MicroRNA Expressions in HPV-induced Human Cervical Dysplasia and Cancer

PhD Thesis

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Introduction

Even though widespread screening programs and the introduction of HPV vaccines decreased morbidity and mortality rates in the past decade, cervical cancer is still the second leading cause of death among women worldwide with an estimated 530,000 deaths per year (age-standardized global incidence rates: 15.3 and mortality rates: 7.8 in 2008). The worldwide prevalence of cervical adenocarcinoma has increased from 5% in 1950-60 to 20-25% in several regions of the world, including Europe and the US. According to the latest data, cervical adenocarcinoma accounts for more than 25% of all newly diagnosed cervical cancers, affecting young women and at the same time it remains a problem even in screened populations. Since HPV negativity is more frequently proved in this tumour subtype than in SCC, incidence rates might even reach higher magnitudes in the upcoming decades of the "post vaccination" era. On the other hand, skip lesions often seen in ACC make early identification of precancerous and cancerous more difficult.

The roll of oncogenic or high-risk HPV viruses in cervical carcinogenesis is inevitable, yet not fully understood. The initial hypothesis concerning the link of human papilloma virus (HPV) infection with the development of cervical cancer was proposed by zur Hausen nearly 40 years ago. HPV typing and especially persisting HPV infection is predictive in the risk assessment of premalignant lesions. The prevalence of HPV infections is the highest in younger women (under 30), especially based on the rate of multiple infections. HPV 16 is the most common genotype worldwide in women without cytological alterations, irrespectively of study design and geographical area. It is also the most common type of HPV infection in adenocarcinomas and squamocellular carcinomas. HPV 18 is also common but its prevalence and incidence is more variable across populations. Other common high-risk (HR) HPV types include: 31, 33, 35, 45, 52 and 58. Together with 16 and 18, these HR HPVs show a combined worldwide relative contribution of above 90%. Oncogenic HPV positivity is registered in more than 99% of cervical cancer cases. Through the course of this virus-mediated carcinogenesis, the initial, persistent oncogenic or high-risk HPV infection can progress to well-defined precursor lesions of different grade, known as cervical intraepithelial neoplasia (CIN). More than 80% of cervical cancer (CC) cases are histologically squamous cell carcinomas (SCC) preceded by high grade dysplasia (CIN2-3).

The Pap smear (cytology) was a revolutionary technique with a major public health impact - that has changed dramatically the incidence of cervical cancer since its widespread introduction in screening programs in developed countries. One of its limitations is the lack of sufficient information regarding infection and disease outcome (6) besides the frequently mentioned inter-observer variation (7). On the other hand, with extensive HPV-testing a high rate of insignificant HPV infections are revealed. Even though co-testing (HPV testing combined with cytology) is a reasonable alternative, the need for

alternative molecular biomarkers with prognostic value, especially regarding the HPV infection outcome, is overwhelming.

What we also need to consider, is that HPV-infection alone is insufficient to induce malignant transformation. Other, for example genetic variations (individual susceptibility), alterations in regulatory mechanisms are also involved in the process. The focus has already turned on regulatory networks in the field of cancer research, with an emphasis on microRNAs, since they play key roles in vital biological functions, including development, differentiation, metabolism, apoptosis. MicroRNAs (miRNAs) are small, non-protein-coding RNAs representing a new class of regulatory mechanisms with important functions in a wide range of biological processes. Aberrant or altered miRNA expression has been reported in a variety of human cancers with a wide range of clinical potential. miRNAs are not only tissue-, organ- or cell-specific. In the process of understanding carcinogenesis, the analysis of miRNA expressions has become one of the focal points in molecular biology. Genome-wide profiling of miRNA signatures has indicated that altered miRNA expression is common in most human tumors. These small, noncoding RNA sequences that regulate gene expression on the posttranscriptional level in a diversified network, might function as oncogenes or tumour suppressors by modulating oncogenic or tumour suppressive pathways. Deregulation of miRNA expression and their contribution to cancer development and progression have already been proven in a lot of malignancies. A more detailed analysis of miRNA alterations occurring during high-risk HPV transformation will increase our current understanding on cervical carcinogenesis. In addition, miRNAs are considered to be a better choice for expression studies since they are more accurate for distinguishing disease states than mRNA expression analysis. Archival collection of formalin-fixed, paraffin-embedded (FFPE) tissues is widely used for biomarker research due to the link to rich clinical databases and utility. Previous studies have already demonstrated that miRNAs are minimally affected and display reliable expression levels regardless of fixation time or age of tissue blocks. Their small size and a possible protein protection by the RNA-induced silencing (RISC) complex are thought to contribute to their stability during fixation and processing Furthermore, a high correlation has been shown by Goswani et al. between miRNA expression profiles in FFPE and fresh-frozen samples.

In high-risk HPV infections the regulation of cellular oncogenic and tumour-suppressive miRNAs is altered, leading to differences in expression profiles. This is most probably generated by viral proteins, mainly E6 and E7. Changes in the expressions of miR-34a, miR-21, miR-203 and miR-218 are all attributed to interactions of these two viral oncoproteins, although not every detail of the network of events has fully been discovered yet. Many times miRNA genes are observed at HPV integration sites, associated with cancers of various types. On the other hand, cellular miRNAs, like miR-125b and miR-203, may also play an important role in the regulation of viral gene expression and DNA replication. The detailed description of the involvement of specific miRNAs might help in the identification of interactions of viral and cellular miRNAs and in understanding the pathogenesis of

cervical cancer. The fact that miRNA profiling overcomes the limitation of age and consequent degradation in FFPE samples, unlike mRNAs, makes the approach even more appealing.

The role of miRNAs has already been investigated and results are diverse, showing high variability especially in normal cervical issues. Data on the level of deregulated miRNAs is sometimes also distinct. In 2007 Lui et al showed significantly reduced expression of miR-143 and increased expression of miR-21 in a panel of matched pairs of human cervical cancer and normal cervical samples using direct sequencing. Also in cervical tissue samples Lee et al. highlighted the upregulation of miR-21, miR-29a, mir-146a, miR-155 and the downregulation of miR-203 among others using TaqMan quantitative real-time PCR. When studying expressions in cervical tissues and in cervical cell lines Wang et al found, that miR-29a, miR-143, miR-145, miR-146a, miR-199a, miR-218 were down-regulated and miR-21, miR-155 were up-regulated. Pereira et al indicated lower expressions of miR-29a, miR-143, miR-145, miR-199a and miR-203 with the simultaneous upregulation of miR-196a. They observed high expression variability between their samples of SCC, H-SIL, L-SIL and normal cervical epithelial tissues, but they were able to identify deregulated miRNAs. The panel of miRNAs was systematically chosen, based on published data and previous experience regarding expression profiles in epithelial tumors.

