# Unique MicroRNA Expression Profiles in Cervical Cancer

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**Abstract.** *Cervical cancer is the second leading cause of death among female patients with cancer in the world. Our aim was to analyze cervical cancer cases, in the Southwestern Transdanubian Region of Hungary, with regard to human papillomavirus (HPV) genotype and histological and clinical grading. After HPV testing and genotyping, the expressions of eight different pre-microRNAs (miR-21, miR-27a, miR-34a, miR-146a, miR-155, miR-196a, miR-203, miR-221) in formalin-fixed paraffin-embedded (FFPE) primary human cervical cancer samples were evaluated with the help of the LightCycler 480 PCR System (Roche). Statistically significant overexpression of miR-21 (p=0.004), miR-27a (p=0.018), miR-34a (p<0.001), miR-155 (p=0.021), miR-196a (p=0.032), miR-203 (p=0.037) and miR-221 (p=0.017) were observed in squamous cell carcinoma, regardless of HPV status and clinical grading. Significant overexpression of miR-21 (p=0.004), miR-27a (p=0.02), miR-34a (p<0.001), miR-196a (p=0.027) and miR-221 (p=0.031) was characteristic of HPV-positive squamous cell carcinomas in contrast to adenocarcinomas of the same HPV status.*

Even though widespread screening programs and the introduction of HPV vaccines have reduced its 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) (1). 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 (2, 3). According to the latest data from 2011, cervical adenocarcinoma accounts for more than 25% of all newly-diagnosed cases of cervical cancer, particularly affecting young women, and at the same time it remains a problem even in screened populations (4). It needs to be emphasized that many women diagnosed with cervical adenocarcinoma (ACC) are young (5). Since human papillomavirus (HPV) negativity is more frequently proven in this tumor subtype than in squamous cell carcinoma (SCC), incidence rates might reach even 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 pre-cancerous and cancerous lesions more difficult.

The role of oncogenic or high-risk HPV viruses in cervical carcinogenesis is indisputible, yet not fully understood. Oncogenic HPV positivity is registered in more than 99% of cervical cancer cases. HPV positivity and especially persistant HPV infection, is predictive in the risk assessment of pre-malignant lesions (6). The prevalence of HPV infection is the highest in younger women (under 30 years old) and current attempts focus on reducing the number of transient infections identified. HPV 16 is the most common genotype in women without cytological alterations worldwide, although it is also the most common type of HPV infection in ACC and SCC (7). The current situation in Hungary is best described by Galamb *et al.* in their analysis of data from 2007-2011 on the first HPV center in Hungary. It is worth mentioning that HPV type was unidentifiable in more than 25% of the patients overall, and in more than 10% of the patients with cytological abnormalities (8).

What we also need to consider is that HPV infection alone is insufficient to induce malignant transformation, other, as yet unidentified genetic alterations (individual susceptibility) are also involved in the process. The focus has already turned to 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,

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*Key Words:* Cervical cancer, microRNA expression, HPV, clinical grading.

metabolism and apoptosis (9, 10). In the process of understanding carcinogenesis, the analysis of miRNA expression 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, non-coding RNA sequences that regulate gene expression at the post-transcriptional level in a diversified network, might function as oncogenes or tumor suppressors by modulating oncogenic or tumor suppressive pathways (9, 10). De-regulation of miRNA expression and their contribution to cancer development and progression have already been proven in many malignancies (11, 12).

In high-risk HPV infections, the regulation of cellular oncogenic and tumor-suppressive miRNAs is altered, leading to differences in expression profiles. This is most probably generated by viral proteins, mainly E6 and E7 (13-15). 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 been fully discovered yet (16-22). MiRNA genes are often observed at HPV integration sites, associated with cancer of various types (23). On the other hand, cellular miRNAs, such as miR-125b and miR-203, may also play an important role in the regulation of viral gene expression and DNA replication (24, 25). 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 formalin-fixed paraffin-embedded (FFPE) samples, unlike mRNAs, makes the approach even more appealing.

