CircCASC15-miR-100-mTOR may Influence the Cervical Cancer Radioresistance

Tingting Yao (yaotting@mail.sysu.edu.cn)
Sun Yat-Sen University 2nd Affiliated Hospital: Sun Yat-Sen Memorial Hospital https://orcid.org/0000-0002-0231-6047

Zhiliao Chen
Sun Yat-Sen University 2nd Affiliated Hospital: Sun Yat-Sen Memorial Hospital

Yongpai Peng
Sun Yat-Sen University 2nd Affiliated Hospital: Sun Yat-Sen Memorial Hospital

Guanglei Zhong
Sun Yat-Sen Memorial Hospital

Chunxian Huang
Sun Yat-Sen Memorial Hospital

Jing(F) Li
Sun Yat-Sen Memorial Hospital

Ruixin Li
Sun Yat-Sen Memorial Hospital

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Abstract

Background

Cervical cancer has ranked the top one in gynecological malignancies for incidence. Radioresistance is now becoming a leading reason of recurrence.

Methods

Our microRNA array data indicated that the miRNA-100 level decreased significantly during radioresistance. In this study, we up-regulated miR-100 in Hela and Siha cells by using miR-100 mimics and observed apoptosis, proliferation, cell cycle and invasion.

Results

It turned out that with overexpression of miR-100, the cells had more apoptosis and less invasiveness as well as proliferation. It may also influence cell cycle via target gene mTOR, and it deed reduced EMT. To examine the role of miR-100 in radioresistance, there was no significant result showed by BSP.While the circCASC15 has been identified with sponge function according to RNA pull down and ISH.

Conclusion

The conclusions indicate miR-100 is a tumor suppressor gene and could be a therapeutic target in radio-resistant cervical cancers.

Introduction

Radiotherapy (RT) has been widely applied from stage I to stage IV cervical cancer as primary or preoperative or postoperative adjuvant treatment. However, recurrence after radiotherapy remains significant. Approximately 20% of patients diagnosed with pelvic recurrence, while the cure rate for early-stage is more than 80% [1]. The 5-year survival rates of recurrenceis between 10.1% and 22.3% [2–3]. Therefore, elimination of RT was important for patients diagnosed with cervical cancer.

MicroRNAs (miRNAs) belong to non-coding RNA family that function at the post-transcriptional level. Their deregulated expression can influence both the treatment response[4] and the development of drug resistance [5–6].

MiR-100 belongs to miR-99 family, which located in chromosome 11. It has been proved playing a role in proliferation, metastasis, as well as sensitivity of to radiotherapy and chemotherapy [7–10].We proved miR-100 downregulated in radioresistant cervical cancer.

Circular RNA (circRNA) is another kind of non-coding RNA because it is a closed covalent loop without poly A tail or 5'to 3'polarity [11]. At present, more than 30,000 circular RNAs have been identified [12]. A variety of circular RNAs are believed to have relationship with occurrence of many tumors [13].Several reports have showed that miRNA response elements exist in circular RNAs, which act as competitive endogenous RNAs (ceRNAs) to sponge miRNAs or transcriptional regulators to. [14].We found circCASC15 may be the sponge of miR-100.

Materials And Methods

Patient selection and human tissues

Group 1 (for miRNA array)

Three patients have been clarified as stage IIB-IVA according to International Federation of Gynecology and Obstetrics (FIGO),, who had received radiotherapy in our hospital between 2000 and 2008. According to the 2013 NCCN cervical cancer clinical practice guidelines, patients received standard radiotherapy, pelvic radiotherapy and branch radiotherapy, with a total dose of 70 Gy at point A [15]. Two pathologists evaluated histology independently. According to the hospital's internal review and ethics committee, all samples were collected with informed consent.

Group 2 for QPCR. 84 cervical tumors were obtained from our department, between January 2002 and June 2011. None of themreceived any treatment before surgery. All patients have been confirmed HPV infection. Two patients without HPV infection were chosen as normal controls.

Mirnas Real Time Polymerase Chain Reaction Array

After isolation total RNA from samples, Universal cDNA synthesis kit (Exiqon) was used. MiRNAs were detected by the miRCURY LNA™ Universal real time microRNA polymerase chain reaction system(Exiqon, kangchen, China).

