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Molecular cytogenetic analyses of *hTERC* (3q26) and *MYC* (8q24) genes amplifications in correlation with oncogenic human papillomavirus infection in Czech patients with cervical intraepithelial neoplasia and cervical carcinomas

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It is known that cervical cancer develop from precancerous intraepithelial neoplasia (CIN) which is characterized by series of genetic abnormalities. The progression of CIN to cervical carcinoma has been associated especially with the genomic integration of oncogenic human papilloma virus (HPV) and gain of the human telomerase RNA gene *hTERC* (3q26) and *MYC* (8q24).

In this study, cytology specimens of cervical intraepithelial neoplasia and cervical carcinoma from 74 Czech women were analyzed using the triple-color Cervical FISH Probe Kit designed for identification of HPV infected cells and copy number aberration of the *hTERC* and *MYC* genes. HPV-positivity exhibited 70% of patients with premalignant lesions (CIN I – CIN III, carcinoma *in situ*), chromosomal changes were found in 53.3% of cases – *MYC* amplification had 33.3% of women with CIN I – CIN III and 50% with carcinoma *in situ*. Amplification of *hTERC* was detected in 16.7% of patient with CIN I, in 50% with CIN II, in 58.3% with CIN III and in 66.7% with carcinoma *in situ*. Based on HPV-positivity and the occurrence of chromosomal aberrations, patients were divided into high-, intermediate- and low-risk group.

Among women with cervical carcinomas, HPV infection was detected in 90.1% of specimens and chromosomal aberrations were found in 87.5% of samples. Amplification of *MYC* gene was detected in 25% and *hTERC* gene in 62.5% of patients. According to the histopathological grade of tumors, *MYC* gene amplification occurred more frequently in specimens of spinocellular carcinoma than adenocarcinoma (p=0.029). We found no association between the frequency of cytogenetic lesions and the incidence of lymphangiogenesis or lymph node metastases in cervical carcinoma patients. Simultaneous *hTERC* and *MYC* genes amplification was significantly more frequent in samples of cervical carcinomas than in premalignant lesions (p=0.008).

In a cohort of 26 patients with cervical carcinoma we used oligo-based GGH+SNP microarray technique for the high resolution mapping of copy number changes of *hTERC* and *MYC* genes. We found that recurrent gain of genetic material in chromosome 3q26 area carrying *hTERC* gene of size 43.6 Mb between 3q25.1-3qter and duplication of 3q were the most common genomic identifications of amplified gene. In *MYC* locus array-CGH profiling identified duplication of 8q and trisomy 8 as frequent genomic changes.

Our work confirmed that in cervical carcinoma gains of *hTERC* and *MYC* genes are specific genomic changes associated with developing of malignant phenotype. We also showed that in premalignant stages HPV-FISH assay can be used as an effective diagnostic procedure to identify patients carrying highly risking HPV infection and chromosomal aberrations associated with this malignancy.

Key words: cervical cancer, cervical dysplasia, HPV infection, hTERC amplification, MYC amplification, FISH, array-CGH

Cervical carcinoma is one of the most common malignant diseases in women with an estimated 528,000 new cases in 2012 worldwide. Mortality varies between the different regions of the world, with rates ranging from less than 2 per 100,000 in Western Asia, Western Europe and Australia/New Zealand to more than 20 per 100,000 in Melanesia, Middle and Eastern Africa [1]. Most cases (85 – 90%) of cervical carcinomas are squamous cell carcinomas, the second largest group is ad-

enocarcinoma (10–15%) coming from glandular epithelium, affecting younger women and having worse prognosis. The development of cervical cancer is preceded by cytological changes of cervical cells called cervical intraepithelial neoplasia (CIN). Cervical cytopathology is the routine method for characterization of cells in cervical samples. In mild dysplasia (CIN I) changes occur in low third of epithelial cells; in moderate to marked dysplasia (CIN II), defect of maturation and nucleocytoplasmic ratio changes with numerous mitosis are detected in two thirds of epithelial cells. Defect of maturation with numerous atypical mitoses in the entire width of epithelium is found in severe dysplasia to carcinoma *in situ* (CIN III).

It is well known that the human papillomavirus (HPV) infection plays an initial role in the development of cervical lesions [2]. However, the high-grade lesions (CIN II – III) and cervical cancer are developed only in a part of infected patients despite the high incidence of HPV infection in dysplastic samples. Therefore, the infection alone is not able to activate the malignant transformation. Genetic abnormalities, the deregulation of oncogenes and tumor suppressor genes, are essential in progress of cancerogenesis [3,4]. In malignant and premalignant lesions and cervical carcinoma cell lines, specific chromosomal changes were observed.

The most frequent alteration, detected in approximately 70% of cervical carcinomas, is amplification of chromosome 3q [5,6]. The region 3q26 contains a RNA subunit of human telomerase RNA gene *hTERC*. Many studies show the rate of *hTERC* amplification correlates with development of CIN lesions to carcinoma and thus it can be a predictive factor of malignant transformation and progression [7-10].

Other frequently observed change is an amplification of protooncogene *MYC* (8q24). This gene encodes transcription factor that plays a role in cell cycle progression, proliferation, apoptosis and cellular transformation. Expression of *MYC* in cells of invasive carcinomas is increased more than in CIN cells [11]. According to published data, the amplification of both genes, *hTERC* and *MYC* can serve as an important marker of chromosomal instability of cervical cells infected with HPV [12] predicting the progression of precancerous cervical lesions into higher stages or into invasive cervical carcinomas.

