# RESEARCH ARTICLE

# Expression of MiR200a, miR93, Metastasis-related Gene RECK and MMP2/MMP9 in Human Cervical Carcinoma - Relationship with Prognosis

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## Abstract

<u>Aim and Background</u>: Cervical cancer remains the third most common cancer in women globally after breast and colorectal cancer. Well-characterized biomarkers are necessary for early diagnosis and to predict metastatic progression and effective therapy. MiRNAs can regulate gene expression, cell growth, differentiation and apoptosis by targeting mRNAs for translational repression or degradation in tumor cells. The present study was conducted to assess expression of miR93, miR200a, RECK, MMP2, MMP9 in invasive cervical carcinoma, and analyze their clinical significance. <u>Method</u>: A total of 116 patients with invasive cervical carcinoma and 100 patients undergoing hysterectomy for benign lesions were retrospectively examined. Quantitative real-time PCR was performed to determine expression of miR93 and miR200a while RECK, MMP2, MMP9 and MVD were assessed by immunohistochemical staining. <u>Results</u>: Cervical carcinoma patients demonstrated up-regulation of miR-93, miR-200a, MMP2 and MMP9, with down-regulation of RECK as compared to benign lesion tissues. RECK was significantly inversely related to invasion and lymphatic metastasis. The 5-year survival rate for patients with strong RECK expression was significantly higher than that with weakly expressing tumors. <u>Conclusion</u>: MiR-93 and miR-200a are associated with metastasis and invasion of cervical carcinoma. Thus together with RECK they are potential prognostic markers for cervical carcinoma. RECK cooperating with MMP2, MMP9 expression is a significant prognostic factor correlated with long-term survival for patients with invasive cervical carcinoma.

Keywords: miR-93 - miR-200a - RECK - MMP2 - MMP9 - MVD

## Introduction

In the worldwide, cervical cancer remains the third most common cancer in women globally after breast and colorectal cancer. However, 86% of all deaths caused by cervical cancer occur in developing countries (Arbyn et al., 2011). Data from the IARC GLOBOCAN 2008 database (http://globocan.iarc.fr/fact sheets/cancers/cervix.asp) estimate that there are 529, 512 new cases of cervical cancer diagnosed per year globally, corresponding to an age standardized incidence rate (ASIR) of 15.2/100,000 and 274,967 deaths. There is a striking difference in incidence of and mortality from cervical cancer in different regions of the world (Denny, 2012) So well-characteristic biomarkers are necessary for early diagnosis, to predict metastatic progression.

Metastatic disease, rather than the primary tumor itself, is responsible for the death in most solid tumors, including cervical carcinoma (Lee et al., 1997; Welch et al., 2000; Yang et al., 2004). Degradation of basal membranes and the extracellular matrix (ECM) is essential for angiogenesis, invasion metastasis, and matrix metalloproteinases (MMPs) are potent enzymes that play a key role in these processes (Sabrina et al., 2012).Matrix Metalloproteinase 2 (MMP-2) (gelatinase A, 72 kDa) and Matrix Metalloproteinase 9 (MMP-9) (gelatinase B, 92 kDa) cleave type IV collagen and gelatin, which are the main structural components of the basal membrane (Toi et al., 1998). Expression of MMP-9 and MMP-2 has been implicated in the development and progression of many tumors, such as prostate, colorectal, breast cancer and cervical cancer (Liabakk et al., 1996; Kodate et al., 1997; Eissa et al., 2007; Rita et al., 2009).

Several miRNAs are reported be associated with cervical carcinoma, Up-regulation of miR-200a and miR-93 promotes metastasis and tumor invasion. According to computational methodology current predictions-MicroCosm MMP2 is target gene to miR93, and TIMP1 is target gene to miR200a, while TIMP3 is target gene to miR93.

