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ORIGINAL ARTICLE

Amplification of TERT and TERC genes in cervical intraepithelial neoplasia and cervical cancer

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Abstract

OBJECTIVES: Telomerase is activated in various stages of oncogenesis. For cervical cancer, telomerase is already active in precancerous lesions. In our study we focused on the analysis of the amplification patterns of telomerase genes TERT and TERC.

DESIGN AND SETTING: We included 39 patients in our study between January 2012 and April 2013. Each patient underwent a classical gynaecological examination and a colposcopy. During the colposcopic examination we collected material for a Pap smear, HPV DNA test (HC2) and LBC (LiquiPrep™), and performed punch biopsies for histopathological evaluation. Residual cytologic sample was hybridized with the FISH probe and telomerase genes were analysed.

RESULTS: The amplification of the TERT gene showed us a very similar amplification pattern as TERC and gradually corresponded with both histolopathological ($p<0.001$) and cytopathological findings ($p<0.001$). The specificity and sensitivity of TERC gene amplification for the detection of CIN2+ lesions (cut off value 2.3) was 88.2% and 95.5% respectively (PPV 91.3%, NPV 93.8%).

CONCLUSIONS: We identified increasing amplification pattern of telomerase genes in cervical lesions. According to our results telomerase genes could help in the future to determine the malignant potential of cervical lesions and could be tested together with cytology and HPV DNA in order to obtain the highest combined sensitivity and specificity for CIN2+ lesion detection.

INTRODUCTION

Cervical cancer was the third most commonly diagnosed cancer in women in 2008 with an estimated 529 800 new cases worldwide, more than 85% of which were in developing countries (American Cancer Society 2011). The large regional variation in cervical cancer rate primarily reflects the availability of organized screening (Papanicolaou (Pap) test). There is a continuously decreasing trend in cervical cancer in developed countries during the last 50 years. In contrast to these favourable trends in all ages combined, however, cervical cancer rates have been increasing among younger generations in several countries including Finland, the United Kingdom, Denmark and China (American Cancer Society 2011).

The Pap test and HPV DNA testing are the most common methods for screening cervical cancer worldwide. Although these tests are responsible for these regressive trends, they will not tell us anything about the malignant potential of particular cervical lesions and what treatment is the most suitable. The authors Nanda and colleagues (2000) included in their analysis 94 studies dealing with the conventional Pap test and its relationship to colposcopic findings and definite histology. Sensitivity ranged from 30–87% and specificity between 86–100%. Another meta-analysis from the year 1995, by the authors Fahey and colleagues compared the Pap test results with histology. It also confirmed that the high specificity (90–95%) does not correspond with the significantly lower sensitivity (20–35%).

Due to the low sensitivity of tests new diagnostic methods have been developed. Arbyn and colleagues (2009) divided these methods into four groups:

A. Alternative forms of cytology – e.g. LBC, computer assisted cytology
B. Molecular detection of high risk HPV DNA and RNA
C. Biomarkers related to regression of HPV infection such as immunohistochemical staining of cell cycle regulators, methylation changes in genes, proteomic, etc.
D. Identifiable changes in biophysical spectroscopy

The study comparing normal PAP smears and liquid based cytology did not find any relevant difference between these methods, however. Szarewski and colleagues compared the sensitivity and specificity of HPV DNA testing as a modern method that should replace or be equal to cytology according to the latest guidelines. The specificity of various HPV DNA tests is around 30% and sensitivity 99%. The low values of specificity are caused by the fact that the great majority of HPV infections regress spontaneously and do not lead to cervical cancer. Nowadays HPV mRNA E6 E7 tests are very promising in the area of higher specificity and comparable sensitivity as HPV tests (Burger *et al.* 2011).

During the last two decades, telomeres, enzyme telomerase and their implications for medicine itself have been the subject of scientists´ increasing interest. Telomeres consist of repetitive DNA sequences (TTAGGG)n (Blackburn *et al.* 1978). Telomeres continuously shorten as the cell undergoes divisions. The very end of each chromosome is not blind but single stranded DNA consisting of 200 nucleotides at the 3´end (Griffith *et al.* 1999). Telomerase is an integrated complex consisting of the reverse transcriptase enzyme (TERT) and RNA component (TR), which acts as a template for the DNA synthesis (Greider *et al.* 1987).