The application of molecular cytogenetic techniques to study genomic alterations as a biomarker for progression during uterine tumorigenesis provided the rationale for the development of fluorescence *in situ* hybridization (FISH) assay as a cytogenetic diagnostic tool for the direct detection of HPV infected cells and alterations of selected genes in cytology specimens. Particularly, triple-color Vysis Cervical FISH Probe Kit was designed to identify HPV infected cells and determine copy number changes of the *hTERC* and *MYC* gene via FISH (HPV-FISH) [12,13].

In this study, cytologic specimens of 74 women from Dept. of Gynecological Oncology of Masaryk Memorial Cancer Institute Brno with histologically confirmed diagnosis of CIN I-CIN III, carcinoma *in situ* or cervical cancers were analyzed using HPV-FISH assay. We evaluated HPV infection/*hTERC*/*MYC* gene amplification in routinely collected cytology samples (PAP smears) and compared the cytogenetic findings with the histological diagnose and clinical outcome. We also set the prognostic risk for each patient according to recommended settings. In addition, 26 cervical carcinomas samples were subjected to array-CGH analysis using Agilent SurePrint G3 Human CGH+SNP 4x180K microarray for the high resolution mapping of copy number changes of *hTERC* and *MYC* genes.

Patients and methods

Samples. Cervical specimens were obtained from Dept. of Gynecological Oncology, Masaryk Memorial Cancer Institute (MMCI) Brno, Czech Republic. Totally 74 patients were included in the study (median age 40.5 years, range 22 – 86 years). The distribution of patients based on histology classification and the clinical characteristics are presented in Table 1. All samples were obtained only after the patients signed the informed consent approved by the Ethical committee of the MMCI. Patients with cervical carcinomas after surgical procedures and any adjuvant treatment were monitored in regular intervals according to oncogynecological guidelines. The follow-up period was 6 – 36 months, and overall survival was not reached as all patients in our cohort are still alive without any sign of tumor recurrence.

PAP smears were prepared according to standard procedure. Slides were fixed in methanol for 20 minutes at 4 °C and in methanol with acetic acid (3:1) for another 20 minutes at 4 °C. Slides were dried at room temperature and then stored at -20 °C before hybridization.

Probe description. To identify human papillomavirus (HPV) infected cells and determine copy number of the chromosomal regions 3q26 (*hTERC*) and 8q24 (*MYC*) via fluorescence *in situ* hybridization (HPV-FISH), Vysis Cervical FISH Probe Kit was used. This kit enables the identification of high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68) using biotin labeling and tyramide signal amplification assay. Detection of copy number alteration of *hTERC* and *MYC* genes is done using standard FISH. The *hTERC* probe is labeled with SpectrumGold fluorescent label and covers approximately 495 kb region; the *MYC* probe is labeled with SpectrumRed fluorescent label and covers approximately 821 kb region.

Slide pretreatment and hybridization. Slide pretreatment and FISH were performed according to the manufacturer's protocols (Abbott-Vysis). Briefly, slides were soaked in 2x SSC at 73 °C for 2 minutes and incubated in pepsin (0.5 mg/ml in 10 mmol/l HCl) at 37 °C for 10 minutes. Slides were soaked in 1x PBS at RT for 5 minutes, fixed in 1% neutral-buffered formalin at RT for 5 minutes and soaked in 1x PBS at RT for 5 minutes. Slides were dehydrated in ethanol series of 70, 80 and 96% for 1 minute and air dried. The probe mixture (10 μl) was applied on the slides; samples were codenatured at 72 °C

Table 1. Clinical characteristics of the patients' cohort

N (%)		Without pathology (N=12) [1]
Histology	inflammation	3 (25.0%)
	precancerosis in CE [2]	9 (75.0%)
		Precancerosis (N=30) [1]
Histology	CIN I	6 (20.0%)
	CIN II	6 (20.0%)
	CIN III	12 (40.0%)
	Ca *in situ*	6 (20.0%)
		Carcinoma (N=32) [1]
Histology	spinocellular carcinoma	25 (78.1%)
	adenocarcinoma	7 (21.9%)
Stage	IA1	2 (6.3%)
	IA2	2 (6.3%)
	IB1	17 (53.3%)
	IB2	3 (9.4%)
	IIA	0
	IIB	0
	IIIA	0
	IIIB	6 (18.7%)
	IVA	2 (6.3%)
T classification	T1a1	2 (6.3%)
	T1a2	2 (6.3%)
	T1b1	21 (65,5%)
	T1b2	4 (12.5%)
	T2a	0
	T2b	1 (3.1%)
	T3b	0
	T4a	2 (6.3%)
Metastasis	LN not affected	21 (77.8%)
	MTS 1 LN	1 (3.7%)
	MTS 2 LN	1 (3.7%)
	MTS 3 LN	1 (3.7%)
	MTS 4 LN	1 (3.7%)
	MTS 7 LN	1 (3.7%)
	MTS 13 LN	1 (3.7%)
	unclassified	5(15.6%)
	negative	21 (77.8%)
	positive	6 (22.2%)
Grade of carcinoma	G1	4 (12.5%)
	G2	12 (37.5%)
	G3	16 (50.0%)
Lymphangioinvasion	negative	19 (59.4%)
	positive	13 (40.6%)

[1] described by absolute and relative frequencies - N (%)
[2] CE...cytological examination

for 2 minutes and hybridized at 37 °C overnight. After hybridization, slides were washed in 2x SSC at 48 °C for 2 minutes and in 2x SSC at RT for 1 minute.