Cell Culture

HeLa and SiHa were purchased from China Academic Sinica Cell Repository, Shanghai, China. They were in MEM medium(Gibco, Los Angeles, CA, USA) with 10% fetal bovine serum (Gibco,Australia)in an incubator under humidity at 37°Cwith 5% CO_2.

Transient Transfection

Mir-100-5p mimics purchased from Genepharma (Shanghai, China), were transient transfected into cells with Lipofectamine™ 2000 (Invitrogen). Mock-transfected condition was made by negative control

mimics with fluorescence. All the cells were collected at 48h post-transfection, and the levels of mir-100 were elevated by RT-PCR assay as follow. The sequences of miR-100-5p mimics and negative control were shown below:

Mimics(forward 5’-AACCCGUAGAUCCGAACUUGUG-3’

Reverse 5’-CAAGUUCGGAUCUACGGGUUUU-3’);

Negative control(forward 5’-UUCUCCGAACGUGUCACGUTT -3’

Reverse 5’-ACGUGACACGUUCGGAGAATT -3’);

Mirna Expression Analysis And Rt-pcr Assay

The RNAs were collected from cells by Trizol extraction (Invitrogen), and cDNAs were acquired using an RNA reverse transcription amplification kit (Takara). RT-PCR assays were performed by SYBR Green Real-time PCR Universal Reagent (GenePharma Co.,Ltd.) and analyzed by BIO-RAD fluorescence quantitative PCR machine. Primers were as follows:

miR-100-5p (5’-AACCCGTAGATCCGAACTTGTG-3’);

u6(5’-ACGCAAATTCGTGAAGCGTT-3’);

GAPDH(forward5’-TGGAAATGACAGTGAAGCACCTC-3’

reverse5’-TCGTTCATGCACTCGCTGAAG-3’);

u6 was used as control for miRNA, GAPDH was used as control for mRNA. Both of them was calculated with the $2 - \Delta\Delta CT$ method.

Proliferation Assay

After cultured (1×10^4 cells/well) in separate 96-well plates for 24h, cells were transfected with miR-100-5p mimics. At day 1, day 2, day 3, day 4 after transfection, cells activation were evaluated by Cell Counting Kit-8 assay, the absorbance were read at 450 nm.

Cell Apoptosis Assay

After digestion with EDTA-free trypsin, incubation with Annexin-V and PI for 10 min at20°C, flow cytometry was performed (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA)

Tumor Xenografts

Siha cells (2×10^6) were transfected with miR-100 or Negative Control for 2 days following animal experiments institutional ethical guidelines. Cells were digested and resuspended in 200μL PBS .PBS with cells or without cells were injected into female BALB/c athymic nude mouse(four-week-old). Six nude mice were used. Mice were anesthetized with Isoflurane (Forene, Abbott GmbH & Co.KG, Wiesbaden, Germany) with 1 $L*min^{-1}$ O2 flow for euthanasia.

Bioinformatics

miRanda,TargetScan were chosen for predicting targetgene.

Immunohistochemistry

After embedded by paraffin- and fixed with formalin, tissue was cut into 5μm section, deparaffinization, rehydration, quenche, and retrieve.Incubation at 4°C overnight with Anti-mTOR (phospho S2448) antibody (HRP) (ab196914) (Abcam).

Establishment Of Radioresistant (Rr) Cell Lines

Cells were exposed to two Gy 3 times, four Gy 3 times, six Gy 3 times, eight Gy 2 times and ten Gy 2 times at 300 cGy/min with a linear accelerator (SIMEN, Germany). [16–17].

5-aza-2-deoxycytidine Treatment

HeLa and SiHa cells(3×10^5cells/well) were cultured in 6-well plates overnight, then 10μM DAC(5-aza-2-deoxycytidine**)** or 10μM DMSO (dimethyl sulfoxide) was separately added to cells per 24h. Cells were harvested after 5 days, and RNAs were extracted for detecting the level of mir-100 as mentioned above.

Bisulfite Sequencing Pcr(Bsp)

Following extraction (QIAamp DNA Mini Kit),Genomic DNA was subjected to EpiTect® Plus DNA Bisulfite Kit. After purifiration, modified DNA was chosen as nested PCR reactions template. The primers were: outer primers, 5’-ATTCGAATTTAGTGGAATTAGAATC-3' (forward) and 5'-AACCTACAACAACAACAACAACG-3'(reverse); nested primers, 5'-TTAGTA ATTTTAGGTTAGAGGGTTATCG-3' (forward) and 5'-ACTCCAAAAACCC ATAACTAACCG-3' (reverse). The second-round PCR products were subjected to electrophoresis on agarose gels, excised, and inserted into the TOPO cloning vector (Invitrogen, K4600-01).Clones were used for DNA sequencing randomly.

