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The role of miR-409-3p in regulation of HPV16/18-E6 mRNA in human cervical high-grade squamous intraepithelial lesions

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ABSTRACT

Cervical cancer is one of the most common malignancies in women. MicroRNAs (miRNAs) are involved in a variety of fundamental cellular processes, including carcinogenesis. The potential utilization of aberrantly expressed miRNAs as novel biomarkers in cervical cancer diagnostics is growing. We investigated miRNA expression profiles during the progression of dysplasia in cervical epithelium to identify aberrantly expressed miRNAs. High-throughput miRNA profiling of high-grade precancerous lesions identified 79 miRNAs showing significant difference in expression values compared to normal cervical epithelium. Ten selected miRNAs were subsequently measured in an independent group of samples to validate them as promising biomarkers of cervical carcinogenesis. MicroRNAs miR-10b-5p, miR-34c-5p, miR-409-3p and miR-411-5p were confirmed as downregulated, while miR-10a-5p, miR-132-3p, miR-141-5p were significantly upregulated in dysplastic cervical tissues. Further investigation revealed an inverse correlation of miR-409-3p with *E6* mRNA levels in precancerous cervical lesions. Subsequent *in vitro* analyses showed a direct involvement of this miRNA in the regulation of *E6* oncogene levels, thus confirming a potential tumor suppressor function of miR-409-3p in cervical malignancies. Hence, miR-409-3p may represent a useful early marker and a potential therapeutic target for cervical cancer.

1. Introduction

The global gynecological cancer burden in 2002 accounted for 19% of all tumors in women (Sankaranarayanan and Ferlay, 2006). However, significant differences could be found across the world, where gynecological malignancies in USA represent about 12.5% of all tumors in women (Society, 2018), in contrast to developing countries where approximately one quarter of cancers in women are gynecological cancers (Iyoke and Ugwu, 2013). Although surgery and chemotherapy improved outcomes for patients with gynecologic malignancies over the last twenty years, they still account for over 11% of cancer-related deaths annually even in developed countries (Society, 2018). The most frequent malignancies comprise endometrial, cervical and ovarian tumors. Importantly, more than 85% of the cervical cancer global burden occurs in developing countries, where it accounts for 13% of all female cancers indicating insufficient prevention and screening programs in comparison with developed western countries (Pedroza-Torres et al., 2014). Virtually all cervical cancers are caused by persistent infection with high risk human papilloma viruses (HPVs) that deregulate normal proliferation and differentiation of cervical squamous epithelial cells. HPV16 and HPV18 are the most common carcinogenic HPV types, and are responsible for approximately 70% of cervical cancer cases and about 52% of high-grade squamous intraepithelial lesions (HSILs) (Smith et al., 2007). Although HPV persistence was established as the initial step of cervical carcinogenesis, the overall process is complex (Bosch and de Sanjose, 2003; zur Hausen, 2000) and involves other factors including (a) inactivation of tumor suppressor genes *TP53* and *RB1* by viral oncoproteins E6 and E7, respectively; (b) deregulation of cell cycle regulators (Jayshree et al., 2009); (c) altered DNA methylation patterns in gene promoter regions (Steenbergen et al., 2014); (d) enhanced telomerase activity (Nachajova et al., 2015); (e) factors contributing to immune tolerance, such as higher activity of indoleamine 2,3-dioxygenase (Mittal et al., 2013) or tobacco use (Castle, 2008).

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Table 1
Selected microRNAs.

Human miRNA	p-level[a]	Profile in cancer[b]	
		down	up
hsa-miR-10a-5p	< 0.0001	NA[c]	Cervical
hsa-miR-10b-5p	0.0052	NA	Breast, colorectal, gastric, glioblastoma, hepatocellular, non-small cell lung cancer, pancreatic
hsa-miR-34c-5p	0.0075	Breast, colorectal, endometrial, lung, nasopharyngeal, osteosarcoma, ovarian	NA
hsa-miR-132-3p	0.0002	Breast, colorectal, lung, osteosarcoma, liver	Gastric
hsa-miR-135b-5p	0.2525	NA	Colorectal, gastric
hsa-miR-141-5p	0.0337	Colorectal, gastric, glioma, hepatocellular, lung, prostate	Non-small cell lung cancer, ovarian, prostate
hsa-miR-200a-3p	0.0560	Breast, gastric, hepatocellular, pancreatic, renal	Breast, esophageal, ovarian
hsa-miR-409-3p	0.0242	Colorectal, gastric, osteosarcoma	NA
hsa-miR-411-5p	0.0013	NA	NA
hsa-miR-429	0.1250	Colorectal, gastric, oral, ovarian, osteosarcoma	Colorectal, hepatocellular, bladder

[a] Mann-Whitney test.
[b] Tumor tissues showing the same miRNA expression profile mentioned in at least two papers according to miRCancer database (Xie et al., 2013).
[c] Not available.

