

# Characterization of the microRNA profile in early-stage cervical squamous cell carcinoma by next-generation sequencing

WANSONG LIN<sup>1,2</sup>, MEI FENG<sup>3</sup>, GUILIN CHEN<sup>3</sup>, ZHIFENG ZHOU<sup>1,2</sup>, JIEYU LI<sup>1,2</sup> and YUNBIN YE<sup>1,2</sup>

<sup>1</sup>Laboratory of Immuno-Oncology, Fujian Cancer Hospital and Fujian Medical University Cancer Hospital;

<sup>2</sup>Fujian Key Laboratory of Translational Cancer Medicine; <sup>3</sup>Department of Gynecologic Oncology, Fujian Cancer Hospital and Fujian Medical University Cancer Hospital, Fuzhou, Fujian 350014, P.R. China

Received July 6, 2016; Accepted September 1, 2016

DOI: 10.3892/or.2017.5372

**Abstract.** Squamous cell carcinoma (SCC) is histologically the most prominent type of cervical cancer. There is accumulating evidence suggesting that microRNAs (miRNAs) play important regulatory roles in the biological processes of cervical squamous cell carcinoma (CSCC). Deciphering the miRNA regulatory network in CSCC could deepen our understanding at the molecular level of CSCC initiation and progression. In the present study, we performed next-generation sequencing (NGS) to profile miRNA expression in 3 pairs of early-stage CSCC samples. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to verify primary findings in another 20 pairs of CSCC samples. We identified 37 known miRNAs that exhibited significant alterations in expression (2-fold change or greater), among which 8 miRNAs were upregulated and 29 miRNAs were downregulated. Nine of these miRNAs were selected for further qRT-PCR validation. A novel miRNA candidate was also reported for the first time in the present study to be upregulated. The Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that its target genes were involved in MAPK, calcium and adherent junction signaling pathways. The present study systematically characterized the miRNA expression variation in early-stage CSCC and provides novel biomarkers for diagnosis and treatment as well as an opportunity for further investigation of the molecular mechanisms underlying the pathogenesis and development of CSCC.

## Introduction

In less developed countries, cervical cancer is the second most common cancer and the third leading cause of cancer-related

deaths in females. There were an estimated 444,500 new cervical cancer cases and 230,200 deaths in these countries in 2012 (1). Almost 90% of the deaths occurred in developing countries and for these countries it is a huge economic health care burden. The highest cervical cancer death rates are in Asia, with an estimated 144,400 deaths occurring in 2012 (1). Squamous cell carcinoma (SCC) is the most common histological type of cervical cancer (~80% of cases) and is closely correlated with human papillomavirus (HPV) infection (2). Cervical screening can prevent SCC, as it allows for detection and treatment of precancerous lesions, and application of the HPV vaccine also reduces the risk of SCC (3). However, the high cost is a major barrier to making screening and vaccination widely available to all women in developing countries, including China.

Cervical squamous cell carcinoma (CSCC) cases that are classified as International Federation of Gynecology and Obstetrics (FIGO) stages IB and IIA have a greater possibility of being cured and patients have better prognosis (4). Since early detection results in a better prognosis and cervical screening and vaccines are extremely expensive, there is an urgent need to identify more efficient early diagnostic biomarkers and therapeutic targets for CSCC. Therefore, it is necessary to augment our understanding of the molecular mechanisms underlying the pathogenesis and development of CSCC.

Although previous studies have identified chromosomal variations, abnormal expression of oncogenes or tumor-suppressor genes and aberrant promoter methylation in CSCC, few are relevant to microRNAs (miRNAs). miRNAs are a class of small non-coding RNAs (ncRNAs) ~19-25 nucleotides (nts) in length that can regulate gene expression by known classic mechanisms such as binding to messenger RNA (mRNA) at the 3'-untranslated region (3'-UTR) to downregulate protein-coding genes by increasing mRNA degradation (5). They also act through other newly found mechanisms such as binding to promoters, proteins, and by directly interacting with other ncRNAs (5,6). miRNAs play important regulatory roles in various biological processes e.g. differentiation, development, proliferation, apoptosis, cell cycle control and metabolism (7,8). Changes in miRNA expression have been identified in many diseases, such as cardiac and autoimmune disorders, schizophrenia and cancer (9). miRNAs have been found to be differentially expressed between almost all types of

---

*Correspondence to:* Dr Yunbin Ye, Laboratory of Immuno-Oncology, Fujian Cancer Hospital and Fujian Medical University Cancer Hospital, 420 Fuma Road, Fuzhou, Fujian 350014, P.R. China  
E-mail: zjyunbin@sina.com

**Key words:** microRNA, cervical cancer, squamous cell carcinoma, early-stage, next-generation sequencing

analyzed benign and malignant tumors and non-tumor tissues, including CSCC (10). In addition, miRNAs participate in regulating a variety of neoplastic biological capabilities acquired during the multistep process of tumor development. Using miRNA microarray, Wilting *et al* observed that 46 miRNAs showed significantly differential expression between normal cervical squamous epithelium and CSCC (11). Both miR-19a and miR-19b were reported to promote cervical cancer cell proliferation (12), whereas miR-125b inhibited cervical cancer cell apoptosis (13); both processes are involved in cervical carcinogenesis. Other studies reported that miR-9 (14) and miR-135a (15) contributed to HPV-induced cervical cancer formation, and that miR-133b (16) and miR-10a (17) promoted the progression and development of cervical cancer by regulating tumor cell migration, invasion and metastasis. However, miR-424 (18), miR-124 (19) and miR-218 (20) have opposite roles. All of these cited studies analyzed the differences in expression of cervical cancer-associated miRNAs by reverse transcription-polymerase chain reaction (RT-PCR) and hybridization-based microarray that only measure known and relatively abundant miRNAs, but are unable to identify novel miRNAs. To date, no studies have focused on the miRNA profile in early-stage CSCC. Thus, investigating the miRNA profile in early-stage CSCC by high-throughput sequencing may facilitate the analysis of expression of all annotated miRNAs, detection of novel miRNAs and identification of candidate markers for early diagnosis, and treatment of CSCC.

In the present study, we applied next-generation sequencing (NGS) to globally and systematically characterize miRNA alterations in early-stage CSCC by a comparison with paired normal samples. Furthermore, we validated these miRNAs by qRT-PCR and analyzed miRNA target genes and performed function annotation and pathway analysis.

