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Performance of a DNA methylation marker panel using liquid-based cervical scrapes to detect cervical cancer and its precancerous stages

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Aim: An essential event in early carcinogenesis is the hypermethylation of so-called CpG islands, which are predominantly located in promoter/5' regions of genes in the human genome. Specific patterns of hypermethylation may thus be indicative for carcinogenesis and provide tools for diagnostics. In the current study the performance of a panel of six DNA methylation marker regions for the detection of cervical precancerous lesions and cancer was assessed using cervical scrapes from corresponding patients.

Methods: A series of cervical scrapes from women with cervical cancer (n=5), cervical intraepithelial neoplasia grade 3 (CIN3) (n=26) or CIN1/2 (n=14), and women with normal cytology (n=60) were assessed for methylation of the marker regions ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671. Methylation analysis was done using the GynTect® assay.

Results: All samples from women with cervical cancer (5/5) were scored positive for the methylation assay. Of the CIN3 cases, 62% (16/26), of the CIN1/2 cases 50% (7/14) were positive for the assay. Only 1.7% of the cytology-normal samples (1/60) were positive for the methylation assay. Overall, the number of methylated marker regions increased proportionally to the lesion severity.

Conclusion: DNA methylation analysis of ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671 in cervical scrapes consistently detects cervical cancer and the majority of CIN3 as well as a subset of CIN1/2 lesions, whereas the detection rate among cytology-normal samples is extraordinarily low. Thus, the GynTect® assay based on detection of these six methylation markers may provide an excellent tool within cervical cancer screening.

Keywords: DNA methylation; human Papillomavirus (HPV); biomarkers; cervical cancer

Využiteľnosť analýzy zmeny metylácie DNA pre detekciu rakoviny krčka maternice a jej prekanceróznych štádií

Cieľ: Zásadnou udalosťou včasnej karcinogenézy je hypermetylácia takzvaných CpG ostrovčekov, ktoré sa prevažne nachádzajú v promótorových/5'-oblastiach génov v ľudskom genóme. Konkrétne vzory hypermetylácie tak môžu indikovať karcinogenézu a slúžiť ako nástroj pre diagnostiku. V aktuálnej štúdii posudzujeme využiteľnosť panelu šiestich vybraných markerov metylácie DNA za účelom detekcie prekanceróznych lézií krčka maternice a jej karcinómu pomocou analýzy DNA získanej z buniek sterov krčka maternice príslušných pacientiek.

Metódy: Sada cervikálnych sterov od žien s rakovinou krčka maternice (n=5), s cervikálnou intraepiteliálnou neopláziou stupňa 3 (CIN3) (n=26) alebo CIN1/2 (n=14), a od žien s normálnym cytologickým nálezom (n=60) bola hodnotená po analýze stavu metylácie oblastí ASTN1, DLX1, ITGA4, RXFP3, SOX17 a ZNF671. Analýza metylačného stavu bola vykonaná pomocou testu GynTect®.

Výsledky: Všetky vzorky od žien s rakovinou krčka maternice (5/5) boli pozitívne v teste metylačného stavu. Z CIN3 prípadov bolo v teste pozitívnych 62% (16/26) a z CIN1/2 prípadov to bolo 50% (7/14). Iba 1,7% zo vzoriek s normálnym cytológickým nálezom (1/60) bolo pozitívnych v teste metylačného stavu. Celkovo sa počet regiónov metylovaných markerov zvyšoval úmerne so závažnosťou lézií.

Záver: Analýza metylácie DNA buniek zo sterov krčka maternice ASTN1, DLX1, ITGA4, RXFP3, SOX17 a ZNF671 spoľahlivo deteguje vzorky s karcinómom krčka maternice a väčšinu CIN3, ako aj podmnožinu CIN1/2 lézií, zatiaľ čo miera detekcie medzi vzorkami s normálnym cytologickým nálezom je mimoriadne nízka. Test GynTect® na základe detekcie vybraných šiestich metylačných markerov môže byť použitý ako vynikajúci nástroj pre skríning rakoviny krčka maternice.

