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Methylation of human papillomavirus 16, 18, 31, and 45 L2 and L1 genes and the cellular DAPK gene: Considerations for use as biomarkers of the progression of cervical neoplasia

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ABSTRACT

During progression of cervical cancer, human papillomavirus genomes and cellular tumor suppressor genes can become methylated. Toward a better understanding of these biomarkers, we studied 104 samples with HPV16, 18, 31, and 45 representing five pathological categories from asymptomatic infection to cancer. We grouped all samples by HPV type and pathology and measured the overall methylation of informative amplicons of HPV late genes and the cellular DAPK gene. Methylation of all four HPV types as well as of the DAPK gene is lowest in asymptomatic infection and increases successively in all four pathological categories during progression to cancer. 27 out of 28 cancer samples showed methylation both in the L2/L1 genes as well as in DAPK, but a much lower fraction in all other pathological categories. We discuss the problem to develop diagnostic tests based on complex methylation patterns that make it difficult to classify amplicons as "methylated" or "unmethylated".

Introduction

Cervical cancer, premalignant cervical lesions and non-neoplastic HPV infections, i.e. atypical cells of undetermined significance (ASCUS) and cervical intraepithelial neoplasia (CIN), are diagnosed by cytology (Papanicolaou test, Pap test), colposcopic inspection, and histological examination of biopsies. These tests and procedures are successful at decreasing the incidence of cervical cancer, but their rate of false diagnoses is a matter of concern (Nanda et al., 2000; Stoler and Schiffman, 2001). Detection of the DNA of high-risk human papillomavirus (HPV) types (Munoz et al., 2003; Bernard et al., 2010), the primary cause of cervical cancer, has become a powerful criterion to amend these procedures, and has greatly increased the sensitivity of screening (Bulkmans et al., 2007; Mayrand et al., 2007; Naucler et al., 2007). However, since the fraction of women being infected by HPVs at some time of their lives (> 80%) vastly exceed the incidence rate of cervical cancer (about 1%), and since a positive HPV DNA test often indicates a transient infection rather than a developing cervical cancer, HPV DNA diagnosis alone is not sufficient to distinguish women with benign infections from those requiring intensive management. In order to prevent unnecessary procedures on patients with abnormal Pap smears who are not at risk for developing cervical cancer, gynecologic practice needs tests that are sensitive and specific to detect high-risk patients. Numerous attempts have been made to measure markers that change as the result of HPV-dependent carcinogenesis, but these tests are still of limited benefit (von Knebel Doeberitz, 2002).

The molecular mechanisms involved in the progression of asymptomatic or low-grade HPV infections to cervical cancer are yet poorly understood, but include the methylation of many of those cellular genes that are also epigenetically affected in cancers of other organ sites and without an HPV etiology. The search of clinically useful epigenetic biomarkers of cervical cancer that may allow risk stratification in patients began relatively recently, but this field of research expanded rapidly, and a review (Wentzensen et al., 2009) compared studies of more than 60 cellular genes. Unfortunately, this meta-analysis came to the conclusion that there is currently no single methylation marker that that has the appropriate performance to serve as cervical cancer biomarker. The reviewed studies point only to

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few genes, notably DAPK (death associated protein kinase 1) and RARB (retinoic acid receptor beta), which might be attractive targets of further evaluations. Notably, these two markers stood out in a large epidemiological study comparing a panel of twenty cellular methylation targets (Feng et al., 2005).

Independently of these studies of cellular genes, our group has investigated how methylation affects HPV genomes in different stages of cervical neoplastic disease (Kalantari et al., 2004, 2008a, 2010; Badal et al., 2004; Turan et al., 2006, 2007), and our findings have been confirmed and expanded by others (Brandsma et al., 2009; Fernandez et al., 2009; Sun et al., 2011; Clarke et al., 2012; Mirabello et al., 2012a). A recent review summarizes this field (Johannsen and Lambert, 2013). Methylation of HPV16 and 18 increases among viral infections progressing from asymptomatic infection through low-grade and high-grade disease and malignancy. This effect is particularly pronounced in the late genes L2 and L1, whose products are not required for neoplastic processes. Methylation may affect the whole viral genome, however, although methylation is a repression mechanism (Bird, 2002). This is possible since neoplastic cells normally contain numerous HPV genomes. As long as one single HPV genome is spared from methylation, it maintains the carcinogenic process, although the other HPV genomes in the same cell may be transcriptionally silenced by methylation (Van Tine et al., 2004). The exact trigger of HPV methylation is not well understood, but there is evidence that methylation correlates with recombination between the HPV genome and chromosomal DNA (Kalantari et al., 2008a, 2008b, 2010). Studies not related to methylation have shown that HPV genomes frequently integrate into the cellular DNA in cancer, but it is disputed whether this mechanism is only a frequent event or mechanistically necessary (Daniel et al., 1997; Ueda et al., 2003; Hudelist et al., 2004; Arias-Pulido et al., 2006; Kulmala et al., 2006; Briolat et al., 2007; Pett and Coleman, 2007; Häfner et al., 2008; Vinokurova et al., 2008; Campitelli et al., 2012; Xu et al., 2013). Foreign DNA that integrates into mammalian chromosomal DNA is known to be a preferred methylation target, and therefore a correlation between HPV recombination and HPV DNA methylation may have nothing to do with the properties of the HPV genome and the biology of the virus (Doerfler et al., 2001). There is evidence that integration of HPV genomes favors the carcinogenic process as it leads to increased E6 and E7 oncoproteins transcription by interference with negative feedback by E2 proteins (Tan et al., 1994); transcriptional induction by the nuclear matrix (Stünkel et al., 2000), and stabilized E6/E7 transcripts (Jeon et al., 1995; Häfner et al., 2008).