## Materials and methods

**Clinical sample collection.** For sequencing, 3 pairs of human CSCC tumor (T) and paired normal tissue (N) samples in FIGO stages I-II were obtained from patients undergoing surgery of the cervix. Additional, 20 pairs of CSCC and normal tissues were also collected for quantitative real-time polymerase chain reaction (qRT-PCR) validation. All tumor and paired normal tissues were examined by hematoxylin and eosin (H&E) staining, and all pathologic diagnosis were confirmed by 2 independent pathologists; tumor tissues containing >90% tumor cells were selected for further study (Fig. 1). The study protocol was approved by the Ethics Committees on Human Research of the Fujian Cancer Hospital. All patients agreed to join the study and signed a written informed consent. None of the patients had received preoperative adjuvant radiotherapy or chemotherapy. All samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C until RNA isolation.

**RNA isolation.** Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, and quantified using a ND-1000 spectrophotometer (NanoDrop, Rockland, DE, USA). The RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA)

and the 28S/18S ratio was determined on 1% agarose gel. Only RNA extracts with an RNA integrity number (RIN) value  $\geq 7$  and 28S/18S ratio  $>1.8$  were used for further analysis.

**Deep sequencing and bioinformatic analysis.** RNA sequencing and bioinformatic analysis were performed at the Beijing Genomics Institute (BGI; Shenzhen, China). In brief, total RNA was ligated to the 5'- and 3'-end adaptors, and sequentially amplified by an RT-PCR reaction. The fragments of ~62-75 bp (small RNA plus adaptors) were isolated from the agarose gel and then purified. These products were directly sequenced with Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After trimming adaptor sequences, removing contaminated reads and filtering low quality reads, the remaining clean reads were mapped to the human genome using short oligonucleotide alignment program (SOAP). The other small RNAs (rRNA, tRNA, snRNA, snoRNA and piRNA) were detected by screening against Rfam 10.1 and the GenBank database. The known miRNAs were identified by aligning them to the miRBase. The expression differences of the known miRNAs between the CSCCs and paired normal samples were evaluated by comparing the  $\log_2$  ratio of the 2 groups. The miRNAs with at least a 2-fold-change of expression and a P-value  $<0.05$  between the 2 groups in all three patients were selected for further study. The novel miRNAs were predicted using MIREAP software.

**Validation of mature miRNA expression by qRT-PCR.** For validation of the selected miRNAs that showed differential expression and predicted novel miRNAs, qRT-PCR was applied using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. In brief, the miRNAs were reverse transcribed into cDNA using the miScript II RT kit (Qiagen, Valencia, CA, USA) with 5X miScript HiSpec buffer. The mixture was incubated at 37°C for 60 min, and then at 95°C for 5 min and then placed on ice. Quantitative PCR was performed using the miScript SYBR-Green PCR kit and 10X miScript Primer Assay (both from Qiagen). The reaction consisted of 1 cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec and 55°C for 30 sec and 70°C for 30 sec. All reactions were repeated 3 times. The relative expression levels of miRNAs were calculated using the  $2^{-\Delta\Delta Ct}$  method with snRNA U6 as the internal reference. Quantitative PCR data were analyzed by SPSS statistics software (version 19.0; IBM, Armonk, NY, USA). A Student's t-test was used to compare the difference in expression between CSCC and normal tissues.  $P<0.05$  was considered to indicate a statistically significant result.

**miRNA target prediction, functional annotation and pathway analysis.** The target genes of the selected miRNAs with differential expression and novel miRNAs were predicted using RNAhybrid. For determination of the main biological functions of the candidate target genes, Gene Ontology (GO) enrichment analysis was applied. We mapped all candidates to GO terms in the database (<http://www.geneontology.org/>), using a hypergeometric test to find significantly enriched GO terms. The GO terms with corrected P-values with an

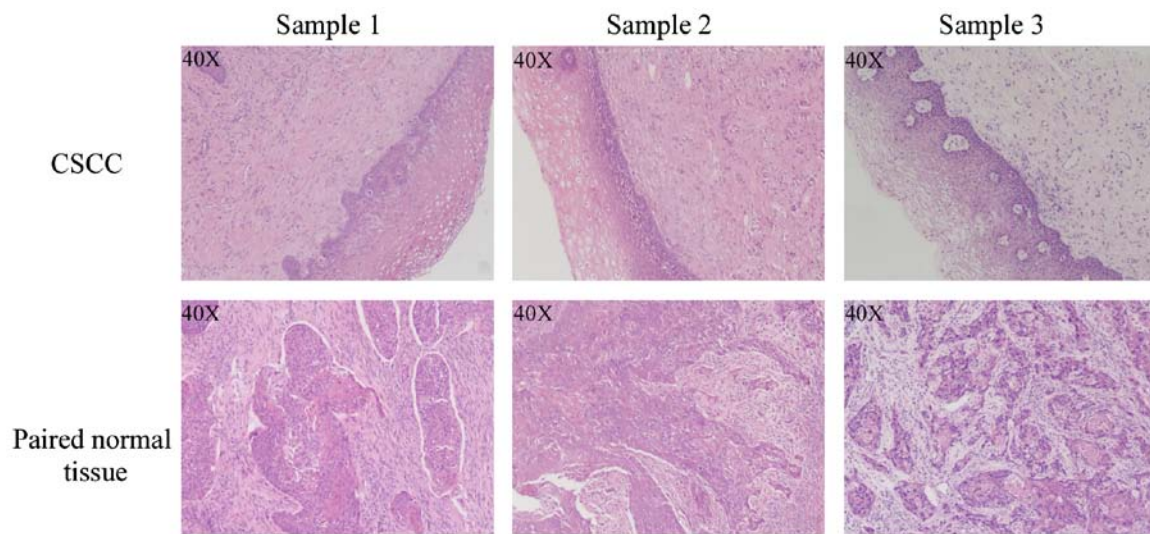


Figure 1. H&E staining of CSCC and paired normal tissues. The H&E staining results of CSCC and paired normal tissues at a magnification of x40 under a microscope.

Table I. Summary of the small RNA sequencing.

Category	Number					
	T-1	T-2	T-3	N-1	N-2	N-3
Total reads	12,000,000	10,523,729	12,000,000	12,000,000	12,000,000	12,000,000
Clean reads	11,713,704	10,313,021	11,519,252	11,799,688	11,820,021	11,704,777
%	98.40	98.73	96.70	99.02	99.18	98.25
Unique sRNAs	884,315	673,581	867,499	615,904	466,839	658,536
miRNAs	4,196	4,545	4,346	3,634	4,035	4,173
Known miRNAs in miRBase	1,708	1,881	1,810	1,571	1,659	1,724
miRNAs	200	225	229	171	182	200
miRNA-5p	414	431	418	385	394	400
miRNA-3p	306	351	325	298	330	335
miRNA precursors	788	874	838	717	753	789
Unannotated sRNAs	232,043	205,460	255,885	291,651	156,254	240,371
Novel miRNA candidates	28	24	31	20	22	26

T, CSCC tumor tissue; N, paired normal tissue.

enrichment ratio  $\leq 0.05$  were defined as significantly enriched target gene candidates. For identification of significantly enriched metabolic pathways or signal transduction pathways in target gene candidates, we mapped these candidates to the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (21). The calculation method is the same as that in the GO analysis. Pathways with corrected P-values with an enrichment ratio  $\leq 0.05$  were considered as significantly enriched target gene candidates.

