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Different amplification patterns of 3q26 and 5p15 regions in cervical intraepithelial neoplasia and cervical cancer

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ABSTRACT

Purpose: The aim of this study was to evaluate and correlate the amplification of chromosomal regions 3q26 and 5p15 in different cytological and histological subgroups of patients and to compare the sensitivity and specificity of amplification tests with cytology, colposcopy and HPV status.

Methods: The work was conducted at the Department of Obstetrics and Gynaecology in cooperation with the Institute of Pathological Anatomy, JFM CU in Martin and UNM during years 2013–2016. Prospective longitudinal study included 131 patients. We focused on the FISH diagnosis of the amplification of regions encoding the components of telomerase enzyme (3q26, 5p15) in cytology specimens. We manually evaluated 100 atypical cells per slide and analysed the amplification patterns. Correlations between cytological, histological, HPV DNA results and amplification patterns of chromosomal regions 3q26 and 5p15 were analysed by chi-squared test and non-parametric Man - Whitney *U* test.

Results: The results showed that the amplification of chromosomal regions increases with the degree of dysplasia toward the invasive disease ($p < 0.001$). Whereas the increase in the amplification of 3q26 is noticeable already at CIN 2 + lesions ($p < 0.01$), 5p15 amplification is shifted up toward CIN 3/CIS ($p < 0.001$) and cervical cancer. Amplification of selected regions correlated with each other and also with hrHPV-positive status ($p < 0.01$).

Conclusion: The analysis of the amplification of 3q26 and 5p15 regions may serve in the future for the differential diagnosis of cervical lesions and to determine their malignant potential. High specificity of these tests can improve the excellent sensitivity of HPV DNA test.

1. Introduction

Cervical cancer is one of the most common malignancies in the female population. The worldwide incidence in 2012 reached 528,000 new cases and that mortality exceeded the 266,000 [1]. Currently, standard screening for cervical precanceroses includes cytological examination of cervical smears in combination with HPV DNA diagnostics. E6, E7 mRNA, p16 protein, methylation markers, glycomics, proteomics and the telomerase activity are the new diagnostic tools for cervical dysplasia and cervical cancer.

In our study we focus on amplification patterns of chromosomal regions 3q26 and 5p15 tightly connected with the telomerase activity. 3q26 chromosomal region contains TERC gene that codes the RNA template of telomerase enzyme. The second gene localised in this region is PIK3CA encoding the catalytic subunit of phosphatidylinositol-3-kinase which represents the intracellular messenger. On the other hand, region 5p15 contains TERT gene responsible for the synthesis of catalytic subunit of telomerase enzyme. Increased telomerase activity is an early process in cervical carcinogenesis and its activity and expression could be a valuable marker for the diagnosis and prognosis of patients with cervical neoplasia [2].

2. Material and methods

The study was carried out between years 2013–2016 at the Department of Gynaecology and Obstetrics and at the Institute of Pathology, Jessenius Faculty of Medicine in Martin, Comenius University, Slovakia. The prospective study involved 131 patients. Twenty patients formed the control group with negative cytology (NILM) and negative high-risk human papillomavirus (hrHPV) finding.

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Patients younger than eighteen years, pregnant women and patients who underwent the operative treatment for cervical lesions in the past were excluded from the study. The study was approved by the ethics committee (27 January 2012) and before each examination the informed consent was signed.

The cytological samples for HPV and fluorescent in situ hybridization (FISH) were obtained during colposcopic examination. The brush was immersed in a liquid fixation medium (Liqui-Prep vial, LiquiPrep™, LGM International Inc.). We continued with advanced colposcopy with 3% acetic acid solution and. Schiller test. In case of insufficient finding we used endocervical speculum (Kevorkian - 3 and 7 mm) for cervical channel visualisation. Colposcopic findings were evaluated according to the latest international nomenclature approved in Rio de Janeiro in 2011. Based on the biopsy results the patients were further managed either conservatively or surgically. The more severe diagnosis (from the biopsy or conisation) was categorized as the definite one. Cytological specimens were classified according to the Bethesda system (2001) whereas HPV status was analysed by the method HC2 (Digene Hybrid Capture 2 High-Risk HPV DNA Test, Gaithersburg, MD).