Telomerase function

Telomerase is activated in various stages of oncogenesis. For tumours whose telomerase is already activated in premalignant lesions, detection of telomerase activity is suitable for screening high-risk patients. If the level of telomerase gradually increases with disease progression or the telomerase is activated in the late stages of disease, telomerase becomes a good prognostic marker. For cervical cancer, telomerase is already active in precancerous lesions (Hiyama *et al.* 2009). Detection of telomerase activity either by the means of PCR or FISH is a part of extensive research in the area of oncology and screening. As far as the cervical cancer is concerned there are many studies containing telomerase that prove the amplification patterns of both parts of the enzyme, especially TERC that correlates with the grade of cervical lesions.

MATERIAL AND METHODS

We included 39 patients in our study between January 2012 and April 2013. Each patient underwent a classical gynaecological examination and a colposcopy. During the colposcopic examination we collected material for a Pap smear, HPV DNA test (HC2) and LBC (LiquiPrep™), and performed punch biopsies for histopathological evaluation. Cytological samples were classified according to the Bethesda classification (2001) (Solomon *et al.* 2002) into six categories: NILM (negative for intraepithelial lesion or malignancy), ASCUS (atypical squamous cells of undetermined significance),ASC-H (atypic squamous cells – cannot exclude high-grade), LSIL (low-grade squamous intraepithelial lesion HSIL (high-grade squamous intraepithelial lesion) and SCC+AC (squamous carcinoma + adenocarcinoma). Residual liquid-based samples were stored at room temperature for the FISH analysis. Histopathological diagnosis followed the WHO criteria (Tavassoeli *et al.* 2003) and included the following sub-classes: WNL (with no lesion), CIN1, CIN2, CIN3, and carcinoma either squamocellular or adenocarcinoma.

Residual LiquiPrep specimens were processed according to the producer´s manual, centrifuged at 1 000 g for ten minutes, mixed with a cellular base LiquiPrep in ratio 1:3, pipetted manually on SuperFrost slides in very thin layer and dried at room temperature.

Slides were pre-treated and incubated for two minutes at 37 °C in 2×SSC solution (pH 7.0) and 1 minute with the pepsin solution (concentration of 0.5 mg/ml) afterwards. After incubation the slides were washed twice in 1×PBS at room temperature each for 3 minutes (pH 7.4), dehydrated in 70%, 85%, and 95% ethanol at room temperature for 1 minute each and air dried. The FISH probe FHACT™ (Cancer Genetics Italia) was vortexed briefly in a microcentrifuge. Subsequently 10 ml of the probe were applied on a slide and covered with a cover glass (24×24 mm). The edges of the cover slide were sealed thoroughly with rubber cement. Afterwards the slides were co-denatured for 3 minutes at 80 °C on a temperature controlled hot plate protected from direct light, incubated for 48 hours in a humidified environment at 37 °C and protected against the light. After the hybridisation process had ended, the

cover glass was removed and the slide was washed for 2 min in 2×SCC/0.1% lgepal at RT, 2 min at 72°C in 0.4×SCC/0.3% lgepal, 1 min. at RT in 2×SCC/0.1% lgepal, dehydrated in 70% , 85%, and 95% ethanol at room temperature and air dried. The slide was briefly rinsed in distilled water and air dried. In the end 10 µl of DAPI (antifade solution, 0.1 µg/ml) was applied to the hybridized area and covered with a cover glass (24×24 mm).

We used an Olympus BX61 fluorescent microscope for the slides evaluation and picture acquisition. The slides were screened with a 100× objective. The FISH probe FHACT™ (The FISH-based HPV-Associated Cancer Test – Cancer Genetics Italia) is designed to determine copy number changes of the 3q26 (TERC), 5p15 (TERT), 20q13, and Cen7 regions. We enumerated TERC signals (red), TERT signals (green) and Cen7 signals (blue). On each slide 100 cells were evaluated manually. A cell with the ratio of 2:2:2 (TERC:TERT:Cen7) was determined as a healthy diploid cell (Figure 1). A cell was considered to be chromosomally abnormal if either the TERC or TERT probe showed 3 or more signals per cell. The mean count of signals per cell was determined for each specimen.

Statistics

Calculations were performed with PASW statistics 18 (IBM®) software. For the correlations between the cytologic, histologic diagnosis and FISH pattern of TERC and TERT Student *t*-test was used (statistically significant values $p<0.05$). Receiver Operatory Characteristics (ROC) were used to determine the threshold of the positive amplification pattern.

