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Detection of oncogenic mutations in cervical carcinoma using method High Resolution Melting (HRM)

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Oncogenic mutations in proto-oncogenes and tumor suppressor genes represent one of key events in cancerogenesis. In this study, we analysed mutation status in *PIK3CA, KRAS* and *EGFR* proto-oncogenes and *TP53* tumor suppressor gene in a cohort of twenty-four patients diagnosed with squamous cell carcinoma or adenocarcinoma using the screening method "High Resolution Melting" (HRM). Positive findings were confirmed and identified by Sanger sequencing. Totally, we detected DNA sequence changes in targeted regions in seven patients (7/24, 29.2%). In *PIK3CA* gene, we found six sequence changes in four patients (4/24, 16.7%) and four of them were confirmed as oncogenic mutations. In *KRAS* gene, we detected sequence changes in four patients (4/24, 16.7%). Conversely, we identified pathogenic or potentially pathogenic sequence changes neither in *EGFR* nor *TP53* genes. Our results suggest that sequence changes are specific neither for a certain histological subtype, clinical stage nor lymph node involvement and they appear independently on the presence of HPV (human papillomavirus) infection since early clinical stages. We observed the correlation between the presence of DNA sequence changes and *hTERC* gene amplification, but we did not find a significant relationship between the identified DNA sequence changes and detected copy-number alterations using the technique of array-CGH (array-based comparative genomic hybridization).

Regardless our results confirmed an important role of oncogenic mutations in *PIK3CA* and *KRAS* genes in the neoplastic transformation process in the cervical carcinoma pathogenesis. Their identification in the early clinical stages should encourage further studies to better understand these mutations and exploit them for more detailed diagnostics.

Key words: cervical carcinoma, mutation, high resolution melting, Sanger sequencing

Cervical carcinoma represents one of the most frequent causes of death due to malignancy in women. The overall incidence rate is around 10.6/100 000, its mortality reaches 7/100 000 women in Europe, however, these numbers differ in various regions [1]. Thanks to the general preventive screening programme and early detection of this disease, especially in its precancerous condition, we can observe a slight decrease of the mortality rate [2]. The early detection of a malignant disease especially in its precancerous condition enables to apply an effective therapeutic strategy with a minimum of side effects and to prevent the tumor development to its metastatic phase.

In 70's, the causality between the human papillomavirus infection (HPV) and neoplastic transformation of human cervical epithelial cells was described. More than 100 subtypes of HPV were described and based on their association with the disease development, they can be divided into high-risk and low-risk subtypes. The presence of high-risk subtypes HPV 16 and 18 is detected in about 70% of patients with cervical carcinoma [3]. Viral proteins deregulate the cell cycle through their binds to proteins which act as tumor suppressors, cyclins and cyclin-dependent kinases [4]. After the elimination of these regulation proteins, damaged and genetically instable cells can survive and this leads to the higher risk for their malignant transformation and progression to more aggressive disease phases.

The process of the malignant transformation affects the cells of the squamous epithelium (squamous cell carcinomas) and adenomatous epithelium (adenocarcinomas) in the uterine cervix. Both the groups differ in their genetic and molecular profiles which results in different clinical characteristics [5]. Adenocarcinomas appear to be related to adverse prognosis with a larger number of distant metastases [6].

Cancer cells in both types of cervical carcinoma are characterized by a wide spectrum of genetic abnormalities, from chromosomal copy-number changes to changes in DNA sequence in single genes [7, 8]. The simultaneous presence of *hTERC* gene amplification (3q26) and *MYC* gene amplification (8q24) was detected recurrently in multiple samples. In previous studies, the significance of mutations in proto-oncogenes and tumor suppressor genes in the initiation and progression of cervical carcinoma was proved, whereas recurrent mutations were identified in mutation "hotspots" in *PIK3CA* (p.R88Q in exon 1, p.E542K, p.542K, p.Q546E, p.Q546H in exon 9, p.M1043I and p.H1047R in exon 20) [9, 10], *KRAS* (p.G12A, p.G12D, p.G12V, p.G13D in exon 1) [10], *EGFR* proto-oncogenes (exons 18-21) [11] and in *TP53* tumor suppressor gene (codons 173-181, 245-249, 273-282) [12, 13].

Mutations in *PIK3CA* gene have an activating oncogenic character with the frequency between 8-31% in invasive cervical carcinomas [10, 14, 15]. They are typically detected in older patients and therefore they represent a risk factor for the malignant transformed and HPV-infected cell survival. They have not been detected in precancerous cervical cells so far, which may indicate their possible role in progression in the course of early and late disease stages [16]. Moreover, the correlation between the HPV presence and mutation in *PIK3CA* gene and between the mutation presence and histological subtype of the tumor has not been proved so far [9, 10].

Mutations in *EGFR* gene lead to the signalling pathways activation [17] but they rather represent a rare genetic hit in the cervical carcinoma pathogenesis. They have been identified exclusively in squamous cell carcinomas so far, which may indicate the existence of various molecular mechanisms of the cancerogenesis in certain histological subtypes. The important role of the increased activation of *EGFR* gene was described in multiple studies but predominantly as a result of gene amplification [18].