2.1. FISH hybridization

LiquiPrep™ specimens were processed according to the producer's manual, centrifuged at 1000*g* for 10 min, mixed with a LiquiPrep™ cellular base with a ratio of 1:3, pipetted manually on SuperFrost slides (Menzel-Glaser, Braunschweig, Germany) and dried at room temperature.

Slides were pre-treated and incubated for 2 min at 37 °C in 2× saline-sodium citrate (SSC) buffer (pH 7.0) and 1 min with the pepsin solution (concentration of 0.5 mg/mL) afterwards. After incubation the slides were washed twice in 1× phosphate-buffered saline at room temperature each for 3 min (pH 7.4), dehydrated in 70, 85 and 95% ethanol at room temperature for 1 min each and air-dried. The FISH probe FHACT™ (Cancer Genetics Inc., Rutherford, NJ, USA) was vortexed briefly in a microcentrifuge. Subsequently, 10 µL of the probe was 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 min at 80 °C on a temperature-controlled hot plate protected from direct light, incubated for 48 h in a humidified environment at 37 °C and protected against the light. After the hybridization process the cover glass was removed and the slide was washed for 2 min in 2× SSC/0.1% Igepal at room temperature, 2 min at 72 °C in 0.4× SSC/0.3% Igepal, 1 min at room temperature in 2× SSC/0.1% Igepal, 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 diamidino-2-phenylindole solution (DAPI, 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 slide 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 Inc., Rutherford, NJ, USA) is designed to determine copy number changes of the 3q26, 5p15, 20q13 and Cen7 regions. We evaluated 100 most atypical cells on each slide and analysed the amplification patterns of 3q26 and 5p15 regions.

Statistical analysis was performed by PASW Statistics 18 (IBM ®) software. Nonparametric Man-Whitney *U* test was used for the correlation between cytological and histological results, HPV DNA status and amplification patterns of 3q26 and 5p15 amplification ROC (Receiver Characteristics Operators) curves were used to determine the cut-off values for the determination of sensitivity, specificity, positive and negative predictive values of amplification tests.

3. Results

The average age of patients included in our study was 39.4 years.

Table 1
Summary of definite histological, cytological and hrHPV results.

		WNL	CIN1	CIN2	CIN3/CIS	SCCA/AC
hrHPV negat	ASCUS	6	4	–	–	–
	LSIL	2	3	–	–	–
	ASC-H	5	0	–	–	–
	HSIL	0	1	–	–	–
	SCCA/AC	0	0	–	–	1
hrHPV pozit	ASCUS	2	1	5	2	–
	LSIL	11	4	11	6	–
	ASC-H	1	1	0	3	2
	HSIL	0	1	8	14	4
	SCCA/AC	0	0	0	0	13

The definite cytological, histological and hrHPV findings are summarised in Table 1.

(WNL – within normal limit, CIN –cervical intraepithelial neoplasia, CIS – carcinoma in situ, SCCA – squamocellular carcinoma, AC – adenocarcinoma, NILM – negative for intraepithelial lesion or malignancy, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepithelial lesion, ASC-H – atypical squamous cells, cannot exclude high-grade, HSIL – high-grade squamous intraepithelial lesion, hrHPV – high-risk human papillomavirus)

3.1. FISH analysis

By the ROC curves, we determined the most appropriate cut-off values for amplification tests of chromosomal region 3q26 and 5p15 in detecting CIN 2+ lesions. The results showed that the amplification increases with the degree of dysplasia ($p < 0.001$). While the increase in the 3q26 amplification is evident even at CIN 2+ lesions ($p < 0.01$), 5p15 amplification is shifted to CIN3+ lesions ($p < 0.001$). The absolute number of positive cases for 3q26 (cut off > 11 cells with > 2 signals per case) and 5p15 (cut-off > 3 cells with > 2 signals per case) is shown in Tables 2 and 3.