The role of miRNAs has already been investigated to an extent and results are diverse, showing high variability, especially in normal cervical tissues. Data regarding the magnitude of deregulation is sometimes also heterogeneous. In 2007, Lui *et al*. showed significantly reduced expression of miR-143 and increased expression of miR-21 in human cervical cancer samples of different histotypes matched to normal cervical samples using direct sequencing (21). In cervical tissue samples Lee *et al*. highlighted the up-regulation of miR-21, miR-29a, mir-146a, miR-155 and the down-regulation of miR-203, among others, using TaqMan quantitative real-time polymerase chain reaction (PCR) (19). 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 (18). Pereira *et al*. indicated lower expressions of miR-29a, miR-143, miR-145, miR-199a and miR-203, with the simultaneous up-regulation of miR-196a with transition from normal cervix to atypical dysplasia to cancer. They observed high expression variability between their samples of SCC, high-grade cervical squamous intraepithelial lesion (H-SIL), low-grade cervical squamous intraepithelial lesion (L-SIL) and normal cervical epithelial tissues, but they were able to identify de-regulated miRNAs (26).

## Materials and Methods

The study was approved by the Ethics Committee of Pécs University (3440.316-8331/KK41/2009). FFPE human primary cervical carcinoma tissue samples (ACC: n=22 and SCC: n=25) from archives of the Pathology Department of Pécs University were randomly selected for further analysis. 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 Laboratory, our aim was to compare the expression of eight different pre-microRNAs (miR-21, miR-27a, miR-34a, miR-146a, miR-155, miR-196a, miR-203, miR-221) in the two most common histological subtypes of cervical cancer. The panel of miRNAs was systematically chosen, based on published data and previous experience regarding expression profiles in epithelial tumors. To investigate miRNA expression profiles in cervical cancer, the first step taken was deparaffination of sample materials (three sections 8-10 μm each) using xylol and absolute alcohol in 1.5 ml volume. After deparaffination, we isolated total miRNA using High Pure microRNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, followed by reverse transcription of 5 μl of extracted miRNA with random hexamer primers using Transcriptor First Strand cDNA Synthesis Kit (Roche), resulting in a total volume of 50 μl in a 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 miRNA precursors 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 water. 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 one cycle melting curve analysis at 95˚C for 5 s, at 65˚C for 1 min, with continuous acquisition 97˚C. All assays, including no template controls, were carried out in triplicate. Inter-run calibrators were used to calculate correction factors to remove the run-to-run differences. Data normalization was performed using 5S rRNA and *U6* snRNA as endogenous references. A normalization factor was calculated based on the arithmetic mean quantification cycle (Cq) value of reference genes. Relative quantification of miRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method. Normalized relative quantities were used for further statistical analysis regarding the effects of several variables, carried out in IBM SPSS Statistics Version 20 with level of significance set at $p<0.05$.

## Results

The median age was lower for patients with AC than for those with SCC and the rate of high-risk HPV in SCC and AC was 76% and 68.18%, respectively, which was lower than

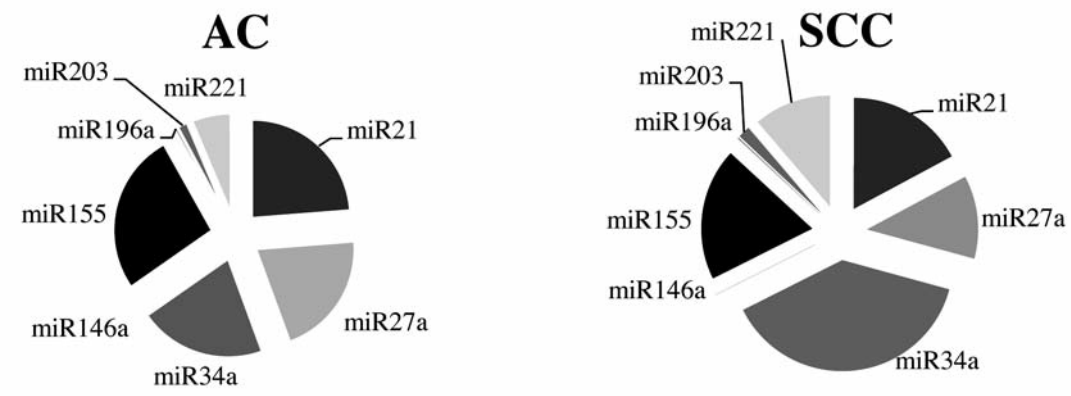


Figure 1. *Overview of expression profiles of the targeted miRNAs in adenocarcinoma (AC) and squamous cell carcinoma (SCC).*

