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A correlational study on MiR-34s and cervical lesions

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Summary

Objective: The aims of this study were to explore the correlation between MiR-34s and cervical lesions. *Materials and Methods:* A case-control study was conducted. From 2014 to 2015, 120 cases were included and divided into four groups depending on cervical biopsy, including group 0 (cervicitis or normal cervix), group 1 (CINI), group 2 (CINII-III), and group C (cervical cancer); each group included 30 cases. Then the relationship between miRNA-34 family (including miRNA-34a, miRNA-34b, miRNA-34c) and clinical pathological features was analysed based on detection of miRNA-34 by real-time polymerase chain reaction (RT-PCR). *Results:* The expression level of miRNA-34a in group 0 was 0.19 ± 0.1073, in group 1 it was 0.1507 ±0. 2124, in group 2 it was 0.0766 ± 0.0948 (*vs.* group 0: $p < 0.05$), and in group C it was 0.0501 ± 0.0271 (*vs.* Group 0: $p < 0.05$). The correlation analysis showed that the expression of miRNA-34a decreased with the aggravating of the cervical lesion ($r = -0.4782$, $p < 0.05$), while there was no difference of miRNA-34b ($F = 0.5835$, $p = 0.6282$) or miRNA-34c ($F = 0.1167$, $p = 0.9500$) in each group. *Conclusion:* The decrease of miRNA-34a is related with cervical malignant lesion, which may be involved in the pathogenesis by regulating target genes, that may be a new molecular marker for diagnosis and prognosis of cervical cancer.

Key words: miRNA-34s; Cervical lesions; Cervical cancer.

Introduction

Cervical cancer is the fourth most common cancer worldwide in women [1]. In 2012, there were 528,000 cases of cervical cancer worldwide and 266,000 people died of it [2]; 85% of cervical cancers occurred in developing countries [3]. Moreover, cervical cancer is the main cause of cancer death in those developing countries [4]. The occurrence and development of cervical cancer is a continuous pathological process from cervical precancerous lesions to cervical cancer, including low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia [5]. When diagnosed in precancerous or situ carcinoma stage, the effect of treatment can be improved and prognosis can be predicted more accurately [6]. With the depth of research, there is an increasing number of medical studies that indicate the existence of important epigenetic abnormalities in different stage of cervical cancer. MicroRNA (miRNA) plays a key role in the occurrence, development, diagnosis, treatment, and prognosis of cervical cancer [7] The miR-34 family (miR-34s) is a highly conserved family of miRNAs that are found in arthropods, nematodes, and mammals, as well as in almost all vertebrates [8]. Currently, 80% of the miR-34 family genes were found in the intergenic region of the gene, with a few found in the intron region and 3'-UTR of the protein-coding gene [9]. The miR-34 family consists of miR-34a, miR-34b, and miR-34c [10]. While the functions of them are similar, the expression patterns are different in different tissues. The study shows that gene encoding miR-34a is located on human chromosome 1p36, while miR-34b and miR-34c are co-transcribed from one transcription unit [11]. In recent years, a number of studies showed that the abnormal expression of miR-34 family can be found in many diseases. It was found that the expression of miR-34 family was significantly reduced in nasopharyngeal carcinoma, gastric cancer, pancreatic cancer, prostate cancer, and in eye and leukemia cells associated with the deletion of the p53 genes [12]. In addition, miR-34a was found to be significantly reduced in leukemia-associated thrombocytosis, polycythemia vera, and primary myelofibrosis cells [13]. Therefore, patients with cervical cancer were selected as the research object. Molecular biology, genetics, immunology, and other technical methods were used to explore the possible new way of diagnosis and treatment.

Materials and Methods

According to the cervical biopsy results, patients who were diagnosed in the present hospital from 2010 to 2015 were divided into four groups, including a cervicitis or normal group of 30 cases (they were selected among the patients who underwent hysterectomy due to uterine myoma or adenomyosis), a CINI group of 30 cases, a CINII-III group of 30 cases, and a cervical cancer group of 30 cases. The patients are in the age range of 28-75 years with the average age of 45.77 (45.77±10.73) years. Exclusion criteria: (1) Subjects with a previous history of cervical cancer or unidentified tumour on cervix, (2) subjects who were pregnant or with termination within three months, (3) subjects who had used hormones within three months, (4) subjects with double cervix or