The importance of *KRAS* gene and its activating mutations have been described in multiple tumor pathogeneses including cervical carcinoma. Based on the results of multiple studies, its mutation frequency in cervical carcinoma ranges between 8-18% whereas considering the histological subtype mutations in *KRAS* gene have been identified predominantly in adenocarcinomas [10, 19, 20]. In squamous cell carcinomas *KRAS* gene mutations have been detected rarely. Due to their rare detection the correlation between mutations in *KRAS* gene and the adverse survival has not been unfailingly observed so far [10].

Somatic mutations in *TP53* gene have been identified in a majority of human cancer types with variable frequency. More than 80% of all mutations occur in exons forming DNA-binding domain. In contrast to ovarian, colorectal or oesophageal cancer with *TP53* mutation frequency about 50%, they represent a rather rare genetic event in cervical carcinoma [21]. Due to generally low mutation frequency, its inactivation is mediated by more mechanisms, e.g. the interaction between protein p53 and HPV protein E6 and after that p53 is degraded. Acquired mutations in *TP53* gene are related to the gain of the aggressive tumor cell phenotype [22, 23]. Considering the histological subtype of the tumor, a higher mutation frequency of *TP53* gene in adenocarcinomas (about 13%) in comparison to squamous cell carcinomas (about 6%) have been proved.

Activating mutations in *PIK3CA, KRAS* proto-oncogenes, an increased expression of *EGFR* proto-oncogene and inactivating mutations in *TP53* gene are related to the adverse prognosis of the disease [15, 16, 24]. Their identification means potential targets in the research, development and improvement of targeted and personalized therapy.

The aim of this pilot study was to analyse mutation status in *PIK3CA, KRAS, EGFR* proto-oncogenes and *TP53* tumor suppressor gene using a screening method "High Resolution Melting" (HRM). Consequently we evaluated the results of mutation analysis in the relationship to clinical characteristics of the disease. Simultaneously, we studied possible correlations between the gene mutations or DNA sequence changes and the presence of chromosomal abnormalities which were detected by methods of HPV-FISH (fluorescence *in situ* hybridization for the detection of human papillomavirus in cells) and array-CGH (array-based comparative genomic hybridization) realized in the scope of our previous studies [7, 25].

Materials and methods

In our study, we analysed cervical carcinoma samples from twenty-four patients diagnosed at the Department of Gynecologic Oncology in the Masaryk Memorial Cancer Institute (median age 42 years, range 38-63 years) which were obtained since 2009 to 2013.

All samples were obtained only after the patients signed the informed consent approved by the Ethical committee of the MMCI and were immediately frozen in liquid nitrogen. Patients after surgical procedures and any adjuvant treatment were monitored in regular intervals according to onco-gynecological guidelines [26]. The basic clinical characteristics of the analysed cohort of patients are summarized in Table 1.

Genomic DNA samples for the molecular cytogenetic and molecular genetic purposes were isolated using standard phenol extraction and consequently, we evaluated their quality and quantity by DNA agarose electrophoresis with TrackIt™ 1 Kb Plus DNA Ladder (Thermo Fisher Scientific) (Figure 1) and NanoDrop® ND-1000 (Thermo Fisher Scientific) and Qubit® 1.0 (Thermo Fisher Scientific).

Molecular cytogenetic analysis by FISH technique was accomplished using the commercial kit Vysis Cervical FISH Probe (Abbott Molecular, Illinois, USA). The kit enables the simultaneous detection of HPV-infected cells and the amplification of chromosomal regions 3q26 (*hTERC* gene) and 8q24 (*MYC* gene) as described previously [7, 25]. For the whole-genome screening of chromosomal abnormalities by array-CGH, we used a platform of oligonucleotide-based

DNA microarrays SurePrint G3 CGH+SNP Array 180K (Agilent Technologies, Santa Clara, CA, USA). The methodology and complete results are described in details in Kuglik *et al* (2014) [7].

Mutation status of given genes was analysed using the screening method "High Resolution Melting" (HRM) followed by Sanger sequencing as a routinely used method for the confirmation and exact identification of DNA sequence changes. For our purpose, we used the commercially available reaction kit MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and termocycler StepOne™ Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). For the primer design for real-time PCR with HRM analysis and their verification, we used bioinformatic databases NCBI and UCSC Genome Browser and online tools Primer3, UCSC In-Silico PCR, PrimerBlast and uMelt℠ – DNA Melting Curve Prediction. Using these databases and online tools, we designed twelve primer pairs (three pairs for mutation "hotspots" in *PIK3CA* gene, one pair for mutation "hotspot" in exon 1 in *KRAS* gene, five pairs for exons 18-21 in *EGFR* gene and three pairs for mutation "hotspots" in *TP53* gene). Primers were synthetized by Sigma Aldrich (St. Louis, MO, USA). Consequently, we confirmed their specificity by DNA agarose electrophoresis (1,2% gel, 100 V/60 min). Their overview is presented in Supplementary Table 1.

