Prevention and Epidemiology

Diagnosing Cervical Cancer and High-Grade Precursors by HPV16 Transcription Patterns

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Abstract

Infections with high-risk human papillomaviruses (HPV), mainly HPV type 16, can cause malignant transformation of the human cervical epithelium and the development of cervical cancer (CxCa). A rapid and precise diagnosis of the precancerous lesions by conventional cytology or HPV DNA tests remains difficult and often leads to overtreatment. We quantitatively analyzed the HPV16 transcriptome of 80 HPV16 DNA-positive cervical scrapes classified as mild cytologic grade, including no intraepithelial lesion or malignancy (NIL/M; normal, n = 25) and low-grade squamous intraepithelial lesion (LSIL; n = 24), and severe cytologic grade, including high-grade squamous intraepithelial lesion (HSIL; n = 24) and CxCa (n = 7), with novel nucleic acid sequence-based amplification–Luminex assays. In severe lesions, HPV16 E6*II and E1C encoding transcripts were strongly upregulated, whereas spliced E1*E4 and L1 encoding transcripts were markedly downregulated. Using a combination of the four marker transcripts, 100% of CxCa and 67% of HSIL cases were correctly identified as severe, and 74% of LSIL and 92% of NIL/M samples as mild cytologic grade. Compared with a commercially available HPV E6/E7 mRNA assay, the specificity of the marker combination for discriminating severe and mild cytologic lesions increased from 23% to 83%. In conclusion, we identified a novel HPV16 RNA pattern for grading of cervical lesions with a potentially high diagnostic value for the primary screening of CxCa precursors and the triage of cervical lesions. Cancer Res; 70(1); 249–56. ©2010 AACR.

Introduction

Persistent infections with 1 of the 15 so-called high-risk mucosal human papillomavirus types (hrHPV) can cause cervical malignancies (1). Being found in 55% of all cases, HPV16 plays a central role in cervical cancer (CxCa) development (2). During carcinogenesis, integration of viral DNA often occurs, causing a disruption of the E2 open reading frame, and thus leading to an upregulation of the viral oncogenes E6 and E7 (3, 4).

CxCa is the second most common cancer among women worldwide, annually with ~470,000 newly diagnosed cases and almost 250,000 deaths (5). In the United States, around 12,000 women are diagnosed and around 4,000 die from CxCa every year (6). CxCa develops from persistent hrHPV infections through a series of stages histologically classified as cervical intraepithelial neoplasia 1 to 3 (CIN1–CIN3), or cytologically defined as low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL). The majority of low-grade and a minority of high-grade lesions regress spontaneously (7); hence, most patients with CIN2/3 lesions require therapy.

In countries with CxCa precursor screening programs (CCPSP), the incidence of CxCa has been reduced since introduction of the Pap test in the 1970s. Despite this success, the CCPSP is far from being perfect. The Pap test depends on subjective judgment of the degree of mitotic activity and nuclear atypia. A single Pap test fails to detect cervical abnormalities in ~50% of women compared with colposcopy (8) and the inter- and intraobserver variability is significant (9, 10). The moderate specificity of cytology results in a substantial number of patients that are unnecessarily treated for a nonexisting disease.

HPV testing is accepted as an adjunct modality to cytologic testing for women over 30 years of age in the United States (11). However, the high sensitivity of HPV tests also leads to the identification of transient HPV infections or clinically irrelevant, regressing lesions. As a consequence, the positive predictive value for the development of CxCa even after repeated hrHPV DNA-positive results remains low.

Unlike HPV DNA testing, RNA detection allows the identification of transcriptionally active viruses. Commercially available RNA detection assays, e.g., the PreTect HPV...
Proofer (NorChip) or its equivalent, the NucliSens Easy-Q HPV (bioMérieux), that qualitatively detects early full-length (fl) transcripts targeting E6 and E7 sequences (E6/E7) from individual hrHPV types 16, 18, 31, 33, and 45 (12) or the Aptima HPV-Test from GenProbe, a broad-spectrum isothermal E6/E7 mRNA amplification method detecting a cocktail of 14 hrHPV types (13), have recently been developed. Limited data using the HPV Proofer test indicated that a substantial number of HPV DNA-positive women with normal cytology will test positive for HPV E6/E7 mRNA resulting in "false-positive" results (12, 14, 15). Thus, like DNA-tests, E6/E7 transcript-based RNA tests will lead to overtreatment, additional costs and considerable anxiety for women concerned.

