Published in final edited form as:

Cancer Res. 2010 February 15; 70(4): 1441–1448. doi:10.1158/0008-5472.CAN-09-3289.

A MicroRNA Expression Signature for Cervical Cancer Prognosis

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Abstract

Invasive cervical cancer is a leading cause of cancer death in women worldwide, resulting in about 300,000 deaths each year. The clinical outcomes of cervical cancer vary significantly and are difficult to predict. Thus, a method to reliably predict disease outcome would be important for individualized therapy by identifying patients with high-risk of treatment failures prior to therapy. In this study, we have identified a microRNA-based signature for the prediction of cervical cancer survival.

MicroRNAs (miRNAs) are a newly identified family of small non-coding RNAs that are extensively involved in human cancers. Using our recently established PCR-based miRNA assays, we have analyzed 102 cervical cancers and identified two miRNAs (miR-200a and miR-9) that are likely to predict patient survival. A logistic regression model was developed based on these two miRNAs and the prognostic value of the model was subsequently validated with 42 independent cervical cancers. Furthermore, functional studies were performed to characterize the effect of miRNAs in cervical cancer cells. Our results suggest that both miR-200a and miR-9 could play important regulatory roles in cervical cancer control. In particular, miR-200a is likely to affect the metastatic potential of cervical cancer cells by simultaneously suppressing the expression of multiple genes that are important to cell motility.

Keywords

microRNA; cervical cancer; prognosis; expression profiling; target regulation

Introduction

It is estimated that there will be about 11,270 new cases of cervical cancer and that 4,070 women will die of this disease in the United States in 2009 (statistics provided by American Cancer Society). Worldwide, invasive cervical cancer is an even greater problem and is a leading cause of cancer death in women. Most cervical cancer patients receive standard radiotherapy and chemotherapy. However, clinical outcomes vary significantly and are difficult to predict. The lack of effective outcome prediction models makes it difficult to apply

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individualized treatment protocols to cervical cancer patients. A method to accurately predict disease outcome prior to standard therapy would be important for the early identification of patients with a high risk of treatment failures. For these high-risk patients, modified therapy, involving different radiation doses or adjuvant therapy, could potentially be applied to improve patient survival.

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules that downregulate the expression of their protein-coding gene targets (1). As of August 2009, 706 human miRNAs have been identified. Despite the relatively small number of miRNAs, both computational and experimental studies have shown that thousands of human protein-coding genes are collectively regulated by miRNAs (2-4). Thus, miRNAs are considered to be master regulators of many important biological processes, such as cell growth, apoptosis, viral infection and cancer development (1,5-8). Genome-wide analyses indicate that about half of miRNA genes are located at fragile sites and genomic regions with frequently dysregulated expression in cancers (9,10). Thus, one major mechanism that underlies the roles of miRNAs in cancer development could be deregulated miRNA expression as compared to normal cells.

miRNA expression signatures have been shown to be promising biomarkers for the classification or outcome prediction of a wide array of human cancers [reviewed in refs (11, 12)]. For example, a miRNA signature has been identified by expression profiling for the prediction of lung cancer outcome (13). However, to date the prognostic value of miRNAs in cervical cancer has not been investigated. Recent studies indicate that multiple miRNAs have altered expression in HPV-integrated cervical cancer cells compared to HPV-negative cervical cancer cells or normal cervical tissues (14,15). In addition, altered miRNA expression profiles have also been reported in cervical carcinomas as compared to normal cervix (16-18).

Here, we present a miRNA-based prediction model to identify cervical cancer patients who are likely to fail radiation and chemotherapy. In addition, we also characterized the functions of two prognostic miRNAs in cervical cancer cells. Our functional studies on the involvement of miRNAs in cervical cancer could be a promising starting point for developing future miRNA-based cervical cancer therapy.