The primer design, sample preparation and experimental conditions were set according to the manufactory recommendation (Thermo Fisher Scientific, Waltham, MA, USA). Data were processed using the StepOneTM Software v2.3 and High Resolution Melt Software v3.0.1 (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples tested positively via mutation screening by HRM were consequently processed for Sanger sequencing. First, we prepared long DNA amplicons using the second primer set which were designed for the purposes of standard PCR (Promega, Madison, WI, USA) and Sanger sequencing. PCR products were purified using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase following manufactory recommendation (Thermo Fisher Scientific, Waltham, MA, USA). Primers were synthetized by Sigma Aldrich (St. Louis, MO, USA). The overview of primer pairs for standard PCR and Sanger sequencing is presented in Supplementary Table 2.

Consequently, we performed the termination reaction to obtain ssDNA fragment library for Sanger sequencing (BigDye® Terminator v3.1 Cycle Sequencing Kit following manufactory recommendation, Thermo Fisher Scientific, Waltham, MA, USA). Sequencing reactions were performed in

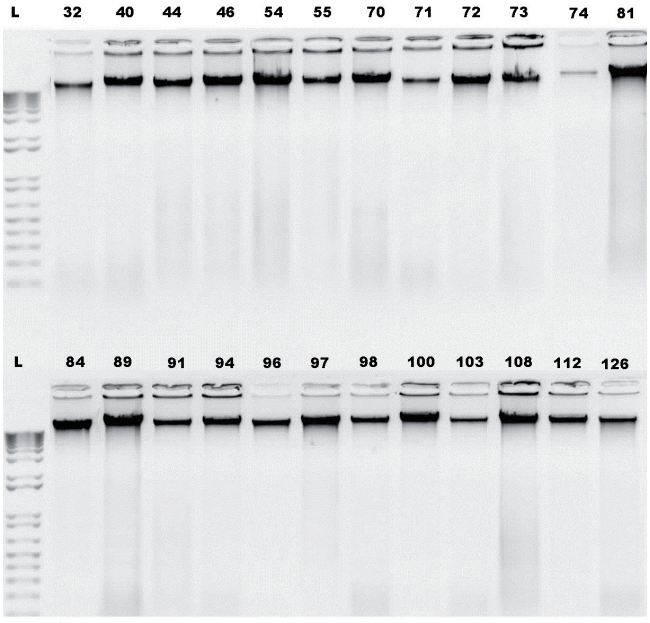

Figure 1. DNA agarose electrophoresis with genomic DNA samples. The DNA samples integrity was estimated to be sufficient for HRM and PCR-based analyses. Glossary: L – TrackIt™ 1 kb Plus DNA Ladder, 32-126 – DNA samples.

Table 1. The clinical and molecular cytogenetic characteristics of the cohort of 24 patients with cervical carcinoma.

Feature	Parameter		Incidence
histological subtype	adenocarcinoma		6 (25%)
	squamous cell carcinoma		18 (75%)
HPV status	positive		20 (83.3%)
	negative		4 (16.7%)
clinical stage (FIGO)	I	IA1	1 (4.2%)
		IA2	2 (8.3%)
		IB1	11 (45.8%)
		IB2	2 (8.3%)
	II		0 (0%)
	III	IIIB	7 (29.2%)
	IV	IVA	1 (4.2%)
lymph node with metastases	0		14 (58.3%)
	1		2 (8.3%)
	2		1 (4.2%)
	3		1 (4.2%)
	4		1 (4.2%)
	7		1 (4.2%)
	13		1 (4.2%)
	not investigated		3 (12.5%)
MYC gene amplification (FISH)	amplification		15 (62.5%)
	no amplification		8 (33.3%)
	not investigated		1 (4.2%)
hTERC gene amplification (FISH)	amplification		20 (83.3%)
	no amplification		3 (12.5%)
	not investigated		1 (4.2%)
copy-number alterations (CNAs) (array-CGH)	0-5		14 (58.3%)
	6-10		6 (25%)
	11-15		1 (4.2%)
	more than 15		3 (12.5%)

