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ScienceDirect
Virology 361 (2007) 185–193
VIROLOGY
www.elsevier.com/locate/yviro

High-throughput detection of human papillomavirus-18 L1 gene methylation, a candidate biomarker for the progression of cervical neoplasia

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Received 19 September 2006; returned to author for revision 31 October 2006; accepted 10 November 2006
Available online 18 December 2006

Abstract

The L1 gene of human papillomavirus-18 (HPV-18) is consistently hypermethylated in cervical carcinomas, but frequently hypo- or unmethylated in exfoliated cells from asymptomatic patients. In precancerous lesions, L1 is sporadically hypermethylated, correlating with the severity of the neoplasia. In order to explore the potential of using L1 methylation as a workable biomarker for carcinogenic progression of HPV-18 infections in routinely taken samples, our aim was to develop methylation–detection techniques that were sensitive and rapid without being overly complex technically. Therein, we developed a methylation-specific PCR (MSP) through the design of primer sets that specifically amplify either methylated or unmethylated HPV-18 L1 DNA within bisulfite-modified sample DNA. Amplification of unmethylated and in vitro methylated HPV-18 DNA by MSP resulted in 2500 copies of either of the two L1 DNA species being detected, a satisfactory sensitivity considering that bisulfite treatment leads to the fragmentation of about 99% of sample DNA. The primers proved specific and did not generate false positive results at concentrations exceeding the lowest limit of detection by a factor of 400. DNA from carcinomas yielded PCR signals only with the methylation-specific primers, and not with primers specific for unmethylated L1 genes. The inverse result was obtained with DNA from precursor lesions that contained only hypomethylated DNA. High-grade precursor lesions and carcinomas that contained hyper- as well as hypomethylated L1 DNA yielded PCR signals with both primers. By developing a fluorescence based real-time PCR, we quantitatively analyzed samples with in vitro methylated and unmethylated L1 DNA, and could distinguish clinical samples with hyper- and hypomethylated DNA or mixtures of both DNAs. The methylation-specific and real-time PCR techniques permitted efficient HPV-18 L1 methylation analyses and open the door for larger-scale clinical studies where the utility of methylation status to predict the progression of HPV-18 infection and HPV-18 associated lesions is assessed.

Keywords: Papillomavirus; DNA methylation; Tumor progression; DNA diagnosis; Polymerase chain reaction

Introduction

Infection with human papillomaviruses (HPVs) is necessary but not sufficient to cause cervical cancer (International Agency for Research on Cancer, 1995). Many molecular principles of the transformation by HPVs are well researched, notably the multiple functions of the oncoproteins E6 and E7, which include abrogation of the normal functioning of tumor suppressor proteins p53 and pRB (Dyson et al., 1989; Werness et al., 1990). However, the mechanisms that determine whether an HPV infection remains latent, progresses to a dysplasia, or worse, carcinoma, remain largely undefined. They likely include immune surveillance as well as mutations of cellular genes that synergize with the HPV infection. Some of these events occur, however, unquestionably on the level of the virus, notably the recombination between the HPV genomic DNA and chromosomal sequences of the host cell. HPV genomes are very often integrated in the host DNA in cancer but are episomal in precursor lesions (Daniel et al., 1995; Schwarz et al., 1985).

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doi:10.1016/j.virol.2006.11.010

This generally accepted observation recently required a slight modification, as stepwise increments of chromosomally integrated HPV genomes rather than singular complete transitions were observed in large-scale studies of disease progression (Arias-Pulido et al., 2006). Integration appears to favor carcinogenesis, as it interferes with negative feedback repression of the transcription of the E6 and E7 oncogenes by the E2 protein (Tan et al., 1994; Demeret et al., 1997) and activates increased transcription of the oncogenes by a nuclear matrix dependent mechanism (Stünkel et al., 2000). Therefore, development of tests that detect HPV genome integration is desirable to improve molecular and etiological diagnosis in both basic research and clinical applications.