Materials and Methods

Patients and tumor RNA samples

One hundred and two cervical carcinomas were included in this study, including 60 for training and 42 for testing the outcome prediction model, respectively. These cancer patients had been treated with standard chemoradiation at Washington University School of Medicine in St. Louis. The patient characteristics are summarized in Table 1. This study was approved by the Human Research Protection Office at the Washington University. For the sixty cervical cancers for model training, formalin-fixed paraffin-embedded (FFPE) tumor tissue biopsies were collected prior to radiotherapy. First, sections from the FFPE tumor blocks were stained with hematoxylin and eosin (H&E) to identify the tumor regions. Then, one 0.6 mm tissue core was obtained from the tumor region of each FFPE block. Total RNAs were extracted from these tumor tissue cores for miRNA expression profiling. In this way, we were able to focus on tumor tissue analysis with minimal contamination from normal adjacent tissues. Total RNAs were isolated with miRNeasy FFPE Kit (QIAGEN) according to the manufacturer's protocol.

The 42 independent cervical tumor samples for prediction model testing included 22 FFPE tumor samples and 20 snap-frozen tumor samples collected from 42 patients in the testing cohort. The testing FFPE samples were processed in the same way as the training FFPE samples described above. The frozen samples were prepared by snap-freezing fresh tumor samples in Tissue Tek O.C.T compound (EMS) prior to radiotherapy. Specimens were sectioned on a

cryostat and stained with H&E for pathologic review. The percentage of tumor cells from each individual tumor sample was assessed by a single pathologist at Washington University specializing in gynecologic oncology. All 20 frozen tumor samples included in this study had at least 75% tumor content. Total RNA was extracted from each sample by the Tissue Core Procurement Facility at Washington University using the Trizol reagent (Invitrogen) according to manufacturer's protocol.

miRNA expression profiling with real-time RT-PCR

miRNA expression profiling of 96 cancer-related miRNAs was performed using our recently developed assays, which are based on real-time RT-PCR, for both the training and testing tumor samples (19). All the RT and PCR primers were synthesized by Integrated DNA Technologies (IDT). The detailed experimental procedure has been described previously (19). In brief, the RT reactions were performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each RT reaction included 150 ng RNA as the template and a pool of RT primers. Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems). Raw threshold cycle (Ct) values from real-time PCR were normalized using a quantile-based scaling method as described previously (19).

Survival analysis

Statistical data analyses were performed with the R package (<http://www.r-project.org/>) and MATLAB. Univariate Cox proportional hazards regression analyses were performed to evaluate the association of each miRNA to overall patient survival. The p-values were calculated using the Wald test, and then corrected by a permutation test as previously described (13). Multivariate Cox proportional hazards analyses were performed to evaluate the independent prognostic value of the miRNA signature. The Kaplan-Meier estimator was employed to evaluate the significance of the outcome prediction model which was based on miRNA expression signature. The p-values of the model prediction in the Kaplan-Meier analysis were calculated with the log-rank test. Overall patient survival was used as the end point to evaluate cervical cancer outcome. Overall survival was defined as the time interval between the date of diagnosis and the date of patient death. Recurrence-free survival was defined as the time interval between the date of diagnosis and the date of first failure.

Cell culture and miRNA transfection

The human cervical cancer cell line HeLa (purchased from ATCC) was used in all miRNA transfection experiments. HeLa cells were seeded 24 h prior to transfection. Transfection experiments were performed with 60 nM miRNA mimics (pre-miR-200a or pre-miR-9) from Ambion and Lipofectamine 2000 from Invitrogen. Negative controls were included in each transfection experiment, including both the negative control miRNA (Ambion) and mock transfection (no RNA added). A parallel GAPDH siRNA (Ambion) transfection experiment was performed as a positive control to monitor transfection efficiency as determined by real-time RT-PCR. In addition, the ectopic expression of transfected miR-200a or miR-9 in HeLa cells was also confirmed directly by real-time RT-PCR. Total RNA was extracted from the cells using the mirVana RNA Isolation Kit (Ambion).