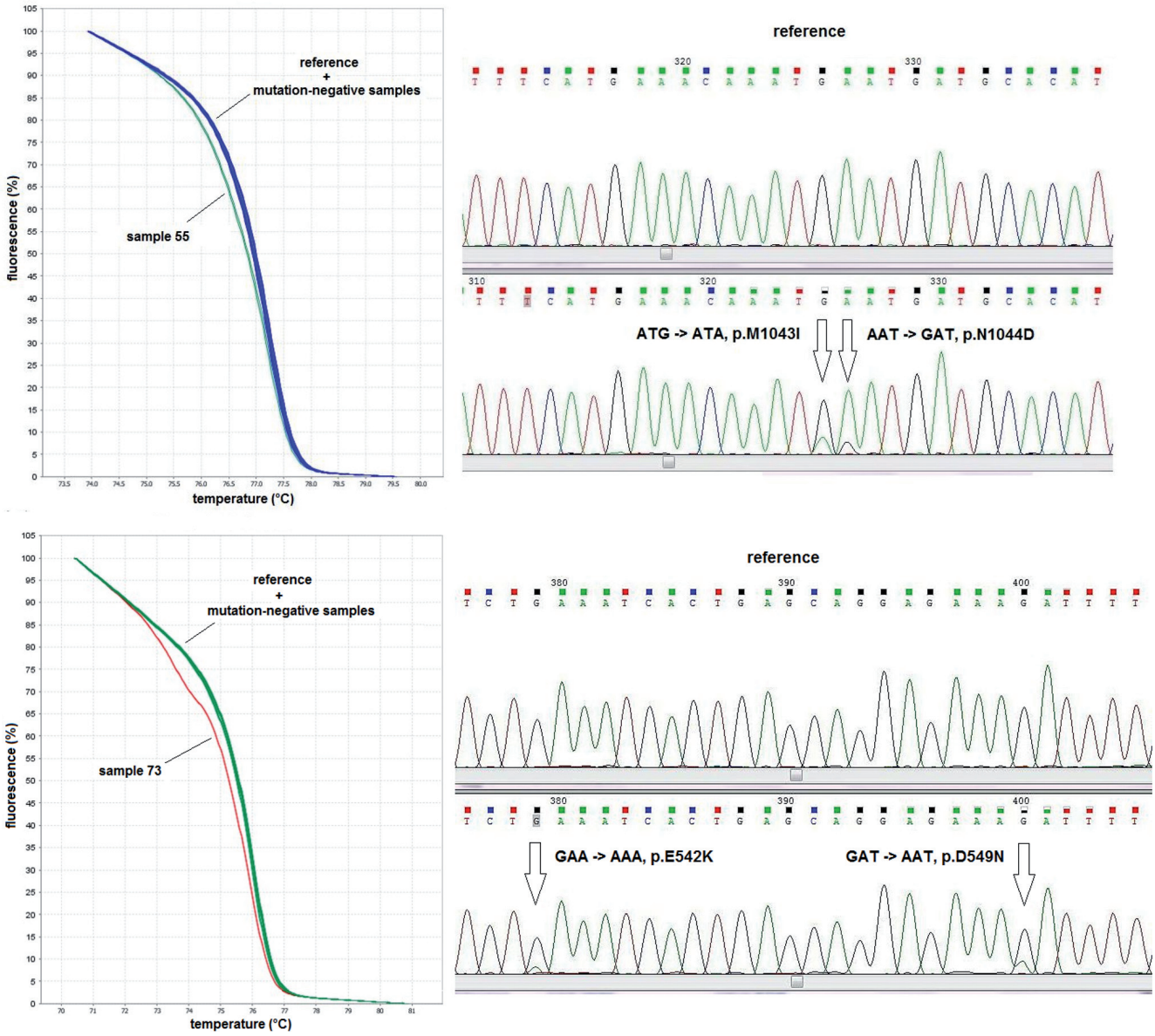

Figure 2. Mutation analyses of "hotspot" regions in *PIK3CA* gene using methods HRM (left) and Sanger sequencing (right). There is shown a melt curves shift in samples 55 (up) and 73 (down) in comparison with reference DNA sample and patients' samples with no DNA sequence changes. On the right there are shown graphical results from Sanger sequencing.

the capillary electrophoresis DNA sequencer ABI 3130 (Thermo Fisher Scientific, Waltham, MA, USA). Chromatograms were analysed using the software Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and Chromas Lite v2.1 (Technelysioum Pty Ltd, South Brisbane, Australia). To predict an effect of mutation, we used online tool PROVEAN (http://provean.jcvi.org/index.php) with threshold -2.5 (a higher value predicts a neutral effect and a lower or threshold value predicts a pathogenic effect, http://provean.jcvi.org/about.php#about_1b).

Results

In this pilot study, we analysed DNA sequence changes in mutation "hotspots" in *PIK3CA, KRAS, EGFR* proto-oncogenes and *TP53* tumor suppressor gene in the tumor DNA samples obtained from twenty-four patients with cervical carcinoma.

In the course of mutation "hotspots" analyses in *PIK3CA* gene, we detected differences in melting curve profiles compared to reference DNA melting curve profile in four patients.

Consequently, we managed to identify four point heterozygous mutations by Sanger sequencing. In one patient, we detected two DNA sequence changes resulting in the substitution of glutamic acid to lysine in the position 542 on the protein level (p.E542K) and aspartic acid to asparagine in the position 549 on the protein level (p.D549N). In the other patient, again, we detected two DNA sequence changes characterized by the substitution of methionine to isoleucine in the position 1043 on the protein level (p.M1043I) and asparagine to aspartic acid in the position 1044 on the protein level (p.N1044D). Based on the result of Sanger sequencing, it is clear that all the detected point mutations are likely to occur in minor clones of tumor cells (Figure 2).

We also analysed mutation "hotspots" in *KRAS* and *TP53* genes and mutation occurrence in exons 18, 19, 20 and 21 in *EGFR* gene using HRM method. We detected DNA sequence changes in exon 1 of *KRAS* gene in four patients' tumor DNA samples (4/24, 16.7%). However, we confirmed none of them by Sanger sequencing. In *EGFR* and *TP53* genes, we detected only common benign SNPs and consequently, all of them were proved by Sanger sequencing.