(WNL – within normal limit, CIN –cervical intraepithelial neoplasia, CIS – carcinoma in situ, SCCA – squamocellular carcinoma, AC – adenocarcinoma, NILM – negative for intraepithelial lesion or malignancy, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepithelial lesion, ASC-H – atypical squamous cells, cannot exclude high-grade, HSIL – high-grade squamous intraepithelial lesion)

(WNL – within normal limit, CIN –cervical intraepithelial neoplasia, CIS – carcinoma in situ, SCCA – squamocellular carcinoma, AC – adenocarcinoma, NILM – negative for intraepithelial lesion or malignancy, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepithelial lesion, ASC-H – atypical squamous cells, cannot exclude high-grade, HSIL – high-grade squamous intraepithelial lesion)

Significant differences were observed between the negative histology and light dysplasia for 3q26 amplification. We did not detected statistically significant difference between ASCUS and LSIL cytology group for the amplification pattern of both genes ($p = 0.910$ (3q26),

Table 2
Number of positive cases of 3q26 amplification in cytological and histological categories (cut-off for 3q26 amplification: > 11 cells with > 2 signals).

	WNL	CIN1	CIN2	CIN3	CA	Total
NILM						1/20
ASCUS	0/8	1/5	2/5	1/2	–	4/20
LSIL	1/13	1/7	5/11	6/6	–	13/37
ASC-H	0/6	0/1	–	3/3	2/2	5/12
HSIL	–	2/2	4/8	11/14	4/4	21/28
CA	–	–	–	–	14/14	14/14
Total	1/27	4/15	11/24	21/25	20/20	

Table 3
Number of positive cases of 5p15 amplification in cytological and histological categories (cut-off for 5p15 amplification: > 3 cells with > 2 signals).

	WNL	CIN1	CIN2	CIN3	SCCA/AC	Total
NILM						1/20
ASCUS	0/8	0/5	0/5	1/2	–	1/20
LSIL	1/13	1/7	4/11	4/6	–	10/37
ASC-H	1/6	0/1	–	3/3	2/2	6/12
HSIL	–	1/2	4/8	9/14	4/4	18/28
SCCA/AC	–	–	–	–	13/14	13/14
Total	2/27	2/15	8/24	17/25	19/20	

$p = 0.359$ (5p15)). On the other hand, we have shown a statistically significant difference between LSIL and HSIL lesions and between ASCUS and control group in the amplification of both genes ($p < 0.05$) (Fig. 1). A significant difference was seen also between hrHPV-positive and negative patients ($p < 0.001$). Positive amplification of 3q26/5p15 was present in 9.5%/4.76% hrHPV negative patients and in 60.67%/52.81% hrHPV positive patients (Fig. 2). The actual amplification of 3q26 and 5p15 correlate with each other; underlining the linkage between the amplification pattern of the chromosomal regions ($p < 0.01$). Amplification patterns of 3q26 and 5p15 showed very high combined sensitivity and specificity compared to HPV test and cytological results (Table 4).

4. Discussion

In our study, we focused on chromosomal changes in the process of cervical carcinogenesis. Chromosomal instability is a crucial sign of malignancy. Fluorescent in-situ hybridization is an effective method for the identification of structural and numerical chromosome aberrations. Many studies have failed to prove the overexpression of telomerase and its correlation with clinical and pathological parameters using the TRAP assay, IHC testing or RT-PCR. False-negative results of the tests could be due to the degeneration of RNA and protein extraction, or due to tissue inhibitors of telomerase [3].

In the most extensive study the authors analysed 35 hybridization probes: 13 centromeric (1, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, X), and 22 locus specific probes (LSI) (1p31, 1q41. 2p24, 2q26, 2q33, 3p14, 3p21, 3q26, 4p15, 4p16, 5p13, 5P15, 6p21, 6q16, 7P1, 8q24, 11p15, 11p23, 11q23, 11q13, 20q13, and Xp22 Xq11) [4]. Twenty-five abnormal cells were evaluated per case with a cut-off value > 4 cells with amplification. The authors observed the highest correlation with high-grade lesions in region 3q26 and 8q24. 8q24 amplification perfectly reflected CIN2 lesions (96.3% positivity compared to 26.3% positive cases of CIN1 and 4.8% WNL), whereas the most appropriate locus for the detection of CIN3 appeared to be 3q26 region (95% positivity compared to 84, 5% CIN2 and 31.6% CIN1 lesions).