Microarrays

Microarrays were performed using the Illumina BeadChip platform at the Washington University Genome Center. Total RNA quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies) before the microarray experiments. Total RNA samples were first amplified with the MessageAmp TotalPrep kit (Ambion). Amplified RNA samples were applied to HumanHT12 Expression BeadChip arrays according to Illumina standard protocol for hybridization and washing. Arrays were scanned on an Illumina BeadArray Reader, and

images were quantitated by Illumina Beadscan and analyzed by the Beadstudio software. On-slide spot replicates were averaged and individual spot data was reported. Raw array data were normalized by the quantile normalization method. The microarray data were deposited in the NCBI GEO database (accession# GSE18545).

Gene Ontology analysis

The Gene Ontology (GO) annotation database was downloaded from <http://geneontology.org>. The gene IDs were mapped to GO annotations using the NCBI Gene database downloaded from <ftp://ftp.ncbi.nih.gov/gene>. Since the Gene IDs were mapped only to the lowest child level of GO categories, a recursive search was performed to associate the Gene IDs to all parent GO categories based on the GO hierarchical structure. To reduce the redundancy in GO annotation, only GO functional categories with 5 - 300 expressed genes in HeLa cells, as determined by microarrays, were included for further analysis. The hypergeometric test was performed to identify gene enrichment in the GO functional categories, using all 9500 HeLa-expressing genes as the background.

miRNA target validation with real-time RT-PCR

Total RNA was reverse transcribed using the High Capacity RT kit (Applied Biosystems). All PCR primers for gene target candidates were retrieved from PrimerBank (20). Real-time PCR was performed to validate the downregulation of candidate miRNA targets with our previously described protocol (20). GAPDH and β -actin were used as internal controls for expression data normalization. Potential target expression changes were determined by comparison with the negative control RNA experiment.

Transwell migration assay

HeLa cells were transfected with miRNA mimics, pre-miR-200a (Ambion) for 48 h, followed by resuspension and washing with PBS buffer. Fifty thousand cells in 100 μ L serum-free medium were added to the upper chamber of each 8 μ m Transwell (Costar). The lower chamber was filled with 600 μ L medium with 10% bovine serum. The cells were incubated for 6 h and then cells on top of the Transwell membrane were removed with a cotton-tipped swab. Migrated cells (through the membrane to the bottom) were fixed and stained with Hema-3 (Fisher Scientific) for counting. The percentage of migrated cells with overexpressed miR-200a was normalized to that of negative control cells with overexpressed negative control RNA.

Results

miRNA expression profiles were associated with cervical cancer survival

Sixty cervical tumors were analyzed as training samples to identify prognostic factors for outcome prediction. The characteristics of this patient cohort are summarized in Table 1. The prognostic value of multiple commonly used clinicopathologic features were analyzed with univariate Cox regression analysis. Only lymph node status was significantly associated with survival ($p=0.03$), while other features, including tumor grade, stage, hemoglobin level at diagnosis, glucose uptake level at diagnosis and patient age at diagnosis, were not (Table 2).

Next, we evaluated the prognostic value of miRNAs by expression profiling. Total RNA samples were isolated from 60 formalin-fixed paraffin-embedded (FFPE) cervical tumor tissues. One RNA sample did not pass the quality control check and was excluded from further profiling analysis. The expression profiles of 96 cancer-related miRNAs across the remaining 59 cervical cancers were determined using our recently established real-time PCR-based miRNA assays (19). These 96 cancer-related miRNAs were selected based on bioinformatics analysis of public miRNA microarray data as well as mining of public literature. These selected

miRNAs (listed in Supplementary Table 1) have been implicated in a variety of human cancers based on many previous studies and are not necessarily cervical cancer specific. The PCR-based profiling assays designed for these miRNAs have been extensively validated in our recent study [details available in ref (19)]. The expression profiles of individual miRNAs were correlated to overall patient survival with univariate Cox proportional hazards regression analysis. P-values were calculated with the Wald test and further corrected by a permutation test as previously described (13). Among all miRNAs included in the profiling experiment, five were significantly associated with cancer survival (miR-9, miR-21, miR-200a, miR-218, and miR-203 with corrected $p=0.02-0.05$). Among these five miRNAs, three have been reported to be differentially expressed (upregulation of miR-21; downregulation of miR-203 and miR-218) in cervical cancers compared to normal cervix (15-17). None of these five miRNAs were significantly associated with the lymph node status as determined by the log-rank test.