To solve the above-mentioned limitations of the CCPSP, quantitative HPV16 transcriptome analyses were undertaken to identify novel RNA patterns for the intelligible grading of LSIL and HSIL and ultimately to improve the positive predictive value of the HPV DNA testing. To this end, RNA originating from 25 no intraepithelial lesion or malignancy (NIL/M), 24 LSIL, 24 HSIL, and 7 CxCa cytologic samples were quantitatively analyzed for 15 spliced and fl HPV16 transcripts and cellular p16INK4A, as well as two housekeeping transcripts.

Materials and Methods

Ethics statement. The study has been approved by the French committee "Comité de Protection des Personnes" (CPP-Est III, Nancy), statement no. DC-2008-374. All women were informed and gave their written consent to participate in the study.

Patients. Samples were selected from patients participating in the Reims HPV Primary Screening Cohort Study, which has been already described (16, 17). Briefly, this population was offered a systematic combined CxCa screening with cytology and hrHPV testing, when they had their routine cervical smear performed either in the gynecology departments of
several French public hospitals (University Hospital of Reims, General Hospital of Sedan, General Hospital of Soissons) or in several private French gynecology offices (Reims, Soissons, Épernay). In case of cytologic abnormalities or hrHPV positivity, a well-defined follow-up algorithm was applied. Since 2006, residual material has also been prospectively stored for DNA and RNA isolation. The mean age of this studied subgroup was 31 years (range, 18–75).

**Samples.** We selected 80 HPV16 DNA-positive cervical scrapes, harboring (i) no cytologic abnormality (25 NIL/M) and (ii) lesions ranging from LSIL to CxCa (24 LSIL, 24 HSIL, 7 CxCa). Cytologic classification was done according to the Bethesda system. All CxCa cases were histologically confirmed. As histologic data were available for most HSIL, for only few LSIL, and for quite none of NIL/M cases (which is in accordance with French guidelines for the management of cytologic abnormalities), cytologic classification alone was retained for all of these samples. Three to 12 mL of exfoliated cervical cells stored in PreservCyt medium (Cytyc Corp.) were used for RNA isolation (see Supplementary Appendix). The 5′ end of the P1 primer contained a T7 RNA polymerase promoter sequence consis-
tent to the target RNA sequence (see Supplementary Table S1 and Supplementary Appendix). RNA and DNA analyses were performed on the liquid-based cervical samples collected in PreservCyt Medium (ThinPrep, Cytyc Corp.). HPV16 DNA positivity was determined by the Linear Array HPV Genotyping Test (Roche) and confirmed by the BSGP5+/6−-PCR-MPG assay (18, 19). The HPV E6/E7 mRNA status was assessed using the NucliSENS EasyQ HPV assay according to the manufacturer’s instructions (BioMérieux).

**HPV16 RNA profiling.** The novel singleplex assays quantified 10 spliced HPV16 RNA sequences [226^409 (E6*I), 226^526 (E6*I1), 226^3358 (E6*I11), 226^2709 (E6*IV), 880^2582, 880^2709, 880^3358 (E1^E4), 1302^3358, 1302^5639, 3632^5639; reviewed in ref. 20], 5 fl sequences (E6 fl, E7 fl, E1 fl, E5 fl, L1 fl), cellular p16^INK4A, and two housekeeping transcripts [Ubiquitin C (UbC) and U1A]. All fl targets were located outside of splice junctions in the respective open reading frames and thus were detected in spliced and unspliced transcripts.

Purified mRNA was quantified using the principle of competitive nucleic acid sequence–based amplification (NASBA; refs. 21–23), which is based on the simultaneous coamplification of calibrator (Q) RNA [primer binding sites and amplifier size identical to wild-type (wt) RNA, but unique probe binding site], combined with subsequent hybridization to oligonucleotide probes coupled to Luminex beads (18).

