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Commentary

New HPV16 viral biomarkers to understand the progression of cervical lesions towards cancer

In this issue, Shukla and collaborators[1] report a comprehensive description of the physical state of human papilloma virus (HPV)16 DNA genome (viral load and integration) in cervical samples from Indian women and representative of the natural history of cervical cancer. They have analyzed the DNA from 130 monotypic HPV16-infected biopsies including 30 cases of cytology-confirmed low grade squamous intraepithelial lesions (LSIL), 30 cases of high grade squamous intraepithelial lesions (HSIL) and 70 cases of invasive squamous cell carcinoma (SCC). The HPV16 DNA load was determined by real-time quantitative PCR targeting the HPV16 URR and was normalized to input diploid cellular DNA by quantifying a cellular gene. As for the physical state of HPV16 genome, it was evaluated by a combination of two PCR targeting *(i)* the entire HPV16 *E2* open reading frame to assess the presence (episomes) or the absence (integrated viral DNA) of the *E2* gene, and *(ii)* the HPV16 *E6* gene followed by the densitometric analysis of *E2* and E6 amplicons after gel electrophoresis as previously described[2]. The authors show an increasing HPV16 DNA load as well as an increasing proportion of samples with integrated HPV16 DNA with the severity of the lesions.

It is now well known that persistent high risk (HR) HPV infection is the major risk factor for the development of precancerous and cancerous lesions of the cervix[3]. Furthermore, HPV16, the most frequently detected HPV worldwide at the cervix level, both in patients with normal smear or with (pre-) cancerous lesion[4], is the most carcinogenic genotype among HR HPV[5]. The strength of the association between HR HPV infection and cervical cancer led to propose HPV testing for primary screening to improve cervical cancer screening[6]. We need to remind that HR HPV infection is also the most frequent sexually transmitted infection with up to 80 per cent of women being infected during their lifetime[7]. Thus only clinically relevant or transforming infections (*i.e*. those associated with a risk of development of HSIL or worst) deserve special attention to correctly triage infected women[8]. One way to meet these expectations is to better describe the natural history of HPV infection linked to the development of cervical lesions to highlight new viral biomarkers. Numerous publications relate HPV DNA load and/or HPV DNA integration assessment in series of cervical samples representative of the natural history of cervical cancer. Most of the studies focused their analyses on HPV16 and as a whole report an increasing HPV DNA load and HPV DNA integration with the lesion grade[9-14]. However, the use of various specimens (cervical scrapes, fresh, frozen or formalin fixed biopsies), the use of samples infected by multiple HPV types, the quantification or not of a cellular gene for normalization could lead to inconsistencies between studies. Although some authors propose clinical thresholds for viral load and integration that would allow the identification of prevalent or incident lesions, no consensus about cut-off values has emerged from the literature. This is why Shukla and collaborators[1] have decided to analyze the HPV16 viral load and the physical status of HPV16 DNA in a perfectly well defined cohort of Indian women. Especially, the authors took care to select cervical samples infected only by HPV16 to ensure that the lesion they have analyzed is attributable to the HPV16 genotype only. The data they have generated confirm the link between HPV16 DNA load and integration and the lesion grade. Consistent with previous observations, they suggest that integration is an early event of cervical carcinogenesis because integrated HPV16 DNA was observed in 10 per cent of LSIL. They also confirm that the majority of samples with integrated HPV16 DNA also present episomes, especially in SCC, raising the

issue of E2 disruption as a necessary event for cervical carcinogenesis. This question was recently addressed in an elegant study that demonstrated the loss of the E2 protein expression in high grade lesions/cervical cancers even in the presence of the *E2* ORF and elevated levels of transcripts[15]. In this regard, the pattern of viral transcript and protein expression in cancerous lesions analyzed by Xue *et al*[15] is very similar to the one observed in CaSki cells, a cervical cancer cell line that harbour multiple HPV16 DNA copies integrated in concatemers with an intact *E2* ORF. Thus, the intrinsic value of HPV DNA integration as a relevant biomarker can be questionable. In addition, we now have some evidences that epigenetic modifications of viral DNA, such as the methylation of the HPV16 promoter which is preferentially observed in cancer[16], also contribute to the deregulation of *E6* and *E7* viral oncogene expression even in the presence of *E2*[17].

Shukla and collaborators[1] further queried whether HPV16 DNA load could be linked to the HPV16 genome physical status. They found that viral loads were higher in samples harbouring mixed forms than in samples with only episomes or with fully integrated HPV16 DNA whatever the lesion grade was (LSIL, HSIL, SCC). The reason why such an association was found is not clear. It can be hypothesized that in the presence of episomes with intact *E1*/*E2* ORF, the capacity of viral DNA replication is maintained not only from episomes but also from integrated HPV16 DNA, leading to the formation of "onion skin"-type replication intermediates as proposed by Kadaja *et al*[18]. This is consistent with the fact that HPV16 load is significantly decreased in cancer samples harbouring only integrated viral DNA.

The work conducted by Shukla and collaborators[1] argues in favour of an intimate link between the natural history of HPV16 infection and the natural history of cervical cancer development. Because HPV infection is a necessary cause for cervical cancer, sensitive and specific viral biomarkers should be identified. In a practical point of view, new biomarkers that will permit to correctly manage women with HPV infection need to fulfill numerous criteria. Among these, clinical sensitivity and specificity are of utmost importance to avoid any unnecessary treatments as well as the risk to misdiagnose an invasive lesion. Now, many challenges must be met and there is a need to standardize procedures both at the pre-analytical, analytical and post-analytical steps. In this regard, specific guidelines have been published[19,20] to ensure the sharing of best experimental practices that need to be implemented to generate high quality biomarkers. Then, the clinical relevance of new biomarkers should be tested in population-based studies before implementation in clinical practice.

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