HPV detection using tyramide signal amplification assay. Detection of the biotin-labeled HPV probes was performed using Alexa Fluor 488 TSA (tyramide signal amplification) kit (Invitrogen) according to Cervical FISH Probe Kit directions. Slides were incubated in 3% H_2O_2 at RT for 30 minutes to block endogenous peroxidase activity and soaked in 1x PBS at RT for 5 minutes. Before application of streptavidin-HRP (SA-HRP) conjugate (diluted 1:100 in blocking reagent), slides were incubated with 1% blocking reagent in PBS (both incubations were performed in a humified chamber at 37 °C for 25 minutes). After washing the slides three times in 1x PBS at 37 °C for 5 minutes, the biotin-labeled HPV probe-SA-HRP complex was visualized by incubation with Alexa Fluor 488 labeled tyramide (1:100 dilution) for 10 minutes at RT. The slides were washed three times in 1x PBS at 37 °C for 5 minutes and nuclear counterstain DAPI was applied.

Slide analysis. Slides were analyzed under the fluorescent microscope BX-61 (Olympus) using DAPI, green, gold and red single bandpass filter sets. The 1300D CCD camera (Vds Vosskühler, Osnabrück, Germany) was used for image acquisition. Image analysis was performed using the LUCIA-KARYO/FISH software (Laboratory Imaging, Prague, Czech Republic). The entire hybridized surface area was analyzed in all cases. HPV staining was visualized with a green filter and localized to the nucleus as confirmed by DAPI staining. HPV staining pattern was classified as diffuse, mixed and punctate (Figure 1) as described previously [12,13]. The sample was considered positive for HPV infection if at least one HPV-positive cell was found.

All HPV-positive cells and their pattern were recorded and number of *hTERC* and *MYC* signals was determined for each HPV-positive cell. According to the manufacturer´s instruction (Abbott Molecular, Inc., Des Plaines, IL), the case was considered positive for chromosomal aberration if 4 or more HPV-positive cells demonstrated copy number gain (more than 2 fluorescent signals) of at least one chromosome locus (*hTERC* or *MYC*). Otherwise, case was considered HPV positive but chromosome negative.

In the absence of HPV-positive cells, slide was evaluated for the presence of cells with amplified *hTERC* and/or *MYC* genes. The case was considered positive for chromosomal aberration if more than 5.8% (cut-off value from negative controls at the 95% confidence limit) of cells demonstrated copy number gain (more than 2 fluorescent signals) of at least one chromosome locus (*hTERC* or *MYC*). Otherwise, the case was considered HPV and chromosome negative. According to the HPV and chromosomal findings, patients were classified to the risk group (Figure 2).

Patients with HPV-negative cells and no copy number changes of *hTERC* and *MYC* were classified as low risk. Women who had HPV-positive cells without chromosomal aberration belonged to intermedium risk group and patients with HPV-positive cells and amplification of *hTERC* or *MYC* genes were classified as high risk.

Array-comparative genomic hybridization. Tumor samples were stored at -70°C until DNA isolation. Genomic DNA

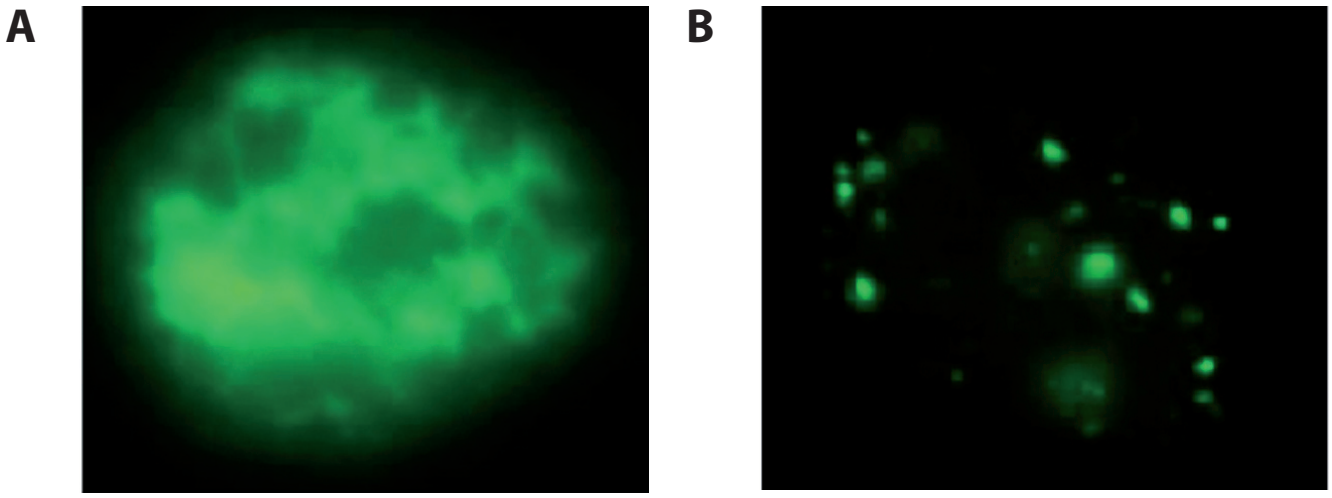

Figure 1. HPV staining pattern using Vysis Cervical FISH Probe Kit
A) Diffuse staining pattern: episomal HPV state
B) Punctate staining pattern: integrated HPV state