Growing evidence shows that cervical carcinogenesis is associated also with microRNAs (miRNAs) (He et al., 2016), which belong to a class of short non-coding RNAs representing trans-acting elements that regulate most human protein-coding genes at the post-transcriptional level (Michalova et al., 2013); the same class as small interfering RNAs (siRNAs) or piwi-interacting RNAs (piRNAs). This rapidly growing area of research has led to numerous discoveries, highlighting miRNA roles in fundamental processes such as cell proliferation, differentiation, metabolism or survival (Bartel, 2009), the onset of cardiovascular (Sun et al., 2017), neurodegenerative (Quinlan et al., 2017) and autoimmune diseases (Chen et al., 2016), and with many types of cancer, where they function as oncogenes or as tumor suppressors (Iorio and Croce, 2012).

The Cancer Genome Atlas (TCGA) project has profiled a number of different tumor types using various "-omic" platforms, generating raw as well as processed data that are publicly accessible (Chandran et al., 2016). Recently, integrated genomic and molecular characterization of cervical cancer was released by TCGA, including miRNA sequencing data for 192 samples from cervical cancer patients. Unsupervised clustering revealed several miRNA clusters that were associated predominately with histology, keratin levels, epithelial-mesenchymal transition (EMT), receptor tyrosine kinases (RTKs) and estrogen receptor (2017). A study by Liang et al. focused on TCGA miRNA sequencing data from 254 samples consisting of 251 cervical cancer tissues and three matched normal tissues, and identified miR-145, miR-200c and miR-218 as potential prognostic factors for cervical cancer patients (Liang et al., 2017). However, it should be stressed that precancerous lesions and early cervical cancer can be successfully treated with surgery or radiation, while women diagnosed with invasive and especially metastatic cervical cancer are often incurable (Uyar and Rader, 2014). Therefore, it is particularly important to identify factors associated with increased risk of cervical cancer for application in screening programs. In this spirit, miRNAs as master regulators of a wide spectrum of cellular processes may represent a promising tool.

2. Materials and methods

2.1. Clinical samples

All clinical specimens were obtained during cervical conization at the Clinic of Obstetrics and Gynecology at University Hospital, Brno. Excised tissues were received within 30 min of surgical removal according to standardized hospital protocols and immediately fixed in 4% neutral formaldehyde for approximately 24 h before processing into paraffin wax. According to histopathological evaluation based on hematoxylin/eosin staining, endocervical lesions and adjacent normal tissues were marked by an experienced pathologist for subsequent RNA isolation. The first cohort of 27 HSIL cases with median patient age 44 years (mean 46 ± 18) was subjected to miRNome profiling. Corresponding controls were 12 adjacent normal tissues taken from the same formalin fixed paraffin embedded (FFPE) blocks. The second cohort consisted of 63 FFPE HSIL blocks taken from patients with median age 36 years (mean 33 ± 9.3). Precancerous tissue was marked in accordance with histopathological examination and used for isolation of both DNA (to determine HPV 16 and 18 status) and total RNA for miRNA analysis. In parallel, adjacent normal tissue from the same FFPE blocks (42 samples) served as a control. The study was approved by the Ethical committee of University Hospital Brno, reference number 02-251017. Signed informed consent was obtained from all individual participants included in the study.

2.2. MicroRNA determination

All tissue blocks used for RNA preparation have been verified by a pathologist according to corresponding hematoxylin/eosin stained tissue section. Two or three 1 mm tissue cores containing malignant or normal content with a minimum tissue depth of 3–4 mm were collected and used for extraction of total cellular RNA by TRI Reagent (MRC). The RNA integrity number (RIN), an indicator of the intactness of two ribosomal RNAs, was determined to assess RNA quality using an Agilent RNA 6000 Nano Kit (Agilent Technologies). RIN values varied within the range 1.5–3.5. Although RIN values are typically low in FFPE blocks due to RNA degradation/fragmentation, the RNA thus prepared is sufficient for RT-PCR of fragments up to 151 bp (Ribeiro-Silva et al., 2007) including miRNAs showing enhanced stability (Hall et al., 2012), and we have successfully isolated RNA from FFPE blocks for gene expression analyses previously (Hrstka et al., 2013, 2017; Slaby et al., 2010). Megaplex Primer Pools were used for reverse transcription of 0.5 µg RNA with MicroRNA Reverse Transcription Kit (both ThermoFisher Scientific). 0.9 µl reverse transcription product was used per port of TaqMan Array MicroRNA Cards A and B that were used for miRNA profiling using 7900HT Fast Real-Time PCR System (all ThermoFisher Scientific). U6 snRNA was used for data normalization and served also as an endogenous control for ddPCR. The detection of selected hsa-miRNAs (Table 1, hereafter referred to as miRNA) in the second cohort of patients was carried out using TaqMan miRNA assays (ThermoFisher Scientific). As an alternative to real-time PCR we also used droplet digital PCR (ddPCR), a technology that overcomes normalization issues and may facilitate miRNA measurement (Campomenosi et al., 2016). 10 ng total RNA for each sample was transcribed using M-MLV Reverse Transcriptase (Promega). The reverse transcription product was diluted

20-fold and triplicate 2 µl samples were used to generate droplets by automated droplet generator. Droplets were transferred into 96-well plates and subjected to PCR amplification using T100 thermal cycler. Droplets containing PCR products were counted by QX200 (all instrumentation from Bio-Rad). Data from at least 12,000 droplets per sample were used for concentration calculations using QuantaSoft software (Bio-Rad).