MicroRNAs (miRNAs) is a novel class of small non-coding RNA molecules, 20-25 nucleotides in length, were shown to have important posttranscriptional gene regulatory functions. While miRNAs seed region

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which comprised of 2-8 nucleotides at 5' end, target to special mRNA at 3' untranslated region (UTR) (Bartel et al., 2009; Kim et al., 2009). If the complementarity of the miRNA-mRNA complex is perfect, miRNAs can exert translational repression function. However, if the complementarity is not perfect, the translation of the target mRNA is suppressed. To date, more than 1900 human mature miRNAs have been identified (http://www.miRbase. org/index. shtml), which are supposed to regulate more than 10% of protein coding genes (Wu et al., 2008), approximately one-third of expressed human genes contain miRNA regulatory target sites. Thus, this suggests that different clusters of miRNAs can regulate the cassette of specific genes which involve in one specific kind of cellular function together (Yang et al., 2003).

It has been reported that RECK over-expression decreases the amount of active MMP-2 and MMP-9 and inhibits metastatic activity in vitro (Oh et al., 2001) and in vivo (Chang et al., 2008). RECK is a membrane-anchored glycoprotein of approximately 110 kDa containing multiple epidermal growth factor-like repeats and serine protease inhibitor-like domains. Down-regulation of RECK in several tumor cell lines and oncogene-transformed fibroblasts identified RECK as a common negative target for oncogenic signals. RECK low-expression, a hallmark of cancer, has been demonstrated to create a hypoxic tumor microenvironment.

The aim of our study was to test the expression of miR200a and miR93 in cervical carcinoma, we propose the induction of the correlation between the expression of miR200a, miR93 and MMP2/9,RECK genes and to investigate whether miR200a, miR93 and MMP-2, MMP2/9 and RECK are expressed in a related pattern respectively in cervical carcinoma. Furthermore, we evaluate important prognostic parameters, analyzed the expression of RECK with 5-year survival rate, to conclude whether RECK is an independent factor to evaluate prognosis.

## Materials and Methods

### Tissue specimens

Cervical carcinoma specimens were obtained from patients undergone primary hysterectomy at the Department of Gynecology from Jan 2005 to Sep 2007, while control group were obtained from patients undergone hysterectomy for benign lesion. The specimens were frozen in liquid nitrogen at -80 °C within 30 minutes after isolated, and all cases were obtained from archives of the Department of Pathology in the Second Affiliated Hospital of Jilin University. The H&E stained slides of the cases were reviewed by gynecological pathologist. Morphology and protein expression were evaluated in consecutive sections. All protocols were reviewed and approved by the Ethical Committee of Second Affiliated Hospital of Jilin University. Written consent was obtained from all participating patients.

### Follow-up

Patients were followed regularly for 5 years at the Second Affiliated Hospital of Jilin University. All patients were followed until death or the study closing date (September 30, 2012). Disease-free survival (DFS) rate, which measured the first recurrence at any site, and overall survival (OS), measuring death from any case, were the two assessments used for prognostic analyses. Patients were re-examined (history, ultrasound examination, cervical screening test) once every 3 months during the first year, once every 6 months from the second year to the third year, and once every year after that. During the follow-up period, 6 patients were loss of follow up. 26 patients had disease recurrence and 36 patients died.

### miRNA isolation

MiRNA was extracted from the tissue using the mirVana miRNA Isolation Kit (AM1561, Ambion) for hysterectomy specimen according to the protocols. The quantity and quality of the miRNA was verified with the NanoDrop spectrophotometer (Thermo Fisher Scientific Incorporated, Wilmington DE, USA) according to the manufacturer's instructions.

### Quantitative real-time PCR (QPCR)

miRNAs was reverse transcribed in a 20 μl reaction using the one step primescript miRNA cDNA Synthesis Kit (Takara, D350A). Forward primer sequences miR93: CAAAGTGCTGTTCGTGCAGGTAG, miR200a: GTAA CACTGTCTGGTAACGATGQPCR was performed on a BioMad Real-Time PCR System (ABI) using Power-SYBR Green PCR Master Mix (Takara, DRR081) in a 20 μl reaction and U6 as an endogenous control, miRlet-7 as positive control, result was determined using the $2^{-\Delta\Delta CT}$. The QPCR experiments were run triply within each experiment run, relative expression values were normalized to standard deviations from the mean.