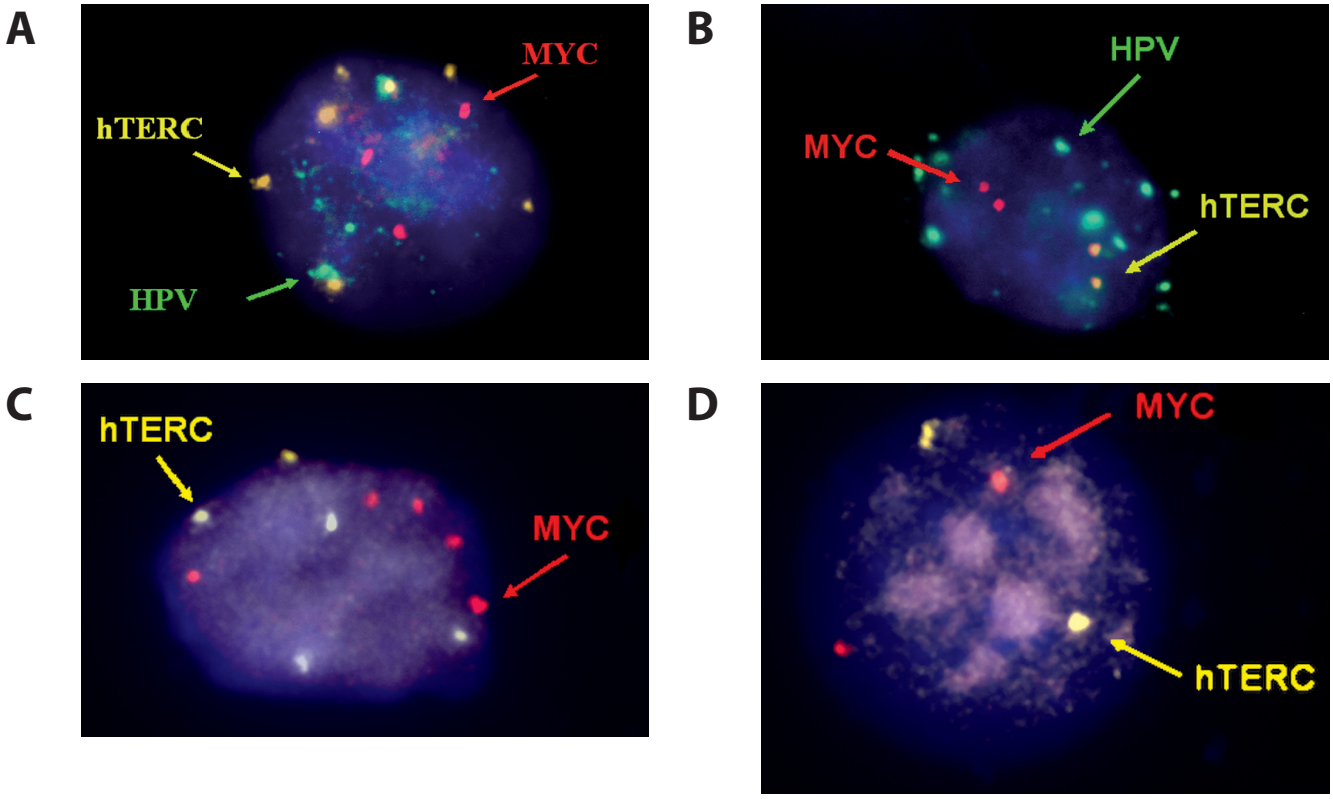

Figure 2. Representative images of HPV staining (green) and chromosome probes for *hTERC* (yellow) and *MYC* (red) signals observed after HPV-FISH assay.
A) HPV-positive cell (green spots) with amplification of *hTERC* (more than 2 yellow spots) and *MYC* (more than 2 red spots) genes – classified as HIGH RISK
B) HPV-positive cell (green spots) without copy number changes of *hTERC* (only 2 yellow spots) and *MYC* (only 2 red spots) genes – classified as INTERMEDIUM RISK
C) HPV-negative cell (no green spots) with amplification of *hTERC* (more than 2 yellow spots) and *MYC* (more than 2 red spots) genes – classified as HIGH RISK
D) HPV-negative cell (no green spots) without copy number changes of *hTERC* (only 2 yellow spots – one is split) and *MYC* (only 2 red spots) – classified as LOW RISK

Table 2. The summary of HPV positivity and the amplifications of the *hTERC* and *MYC* genes in 72 women with a cervical precancerous and cervical carcinomas analyzed by HPV-FISH technique

N (%)	Without pathology (N=12)	precancerous (N=30)	Carcinoma (N=32)
HPV by FISH			
negative	2 (16.7%)	9 (30.0%)	3 (9.4%)
positive	10 (83.3%)	21 (70.0%)	29 (90.6%)
Positive HPV by FISH			
CIN I	-	3 (50.0%) / 6	-
CIN II	-	3 (50.0%) / 6	-
CIN III	-	10 (83.3%) / 12	-
Ca in situ	-	5 (83.3%) / 6	-
Amplification total			
none	9 (75.0%)	14 (46.7%)	4 (12.5%)
MYC	0	1 (3.3%)	0
hTERC	0	5 (16.7%)	8 (25.0%)
MYC and *hTERC*	3 (25.0%)	10 (33.3%)	20 (62.5%)
Risk groups			
Low risk	2 (16.7%)	6 (20.0%)	0
Intermediate risk	7 (58.3%)	8 (26.7%)	4 (12.5%)
High risk	3 (25%)	16 (53.3%)	28 (87.5%)

(gDNA) for array-CGH experiments was isolated using standard phenol extraction. Simultaneous whole-genome analysis of unbalanced chromosomal changes (CNAs) and copy-neutral regions of loss of heterozygosity (cnLOH) in cervical tumors was done using oligonucleotide-based or SurePrint G3 CGH+SNP Array 180K platform (Agilent Technologies, Santa Clara, CA, USA), as described elsewhere. Briefly, 1 – 1.5 μg of reference DNA (Agilent Euro Female) and patient DNA were digested with Alu1 and Rsa1 (Promega, Madison, WI, USA) for 2 hours at 37°C. Fluorescent labeling was done by the SureTag DNA Labeling Kit (Agilent Technologies). Purified and differentially labeled sample and reference DNA were co-hybridized at 65°C for 24 hours to the array. Microarrays were scanned with Agilent SureScan C Scanner with 3 μm resolution, features were extracted using Feature Extraction software (v11.1) and normalized data were analyzed and visualized by Agilent Genomic Workbench v. 7.0.1.4

Statistical analyses. Fisher's exact test was applied when two categorical variables were compared. Mann-Whitney test was used for testing differences in continuous parameters between groups. Statistical analyses were performed using the software IBM® SPSS® Statistics 21.