Kľúčové slová: metylácia DNA; ľudský *Papillomavirus* (HPV); biomarkery; rakovina krčka maternice

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Introduction

Cervical cancer is still among the most frequent cancers in women world-wide (Torre et al., 2015). With the availability of screening programs, however, cervical cancer incidence and mortality have markedly decreased, especially in developed countries (Anttila et al., 2009). The effects of the cytology-based diagnostics – the so-called Pap test, the most prominent screening tool applied even nowadays – have, however, levelled-off the last decade, mainly because of the limited sensitivity for precancerous lesions, as well as limited participation of the women. On the other hand, limited specificity of the Pap test also leads to over-diagnosis and over-treatment, mainly among young women. Therefore alternative screening tools, which may lead to an overcome of these limitations of cytology are discussed since several years.

Testing for the human papillomaviruses (hrHPV) that evoke cervical cancer improves the sensitivity of screening (Ronco et al., 2014). In some countries (e.g. the Netherlands, USA) HPV testing has already been implemented in screening. Infection with one of the high-risk HPV strains is the prerequisite for the development of cervical cancer. Therefore, HPV screening has high sensitivity. It lacks, however, specificity, since most women infected with HPV will clear such an infection without symptoms. Therefore, HPV-based cervical cancer screening only makes sense with the availability of triage methods that allow the detection of precancerous lesions and cancer cases among women tested HPV-positive (Wentzensen et al., 2015).

In this context, hypermethylation of certain DNA regions during the course of carcinogenesis may provide a promising tool for triage of a highly sensitive screening, which finds virtually all disease cases, but lacks specificity, as is the case if testing for HPV infection (Lorincz et al., 2013; Wentzensen et al., 2015). We have previously shown that detection of a DNA hypermethylation marker panel consisting of the five marker regions DLX1, ITGA4, RXFP3, SOX17, and ZNF671 may be a useful tool for triaging HPV-positive women (Hansel et al., 2014). Here we show that a molecular diagnostic test based on the marker regions ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671, termed GynTect, which received CE IVD mark in October 2015, can easily be adapted for liquid-based cytology samples triage.

Materials and Methods

Informed consent

All samples utilized in this study were collected only after informed consent of patient was gained.

Patient samples

Residual liquid-based cytology (LBC) samples from both, routine cervical cancer screening as well as more accurate diagnostics for further triage following an abnormal cytology result (Pap III or higher) were used for the study. The collection consisted of 60 screening samples from women with normal cytology (PapI), and 45 screening and triage samples from women with histopathology diagnosis CIN1/2 (14 samples), CIN3 (26 samples), and cervical cancer (5 samples). All samples were collected in PreservCyt medium (Hologic). For all samples cytology results were available. For the samples with abnormal Pap smear finding histopathology results were available, classifying them into the different CIN stages.

Sample preparation

For sample preparation and lysis the LBC samples were vortexed for a few seconds, and 1 ml of each sample were immediately transferred into 1.5-ml microcentrifuge vials. Cellular material was pelleted by centrifugation at 10,000 x g for 5 min, and the supernatant was removed carefully by pipetting. Pellets were then resuspended in 40 µl of sample lysis buffer (GynTect, oncgnotics), and incubated at 60°C for 30 min at 1,000 rpm in a thermoshaker (Thermomixer, Eppendorf).