The positive findings, obtained by the mutation screening by HRM and Sanger sequencing, respectively, were analysed in the context to clinical characteristics and results of molecular cytogenetic investigations. DNA sequence changes in *PIK3CA* and *KRAS* proto-oncogenes were detected in five patients with squamous cell carcinoma (5/18, 27.8%) and in two patients with adenocarcinoma (2/6, 33.3%), whereas DNA sequence changes occurring in gene *PIK3CA* were identified only in patients with squamous cell carcinoma. In contrast to gene *PIK3CA* gene mutations in mutation "hotspot" in exon 1 of *KRAS* gene were detected in two patients with squamous cell carcinoma and in two patients with adenocarcinoma as well. Moreover, in these two patients with adenocarcinoma, we did not detect the presence of HPV infection.

The results were consequently evaluated with regard to the clinical stage of the disease according to FIGO classification. Based on the clinical investigation, three patients harbouring either DNA sequence changes or point mutations in *PIK3CA* gene were grouped to the stage I (IA2, IB1) and one patient was grouped to the stage IIIB. Two patients harbouring DNA sequence changes in mutation "hotspot" in *KRAS* gene were grouped to stage I (IA2, IB1) and IIIB, respectively. None of the patients with positive HRM screening results were grouped neither to stages II nor IV. Despite a small group of patients, we can observe a tendency to occurrence of pathogenic/potentially pathogenic mutations in genes *PIK3CA* and *KRAS* since the early stages of cervical carcinomas.

In both patients in the clinical stage IIIB, we observed metastases in two and three lymph nodes, respectively. In the rest of the patients harbouring mutations or DNA sequence changes in *PIK3CA* and/or *KRAS* genes, we did not detect the metastases in any lymph nodes.

Consequently we studied an association between the cytogenetic findings and results obtained by mutation analyses. The *hTERC* gene amplification was observed in all the seven

Table 2. The summary of mutation analysis results in individual patients and their context to clinical data and to results obtained by molecular cytogenetic analyses.

Clinical characteristics					Molecular cytogenetic analyses					Mutation screening by HRM and Sanger sequencing		
ID patient	Histological type	HPV status	FIGO classification	Lymph nodes with metastases	*MYC* gene amplification	*hTERC* gene amplification	Total number of CNAs	Number of losses	Number of gains	Gene	Mutation	Mutation status
54	S	pos	IIIB	2	neg	pos	19	10	9	*KRAS*	nd	-
55	S	pos	IB1	nd	pos	pos	1	0	1	*PIK3CA*	p.M1043I	het
											p.N1044D	het
73	S	pos	IB1	0	pos	pos	19	0	19	*PIK3CA*	p.E542K	het
											p.D549N	het
74	A	neg	IIIB	3	pos	pos	0	0	0	*KRAS*	nd	-
98	S	pos	IA2	0	neg	pos	0	0	0	*PIK3CA*	nd	-
										KRAS	nd	-
100	A	neg	IB1	0	pos	pos	0	0	0	*KRAS*	nd	-
103	S	pos	IIIB	1	neg	pos	1	0	1	*PIK3CA*	nd	-

Glossary: S – squamous cell carcinoma, A – adenocarcinoma, neg – negative, pos – positive, CNAs – copy-number alterations, nd – not defined, het – heterozygous mutation

patients with positive results obtained by mutation analyses (7/7, 100%) as *MYC* gene amplification was detected in four patients (4/7, 57.1%). The simultaneous amplification of *MYC* and *hTERC* genes was identified in both the patients with the closely specified *PIK3CA* gene mutations. In other two patients harbouring DNA sequence changes in *PIK3CA* gene, we did not detect *MYC* gene amplification. Simultaneous *MYC* and *hTERC* genes amplification was detected in two patients harbouring DNA sequence change in mutation "hotspot" in *KRAS* gene. The results obtained by mutation analyses in the context to clinical and molecular cytogenetic data are summarized in Table 2.

All the patients underwent whole-genome screening by array-CGH method for detection of unbalanced chromosomal aberrations (CNAs; Copy-Number Alterations), i.e. losses and gains of genetic material. We assessed the whole-genome profile of losses and gains of DNA sequences for every individual patient. These results were evaluated in context to results obtained using mutation analyses.

We did not find a statistical significance between the number of CNAs and DNA sequence changes in *PIK3CA* and *KRAS* genes. In three patients with detected DNA sequence changes in the course mutation analyses, no CNAs was found and we detected one CNA (gain 8q and gain 17q, respectively) in two patients. In other two patients, we found more than fifteen CNAs whereas in one patient harbouring DNA sequence change in *KRAS* gene, we identified ten losses and nine gains of genetic material in chromosomal regions and in the other patient harbouring point mutations in *PIK3CA* gene (p.E542K a p.D549N), we detected nineteen gains of genetic material in chromosomal regions. The complete results of genomic profiling of our cohort of patients by array-CGH method was described elsewhere [7]. The summary of results of mutation analyses and their context to clinical data and results obtained by HPV-FISH and whole-genome screening by array-CGH as well is listed in Table 2.