Results

Molecular cytogenetic analyses of hTERC (3q26) and MYC (8q24) genes amplifications using HPV-FISH

Patients without pathology. The first group of studied women were twelve patients without pathology, three of them (25%) had chronic inflammation and nine (75%) had precancerosis in cytological, not in histological examination. HPV infection was found in 83.3% (10/12) of samples. Chromosomal aberrations were detected in 25% (3/12) of patients. In all 3 cases the amplification of both genes *MYC* and *hTERC* together was found (Table 2).

Following HPV positivity and chromosomal alterations, 16.7% (2/12) of patients were classified as low-risk, 58.3% (7/12) of women as intermediate-risk and 25% (3/12) of patients were put into high risk group.

Patients with premalignant dysplasia. Based on histological findings, specimens of 6 patients with CIN I, 6 patients with CIN II, 12 patients with CIN III and 6 patients with carcinoma *in situ* were examined.

HPV-positive cells were found in 70% (21/30) of women from this subgroup. According to histology, HPV-positive samples were detected in 50% of patients with CIN I and CIN II, and in 83.3% of patients with CIN III and with carcinoma *in situ* (Table 2).

Among thirty patients with precancerosis, copy number changes of *MYC* and *hTERC* genes were found in 53.3% (16/30) of cases. Totally, the amplification of *MYC* gene occurred in 36.6% (11/30) of women and the amplification of *hTERC* gene was detected in 50% (15/30) of patients.

Single amplification of *MYC* gene was detected in 3.3% (1/30) of woman. Solely amplification of *hTERC* gene was detected in 16.7% (5/30) of specimens and the amplification of both genes *MYC* and *hTERC* together was found in 33.3% (10/30) of patients (Table2).

With regards to histological grades, the amplification of *MYC* gene had 33.3% of women with CIN I – CIN III and 50% of women with carcinoma *in situ*. Amplification of *hTERC* gene was detected in 16.7% of patient with CIN I, in 50% with CIN II, in 58.3% with CIN III and in 66.7% of women with carcinoma *in situ* (Table 3).

According to the HPV and chromosomal findings, the risk assessment was set down – 20% of patients were categorized as low-risk, 26.7% of patients as intermediate-risk and 53.3% of patients as high-risk.

Patients with cervical cancer. Furthermore, the 32 women with cervical cancer were also examined by HPV-FISH and HPV infection was found in 90.1% (29/32) of samples.

Chromosomal abnormalities were present in 87.5% (28/32) of patients from this subgroup. Amplification of *MYC* gene occurred in 62.5% (20/32) and amplification of *hTERC* gene was detected in 87.5% (28/32) of patients (Table 2). Totally, no solely amplification of *MYC* gene was detected, 25% (8/32) of specimens had single amplification of *hTERC* gene and the amplification of both *MYC* and *hTERC* genes together was noticed in 62.5% (20/32) of patients.

Following HPV-positivity and chromosomal alterations 87.5% of patients were classified as high-risk and 12.5% of women as intermediate-risk. No patient with cervical cancer was included in the low-risk category.

Table 3. Comparison of *hTERC* and *MYC* amplifications in 30 patients with a cervical precancerous according to histological grade

N (%)	Histology of precancerous (N=30)				
	CIN I (N=6)	CIN II (N=6)	CIN III (N=12)	Ca in situ (N=6)	p[1]
Amplification					
none	4 (66.7%)	3 (50.0%)	5 (41.7%)	2 (33.3%)	0.225
MYC	1 (16.7%)	0	0	0	
hTERC	0	1 (16.7%)	3 (25.0%)	1 (16.7%)	
MYC and *hTERC*	1 (16.7%)	2 (33.3%)	4 (33.3%)	3 (50.0%)	
none *MYC*	4 (66.7%)	4 (66.7%)	8 (66.7%)	3 (50.0%)	0.663
MYC	2 (33.3%)	2 (33.3%)	4 (33.3%)	3 (50.0%)	
none *hTERC*	5 (83.3%)	3 (50.0%)	5 (41.7%)	2 (33.3%)	0.109
hTERC	1 (16.7%)	3 (50.0%)	7 (58.3%)	4 (66.7%)	
Risk groups					
Low risk	2 (33.3%)	2 (33.3%)	1 (8.3%)	1 (16.7%)	0.431
Intermediate risk	2 (33.3%)	1 (16.7%)	4 (33.3%)	1 (16.7%)	
High risk	2 (33.3%)	3 (50.0%)	7 (58.3%)	4 (66.7%)	

[1] p-value of a Kruskal-Wallis test with histology as continuous variable coded as CIN I=0, CIN II=1, CIN III=2, Ca in situ=3

We also analyzed *MYC* and *hTERC* genes amplifications in relation to standard clinical parameters. According to the histopathological grade of tumors, *MYC* amplification occurred more frequently in specimens of spinocellular carcinoma (65.0%; 13/20) than adenocarcinoma (35.0%; 7/20) and this association was statistically significant (p=0.029) (Table 4). Also a trend between *MYC* gene amplification and tumor size was detected – carcinomas in T1b1, T1b2b and T2b stage had gain of *MYC* more often (p=0.092).