A miRNA-based model to predict cervical cancer survival

Given the significant association of miRNA expression profiles with patient survival, it is likely that a selected set of miRNAs could be used to build a predictive model for cervical cancer prognosis. In order to identify the most predictive miRNAs, we used the recursive feature elimination (RFE) technique to rank the relative importance of each miRNA on disease outcome classification. In this RFE analysis, a machine learning framework, support vector machine (SVM), was used to evaluate all the miRNAs collectively. The least important miRNA was first identified by the SVM and subsequently eliminated. The remaining miRNAs were then evaluated collectively again using the SVM framework to identify the next least important miRNA for elimination. The process was repeated with one miRNA eliminated from each cycle until only one miRNA was left. SVM-RFE is especially useful to determine the independent contribution of each miRNA for model performance. To reduce potential overtraining risk, 10-fold cross-validation was performed during the miRNA selection process. Top-ranking miRNAs selected by SVM-RFE were: miR-200a, miR-9, miR-10b, miR-183, miR-204, miR-24, miR-181a, miR-193b, miR-146b and miR-10a (ranked #1-10 in order).

Among all the miRNAs that we analyzed, miR-200a and miR-9 were especially promising. Both miRNAs were significantly associated with disease outcome by univariate Cox regression analysis (corrected p-values of 0.03 and 0.02, respectively), and had the highest rankings for disease outcome classification as revealed by SVM-RFE (ranked #1 and #2, respectively). Therefore, miR-200a and miR-9 were used to build a logistic regression model for outcome prediction as the following:

$S = 17.9 - 0.284 \times E_{\text{miR-9}} - 0.376 \times E_{\text{miR-200a}}$, where S represents the risk score for each patient; $E_{\text{miR-9}}$ and $E_{\text{miR-200a}}$ represent the normalized expression levels of miR-9 and miR-200a in each patient, respectively. In this prediction model, a high risk score (>0) predicts poor survival and a low risk score (<0) predicts good survival. Kaplan-Meier survival analysis indicated that our miRNA-based model was significantly predictive of overall patient survival for the 59 cervical cancers (Figure 1A, $p=0.0003$ with the log-rank test). The majority of the cancers (45 out of 59) were predicted to be of low risk, which was consistent with the fact that the majority of the patients (36 out of 59) survived at the end of the study period. This miRNA signature was also significantly predictive of local-recurrence-free survival ($p=0.036$ with the log-rank test). The model prediction was still statistically significant after controlling lymph node status, tumor stage and patient age with multivariate Cox proportional hazards regression analysis, indicating its significant independent prognostic value from clinicopathologic features (Table 3, $p=0.001$).

Model validation with independent cervical cancers

The prediction model was further validated with 42 independent cervical cancer samples, including 22 FFPE samples and 20 frozen samples. First, the expression profiles of miR-200a and miR-9 in the 42 validation cervical cancers were determined using our recently established PCR-based assays (19). Then, a risk score was calculated for each of the 42 cancer patients by applying the miRNA expression values to the outcome prediction model. Based on the risk score, the patients were classified into either the high risk group (score >0) or the low risk group (score <0). Kaplan-Meier analysis indicated that these two patient groups had significantly different overall survival rates (Figure 1B, $p=0.002$ with the log-rank test). The model prediction was still significant when applied to the frozen and FFPE samples separately for predicting overall survival ($p=0.02$ and 0.05 for 20 frozen testing samples and 22 FFPE testing samples, respectively, with the log-rank test). These two patient groups were also significantly different for recurrence-free survival ($p=0.002$ with the log-rank test). Thus, our logistic regression model was predictive of patient survival when applied to 42 independent cervical cancers.