**NASBA primer and probe design.** For each target, one primer pair was designed following the guidelines of the NucliSens EasyQ Basic kit (BioMérieux). The P2 primer consisted of a 5′ generic sequence (5′-ATA TAC TAC GGA TGG CCT G-3′) required for the hybridization with the decorator probe and a 3′ stretch of nucleotides that was identical to the target RNA sequence (see Supplementary Table S1 and Supplementary Appendix). The 5′ end of the P1 primer contained a T7 RNA polymerase promoter sequence consisting of 25 nucleotides (5′-ATT TCT AAT ACG ACT CAC TAT AGG G-3′). The location of amplimers within the HPV16 genome is shown in Fig. 1. For detection of U1A housekeeping transcripts, validated sensitive primer sequences were used as published (24).

Probes detecting spliced transcripts spanned splice junctions (see Supplementary Table S2 and Supplementary Appendix). For probes detecting fl transcripts, genomic sequences of HPV16 isolates and other genotypes were obtained from Genbank, and alignments performed by using ClustalW software (25) enabled the identification of HPV16 specific regions. For specificity evaluation, in vitro transcripts containing spliced and fl RNA sequences were used (Fig. 1). In vitro transcripts were generated from Bluescript M13-KS vectors (Stratagene) containing spliced and fl sequences (see Supplementary Appendix). All primers and probes were purchased from MWG-Biotech AG.

**Quantitative NASBA.** Isothermal nucleic acid amplification of target RNA is accomplished by the simultaneous enzymatic activity of avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase, and RNase H. NASBA amplification was performed by the NucliSens EasyQ Basic kit V.2 (BioMérieux) using RNA samples that had been stored at –80°C. Quantification and internal performance control required the addition of in vitro–transcribed calibrator RNA.
(Q-RNA) of known concentration to the NASBA mix. Wt and Q-RNA were converted into cDNA and coamplified with the same primers, allowing competitive amplification of both RNAs. As RNA amplification by NASBA follows linear kinetics for a wide range of RNA input, the ratio of the two amplimers at the end of amplification, as determined by wt and Q probes, reflected the ratio of the two targets present at the beginning of amplification (26).

The Q-RNA amount that was included in each NASBA reaction was titrated for each NASBA target. In general, optimal Q-RNA quantities were 100-fold to 1,000-fold above the detection limit. In addition, Q-RNA served as internal performance control indicating the presence of potential NASBA inhibitors. The quantitative NASBA-Luminex assay was able to quantify 10-fold differences in wt-RNA quantities over five orders of magnitude (Fig. 2).

In contrast to the manufacturer’s instructions, singleplex NASBA amplification was carried out in a 10.0-μL reaction volume consisting of 2.5 μL RNA template solution, 5.0 μL primer reagent mix, and 2.5 μL enzyme solution. A final concentration of 80 mmol/L KCl and 0.2 μmol/L of primers were used. Amplification took place in eight-tube strips from Nerbe plus and an Eppendorf Mastercycler with heated lid (60°C). Each experiment included a series of diluted standard in vitro RNA and water controls.

**Luminex hybridization of NASBA amplimers.** After NASBA amplification, amplimers were analyzed as described (18, 19) with some modifications. All solutions and buffers were certified DNase/RNase free. Of the NASBA reactions, 1.0 to 0.1 μL was transferred to PCR plates. Using a multichannel pipette, 49 μL hybridization solution were added composed of 33 μL 4.5 mol/L TMAC, 75 mmol/L Tris-HCl (pH 8.0), 6 mmol/L EDTA (pH 8.0), 1.5 g/L Sarkosyl, 16 μL TE buffer, a mixture of 2,000 probe-coupled beads per set, and 0.2 μmol/L 5′-biotinylated decorator probe. The whole mixture was denatured at 95°C for 5 min and immediately placed on ice for 1 min. The hybridization plate was transferred to a heated block shaker, and the hybridization was performed at 41°C for 30 min. The content of each well was transferred to a wash plate by using a multichannel pipette. Subsequently, the wells were washed with 100 μL washing buffer (1× PBS, 0.02% Tween) on a vacuum manifold. Beads were resuspended for 20 min on a shaker at room temperature in 50 μL of detection solution [2 mol/L TMAC, 75 mmol/L Tris-HCl (pH 8.0), 6 mmol/L EDTA (pH 8.0), 1.5 g/L Sarkosyl] containing 1/1,000 diluted streptavidin-R-phycoerythrin (Strep-PE; Molecular Probes). Beads were washed twice with 100 μL washing buffer and resuspended in 100 μL washing buffer for 2 min on a shaker. Analysis was performed on a Luminex 100 analyzer.