In our work, we used the four-colour probe for amplification analysis. 20q13 region due to the unavailability of specific gold-filter for fluorescence microscope was not evaluated. 20q13 region contains a number of important genes, including aurora kinase A, which is essential in the development of aneuploidies by the deregulation of centrosome formation. The increased copy number of 20q13 study was linked to the development of cervical carcinoma. However, the specificity of the test in CIN 3 lesions was not as high as in the case of 3q26 [4]. The analysis was therefore focused on the chromosomal region encoding the essential parts of telomerase enzyme: 3q26 (TERC), 5p15 (TERT) and control centromeric probe Cen7. Centromeric probes did not show statistically significant values except CEP15 [4].

The methodology is a weakness of new hybridization techniques. About thirty studies were published that evaluated the amplification of chromosomal regions by comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). Specimen varied between studies including the biopsy samples, conventional cytology and "liquid-based" samples [5]. The evaluation of the cervical cells is either based on automatic software by using a microscope scan of the entire area of cytological specimen [2,6,7] or on the manual evaluation which is connected with subjective errors [8,9]. The FISH method is slow in contrast to the rapid polymerase chain reaction (PCR). On the other hand we could obtain a qualitative data regarding the particular cells by the hybridization method (aneuploidy, polyploidy).

In our work, we selected the manual counting of signals in 100 atypical cells per slide. Heselmeyer-Haddad et al. in 2003 evaluated 209 to 3903 cells per sample using the software, but the process itself was very lengthy, as the evaluation of the entire sample took 60 min. On the other hand only a minority of studies analysed a small number of atypical cells [3,10]. Nowadays the whole process of evaluation is still a luxury and makes the screening 5-times more expensive compared to classical cytology [11]. Automated sample preparation as well as the overall evaluation would get the methodology cheaper. Despite the high price of examination the amplification of selected

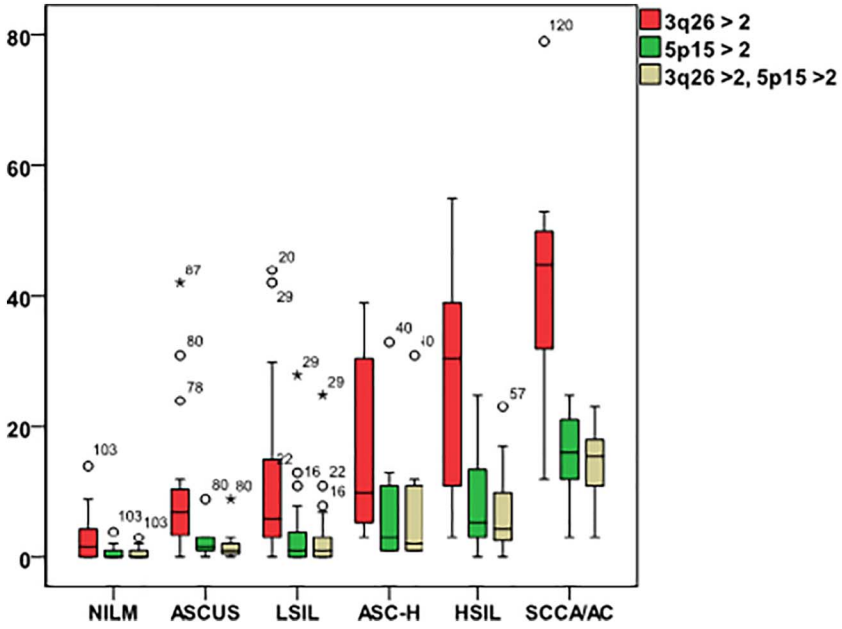

Fig. 1. Amplification pattern of 3q26 (red) and 5p15 (green) and their combination (grey) according to cytological result. The Y axis represents absolute numbers of cells with amplification. (NILM – negative for intraepithelial lesion or malignancy, ASCUS –atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepithelial lesion, ASC-H – atypical squamous cells, cannot exclude high-grade, HSIL – high-grade squamous intraepithelial lesion, hrHPV – high-risk human papillomavirus). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

