Cervical atypical glandular cells and false negative HPV testing: a dramatic reality of the wrong test at the right place

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Summary

Background: Due to cervical cancer screening the number of squamous cancer have declined. The number of adenocarcinomas (ADCs) does appear to be rising. ADCs are often missed and human papillomavirus (HPV) testing could be helpful in detecting these abnormalities earlier. *Case:* A 36-year-old woman, who had a normal smear three years earlier, had a pap smear with atypical glandular cells. The L1 HPV test showed that there was no HPV infection. Other HPV tests which looked at E6 and E7 showed an infection with HPV 16. Due to unknown reasons, no action was taken regarding the atypical glandular cells. Two years later the patient was diagnosed with a FIGO Stage IVb ADC of the cervix. The L1 HPV test was still negative and the E6/E7 HPV test was still positive. Despite several multiple treatment modalities she succumbed of her disease two years later leaving behind a young family. *Conclusion:* HPV test looking only at L1 can give false negative results if the virus is integrated in the human genome.

Key words: Human papillomavirus; HPV; Adenocarcinoma; L1; E6; E7; Integration; False negative; Cervical cancer; Prophylactic vaccination; Screening; Cross-protection; HPV testing.

Introduction

More than 500,000 cervical cancers are diagnosed each year and around 275,000 women die annually of this disease [1]. Luckily cervical cancer is also a preventable disease. It is the only cancer, which can be detected by screening in a precancer stage. Literally one can see the development of the cancer by the naked eye if it is not treat it. The authors recently showed that every high-grade cervical intraepithelial neoplasia (CIN) and cancer (CIN3+) lesion is characterized by a linear increase of type specific HPV viral load in time [2]. Enabling measurement and prediction towards CIN3+ long before cervical cancer would occur. Nevertheless it is still the third cancer among women and the fourth cause of cancer death [1].

The introduction of cytological screening, the so-called PAP smear, based on the findings of George Papanicolaou has lead to a decline in cervical cancer incidence. The decline is mainly attributed to a decrease in squamous cell carcinoma (SCC) and not to other cancer types. In fact, there is a steady rise in the absolute and relative incidence of cervical adenocarcinoma (ADC) among younger women in many countries [3]. It is unclear what the reason is for the increase of cervical ADC. It could be a true rise or a failure in detection of the precancer ADC lesions by cytological screening. The failure in detection of the precancer stage could explain why ADC is often diagnosed in an advanced stage.

The top five human papillomavirus (HPV) types for squamous and ADC of the cervix are the same but the distribution of the HPV types is different. Primary cervical cancer prevention by prophylactic HPV vaccination with a broad cross protection could therefore prevent almost 80% of all squamous cancers and more than 90% of all adenocancers of the cervix [4, 5]. If adolescences are vaccinated today, then cervical cancer landscape will be completely different over 30 years. Not only will there be an reduction in cancer by more then 80%, but it is likely that other HPV types will be responsible for the cancer.

Secondary cancer prevention by screening is already in place for many decades. The cytological screening has been a large success, and millions of women lives have been saved despite the 53% sensitivity of the test [6]. Because HPV testing has a 30%-43% higher sensitivity, the introduction of HPV testing in cervical screening would mean that more precancer lesions, especially the adeno lesions, would be detected earlier. Resulting in a broadening of the screening intervals to at least five years.

There are two distinct pathways that can be measured in cervical cells during natural history of an HPV infection. In transient infections, HPV tests measure the viral reproduction (or clearance) in desquamating cervical cells. These new-formed viruses always contain all the genomic information including L1. In persistent infections leading to CIN3+ the HPV measured represents HPV DNA present in dividing cervical basal cells, which have been transformed (linear increase in time) [2]. Because only E6 and E7 are needed for the immortalization of the basal cells, other parts of the viral genome can be missing (L1-E2). Another difference is in the amount of virus detected per cell (viral load), which is very high in transient infections (reproduc-

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tion of virus) and very low in cancer (only limited number of HPV copies who transformed the initial basal cell). Because HPV tests have a fixed sensitivity cutoff, the same amount of measurable HPV takes more time to accumulate in cancer (viral load doubling every 289 days) compared to transient infections (viral load doubling every three days), resulting in older (larger) cancers upon detection. HPV tests that only focus on L1 favor detection of transient infections while HPV tests targeting E6 and E7 can both measure transient and persistent infections [7]. When during viral DNA integration L1 is lost, this could lead to false negative HPV results in test targeting only the L1 region.