Objectives

1. To determine the prevalence of specific oncogenic HPV types in the studied specimens of the two most common types of cervical cancer with a special focus on and the characterisation of multiple HPV infections
2. Targeted miRNA expression profile analysis in cervical cancer specimens of different HPV status and histological type
    a. determination of possible correlations with histopathology (SCC, ACC)
    b. determination of possible correlations with HPV status
3. To determine and analyze specific differences in the miRNA expression profiles of cervical cancer according to clinical grading with a special focus on single and multiple HPV positivity
    a. determination of possible correlations with clinical grade (FIGO)
    b. determination of possible correlation with mono- and multiple HPV positivity
4. Analysis of targeted miRNA expression profiles of HPV positive dysplastic and neoplastic cervical tissue
    a. comparison of HR HPV induced squamous intraepithelial lesions and early stage (FIGO I) cervical cancer
    b. characterization and analysis of miRNA expression profiles based on specific features of HPV infection (mono/multiple HPV, HPV16 positivity)

c. possible correlations with demographic, socioeconomical and lifestyle factors

5. Identification of potential miRNA biomarkers or their combination to differentiate HPV induced dysplastic alterations from malignant cervical cancer with the definition of possible predictive value.

Materials and methods

Samples

Formalin-fixed paraffin embedded human primary cervical carcinoma tissue samples (adenocarcinoma: n=24 and squamocellular carcinoma: n=26) were selected for further analysis from the archives of the Pathology Department of Pécs University. Samples were obtained from patients diagnosed and treated between 2007-2010 at the Department of Obstetrics and Gynaecology, Pécs University. Following HPV genotyping, performed by Genoid Laboratorium, our aim was to compare the expression of 8 different microRNAs (miR-21, miR-27a, miR-34a, miR-146a, miR-155, miR-196a, miR-203, miR-221) in the 2 most common histological subtypes of cervical cancer. Overall, the selected miRNAs were reported to be involved in oncogenesis and progression in cervical and other neoplasms (over- or under-expression).

A total of 100 female patients' (admitted between 2009-2014 to the Department of Obstetrics and Gynecology, University of Pécs) cervical FFPE tissue samples (obtained either by conisation or hysterectomy), known to be high-risk HPV-positive (pre-selection criteria based on previous HPV genotyping) with definitive histological diagnosis of cervical lesions (CIN1, CIN2-3 and invasive cervical cancer), were used in the second phase of our study. Histological diagnoses were produced by the same gynecological pathologist. Tissue blocks were retrieved from the Department of Pathology, University of Pécs, according to approved protocols and were micro dissected restraining to selected morphologically distinctive areas. A total of 4 pieces of 10 μm thick FFPE sections were collected and used for further analysis. The miRNAs investigated in this phase (miR-21, miR-27a, miR-34a, miR-155, miR-196a, miR-203) were systematically chosen based on our previous results and published literature (cell lines, clinical specimen).

Clinical specimens were grouped according to histological diagnosis: 1, mildly dysplastic lesions (CIN1: n=30), which typically spontaneously regress; 2, precancerous lesions (CIN2: n=10 and CIN3: n=20), about 20% of these cases would progress to invasive cervical cancer; and 3, squamous cell cervical carcinoma (SCC: n=38). Single (n=65) and multiple (n=33) HR HPV-positive samples were found in every category. Low-risk HPV types (6 and 11) were identified in one case simultaneously with HR HPV positivity.

Deparaffination

To investigate miRNA expression profiles in cervical cancer the first step taken was deparaffination of sample materials (3 sections 8-10μm each), using xylol and absolute alcohol in 1.5 ml reaction volume.

RNA extraction and microRNA real-time polymerase chain reaction (qPCR) expression analysis

After deparaffination we isolated total miRNA according to manufacturer's instructions (High Pure microRNA Isolation Kit, Roche), followed by reverse transcription of 5 μl of extracted miRNA with random hexamer primers using Transcriptor First Strand cDNA Synthesis Kit (Roche) resulting in total volume of 50 μl in LightCycler® 2.0 (Roche). RNA purity was measured using the A_{260}/A_{280} ratio and was found to be between 1.9 and 2.1. The expression of miRNAs was determined using quantitative real-time PCR using a standard LightCycler® 480 SYBR Green I Master PCR kit protocol in the LightCycler® 480 Instrument (Roche). The 20 μl PCR mix included 5 μl template cDNA, 10 μl PCR Master Mix, 2 μl pre-miRNA-specific primer (10 μM solution of each pair of primers - Exiqon, diluted and stored at 4°C in PCR tubes) and 3 μl PCR grade H_2O. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 55 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s and extension at 72°C for 15 s. Runs were concluded with quantification and 1 cycle melting curve analysis at 95 ^{o}C for 5 s, at 65 ^{o}C for 1 min, Continuous acquisition 97 ^{o}C. All assays, including no template controls (nuclease-free water) were done in triplicate. Inter-run calibrators were used to calculate correction factors to remove run-to-run differences. Data normalization was done using 5S rRNA and U6 snRNA as endogenous references. A normalization factor was calculated based on the arithmetic mean Cq value of reference genes. Relative quantification of miRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical methods

Statistical analyses were performed using IBM SPSS Version 21 (Armonk, New York, USA). Mann-Whitney U-test, unpaired t-test and one-way analysis of variance (ANOVA) were used to compare continuous and categorical variables based on data distribution. Logistic regression analyses were conducted to identify variables (miRNA expressions, sociodemographic and life-style factors) associated with worse pathological outcomes. The level of significance was set at $p<0.05$.

Results

Prevalence of specific oncogenic HPV types in the studied specimens of the two most common types of cervical cancer with a focus on multiple HPV infections, including characterisation

The rate of high risk HPV positivity in SCC and ACC was 76% and 68.18% respectively, which was lower than previously expected, based on published data. Out of the high risk HPV types 16 and 18 were the most frequently registered, with an overall prevalence of 78.95% in SCC and 86.67 % in ACC. In SCC type 16 was more abundant in SCC (14 : 1), than in ACC, where the ratio of 16 and 18 was almost equal (7 : 6). The difference was statistically significant between the two histopathological subtypes (p=0.041 using Pearson χ^2 test).

miRNA Expression Profile in correlation with histopathology

The miRNA profiling data were analysed to identify individual or multiple miRNAs that significantly correlated with histopathological findings. The overall expression profiles based on the chosen eight microRNAs were distinctive of the histological characteristics in cervical cancer samples. The comparison of targeted miRNA expression profiles of ACC and SCC displays distinctive characteristics. The expression levels of all miRNAs were higher in SCC, than in ACC. The magnitude of difference was the highest (4.86x) in the case of miR-34a with a p=0.001. Based on mean values, the differences, expressed in "n times" form, have reached the level of statistical significance in almost all the cases, using independent samples T-test: miR-21: 2.62 (p=0.004), miR-27a: 1.88 (p=0.018), miR-146a: 3.98, miR-155: 2.06 (p=0.021), miR-196a: 1.44 (p=0.032), miR-203: 2.75 (p=0.037) and miR-221: 3.35 (p=0.017). The only exception was miR-146a, where the difference lacked significance.