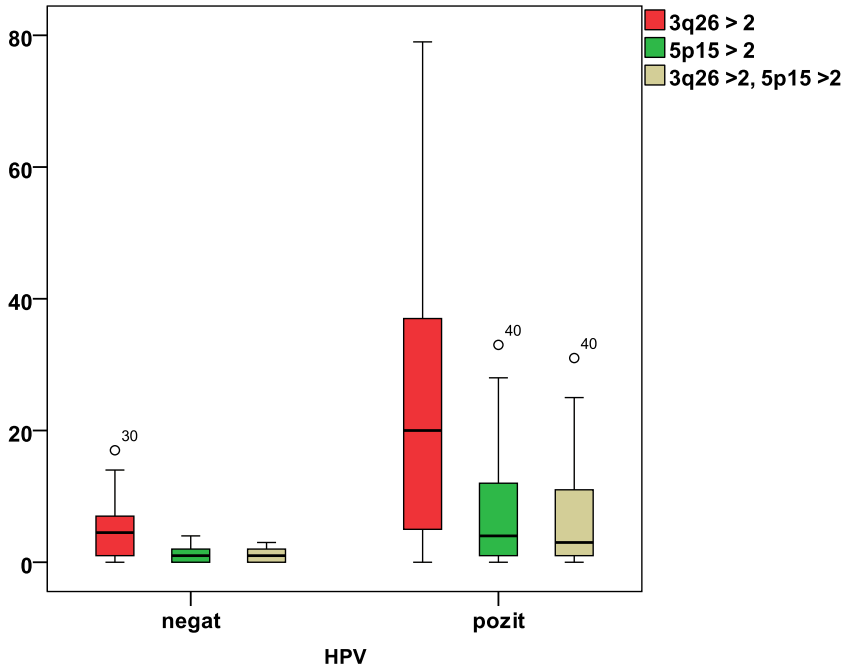

Fig. 2. Amplification pattern of 3q26 (red) and 5p15 (green) and their combination (grey) according to the hrHPV status (negat – negative, posit – positive). The Y axis represents absolute numbers of cells with amplification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Sensitivity, specificity, NPV (negative predictive value), PPV (positive predictive value) for particular screening tests for CIN2+ detection.

	Sensitivity in % (95% CI)	Specificity in % (95% CI)	NPV v % (95% CI)	PPV v % (95% CI)
Cytology HSIL+	65.22 (52,71–76,02)	85.48 (57,13–92,75)	68.83 (57,13–78,64)	83.33 (70,21–91,64)
hrHPV DNA	98.55 (91,11–99,92)	66.13 (52,90–77,35)	97.62 (85,91–99,88)	76.40 (66,00–84,49)
3q26 > 2	75.36 (63,5–84,9)	90.32 (80,1–96,3)	76.71 (65,09–85,48)	89.66 (78,16–95,72)
5p15 > 2	63.77 (51,3–75,0)	91.94 (82,2–97,3)	69.51 (58,22–78,95)	89.80 (76,99–96,18)
3q26 > 2, 5p15 > 2	69,57 (57,3–80,1)	90,32 (80,1–96,3)	72,72 (61,19–91,96)	88,89 (76,69–95,40)

chromosomal regions could help in managing borderline cases. The amplification of 3q26 and 5p15 could represent a marker for the use of telomerase inhibitors in the treatment of cervical cancer.

Ramsaroop et al. defined the equivalent amplification of hTERC and CSP7 as a polyploidy. Aneuploidy was represented by the amplification ratio hTERC:CSP7 > 1 [3]. They demonstrated that the polysomy was a common finding in low-grade and high-grade lesions. Aneusomy was more common in high-grade lesions and this result was also confirmed in our study. Polysomic cells with the pattern of 4:4:4 (3q24:5p15: Cen7) were excluded from the evaluation because they may represent healthy cells during division.