Bisulfite treatment and marker methylation analysis

Bisulfite treatment of cervical samples was performed using the EpiTect Fast Bisulfite Kit (Qiagen) following the supplier's manual. 40 µl of the cervical sample was directly used for bisulfite treatment without prior DNA isolation. After elution in 20 µl Elution Buffer, 70 µl of water was added, and 10 µl of the diluted DNA were used for each single reaction in the GynTect real-time methylation-specific PCR (qMSP) assay as described in the manual of the GynTect kit. The qMSPs were run on a ABI 7500 Real-Time PCR System (Life technologies, Thermo Scientific). Ct values for each marker and each internal control were recorded, and their validity was controlled by comparing the melting curve characteristics of each PCR fragment produced with corresponding positive controls. For such positive controls, which were included in each PCR run, DNA known to be methylated in the marker regions was used. A no template control using water as template was also included in each qMSP run. For evaluation, the difference of the Ct values of each marker with the internal control ACHE was calculated. qMSPs for ASTN1, DLX1, ITGA4, RXFP3 ans SOX17 yielding a difference between sample and internal control ≤ 9.0 were scored positive, for ZNF671 ≤10 was scored positive. A GynTect assay was scored positive, if the sum of the factors attributed to each marker was 0.5 or higher ***(Table 1)***.

Results

For assessing methylation of the GynTect markers ASTN1, DLX1, ITGA4, RXFP3, SOX17, ZNF671 as well as the two internal marker regions ACHE and IDS an intercalating dye-based qMSP assay was performed for each of the 105 patient samples included in this study. The results obtained for the 60 samples from patients with a cytology result Pap I were the basis for setting the delta Ct value limit ≤ 9 for all markers but ZNF671, for which the delta Ct value limit was set to ≤ 10.

Table 1. *Factors and DeltaCt values for the GynTect markers*

Marker	Delta Ct ACHE	Factor
ASTN1	≤9	0.2
DLX1	≤9	0.1
ITGA4	≤9	0.2
RXFP3	≤9	0.2
SOX17	≤9	0.2
ZNF671	≤10	0.5

To be scored valid, the Ct value for the control marker ACHE had to be below 32. At these settings, 59 of the 60 Pap I samples were scored negative for the GynTect test. This evaluation was then the basis for scoring the data obtained for the 45 CIN1+ samples.

All five carcinomas included in the study were scored GynTect-positive. Of the 26 samples with histopathology-confirmed CIN3, 16 (= 61.5%) turned out to be GynTect-positive, whereas of the 14 CIN1/2 samples seven (= 50%) were GynTect-positive ***(Figure 1A)***. When related to cytology findings, the following results were obtained for the CIN samples: of the 17 samples scored Pap III or Pap IIID in cytology, seven (= 41%) turned out to be GynTect-positive; of the 23 Pap IVa samples, 16 (= 69.6%) were GynTect-positive ***(Figure 1B)***.

Discussion

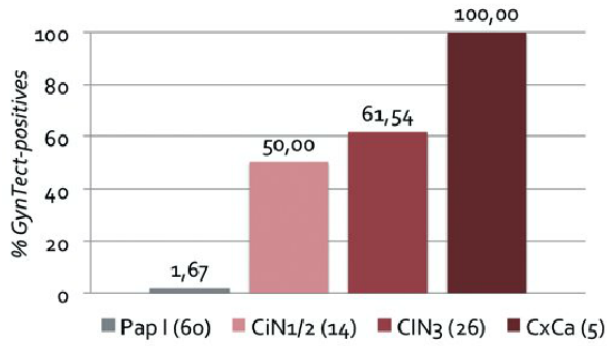
In previous studies we have shown that hypermethylation of CpG islands in proximity to the genes *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671* correlated with the presence of precancerous cervical lesions and cervical cancer (Hansel et al., 2014). The molecular diagnostic test GynTect based on these results allows the detection of these marker regions in cervical smears collected in the denaturing specimen transport medium (STM), which is originally used for QIAGEN's DIGENE HPV test. Utilization of this medium has, however, its limitations, the most important being that from STM only molecular test formats can be performed. In contrast, cervical smear material collected in liquid-based cytology media can be used more flexibly. As a main advantage, the cellular material preserved in this medium can be used for cytology as well as molecular biology tests. This enables the performance of triage tests from the same sample as the initial screening test, a feature which increasingly is demanded as prerequisite for diagnostics.