This article is written to highlight the value of HPV testing in general and to point out the value of E6 and E7 HPV testing in case of integration. It is important to detect the integrated HPV because those are the lesions, which are mostly likely to progress to an invasive cancer.

Case Report

This case is a description of a 36-year-old woman, G2P2, who had a normal smear in September 2002 and again in March 2004. Three years later she had a repeated smear that showed atypical glandular cells (AGC). The L1 HPV test showed no signs of a HPV infection. The E6/E7 HPV test on the other hand showed an infection with HPV 16. The viral load for HPV 16 E6 was ten copies per cell, while the viral load for HPV 16 E7 was 13 copies per cell. Due to unknown reasons, no further action was undertaken regarding the AGC. Two years later she had a repeated smear that still showed AGC. The L1 HPV test was still negative and the E6/E7 HPV test was still positive. The viral load increased both for E6 and E7 and was at this time, respectively, 24 in 2,755 copies per cell. The increase in HPV 16 E7 load was 0.0031 HPV 16 E7 copies per cell per day. Independent HPV 16 E2 PCR was also negative for all three PAP smears. In 2009, retesting of the liquid based cytology leftover from the 2004 normal smear also already showed the presence of HPV 16 E6 (one copy/cell) and E7 (seven copies/cell). A biopsy taken of the cervix showed an invasive ADC and subsequent staging revealed positive lymph nodes in the groin. The lesion was negative for HPV L1 on immunostaining. The FIGO Stage was therefore IVb. Despite multiple treatment modalities, the patient succumbed two years later, leaving behind a young family.

Discussion

This case clearly illustrates that HPV L1 based tests can miss cervical cancer, although E6/E7 based test could detect HPV many years earlier, leading to a delayed detection and treatment of the cancer. Invasive cervical cancers (ICC) can be divided in SCC (75%-90%), ADC (10%-25%) and a rest group containing adenosquamous cell carcinoma and rare types like melanoma, sarcoma, lymphoma, neuroendocrine tumors, and cancers of unspecified histology [3,8,9]. SCC occurs mainly at the ectocervix, while ADC will appear at the endocervix with a normal ectocervix. The latter probably responsible for the often false negative smear. ADC *in situ* (AIS) is multifocal in 15% of women [10]. Misdiagnoses between ADC of the endocervix and of the endometrium occur [4,5]. Misdiagnosis leads to mismanagement because surgery, chemotherapy, and radiotherapy differ for the two tumor types. The use of HPV is often helpful in the distinction between the tumor types. However there are some types of ADC, which are known to be HPV negative [11].

The top five HPV types for squamous cancer are the same as the top five for ADC [4,5,12]. The top five HPV types are HPV 16, HPV 18, HPV 31, HPV 33, and HPV 45. The distribution of the HPV types for the two histological cancer types are however different. HPV 16 infection results in predominantly squamous cervical neoplasia, while HPV 18 and HPV 45 have greater tendency to induce glandular cervical neoplasia [12]. ICC caused by HPV 16, 18, and 45 tended to be at an earlier age (average of 47 years) than ICC associated with other HPV types (average age 56 years) [4,5].

Together HPV 16, 18, and 45 account for approximately 90% of ADC and 70% of SCC worldwide [3-5, 8, 13]. This has major implication for primary prevention. There are two commercially available HPV vaccines Gardasil and Cervarix both are targeted on the high-risk types 16 and 18. Gardasil also targets the low risk types 6 and 11, and has a cross protection against high risk type HPV 31 [14]. Cervarix on the other hand has cross protection not only against high-risk type HPV 31 but also against the high-risk types HPV 33, HPV 45, and HPV 51 [14-16]. Due to this broadened cross protection there is an increase of prevention between ten to 15% against cancer. Translating the efficacy, in which the percentage of cancer could be prevented, would mean that roughly 70% of the SCC and more than 90% of the ADC could be prevented. Especially the impact on ADC is important because those are the cancers, which are often missed by the classical cytological screening.

AGC are reported in 0.4% of all cervical smears [17-21]. Regardless of HPV status, cytological results of AGC requires further investigations. Because these cytological abnormalities are associated with significant risk of an underlying precancerous (9%-38%) or malignant neoplastic processes (3%-17%). The ASCCP clinical follow-up guideline of 2001 and subsequently 2006 are quite clear they recommend colposcopic evaluations and endocervical sampling on all patients with AGC Pap results, regardless of age [17-21].