Although it is difficult to compare the results between particular studies due to differences in cut-off values and sources of the data, our results are consistent with the previous research. Heselmeyer-Haddad et al. in 2003 demonstrated the first detection of hTERC amplification in LBC samples in 63% cases of CIN2, and in 76% cases of CIN3 [5]. The percentage of positive amplification test results depends on the determination of its cut-off value. CIN3 lesions were positive in 85.7% cases and squamous cell carcinoma in 90.6% of cases for the cut-off value of 5.89 cells with amplification pattern [12]. The proportion of positive cases in CIN3 and carcinoma groups in our study was similar: for the amplification of 3q26 (cut-off > 11 cells) it was 84% and 100%, for the amplification of 5p15 (cut-off > 3 cells) 68% and 95%. Our results showed that the best combination of sensitivity and specificity for detection of CIN2+ lesions showed the amplification of 3q26: 75.36% and 90.32% respectively. Comparable results were also observed in the amplification of 5p15: 63.77% and 91.94%. In the control group with negative cytology and HPV status only one case showed positive amplification of 3q26.

In ASCUS group the percentage of positive amplification was only 20%, compared to slightly higher results in LSIL group (35.1%). The increasing trend of amplification was showed in HSIL group (75%) and in cancer specimen (100%). The only study that analysed a similar spectrum of genes is from 2013. The sample analysis consisted of 1000 cells that improved the specificity of hybridization test. The authors observed an association of amplified number of signals per cell (> 3 and > 4) with increasing severity of cervical lesions, which may help to differentiate LSIL and HSIL lesions. The cut-off value was determined as 39 cells with amplification per cytological slide. The amplification of 3q26 showed sensitivity of 82% and a specificity of 53% for the detection of CIN2 + lesions. Two probes with the lowest correlation coefficient were 20q and cen7. Amplification of 3q26 appears in the earlier stages of precancerous lesions, while 20q13 is amplified in CIN3+ lesions. The differences in chromosomal abnormalities could be also based on different types of HPV infection (Luhn et al. 2013). The point of interest is histological category CIN2, where the percentage of

amplification in our study did not reach even 50% (3q26–45.8%, 5p15–33.3%), showing the heterogeneity of this subgroup. Low level of amplification could potentially support the conservative treatment.

The case with the highest value of 3q26 amplification was represented by the adenocarcinoma in clinical stage II (79% of cells with the average number of 3q26 signals 3.31 per cell). Other samples of squamous cell carcinoma did not reach this extreme value. The amplification of 5p15 region showed the maximum of 25% of positive cells in the case of squamous cell carcinoma. Andersson et al. in 2006 detected hTERC amplification in paraffin embedded samples in 100% adenocarcinomas using FISH analysis. However, in this study, hTERC amplification was detected only in 40% of LBC samples; significantly less than in squamous cell carcinoma (90.6% SCC, 85.7% CIN 3). Adenocarcinomas are derived from glandular cells of the endocervix and are often presented as endophytic lesions that are not fully identified by cytological sampling. Therefore, the use of LBC samples for the diagnosis of similar lesions can be limited. 3q26 amplification was not associated with lymphovascular invasion (LVSI), tumour size and myometrial invasion in cervical cancer stages I-IIA. However, increased 3q26 amplification was observed in cases with positive lymph nodes [13]. Pelvic lymph node involvement was diagnosed only in nine out of 53 squamous cell carcinomas in this study. We analysed the samples of twenty carcinomas and only two cases with histologically proven metastases in the lymph nodes. We did not record notable increase in amplification of 3q26 or 5p15 compared to negative lymph node status. The literature shows that the amplification of both chromosomal regions is not a useful prognostic marker for cervical cancer.

5. Conclusion

Telomerase enzyme is essential in the process of carcinogenesis, providing the cells by immortality. Analysis of telomerase activity may not only be used in the differential diagnosis of cervical lesions, but also in the search of appropriate therapy which would block the enzyme activity in the tumour tissue. Based on available knowledge, the most promising therapeutic modalities are the following: an antisense oligonucleotide inhibitor of GRN163L, dendritic cell immunotherapy (GRVAC1), hTERT peptide (GV1001) and cryptic peptides (*H*-001) [14]. The analysis of 3q26 amplification will not probably replace the HPV testing indicated especially in ASC-US category. However, the potential is in the identification of lesions in categories LSIL and ASC-H which could have more aggressive behaviour. On the other hand the potential of fluorescent in situ hybridization is limited by a small number of studies for every cytological subgroup and by the insufficient stratification according to HPV status [15]. That is why there is a need of more complex studies for the determination of sensitivity and specificity of amplification tests.

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Conflict of interest

The authors claim that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all women included in the study.

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