**Statistical analysis.** All quantitative data are expressed as the mean  $\pm$  standard deviation (mean  $\pm$  SD) from at least 3 samples or experiments/data point. Significant differences were analyzed by the Student's t-test to compare 2 groups of independent samples.

## Results

**Overview of small RNA sequencing results.** To determine the miRNA profile of early-stage CSCC, we performed high-throughput NGS in 3 CSCC and their paired normal samples using Illumina HiSeq 2500. After removing adaptors, low quality tags and contaminants, an average of 11,478,411 clean reads (ranging from 10,313,021 to 11,820,021) remained (Table I). The majority of these clean reads were 18-28 nt in length, with 22 nt RNAs the most abundant. These clean reads were mapped to the Human Genome and rRNA, tRNA and snRNA were identified by the alignment to Rfam 10.1 and GenBank databases. Subsequently, an average of 1,726 mature miRNAs (ranging from 1,571 to 1,881) were successfully annotated in the miRBase, and thus identified

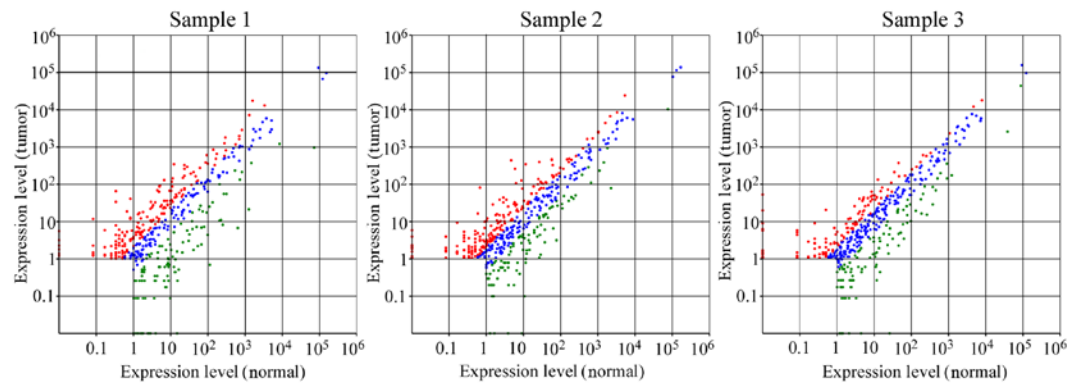


Figure 2. Scatter diagrams of miRNA expression between CSCC and normal tissues (x-axis, normal and y-axis, tumor). Red points represent miRNAs with ratio >2, blue points represent miRNAs with ratio >1/2 and ≤2 and green points represent miRNAs with ratio <1/2. Ratio, normalized expression in the tumor/normalized expression in the control tissue.

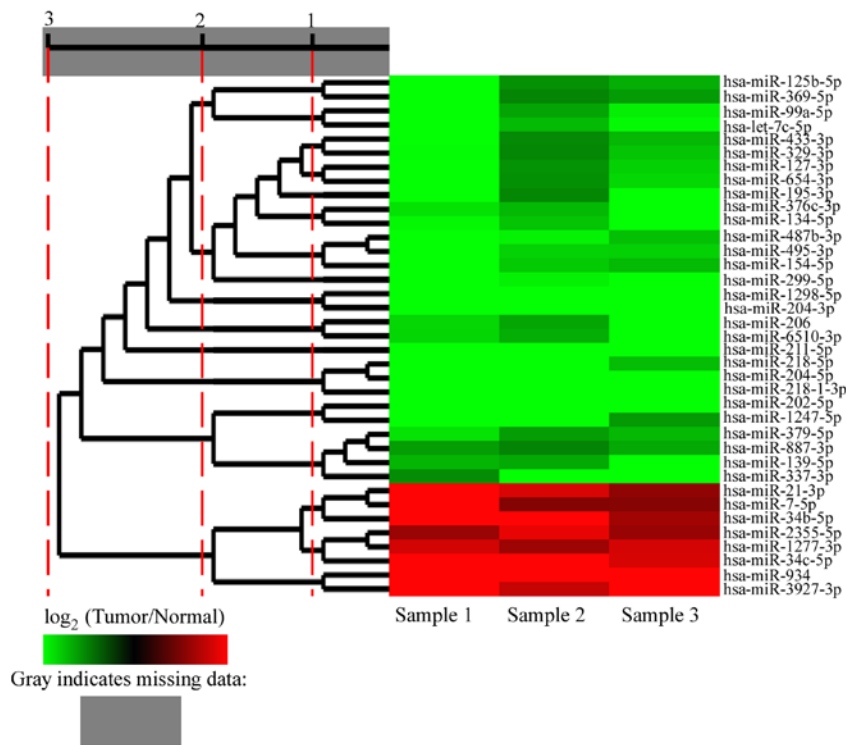


Figure 3. Heatmap of miRNA expression varied significantly between CSCC and normal tissues in the 3 pairs of samples.

as known miRNAs (Table I). The sequences which did not match with any database were considered as unannotated sRNAs (Table I).

*Identification and validation of differentially expressed known miRNAs between CSCC and normal tissues.* To detect the known miRNAs which were differentially expressed between the CSCC and normal tissues, the expression level of each miRNA was first normalized to provide the expression of transcripts per million (TPM), and then the fold-change and P-values were calculated from the normalized expression. There were 424 differentially expressed miRNAs between T1 and N1, 480 between T2 and N2 and 440 between T3 and N3 (Fig. 2). Subsequently, on the basis of the cut-off criteria (fold-change >2 and p-value <0.05),

186, 149 and 126 miRNAs were selected from the data from each of the 3 pairs of patient samples. Finally, only 37 miRNAs exhibited consistent changes in all 3 pairs of samples; among these 37 differentially expressed miRNAs, 8 were significantly upregulated and 29 were significantly downregulated (Fig. 3; Table II).

To further validate the deep sequencing results, qRT-PCR was applied to assess the significantly differentially expressed miRNAs in 3 pairs of samples used for sequencing and the additional 20 pairs of samples. Nine miRNAs were selected from the miRNAs with significantly modified expression and measured by qRT-PCR. All of the 9 miRNAs displayed the same expression pattern consistent with the sequencing data (Fig. 4). This indicated that our high through-put sequencing results were reliable.