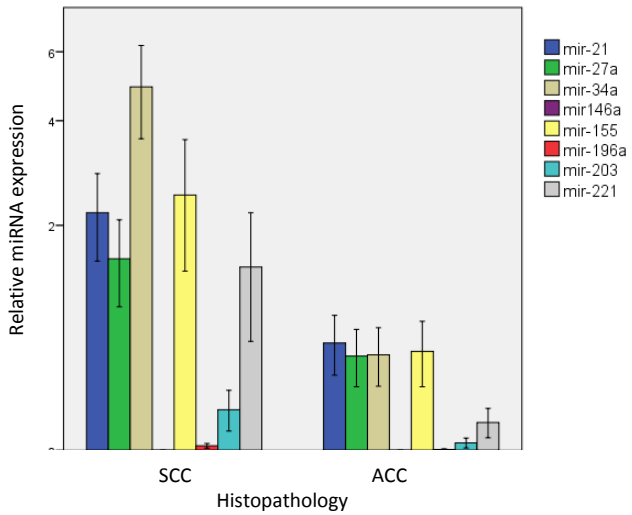


Figure 1. Relative miRNA expressions (5SrRNA) in correlation with histopathology (SCC, ACC)

Correlation of miRNA expression with HPV status

On comparing the microRNA expression of HPV negative and positive cervical cancers, the expressions of miR-21, miR-27a, miR-146a, miR-196a, miR-221 were higher and the levels of miR-34a, miR-155 and miR-203 were lower in the HPV positive group (Figure 2.), even though after statistical analysis the differences were not significant.

As we assessed the histopathological diagnosis parallel with the HPV status, the patterns of the expressions of the selected miRNAs showed greater variability. In the case of SCC with HPV positivity we found higher levels of miR-21, miR-146a, miR-196a, miR-221 than in HPV negative patients. None of the differences reached statistical significance. As for miR-34a, miR-155 and miR-203, HPV positive patients showed lower levels of expression. The difference was statistically not significant.

In ACC HPV positivity featured higher miRNA expressions, than HPV negativity. The only exception was miR-146a. The differences have not reached the level of statistical significance.

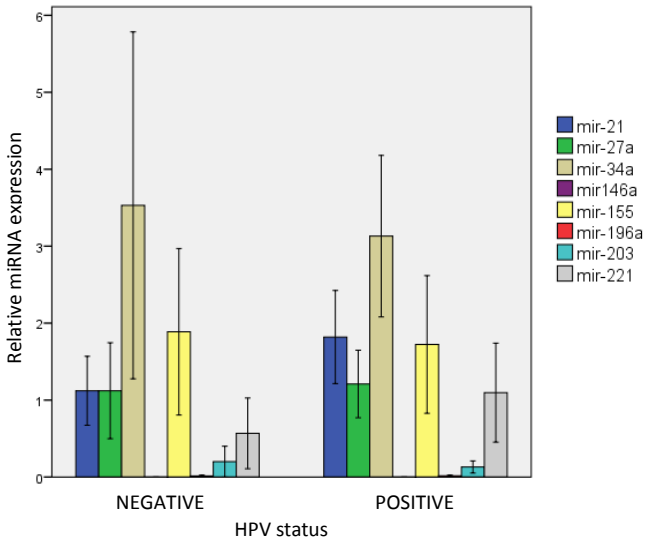


Figure 2.

We also differentiated mono- and multiple HPV positivity and analysed the expression profile of the studied miRNA panel in these two groups. In ACC the expressions of miR-21 and miR-27a were independent of HPV status. In SCC miR-21 expression was similar in HPV negative and single HPV positive samples. On the other hand expression level was 3.11 times higher in multiple HPV positive samples. The level of miR-27a was 4.37 times higher in multiple HPV positive samples (median: 2.463) compared to mono-HPV positives (median: 0.563). The expression of miR-34a showed continuous decrease in both SCC and ACC starting out from HPV negative through mono- and multiple HPV positivity. In mono HPV positive SCC the expression of miR-146a was exceptionally high, while in ACC its expression was lowest in HPV negative samples. As for miR-155 a gradual decrease in expression levels was determined in SCC. We measured the highest expressions in ACC

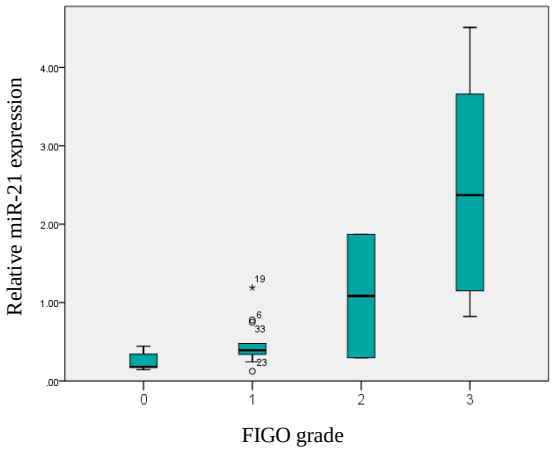
also in HPV negative cases, but on comparing mono and multiple HPV positivity we saw slight increase. The expression of miR-196a was steady, independent of HPV status in ACC. Highest miR-196a expression in SCC was in single HPV positivity. We determined a slight, but gradual decrease in tendency for the expression of miR-203 in SCC. As for ACC the trend was different. Highest miR-203 expression in ACC was in HPV negative samples, but after a lower level in mono HPV positivity expression slightly increased in multiple HPV positivity. The expression of miR-221 was the highest in mono HPV positive ACC, while it showed relatively constant in SCC with the only difference in variance.

The miRNA expression patterns observed in SCC with HPV16 positivity were also characteristic in the case of miR-27a (p=0.001), miR-203 (p=0.003) and miR-221 (p=0.009). No significant differences were found in ACC in correlation with HPV16 positivity.

After detailed statistical analysis of the two most prevalent types of cervical cancer the use of miR-21 (p=0.007), miR-34a (p=0.002), miR-155 (p=0.003), miR-203 (p=0.01) and miR-221 (p=0.003) as potential future biomarkers look the most probable.

Specific differences in miRNA expression profiles of cervical cancer according to clinical grading with a special focus on single and multiple HPV positivity

We compared the different clinical stages of cervical cancer according to FIGO in the next phase of the statistical analysis. Distinctive and statistically significant differences were registered. The expression levels of miR-21 and miR-27a (Figure 3.), miR-146a (Figure 4.), miR-155 and miR-196a (Figure 5.) and miR-221 (Figure 6.) showed continuous increase with disease progression. At the same time the expression levels of miR-34a and miR-203 (Figures 4. and 6.) displayed a progressively decreasing tendency.