Discussion

In the course of our previous studies, we proved that specific genomic alterations, especially gains of genetic material in *MYC* and *hTERC* gene loci together with HPV infection play an important role in the neoplastic transformation of epithelial cells of uterine cervix[7]. We steadily continue with this study which is focused on mutation status analyses of *PIK3CA, KRAS* and *EGFR* proto-oncogenes and *TP53* tumor suppressor gene using HRM screening method. Totally, we found point mutations or potentially pathogenic DNA sequence changes in seven patients. In four patients, we detected DNA sequence changes in two mutation "hotspots" in exons 9 and 20 of *PIK3CA* gene and in four patients, we identified DNA sequence changes in mutation "hotspot" in exon 1 of *KRAS* gene.

PIK3CA gene encodes p110α catalytic subunit (110kDa) of phosphatidylinositol 3-kinase (PI3K), an essential part of signalling pathway PI3K/AKT/mTOR which is important for cell cycle progression, cell proliferation and survival, as well. It was proved that dysregulation of this signalling pathway by various genetic mechanisms results in its constitutive activation leading to changes in regulation of essential cell processes. Beside activating point mutations affecting *PIK3CA* gene, its amplification was observed as an activating mechanism in some types of tumors, as well [27]. In this study, we analysed its three mutation "hotspots" and their adjacent DNA sequences. We detected DNA sequence changes in four patients (16.7%) and in two of them, we described these DNA sequence changes as four point heterozygous mutations p.E524K, p. D549N, p.M1043I a p.N1044D. Mutation p.E542K has been already proved as pathogenic with constitutively activating effect in PI3K/AKT/mTOR signalling pathway in a large number of tumor diseases [28]. In contrast to this well characterized point mutation, the other mutation p.D549N has been described only in a few cases of colorectal carcinoma, breast or endometrial carcinoma so far [29-31]. Concerning to its low frequency, the information about its effect in the protein function is still absent. Therefore, based on *in silico* prediction by PROVEAN, we evaluated the mutation effect as neutral on the protein function (PROVEAN score -2,355). Whyte *et al.* (2006) identified this mutation in tumor cell line HCT-15 derived from large intestine and in the same sample, they detected point mutation p.E545K previously characterized as pathogenic, with constitutively activating effect on kinase AKT [32-34]. In our study, we detected mutation p.D549N in one tumor DNA sample together with oncogenic mutation p.E542K. According to Whyte *et al.* (2006), this mutation p.D549N may function as a "passenger" in the process of malignant transformation without a direct contribution to the gain of the malignant phenotype of the cell clone [32]. In the other patient, we also detected two point mutations in *PIK3CA* gene. Mutation p.M1043I was described in previous studies as oncogenic [9]. Mutation p.N1044D has been detected only in a few cases of large intestine carcinoma, endometrial carcinoma and glioblastoma so far [34-36]. Using *in silico* tool PROVEAN, we evaluated its effect on the protein function as neutral (PROVEAN score -0.458).

In the context to the histological subtype, we identified point mutations and potentially pathogenic DNA sequence changes, respectively, in *PIK3CA* gene in four patients with squamous cell carcinoma but not in patients with adenocarcinoma. The study by Miyake *et al.* (2008) did not prove the occurrence of *PIK3CA* gene mutation exclusively in a particular histological subtype, as well [34]. Simultaneously, our results agree with the results obtained in study by Cui et *al.* (2009) and indicate that *PIK3CA* gene mutations are specific neither for HPV-positive nor HPV-negative cervical carcinoma samples [9]. We observed the correlation between the presence of *PIK3CA* gene mutations and *hTERC* (3q26) gene amplification. This fact indicates that gene mutations contribute to the development of malignant phenotype. Conversely, we cannot prove a significant context between

PIK3CA gene mutation presence, the number of CNAs or *MYC* (8q24) gene amplification. No study has analysed the relationship between *PIK3CA* gene mutations and numeric changes in chromosomal region 3q harbouring *PIK3CA* gene (3q26.3) so far.

Based on the data in the study by Cui *et al.* (2009) mentioned above, the presence of *PIK3CA* gene mutation may represent a risk factor in combination with an invasive type of carcinoma for older patients (above 60 years). In younger patients, cervical carcinomas are more frequently related to HPV infection. This fact indicates a possible evidence for more mechanisms of cancerogenesis and risk factors in cervical carcinoma. In our patients' group, this hypothesis is declared by a presence of two *PIK3CA* gene mutations (p.M1043I a p.N1044D) in one older patient (68 years) but without an invasive type of cervical carcinoma and with HPV infection positivity. Point mutations and potentially pathogenic DNA sequence changes in *PIK3CA* gene were detected in three patients in clinical stage I (IA2, IB1) and in one patient in clinical stage IIIB. The study by Tornesello *et al.* (2014) showed impaired effect of *PIK3CA* gene mutations in the context to shorter survival in patients in clinical stage IB/II who had undergone radical chemotherapy [16]. This effect was not observed in patients in clinical stage III/IV probably due to generally impaired survival in patients in advanced clinical stages.