2.3. Nucleic acid isolation and E6 mRNA determination

Genomic DNA was purified from FFPE tissue sections using QIAamp DNA FFPE Tissue Kit (Qiagen). HPV 16/18 was determined as described previously (Hublarova et al., 2009; Bartosik et al., 2016). Total RNA was isolated using TRIzol reagent (Invitrogen). The cDNA was synthesized by RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific). Either SYBR Green MasterMix (Roche) or TaqMan Universal PCR MasterMix (ThermoFisher Scientific) was used for quantitative PCR. 18S rRNA and GAPDH served as parallel endogenous controls. All samples were analyzed in triplicate. The primer sequences were: HPV-16 E6 forward 5′-ATGGTACAATGGGCCTACGA-3'; reverse 5′-ACTCAT TTGTTTTTTTTCTGCTCGT-3'; HPV-18 E6 forward 5′-AATGGTACAAT GGGCATTTG-3'; reverse 5′-GTTTTTGGGCTCGCCTATAA-3'. The relative mRNA levels of each gene were calculated according to the $2^{-\Delta\Delta CT}$ method.

2.4. Cell culture methods

Human cervical carcinoma cell lines Ca Ski and SiHa were maintained in RPMI-1640, C-4I cells in McCoy's and HeLa cells in high glucose Dulbecco's modified Eagle's medium. Media were supplemented with 10% fetal bovine serum, 300 µg/ml L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were grown to 60–80% confluence in media without antibiotics prior to transfection. Negative miRNA control, miR-409-3p mimic or inhibitor (all mirVana, Ambion) were diluted to 30 pmol in serum-free medium and mixed with polyethylenimine (PEI) transfection reagent at a ratio of 1:3. The mixture was vortexed and left for at least 15 min at room temperature before adding to the cells. After 48 h, cells were used in the following experiments.

In scratch assays, cells were grown to 100% confluence and scratched in serum-free medium to avoid cellular proliferation as described previously (Jonkman et al., 2014). Cells were left to re-populate the scratched area for 24 h and photographs were taken using a digital camera mounted on an inverted microscope Nikon. Wound closure rates were analyzed by TScratch software (Geback et al., 2009). Cellular proliferation was measured using real time xCELLigence system RTCA DP (Roche). Cells were transfected, trypsinized and 10,000 cells were seeded into each well of an E-plate according to the manufacturer's instructions and changes in impedance were monitored. Cell viability was assessed by MTT. Briefly, 5000 cells per well were seeded in 96-well plates and allowed to adhere overnight. Next day, chemotherapeutic drugs were added. After 72 h of incubation, MTT was added and incubated for 3 h at 37 °C, crystals of formazan were dissolved in DMSO and measured at 595 nm.

2.5. Statistics

Statistical analysis was carried out using the R software version 3.2.4 and Statistica version 12. Differential expression between precancerous tissue (HSIL) and adjacent normal tissue (Norm) was assessed by a non-parametric Mann–Whitney *U* test. To address the issue of multiple testing and controlling false discovery rate, adjusted p values were obtained by Benjamini and Hochberg's method (Benjamini and Hochberg, 1995). miRNAs with adjusted $p < 0.05$ were selected for supervised hierarchical clustering. Both unsupervised and supervised hierarchical clustering analyses were performed using the complete linkage method and Euclidean distance. miRNA expression values were standardized for visualization purposes.

To predict potential binding sites for miR-409-3p within E6 mRNA of both HPV16 and HPV18 we used STarMir tool (Kanoria et al., 2016; Rennie et al., 2014) based on the logistic prediction models developed with miRNA binding data from CLIP studies. Each of the candidate sites is assigned a logistic probability as a measure of confidence in the predicted site (Liu et al., 2013).