Western blotting

Protein was extracted from cells with ice-cold radioimmunoprecipitation lysis solution, then centrifugated to remove cell debris. N-cadherin (ab18203) and E-cadherin (ab194982) from Abcam(Cambridge, MA, USA) were chosen as primary antibodies and HRP-conjugated secondary antibody was purchased from Sangon (Shanghai, China).GAPDH and β-actin were employed as loading controls.

CircRNA expression profiles in CC

We enriched the contained circRNAs and digested them with RNase A, then reversely transcribed them to RNA by fluorescent reagents and random primers. We used Human circRNA Arrays (8 × 15 K, Arraystar, Rockville, MD, USA) to determine the circRNA profile. Those circRNAs with fold change ≥ 2 were identified as differentially expression circRNAs. The data were analyzed by using R software limma and Arraystar program (Arraystar).

Biotin-coupled probe pull-down assay

Hela and Siha cells were transfected by biotinylated miR-100-5p mimics or its mutant (Genepharma, Suzhou, China). Then cells underwent harvest, lysis, sonicate, and incubation with magnetic beads at 4℃ overnight. The RNA mix bound to the magnetic beads was eluted and treated with Trizol for further qRT-PCR.

Florescent in situ hybridization

Fluorescent in situ hybridization probes for circCASC15, miR-100, and 18S RNA were designed and synthesized by Synbio Technologies (Suzhou, China). 18S RNA was used as a positive control. The signals of the probe were examined by the Florescent in Situ Hybridization Kit (RiboBio, Guangzhou, China).

Data Analysis

All data were based onthree independent replicates. Statistical analyses were analyzed by SPSS 13.0. Data were presented as the mean values ± standard error. $P<0.05$ was considered statistically significant.

Results

MiR-100 is down-regulated in radioresistant human cervical cancer.

To investigate miRNAs differential expressions in human radioresistant cervical cancer and to seek their roles in radioresistance, we performed miRNA microarray profiling analysis. At last we confirmed a set of miRNAs that are down-regulated in invasive cervical cancer. The most deregulated miRNA is miR-100 at 5 folds($P=0.028$) (Fig. 1).

MiR-100 is correlated with poor clinical outcomes in cervical cancer.

The expression of miR-100 in 84 samples of cervical cancer and normal cervical tissues from hysterectomy including uterine myoma or prolapse.In Fig. 2, the average expression of miR-100 in cancer samples was lower than normal samples ($P< 0.01$). We found that miR-100 expression was correlated with tumor size ($P<0.001$), lymph node (LN) status ($P<0.001$) and TNM stage ($P<0.001$).

MiR-100 inhibits invasion and reduces EMT.

Since the fact that miR-100 was decreased at 5 folds in invasive cervical cancer according to the array, miR-100 mimics was used to up-regulate its expressions in Hela and Siha(Fig. 3A). Cell invasiveness was tested by transwell assay in 24-well-plate which illustrated that miR-100 could inhibit invasion at 60% in Hela and 50% in Siha cells (Fig. 3B). Wondering whether the inhibition of invasiveness was mediated via EMT, Western Blot was performed. As expected, up-regulation of miR-100 reduced EMT in protein levels(Fig. 3C).

MiR-100 promotes cell apoptosis and inhibits proliferation in cervical cancer.

The observed down-regulation of miR-100 in human invasive cervical cancer tissues prompted us to detect whether it would be a tumor suppressor. Proliferation was measured by CCK8 kit after cells were transfected miR-100 mimics for 24, 48, 72 and 96h. No significant difference of proliferation of Hela(Fig. 4A) and Siha(Fig. 4B) were observed at the point of 24h after transfection. While at the other time points, proliferations were inhibited in cells treated with mimics.

Furthermore, the early apoptosis rates of Hela and Siha which transfected mimic and NC sequence were tested by annexin-V and PI assay and revealed together in Fig. 5A.

At last, cell cycle assay demonstrated that cell accounts in G2/M phase were decreased in Hela and Siha transfected miR-100 mimics respectively. Detail rates were shown in Fig. 5B.

MiR-100 inhibits tumorigenesis in SCC xenografts.