Lymph node space invasion (LVSI) was observed in 40.6% (13/32) of patients with cervical carcinomas.

Amplification of *MYC* gene in this subgroup was found in 53.8% (7/13) of women and amplification of *hTERC* gene occurred in 92.8% (12/13) of patients while in patients without LVSI the incidence was 68.4% (13/19) for *MYC* gene and 84.2% (16/19) for *hTERC* gene amplification. Comparison of *MYC* and *hTERC* genes amplification between LVSI positive and negative cohort showed no statistical significance (p=0.434).

Similar results were obtained in patients affected with metastases in regional lymph nodes (LNM). We observed amplification of *MYC* and *hTERC* genes in patients with (6) and without (21) LMN (50.0% vs. 67.0%; 100% vs. 86%, respectively), however the differences did not meet statistical significance (p=0.371). Thus, the frequency of amplification was not associated with metastatic behavior.

Comparison of hTERC and MYC genes amplification between patients with premalignant lesions and patients with cervical cancer. Detailed analysis of chromosomal findings in patients with premalignant lesions and cervical cancer showed that simultaneous *hTERC* and *MYC* genes amplification was significantly more frequent in samples of cervical carcinomas than in specimens of premalignant lesions (62.5% vs. 33.3%; p=0.008). Likewise, solely amplification of *hTERC* gene was significantly (p=0.002) more frequently detected in patients with cancer (87.5%) than in women with precancerous stage (50%). The analysis of risk group distribution (Table 2) revealed that patients with premalignant dysplasia fell within all three groups, while majority of women with cervical cancer belonged to high-risk group (87.5%; p=0.003).

Mapping hTERC and MYC genes amplification by High-Density Oligonucleotide Array-CGH. A subset of 26 cervical carcinoma cases was also analyzed using high-density oligonucleotide array-CGH for the detection of *hTERC* and *MYC* genes amplifications. These arrays were performed to confirm the presence of genetic abnormalities detected by

Table 4. Statistically significant associations of the histology of a carcinoma and the amplification of *MYC* gene

	Amplification total[1]				Amplification of *MYC* gene[1]		
	non (N=4)	*hTERC* (N=8)	*MYC* and *hTERC* (N=20)	p[2]	negative (N=12)	positive (N=20)	p[2]
Histology of carcinoma							
spinocellular carcinoma	4 (100.0%)	8 (100.0%)	13 (65.0%)	0.090	12 (100.0%)	13 (65.0%)	**0.029**
adenocarcinoma	0	0	7 (35.0%)		0	7 (35.0%)	

[1] described by absolute count and relative frequencies – N (%)
[2] p-value of a Fisher's exact test

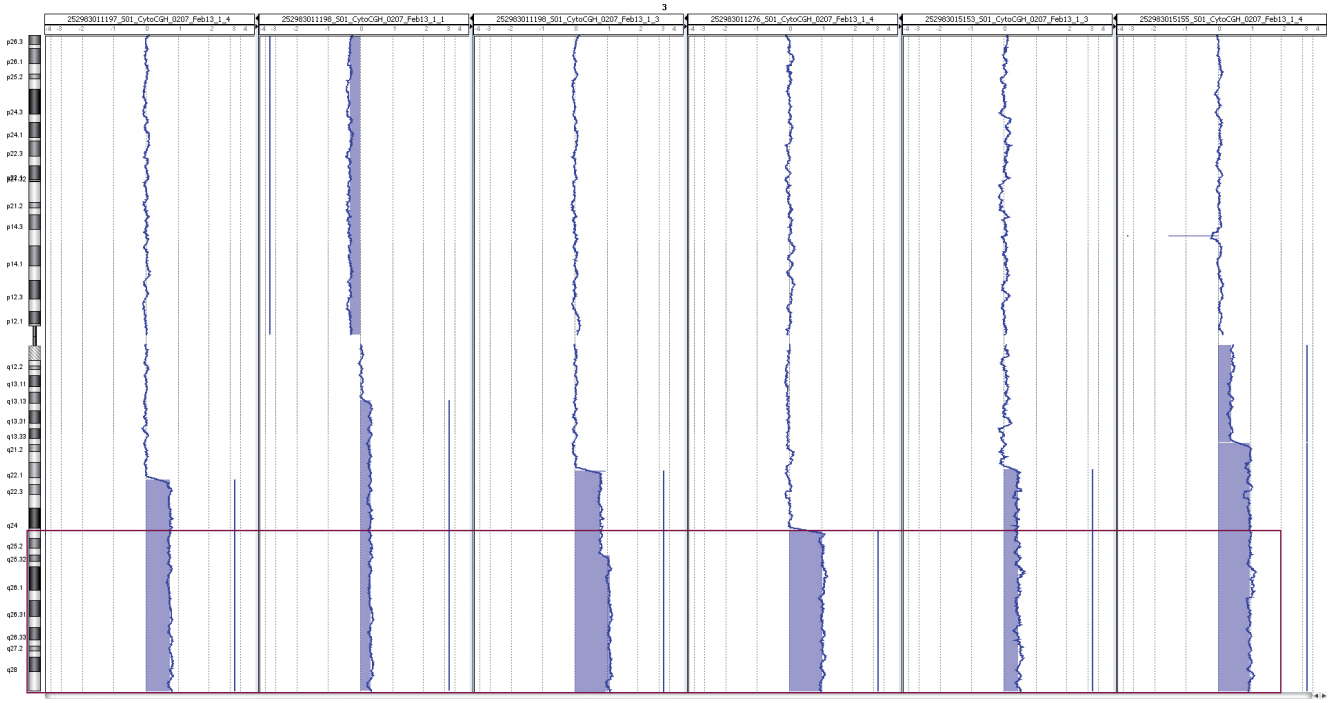

Figure 3. Graphical overview of minimal region of gain (MDR) in chromosome 3q in 6 patients with cervical carcinoma. Highlighted box shows minimally gained region 43.6 Mb of size between 3q25.1-3qter carrying *hTERC* locus.