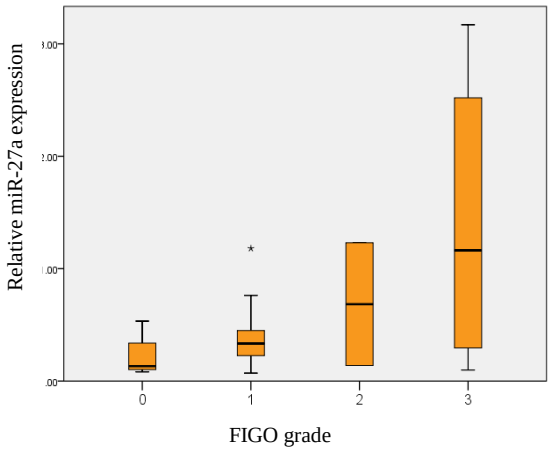


Figure 3. Relative expression of miR-21 and miR-27a (median) in different clinical grades of cervical cancer (FIGO 0-3)

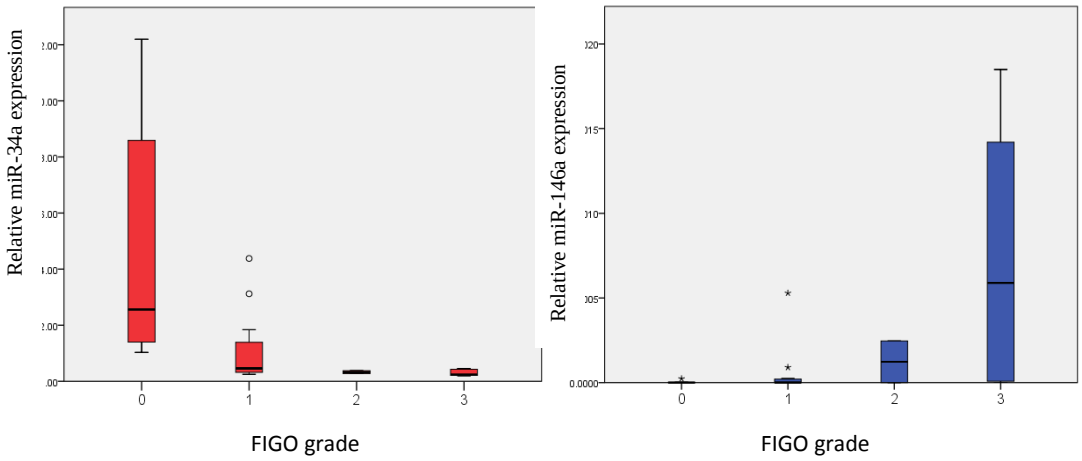


Figure 4. Median values of relative miR-34a and miR-146a expression in different clinical grades of cervical cancer (FIGO 0-3)

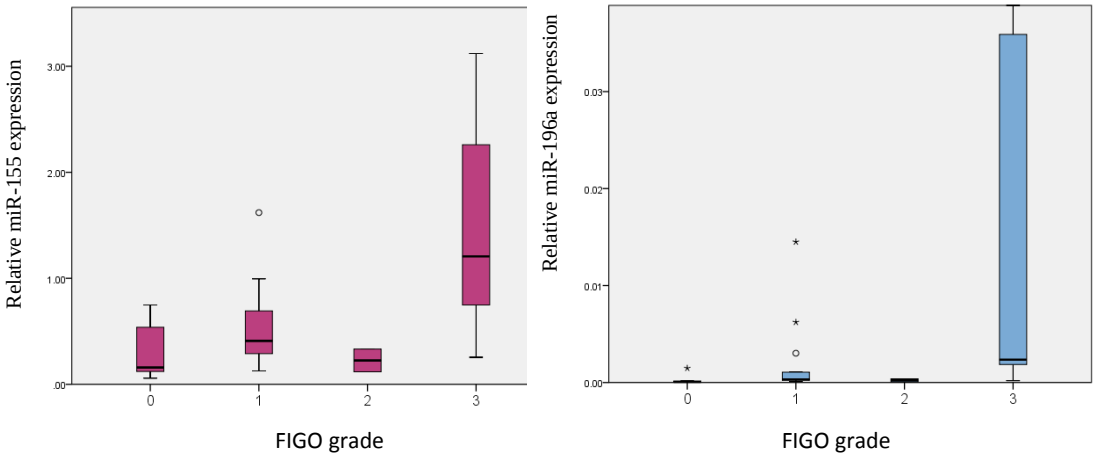


Figure 5. Median values of relative miR-155 and miR-196a expression in different clinical grades of cervical cancer (FIGO 0-3)

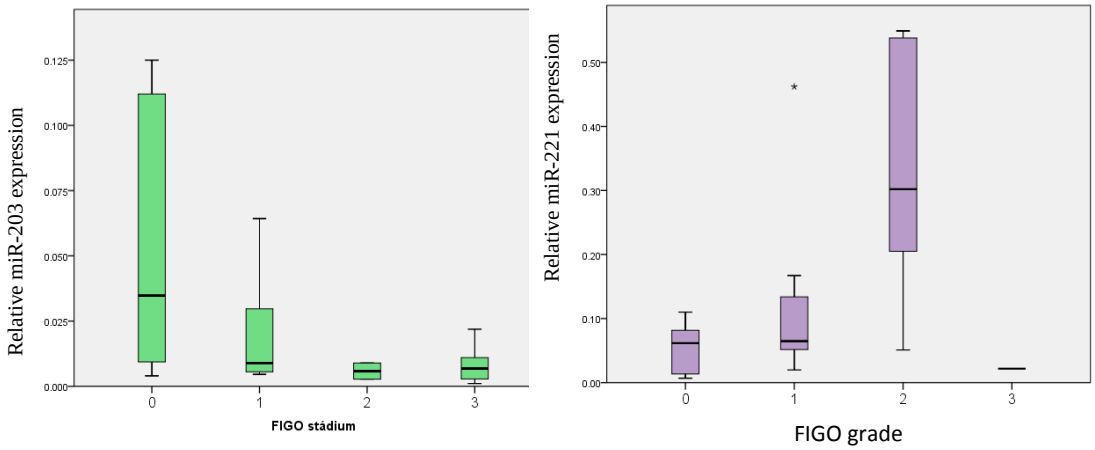


Figure 6. Median values of relative miR-203 and miR-221 expression in different clinical grades of cervical cancer (FIGO 0-3)

Statistical evaluation (one-way ANOVA) identified distinct correlations between the following targeted miRNA expression levels and the extent of cervical cancer regardless of histopathology: miR-21 (F=9.656, p=0.001), miR-27a (F=3.262, p=0.042), miR-34a (F=4.275, p=0.016), miR-146a (F=4.44, p=0.014) and miR-221 (F=3.380, p=0.036). The Tukey post-hoc test proved significantly higher miR-21 expression in FIGO III (2.48±0.65) on comparison with FIGO 0 (0.256±0.058, p=0.001) and FIGO I (0.474±0.78, p=0.001). In the case of miR-27a significant association was found in FIGO III (1.401±0.54) and FIGO I (0.401±0.083. As for miR-34a we found significantly lower expression in FIGO III (0.307±0.057) related to FIGO 0 (4.723±1.87, p=0.028) and FIGO I (1.092±0.359, p=0.026). We observed significantly higher expression levels in FIGO III (0.00074±0.0003) compared to FIGO 0 ($5.21x10^{-6}$±$4.5x10^{-6}$, p=0.033) and FIGO I (0.000053±0.00004, p=0.013). Multivariate tests (two- and three-way ANOVA, logistic regression) found no significant interaction between the examined parameters.