RESULTS

The amplification pattern of hTERC increased gradually corresponding to the severity of cervical lesions ($p<0.001$). When we considered the cut off value for TERC> 2.3, the numbers of patients in particular cytology groups positive for TERC amplification were as followed: 0/9 (NILM), 1/5 (ASCUS), 0/1 (ASC-H), 8/8 (LSIL), 12/12 (HSIL) and 4/4 (carcinoma). As far as the histology results are concerned, the numbers of patients positive for TERC amplification (cut off value >2.3) were: 2/12 (WNL), 2/5 (CIN1), 9/10 (CIN2), 8/8 (CIN3) and 4/4 (carcinoma). The specificity and sensitivity for the detection of CIN2+ lesions (cut off value 2.3) was 88.2% and 95.5% respectively (PPV 91.3%, NPV 93.8%). From 100 evaluated cells per slide we counted those cells that had ≥4 copies of the TERC gene. (Tables 1 and 2). These conditions also showed us statistically highly significant values in relation to cytology (TERC≥4 ($p<0.01$), and histology (TERC≥4 ($p<0.01$).

The amplification of the TERT gene showed us a very similar amplification pattern as TERC and gradually corresponded with both histolopathological ($p<0.001$) and cytopathological findings ($p<0.001$). With the cut off value 2.08 TERT positive patients were as follows: 1/9 (NILM), 0/5 (ASCUS), 0/1 (ASC-H), 4/8 (LSIL), 10/12 (HSIL) and 4/4 (carcinoma). According to the histopathological classification, positive TERT amplification was present in 1/12 of WNL, 0/5 CIN1, 6/10 CIN2, 7/8 CIN3, and 4/4 of carcinomas. The specificity and sensitivity for the detection of CIN2+ lesions (cut off value >2.05) was 94.1% and 77.3% respectively (PPV 94.4%, NPV 76.2%). What is more, the amplifica-

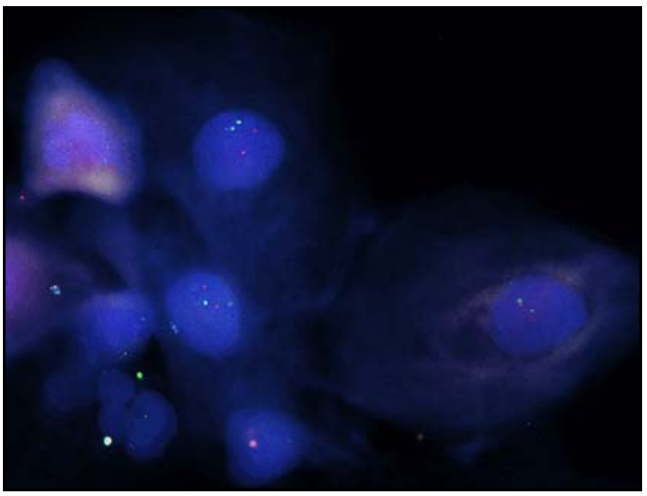

Fig. 1. Squamocellular cells with physiological number of TERC and TERT copies (FISH, 100× objective).

Tab. 1. Average value of amplification pattern of TERC and TERT per cell in histological specimens.

Histology	No.	TERC	TERT	TERC≥4
WNL	12	2.19	2.03	3
CIN1	5	2.24	2.05	6
CIN2	10	2.44	2.16	10
CIN3	8	2.57	2.19	18
Ca	4	2.93	2.20	31

No. – number of patients, TERC – average value of TERC amplification per cell, TERT – average value of TERT amplification per cell, TERC≥4 – number of cells with ≥4 copies of TERC.

Tab. 2. Average value of amplification pattern of TERC and TERT per cell in histological specimens.

Cytology	No.	TERC	TERT	TERC≥4
NILM	9	2.15	2.03	3
ASCUS	5	2.29	2.04	4
ASC-H	1	2.14	2.03	1
LSIL	8	2.41	2.14	9
HSIL	12	2.56	2.18	17
Ca	4	2.82	2.20	31

No. – number of patients, TERC – average value of TERC amplification per cell, TERT – average value of TERT amplification per cell, TERC≥4 – number of cells with ≥4 copies of TERC

tion of the TERC gene correlates with the amplification of the TERT gene ($p<0.02$).