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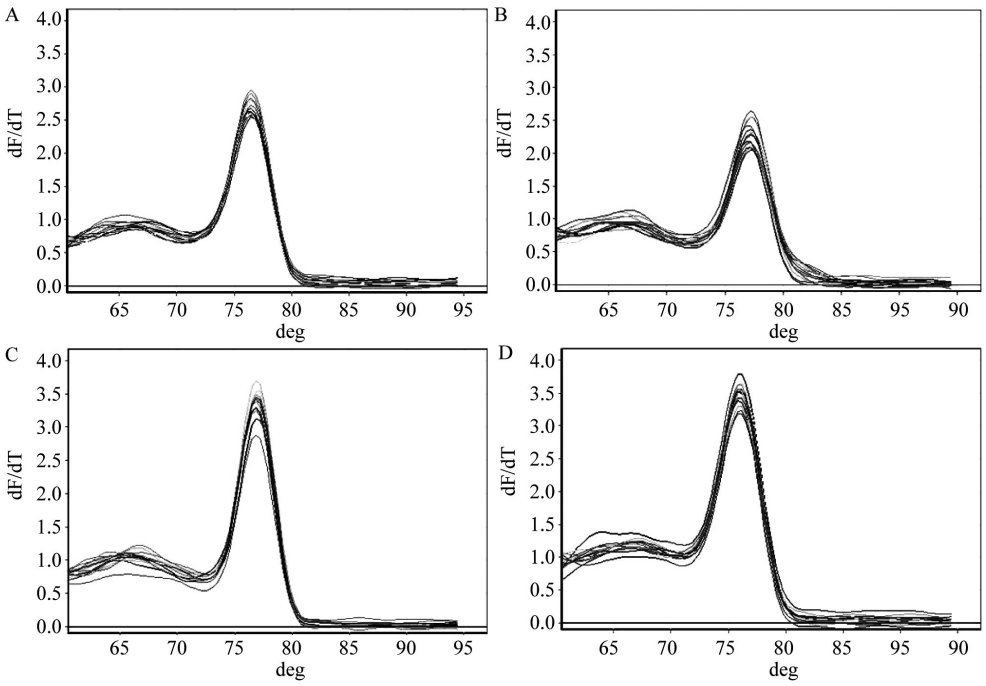

Figure 1. — A) 34a dissolution curve. B) 34b dissolution curve. C) 34c dissolution curve. D) U6 dissolution curve.

Mayer-Rokitansky-Küster-Hauser syndrome (MRKH), (5) subjects with a previous history of a local excision of the cervix or radiotherapy or chemotherapy, and (6) subjects that had a hysterectomy. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region. Written informed consent was also obtained from all participants.

All fresh tissues were stored in liquid nitrogen or kept at -80°C for long-term preservation. All specimens are stored properly and all clinical data were archived. Total RNA extraction: the samples were frozen in liquid nitrogen, then were grounded and transferred to Trizol for grinding and digestion. The standard procedures for RNA extraction and purification were performed according to the following: RNA concentration and purity were measured by using an ultra-violet spectrophotometer, and OD260 values were recorded. In the reverse transcription (RT) with reference made to the miScript II RT kit handbook, a total of 20 µl of reaction system was prepared, including 4 µl of HIFlex buffer, 2 µl of nucleics mix, 2 µl of RT enzyme mix, and 12 µl of RNA (concentration: about 20 ng/µl) + RNase-free water. The samples were then incubated for 60 minutes at 37°C and for five minutes at 95°C.

MiR-34a-specific primers, MiR-34b-specific primers, MiR-34c-specific primers, and U6-specific primers (MS00033740) were all designed and synthesized. The reaction was performed using a miScript SYBR Green PCR kit. A total of 25 µl of reaction system was prepared, including 10 µl of SYBR Green PCR master mix, 2 µl of miScript primer assay, 2 µl of miScript universal primer, 10 µl of RNase-free water, and 1 µl of template cDNA. The samples were then incubated for one cycle at 95°C for 15 minutes and then 35 cycles of 94°C for 15 seconds, 55°C for 45 seconds, and 70°C for 30 seconds. Three replications were made for each sample. MiR-34a, MiR-34c and U6 were detected under the same reaction conditions. Data were analysed by Rotor-Gene Q Series Software after the program was run. At last, the amplification curve, melting curve, and Ct value of each sample was obtained.

In the statistical analysis, the relative quantification of the gene was represented by the value of ΔCt. ΔCt equals the Ct value of the target gene minus the Ct value of the target group and ΔΔCt equals to the ΔCt value of each sample minus the minimum value of ΔCt. The difference of the gene expression is expressed as $2^{-\Delta\Delta Ct}$ value. All data were analysed using SAS JMP9.0. The mean rates were compared using the q-test. In the correlation analysis, statistical significance was set at $p < 0.05$.