Analysis of targeted miRNA expression profiles of HPV positive preneoplastic and neoplastic cervical tissue

As far as for the distribution of HR HPV, HPV 16 was the most prevalent (57.53%) considering both single and multiple infections; types 18 and 31 were the second most frequent (10.96%) followed by 56 (9.59%) and types 51, 52 and 58 (8.22%). These findings correspond with the results of the first Hungarian HPV-Centre in Budapest from 2007-2011. Galamb et al. detected a relatively low representation (compared to data from developed countries) of HPV 18 along with high prevalence of other, not so common, HPV types. At the same time, the prevalence of types 51 and 31 among patients with cytological abnormality exceeded the expectations based on international data reflecting dominance of types 16, 18 and 45. The prevalence of multiple HPV infection was 33.67%, much lower than expected based on previous data.

As described previously, the total of 100 samples were grouped according to histological diagnosis (CIN1: 23%, CIN2-3: 34% and SCC: 43%) and the presence of single or multiple HPV positivity. The mean age of the studied population was 36.17 (range, 18-65). Mean age was significantly lower among multi-HPV-positive patients than among mono-HPV-positive individuals (33.30±7.994 compared to 37.98±10.516, p=0.039). The mean age for patients infected with multiple HPV types was lower in every disease stage than the mean age for patients with single HPV infection, with the only exception being the CIN1 group where the mean age and the age range were the same or quite similar for both subcategories (single: 33.2±8.804 and multiple: 33.2±10.207).

The expressions of oncomiRs miR-21 and miR-155 increased parallel with severity and progression of squamosus lesions, while the expression of miR-34a, a tumour suppressor, continually decreased (Figure 7.). The expressions of the other miRNAs (miR-27a, miR-196a, miR-203) were higher in CIN2-3 than in CIN1, but lower levels were determined in early stage SCC than in CIN2-3 (Figure 7.).

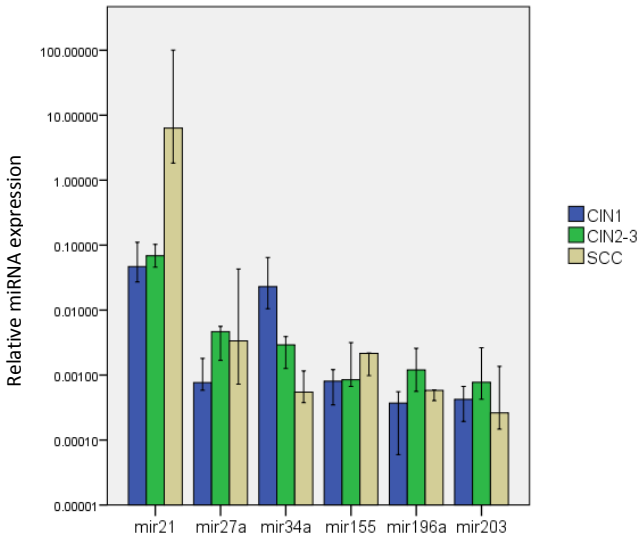


Figure 7. Relative median expressions of the studies miRNAs
in HR HPV positive cervical samples of different grade

Significant associations were found as the expression of miR-27a increased with progression: CIN2-3 related to CIN1 (p=0.023) and SCC to CIN2-3 (p=0.033). MiR-21 and 155 also showed an increase in the transition from CIN1 to SCC with statistically significant correlation in CIN2-3 over CIN1 (p=0.023). MiR-196a expression showed an increase from CIN1 to CIN2-3 but was significantly lower in SCC than in CIN2-3 (p=0.016). These associations were unaffected by differences in the type or number of HPV. Even though we registered elevations in miR-21 expression through every disease stage from the progression of CIN1 to CC, the differences were not significant. On the other hand, significantly lower levels of miR-34a were detected in CIN2-3 than in CIN1 (p=0.041) and in SCC than in CIN2-3 (p=0.025).

Analysis of targeted miRNA expression profiles of HPV positive preneoplastic and neoplastic cervical tissue with specific focus on mono/multiple HPV and HPV16 positivity

Furthermore, we found significant differences in subjects with multiple HPV for miR-27a (p=0.001), miR-34a (p<0.001) and miR-203 (p=0.025) in CIN2-3 compared to CIN1 and for miR-21 (p=0.002) and mir-27a (p<0.001) in SCC/CIN2-3 with miR-21 (p=0.001), miR-27a (p=0.001) and miR-34a (p=0.001) in CIN1/SCC. The statistical analysis (logistic regression) regarding the correlation between

miRNA expression and the role of single or multiple HPV showed significant association with disease outcome in the case of miR-27a (p=0.029) and miR-34a (p=0.045) (Figures 8 and 9, respectively).

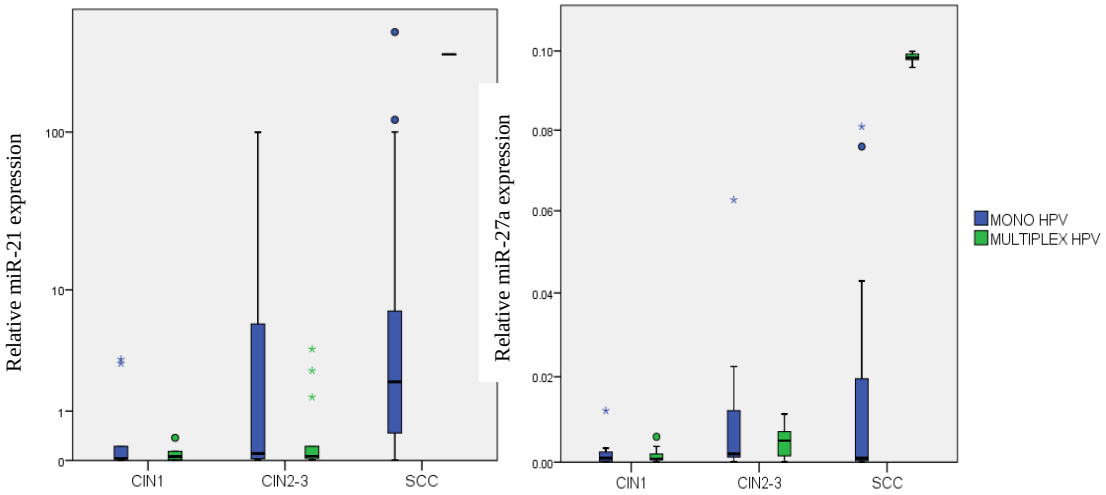


Figure 8. Relative expression of miR-21 (on a $\log_{10}$ scale on the y-axis) and miR-27a in different grades of squamous intraepithelial dysplasia and early stage (FIGO I) cervical cancer