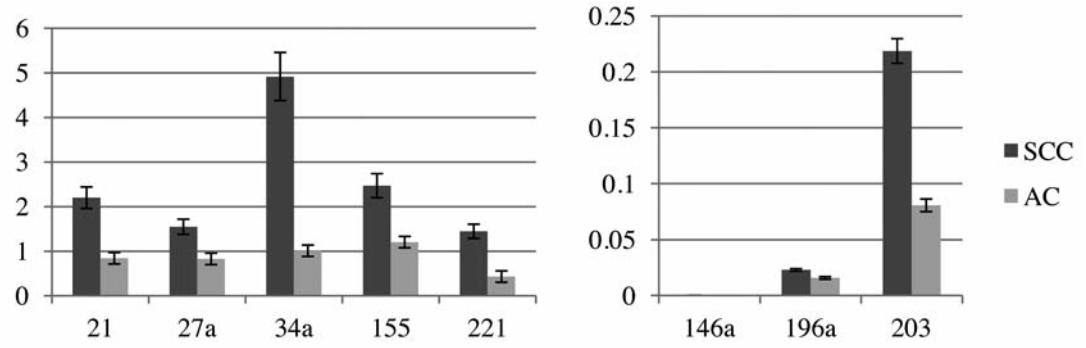


Figure 2. *Expression (relative to U6 snRNA, after normalisation) in squamous cell carcinoma (SCC) and adenocarcinoma (AC) for the targeted miRNAs.*

previously expected, based on published data (7). 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 AC. Type 16 was more abundant (14:1) in SCC, than in AC, 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 $\chi^2$ test).

*miRNA expression profile in correlation with histopathology.* The miRNA profiling data were analyzed to identify miRNAs that significantly correlated with histopathological findings. The overall expression profiles based on the eight chosen microRNAs were distinctive of the histological characteristics in cervical cancer samples. The comparison of targeted miRNA expression profiles of AC and SCC reveals distinctive characteristics (Figure 1). The expression levels of all miRNAs were higher in SCC than in AC. The difference reached the level of statistical significance in almost all cases using independent sample $t$-tests: miR-21, $p$=0.004; miR-27a, $p$=0.018; miR-34a, $p$<0.001; miR-155, $p$=0.021; miR-196a, $p$=0.032; miR-203, $p$=0.037; and miR-221, $p$=0.017; the only exception was miR-146a, where the difference lacked significance (Figure 2).

*Correlation of miRNA expression with HPV status.* On comparing the microRNA expression of HPV-negative and -positive cervical carcinomas, the expressions of miR-21, miR-27a, miR-146a, miR-196a, miR-203, miR-221 were higher and the levels of miR-34a and miR-155 were lower in the HPV-positive group, although the difference was not significant after statistical analysis (Figure 3). After we summarized the targeted miRNA profiles of AC and SCC in conjunction with HPV status, relevant alterations stood out. On detailed statistical analysis, we found statistically significant differences between HPV-positive AC and SCC in the case of miR-21 ($p$=0.004), miR-27a ($p$=0.02), miR-34a ($p$<0.001), miR-196a ($p$=0.027) and miR-221 ($p$=0.031) (independent samples t-test). As for the statistical analysis of HPV-negative AC and SCC, the differences did not reach the level of statistical significance (Figure 4). As we assessed the