There mice were injected cells transfected with miR-100, while others injected without transfection. Compared to control group, the volumn of tumor was reduced (Fig. 6A&6B**).**

MiR-100 targets mTOR.

MiRanda, TargetScan, and miRbase predicted mTOR to be the target of miR-100.

According to sequence analysis, mTOR was found contains a putative binding site of miR-100 (Fig. 7A). To verify whether mTOR is the target of miR-100, a wild-type mTOR 3'UTR fragment was cloned downstream of the firefly luciferase reporter gene. When pc3-miR-100 was co-transfected, the relative luciferase activity of the reporter gene containing the wild-type 3'UTR was inhibited significantly Fig. 7B.And the expression of p-mTOR on cervical cancer was significantly higher than normal cervical Fig. 7C.

Mir-100 Was Provoked By 5-aza-cdr In Hela And Siha

We applied 10μM 5-Aza-CdR to HeLa and SiHa, to evaluate the effects on miR-100. As shown in Fig. 8A the treatment significantly up-regulated the level of miR-100 in both cell lines, suggesting a possible role of methylation in the regulation of miR-100 during the process of radiotherapy(P<0.05).

The level of miR-100 regulated by radiotherapy may not be mediated by miR-100HG

Bisulfite sequencing PCR was used to elevate the methylation of MIR100HG in HeLa, HRR, SiHa and SRR. The percentage of CpG Fig. 8B methylation was 0.207±0.050, 0.148±0.042,0.067±0.014, 0.045±0.021, respectively. As shown in Figure8B, the methylation of MIR100HG decreased after radiotherapy, though there was no statistic significance.

Circcasc15 May Sponge Mir100 In Cerna Manner

Three cervical cancer tissues and three normal cervical tissues were used for circRNAs expression profiling. The scatter and volcano plots demostrated the circRNA expression Fig. 9A. The cluster heat map showed circRNAs that expressed over 2 fold change. qPCR was performed to verify the microarray results. Consistent with the microarray, qPCR showed that circCASC15 was increased in Hela and Siha cells compared to normal cervical tissues (Fig. 9B**)**.

With bioinformatic prediction, miR-100-5p was predicted as the sponge target of has-circ-CASC15 (Fig. 9C). To find the regulatory mechanism of miR-100, RNA pulldown assay was performed. CircCASC15 was found to be combined with miR-100(Fig. 9D). CircCasc15 and miR-100 were found mainly expressed in cytoplasm. Both in our microarray analysis and tissues' validation, circCASC15 was downregulated (Fig. 9E).

Discussion

In our study, the increase of miR-100 in the radio-resistant cell lines was detected, which was in accordance with the assay of breast cancer [18].While miR-100 was considered to be a suppressor gene, which used to decrease in cervical cancer. In this assay, its provoked expression suggested a potential role in sensitivity of radiotherapy. In oral cancer, the increase of miR-100 inhibited FGFBP1 and FGFR3, which over-expressed in radio-resistant cells [8], indirectly corroborate our hypothesis.

The accumulation of miR-100 altered in different fractionated radiation exposure patterns, namely longer interval and larger dose had better effect on inducing the expression, while the mechanism is still unknown.

It is recorded that DNA methylation may play an important role in silencing microRNA in tumors. A meta-analysis of 122 microRNAs showed that microRNAs regulated by methylation were mainly located in chromosome 1, 7, 11, 14 and 19[19]. As miR-100 locates in chromosome 11, we inferred that methylation

may contribute to the regulation of it. Thereby we detected the effects of 5-Aza-CdR on HeLa and SiHa. With the treatment, the expressions of miR-100 were significantly induced. Our evidence linked methylation to irradiation on the increase of miR-100 in HeLa and SiHa, suggesting the level of methylation may have changed in the process of radiotherapy.

In breast cancer, miR-100 has been proved to be regulated by the methylation of MiR100HG, a host gene, where the miR-100 gene embedded[20]. Furthermore, we elevated the methylation of MiR100HG in SiHa, SR6, HeLa and HR6 to validate our hypothesis. The Bisulfite sequencing PCR showed that the percentage of CpG methylation slightly decreased in HR6 and SR6, compared to Hela and SiHa. Accordingly, the level of miR-100 regulated by radiotherapy may not be directly mediated by the methylation of MIR100HG. As the loss of chromosome 11 also can lead to the low expression of miR-100[21], the regulation of miR-100 remains to further research.