Table II. Summary of the significantly upregulated and downregulated miRNAs in CSCC.

miRNA	Fold-change (log <sub>2</sub> tumor/normal)		
	T1 vs. N1	T2 vs. N2	T3 vs. N3
hsa-miR-934	8.30318711	4.2842649	7.20535341
hsa-miR-3927-3p	4.51831602	3.10372182	9.11787682
hsa-miR-34b-5p	9.09373446	4.03811041	2.54681543
hsa-miR-34c-5p	4.78205816	4.92246647	3.3850871
hsa-miR-21-3p	6.62522824	3.4080715	2.26011983
hsa-miR-7-5p	5.56817204	2.07123043	2.11570518
hsa-miR-1277-3p	3.30345745	2.463592	3.34499026
hsa-miR-2355-5p	2.42581593	3.65621776	2.39229062
hsa-miR-211-5p	-7.08321336	-6.80271289	-11.34275801
hsa-miR-204-5p	-6.85930564	-9.83557679	-5.26254955
hsa-miR-218-1-3p	-7.33109609	-7.40258575	-6.9021942
hsa-miR-202-5p	-10.57502857	-4.68366909	-5.0642676
hsa-miR-218-5p	-7.941165	-8.60425671	-2.97717439
hsa-miR-1298-5p	-5.92810297	-4.54163652	-7.7387003
hsa-miR-204-3p	-6.66817587	-4.05060043	-7.09486942
hsa-miR-6510-3p	-3.36793893	-2.73610137	-8.822762
hsa-miR-299-5p	-5.52895357	-3.78516379	-5.511115
hsa-miR-1247-5p	-7.39024513	-4.67666656	-2.41593802
hsa-miR-206	-3.37648276	-2.61057144	-7.18229503
hsa-let-7c-5p	-6.26600709	-2.93147207	-3.93093655
hsa-miR-99a-5p	-6.62465962	-2.58200581	-3.74953697
hsa-miR-487b-3p	-5.00445576	-3.79678741	-3.01875682
hsa-miR-154-5p	-5.43273636	-3.17579365	-2.93133666
hsa-miR-337-3p	-2.21193294	-4.38841479	-4.62100525
hsa-miR-495-3p	-4.59233357	-3.30958264	-3.28815361
hsa-miR-134-5p	-3.89631011	-3.08449386	-4.1468506
hsa-miR-125b-5p	-5.94077162	-2.27910623	-2.73045243
hsa-miR-376c-3p	-3.55711379	-2.91544225	-4.19191414
hsa-miR-195-5p	-4.33482863	-2.13708218	-4.13398935
hsa-miR-654-3p	-4.63279944	-2.26265207	-3.39215912
hsa-miR-127-3p	-4.56838942	-2.30793519	-3.28638674
hsa-miR-369-5p	-5.41522226	-2.08867671	-2.46223868
hsa-miR-139-5p	-2.84273417	-2.67661687	-4.37453647
hsa-miR-433-3p	-4.37189471	-2.16954783	-2.89020567
hsa-miR-329-3p	-3.95914876	-2.12515371	-3.0926029
hsa-miR-379-5p	-3.57432673	-2.49126887	-2.8998286
hsa-miR-887-3p	-2.49199079	-2.07624607	-2.66140231

Each miRNA is listed in the Table with P<0.01 in all 3 pairs of samples. CSCC, cervical squamous cell carcinoma.

**Target genes of the significantly altered miRNAs.** To demonstrate the differences in gene expression associated with the miRNA profile in CSCC, the putative target genes of the miRNAs that were expected to be significantly differently expressed between CSCC and normal tissues were predicted using RNAhybrid. GO and KEGG pathway analyses were performed to enrich the main biological processes and pathways in which the target gene candidates were involved.

However, the P-value of the enrichment ratio failed to achieve filter conditions and the analysis was not continued.

**Prediction of novel miRNAs, their target genes and pathways.** To detect novel miRNAs, unannotated sRNAs were further processed using MIREAP. The number of novel miRNA candidates obtained from the 6 small RNA libraries ranged from 20 to 31 (Table I). There were 5 common novel miRNA

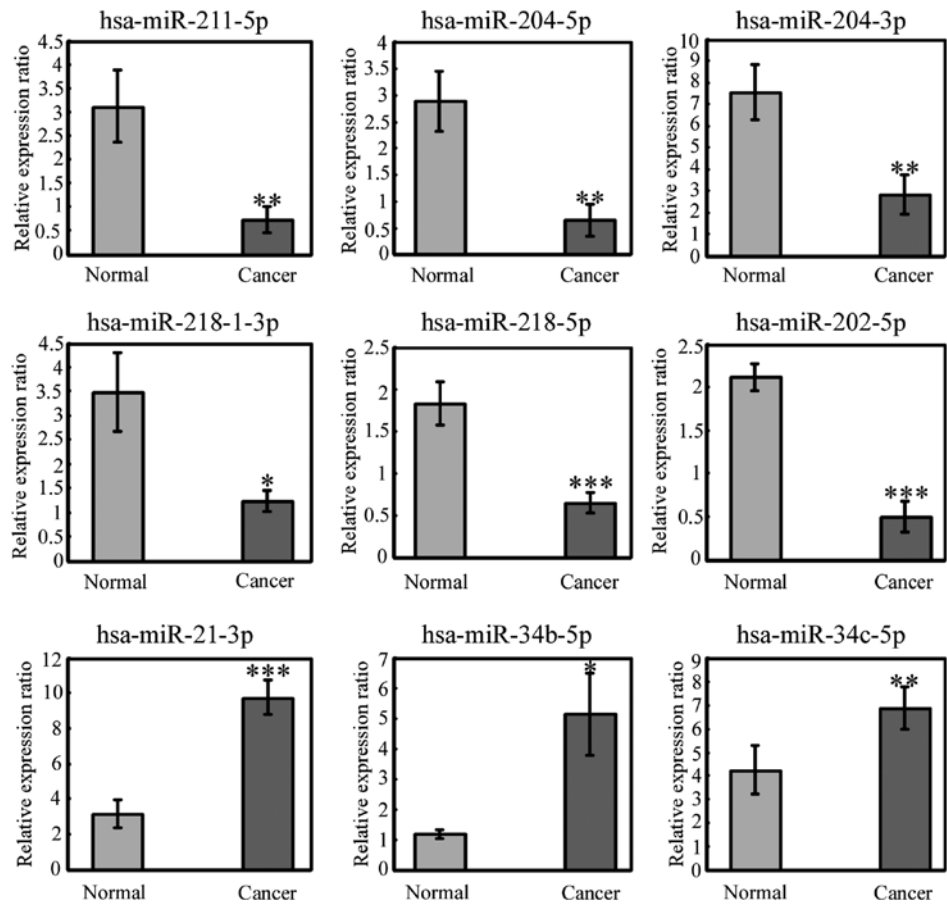


Figure 4. Validation of the significantly different miRNAs between CSCC and normal tissues by qRT-PCR in 20 pairs of samples (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).