### Immunohistochemical stain

To determine the expression of RECK, MMP2, MMP9 and MVD, immunohistochemical staining was carried out using the two-step plus poly-HRP method as described previously. After blocking with 3% hydrogen peroxide, the slides were incubated with primary anti-RECK antibody, anti-MMP2 antibody, anti-MMP9 antibody and CD34 antibody (1:50 goat mAb respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, the slides were stained with the two-step plus poly-HRP antigoat IgG detection system (ZSGB-Bio, Beijing, China). For negative controls, the primary antibody was substituted with PBS in order to confirm the specificity of the primary antibody.

### Evaluation of immunohistochemical staining

Two experienced investigators, who provided a consensus opinion of stain patterns by light microscopy, evaluated sections. RECK, MMP2 and MMP9 expression was estimated from the staining intensity and graded as follows: Grade 0, no staining (-); Grade 1, faint staining (+); Grade 2, moderate staining (++); and Grade 3, strong staining (+++). The positively stained area (distribution) was expressed as the percentage of the whole area under evaluation and scored as follows: 0, no staining; 1, 1~25% positive cells; 2, 26~50% positive cells; 3, 51~75%

**Table 1. Expression of RECK, MMP2, MMP9 in Cervical Carcinoma and Control Tissue**

| | N | RECK | | | | MMP-2 | | | | MP-9 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | — | + | ++ | +++ | — | + | ++ | +++ | — | + | ++ | +++ |
| Cervical carcinoma | 116 | 68 | 38 | 7 | 3 | 49 | 31 | 22 | 14 | 54 | 9 | 22 | 31 |
| Control group | 100 | 14 | 18 | 44 | 24 | 75 | 17 | 8 | 0 | 83 | 8 | 7 | 2 |
| $X^2$ | | 13.5495 | | | | 5.4765 | | | | 7.4875 | | | |
| $P$ | | 0.0002 | | | | 0.0193 | | | | 0.0062 | | | |

**Table 2. Correlation Between RECK Expression and Various Clinicopathological Features in Cervical Cancer Patients**

| | N | RECK | | positive | $\chi^2$ | $P$ |
|---|---|---|---|---|---|---|
| | | negative | positive | (%) | | |
| Stage | | | | | | |
| I | 64 | 33 | 31 | 48.44 | 6.198 | 0.1024 |
| II | 33 | 21 | 12 | 36.36 | | |
| III | 17 | 12 | 5 | 29.41 | | |
| IV | 2 | 2 | 0 | 0 | | |
| Grade | | | | | | |
| I | 23 | 13 | 10 | 43.48 | 3.6204 | 0.1636 |
| II | 63 | 42 | 21 | 33.33 | | |
| III | 30 | 15 | 15 | 50 | | |
| Invasive depth | | | | | | |
| T2 | 66 | 43 | 23 | 34.85 | | 0.0184 |
| T3-T4 | 50 | 36 | 24 | 48 | | |
| Lymph node status | | | | | | |
| N1-N3 | 15 | 14 | 1 | 6.67 | | 0.0237 |
| N0 | 101 | 58 | 53 | 52.48 | | |
| Squamous carcinoma | 99 | 60 | 39 | 39.4 | | 1 |
| adenocarcinoma | 17 | 11 | 6 | 35.29 | | |