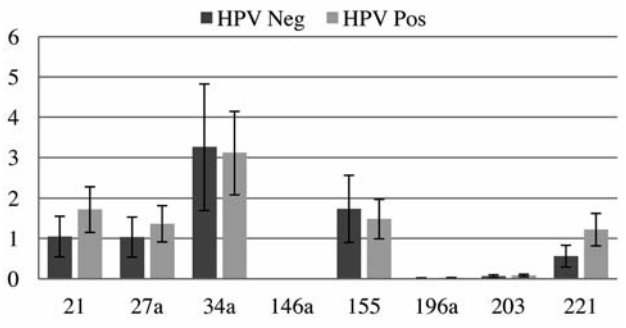


Figure 3. *Overview of expression profiles of the targeted miRNAs (relative to U6 snRNA, after normalisation) in human papillomavirus (HPV)-positive and -negative cervical cancer.*

histopathological diagnosis in 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 SCC. None of the differences reached statistical significance, however. As for miR-34a, miR-155 and miR-203, HPV-positive SCC cases had lower levels of expression. The differences, again, were not statistically significant. In AC, HPV positivity featured higher miRNA expression than HPV negativity. The only exception was miR-146a. The differences did not reach the required level of statistical significance.

*MiRNA expression according to clinical grading*. The clinical status was assessed using the International Federation of Gynecology and Obstetrics (FIGO) grades. The studied samples were from patients with various clinical stages of cervical cancer (FIGO 0: 5, FIGO I: 23, FIGO II: 7, FIGO III: 9, FIGO IV: 2). Statistical evaluation identified distinct correlations between the following targeted miRNA expression levels and the extent of cervical cancer regardless of histopathology: miR-21, $p<0.001$; miR-27a, $p=0.042$; miR-34a, $p=0.016$; miR-146a, $p=0.014$; and miR-221, $p=0.036$ (one-way ANOVA) (Figure 5). We compared the different stages in the next phase of the statistical analysis (independent samples *t*-test). A statistically significant difference was obtained in the case of miR-21 ($p=0.001$) and miR-203 ($p=0.002$), comparing FIGO 0 with FIGO I, with each miRNA having a higher level of expression in FIGO I (Figure 6A). The same pattern was distinguishable for the relationship of FIGO I and II, where the expressions were consistently higher in FIGO II. Statistical significance was detectable for miR-221 ($p=0.015$) (Figure 6B). Only the expressions of miR-34a and miR-146a were higher in FIGO III than in FIGO II and none of the deviations had statistical significance (Figure 6C). In FIGO IV, most miRNA expressions (miR-27a, miR-34a, miR-155, miR-196a, miR-203 and miR-221) were higher than in FIGO III. Relatively lower expressions of miR-21 and miR-146a characterized FIGO IV from FIGO III. The degree of divergence reached the level of significance in the case of miR-34a ($p=0.037$) and miR-196a ($p=0.007$) (Figure 6D). Multivariate tests (two- and three-way ANOVA, logistic regression) found no significant interaction between the examined parameters.

## Discussion

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 tumor type distinction and maybe even in prediction in the case of pre-neoplastic lesions. There are 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 (13). HPV-associated miRNAs (miR-34a, miR-146a, miR-203), directly or indirectly regulated by E5-E7 oncogenic proteins (17, 24), 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 between the two histotypes. We verified significant differences between the HPV-positive two 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 tumors (27), 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 (28, 29), suggesting that the expression of cellular miRNA genes at or near HPV integration sites may contribute to the tumor phenotype. Here, we have shown that the expression of miR-21 was up-regulated 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 is 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