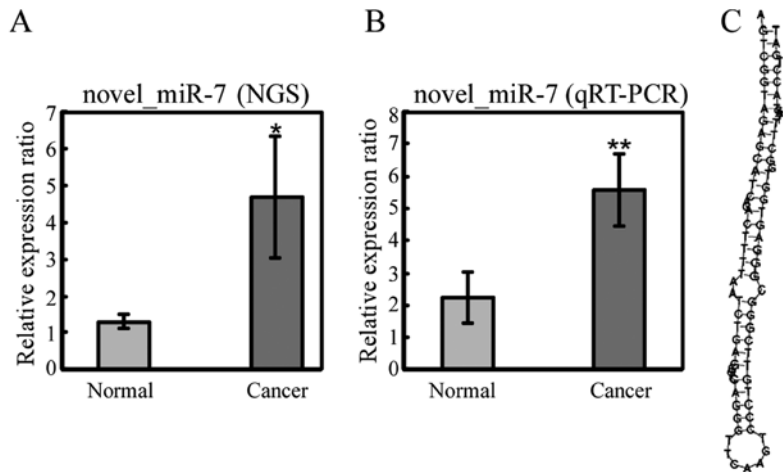


Figure 5. Prediction of novel miRNAs in CSCC. (A) Expression of novel\_mir\_7 by NGS. (B) Expression of novel\_mir\_7 by qRT-PCR validation in 20 cancer samples. (C) The sequence and the stem loop structure of novel\_mir\_7 (\*P<0.05, \*\*P<0.01).

candidates in the 3 pairs of CSCC and normal samples. Among the 5 common miRNAs, only 1 miRNA, novel\_mir\_7, displayed a significant difference in expression (fold-change >1 and P-value <0.05) in all 3 pairs of samples: novel\_mir\_7 was selected as a putative novel miRNA and verified by qRT-PCR (Fig. 5). Its sequence and stem-loop secondary structure are shown in Fig. 5. To further understand the function

of the novel miRNA, its target genes were predicted using RNAhybrid and also assessed by GO and KEGG pathway analyses. The GO analysis revealed the main biological processes in which the target genes are involved and is shown in Fig. 6. The KEGG pathway analysis indicated that 5 pathways were enriched; including the MAPK, calcium and adherent junction signaling pathways (Table III).

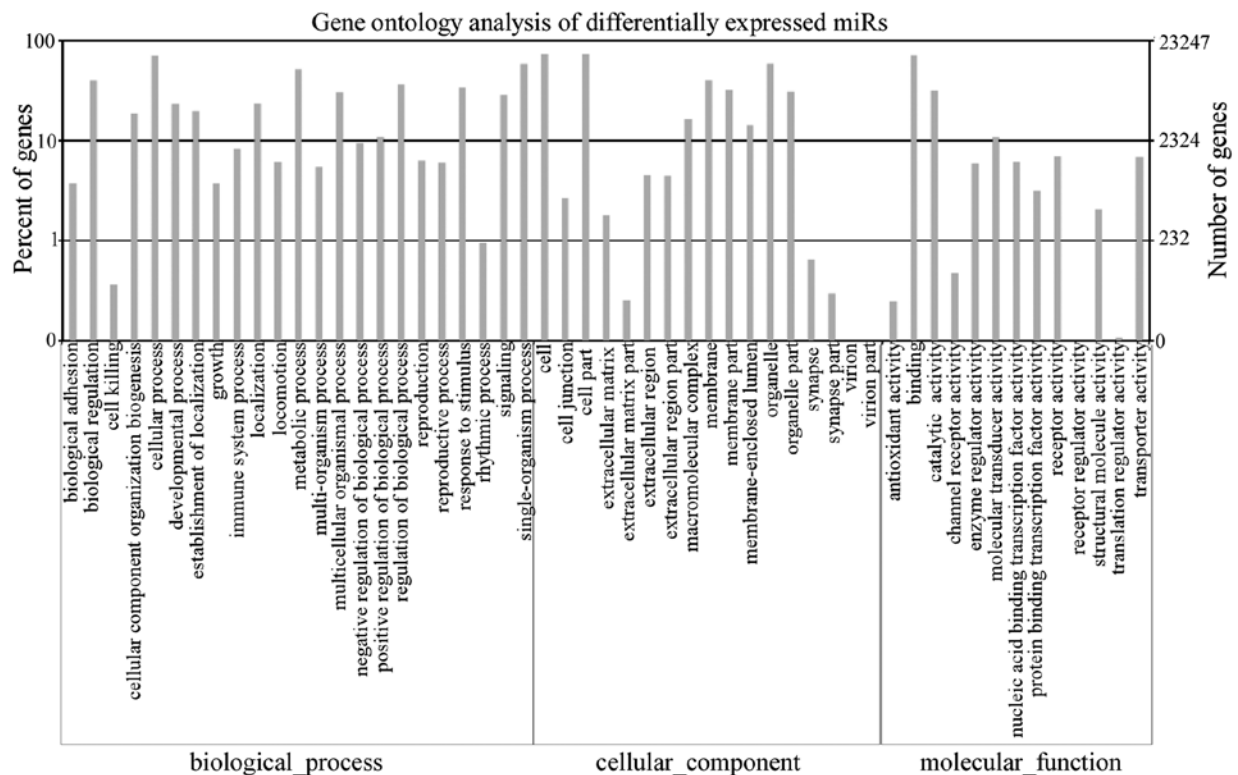


Figure 6. Functional annotation for predicted target genes of the novel miRNA in CSCC.

Table III. KEGG pathway analysis for predicted target genes of the novel miRNA in CSCC.

KEGG no.	KEGG pathway	Significance
KO:04010	MAPK signaling pathway	<sup>a</sup>
KO:04020	Calcium signaling pathway	<sup>b</sup>
KO:04520	Adherent junction	<sup>b</sup>
KO:05217	Basal cell carcinoma	<sup>b</sup>
KO:04730	Long-term depression	<sup>b</sup>

<sup>a</sup>Q-value <0.05 in all 3 pairs of samples; <sup>b</sup>Q-value <0.01 in all 3 pairs of samples. Q-value, corrected P-value. KEGG, Kyoto Encyclopedia of Genes and Genomes.