Primary HPV testing will increase the detection of adenocarcinomas, because cytology is frequently normal while HPV testing is positive in these cases. HPV testing can however also become false negative. In low-grade lesions the percentage of integration is very low, while there is a high percentage of integration of HPV into the host genome in high-grade lesions and invasive cancers. The integration frequency is different for the different HPV types. The integration in cancer for HPV 18 is 92%-100%, HPV 45 is 83%, HPV 16 is 55%-80%, HPV 33 is 37%, and HPV 31 is 14%

[22-24]. Due to the integration, there is a loss of the L1 sequences amplified by the SPF_{10} primers [25]. There is however never a loss of E6 and E7. Current HPV test are based on primers for either L1 or E6/E7 or all three regions. If a HPV test is solely based on the L1 region, one will miss about 15% of the integrated HPV [26]. As current case, these patients can become L1 negative. Together with the fact that cytology will miss almost half of the abnormities, one will have the wrong test at the right place. The sensitivity of an arbitrary HPV testing is at least 30% better then cytology in cervical cancer screening. One should therefore opt for HPV cervical cancer screening. Lesions that progress from low grade, to high grade, to ICC are more likely to have HPV integration. In order not to miss these cancers a HPV test based on the E6/E7 region should be used, instead of L1. This will further increase the sensitivity by at least 10%. The latter is especially important for cervical ADCs because there are frequently false negative on cytology.

The role of viral load in cervical cancer screening is gaining more and more interest. The viral load threshold cannot be used to distinguish between a clinically relevant (leading to CIN3+) and an irrelevant (transient) HPV infection. The viral load course (for a specific HPV type) can (or is) be the sum of transient infections (limited in time) and linearly progressing infections leading to CIN3+. There can be one or more infections at the same time. In a single infection, there is a transient prophase preceding the linear increase, meaning that a given threshold can be reached three times during the natural course of an HPV infection leading to CIN3+. The first time in the beginning of the reproductive transient prophase, the second time when the transient infection is clearing, and a third time when the linear increase underneath reaches the threshold level again.

The viral load in the transient (LSIL) (pro) phase can be very high, because it represents the summit of the reproductive phase of the virus infection. This very high level of viral load can in most cases never be reached in many of the CIN3+ lesions, because the size of the lesion is limited by the law of universal growth, and because each of the tumor cells which are derived from one clonal cell is limited by the number of HPV copies present in this cell. An example, the measured viral load of a CIN3+ lesion comprising of Hela cells (+/-50 HPV 18 copies/cell) would have a lower load (per CIN3+ cell) than a Caski tumor with the same amount of cells (+/-600 HPV copies/ Caski cell).

The load per scrape cannot always be reduced (calculated) to the load per cell. A better sampling with a Cervex-brush combi could increase the number of squamo-columnar junction cells (containing non-reproducing HPV virus in CIN3+ vs. reproducing HPV virus in transient infections). When a fixed volume of the cervical sample is taken to perform DNA extraction on (two ml from ten ml), the viral load per cell can be representative for the whole scrape. When a fixed volume is taken from a vial (eight ml) which is enriched (density gradient, BD-SurePath) and then extracted, there is a change in the proportion of squamo-columnar junction cells vs. non squamo-columnar junction cells (discarded after density gradient), making the assumption that load per scrape can be reduced (calculated) to the load per cell. Also load per scrape and the load per cell does not correct for the number of HPV copies present per cell, whereas assessing the type specific viral load over time does by eliminating it from the equation.

It is not the viral load threshold in se that is primordial in cervical cancer detection, but how fast the type specific (E6/E7) load increases or decreases over time [2]. The road to cervical cancer lies on well predestined line (HPV type specific E6/E7 load $=1e\text{-}5e^{0.0068 \text{ number of days}}$) the lower the analytical sensitivity of the test (HPV), the sooner the process of doubling basal cells carrying the cervical cancer marker (type specific HPV E6/E7) can be detected. This implies that type specific HPV slope measuring can detect a malignant process much sooner, and in time will lead to a higher clinical sensitivity. As in many other types of cancers, improving the sensitivity of measuring the malignant process will impact the clinical sensitivity and inevitably the outcome for the patient. A disadvantage of the hc2 is that it introduces a HPV viral load threshold, through a predefined cytology threshold, thereby limiting the action to be undertaken: waiting until a certain threshold is reached denies preventive action.

In conclusion cervical cancer screening should be based on HPV testing with detection in E6/E7 instead in L1, to avoid missing lesions with integrated HPV. The right test in the right place will detect almost all cervical cancers in an early and curable stage.

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