**Cutoff definition and statistics.** For each probe, median fluorescence intensity (MFI) values in reaction with no amplimer added to the hybridization mixture were considered background values. Net MFI values of hybridized amplimers were computed by subtracting 1.2 times the median background value from the raw MFI value. Net MFI values above three MFIs were defined as positive reactions. For each probe, the cutoff value was above the mean background plus three times the SD. For quantification of RNA amplimers, only values above cutoff were normalized by the calibrator MFI signal. Severe and mild cytologic grades comprise CxCa+HSIL and LSIL+NIL/M, respectively. Samples negative for the four viral transcripts but positive for the two housekeeping transcripts (n = 13) were automatically scored as mild cytologic grades (Fig. 5).

Statistical analyses were performed with the SAS software, version 9.1.3 (SAS Institute). Comparisons across all cytologic grades were performed by one-way ANOVA. Pairwise comparisons were performed by the Wilcoxon signed rank sum test to compare median values in different groups. All tests were performed two-sided, and P values below 0.05 were considered statistically significant.

**Results**

**Analytic sensitivity and specificity of RNA detection.** The sensitivity of the novel NASBA-Luminex assays was determined using serially diluted, in vitro–generated transcripts (Fig. 1) with optimized calibrator Q-RNA input. The detection limits ranged from 25 to 2,500 copies for all transcripts, with the exception of p16INK4A reaching a detection limit of 25,000 copies per reaction. In general, optimal Q-RNA quantities were 100-fold to 1,000-fold above the detection limit. Using total RNA purified from HPV16-positive SiHa cells, as little as 0.3 SiHa cell equivalents were detected using E6*I and E7-specific NASBA primers, whereas the detection limit with E6*II, Ubc, or U1A NASBA primers was three or more cells. The quantitative NASBA-Luminex assays were able to quantify 10-fold differences in wt RNA quantities over four to five orders of magnitude (Fig. 2).

Specificity of the singleplex NASBA-Luminex assays were a sum of HPV16 type-specific primers and splice site– and type-specific hybridization probes. No cross-reactivity was observed in clinical specimens containing E6/E7 mRNA of the highly HPV16-related HPV types 31 and 33. Complete splice
site specificity of probes was confirmed by hybridizing fl E6 amplimers with the respective E6 fl and E6*I probes (Fig. 3).

Cross-sectional study. We analyzed the HPV16 transcriptome and the cellular p16INK4A transcript in HPV16 DNA-positive smears with normal (NIL/M, n = 25), LSIL (n = 24), HSIL (n = 24), and CxCa (n = 7) cytologic diagnoses. When assessing the RNA integrity, U1A and UbC housekeeping transcripts showed concordant results in 79 of 80 cases. One LSIL sample had to be excluded due to negative values with all transcripts analyzed. Three LSIL and two NIL/M samples were positive for both housekeeping transcripts but negative for any HPV16 transcript and were excluded in the following two paragraphs. In accordance with published data (27), the 1302^5639 spliced transcript was not detected.

Overall prevalence. Among the 74 specimens, highest prevalence (at least 87% in each cytologic group) was observed for fl RNA sequences E1, E7, E6, L1, and E5, irrespective of the cytologic grade (Table 1). E5 expression was absent in one CxCa case, suggesting the presence of an integrated HPV16 genome.

Quantitative expression levels of single transcripts. E6*I, E6*II, E6 fl, and E7 fl oncogene encoding transcripts showed a significant upregulation in their expression in CxCa compared with NIL/M (P < 0.01; exemplarily shown for E6*I, Fig. 4A). E7 fl and E6*II expression levels were also significantly increased in HSIL versus NIL/M (P < 0.05), whereas only E6*II transcripts were significantly augmented in CxCa versus LSIL (P < 0.01; Fig. 4A). In addition, 880^2582 containing transcripts were significantly upregulated in HSIL and CxCa compared with NIL/M (P < 0.01), respectively (Fig. 4B). The cellular p16INK4A transcripts showed a significant upregulation in HSIL and CxCa versus NIL/M (P < 0.05), respectively. Conversely, the expression of transcripts, encoding proteins required for virus capsid formation and release, including 880^3358, 3632^5639, and L1 fl, was downregulated in CxCa compared with LSIL (exemplarily shown for L1 fl and 880^3358 transcripts; Fig. 4C, D). This effect was particularly strong for the 880^3358 transcript (P < 0.01).