## Discussion

Although CSCC caused by HPV infection can be prevented, it is still a common cancer in women. There is accumulating evidence to indicate that HPV infection alone is insufficient to cause the disease and miRNAs may play critical roles in carcinogenesis (22-24). Therefore, global analysis of miRNA expression comparing CSCC with normal tissue may aid in the better understanding of the function of miRNAs in the course of the disease as well as finding novel biomarkers for diagnosis, treatment and prognostic evaluation. To the best of our knowledge, this is the first study to profile miRNA expression in early-stage CSCC on the NGS platform. We identified 37 significantly differentially regulated known miRNAs in CSCC compared with normal tissue and confirmed a sample

of 9 by qRT-PCR. Furthermore, a novel miRNA candidate, novel\_miR\_7, was identified for the first time and its target genes are involved in MAPK, calcium and adherent junction signaling pathways.

miRNAs are rather stable, even in body fluids such as serum, plasma, urine and saliva (25). The expression of miRNAs is unique to tissues or organs and they can be easily detected (26). These characteristics make them ideal cancer biomarkers. Juan *et al* (27) performed deep sequencing on the serum pools of cervical cancer patients and healthy controls to identify putative novel miRNAs. They found that only 1 of 17 common novel miRNA candidates may distinguish cervical cancer patients from healthy controls. Wang *et al* (28) also constructed expression profiles of miRNAs in the serum of CSCC patients by microarray. They discovered that the levels of 291 of 338 detectable circulating miRNAs were changed >2-fold in serum samples from CSCC patients vs. controls. However, they did not validate these results through any other methodologies. Although detection of miRNAs in serum has been proposed to be a simpler and less invasive diagnostic method than sampling tissue, the consequential biological and technical variability, which is mainly assessed by a series of different normalization processes based on the expression of reference genes, is difficult to avoid. Currently, there is no known suitable extracellular reference RNA for a proper normalization, particularly in cell-free miRNA analysis (26). Commonly used references, including U6 small nuclear RNA (RNU6B) and 5S ribosomal RNA, may be degraded or expressed less stably than others in serum (29). Therefore, we decided to analyze miRNA expression in CSCC tissues. In previous studies, a microarray technique, which is biased



towards abundantly expressed known miRNAs, was the major platform for miRNA expression profiling (11,28). We chose a more sensitive and specific method, NGS, which enables high-throughput analysis and novel miRNA discovery, to measure miRNA expression.

In the present study, we identified 9 miRNAs that exhibited differential expression in CSCC using deep sequencing and confirmed by qRT-PCR evaluation. Six were downregulated, including miR-211-5p, miR-204-5p/3p, miR-218-1-3p/5p and miR-202-5p, and 3 were upregulated, including miR-34b-5p, miR-34c-5p and miR-21-3p. Among these miRNAs, miR-218, miR-21 and miR-34b-5p have previously been discovered and their functions had been verified to be associated with cervical cancer. In comparison to normal cervical tissues the expression of miR-218 was reported to be lower in cervical cancer tissues (20), and that finding was concordant with our results. Its downregulation was significantly associated with worse overall survival, worse disease-free survival and pelvic/aortic lymph node recurrence. miR-218 was found to target survivin to inhibit cervical cancer progression by regulating clonogenicity, migration and invasion. Increased miR-21 expression was found to be correlated with poorer histological diagnosis in cervical cancer (30). Meanwhile, miR-21 was found to be mainly expressed in the tumor stromal microenvironment where it may be involved in cervical cancer progression. In addition, expression of miR-21 in HPV-positive samples and SCC was significantly higher than that noted in HPV-negative samples (31). Constitutive activation of aberrant STAT3 signaling via miR-21, which is regulated by the HPV oncoprotein E6, plays a pivotal role in the initiation and progression of HPV-induced cervical carcinogenesis (32,33). Our sequencing results found that miR-21 was upregulated in CSCC and it may act as an oncogene; which is consistent with previous studies (30,31). miR-34b-5p was found to be downregulated in minimal deviation adenocarcinoma (MDA) compared with normal proliferative endocervical tissues (34), but in the present study, it was found to be upregulated in CSCC. The discrepancy suggests different expression patterns of miRNAs in different histological subtypes of tumors.

The other differentially expressed miRNAs we described have seldom been reported in previous studies of cervical cancer, but they have been demonstrated to be correlated with other malignant tumors. Trends seen in the expression of certain miRNAs that were determined by sequencing were in accord with published functional studies. For example, miRNA-211 functions as a tumor-suppressor in melanoma (35,36) and glioma (37). It could block tumor invasion and migration by targeting NIAK1 (38), BRN2 (39), KCNA1 (40) or MMP-9 (37). Both miR-204-5p/3p and miR-202 have tumor-suppressive function as well. Downregulation of miR-204-5p in colorectal cancer tissues was associated with poor prognosis: It suppresses colorectal cancer cell growth, migration, and invasion as well as promotes tumor sensitivity to chemotherapy by inhibiting RAB22A (41). In endometrial carcinoma, miR-204-5p exhibits a similar function through the TrkB-STAT3-miR-204-5p regulatory pathways (42) and miR-204-3p inhibits the growth of hepatocellular carcinoma tumor endothelial cells by blocking the adhesion function of FN1 (43). miR-202 can suppress osteosarcoma cell

proliferation and induce cell apoptosis by downregulating Gli2 expression (44). While miR-34c is considered to be a putative tumor suppressor in a majority of tumors (45-48), we found it to be upregulated in CSCC, which is in agreement with previous results that determined expression by miRNA microarray in cervical cancer (11,28). Thus, we hypothesized that miR-34c may play different roles in cervical cancer and other types of malignancy.

NGS technology allows the rapid discovery of novel miRNAs, for some of which the biological function is unknown. We identified 5 common novel miRNA candidates with differential expression between CSCC and normal tissues. Our focus was narrowed to miRNAs that exhibited a significant change of expression in all 3 pairs of samples. We identified novel\_miR\_7 as a candidate novel miRNA and confirmed its presence. Functional annotation revealed that its predicted target genes are in the MAPK signaling pathway, which is closely related to cell proliferation, cell death, and the balance between them. Early in 2006, Engelbrecht *et al* indicated the involvement of MAPK in cervical cancer and demonstrated the correlation between the activity of MAPK and apoptosis in the disease process (49). The HPV 16 E2 protein may induce apoptosis by inhibiting MAPK signaling in CSCC (50). However, playing a role in cervical cancer carcinogenesis, the expression level of MAPK may serve as a marker for cervical cancer progression (51,52) and its inhibitors have shown the potential to be useful for cervical cancer therapy (53). Therefore, our results provide a solid basis for the characterization of the role of novel\_miR\_7 in the pathogenesis and development of CSCC and in the treatment of this disease.

