Identification of a serum three-microRNA signature for cervical cancer diagnosis

Min-Min Cao[1], Qing-Xie Liu[1], Xuan Zou[2], Xing-Chen Fan[1], Cheng Liu[3], Shi-Yu Zhang[1], Tong-Shan Wang[1], Chun-Yu Li[4], Cheng Zhang[5], Xiang-Nan Geng[6], Ping Liu[1], Wei Zhu[1]

[1]Department of Oncology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China;
[2]Department of Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China;
[3]Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China;
[4]Department of Intensive Care Unit, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China;
[5]Department of Science and Technology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China;
[6]Department of Clinical Engineer, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China.

To the Editor: Cervical cancer (CC) is one of the leading causes of cancer deaths in women around the world.[1] In clinical practice, screening methods such as human papillomavirus testing and cytology-based detection have the disadvantages of having a high false rate, being hysteretic or ineffective.[2] Novel non-invasive biomarkers that can help diagnose CC with higher specificity and sensitivity are in great demand. MicroRNA (miRNA) is a type of non-coding small RNA of 18 to 25 nucleotides in length. Circulating miRNAs show the great potential of being cancer biomarkers for the stable existence in peripheral serum or plasma.[3] In this study, we performed a four-stage experiment using a total of 108 CC and 108 normal control (NC) serum samples to explore the diagnostic potential of serum miRNAs in CC. The schematic diagram of the experimental procedure was shown in Supplementary Figure 1, http://links.lww.com/CM9/A429.

All the participants of the experiment were recruited from the First Affiliated Hospital of Nanjing Medical University from 2016 to 2017. The criteria of inclusion and exclusion and the clinical characteristics of the participants were listed in Supplementary Tables 1 and 2, http://links.lww.com/CM9/A430. The study was conducted under the guidelines of the Hospital Ethics Committee and approved by the Institutional Review Board of the First Affiliated Hospital of Nanjing Medical University (No. 2016-SRFA-148).

In the screening stage, 20 serum samples from CC patients and ten from NCs were randomly selected and grouped into two CC pools and one NC pool (per ten samples into one pool). Exiqon miRCURY-Ready-to-Use PCR-Human-panel-I + II-V1.M (Exiqon miRNA qPCR panel, Vedbaek, Denmark), a platform assessing 174 commonly expressed serum/plasma miRNAs, was used for the initial selection of differentially expressed serum miRNAs in CC. In this process, a total of 29 candidate miRNAs were identified [Supplementary Table 3, http://links.lww.com/CM9/A430]. These miRNAs met the following criteria: (i) the Ct value was <37 and 5 lower than negative control; (ii) the Ct value was altered with a fold change (FC) of >1.5 or <0.67 in both CC pool compared with the NC pool. In addition, four miRNAs (miR-196a-5p, miR-218, miR-21-5p, and miR-20a-5p) proposed by previous literature were also analyzed in this study.

These candidate miRNAs were further tested in the training (30 CC *vs.* 30 NC) and testing stages (60 CC *vs.* 60 NC) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In this process, the Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio, Guangzhou, China) and SYBR Premix Ex Taq II (TaKaRa, Kyoto, Japan) were applied. The relative expression levels of miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method[4] (cel-miR-39 as exogenous reference miRNA; miR-16-5p as endogenous reference for serum sample normalization; *RNU6B*[*U6*] as endogenous reference for tissue sample normalization; $\Delta Ct = Ct_{miRNA} - Ct_{normalizer}$). As shown in Figure 1 and Supplementary Table 4, http://links.lww.com/CM9/A430, miR-20a-5p and miR-122-5p showed a significant trend of up-regulation ($P < 0.01$; FC > 1.5) in the serum of CC patients compared with NCs, while miR-133a-3p were conversely down-regulated ($P < 0.01$; FC < 0.67). It was suggested that the three-serum miRNAs

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001327

Correspondence to: Wei Zhu, Department of Oncology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China
E-Mail: zhuwei@njmu.edu.cn

Chinese Medical Journal 2021;134(14)
Received: 06-05-2020 **Edited by:** Li-Shao Guo