3. Results

3.1. miRNA profiling in cervical HSILs

MicroRNA profiling was determined using 384-well microfluidic card containing TaqMan microRNA assays in a cohort of 27 HSILs and 12 adjacent normal tissues taken from 29 patients (Table S1). In total, 768 miRNAs were measured and normalized in relation to U6 snRNA that is an integral part of TaqMan Array MicroRNA Cards for data standardization purposes and has been used in cervical malignancies (Gocze et al., 2013; Peng et al., 2012). In parallel, hsa-miR-1290 showing the highest stability with respect to coefficient of variation was also applied. Importantly, there were no significant differences in the results when comparing these two normalizations. Only miRNAs with C_T value less than 38 in at least one-third of the samples in both cohorts were included in further analyses. Following these criteria, 288 miRNAs passed into unsupervised hierarchical clustering, which did not clearly distinguish between normal and dysplastic (HSIL) cervical tissues (Fig. S1). In following supervised clustering analysis, 31 miRNAs showed differential expression (adjusted $p < 0.05$) between precancerous tissue (HSIL) compared to adjacent normal tissue (Norm). By this analysis we were able to discriminate only 17 HSIL specimens divided into 3 specific clusters (Fig. S2). However, the remaining HSILs were not discriminated from normal tissue samples.

Since supervised clustering analysis was not able to discriminate between dysplastic and normal cervical tissue, 10 miRNAs selected according to p-value were then analyzed using ddPCR in the second cohort. Significantly different expression ($p < 0.05$) was confirmed for seven miRNAs, while miR-200a-3p showed only marginal significance and the remaining two miRNAs were not significant (Table 1). Along with p-values for these miRNAs, previously published expression of these miRNAs in different malignancies is provided (Xie et al., 2013). Boxplots demonstrate the levels of these miRNAs in the second cohort of patients (Fig. S3). According to the level of these miRNAs in precancerous and normal tissue, we can divide them into two groups: potentially oncogenic miRNAs that are upregulated in precancerous tissues (T) and potential tumor suppressor miRNAs showing increased expression in normal tissues (N). Thus miR-10a-5p, miR-132-3p, miR-141-5p, miR-200a-3p and miR-429 represent oncogenic miRNAs and miR-10b-5p, miR-34c-5p, miR-135b-5p, miR-409-3p and miR-411-5p could be characterized as miRNAs with a tumor suppressor role in cervical tissues (Fig. S3).

3.2. Negative correlation between E6 mRNA and microRNA miR-409-3p

The presence of HPV16 and/or HPV18 was detected by PCR in 38 (60.3%) patients; 14 samples were HPV16 positive, 22 were HPV18 positive and 2 samples showed positivity for both HPV16 and HPV18. Based on the type of HPV, the level of either HPV16 *E6* mRNA or HPV18 *E6* mRNA was determined in these 38 HSIL HPV16/18 positive samples along with 25 corresponding normal adjacent tissues. Significantly higher *E6* mRNA levels in HSILs compared to adjacent normal tissues confirmed the involvement of E6 in cervical carcinogenesis (Fig. 1A). Interestingly, the comparison of *E6* mRNA levels with our selected miRNAs revealed an inverse correlation with miR-409-3p (Fig. 1B), indicating a potential regulatory role of miR-409-3p on *E6*.