In summary, for the first time we identified known and novel miRNAs in early-stage CSCC by high-throughput NGS. Moreover, we validated these miRNAs in other independent samples by qRT-PCR. Our analysis of differentially expressed miRNAs not only support existing findings but also uncovered various differentially expressed miRNAs which had not previously been reported in CSCC, thus providing new molecular signatures of the disease. The prediction of novel miRNAs found that novel\_miR\_7 may be a promising biomarker for CSCC. These results have implications for exploring the regulatory network of miRNAs, provide new targets for molecular mechanistic studies of CSCC as well as for early detection, targeted therapy and prognostic evaluation of CSCC patients in the future.

## Acknowledgements

The present study was supported by grants from the Fujian Province Science and Technology Plan Key Project (no. 2013Y0030) and the National Clinical Key Specialty Construction Program of China.

## References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 65: 87-108, 2015.
2. Bosch FX, Lorincz A, Muñoz N, Meijer CJ and Shah KV: The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 55: 244-265, 2002.



3. Vaccarella S, Lortet-Tieulent J, Plummer M, Franceschi S and Bray F: Worldwide trends in cervical cancer incidence: Impact of screening against changes in disease risk factors. *Eur J Cancer* 49: 3262-3273, 2013.
4. Cole L and Stoler MH: Issues and inconsistencies in the revised gynecologic staging systems. *Semin Diagn Pathol* 29: 167-173, 2012.
5. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
6. Berindan-Neagoe I, Monroig PC, Pasculli B and Calin GA: MicroRNAome genome: A treasure for cancer diagnosis and therapy. *CA Cancer J Clin* 64: 311-336, 2014.
7. Gregory RI, Chendrimada TP, Cooch N and Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123: 631-640, 2005.
8. Rossi JJ: New hope for a microRNA therapy for liver cancer. *Cell* 137: 990-992, 2009.
9. Mendell JT and Olson EN: MicroRNAs in stress signaling and human disease. *Cell* 148: 1172-1187, 2012.
10. Calin GA and Croce CM: MicroRNA signatures in human cancers. *Nat Rev Cancer* 6: 857-866, 2006.
11. Wilting SM, Snijders PJ, Verlaet W, Jaspers A, van de Wiel MA, van Wieringen WN, Meijer GA, Kenter GG, Yi Y, le Sage C, *et al*: Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. *Oncogene* 32: 106-116, 2013.
12. Xu XM, Wang XB, Chen MM, Liu T, Li YX, Jia WH, Liu M, Li X and Tang H: MicroRNA-19a and -19b regulate cervical carcinoma cell proliferation and invasion by targeting CUL5. *Cancer Lett* 322: 148-158, 2012.
13. Wang YD, Cai N, Wu XL, Cao HZ, Xie LL and Zheng PS: OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. *Cell Death Dis* 4: e760, 2013.
14. Liu W, Gao G, Hu X, Wang Y, Schwarz JK, Chen JJ, Grigsby PW and Wang X: Activation of miR-9 by human papillomavirus in cervical cancer. *Oncotarget* 5: 11620-11630, 2014.
15. Leung CO, Deng W, Ye TM, Ngan HY, Tsao SW, Cheung AN, Pang RT and Yeung WS: miR-135a leads to cervical cancer cell transformation through regulation of  $\beta$ -catenin via a SIAH1-dependent ubiquitin proteasomal pathway. *Carcinogenesis* 35: 1931-1940, 2014.
16. Qin W, Dong P, Ma C, Mitchelson K, Deng T, Zhang L, Sun Y, Feng X, Ding Y, Lu X, *et al*: MicroRNA-133b is a key promoter of cervical carcinoma development through the activation of the ERK and AKT1 pathways. *Oncogene* 31: 4067-4075, 2012.
17. Long MJ, Wu FX, Li P, Liu M, Li X and Tang H: MicroRNA-10a targets CHL1 and promotes cell growth, migration and invasion in human cervical cancer cells. *Cancer Lett* 324: 186-196, 2012.
18. Xu J, Li Y, Wang F, Wang X, Cheng B, Ye F, Xie X, Zhou C and Lu W: Suppressed miR-424 expression via upregulation of target gene Chk1 contributes to the progression of cervical cancer. *Oncogene* 32: 976-987, 2013.
19. Wan HY, Li QQ, Zhang Y, Tian W, Li YN, Liu M, Li X and Tang H: MiR-124 represses vasculogenic mimicry and cell motility by targeting amotL1 in cervical cancer cells. *Cancer Lett* 355: 148-158, 2014.
20. Kogo R, How C, Chaudary N, Bruce J, Shi W, Hill RP, Zahedi P, Yip KW and Liu FF: The microRNA-218~Survivin axis regulates migration, invasion, and lymph node metastasis in cervical cancer. *Oncotarget* 6: 1090-1100, 2015.
21. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, *et al*: KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 36: D480-D484, 2008.
22. Baranwal S and Alahari SK: miRNA control of tumor cell invasion and metastasis. *Int J Cancer* 126: 1283-1290, 2010.
23. Farazi TA, Spitzer JJ, Morozov P and Tuschl T: miRNAs in human cancer. *J Pathol* 223: 102-115, 2011.
24. Gilabert-Estelles J, Braza-Boils A, Ramon LA, Zorio E, Medina P, Espana F and Estelles A: Role of microRNAs in gynecological pathology. *Curr Med Chem* 19: 2406-2413, 2012.
25. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK and Calin GA: MicroRNAs in body fluids - the mix of hormones and biomarkers. *Nat Rev Clin Oncol* 8: 467-477, 2011.
26. Etheridge A, Lee I, Hood L, Galas D and Wang K: Extracellular microRNA: A new source of biomarkers. *Mutat Res* 717: 85-90, 2011.
27. Juan L, Tong HL, Zhang P, Guo G, Wang Z, Wen X, Dong Z and Tian YP: Identification and characterization of novel serum microRNA candidates from deep sequencing in cervical cancer patients. *Sci Rep* 4: 6277, 2014.
28. Wang WT, Zhao YN, Yan JX, Weng MY, Wang Y, Chen YQ and Hong SJ: Differentially expressed microRNAs in the serum of cervical squamous cell carcinoma patients before and after surgery. *J Hematol Oncol* 7: 6, 2014.
29. Peltier HJ and Latham GJ: Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 14: 844-852, 2008.
30. Deftereos G, Corrie SR, Feng Q, Morihara J, Stern J, Hawes SE and Kiviat NB: Expression of miR-21 and miR-143 in cervical specimens ranging from histologically normal through to invasive cervical cancer. *PLoS One* 6: e28423, 2011.
31. Bumrungrathai S, Ekalaksananan T, Evans MF, Chopjitt P, Tangsiriwatthana T, Patarapadungkit N, Kleebkaow P, Luanratanakorn S, Kongyingyoes B, Worawichawong S, *et al*: Up-regulation of *miR-21* is associated with cervicitis and human papillomavirus infection in cervical tissues. *PLoS One* 10: e0127109, 2015.
32. Shishodia G, Verma G, Srivastava Y, Mehrotra R, Das BC and Bharti AC: Deregulation of microRNAs let-7a and miR-21 mediate aberrant STAT3 signaling during human papillomavirus-induced cervical carcinogenesis: Role of E6 oncoprotein. *BMC Cancer* 14: 996, 2014.
33. Shishodia G, Shukla S, Srivastava Y, Masaldan S, Mehta S, Bhambhani S, Sharma S, Mehrotra R, Das BC and Bharti AC: Alterations in microRNAs miR-21 and let-7a correlate with aberrant STAT3 signaling and downstream effects during cervical carcinogenesis. *Mol Cancer* 14: 116, 2015.
34. Lee H, Kim KR, Cho NH, Hong SR, Jeong H, Kwon SY, Park KH, An HJ, Kim TH, Kim I, *et al*: Gynecological Pathology Study Group of the Korean Society of Pathologists: MicroRNA expression profiling and Notch1 and Notch2 expression in minimal deviation adenocarcinoma of uterine cervix. *World J Surg Oncol* 12: 334, 2014.
35. Levy C, Khaled M, Iliopoulos D, Janas MM, Schubert S, Pinner S, Chen PH, Li S, Fletcher AL, Yokoyama S, *et al*: Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. *Mol Cell* 40: 841-849, 2010.
36. Xu Y, Brenn T, Brown ER, Doherty V and Melton DW: Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. *Br J Cancer* 106: 553-561, 2012.
37. Asuthkar S, Velpula KK, Chetty C, Gorantla B and Rao JS: Epigenetic regulation of miRNA-211 by MMP-9 governs glioma cell apoptosis, chemosensitivity and radiosensitivity. *Oncotarget* 3: 1439-1454, 2012.
38. Bell RE, Khaled M, Netanel D, Schubert S, Golan T, Buxbaum A, Janas MM, Postolsky B, Goldberg MS, Shamir R, *et al*: Transcription factor/microRNA axis blocks melanoma invasion program by miR-211 targeting NUA1. *J Invest Dermatol* 134: 441-451, 2014.
39. Boyle GM, Woods SL, Bonazzi VF, Stark MS, Hacker E, Aoude LG, Dutton-Regester K, Cook AL, Sturm RA and Hayward NK: Melanoma cell invasiveness is regulated by miR-211 suppression of the BRN2 transcription factor. *Pigment Cell Melanoma Res* 24: 525-537, 2011.
40. Mazar J, DeYoung K, Khaitan D, Meister E, Almodovar A, Goydos J, Ray A and Perera RJ: The regulation of miRNA-211 expression and its role in melanoma cell invasiveness. *PLoS One* 5: e13779, 2010.
41. Yin Y, Zhang B, Wang W, Fei B, Quan C, Zhang J, Song M, Bian Z, Wang Q, Ni S, *et al*: miR-204-5p inhibits proliferation and invasion and enhances chemotherapeutic sensitivity of colorectal cancer cells by downregulating RAB22A. *Clin Cancer Res* 20: 6187-6199, 2014.
42. Bao W, Wang HH, Tian FJ, He XY, Qiu MT, Wang JY, Zhang HJ, Wang LH and Wan XP: A TrkB-STAT3-miR-204-5p regulatory circuitry controls proliferation and invasion of endometrial carcinoma cells. *Mol Cancer* 12: 155, 2013.
43. Cui ZH, Shen SQ, Chen ZB and Hu C: Growth inhibition of hepatocellular carcinoma tumor endothelial cells by miR-204-3p and underlying mechanism. *World J Gastroenterol* 20: 5493-5504, 2014.
44. Sun Z, Zhang T, Hong H, Liu Q and Zhang H: miR-202 suppresses proliferation and induces apoptosis of osteosarcoma cells by downregulating Gli2. *Mol Cell Biochem* 357: 277-283, 2014.
45. Yu Z, Kim J, He L, Creighton CJ, Gunaratne PH, Hawkins SM and Matzuk MM: Functional analysis of miR-34c as a putative tumor suppressor in high-grade serous ovarian cancer. *Biol Reprod* 91: 113, 2014.