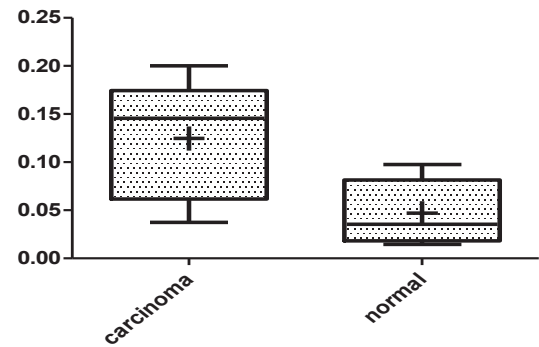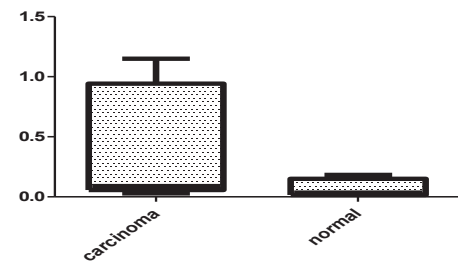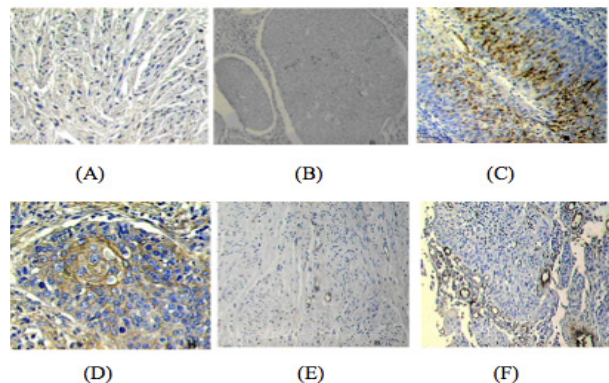**Figure 1. Expression of miR93 (A) and miR200a (B) in Cervical Carcinoma in Relation to Normal Tissue**

positive cells; and 4, 76~100% positive cells. Overall expression was then graded as low expression (score 0~2), intermediate expression (score 3~5), and high expression (score 6~7).

*Statistical analysis*

Analysis were carried out by BioMad CFX system and statistical software SPSS 14.0 (SPSS, Chicago, IL, USA). The correlation of RECK, MMP2/9 expression with patients' clinicopathological factors was analyzed by the Fisher's exact test. The Kaplan-Meier method was used to estimate OS. Survival differences according to RECK expression were analyzed by the log-rank test. The risk ratio and its 95% confidence interval were recorded for each marker. $P$-values $< 0.05$ were considered statistically significant in all of the analysis.

## Results

Mean age of the total 116 patients was $49.3\pm2.39$ years (24~77 years). 36 patients (31.03%) died, and 74 patients (63.79%) were alive at the end of research. Results of qPCR showed that miR93 and miR200a expression was higher in cervical carcinoma tissues (Figure 1A, Figure 1B). RECK was detected in the cytoplasm of normal cells (Figure 2A, Figure 2B), and of cervical carcinoma specimen its expression was much lower than that in control group, MMP9, MMP2 was detected in the cytoplasm of cells (Figure 2C, Figure 2D). MMP2 and MMP9 expression was significantly higher in cervical carcinoma than that in control group (Table 1). According



**Figure 2. Expression of RECK, MMP2, MMP9.** (A) expression of RECK in control group; (B) expression of RECK in cervical carcinoma; (C) expression of MMP2 in cervical carcinoma; (D) expression of MMP9 in cervical carcinoma; (E) MVD expression in control group; (F) expression of MVD in cervical carcinoma

to Cox regression analysis result, the expression of MMP2 was positively related to expression of miR93 ($P=0.0027$) and miR200a ($P=0.0016$). However, higher expression of RECK related to lower expression of MMP2, MMP9.

We also examined positive RECK staining in different clinopathological factors such as stage, grade, invasion depth and lymph node metastasis (Table 2). These data indicated that the frequency of RECK expression in high-grade was much higher than in low-grade cervical carcinoma. RECK expression, however, was significantly associated with lymph node metastasis ($P=0.0237$)

**Table 3. Expression of MVD in Cervical Carcinoma and Control Tissue (n)**

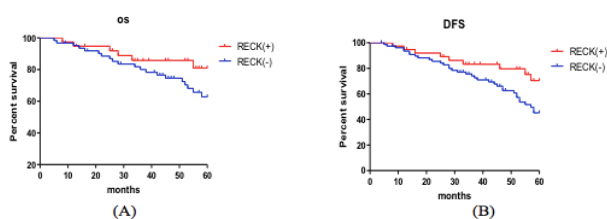| P | n | MVD (n/*400) | P | RECK (positive) [n(%)] | r |
|---|---|---|---|---|---|
| Cervical carcinoma | 116 | 19.4615±3.0718 | 0.000 | 48(40.31) | -0.397 |
| Control group | 100 | 12.0000±2.6629 | 0.0495 | 86(86.0) | |



(A)    (B)