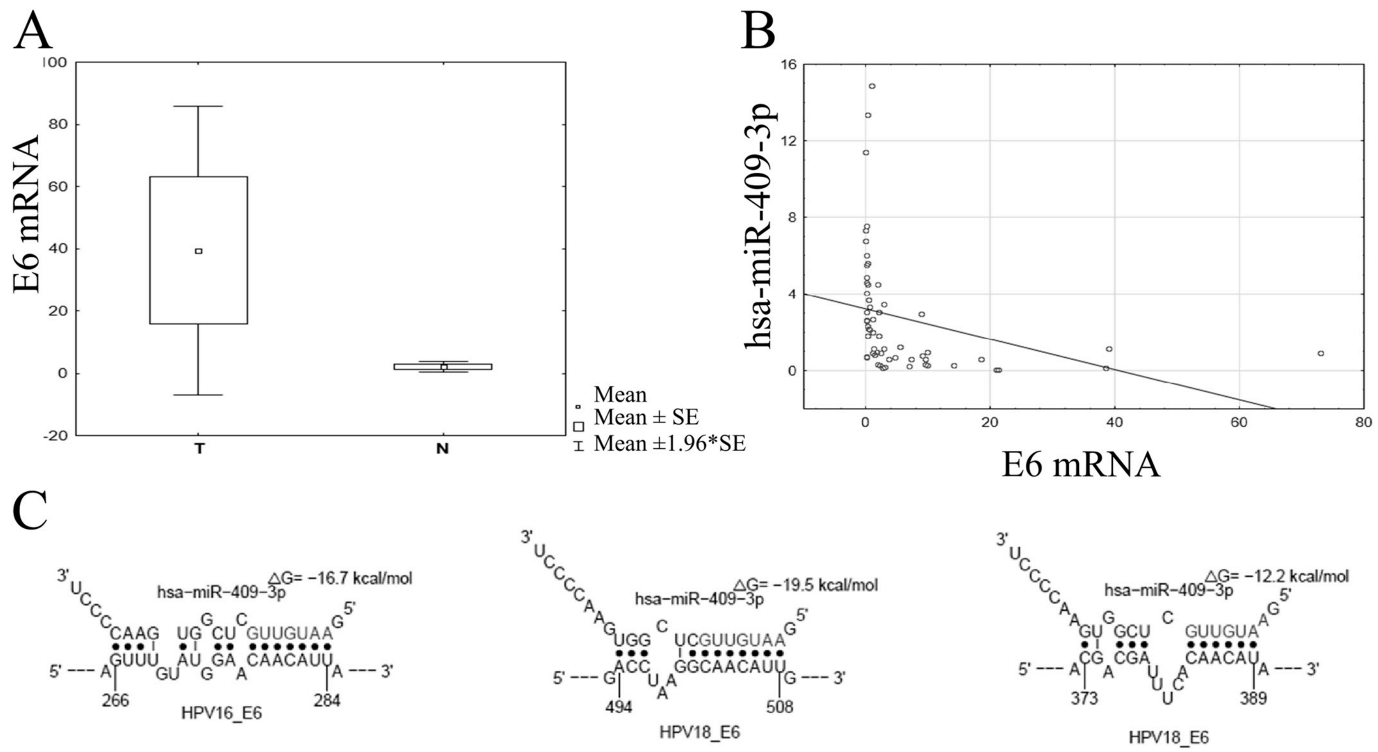

Fig. 1. The relationship between *E6* gene and miR-409-3p. (**A**) Determination of *E6* mRNA levels in HSILs (T) compared to adjacent normal tissue (N). (**B**) Analysis of miR-409-3p in relation to *E6* mRNA levels. (**C**) Prediction for miR-409-3p seed sites within *E6* coding sequence for HPV16 and HPV18.

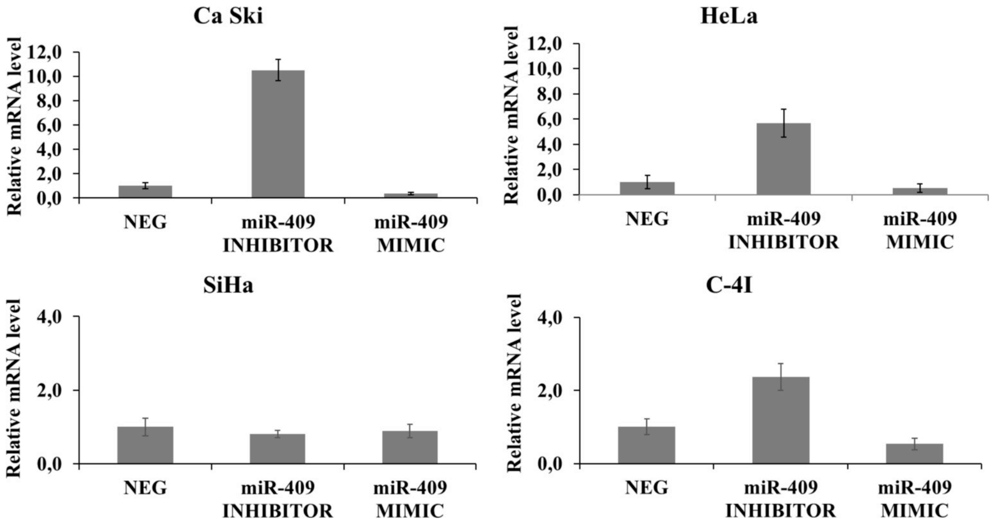

Fig. 2. miR-409-3p regulates *E6* mRNA levels. miR-409-3p was modulated by specific inhibitor or mimic in Ca Ski, SiHa (left part, HPV16 positive) and HeLa, C-4I (right part, HPV18 positive) cells to determine *E6* mRNA levels.

STarMir web server was used to predict potential binding sites for miR-409-3p within *E6* mRNA of both HPV16 and HPV18. The prediction of potential miRNA binding sites is based on RNA hybrid program, which finds the energetically most favorable hybridization sites of a small RNA in a large RNA (Rehmsmeier et al., 2004). For such defined sites, a comprehensive list of sequence and structure-based features is computed as described by Liu et al. (2013). In general, a probability of 0.5 indicates a fairly good chance of miRNA binding, and high likelihood by a probability of 0.75 or higher (Rennie et al., 2014). Interestingly, miR-409-3p seed match with a hybrid stability of −16.7 kcal/mol for HPV16 *E6* mRNA and two matches for HPV18 *E6* mRNA with hybrid stability of −19.5 and −12.2 kcal/mol were obtained (Fig. 1C). Computed logistic probabilities of the predicted site confidence for HPV16-*E6* mRNA (0.878) and for HPV18-*E6* mRNA (0.683 and 0.607) indicate a high likelihood of miR-409-3p binding to these mRNAs. Besides the predicted miR-409-3p binding sites within *E6* coding sequences, binding sites were also identified in downstream sequences representing potential 3′UTR regions at positions 512 nucleotides from the stop codon of HPV16-*E6* and 56 nucleotides from the stop codon of HPV18-*E6* (Fig. S4).