HPV16 transcript patterns. We assumed that the ratio of viral transcripts is similar in the majority of HPV-positive cells in a given sample. To normalize for variable amounts of HPV-positive cells in cervical swabs, the ratio of the expression of two viral transcripts was compared in severe (CxCa+HSIL) versus mild (LSIL+NIL/M) cytologic grades in the 79 cervical swabs with intact RNA (Fig. 5).

In line with observations described above, 880^2582 transcripts normalized with the 3632^5639 or 880^3358 transcripts allowed a highly significant discrimination of severe and mild grades (P < 0.01). Moreover, the putative integration status was assessable by the normalized expression of E6*II versus 880^3358 or E5 fl transcripts that allowed a significant distinction of severe and mild grades (P < 0.05; Fig. 5). Combining the results of the 880^2582/3632^5639 ratio (Fig. 5A) with the results of the E6*II/880^3358 ratio (Fig. 5B), 7 of 7 CxCa (100%) and 16 of 24 HSIL cases (67%) were correctly identified as HSIL+CxCa (sensitivity, 74%), and 17 of 23 LSIL (74%) and 23 of 25 (92%) NIL/M samples were correctly identified as LSIL+NIL/M (specificity, 83%).

Table 1. Prevalence (%) of transcripts in different cytologic groups

<table>
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<tr>
<th>Transcripts</th>
<th>Coding potential</th>
<th>CxCa (n = 7)</th>
<th>HSIL (n = 24)</th>
<th>LSIL (n = 20)</th>
<th>NIL/M (n = 23)</th>
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<tr>
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Comparison to NucliSENS EasyQ HPV. Next, we compared the newly identified HPV16 RNA pattern to the commercially available NucliSENS EasyQ HPV kit (BioMérieux). Of the 79 specimens with intact RNA, one CxCa, two HSIL, and one LSIL had to be excluded due to technically invalid results with the commercial assay. The RNA pattern was less sensitive (71%) compared with the commercial HPV test (96%), classifying eight HSIL patients as LSIL or NIL/M. At the same time, the specificity of the novel RNA pattern for discriminating severe from mild cytologic grades increased from 23% to 83% compared with the commercial test. The NucliSENS EasyQ HPV test gave false-positive results in 17 of 22 LSIL (77%) and 19 of 25 NIL/M (76%) samples.

Discussion

A major limitation of current HPV-based CCPSP is its poor clinical specificity, leading to many "false-positive" women being referred to costly follow-up and/or treatment. Using commercial E6/E7-based RNA tests, the specificity was only slightly improved compared with DNA tests (12, 14, 15), because a substantial fraction of HPV DNA-positive women with normal cytology do also express E6/E7 mRNA. Here, we report that the specificity of HPV-based tests can be substantially improved by quantitatively analyzing a combination of different spliced transcripts from HPV16.

The major finding of this study was the substantial upregulation of 880^2582 transcript expression in HSIL and CxCa patients that was also seen in the HPV16-positive CxCa cell lines SiHa, CasKi, MRIH-186, and MRI-H196. This transcript potentially encodes the COOH terminus of E1 (E1C), but also E2 (28). It remains unclear whether the upregulation of this transcript in CxCa patients is due to the fact that (a) E1C functions as transactivator of the virus long control region (LCR; ref. 29) or (b) the E1C and E2 open reading frames overlap, thereby suppressing E2 translation after translation termination of E1C. For cells containing episomal HPV16 genomes, high E2 expression is known to repress the LCR (29), thereby reducing the expression of E6 and E7 that is required for malignant transformation of cervical cells. Upon viral integration, found in 60% of CxCa cases (30), E2 expression and thus the regulation of the E6/E7 expression is

3 M. Schmitt and M. Pawlita, unpublished data.
frequently lost. In HSIL and cancerous cells with epimonal genomes, however, E2 should be still expressed. As indicated above, the E1C encoding transcript may counteract the E2 protein, thereby promoting the upregulation of E6 and E7. Consistently, two CxCa and one HSIL samples analyzed in our study that presumably contained only integrated virus genomes, as indicated by the absence of \(880\times3358\) transcripts, did not express the \(880\times2582\) transcript. These findings may hint at an integration-independent mechanism for HPV16-mediated transformation of cervical cells by the overexpression of E1C encoding transcripts, a novel promising marker in the CCPSP.