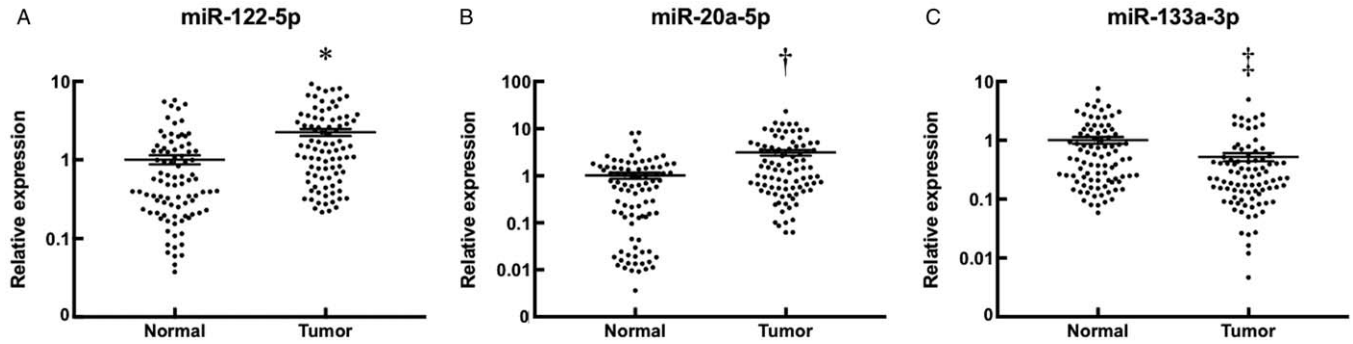

Figure 1: Expression levels of the three identified miRNAs in the combined training and testing cohorts (90 CC *vs.* 90 NC). Horizontal line: mean with 95% confidence interval (CI). (A) miR-122-5p: $^{*}P < 0.001$, $Z = -5.504$; (B) miR-20a-5p: $^{+}P < 0.001$, $Z = -4.746$; (C) miR-133a-3p: $^{‡/†}P < 0.006$, $Z = -3.808$. CC: Cervical cancer; NC: Normal control.

might serve as potential diagnostic biomarkers for CC patients.

Receiver-operating characteristic (ROC) curve analysis was used to evaluate the performance of the three miRNAs in identifying CC patients from NCs. When the data of training and testing stages were combined, the areas under the curve (AUCs) were 0.672 (95% confidence interval [CI]: 0.581–0.763, $P = 0.005$, sensitivity = 67.5%, specificity = 65.4%) for miR-122-5p, 0.681 (95% CI: 0.615 to 0.747, $P < 0.001$, sensitivity = 62.0%, specificity = 79.5%) for miR-20a-5p, and 0.666 (95% CI: 0.620 to 0.712, $P < 0.001$, sensitivity = 68.9%, specificity = 69.3%) for miR-133a-3p [Supplementary Figure 2, http://links.lww.com/CM9/A429]. To improve the diagnostic performance, the three miRNAs were combined into a panel using logistic regression analysis. According to the constructed model, the probability of CC could be calculated using the equation: Logit (P) = 0.541 + 0.396 × miR-20a-5p + 0.368 × miR-122-5p − 0.843 × miR-133a-3p. The AUCs of the panel to identify CC patients were 0.816 (95% CI: 0.717–0.915, $P < 0.001$, sensitivity = 70.5%, specificity = 85.6%; Supplementary Figure 3A, http://links.lww.com/CM9/A429) for the combined training and testing stages, 0.833 (95% CI: 0.730–0.936, $P < 0.001$, sensitivity = 72.2%, specificity = 88.7%; Supplementary Figure 3B, http://links.lww.com/CM9/A429) for the training stage, and 0.813 (95% CI: 0.718–0.908, $P = 0.001$, sensitivity = 76.9%, specificity = 79.5%; Supplementary Figure 3C, http://links.lww.com/CM9/A429) for the testing stage. The diagnostic capability of the panel was further verified in the external validation stage (18 CC *vs.* 18 NC) by qRT-PCR, and the corresponding AUC value remained as high as 0.808 (95% CI: 0.725–0.891, $P = 0.003$, sensitivity = 74.6%, specificity = 72.5%; Supplementary Figure 3D, http://links.lww.com/CM9/A429).