3.3. Alterations in miR-409-3p expression affect cellular proliferation, migration and chemoresistance in vitro

Cervical cancer cell lines were used to investigate the relationship between miR-409-3p and *E6* expression. First, we analyzed miR-409-3p in our panel of cell lines. Real-Time PCR convincingly detected miR-409-3p only in HeLa cells, while Ca Ski and C4-I cells were close to the detection limit and SiHa cells were negative (data not shown). Since ddPCR is best suited for detecting low-abundance targets (Taylor et al., 2017), we used this approach to determine the presence of miR-409-3p. As expected, ddPCR showed relatively high levels of miR-409-3p in HeLa cells and confirmed the presence of miR-409-3p in C4-I and Ca Ski cells, while SiHa cells remained negative (Fig. S5). Then we applied both miR-409-3p miRNA mimic and inhibitor to analyse *E6* mRNA expression. All tested cell lines displayed a similar trend, showing increased *E6* mRNA levels in response to miR-409-3p inhibition, except SiHa cells which showed no detectable miR-409-3p. *Vice versa*, *E6* mRNA levels decreased in response to miR-409-3p miRNA mimic (Fig. 2). Differences in the magnitude of effects of mimic and inhibitor in different cell lines would be explained by their different characteristics such as transfection efficiency, proliferation rate (Barron et al., 2015) and different original levels of miR-409-3p. Nevertheless, these data mechanistically confirm the involvement of miR-409-3p in the regulation of *E6* mRNA levels.

To functionally investigate the role of miR-409-3p during cervical carcinogenesis, the effects of miR-409-3p on cloning efficiency, proliferation and migration were analyzed. Although the modulation of miR-409-3p levels showed no impact on colony development (data not shown), significant effects on cell proliferation were observed. Real time cell measurements showed a decrease in cell proliferation rate after transfection with miR-409 mimic (Fig. 3). On the other hand, transfection with miR-409 inhibitor resulted in increased proliferation, except for HeLa cells, in which the inhibitor had no effect (Fig. 3).

To determine the potential contribution of miR-409-3p to chemosensitivity, we analyzed the viability of cells transfected with either miR-409-3p mimic or inhibitor in combination with the most standard chemotherapy regimens, represented by topotecan, cisplatin and paclitaxel. Although miR-409-3p mimicking was associated with slightly decreased metabolic activity, as shown by MTT assay, in general the alteration in miR-409-3p level had only negligible impact on sensitivity to the tested drugs (Fig. S6). However, the preferred treatment in certain stages of cervical cancer is either radiation alone (brachytherapy) or concurrent chemoradiation. Thus, cells transfected with either miR-409-3p mimic or inhibitor were also exposed to 4 Gy ionizing radiation. Manipulation of miR-409-3p showed no significant effect on radiation-induced cytotoxicity (Fig. S7), indicating that miR-409-3p is not directly involved in the regulation of cervical cancer cell responses to chemo- and radio-therapy. On the other hand, a potential tumor suppressor activity was demonstrated by inhibition of typical aggressiveness features of tumor cells such as proliferation and migration, since an *in vitro* scratch assay revealed that cervical cancer cells transfected with oligonucleotides mimicking miR-409-3p migrate more slowly than cells transfected with control oligonucleotides (Fig. 4, Fig. S8).

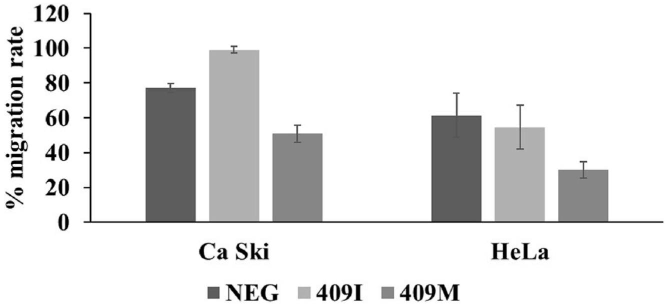

Fig. 4. The involvement of miR-409-3p in cell migration. The effect of miR-409-3p inhibition (409I) and mimicking (409M) on cell migration compared to corresponding miRNA negative controls (NEG) was determined using scratch assay.