Functional characterizations of the prognostic miRNAs in cervical cancer cells

In order to characterize the global cellular functions of the prognostic miRNAs included in the outcome prediction model, miR-200a and miR-9 were individually transfected into cervical cancer HeLa cells, and microarrays were performed to identify genes downregulated by miR-200a or miR-9 overexpression. Based on recent studies (4,5), the majority of miRNA targets are downregulated at both the mRNA and protein levels. The high-throughput nature of microarrays is ideal for systematic analysis of miRNA target regulation even though some miRNA targets are likely to be missed by microarrays. For the genes downregulated by miRNAs, Gene Ontology (GO) enrichment analysis was performed to evaluate the effects of miRNA overexpression on cervical cancer cell functions. The most significantly affected GO functional categories by miRNA overexpression are listed in Table 4. For miR-200a, the most affected functional category was “regulation of cell adhesion”. In addition, the structure formation involved in morphogenesis was also significantly affected. These biological properties are known to be critical for the regulation of the metastatic potential of cancer cells to migrate to distant sites. Thus, miR-200a could potentially be involved in tumor control by regulating cancer cell metastasis. Similarly, the effects of miR-9 overexpression were also evaluated by GO analysis. Interestingly, most of the affected functional categories were related to tumor cell metabolism (Table 4). The maintenance of high metabolic rate is important for the rapid proliferation of cervical cancer cells (21). Thus, miR-9 could potentially be involved in tumor control by regulating tumor cell metabolism.

Detailed target analysis was performed to further characterize the functional involvement of the prognostic miRNAs in cervical cancer. Identification of miRNA gene targets is critical to understanding the direct regulatory roles of miRNAs in cervical cancer. We have recently developed a robust bioinformatics algorithm, MirTarget2, for miRNA target prediction (22). MirTarget2 has been demonstrated to have superior performance over other existing algorithms by comparative analysis (22). In addition, we have also developed a new strategy to systematically identify miRNA targets by combining computational target prediction with microarray expression profiling (23). These methods were used in this study to identify genes targeted by miR-200a and miR-9. Predicted gene targets were retrieved from the miRDB database, which was developed based on the MirTarget2 algorithm (24). There were 9500 genes with detectable expression in HeLa cells as determined by microarrays. Among these genes, 305 were downregulated by miR-200a overexpression, and 370 were predicted to be miR-200a targets (Figure 2A). In this way, we identified 58 genes that were both predicted miR-200a targets and downregulated by miR-200a as revealed by microarrays. Thus, these genes are likely to be directly targeted by miR-200a. Similarly, we also characterized the genes

downregulated by miR-9 overexpression (Figure 2A). Among all the genes expressed in HeLa cells, there were 274 predicted miR-9 targets and 285 miR-9 downregulated genes. Altogether, 52 genes were both predicted to be targeted by miR-9 targets and downregulated by miR-9 as revealed by microarrays. Thus, these 52 genes represent likely direct miR-9 targets.

Based on the bioinformatics target analysis, we further characterized the roles of miRNA-200a in cervical cancer progression. Real-time RT-PCR experiments were performed to validate the microarray results for miR-200a overexpression. Thirteen candidate miR-200a targets, as identified by both microarrays and computational target prediction, were selected for real-time RT-PCR validation. These genes were selected for their known involvement in carcinogenesis. Among these thirteen genes, nine showed downregulated expression by at least 40% as a result of miR-200a overexpression in cervical cancer HeLa cells (Figure 2B). Among these downregulated gene targets, several are related to the metastatic potential of the tumor cells, including ZEB1, ZEB2, TGFB2 and EXOC5 (details available in Discussion). Thus, simultaneous repression of these metastasis-related genes by miR-200a could be important for controlling cervical cancer cell morphology and the ability to migrate to distant sites. This hypothesis is also consistent with the GO analysis results described earlier. To further test the hypothesis, we overexpressed miR-200a in cervical cancer HeLa cells. The effect of miR-200a overexpression on HeLa cell motility was examined by transwell migration assays. As shown in Figure 2C, overexpression of miR-200a led to a significant reduction (46%) of cervical cancer cell mobility.