The study reported here had the primary goal to compare the methylation of HPV late genes with methylation of the DAPK promoter, and with histological or cytological diagnoses among high-risk patients that were referred to a colposcopy clinic based on abnormal cervical cytology. Based on the literature cited above, we considered DAPK the most promising among the cellular epigenetic markers and we intended to compare this diagnosis with that of the viral late gene methylation. Aside from HPV16 and HPV18, our study targeted HPV31 and HPV45, which had not yet been studied when this research was done, but has been reported since then (Wentzensen et al., 2012). Our research targeted the promoter region of the DAPK gene, and two or three amplicons of the L2 and L1 genes of the four high-risk HPV types.

Results

Sample identification, clinical diagnosis and evaluation of DNA methylation

The objective of this study was to establish the methylation of CpG dinucleotides in two or three segments of the L2 and L1 genes of HPV16, 18, 31, and 45, and compare it with the CpG methylation of the promoter of the cellular DAPK gene in order to analyze the viral and cellular epigenetic changes as potentially useful clinical progression markers of cervical cancer.

All samples of precursor lesions of cervical cancers and of asymptomatic HPV infection were selected based on the HPV typing of the DNA of consecutive patients of a colposcopy clinic of the University of California Irvine as described in the Materials and methods section. This cohort yielded 50 samples with HPV16, nine with HPV18, eleven with HPV31, and six with HPV45. As this cohort did not contain patients with invasive cancers, we complemented these samples with material from a Norwegian cervical cancer archive, namely 11 samples with HPV16, four samples with HPV18, four samples with HPV31, and six samples with HPV45. We also included the analysis of C33A and SiHa cells with HPV16, and HeLa cells with HPV18, and report these three cell lines as cancers.

Most Californian patients were diagnosed prior to colposcopy by cytology and if medically indicated as part of the colposcopic examination by histology. Many of these diagnoses confirmed one another, e.g. patients with a low-grade squamous intraepithelial lesion (LSIL) by cytology were often found to have a cervical intraepithelial neoplasia grade I (CIN1) by histology. We sorted our samples according to these cytologic and histologic diagnoses, using in cases of discrepancy the higher grading of a lesion, i.e. a patient with LSIL and CIN3 is included in the category HSIL/CIN2-3. Our molecular data were based on analysis of cytological samples with the exception of cancer biopsies.

Methylation data were established for two or three, respectively, amplicons of the L2 and L1 genes of each HPV types, which had been found by us and others to be among the most highly methylated parts of the HPV genomes (Kalantari et al., 2004; Turan et al., 2006; Brandsma et al., 2009; Sun et al., 2011; Wentzensen et al., 2012), as well as for the promoter sequences of DAPK. Samples may contain cell and viral populations with diverse epigenetic states and histories. Many CpG residues in any particular genomic position can be completely methylated or unmethylated. Alternatively, a sample may contain molecules with mixtures of methylated and unmethylated CpGs in the same position (a sequencing output of overlapping C and T peaks). We report samples with mixtures of methylated and unmethylated CpGs as “methylated”, as they clearly contained HPV or DAPK populations with methylated CpGs.

Previous studies from our lab and others have shown that sporadic and low levels of CpG methylation occur in most HPV16 samples, including those derived from asymptomatic infection, low-grade lesions, and cell cultures with episomal HPV16 genomes. At this point no criterion exists to assign CpGs in any specific genomic position a diagnostically superior status, nor is it possible to define unequivocally a certain percentage of methylation as a diagnostically relevant threshold, making it difficult to classify individual samples as unambiguously “methylated” or “unmethylated”. As the principal output of our study, we therefore measured and reported the total number and percentage of methylated CpGs in all molecules that fall into any specific pathological category. All details of the methylation patterns of all amplicons are reported graphically, and we present statistical analyses as first steps to define quantitative criteria for the use of methylation data.

Methylation of the L2/L1 amplicons and the cellular DAPK promoter in samples containing HPV16

Sixty-three samples contained HPV16, and the methylation of their L2/L1 and DAPK methylation is shown in Fig. 1 and quantitatively summarized in Table 1. In HPV16, only 10–12.2% of all CpGs are methylated in asymptomatic infection and ASCUS (atypical