Furthermore, we describe a novel HPV16 RNA pattern comprising two combinations of each two viral transcripts to specifically predict the presence of mild (including LSIL and NIL/M) or severe cytologic grades (including CxCa and HSIL). The use of viral transcripts allowed normalizing for variable quantities of HPV-infected cells in cervical smears, assuming that the transcript ratio is similar in the majority of HPV-positive cells in a given sample.

The first combination of the RNA pattern comprised the above-mentioned \(880\times2582\) transcript normalized to the L1 protein encoding \(3632\times5639\) transcript. The latter was also detected in four cancer cases and in the HPV16-positive cancer cell lines MRIH-186 and MRI-H196.3 Surprisingly, the L1 fl transcript was even more frequently found in HSIL and CxCa samples (Table 1) and in various HPV16-positive cell lines including CasKi, MRIH-186, and MRI-H196, although L1 protein expression has not been described for CxCa specimens (31). These data may indicate that L1 expression is regulated in differentiating keratinocytes at the translational rather than transcriptional level. Irrespective of the unexpectedly high prevalence of spliced L1 encoding transcripts, their median expression was reduced in CxCa compared with LSIL, enabling its use for the normalization of \(880\times2582\) transcripts.

In the case of samples negative for \(880\times2582\) and \(3632\times5639\) transcripts or samples with the respective ratios below the threshold, a second combination of the viral transcripts was analyzed. Samples were classified as severe lesions if at least one ratio from both combinations was above threshold. The second combination comprised the \(226\times526\) transcript (E6*II), which seemed to be stronger upregulated in CxCa than the \(226\times409\) (E6*I), E6 fl and E7 fl transcripts (data not shown). By normalizing its expression level to the \(880\times3358\) transcript (E1*E4), the putative integration status of HPV16 could be assessed. The \(880\times3358\) transcript was strongly downregulated especially in CxCa cases, whereas at the same time high levels of \(226\times526\) transcript expression were measured. Two CxCa samples (28%) were negative for \(880\times3358\) transcripts indicating the presence of only integrated forms of the virus genome. Four CxCa cases showed a reduced \(226\times526/880\times3358\) ratio suggesting the presence of integrated and episomal, transcriptionally active viruses.

The HPV16-specific RNA pattern reached a thus far unique specificity for discriminating severe and mild cytologic grades compared with DNA tests or the NucliSENS EasyQ HPV RNA assay. As a primary screening modality, the latter two would lead to a high proportion of clinically false-positive results in patients with normal cytology, causing wrong management and potentially overtreatment of healthy individuals. Using the novel RNA pattern, the positive predictive value increased from 43% to 71%, and only 26% of LSIL and 8% NIL/M samples showed RNA patterns similar to severe lesions. While one NIL/M sample showed a clear indication for integrated HPV16, as determined by very high \(226\times526\) expression and absence of \(880\times3358\) transcripts, the seven remaining samples were positive for the E1C encoding transcript. This finding may indicate either an upcoming progression into a high-grade lesion or a cytologic misclassification. Large follow-up studies need to be done to verify the hypothesis of a high positive predictive value of RNA pattern analysis.

Using the RNA pattern, one third of the HSIL samples was classified as LSIL+NIL/M. This may be attributed to several factors including cytologic misclassification, future regression of currently present high-grade lesions, and additional infections with high-risk HPV types other than HPV16 being...
the driving force in the carcinogenesis [three of these samples were also DNA-positive for HPV18 (n = 2) and HPV66 (n = 1)].

In conclusion, a combination of four HPV16-based spliced transcript markers has been identified, presenting a novel and improved screening tool for CCPSP.

Disclosure of Potential Conflicts of Interest

M. Schmitt, L. Gissmann, and M. Pawliita are listed on a patent application to the European Patent Office (Europe patent application EP08168608.1–1222). L. Gissmann received consulting fees from Quagen and Abbott. V. Dalstein and C. Clavel received consulting fees from Genprobe. T. Waterboer disclosed no potential conflicts of interest.

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256

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