In conclusion, miR-100 turns out to be a tumor suppressor in cervical cancer for its exceeding ability to invasion /proliferation inhibition and apoptosis promoting. We've found that its inhibition of proliferation was double consequences, on one hand, miR-100 induced G2/M phase arrest which weakened cell mitosis, on the other hand, miR-100 initiated cell apoptosis which promoted cell death.

It's widely shared that miRNAs could bind to 3'UTR leading mRNA cleavage or translation inhibition which negatively regulate downstream mRNA and transcription factors [22]. While as a single miRNA can regulate sorts of mRNAs and in return one individual mRNA could be controlled by several miRNAs. Nevertheless genes regulated by one miRNA often have similar functions. The identified targets of miR-100 include mTOR[7, 23] .They both act as cell cycle activative genes which enable and allow cells entry into mitosis[27–29].

MiR-100 influences cell cycle and apoptosis and it's wildly demonstrated in ovary cancer[7], mammary carcinoma[20] and hepatocellular cancers[29] but its relationship to invasion is rarely reported. Our research found out miR-100 down-regulated invasiveness in cervical cancer cell lines for the first time. In the meanwhile, miR-100 could also reduce EMT, which explained its invasion cut down function. EMT is the characteristics that epithelial cells gaining mesenchyma transition, in this transforming cancer cells express MMPs including MMP2, MMP9, which is a family of key factors melting basement membrane and enable cells accessing circulation and mediate metastatic.

MTOR is a protein kinase belonging to PI3K family [30]. As the core component of many different complexes, mTOR plays a key role in a variety of biological processes [30–31]. Up-regulating mTOR signaling can promote tumor growth and progression through a variety of mechanisms [32]. One study has revealed increased expression of mTOR in cervical cancer [33]. In all cases, SCCs showed high translocation of p-mTOR and p-p70S6K in nuclear [34]. Faried et al. found activated AKT and mTOR related with poorer prognosis. The same authors [35] also investigated the expression of phosphorylated mTOR was independentand was a significant prognostic marker in cervical adenocarcinoma. In addition, the expression of p-mTORcan be used as a marker to predict chemotherapy response and survival of CC [36]. Moreover, Kim et al. [37] have also reported that p-mTOR was associated with poor response to

radiotherapy. They [35] also independently studied the expression of p-mTOR as an important prognostic marker of cervical adenocarcinoma. In addition, the expression of phosphorylated mTOR can be used to predict the chemotherapy response and survival [36]. In addition, the cytoplasmic expression of p-mTOR was also related to the adverse reactions of radiotherapy [37].

CircCASC15 was also named hsa_circ_0075828. The circCASC15, generated from CASC15 gene, was 204 nucleotides in length. Divergent primers were designed and confirmed by Sanger sequencing. MRE (miRNA response element) has been proved existing in circRNA and acts as a competitive endogenous RNA (ceRNA) sponge miRNA or transcription regulator

In conclusion, circCASC15-miR-100-mTOR might influnce the EMT of cervical cancer. And this loop will be the target of therapy in the future.

Declarations

Ethics approval and consent to participate

Use of patient tissue samples and nude mice were approved by the ethics committee of Sun Yat-sen Memorial Hospital

Consent to publish

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Tingting Yaooriginally designed the project and wrote this article; Zhiliao Chenconceptualized the research; Yongpai Pengprepared the tables and figures; Guanglei ZhongFlorescent in situ hybridization. Chunxian Huangdid Transient transfection, Jing Li (F)did Tumor xenografts , Ruixin Li did 5-aza-2-deoxycytidine Treatment and BSP.All authors have read and approved the manuscript.

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Figures

Figure 1

MiRNA microarray of radioresistant cervical cancer.