PIK3CA gene mutations or potentially pathogenic DNA sequence changes in this gene were analysed in the context to the presence of lymph nodes affected by metastases and their number. We did not observe any metastatically affected lymph nodes in these patients but the results of a recent study by Hou *et al.* (2014) confirm the outcomes from the previous studies indicating the correlation between the higher frequency of *PIK3CA* gene mutations and metastases presence [37]. High mutation prevalence in *PIK3CA* gene enables to use this gene as a potential molecular target for targeted therapy with PI3K/AKT/mTOR inhibitors, leading to a higher therapeutic response [10, 38]. Our analytical approach and results could help to define a group of patients with a higher risk of metastatic disease. These patients require an individual attention in clinical monitoring of the disease progression and may compose a target group for targeted therapy application.

KRAS gene encodes a small GTPase protein which creates an essential part of RAS/MAPK signalling pathway. Similarly to PI3K/AKT/mTOR, it represents one of the key signalling pathways which plays an important role in cell proliferation, cell cycle progression and cell survival. The dysregulation of these cell signalizations has been shown to be one of the significant molecular causes in cancerogenesis in a large number of tumor diseases. *KRAS* gene mutations had been proved in less than 10% cases of cervical carcinoma and associated with adverse prognosis, especially in patients with squamous cell carcinoma [39]. In the context to the histological subtype, we identified potentially pathogenic DNA sequence changes in *KRAS* gene in two patients with adenocarcinoma and in two patients with squamous cell carcinoma, as well. Based on the results obtained by several extensive studies, it is not possible to determine the specificity of *KRAS* gene mutations for a particular histological subtype. Nevertheless, there is a tendency to occur in adenocarcinoma more frequently than in squamous cell carcinoma [10, 40]. Moreover, our data confirm that *KRAS* gene mutation does not correlate with a particular clinical stage or the presence of HPV infection. In two cases, *KRAS* mutation was detected in HPV-negative cervical carcinoma samples. This may indicate that the presence of *KRAS* mutations and HPV infection presumably represent two independent events in cancerogenesis as suggested in the study of Buyru *et al.* (2006) [41]. Further studies with large cohorts of patients to clarify this statement are necessary. As well as in the course of *PIK3CA* gene mutation analysis, we found the correlation between the presence of potentially pathogenic DNA sequence changes in *KRAS* gene and *hTERC* (3q26) gene amplification. Simultaneously, we did not observe a significant correlation between the presence of potentially pathogenic DNA sequence changes in *KRAS*, the number of CNAs or *MYC* (8q24) gene amplification.

Janku *et al.* (2012) proved an increased coincidence of *KRAS* and *PIK3CA* genes mutations in cervical carcinoma samples and determined its adverse outcome in therapeutic response, resulting in the resistance to PI3K/AKT/mTOR pathway inhibitors [38]. In our group of patient we detected a coincidence of potentially pathogenic DNA sequence changes in *KRAS* and *PIK3CA* genes in one patient with squamous cell carcinoma. DNA sequence changes in mutation "hotspot" in *KRAS* gene were found in two patients in clinical stage I (IA2, IB1) and in two patients in stage IIIB. Similarly to mutations in *PIK3CA* gene, we can observe DNA sequence changes in *KRAS* gene since the early stages of disease. Based on previous studies on cervical carcinoma and other types of solid tumors, it is clear that oncogenic mutations may contribute to the metastatic potential of cancer cells. This finding may serve for more specific stratification of patients based on the risk of metastatic progression of the disease, for clinical monitoring of individual defined patients. Moreover it may help to profile a group of patients who could be candidates for the application of the targeted therapy by RAS/MAPK pathway inhibitors.

EGFR gene encodes cell-surface receptor for a family of epidermal growth factors. It is a key member of many signalling pathways which stimulate the cell cycle and pathways which stimulate the cell proliferation and survival. The changes in its structure or in a number of its copies were proved as an important aspect in cancerogenesis in a wide spectrum of tumors. In our group of patients, we did not detect a potentially pathogenic DNA sequence change leading to constitutional EGFR protein activity. We detected only common benign SNPs in studied DNA regions and all of them were confirmed by Sanger sequencing. Our results agree with other studies which indicate a rarity of *EGFR* gene mutations and *EGFR* gene amplification as more common mechanism for upregulation of *EGFR* gene expression in cervical carcinoma [24, 42].

TP53 gene mutations were described as a key aspect in neoplastic cell transformation in a majority of human tumors, with varying mutation frequency and tumor stages, as well. In cervical carcinoma, they are detected rather rarely, therefore, there has been clearly described neither their prognostic nor predictive role so far [20]. Unsurprisingly, in our group of patients we did not identify any potentially pathogenic DNA sequence changes using HRM.

Generally, the identification of point mutations in prognostically important proto-oncogenes and tumor suppressor genes represents a key aspect for possible application for the targeted therapeutic approach in a defined group of patients. Activating oncogenic mutations are potential molecular targets for design of a specific signalling pathway inhibitor. The implementation of HRM method with positive reference DNA, i.e. reference DNA sample harbouring a known mutation, could offer a fast screening of specific mutations in genes of interest. This could define a group of patients which may profit from the personalized targeted therapy [43-45]. In our study using HRM method lacking positive control DNA samples, Sanger sequencing showed to be insufficient for the confirmation and identification of DNA sequence changes if they appear in minor tumor cell clones under the detection limit.

