. HPV subtypes were identified based on restriction fragment length polymorphism (RFLP) analysis.

We estimated the copy number of HPV16 DNA molecules present in a smear sample by quantitative PCR that utilized the 5′-exonuclease assay and real-time detection of the accumulation of fluorescence (Taqman) [15]. The 180 bp fragment of the E1 open reading frame in the presence of a HPV16-specific hybridization probe was amplified for quantification of the amount of HPV16 DNA. An amount of 500 ng isolated DNA from the exfoliated cells as a template, 0.25 μmol/L of 5′ primer mix, 0.5 μmol/L of 3′ primer mix, and 0.1 pmol of the probe were mixed with a final volume 25 μL of PCR solution. The primer and the probe were constructed as described previously [15]. Fluorescence was detected by an ABI Prism 7700, Sequence Detection System (Perkin–Elmer). The amplification ramp included two hold programs followed by a two-step PCR cycle (for 15 s at 95 °C and for 1 min at 55 °C) for a total of 50 cycles. To adjust for variation in the number of cells in different samples, the copy number of β-actin was estimated by the same method using the commercially available primer and hybridization probe (Perkin–Elmer).

Detection of NAs against HPV16. Neutralizing activity in serum was determined as an activity to inhibit the infection of cultured COS-1 cells with reassembled HPV16 pseudovirions containing expression plasmid for β-galactosidase as described previously [11]. The pseudovirions of 50 infectious units were mixed with 0.1 ml of 100-fold dilutions of sera in PBS (pH 6.8), incubated for 1 h, and then mixed with COS-1 cells. Cells expressing β-galactosidase were stained using X-gal as a substrate (In Situ β-Galactosidase Staining Kit, Promega, Madison, WI). The number of blue-stained cells was counted and positivity for NAs was determined by an activity to reduce the number of the blue cells to less than half of that in the sample treated with PBS (mock).

Statistical analysis. Relations between cervical pathologies and positivity for NAs were assessed by Fisher's exact test. Time from the detection of abnormal cytology to the regression was analyzed with survival analysis methods (Kaplan–Meier analysis). Log-rank test was used to compare survival curves between the NA-positive vs negative group.

Results

We compared the incidence of the presence of the NAs against HPV16 among women who had HPV16-positive cervices with or without varying degree of cervical pathology (Table 1). The NAs were detected in 6 out of 7 non-CIN cases (85.7%). On the other hand, in women with cervical pathologies, the NAs were found in 8 out of 37 CIN1 cases (21.6%), 3 out of 19 CIN2/3 cases (15.8%), and in none of 13 invasive cancers (0%), with statistical significance being achieved between the non-CIN cases vs the CIN cases or the invasive cancer cases ($p < 0.0001$).

Among the 37 HPV16-positive CIN1 cases, the 33 cases could be followed up for more than a year, ranging from 16 to 40 months (median follow-up period: 24 months), at four-months intervals. The regression of CIN1 was estimated by confirming normal Pap smears consecutively for one year or more. Kaplan–Meier curves showed that the regression of CIN1 occurred more frequently in the NA-positive group against HPV16 compared with the negative group (Fig. 1) (Log-rank test:

Table 1
Comparison of positivities for NAs against HPV16 between non-CIN women with HPV16-positive cervices and HPV16-positive cervical neoplasia cases

Cervical status of seropositive women	No. cases	NA-positive[a]	p-value[b] vs non-CIN
Non-CIN	7	6 [85.7%]	
CIN1	37	8 [21.6%]	$p < 0.0001$
CIN2/3	19	3 [15.8%]	$p < 0.0001$
Invasive cancer	13	0 [0.0%]	$p < 0.0001$

[a] NA (neutralizing antibodies against HPV16) positive; 100-fold dilution of serum could reduce the number of blue cells to half of that in the sample treated with PBS in a neutralization assay with pseudovirions.
[b] p-value; Fisher's exact test.

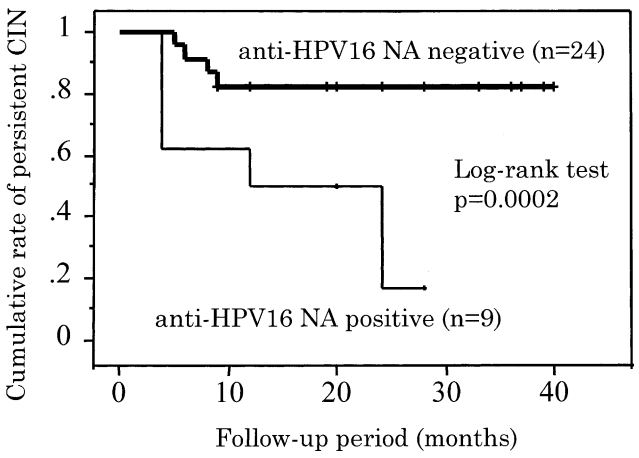

Fig. 1. The cumulative persistence rates of HPV16-positive CIN1 in NA-positive and -negative groups. p-value was estimated by log-rank test.