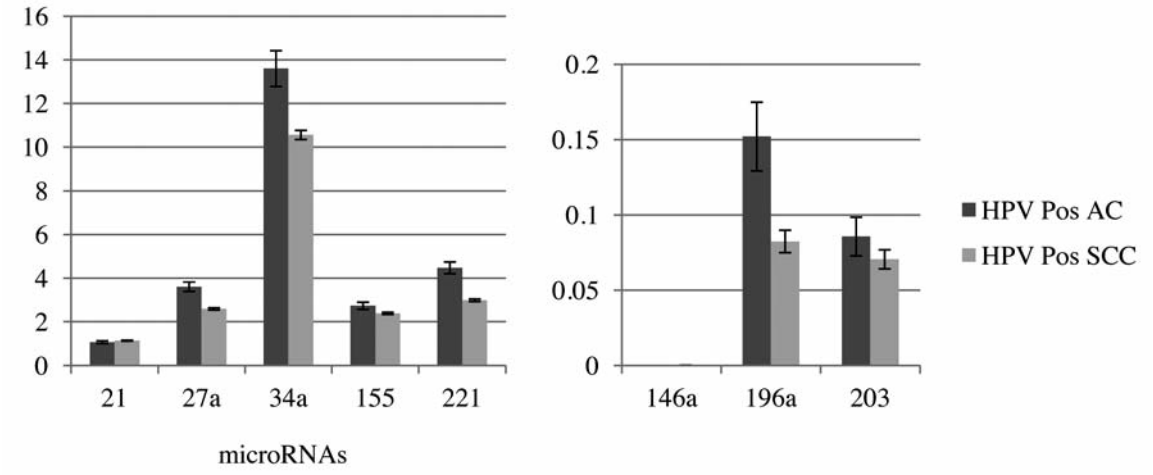


Figure 4. *Targeted miRNA expressions (relative to U6 snRNA, after normalisation) in HPV-positive adenocarcinoma (AC) and squamous cell carcinoma (SCC).*

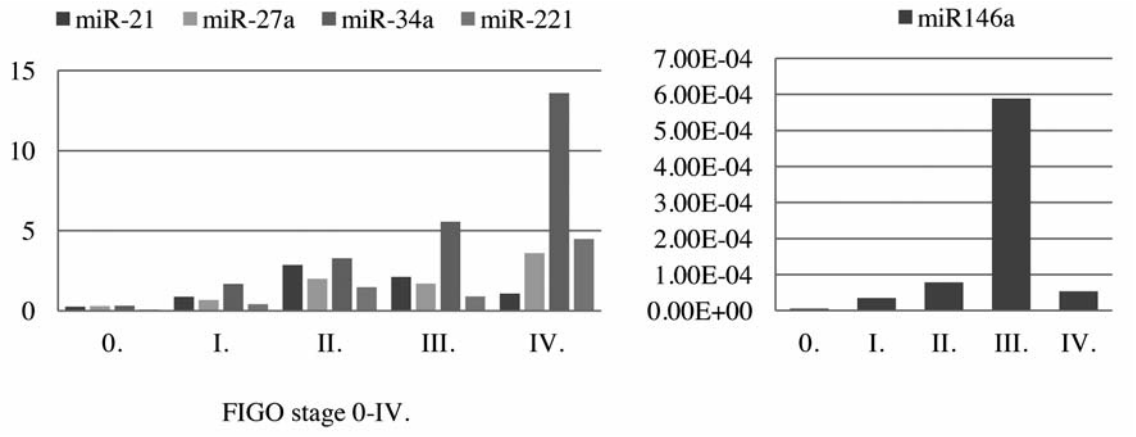


Figure 5. *Targeted miRNA expressions (relative to U6 snRNA, after normalisation) in correlation with FIGO grade.*

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 (30). Scmitz *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. Out 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 (31). Previous studies indicated that the HPV E7 protein down-regulates miR-203 expression upon differentiation, which may occur through the mitogen-activated protein (MAP) kinase/protein kinase C (PKC) pathway (32). One target of miR-203 is the p63 family of transcription factors, and 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 (33), 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*. (18).