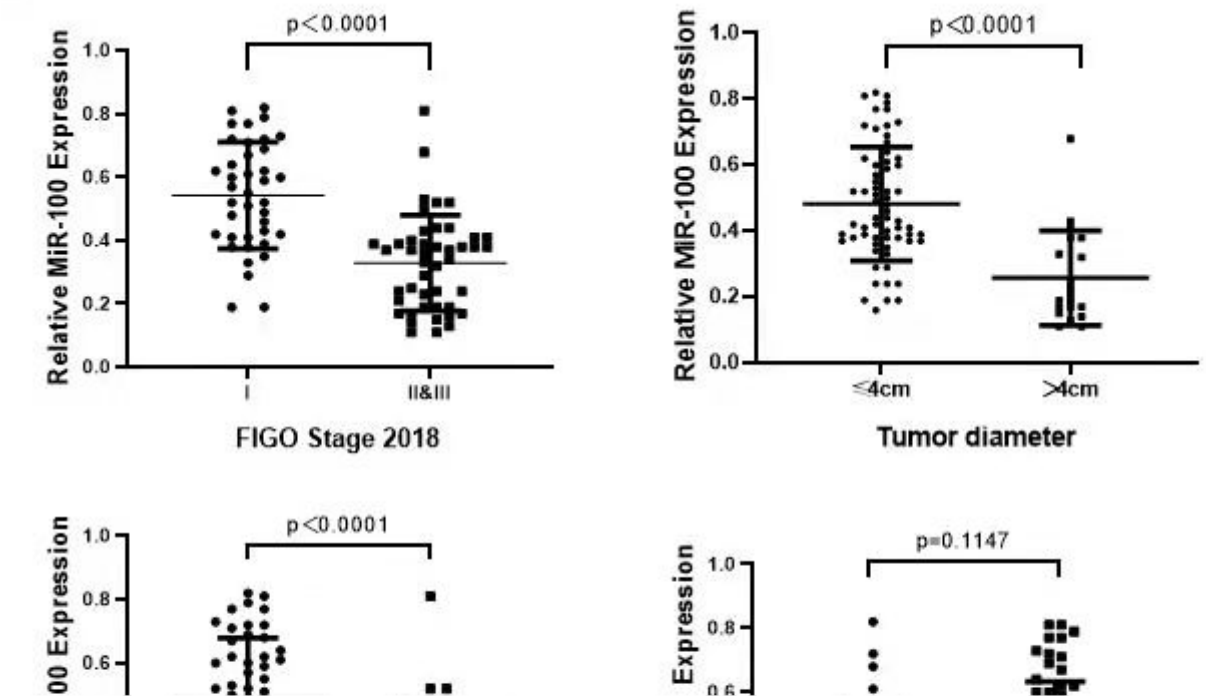

Figure 2

MiR-100 in cervical cancer samples was significantly lower than the mean level in normal tissues samples. Low level of miR-100 is correlated with poor clinical outcomes in cervical cancer patients tumor size,lymph node status , TNM stage disease-free survival.

Figure 3

(A) miR-100 mimics was used to up-regulate its expressions in Hela and Siha. Cell invasiveness was tested by transwell assay in 24-well-plate which illustrated that miR-100 could inhibit invasion at 60% in Hela and 50% in Siha cells(B).(C) Western Blot showed the expression of N-cadherine and E-cadherine.

Figure 4

Proliferation was measured by CCK8 kit after cells were transfected miR-100 mimics for 24, 48, 72 and 96h. No significant difference of proliferation of Hela and Siha were observed at the point of 24h after

transfection. While at the other time points, proliferations were inhibited in cells treated with mimics(A)Hela&(B)Siha.

Figure 5

miR-100 induced appoptosis (A) and decrease G2/M(B).

Figure 6

MiR-100 inhibits tumorigenesis of SCC xenografts(A&B).

Figure 7

MTOR is a direct target of miR-100. (A) Putative miR-100-binding sequence in the 3'UTR of mTOR mRNA. (B) Luciferase activity on the presence of both wild-type mTOR 3'UTR or mutant and miR-100 was compared with those of the controls . *P□0.05. (C) The expression of p-mTOR on cervical cancer was significantly higher than normal cervical.

Figure 8

Bisulfite sequencing PCR was used to elevate the methylation of MIR100HG in HeLa, HRR, SiHa and SRR(A).5-Aza-CdR provoked mir-100 in HeLa and SiHa(B).

Figure 9

(A)The cluster heat map demonstrates the differentially expressed circRNAs over 2 fold change. (B) A human CCL20 3'UTR fragment containing wild-type or mutated was cloned downstream of the luciferase reporter gene. (C)The expression of circCASC15 in CC was upregulated.Mir-100 was sponged by circCASC15. (D)Pull-down assay for biotin labeled miRNA was used to evaluate binding properties between miR-100-5p and circCASC15 in Hela and Siha. (E)CircCasc15 and mir-100 were found mainly expressed in cytoplasm.