4. Discussion

It has been estimated that viruses are etiological agents in approximately 12% of human cancers (de Martel et al., 2012; Parkin, 2006). One of the best-known examples is the association between cervical malignancies and HPV. Recent studies indicate an important interplay among viral oncoproteins, virus-encoded miRNAs, cellular miRNAs and cellular genes. It is generally accepted that, unlike other DNA viruses, HPVs do not encode any miRNAs (Xu and Ye, 2014). On the other hand, recent studies indicate that cellular miRNAs play important roles in HPV-related cervical oncogenesis (Wang et al., 2011, 2014). Importantly, HPV-encoded genes regulate the levels of cellular miRNAs and, *vice versa*, cellular miRNAs regulate HPV-encoded genes (Chen, 2015; Xu and Ye, 2014). To clarify these complex processes in cervical neoplastic and malignant transformation, specific miRNA signatures of cervical high grade squamous intraepithelial lesions and adjacent normal cervical tissues were obtained. Since no specific miRNA expression pattern enabled discrimination between transformed and normal tissue, we selected 10 miRNAs according to their p-value of differential expression and validated them using an independent data set. Seven miRNAs remained significantly associated (plus one marginally associated) with the presence of HSIL (Table 1) and have been searched in the literature in relation to cervical cancer.

A recent meta-analysis (Li and Hu, 2015) comparing miRNA expression profiles between cervical cancer and normal cervical tissue reported 15 miRNAs significantly upregulated in cervical cancer tissue, including miR-10a, miR-141 and miR-200a that we also identified. Interestingly, miR-10a-5p and miR-132-3p were also identified as miRNAs significantly upregulated in HPV-positive tonsillar tumors compared to HPV-negative counterparts, and miR-200a along with miR-141 were defined as specific determinants of HPV-induced malignancies (Vojtechova et al., 2016). In agreement with these findings, miR-10a-5p and miR-132-3p have increased expression during the transition from normal to atypical precancerous cervical dysplasia

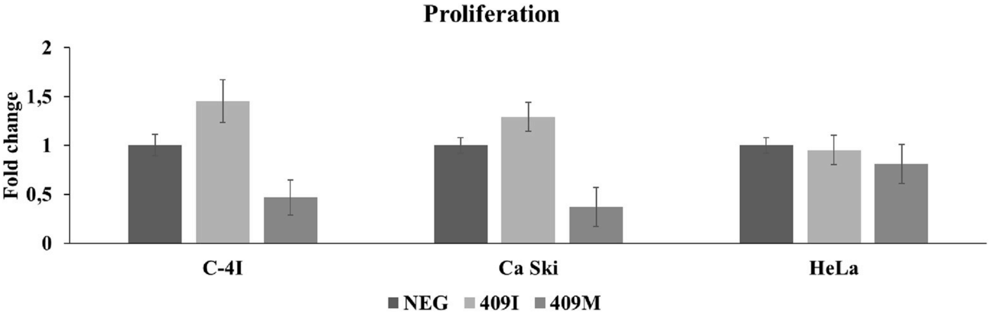

Fig. 3. The impact of miR-409-3p on cell proliferation. Cell proliferation of C-4I, Ca Ski and HeLa cells was analyzed in response to inhibition (409I) and mimicking (409M) of miR-409-3p.

(Pereira et al., 2010).

Altered expression of miRNAs downregulated in our HSIL specimens was also described previously. For instance, miR-10b was found to be downregulated during cervical cancer progression and in association with a more aggressive tumor phenotype. On the other hand, overexpression of miR-10b in cervical cancer cells inhibited cell proliferation and invasion, most probably due to direct targeting of *HOXA1* (Zou et al., 2016). The expression of miR-34c seems to be downregulated by methylation in HPV16-positive cervical malignancies, indicating that the alteration in methylation and expression of this miRNA contributes to the pathogenesis of the disease. A significant association of aberrant miR-411 expression with cervical malignancy has not been described to date; however, its tumor suppressive effects were described in breast (Zhang et al., 2016), colorectal (Zhao et al., 2018) and renal (Zhang et al., 2017) tumors.

Clinical studies have clearly demonstrated that the major cause of cervical cancer worldwide is infection with high risk-HPVs, predominately HPV16 and 18 (Smith et al., 2007; Teguete et al., 2017). The predominance of HPV16 and 18 is also seen in Central Europe; HPV16 and/or HPV18 are present in 63.3% and 79.3% of women with HSILs and cervical cancer respectively, in the Czech Republic (Bruni et al., 2017). The relatively high number of HPV18 positive samples in our study reflects that, although the samples were selected randomly, emphasis was placed on larger volume of histologically verified dysplastic tissue, thus HSILs with severe dysplasia and carcinomas *in situ* were selected. Thus, increased incidence of both HPV16 and 18 could be expected as documented by HERACLES study, in which more than 6000 women diagnosed with high grade-CIN or invasive cervical cancer from 17 European countries were enrolled (Tjalma et al., 2013).