Summary

In the course of this study, we have analysed the mutation status in *PIK3CA, KRAS, EGFR* proto-oncogenes and *TP53* tumor suppressor gene in their mutation "hotspots" using HRM screening method and Sanger sequencing as a confirmatory method. In *PIK3CA* gene we detected two previously described point mutations and other two point mutations without previous evidence in cervical carcinoma. Moreover, we detected two DNA sequence changes in this gene lacking their exact identification using Sanger sequencing. In *KRAS* gene we identified four potentially pathogenic DNA sequence changes affecting mutation "hotspot" in exon 1. Although our study is based on a small group of patients, current knowledge resulting from mutation analyses confirm the results of previous extensive studies that squamous cell carcinoma and adenocarcinoma differ in their molecular profiles on the level of gene mutations. These findings are expected to be confirmed and specified in the course of studies encompassing larger groups of patients.

Supplementary information is available in the online version of the paper.

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Gene	Hotspot	Sequence		Amplicon length
PIK3CA	codon 88	forward	AAGTGTTACTCAAGAAGCAGAAAGG	234 bp
		reverse	ACGAAGGTATTGGTTTAGACAGAAA	
	codons 542-546	forward	AGCTAGAGACAATGAATTAAGGGAAA	133 bp
		reverse	TCTCCATTTTAGCACTTACCTGTGAC	
	codons 1043-1047	forward	TCTTTTGATGACATTGCATACATTC	197 bp
		reverse	CAGAGTGAGCTTTCATTTTCTCAGT	
KRAS	codons 12, 13	forward	CTGAATATAAACTTGTGGTAGTTGGA	166 bp
		reverse	TTTATCTGTATCAAAGAATGGTCCTG	
EGFR	exon 18	forward	TACATTTGTCCTTCCAAATGAGC	298 bp
		reverse	CCTGTGCCAGGGACCTTAC	
	exon 19	forward	TTAACGTCTTCCTTCTCTCTCTGTC	250 bp
		reverse	GAAAGTGAACATTTAGGATGTGGAG	
	exon 20 (1st part)	forward	GTATTTTGAAACTCAAGATCGCATT	250 bp
		reverse	GTCTTTGTGTTCCCGGACATAGT	
	exon 20 (2nd part)	forward	CACCGTGCAGCTCATCAC	140 bp
		reverse	CGTATCTCCCTTCCCTGATTAC	
	exon 21	forward	ATTCGGATGCAGAGCTTCTT	300 bp
		reverse	TTAAACAATACAGCTAGTGGGAAGG	
TP53	codons 173-181	forward	ATCTACAAGCAGTCACAGCACAT	157 bp
		reverse	CTAAGAGCAATCAGTGAGGAATCAG	
	codons 245-249	forward	GTTATCTCCTAGGTTGGCTCTGACT	119 bp
		reverse	GAGTCTTCCAGTGTGATGATGGT	
	codons 273-282	forward	TAGTGGTAATCTACTGGGACGGAAC	149 bp
		reverse	CTTGCTTACCTCGCTTAGTGCT	

Supplementary Table 1.: Primer pairs for HRM analyses.

Gene	Hotspot	Sequence		Amplicon length
PIK3CA	codon 88	forward	TTTGGGACAACCATACATCTAATTC	514 bp
		reverse	ACGAAGGTATTGGTTTAGACAGAAA	
	codons 542-546	forward	GAGGAAAAGTAAATTGTTCACTACCA	468 bp
		reverse	TCTCCATTTTAGCACTTACCTGTGAC	
	codons 1043-1047	forward	TAAAGGGAATCAAAAGATGTTGGTA	551 bp
		reverse	AATTCTAATGCTGTTCATGGATTGT	
KRAS	codons 12, 13	forward	TACAGTTCATTACGATACACGTCTG	560 bp
		reverse	CTCTGAAATACACTTCCAATCAAAA	
EGFR	exon 18	forward	TTTCTACCAACTTCTGTCAAGCTCT	545 bp
		reverse	ATAAAAATGCCTTTGGTCTGTGAAT	
	exon 19	forward	CAGCATCATTAAATTCTGGATGAA	561 bp
		reverse	TGTCTCTAAGGGGAGGGAGTTATAC	
	exon 20	forward	GTATTTTGAAACTCAAGATCGCATT	554 bp
		reverse	CACACTGAGCACTCAATAAAGAGAA	
	exon 21	forward	CTTTCCATTCTTTGGATCAGTAGTC	581 bp
		reverse	GAGAGACTGAAACCTAACATTTGCT	
TP53	codons 173-181	forward	AACTCTGTCTCCTTCCTCTTCCTAC	550 bp
		reverse	GTTAAACCCATTTACTTTGCACATC	
	codons 245-249	forward	CTGTGTTATCTCCTAGGTTGGCTCT	291 bp
		reverse	AGAAAACTGAGTGGGAGCAGTAAG	
	codons 273-282	forward	TTCTTCCATACTACTACCCATCCAC	571 bp
		reverse	AAGACTTAGTACCTGAAGGGTGAAA	

Supplementary Table 2. Primer pairs for standard PCR and Sanger sequencing.