HPV-FISH and to define the size and the character of these genomic changes.

In our dataset, the recurrent gain in chromosome 3q26 area carrying *hTERC* was observed in 50% (13/26) of samples. In 23.1% of cases (6/26), we found minimal region of genetic material gain (MGR) of 43.6 Mb between 3q25.1-3qter, with amplification (>5 copies) of *hTERC* loci in 11.6% (3/26) of samples (Figure 3). In addition, duplication of whole 3q arm was detected in 19.2% (5/26) of cases; in 2 cases we observed trisomy of chromosome 3.

Incidence of genetic material gain in *MYC* locus (8q24.3) was observed in 15. 4% (4/26). In 2 cases we found trisomy of chromosome 8 and 2 cases had gain of whole 8q arm.

Discussion

Cervical cancer remains the second most common malignancy among women in the Czech Republic with the incidence 19/100000. It is known that the infection of cervical epithelial cells with HPV is necessary but still insufficient for neoplastic progression.

Genetic abnormalities play an important role in tumor transformation etiology. Characteristic marker of malignant tumors is chromosomal instability, either numeric or structural, having prognostic and predictive impact in many tumor sites. In cervical malignant and premalignant lesions and cervical carcinoma cell lines, specific chromosomal changes were repeatedly found, mainly in regions 3q26 (*hTERC* gene) [14] and 8q24 (*MYC* gene) [15]. According to published data, 80 – 90% of cervical carcinoma cases are characterized by an amplification of human telomerase RNA gene (*hTERC*) [16]. This specific genetic abnormality was found also in premalignant CIN II/CIN III and is considered as a genetic aberration occurring in early stages of the tumor development with value to predict malignant transformation and progression of the disease [8,14,17].

Another specific genetic abnormality linked to the development of cervical carcinoma is *MYC* gene amplification. Golijow *et al.* [15] showed that there is a difference among the number of *MYC* copies in CIN I, CIN II/CIN III and carcinoma *in situ* and thus the amplification of *MYC* gene is not important only in progression of the tumor, but even in the cell transformation during pre-invasive stages.

The principal aim of this study was to optimize the molecular cytogenetic technique, i.e. HPV-FISH assay, originally developed by Sokolova *et al.* [12] and to evaluate HPV infection/*hTERC*/*MYC* genes amplification in routinely collected cytology samples obtained from 74 patients with cervical dysplasia or cervical carcinomas. The Vysis Cervical FISH Probe Kit contains multi-color FISH probes for *hTERC* (3q26) and *MYC* (8q24) genes together with an HPV probe cocktail containing 6 HPV types with homology to the majority of high-risk types. In our study, we verified utilization of this kit as a robust tool for subclassification of patients into low-, intermediate- and high risk subgroups and identifying risk of progression from mild and moderate stages to high grade dysplasia.

Although HPV-positive cells were observed in our study in the group of women without pathology, 75% (9) patients had no amplification of *MYC* or *hTERC* genes. It suggests that these HPV infected cells will be eventually eliminated by immune system; spontaneous regression comes in about 95% of HPV infected cells [18]. However, simultaneous amplification of *hTERC* and *MYC* detected in 25% (3) of women without pathology indicates that patients with high risk can also be in this group. Jiang *et al.* and Chen *et al.* [19,20] analyzed *hTERC* gene in 178 and 164 patients without oncological finding and the amplification of this gene was found in 6.2% and 9.2% respectively. Our observed results can be caused by small number of tested women. It is highly possible that these double-positive cells will progress to premalignant lesions. Study of Heselmeyer-Haddad *et al.* [7] proved that cells with 3q gain are not able to regress spontaneously and they have persistent infection of HPV. Thus, it is necessary to provide regular check-ups of these women and in case of progression to take therapeutic measures.

In the group of patients with premalignant lesions (CIN I – III, carcinoma *in situ*), the incidence of HPV-positive cells is in correlation with increasing grade of intraepithelial neoplasia. HPV-positive samples were detected in 50% of patients with CIN I, in 50% of women with CIN II, in 83.3% of patients with CIN III and in 83.3% of women with carcinoma *in situ*. Positive correlation between histological stage and incidence of *hTERC* amplification was also found – amplification of *hTERC* was detected in 16.7% of patient with CIN I, in 50% of women with CIN II, in 58.3% of patients with CIN III and in 66.7% of women with carcinoma *in situ*. This trend was also observed by other teams. Heselmeyer-Haddad *et al.* [7] found positivity for extra copies of 3q in 7.1% (1 of 15) of CIN I samples, 62.5% (5 of 8) of CIN II samples and 76.4% (13 of 17) of CIN III samples. Chen *et al.* [20] observed *hTERC* amplification in 17.2% (5/29) of CIN I patients, in 76.2% (16/21) CIN II patients and even in 100% (22/22) patients with CIN III. Another two Chinese studies also confirmed that increase of *hTERC* amplification was correlated with higher histological grade of premalignant lesions. In large study, Jiang *et al.* [19] examined specimens of 4851 women with premalignant lesions, positivity of *hTERC* gain was detected in 20.8% of women with CIN I, in 68.6% patients with CIN II and in 82.4% women with CIN III. Similarly, in study of Jin *et al.* [9] amplification of *hTERC* occurred in 18.2% (6/33) of CIN I patients, in 66.7% (6/9) of CIN II patients and in 84.6% (22/26) of CIN III patients.