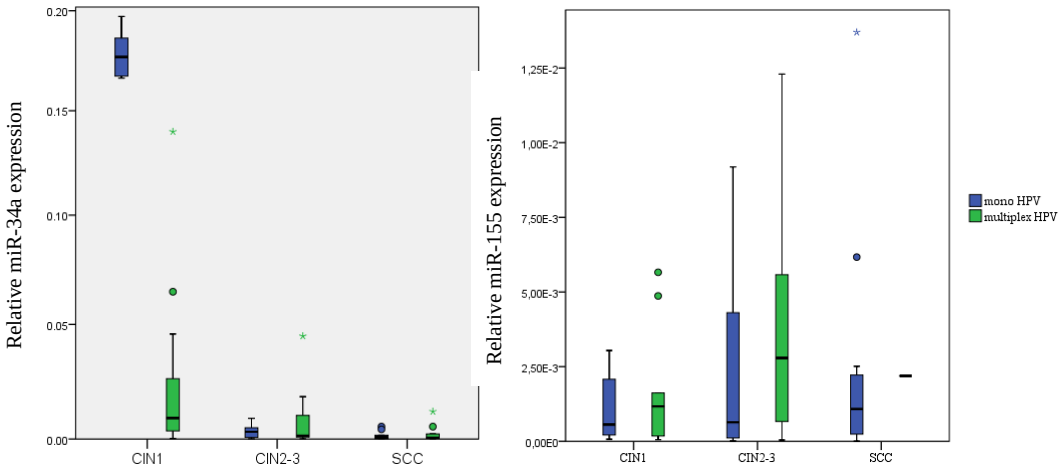


Figure 9. Relative expression of miR-34a (on a $\log_{10}$ scale on the y-axis) and miR-155 in different grades of squamous intraepithelial dysplasia and early stage (FIGO I) cervical cancer

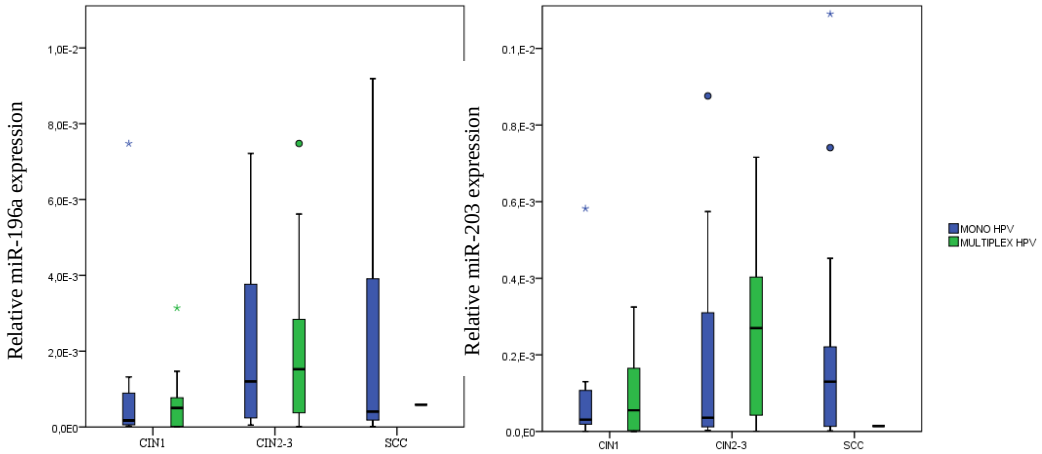


Figure 10. Relative expression of miR-196a and miR-203 in different grades of squamous intraepithelial dysplasia and early stage (FIGO I) cervical cancer

The presence of HPV 16 has a significant effect on disease outcome (p=0.004) (CIN1: 39%; CIN2-3: 65%; SCC: 72%). During the transition from CIN1 to CIN2-3, miR-27a and miR-34a correlated with HPV 16 positivity (p=0.028 and p=0.036, respectively). As for the progression of CIN2-3 to SCC, in correlation with HPV 16, miR-27a and miR-34a reached significance (p=0.027 and p=0.036, respectively). Our study population had a rate of cigarette smoking at 65% among women with CIN1, 46.67% among women with CIN2-3 and 53.33% among CC subjects. Alteration in miRNA expressions between smokers and non-smokers appeared statistically significant in the case of miR-27a and miR-34a among CIN1 subjects (p=0.036 and p=0.029, respectively). The expressions of miR-155, miR-196a and miR-203 were independent of the presence of HPV16.

Identification of potential biomarkers

For the identification of potential biomarkers we carried out ROC Curve analysis. HR HPV induced squamous intraepithelial lesions of different grade were efficiently distinguishable with the analysis of miR-27a and miR-34a expressions. Simultaneous, parallel monitoring of these two miRNAs helped classify the different lesions. Our results suggest that the combination of miR-27a and miR-34a was most effective in the discrimination of high grade dysplasia (CIN2-3 or H-SIL) and early stage SCC. The AUC (Area under the curve) =0.889 (88.9%), with a 95% confidence interval of 0.779-0.999 and p=0.001. The sensitivity and specificity were not as adequate if the same test was used for distinguishing low (CIN1) and high grade (CIN2-3) dysplastic lesions with AUC=0.845 (84.5%). (Figure 11.)

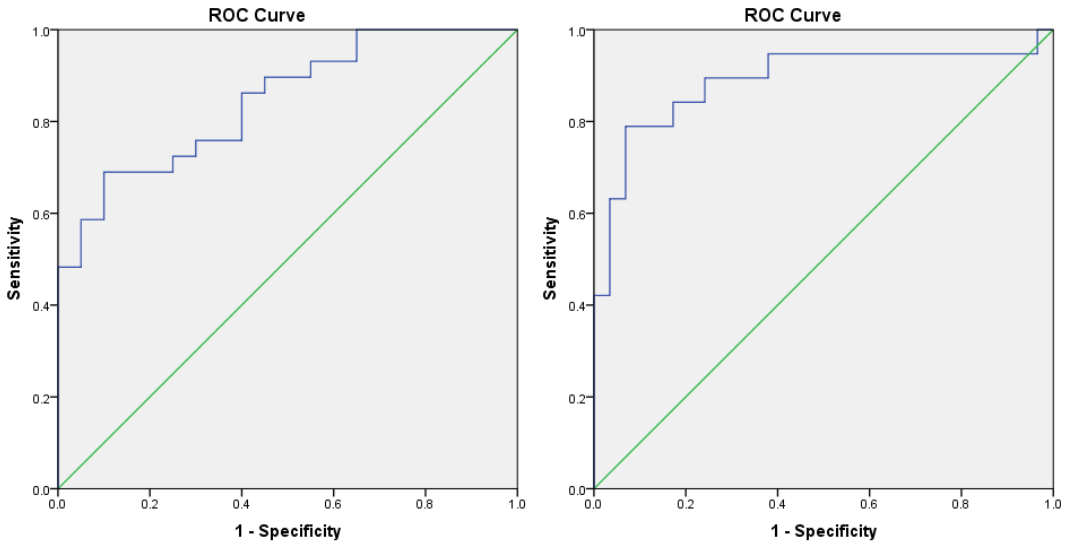


Figure 11.