Discussion

Although miRNA expression signatures have been applied to the outcome prediction of multiple cancers such as lung cancer and breast cancer, no study to date has been reported for the application of miRNAs to cervical cancer prognosis. One unique challenge in cervical cancer biomarker study is the lack of large amounts of tumor tissues, since most cervix biopsies are relatively small. In addition, most existing cervical cancer tissues in hospitals are preserved using the FFPE method, leading to severe degradation of the RNA in tumor cells. These issues pose a significant challenge to miRNA expression profiling analysis. To address this challenge, we have recently developed a new PCR-based profiling method for miRNA expression analysis, which has been demonstrated to have superior performance in detection sensitivity and specificity compared to traditional profiling methods (19). This new method has also been successfully applied to the profiling of minute amounts of highly degraded clinical RNA samples (19). In this study, this new method was applied for the miRNA profiling of FFPE cervical tumors, resulting in the identification of a miRNA signature for predicting cervical cancer survival. Furthermore, this prognostic miRNA signature was validated by both independent FFPE and snap-frozen cervical cancer samples. Interestingly, our new miRNA signature was successfully validated despite the difference in tissue preservation method (FFPE vs. snap-frozen), indicating that miRNAs are robust biomarkers in cervical cancer. This miRNA signature has provided a new biomarker-based strategy for the identification of high-risk cervical cancer patients for potential individualized therapy.

Our functional studies indicated that, by targeting multiple cancer-related genes, miR-200a likely acts as a master regulator that is critical to cervical cancer control. In support of this view, recent studies demonstrate that the miR-200 miRNA family may inhibit the epithelial to mesenchymal transition (viewed as an important initiating step for tumor metastasis) by directly targeting E-cadherin transcriptional repressors ZEB1 and ZEB2 (25-27). Interestingly, ZEB1 and ZEB2 were also among the genes downregulated by miR-200a in our cervical cancer study. Besides ZEB1 and ZEB2, we also identified other miR-200a targets that are involved in metastasis. For example, TGFB2 (TGF-beta 2) has been shown to promote metastasis in multiple cancers (28,29); EXOC5 (exocyst complex component 5) is involved in the actin

cytoskeletal remodeling machinery, and is essential for the biogenesis of epithelial cell surface polarity (30). Thus, our target analysis indicated that miR-200a can simultaneously target multiple genes that are important to the metastatic potential of cervical cancer cells. In this way, miR-200a could potentially act as a master suppressor for cervical cancer metastasis. Indeed, cervical cancer cell motility was significantly reduced when miR-200a was overexpressed (Figure 2C). Thus, one potential tumor control strategy could be the manipulation of miR-200a expression. Recent studies indicate that the therapeutic delivery of miR-26a and miR-31 can suppress liver and breast cancers, respectively (31,32). Similarly, based on our research, it is reasonable to expect that the therapeutic delivery of miR-200a could be a promising new treatment strategy for cervical cancer control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by grants from the American Cancer Society (IRG-58-010-52), the Longer Life Foundation and the National Institutes of Health (CA095713).

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Figure 1.

Kaplan-Meier analysis to evaluate the statistical power of a miRNA-based model on predicting cervical cancer survival. A risk score was assigned to each patient as calculated by the prediction model. Based on the risk score, the patients were classified into either the low risk group (score <0) or the high risk group (score >0). **(A)** Application of the model to 59 training tumor samples. The risk scores generated by the model were significantly predictive of overall patient survival ($p=0.0003$ with the log-rank test). **(B)** Application of the model to 42 independent testing tumor samples. The risk scores generated by the model were significantly predictive of overall patient survival ($p=0.002$ with the log-rank test).