Table 2
Correlation of the copy number of HPV16 DNA with the NAs against HPV16

NA[a]	No. of CIN cases	Copy number of the viral DNA		p-value[b]
		$<10^4$	$\geqslant 10^4$	
Positive	11	6	5	
Negative	26	5	21	0.05

[a] NA (neutralizing antibodies against HPV16) positive; 100-fold dilution of serum could reduce the number of blue cells to half of that in the sample treated with PBS in a neutralization assay with pseudovirions.
[b] p-value; Fisher's exact test.

$p = 0.0002$). When looking at the 24-month regression rates, those of the NA-positive group (85.2%) were much higher than those of the NA-negative group (13.6%).

To see whether the presence of the NAs was associated with HPV16 viral loads, we examined the copy number of viral DNA in CIN1 lesions in the presence or in the absence of the NAs (Table 2). The number of collected cells from both groups being comparable was confirmed by observing the number of β-actin gene (data not shown). Interestingly, a great majority (80.8%) of the NA-negative group had high-level copy number of the viral DNA ($\geqslant 10^4$ copies), whereas a majority (54.5%) of the NA-positive group had low-level copy number ($<10^4$), suggesting a role for the NAs in reducing the number of HPV virus resident in CIN1 lesions.

Discussion

We have demonstrated that the incidences of the presence of NAs against HPV16 in sera are different among women with or without HPV-related pathology in the uterine cervix, a higher incidence being found in women without cervical pathology compared with affected women. Of note, none of invasive cancer cases exhibited the NAs while the incidence of the NAs in CIN cases was somewhere between unaffected women and invasive cancer cases. These data can be interpreted to mean that the NAs seem to have a role in deterring HPV-related cervical lesions from progressing to preinvasive and invasive lesions.

In a cohort study for CIN1, HPV-specific IgG antibodies were constantly detected by ELISA assay in women who had long-standing CIN1, ultimately progressing to CIN2/3 [16]. In this study, as small as 30% of the CIN1 cases positive for HPV16 IgG antibodies had the NAs (data not shown). Moreover, it was shown here that the regression of CIN1 lesions tended to occur in the cases positive for the NAs. Thus, the presence of the NAs appears to be an important predictor for the regression of the lesions in CIN1 cases positive for HPV-specific IgG antibodies.

At present, precise mechanisms of how the NAs work so as to suppress the progression of HPV-related cervical lesions remain to be seen. The production of HPV can occur in CIN lesions [8]. It may be that the NAs against HPV may inhibit the infection with de novo replicated HPV and thus reduce a viral load, as was suggested in this study. The lesions with a diminished viral load is considered to be eliminated in the course of time whereas the lesions with an increased viral load may persist for longer time, resulting in the progression to different stages. Of course, cellular immunity against HPV has an important role in clearance of HPV infection and HPV-induced CIN [17]. In particular, cytotoxic T lymphocyte (CTL) activity to E6 and E7 proteins of HPV16 is involved in the protection of CIN1 progression to high-grade CIN [18]. Putting together, we can speculate that the regression of CIN1 may require CTL for clearance of the established HPV-related lesions on one hand and the NAs to eliminate de novo infection with replicated viruses on the other hand.

In mucosal tissues, such as the uterine cervix, secretory antibodies have the cardinal role in protecting the attack of pathogens. However, we did not test secretory-type NAs. In this regard, recent studies from several laboratories demonstrated the production of secretory-type NAs in the genital tract that was coupled with the presence of NAs in sera using immunized mice with HPV capsid protein [13,19,20]. Thus, it is likely that the secretory-type NAs might be present in cases positive for the NAs as shown here, a reasonable explanation for a link between the NAs detected in sera and the regression of CIN1.

In summary, this study demonstrated that the NAs against HPV16 are associated with the regression of the HPV-related CIN1, suggesting that HPV vaccine to induce the NAs may raise hopes for protecting the progression of the disease, especially in NA-negative CIN1 cases.

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