**Figure 3. Kaplan–eier Analysis for Disease-free Survival (DFS) and Overall Survival (OS) Based on RECK Expression in Cervical Carcinoma Patients.** (A) Kaplan–Meier analysis for OS based on RECK expression in patients with cervical carcinoma ($P =0.0399$, log–rank test); (B) Kaplan–Meier analysis for DFS based on RECK expression in patients with cervical carcinoma ($P = 0.0387$, log–rank test). RECK(+): RECK-positive patients (n =48 ); RECK(-): RECK-negative patients (n = 68)

and invasive depth ($P$=0.0184). Lymph node negative patients had higher RECK expression (53/101, 52.48%) than lymph node positive patients (1/15, 6.67%). Deeper invasive patients had lower RECK expression (24/50, 48%) than lower invasive patients (23/66, 34.85%). We found that histopathological grade, pathological TNM stage have no significance as prognostic predicators (Table 2). Multivariate analysis was carried out on the same set of patients for RECK expression and pathological predictors using the Cox regression model. The results indicated that RECK status (risk ratio, 3.312; $P$<0.05) was independent prognostic factor.

Microscopic observation of MVD staining showed that in cervical carcinoma group, micro-vascular arranged disorderly, size and shape were irregular, thickness of vascular wall was nonuniform (Figure 2F), while in control group, clearer expression of micro-vascular endothelial cells were round or oval and in regular shape (Figure 2E). According to the Cox regression analysis, RECK expression in cervical carcinoma was negatively associated with MVD value (r=-0397, p=0.0495) (Table 3).

To determine the relation between RECK expression and prognosis, Patients were divided into two groups on the basis of their prognosis. Our results indicated that patients with a poor prognosis(recurrence or metastasis) had low levels of RECK expression ($P$<0.05). Kaplan-Meier survival analysis showed that RECK positive patients also had significantly higher OS rates ($P$<0.05, log-rank test; Figure 3A).RECK positive patients had higher DFS rates compared with RECK negative patients ($P$=0.0387, log-rank test; Figure 3B).

## Discussion

Accumulating reports demonstrated that miRNAs have been observed in a variety of human cancers, and miRNA signatures accurately reflect the developmental lineages

and differential expression states of tumors by microarray profiling studies (Lu et al., 2005; Rosenfeld et al., 2008) Furthermore, they certified miRNAs involved in tumor cells invasion, apoptosis, angiogenesis and metastasis through regulation to target genes of corresponding signal pathways (MA et al., 2012). In previous research on human cervical cancer, expression of miR-15a, miR-20b, miR-21 and miR-224 is obviously increased in tissue and let-7c, miR-143, miR-199a-5p, miR-203 and miR-145 is reduced (Pereira et al., 2010; Wang et al., 2008). In our study, miRNAs expressions were quantified by using quantitative real-time PCR in which miR-93, miR-200a were up-regulated in cervical carcinoma tissue. Their expressions were accompanied by over-expression in MMP-2, MMP-9 and suppression in RECK gene.