Subgroup analyses were further performed to evaluate the association between the identified signature and patients' clinicopathological parameters including the Tumor Node Metastasis stage and histological type. The three miRNAs were unspecific to histological subtype since none of the three miRNAs were differentially expressed between squamous cell carcinoma and adenocarcinoma patients ($P > 0.05$). The expression levels of two serum miRNAs (miR-122-5p and miR-20a-5p) were consistently up-regulated in CC patients at early stages (stage I or stage II) and advanced stages (stage III or IV) when compared with NCs ($P < 0.05$; data not shown). Subgroup ROC curve analyses comparing CC patients at each Tumor Node Metastasis stage and NCs were further performed. The three-miRNA signature proved to have reliable performance in discriminating CC patients at any stage from healthy people with the AUCs being 0.705 (95% CI: 0.612–0.797, $P < 0.001$, sensitivity = 73.1%, specificity = 66.6%), 0.718 (95% CI: 0.615–0.747, $P < 0.001$, sensitivity = 70.2%, specificity = 67.0%), 0.722 (95% CI: 0.620–0.712, $P < 0.001$, sensitivity = 65.4%, specificity = 76.5%), and 0.661 (95% CI: 0.620–0.712, $P < 0.001$, sensitivity = 71.2%, specificity = 58.3%) for patients at stage I, II, III, and IV, respectively [Supplementary Figure 4, http://links.lww.com/CM9/A429]. The biomarker showed stable diagnostic performance in identifying CC patients regardless of the disease stage.

To explore the potential forms of the identified miRNAs, the expression levels of the three miRNAs were further detected in tissue samples (24 CC *vs.* 24 NC) and serum-derived exosomes samples (24 CC *vs.* 24 NC) using qRT-PCR. As shown in Supplementary Figure 5, http://links.lww.com/CM9/A429, miR-20a-5p was significantly up-regulated in CC tissues ($P < 0.05$), which to some extent indicated the potential origin of this over-expressed circulating miRNA. In the exosomes samples of CC patients, the expression of exosomal miR-122-5p and miR-20a-5p was significantly increased with $P < 0.05$ [Supplementary Figure 6, http://links.lww.com/CM9/A429]. The findings might give some hints about cell-to-cell communication and cell-to-microenvironment communication mediated by these identified miRNAs in the development and progression of CC.

Moreover, the potential functions of miR-122-5p, miR-20a-5p, and miR-133a-3p were preliminarily explored using the online tool DIANA-miRPath v.3.0 (http://www.microrna.gr/miRPathv3), a software designed for the functional annotation of one or more miRNAs largely based on the experimentally validated miRNA-gene interactions.[5] The Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology terms commonly involved by the identified miRNAs were presented in Supplementary Table 5, http://links.lww.com/CM9/A430. It can be concluded that the identified dysregulated miRNAs were significantly involved in some classic

cancer-related biological processes (false discovery rate < 0.05), such as "cell cycle," "p53 signaling pathway," and "pathways in cancer." More research focusing on the exact role of these miRNAs in CC was in demand according to the results of bioinformatics analysis.

We conclude that we have identified a three-miRNA signature in serum for CC diagnosis. Though there will still be a long way to go before clinical application, the findings of this study might be a hint to assist the diagnosis of CC patients in the future.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81672400 and 81672788).

Conflicts of interest

None.

References

1. Banno K, Iida M, Yanokura M, Kisu I, Iwata T, Tominaga E, *et al*. MicroRNA in cervical cancer: OncomiRs and tumor suppressor miRs in diagnosis and treatment. Scientific World Journal 2014;2014: 178075. doi: 10.1155/2014/178075.
2. Dijkstra MG, Snijders PJ, Arbyn M, Rijkaart DC, Berkhof J, Meijer CJ. Cervical cancer screening: on the way to a shift from cytology to full molecular screening. Ann Oncol 2014;25:927–935. doi: 10.1093/annonc/mdt538.
3. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, *et al*. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105: 10513–10518. doi: 10.1073/pnas.0804549105.
4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–408. doi: 10.1006/meth.2001.1262.
5. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, *et al*. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. Nucleic Acids Res 2015;43:W460–W466. doi: 10.1093/nar/gkv403.

How to cite this article: Cao MM, Liu QX, Zou X, Fan XC, Liu C, Zhang SY, Wang TS, Li CY, Zhang C, Geng XN, Liu P, Zhu W. Identification of a serum three-microRNA signature for cervical cancer diagnosis. Chin Med J 2021;134:1756–1758. doi: 10.1097/CM9.0000000000001327