The proteins encoded by HPV *E6* and *E7* play critical roles in HPV-positive cervical carcinomas. Inactivation of the tumor suppressor proteins p53 and retinoblastoma (pRb) is one of the main mechanisms by which E6 and E7 induce carcinogenesis (Jung et al., 2015). Thus, selected miRNAs were analyzed in relation to *E6* mRNA. An inverse correlation between miR-409-3p and *E6* mRNA was found, indicating a potential regulatory role of this miRNA towards *E6* mRNA levels. Further investigation of this relationship revealed that miR-409-3p directly attenuates *E6* mRNA levels. This effect was reflected by increased proliferation of C-4I and Ca Ski cells in response to miR-409-3p depletion and by attenuated proliferation in all three tested cell lines treated with miR-409-3p mimic. Minor discrepancies in the response of particular cell lines to the manipulations with miR-409-3p may be based in the different transfection efficiency of cell lines and their rate of proliferation, since all four cervical cell lines used in the study show different doubling time (Barron et al., 2015). Similar differences between cervical cell lines were observed in a study in which the authors conclude that the effect of p53 transfection on cell cycle arrest is dependent on the particular cell line (Ahn et al., 2002). Another important aspect is the different level of miR-409-3p in both clinical samples and cell lines, which may be influenced by plethora of *i*) intrinsic mechanisms e.g. single nucleotide polymorphisms, miRNA editing, methylation etc. as well as *ii*) extrinsic mechanisms such as various physiological and pathological stimuli e.g. steroid hormones (Katchy and Williams, 2016), stress conditions (Leung and Sharp, 2010) and also integrated vs. episomal presence of HPVs, different copy number and site of integration (Zheng and Wang, 2011). Accordingly, different original level of miR-409-3p may also significantly influence the effect of inhibitors and mimics, as observed in SiHa cells (miR-409-3p negative) showing no effect of miR-409-3p inhibitor on *E6* levels.

Our data on miR-409-3p correspond with Li et al. (2018), showing that decreased miR-409-3p is associated with cervical cancer tissues compared to normal tissues. Moreover, they identified *AKT* as a prominent miR-409-3p target gene, indicating that miR-409-3p may also interfere with PI3K/AKT signaling that promotes proliferation, invasion and metastasis (Lalaoui et al., 2011; Li et al., 2018). In line with these findings, we observed downregulation of miR-409-3p in precancerous lesions, indicating the involvement of this miRNA in early phases of cervical carcinogenesis. Downregulation of miR-409-3p was also observed in other human tumors such as gastric (Zheng et al., 2012), lung (Wan et al., 2014), breast (Cao et al., 2016) and osteosarcoma (Wu et al., 2016). Functional analyses revealed that this miRNA inhibits tumor cell migration, invasiveness and metastasis development. The potential utilization of miR-409-3p in clinical practice is supported by Tan et al. (2016) showing that miR-409-3p enhances oxaliplatin sensitivity by inhibiting Beclin-1-mediated autophagy in colon cancer cells, and by other studies (Cheng et al., 2018) showing that miR-409-3p sensitizes both cisplatin-sensitive and cisplatin-resistant ovarian cancer cells to chemotherapy by blocking Fip200 mediated autophagy; however, our *in vitro* experiments using specific miR-409-3p inhibitor and mimic showed almost no effect on the chemo- or radio-sensitivity of cervical cancer cells.

Studies on different cancer cell lines reported that E6 regulates the expression of miR-23a, miR-26a and miR-34a (Suzuki et al., 2009) and E7 regulates the expression of miR-17-92, miR-15b/16-2 and miR-106b-25 (Chang et al., 2008). Conversely, Jung et al. (2014) demonstrated that miR-375 directly downregulates E6-associated protein (E6AP) in HPV-positive tumor cells. Cellular E6AP forms a complex with E6 and contributes to ubiquitin-dependent degradation of p53. Therefore, elevated levels of miR-375 increase the levels of p53 in HPV16- and 18-positive cervical cancer cells due to repression of E6 activity, resulting in reduced proliferation of HPV-positive cervical cancer cells. Direct interactions of human miRNAs with HPV encoded genes was recently demonstrated by Lin et al. (2017), showing that miRNA-187 suppresses cervical cancer cell proliferation, migration and invasion *via* HPV16 *E6* mRNA. Furthermore, two human miRNAs, miR-875 and miR-3144, were predicted to bind to the coding region of the *E6* gene. Functional analyses confirmed that these two miRNAs suppress the expression of *E6*, inhibit cell growth and promote apoptosis in HPV16-positive cervical cancer cells (Lin et al., 2015).

In conclusion, these data demonstrate that cellular miRNAs can be involved in HPV regulation and support our results identifying miR-409-3p as a potential tumor suppressor in HPV infected cervical cancer cells. miR-409-3p shows significantly lower expression in cervical dysplastic tissues and inversely correlates with *E6* mRNA level. Tumor suppressor role of miR-409-3p consists in negative regulation of HPV16/18 *E6*, which contain miR-409-3p seeding sites in both the coding sequences and the 3′UTR region. Mimicking miR-409-3p upregulation had a suppressive effect on proliferation and migration of cervical cancer cells. These findings contribute to a better understanding of the role(s) of microRNAs in the pathogenesis and progression of cervical neoplasm at the molecular level. Although further work is required, the field of miRNA regulation in HPV associated malignancies is of clinical relevance and holds promise for the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.019.

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