In our previous studies of HPV DNA methylation, we observed that the L1 gene of HPV-18 is hypermethylated in the carcinomas that were investigated, contrasting with its hypo- or unmethylated state in asymptomatic and a component of precursor lesions. The adjacent long control region (LCR) is hypomethylated in tumors, precursor lesions and in asymptomatic infections (Badal et al., 2004; Turan et al., 2006). Similar studies of HPV-16 suggested a somewhat more complex, two-pronged scenario. In this virus, we observed an intermediate level of methylation of L1 and the LCR in asymptomatic infection, hypomethylation in precursor lesions and hypermethylation that maximally affected L1, just as in HPV-18, in carcinomas (Badal et al., 2003; Kalantari et al., 2004). These findings suggest that L1 DNA methylation may be a powerful biomarker of the clinical progression of HPV-18 associated disease and possibly also HPV-16 associated lesions, although it is still a matter of inference whether L1 DNA is methylated as a consequence of recombination between viral and cellular DNA or for yet unknown reasons. We also cannot yet explain the methylation differences between HPV-16 and HPV-18 that we observed during asymptomatic infection. These two HPV types account for more than two thirds of the cervical cancers worldwide (Clifford et al., 2003; Munoz et al., 2003) and are the most frequently studied types among the 18 or so high-risk HPV types. Being the paradigms for high-risk HPV types, they are sometimes considered to be very similar, although a more thorough analysis reveals that they are only remotely related phylogenetically (de Villiers et al., 2004). While they are associated with cervical malignancies, they differ in a wide array of molecular, biological and pathological properties (for references, see Arias-Pulido et al., 2005).

The term “DNA-methylation” refers to the transfer of a methyl-group to cytosine residues, which are typically stably maintained in methylated form when they are part of the palindromic cytidine-guanidine (CpG) dinucleotides. DNA methylation is of great general interest, as it affects the conformation of nucleosomes in a complex network of epigenetic interactions with histone methylation, histone acetylation and histone deacetylation (reviewed in Bird, 2002; Fuks, 2005; Goll and Bestor, 2005), regulating gene expression on the level of chromatin.

The methylation of HPV-1 genomes and the cottontail rabbit PV was observed more than 20 years ago (Burnett and Sleeman, 1984; Sugawara et al., 1983; Wettstein and Stevens, 1983), but only recently was it shown that methylation of HPV-16 and 18 DNA takes place regularly in vivo in cervical cells, both in clinical samples and in cell cultures (Badal et al., 2003, 2004; Kim et al., 2003; Van Tine et al., 2004; Kalantari et al., 2004; Wiley et al., 2005; Turan et al., 2006; Bhattacharjee and Sengupta, 2006). This phenomenon raises questions of where and how HPV genomes are recognized by the cellular DNA methylation machinery, and about the role of this mechanism during the HPV life cycle and HPV dependent carcinogenesis. During carcinogenesis, HPV DNA may become methylated like any type of foreign DNA in response to the recombination with cellular DNA, pointing toward a correlation of these events, while not yet to the actual cause of methylation (Doerfler et al., 2001). Methylation during the productive HPV-16 life cycle is not yet understood, but may be linked to the binding of sequence-specific transcription factors like CDP, that synergize with histone modification mechanisms (O’Connor et al., 2000).

In this publication, we report on the development of improved diagnostic tests of HPV-18 L1 methylation that could be used within large-scale clinical studies designed to assess the nature and course of HPV-18 mediated cervical disease with respect to methylation. The technical foundation of this work was drawn from our past studies where bisulfite sequencing of HPV genomes was established. This involved the modification of sample DNA with bisulfite, followed by PCR and product cloning into *E. coli* vectors before sequencing. Application of this approach, referred to as “bisulfite sequencing”, leads to cytosines being detected as thymine residues while methyl cytosines are detected as cytosine residues. Our previous strategies involved the sequencing of multiple *E. coli* clones from each clinical sample, which was justified in order to evaluate the complex and diverse methylation patterns of HPV genomes both between and even within individual HPV infections. However, this approach would be too labor intensive and complex to apply to larger scale clinical studies designed to assess the impact of methylation using routinely collected patient samples. In order to overcome this, in the present study, we explored two techniques designed to detect HPV-18 L1 DNA methylation that could be amenable to high-throughput testing. One of these techniques is based on PCR primers specific for methylated and unmethylated HPV DNA (methylation-specific PCR, or “MSP”, Herman et al., 1996). It has the power to detect fairly small amounts of methylated DNA among an excess of unmethylated sequences, and vice versa. Its complexity is reduced compared with the bisulfite sequencing approach described earlier, as it does not require cloning and sequencing. The second technique is based on fluorescence based real-time PCR with TaqMan probes specific for methylated DNA post amplification with primers that do not distinguish between methylated and unmethylated DNA (Eads et al., 2000). This technique is quantitative and even less labor intensive than MSP, as it does not require agarose gel electrophoresis subsequent to the PCR. While neither of these techniques reveals the detailed methylation patterns, they are both useful and suitable tools for the analysis of HPV methylation analyses within the context of large clinical studies.