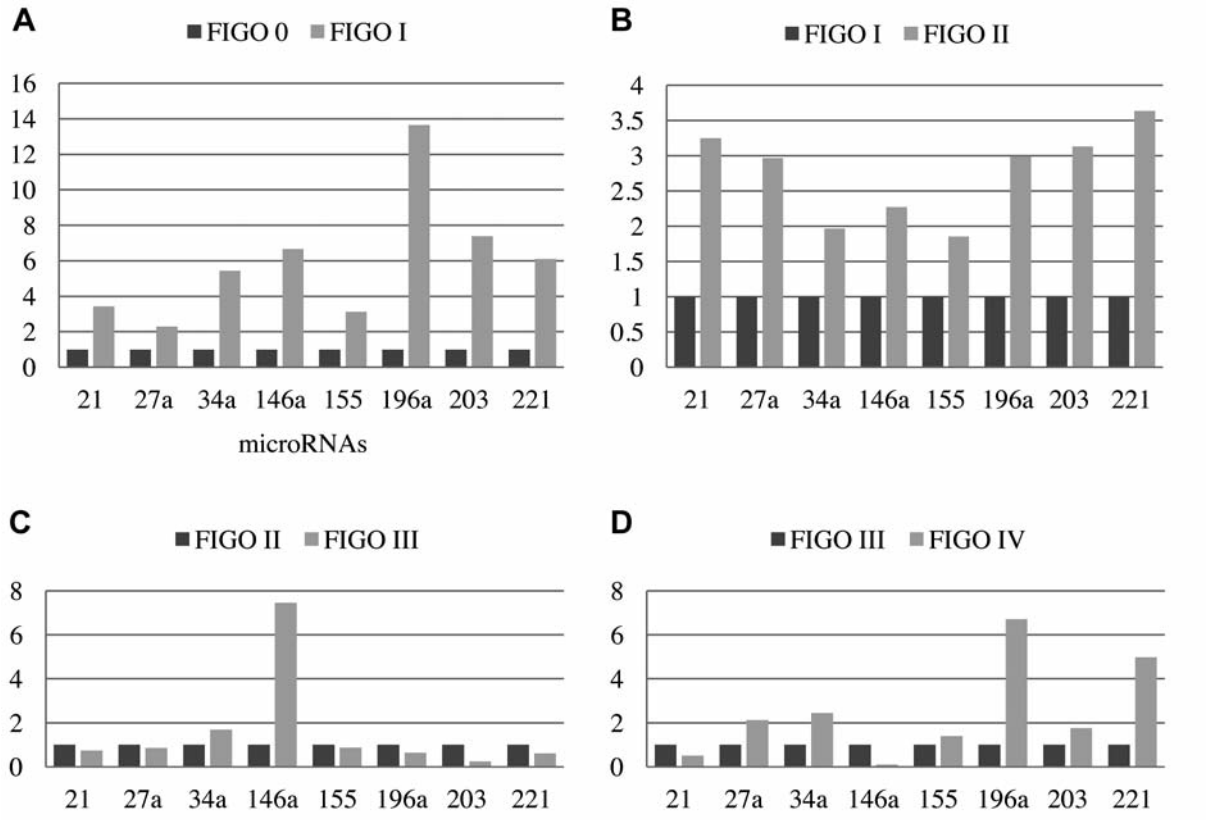


Figure 6. *Part A-D. Relative comparison of different clinical FIGO grades based on miRNA expressions (comparison of consecutive FIGO grades, relative fold difference in miRNA expression is shown).*

## Conclusion

Even though the small sample size was a known limitation, the p53-induced miR-34a together with miR-21, miR-27a, miR-155, miR-203 and miR-221 can be useful predictive and prognostic biomarkers and will induce more detailed investigations for future verification. Since miRNA expression is highly preservative, sequential and tissue specific, the observed alterations in miRNA expressions may 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 urgent need for earlier diagnosis, more precise prognosis and 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 the generations to come, less frequent screening is only acceptable if vaccination uptake is evenly distributed across all social, economic and ethnic 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 expression of a considerable number of cellular miRNAs, both oncogenic and tumor-suppressive. Despite the uncertainty regarding the functional effects of miRNA dysregulation in the pathogenesis of cervical cancer, determining miRNA expressions might help to assess factors and risks playing a part in individual susceptibility and might even help in the clinical projection regarding various characteristics, including histopathological classification.

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*Received March 5, 2013*
*Revised April 22, 2013*
*Accepted April 23, 2013*