Figure 2.

Functional characterization of the prognostic miRNAs by target analysis. **(A)** Identification of miRNA gene targets by combining computational target prediction with microarray expression profiling. Genome-wide miRNA target prediction was performed with the MirTarget2 program. In parallel, microarrays were performed to identify genes downregulated by miRNAs. Fifty-eight genes were both predicted miR-200a targets and downregulated by miR-200a as revealed by microarrays; fifty-two genes were both predicted miR-9 targets and downregulated by miR-9 as revealed by microarrays. **(B)** Real-time RT-PCR validation of miR-200a gene targets. Cervical cancer HeLa cells were transfected with miR-200a, and the expression levels of 13 candidate miR-200 targets were determined. Nine of the 13 genes were downregulated

by at least 40% (<60% remaining expression). (C) The effect of miR-200a overexpression on cervical cancer cell motility. miR-200a was transfected into cervical HeLa cells, and the effect of miRNA overexpression on cell motility was evaluated by Transwell migration assays. The percentage of migrated cells with overexpressed miR-200a was normalized to that of control cells transfected with a negative control RNA.

Table 1

Characteristics of 102 cervical cancer patients

	Training cohort (n=60)	Testing cohort (n=42)
Median age at diagnosis	53	47
Tumor histology		
Squamous	50	39
Adenocarcinoma	7	3
Clear cell	2	
Undifferentiated	1	
FIGO stage *		
I	17	11
II	22	14
III	18	15
IV	3	2
Histological grade		
Well	2	2
Moderate	29	19
Poorly	19	12
Undetermined	10	9

* FIGO represents International Federation of Gynecology and Obstetrics.

Table 2Univariate Cox proportional hazards regression analysis of clinical parameters in relation to disease outcome^{*}

Clinical Variable	Overall survival
Lymph node status	0.03
Histological grade	0.09
Clinical stage	0.15
Hemoglobin level at diagnosis	0.19
Patient age at diagnosis	0.42
Glucose uptake level at diagnosis	0.65

^{*}The numbers in the table represent p-values calculated with the Wald test.

Table 3

Multivariate Cox proportional hazards regression analysis to evaluate the independent prognostic value of miRNA signature and clinical parameters^{*}

Parameters	Overall survival
Lymph node status	0.02
Histological grade	0.11
Clinical stage	0.78
Patient age at diagnosis	0.50
miRNA signature	0.001

^{*} The numbers in the table represent p-values calculated with the Wald test.

Table 4

The most affected GO categories by miR-200a and miR-9 as revealed by microarrays*

GO ID	GO Description	Enrichment P-Value
<i>miR-200a overexpression effects</i>		
GO:0030155	regulation of cell adhesion	7.30E-05
GO:0060348	bone development	1.70E-04
GO:0032940	secretion by cell	1.80E-04
GO:0045646	regulation of erythrocyte differentiation	2.70E-04
GO:0048646	anatomical structure formation involved in morphogenesis	2.80E-04
<i>miR-9 overexpression effects</i>		
GO:0009396	folic acid and derivative biosynthetic process	2.50E-05
GO:0048514	blood vessel morphogenesis	3.70E-04
GO:0006752	group transfer coenzyme metabolic process	5.70E-04
GO:0016887	ATPase activity	7.10E-04
GO:0009064	glutamine family amino acid metabolic process	1.10E-03

* P-values were used to assess the enrichment of genes downregulated by miR-200a or miR-9, as revealed by microarrays, in each GO category. The p-values were calculated with the hypergeometric test using all expressed genes in HeLa cells as background. The five most significantly affected GO categories by miR-200a or miR-9 are presented here.