Results

Compared with the U6 curve, miRNA-34a, miRNA-34c, and miRNA-34c in cervical lesion and normal skin tissue showed a typical S-type smooth curve, which indicated that the amplification efficiency was good (Figure 1). The curve showed no miscellaneous peak and non-specific amplification, indicating good amplification specificity.

The expression level of miRNA-34a in cervical cancer was 0.0501 ± 0.0271, 0.19 ± 0.1073 in cervicitis or normal cervix group, 0.1507 ± 0.2124 in CINI group and 0.0766 ± 0.0948 in CINII-III group. The expression level in cervicitis or normal cervix groups was 3.79 times in cervical cancer group. Therefore, the comparisons were considered statistically significant at q = 4.93 and $p < 0.05$.

The correlation analysis showed that the expression of miRNA-34a was decreased with the aggravating of cervical lesion. The difference is considered statistically significant at $r = -0.4782$ and $p < 0.05$. There was no significant difference in the expression of miRNA-34b and miRNA-34c in each group (F = 0.5835, $p = 0.6282$; F = 0.1167, $p = 0.9500$).

Discussion

Cervical cancer is the most common female reproductive tumour and it also the fourth most common cancer worldwide. In China, the prevalence rate of cervical cancer is about 138/10 million, and the mortality rate of it is 2-20/10 million [14]. The infection of cervical cancer is a multi-factor and multi-step process, which also involves gene inactivation and activation [15]. HPV (high-risk type) is the primary factor in the occurrence and progression of cervical cancer, but not a sensitive criterion for detection [16]. Therefore, using HPV detection as a direct predictor of cervical cancer, as many scholars have proposed, is insufficient and a new diagnostic method should be used [17]. Moreover, because of the low accuracy and sensitivity of current detection, the majority of patients who were diagnosed in the present hospital were in a moderate or advanced stage.

miRNA is a kind of non-coding and small-molecule single-stranded RNA which is involved in many biological processes, including growth and development, metabolism, organogenesis, cell differentiation, proliferation, and apoptosis [18]. With continuing research, there is an increasing number of miRNA that have been found. According to the miRBase 20.0, there are 2,578 types of miRNA.

miR-34, which was first identified in nematodes in 2001, is widely distributed in arthropods, nematodes, and mammals [19]. The miR-34 family is very conserved in evolution, with a homologous conserved sequence from lower organisms to humans [20]. The miR-34 family plays an essential role in the inhibition of many tumours (neuroblastoma, hepatocellular carcinoma, digestive tract cancer, breast cancer, lung cancer, rectal cancer, etc.) [21]. The overexpression of miR-34 apoptosis can cause cell cycle arrest, cell senescence, inhibition of tumour cell regeneration, and migration [22]. With regards to the relationship between Notch and miR-34 in glioblastoma and medulloblastoma, the results showed that miR-34a can downregulate the expression of Notch-1 and Notch-2 at the same time [23]. Moreover, miR-34a can regulate the expression of Jagged-1 and Notch-1 in cervical cancer, reduce the invasive ability of tumour cells, and promote apoptosis [24]. In addition, miR-34 can also participate in the apoptosis of tumour cells through other pathways. Some studies show that miR-34c can significantly inhibit type II endometrial cancer cell proliferation and promote apoptosis. The apoptosis can further inhibit cell proliferation and cell cloning. Thus, miR-34c is considered a potential tumour suppressor gene [25].

The present study showed that the expression level of miRNA-34a in cervicitis or normal cervix groups was 3.79 times that of cervical cancer group. Therefore, the comparisons are considered statistically significant. Furthermore, the expression level in cervicitis or normal cervix groups was 2.48 times in CINII-III group. Thus, the comparisons are considered statistically significant. In addition, the correlation analysis showed that the expression of miRNA-34a decreased with the aggravating of cervical lesion. It should be noted that miRNA-34a may be involved in the pathogenesis of cervical lesions through regulation of target genes. Previous studies showed that NOTCH1 participates in important physiological activities, such as cell proliferation. It was found that the 3'UTR segment of NOTCH1 gene was partly compatible with miRNA-34a sequence. Furthermore, NOTCH1 has a high degree of conservation and lower binding free energy in a variety of species. Therefore, the miR-34a may be involved in the regulation of multiple target genes. NOTCH1 is likely to be a target gene of miR-34a, which requires further validation. The miRNA-34b and miRNA-34c have no correlation with lesions, which also requires further study.

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