Left: Differentiation of low (CIN1) and high grade (CIN2-3) dysplasia based on the combination of miR-27a and miR-34a expression

Right: Differentiation of high grade (CIN2-3) dysplasia and early stage SCC (FIGO I) based on the combination of miR-27a and miR-34a expression

Discussion

Deregulation of microRNAs, in connection with malignant transformation, is well known. The etiological relevance of high-risk HPV types in the development of cervical cancer has been demonstrated by a series of epidemiological and biological studies during the past few decades. Nevertheless, a high-risk HPV infection does not inevitably progress to invasive cervical cancer. Multiple HPV genotypes have been found to be a risk factor for persistent infection in healthy young women and co-infections involving HPV 16 and 58 seemed particularly prone to increased risk. The phenomenon that we observed in the case of miR-27a and miR-34a, in correlation with HPV 16 positivity during the transition from low-grade to high-grade dysplasia, seems to reinforce this previous observation. Alterations in the expressions of both oncogenic and tumour suppressor miRNAs (miR-27a and miR-34a) also verify the opinion that multiple HPV types appear to act synergistically in cervical carcinogenesis.

Previous studies have used different types of samples (cultured cells, cancer-derived cell-lines, human tissue samples - fresh-frozen or FFPE, serum) for the analysis of miRNA expression profiles related to cervical cancer and, despite the overlapping results obtained, there is a considerable amount of variability.

MiRNA expression analysis in cervical cancer in correlation with histopathology, HPV status and clinical grade

In this study, we have shown that it is possible to differentiate the two most frequent histological types of cervical cancer based on miRNA profiles and even though the diagnosis of cervical cancer is currently based on clinical and pathological examination, our findings raise the possibility of using miRNA expression profiles in tumour type distinction and maybe even in prediction in the case of preneoplastic lesions. There is scarcely any published data regarding the differences in microRNA profiles based on histopathology. We also took the relevance of HPV infection into consideration, since it is thought to be the most important factor for transition from normal cervical epithelium to pre-neoplastic cervical intraepithelial neoplasia that subsequently transforms to invasive cervical cancer. However, the pathogenic mechanism is still unknown. Proteins encoded by E6 and E7 genes of high-risk HPVs cause degradation and/or inactivation of p53 and Rb proteins. HPV-associated miRNAs (miR-34a, miR-146a, miR-203), directly or indirectly regulated by E5-E7 oncogenic proteins, play an important role in the initiation and progression of cervical cancer.

Our analysis of expression profiles for the targeted miRNAs indicated significant differences in the case of miR-21, miR-27a, miR-34a, miR-155, mir-196a, miR-203 and miR-221. We verified significant differences between the HPV-positive histotypes in the case of miR-21, miR-27a, miR-34a, miR-196a and miR-221. The miR-21 gene is located on chromosome 17q23.2, which is inside the common fragile site FRA17B. This region has been frequently found to be amplified in several solid tumours, which seems to be consistent with the fact that miR-21 is elevated in these cancers. One of the HPV16 integration loci is at 17q23.2, suggesting that the expression of cellular miRNA genes at or near HPV integration sites may contribute to the tumour phenotype. Here, we have shown that expression of miR-21 was upregulated in patients with HPV infection, implying that HPV infection induces carcinogenesis probably through altering expression of some oncomiRs such as miR-21. We found that miR-21 was abundantly expressed in HPV-positive samples overall and separately in both histotypes. The overexpression of miR-21 was also consistently increasing with clinical grade (FIGO 0-III). Yao and Lin indicated that miR-21 has multiple functions in the development of cervical squamous cancer by showing that miR-21 could dramatically increase cell proliferation, inhibit apoptosis and promote cell migration in HPV16-positive cervical squamous cancer lines. In the same study they also proved that overexpression of miR-21 was associated with advanced disease and lymph node metastasis. Schmitz et al. studied HPV integration sites and they concluded that integration is not an entirely random event but also involves preferred chromosomal sites, including near miRNAs. Of the 75 miRNAs in the neighbourhood of integration sites many have already been associated with cancer and of these miR-34a, miR-21 and miR-27a are expressed in cervical cancer cells. Previous studies indicated that the HPV E7 protein downregulates miR-203 expression upon

differentiation, which may occur through the mitogen-activated protein (MAP) kinase/protein kinase C (PKC) pathway. One target of miR-203 is the p63 family of transcription factors, and we demonstrate that HPV-positive cells maintain significantly higher levels of these factors upon differentiation than do normal keratinocytes. Melar-New and Laimins concluded that high levels of miR-203 are inhibitory to HPV amplification and that HPV proteins act to suppress expression of this microRNA to allow productive replication in differentiating cells. In addition McCluggage has already declared that p63 is a useful marker of squamous neoplasms within the cervix, which might as well imply the importance of miR-203. MiR-146a is also considered to be cervical cancer specific, but it was proved to be independent from histotype or HPV-infection. Latter has already been indicated by Wang et al.

MiRNA expression analysis in HR HPV positive cervical dysplasia and cancer

In our study, we wanted to distinguish characteristic alterations in miR-21, miR-27a, miR-34a, miR-155, miR-196a, miR-203 expression in different stages of cervical dysplasia and SCC. MiR-27a is an oncogenic miRNA modulated by p53, E2F and c-Myc. Both HR HPV E6 and E7 interact with c-Myc and augment c-Myc transactivation activities. We found that in HR HPV positive tissue samples miR-27a expression correlated with the progression of high grade cervical lesions to cancer making it a credible biomarker for HR HPV-associated cervical carcinogenesis. A similar phenomenon was observed with higher level of miR-27a in CC compared with CIN3, CIN1+2 or normal cervical tissues in oncogenic HPV-infected raft tissues by Wang et al. Our results are unique regarding associations with single and multiple HPV positivity.