In this study we evaluated whether our molecular diagnostic test GynTect is suitable for using residual material from liquid-based cytology samples and such fulfils this prerequisite. GynTect provides the possibility to test if a woman who obtained an abnormal cytology finding in the Pap smear and/or a positive HPV test result, has a precancerous lesion that requires follow-up and treatment. For this purpose we used samples for which the cytology findings and, for all Pap-abnormal samples, the histopathology results were available for comparison. GynTect showed an excellent performance, since for all 105 samples valid test results could be obtained. The results obtained for the two internal markers that are tested with each patient sample demonstrate this. In fact, the Ct values for these two internal markers obtained for all 105 LBC samples are much lower than those obtained for samples collected in STM, indicating better preservation of the DNA in these LBC samples. Due to the improved performance, a threshold for the marker Ct values in relation to the controls was set. Using a delta Ct threshold of 9 for the five markers ASTN1, DLX1, ITGA4, RXFP3, and SOX17 as well as a delta Ct threshold of 10 for ZNF671, of the 60 samples with a normal cytology, Pap I, only one sample yielded a GynTect-positive result, implying that the test has a very good specificity within this group. A larger number of such samples will definitely have to be examined to confirm these results and this very high specificity of the test among healthy women.

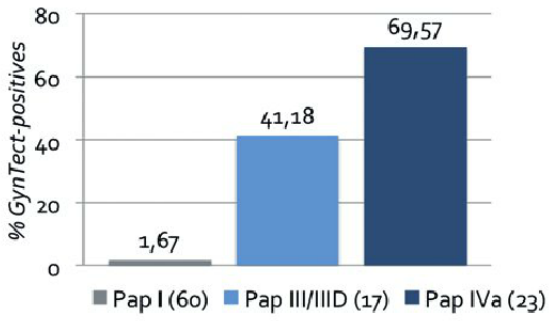
As expected, all 5 cervical cancer samples included in the study were detected by GynTect, and that by at least four GynTect markers. This very high sensitivity for cancer cases was already shown previously (Hansel et al., 2014). A detection rate of >60% among the CIN3 samples examined also confirms results obtained in previous studies (Hansel et al., 2014). It is well-known that not all CIN3 lesions proceed to cervical cancer (McCredie et al., 2008), although these high-grade lesions are considered as precancerous stages. In several observational studies CIN2/3 short-term regression rates around 30% were reported (Trimble et al., 2005, 2010, 2015; Grimm et al., 2012). Very recently, Loopik et al. (2016) have demonstrated in a retrospective study that in women < 25 years the regression rate of CIN2 lesions was as high as 71% (150 of 211 women followed

Figure 1. *Performance of the GynTect assay using PreservCyt samples. In total, residual material from 105 samples was used for the analysis. A. GynTect results compared to histopathology findings for the PreservCyt samples. B GynTect results compared to liquid-based cytology findings for all PreservCyt samples except for the cervical cancer samples. The bar graph in* ***Figure 1A*** *shows the percentage of the GynTect-positive samples in the categories "Pap I", "CIN1/2", "CIN3", and "CxCa", with the numbers of cases given in parentheses. The bar graph in* ***Figure 1B*** *shows the percentage of the GynTect-positive samples in the categories "Pap I", "PapIII/IIID", "PapIVa", with the numbers of cases given in parentheses.*

1A



1B



after CIN2 diagnosis), and the overall progression rate in this study was very low (15%).

The data show that the GynTect score is related to the severity of the lesion confirmed by histopathology. In fact, the higher the CIN grade, the more GynTect markers are positive in the LBC samples. The only GynTect-positive CIN1 case, however, had a score of 1.2, so five of the six markers were positive, with rather low delta Ct values. In cytology this case was graded Pap IVa, which might imply that the biopsy in this case was not taken at the punctum maximum of the lesion. Altogether, the correlation between cytology finding and GynTect result was even higher.

In conclusion, GynTect, a test which provides a triage option for either HPV-based or cytology-based cervical cancer screening, shows excellent results if performed on cervical scrape material in liquid-based cytology media, a prerequisite for employing such a test in new screening programs.

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