46. Yang S, Li WS, Dong F, Sun HM, Wu B, Tan J, Zou WJ and Zhou DS: KITLG is a novel target of *miR-34c* that is associated with the inhibition of growth and invasion in colorectal cancer cells. *J Cell Mol Med* 18: 2092-2102, 2014.
47. Achari C, Winslow S, Ceder Y and Larsson C: Expression of *miR-34c* induces G2/M cell cycle arrest in breast cancer cells. *BMC Cancer* 14: 538, 2014.
48. van der Deen M, Taipaleenmäki H, Zhang Y, Teplyuk NM, Gupta A, Cinghu S, Shogren K, Maran A, Yaszemski MJ, Ling L, *et al*: MicroRNA-34c inversely couples the biological functions of the runt-related transcription factor RUNX2 and the tumor suppressor p53 in osteosarcoma. *J Biol Chem* 288: 21307-21319, 2013.
49. Engelbrecht AM, Gebhardt S and Louw L: Ex vivo study of MAPK profiles correlated with parameters of apoptosis during cervical carcinogenesis. *Cancer Lett* 235: 93-99, 2006.
50. Gao LJ, Gu PQ, Zhao W, Ding WY, Zhao XQ, Guo SY and Zhong TY: The role of globular heads of the Clq receptor in HPV 16 E2-induced human cervical squamous carcinoma cell apoptosis is associated with p38 MAPK/JNK activation. *J Transl Med* 11: 118, 2013.
51. Chen TP, Chen CM, Chang HW, Wang JS, Chang WC, Hsu SI and Cho CL: Increased expression of *SKP2* and phospho-MAPK/ERK1/2 and decreased expression of *p27* during tumor progression of cervical neoplasms. *Gynecol Oncol* 104: 516-523, 2007.
52. Su PH, Lin YW, Huang RL, Liao YP, Lee HY, Wang HC, Chao TK, Chen CK, Chan MW, Chu TY, *et al*: Epigenetic silencing of PTPRR activates MAPK signaling, promotes metastasis and serves as a biomarker of invasive cervical cancer. *Oncogene* 32: 15-26, 2013.
53. Meira DD, de Almeida VH, Mororó JS, Nóbrega I, Bardella L, Silva RL, Albano RM and Ferreira CG: Combination of cetuximab with chemoradiation, trastuzumab or MAPK inhibitors: Mechanisms of sensitisation of cervical cancer cells. *Br J Cancer* 101: 782-791, 2009.