The miR-93 gene is located on chromosome 7q22.1, it can suppress proliferation and differentiation of cancer stem cells, while promoting tumor growth and malignant cells survival (Fang et al., 2011; Yu et al., 2011; Suling et al., 2012). In the present study, mir-93 expression was 5.29 fold higher compared to normal tissue. Our data are consistent with other reports indicating that mir-93 expression increased with cervical carcinoma (Lui et al., 2007). By microcosm predictor system, MMP2 has the target gene in 3' UTR to miR-93, also TIMP3 is the proposal target gene of miR-93 ,whose sequence GAUGGACGUGCUUGUCGUGAAA was relatively complemented with CTTTCTATGTGCAAGGCACTTT in TIMP3 (http://www.ebi.ac.uk/enright-srv /microcosm/ htdocs/targets/v5). Endogenous angiogenesis inhibitors TIMPs are necessary to block the mitogenic stimuli in the vascular endothelium (Curran et al., 2000). TIMP3 is inhibitor of MMP2 and associated with actin and serve to stabilize microfilaments, so it act as tumor suppressor gene (Perry, 2001). miR93 was indentified up-regulated expression of MMP2 in cervical carcinoma, for the up-regulated mir-93, the inhibiting function of tumor suppressor genes TIMP3 maybe suppressed. But this hypothesis has not been certified. The miR-200a gene is located on chromosome 1P36.33, and can enhance invasion and growth of malignant cells. In this study, miR-200a was over-expressed by 3.65 folds in cervical carcinoma compared to normal tissues respectively. Similar studies indicated the miR-200a was up regulated which may supporting the concept that miR-200a functions as oncogene (Cong et al., 2013; Rasheed et al., 2013; Yu et al., 2013). In our study, the over-expression of miR-200a was associated with the over-expression of MMP2, MMP9. According to Microcosm target gene predictor system, TIMP1 is the corresponding target gene of miR-200a. miR-200a has been supposed to involve in down-regulating of TIMP1, whose inhibitor function to MMP2 and MMP9 is weakened and led to over-expression of MMP2 and MMP9. Similar research instructed that miR-200b is overexpressed in endometrial adenocarcinomas and enhances MMP2 activity by down regulating TIMP2 in human endometrial cancer cell Line HEC-1A cells (Dai et al., 2013). But the accurate mechanism in cervical carcinoma still need to certified in vitro until now.

In this research, RECK expression is suppressed

in cervical carcinoma tissue when compared to benign lesion tissue. There are almost certainly pathways by which RECK is down-regulated in cancer. Hypoxia induces RECK down-regulation through the recruitment of HDAC1 and HIF-1α to the rHRE2 site in the promoter and the inhibition of hypoxic RECK silencing would be a therapeutic and preventive target for early tumorigenesis (Zhang et al., 2012). However, the CpG island promoter hypermethylation is associated silencing of tumor suppressor genes, which is the most recognized epigenetic disruption in human tumors (Rodriguez et al., 2011).

Low RECK expression is closely correlated with high MMP2, MMP9 expression. In addition, increased expression of MMP2, MMP9 with decreased expression of RECK in invasive cervical carcinoma irrespective of histological grading supports the fact that RECK has a negative effect on the invasiveness of cervical cancer. Mori had found (Mori T et al., 2007) that MMP-2 activity, but not its mRNA expression, was significantly down-regulated in HT1080 cells after they were transferred into the RECK plasmid (Bin Zhang et al., 2009). Similarly, our results showed a negative correlation between RECK and MMP-2 protein expression. In HUVECs, specific inhibition of MMP-2 significantly antagonized the effect of RECK depletion on β1-integrin signaling, cell proliferation, and tube elongation (Namwat et al., 2011). Moreover, RECK-mediated suppression of MMP-9 promoter activity requires 12-O- tetradecanoylphorbol-13-acetate-responsive element (TRE) and KB sites. Moreover, the binding ability of Fra-1 and c-Jun to TRE within the MMP-9 promoter region was suppressed by RECK (Satoshi et al., 2007). In this research, MVD CD34 for tumors with lower-expression RECK is obviously increased, which indicates that RECK can inhibit angiogenesis. Targeting RECK specifically in tumor-associated vascular endothelial cells resulted in tumor regression (Takao et al., 2010).

RECK positive patients showed higher 5-year survival rates and DFS rates. Furthermore, we found that RECK expression was significantly associated with lymph node metastasis and deeper invasion. HER-2/neu oncogene inhibits the expression of RECK to promote cell invasion (Tsung-Te et al., 2012). Hypermethylation of RECK promoter is also a common event in human ESCC, which occurs concurrently in tumor-adjacent normal mucosa and is correlated with poor prognosis in ESCC patients (Long NK et al., 2008). RECK displays as a metastasis suppressor and up-regulation of RECK expression could provide a potential therapy to improve the prognosis (Namwat et al., 2011).

In conclusion, MiR-93, miR-200a is associated with metastasis and invasion of cervical carcinoma, thus MiR-93, miR-200a, RECK expression is a potentially prognostic marker for cervical carcinoma. RECK cooperating with MMP2, MMP9 expression is a significant prognostic factor correlated with long-term survival for patients with invasive cervical carcinoma.

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