MiR-34a is directly regulated by p53 and acts as a tumour suppressor, while HR HPV E6 induces the inhibition of miR-34a through the p53-pathway. It is a downstream target of the p53-network with key regulatory functions in cellular apoptosis, G1-arrest, DNA repair and senescence, which are essential for the maintenance of cellular stability and stress response. Li et al. have demonstrated that the expression of miR-34a is significantly lower in HR HPV-infected tissues. Our observations clearly confirm that miR-34a acts as a tumour suppressive miRNA in HPV-induced cervical transformation. We also note that alterations in miR-34a expression are associated with the presence of single or multiple HR HPV types supporting the theory of synergism on multiple HPV infection. Cigarette smoke-induced dysregulation of miRNA expression has been demonstrated in both rats and humans but mainly focusing on lung cells or the bronchial airway epithelium. One of the miRNAs significantly dysregulated by cigarette smoke (CS) and implicated in lung carcinogenesis is miR-34a, causing disruption of transcription. Smoke seems to leave a unique “molecular signature” in the p53 gene in smoke-exposed lung tumours. In the light of these observations, we can rightly hypothesize that CS might influence miRNA regulatory mechanisms in other organs, such as the cervix as well. Based on these findings, we can conclude that miR-27a and miR-34a could help to predict HPV infection outcome and assist in distinguishing different cervical lesions.

The fact that the expression of miR-155 correlated with the progression of low grade to high grade cervical lesions makes miR-155 eligible as a possible biomarker for HR HPV-associated cervical carcinogenesis. Even though we did not find a significant correlation, the difference in miR-155 expression linked to smoking history must be mentioned. Functionally miR-155 mediates immune response by shaping the transcriptome of lymphoid cells that control diverse biological functions vital in adaptive immunity. Its altered expression has also been implicated in viral infections and persistence (Epstein Barr Virus). Lao et al. registered up-regulated expression of miR-155 in cervical cancer tissues compared to adjacent non-cancer tissues. They found that over-expression of miR-155 promoted the proliferation of Hela and SiHa cells and the down-regulation of miR-155 inhibited the growth of cervical cancer cells and promoted apoptosis and induced cell cycle arrest. They also suggested that miRNA-155 promoted the proliferation of cervical cancer cells by regulating tumour suppressor liver kinase B1 (LKB1) expression. Functional studies revealed that miR-155 directly targets the tumour protein 53-induced nuclear protein 1 (TP53INP1) and suppresses apoptosis in tumour cells. MiR-155 over-expression has been observed in other types of tumours, including oral SCC (OSCC) and head and neck squamous cell carcinomas (HNSCC), also known to be associated with HPV infection.

With respect to variations of miR-196a expression during HPV-mediated cervical carcinogenesis, we have confirmed previously reported alterations in tissue samples. We found that these alterations were uninfluenced by the dimension of HPV infection (single-multiple, HPV 16 or other). Villegas-Ruiz et al. have validated HOXC8, involved in embryonic development and cellular remodelling, as a target of miR-196a in CC. Hou et al. suggested that the up-regulation of miR-196a enhanced G1/S-phase transition and the proliferative ability of cervical cancer cells through the regulation of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway. It is important to note that miR-196a also plays a distinct role in the regulation of cell proliferation in HNSCC. Wu et al. have demonstrated that miR-196a might be involved in tumour invasion and in the interactions of tumour microenvironment in patients with squamous cell lung carcinoma. In the case of OSCC, high expression of miR-196a was associated with tumour recurrence, nodal metastasis, mortality and poor survival.

We have proven, in conjunction with Pereira et al., that miR-203 shows a relative reduction in expression during the transition from normal tissue to atypical dysplasia and cancer. This observation is due to the assumption that high levels of miR-203 (targeting, amongst others, the p63 family of transcription factors) are inhibitory to HPV amplification and that HPV proteins (E7 in particular) act to suppress expression of this microRNA to allow productive replication in differentiating cells. It has also been indicated that miR-203 down-regulation in cervical cancer is caused by promoter hypermethylation. Investigations regarding invasion and metastasis showed that low expression of miR-203 correlated with lymph node metastasis.

In this study we have shown that miRNA profiles can differentiate the two most frequent histological types of cervical cancer and even though the diagnosis of cervical cancer is currently based on clinical and pathological examination, our findings raise the possibility of using miRNA expression profiles in tumour type distinction and maybe even in prediction in the case of preneoplastic lesions. The alterations we observed in miRNA expressions can be candidate gene targets and might even serve as possible predictive biomarkers in the field of prevention and therapeutic decision support in response to the urging need for an earlier diagnosis, a more precise prognosis and a successful, personalized therapy.

Further reduction in morbidity and mortality in the case of cervical cancer can only be achieved with future improvements in all levels of prevention. In the era of HPV vaccines for generations to come later and less frequent screening is only acceptable, if vaccination uptake is evenly distributed across all social, economic, ethnical levels of populations. Individual risk estimation is a pivotal point in primary prevention, where higher efficacy is crucial. Cervical cancer, like many other human cancer types, displays significantly aberrant expressions of considerable number of cellular miRNAs, both oncogenic and tumour suppressive. Despite the uncertainty regarding the functional effects of miRNA dysregulation in the pathogenesis of cervical cancer, it might help to assess factors, risks playing part in individual susceptibility and might even help in the clinical projection regarding various characteristics, including histopathologic classification.

Summary

1. We determined the HPV status of the studied specimens along with specific features (genotype, single and multiple HPV positivity)
2. On comparing specific miRNA expression levels (miR-21, miR-27a, miR-34a, miR-203, miR-221) in squamocellular cancer and adenocarcinoma of the cervix we found significant differences. These variations were distinctive not only for histopathologic grade but also for HR HPV status (negative, single and multiple HPV positivity and HPV16 positivity). Our results were based on the outcome of qRT-PCR with relative quantification using SYBR-Green-based detection.
3. We identified specific miRNA expression alterations (miR-21, miR-27a, miR-34a, miR-146a, miR-203) in cervical cancer associated with clinical stage (FIGO 0-III).
4. Characteristic differences in miRNA expression levels (miR-21, miR-34a, miR-155) in HR HPV induced dysplastic (CIN1-3) and neoplastic lesions of the cervix (early stage SCC - FIGO I) were distinguished. These expression profiles (miR-27a, miR-34a) were also influenced by specific features of HPV positivity (single or multiple and HPV16 positivity). Based on these results we sustained the oncogenic role of miR-21 and miR-27a and the

tumorsuppressor nature of miR-34a in the cervical carcinogenesis. The synergic effect suspected by multiple HPV positivity was also successfully verified on the level of miRNA expressions (miR-27a, miR-34a).

5. Regarding the connection between the expression levels of the studied miRNAs and demographic, socioeconomical and lifestyle factors of the patients, we were unable to prove any correlation.
6. We proposed the use of miR-27a and miR-34a as possible biomarkers with predictive potential. We base this assumption on our results regarding the expression patterns for miR-27a and miR-34a in HR HPV positive cervical intraepithelial neoplasia and early SCC.

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