In relationship to hrHPV status amplification of TERC and TERT gene showed statistically significant value ($p<0.05$)

DISCUSSION

The study of authors Kirhoff and colleagues using the CGH method showed the following chromosomal changes in the transition from pre-invasive lesions to malignancy: gains (1q,3q,5p,15q) and losses (4p,6q,13q). Heselmeyer and colleagues showed that amplification in the region of the long arm if the third chromosome (3q) is emerging in the transition of premalignant lesions to cervical cancer. Amplification (or gain) is done mainly on the segment 3q26-27. This region incorporates the TERC gene, which encodes the RNA template for the enzyme telomerase, and the PIK3CA gene encoding the catalytic subunit of phosphatidylinositol-3-kinase (cell intracellular messenger). Because telomerase is activated relatively early in the process of cervical carcinogenesis, it can be a useful tool for diagnosis and prognosis of patients with cervical neoplasia (Jiang *et al.* 2010).

We created a preliminary study with a small number of patients (n=20) in order to test our methodology and obtain the first results for our future research. There are approximately 20 studies concerning the TERC gene and its relationship to cervical carcinogenesis. Except for the study by Takac and colleagues (2009) the rest all proved the importance of TERC gene testing. TERC gene amplification detected by the FISH method is present in about 7% of histologically negative lesions, 24% of CIN1, 54% of CIN 2, 91% CIN 3 and 100% of invasive carcinomas. The percentages show that positive TERC amplification increases with the severity of dysplasia (Andersson *et al.* 2009). Given our study concerns only 39 patients, the percentages in particular groups are slightly different. Thresholds for TERC positivity vary among studies and this is the main reason why the results are not similar. Another problem is methodology. In our study we used liquid-based cytology samples for FISH diagnostics as in most studies so far. But some studies evaluated TERC amplification using classical PAP smears (Heselmeyer-Haddad *et al.* 2003). The method for evaluating this amplification is very important to establish. There is automatic software or a manual method for counting the enumerated signals. We did not have the appropriate software for signal count and that is why we tried a manual method, one which is subjective but still sufficiently accurate. After some pitfalls in processing the samples, we created a completely new FISH protocol for evaluating the cytology samples. We counted only 100 cells per slide as in 3 works published so far (Tu *et al.* 2009; Xiang *et al.* 2011; Chen *et al.* 2012). Heselmeyer and colleagues (2003) counted between 209-3903 cells, but enumerating cells on the whole slide might be time consuming and it usually takes 60 minutes to evaluate the specimen from one patient. One part of the studies evaluated only a small number of morphologically abnormal cells (Caraway *et al.* 2008; Ramsaroop *et al.* 2009; Kokalj-Vokac *et al.* 2009). We did not choose absolute cell count with amplification pattern but we took into consideration the average copies of TERC gene per cell. This should lower mistakes from subjective counting of cells. There is no study so far that evaluates both genes coding the active enzyme telomerase: TERC and TERT. There was a significant correlation between the grade of cervical lesion and amplification pattern of both genes ($p<0.001$). What is more, TERT amplification correlated with TERC amplification ($p<0.01$). This correlation supports the fact that both coding genes are needed for a correct function of the enzyme. One patient from our study with aggressive adenocarcinoma G3 (intestinal type) had numerous amplifications in almost every cell with an average number of 3.3 copies per cell. When we compare it to squamocellular carcinoma with 2.82 copies per cell, there is a significant difference. Activation of telomerase by the HPV protein E6 corresponds with our results where we proved the correlation of catalytic subunit TERT and hrHPV status.

Amplification of the targeted gene is crucial for the progression from CIN 1/2 to CIN 3 and predicts the progression of lesions. Amplification of the TERC gene was detected in 33% of women with normal cytological findings who were later diagnosed with CIN 3 lesions or even invasive carcinoma (Heselmeyer-Haddad *et al.* 2005). In our study there was a significant difference between TERC amplification ($p<0.01$) and TERT amplification ($p<0.01$) in CIN2+ lesions compared to less severe lesions. This supports the fact that telomerase genes should serve in the future to determine the malignant potential of every cervical lesion and should be tested together with cytology and HPV DNA in order to obtain the highest combined sensitivity and specificity for CIN2+ lesion detection.

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