These findings confirmed that amplification of *hTERC* gene in patients with precancerous cervical lesions is a strong prognostic marker of progression of the disease and this biomarker should be used in clinical diagnostics as a preventive and routine screening tool.

The *MYC* gene amplification was detected in patients of all precancerous grades (CIN I – CIN III 33.3%; carcinoma *in situ* 50%). The 8q24 gain confirms the integration of HPV into cell genome because the activation and overexpression of *MYC* gene start in cells with integrated virus [21]. Chen *et al.* [10] detected the amplification of *MYC* gene in 31% of patients in CIN I grade, in 71.4% of patients in CIN II grade and in 81.8% of patients in CIN III grade. In our set, increasing incidence of *MYC* amplification correlating with higher CIN grade was not proved; one of the possible explanations could be the small number of examined patients.

Using molecular cytogenetic analyses, HPV infection was found in 90.1% of patients with cervical cancer. Chromosomal change of *MYC* gene occurred in 62.5% and amplification of *hTERC* gene was present in 87.5% of patients from this group. Comparison of *hTERC* and *MYC* genes amplification rates between patients with intraepithelial neoplasia and patients with cervical carcinoma showed significantly higher frequency of these alterations in carcinoma samples.

The amplification of human telomerase RNA gene was shown to be the most frequent aberration in cervical cancer; more than 85% invasive cervical carcinomas carry this gain [7]. Jiang *et al.* [19] observed *hTERC* amplification in 94.9% of patients with squamous cell carcinoma, Yin *el al.* [22] detected this alteration even in 100% of women with invasive carcinoma of cervix uteri. Other subsequent studies [9,16,17,20,23] also proved gain of 3q chromosome arm as the most common aberration.

From the clinical view, we tested the diagnostic and prognostic value *of MYC* and *hTERC* genes alterations in cervical cancer patients detected by HPV-FISH.

In our study, *MYC* gene amplification was found to be significantly more frequent in specimens of spinocellular carcinomas than adenocarcinomas. Similar findings were described by Jin *et al.* [9] and Chen *et al.* [10], who studied the role of *MYC* protooncogene in origin and progression of cervical carcinomas. Recently, Wright *et al.* confirmed [24] that spinocellular carcinomas and adenocarcinomas have distinctly different molecular profiles that may explain the observed clinical differences.

A correlation between *MYC* gene amplification and tumor size was also detected – our samples of carcinomas in T1b1, T1b2b and T2b stages had the gain of *MYC* gene more often. However, this correlation needs to be proved on a larger set of patients. Also Abba *et al.* [25] and Eid *et al.* [26] found significant correlation between *MYC* gene amplification and tumor grading. Lymphovascular space invasion is an important independent prognostic factor in cervical carcinoma diagnosis, and it is in direct correlation with the incidence of lymph node metastases [27,28].

Statistical analysis of our results did not reveal relationship between *hTERC/MYC* genes amplification rate and lymphangioinvasion or presence of metastasis in lymphatic nodes. However, study of Riou *et al.* [29] found significant relation between *MYC* overexpression and higher risk of distant metastases and relapse in set of women with early stages of carcinoma. Kim *et al.* [30] consider *MYC* gene amplification to be a prognostic factor of DFS (disease-free survival) in patients with operable IB – IIB stage of carcinoma.

In patients with cervical cancers we need further studies with *hTERC* and *MYC* genes evaluation to confirm that these genetic biomarkers are associated with a more advanced grade or can predict a risk of spread outside primary localization and determine probability of disease recurrence.

Analysis of *hTERC* and *MYC* loci with array-CGH showed that both changes are presented as a part of complex genetic lesions in genome of tumor cells in cervical carcinoma patients. Our results showed that both *hTERC* and *MYC* genes amplifications are mostly part of larger areas gain in chromosome arms, or even whole chromosomes. Amplification of *hTERC* gene in our cohort was observed most frequently as a minimal region of gain in 3q25 – 3qter in 23.1% (6/26), while in 19% (5/26) we found gain of whole 3q arm and 2 cases with trisomy of chromosome 3. Similar observations were made by other groups when array-CGH technique was used MRG defined slightly different between 3q24–3q26 [31-33]. This discrepancy can be explained by utilization lower resolution BAC microarrays with combination of FFPF samples. Likewise, incidence of *MYC* gene amplification was connected with chromosome 8 trisomy (2 cases) or whole 8q gain (2 cases).

In conclusion, our results showed that analysis of *hTERC* and *MYC* genes as the specific genetic biomarkers is important and has a great diagnostic and prognostic potential both in patients with cervical precancer and cervical carcinoma. The testing of *hTERC* and *MYC* gene amplification by HPV-FISH in cervical lesions might be a supplementary to cytology screening and HPV test, especially in high-risk patients. Based on HPV infection presence and results of molecular cytogenetic examination we can better specify intraepithelial dysplasia, predict its regression or progression to invasive cancer and choose an appropriate therapeutic procedure.

In patients with cervical cancers we need further studies with *hTERC* and *MYC* genes evaluation to confirm that these genetic biomarkers are associated with a more advanced grade or can predict of risk of spread outside primary localization and determine probability of disease recurrence.

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