Clin. Lab. 2020;66:XXX-XXX

ORIGINAL ARTICLE

Application of CircEIF4G2 in Screening of Cervical Lesions

Yifan Mao [1], Liya Zhang [1], Yuan Li [2]

[1] *Department of Gynecology, Second People's Hospital in Wuhu City, Anhui Province, Wuhu, Anhui Province, China*
[2] *Department of Geriatrics, The First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui Province, China*

SUMMARY

Background: **The aim was to investigate the expression of circulating RNA EIF4G2 (CircEIF4G2) in cervical cancer and its correlation with clinicopathological features.**
Methods: **Cervical tissue and peripheral blood serum samples were collected from 148 patients with cervical lesions, including 30 patients with low-grade squamous intraepithelial lesions (LSIL group), 35 patients with high squamous intraepithelial lesion (HSIL group), 28 patients with atypical squamous cells (ASC group), and 55 patients with cervical cancer (CC group). At the same time, cervical biopsy specimens and peripheral blood serum were collected from 40 healthy women (Normal group). RT-PCR was used to detect the expression of CircEIF4G2 in cervical tissues and peripheral blood of each group. Electron microscopy was used to observe the distribution of exosomes CircEIF4G2 in cervical tissues. Meanwhile, the correlation between the expression level of CircEIF4G2 and clinical pathological data of patients was analyzed. *In vitro*, HeLa cells and primary cervical epithelial cells were cultured for 24 hours. Then, the expression levels of CircEIF4G2 in the two kinds of cells were detected by RT-PCR in medium. Furthermore, primary cervical epithelial cells were co-cultured with HeLa cells to observe the effect of exosome CircEIF4G2 on primary cervical epithelial cells.**
Results: **The expression of CircEIF4G2 in the cervical tissue and serum of the normal group was significantly lower than that in the CC group ($p < 0.05$), but there was no significant difference between the LSIL group and the HSIL group in the cervical tissue and serum ($p < 0.05$). The distribution and expression of exosomes CircEIF4G2 in each group were consistent with RT-PCR results under an electron microscope. The results of experiments *in vitro* showed that the expression level of CircEIF4G2 in HeLa cells was significantly higher than that in primary cervical epithelial cells ($p < 0.05$). The medium in which Hela cells were cultured for 24 hours was added to the culture medium of primary cervical epithelial cells. The process of exosomes CircEIF4G2 entering primary cervical cancer cells was observed by electron microscopy.**
Conclusions: **The increased expression of CircEIF4G2 in tissues and serum of cervical lesions may be caused by the secretion of exosomes containing CircEIF4G2 by cervical cancer cells. Therefore, CircEIF4G2 can be used as a marker for the diagnosis of cervical lesions.**
(Clin. Lab. 2020;66:xx-xx. DOI: 10.7754/Clin.Lab.2019.190915)

Correspondence:
Yuan Li
Department of Geriatrics
The First Affiliated Hospital of
Wannan Medical College
241000 Wuhu
Anhui Province
China
Email: liyuanahwhbio@163.com

Manuscript accepted December 1, 2019

KEY WORDS

cervical lesions, exosomes, CircEIF4G2, markers

INTRODUCTION

Cervical cancer is one of the most common malignant tumors among women worldwide, and it is also one of the leading causes of cancer deaths in women [1]. Most cases of cervical cancer are caused by human papillomavirus (HPV) infection of cervical epithelial cells, hu-

man papillomavirus E6, E7 protein. Cervical cancer cell tumor suppressor gene and proto-oncogene interact to induce tumor suppressor gene inactivation. The activation of proto-oncogenes and telomerase and the disorder of the body immune system eventually lead to abnormal proliferation and apoptosis of cervical epithelial cells, resulting in cervical tissue cancer. Current clinical prevention of cervical cancer includes screening and vaccination. However, the molecular mechanism for the occurrence and development of cervical cancer has not been fully clarified [2-5]. Therefore, the search for key pathogenic genes and proteins in the pathogenesis of cervical cancer is of great significance for its future targeted therapy.

Exosomes are cystic vesicles of 30 to 100 nm in size containing specific proteins or RNA secreted by host cells [6]. These exosome-derived RNAs or proteins can be targeted to the recipient cells by fusion with the cell membrane of the recipient cell, and the specific biological effect on the receptor cells can be exerted through a non-contact manner [7]. The eukaryotic translation initiation factor 4G2 (EIF4G2) acts as a "scaffold protein" that interferes with translation of cap-dependent and non-dependent proteins in eukaryotic cells, and thus the proliferation, apoptosis, cell cycle, migration, invasion, and angiogenesis of tumor cells will be affected, which will ultimately affect the occurrence and development of tumor [8,9]. However, at present, the expression of EIF4G2 in cancer tissues and serum of patients with cervical cancer and its correlation with disease have not been reported.

This study examined the expression of CircEIF4G2 in cervical tissue and serum in healthy and cervical lesions, and analyzed the correlation between CircEIF4-G2 expression and LSIL, HSIL, and CC. In addition, *in vitro* primary cervical epithelial cells and HeLa cells were used for 24 hours to detect the contents of exosomes EIF4G2 in the culture medium. The exosome EIF4G2 was added to the culture medium of primary cervical epithelial cells after exosomes were separated. The endogenous process of exosomes EIF4G2 in primary cervical epithelial cells was observed by electron microscopy.

MATERIALS AND METHODS

Disease sample data

Cervical cancer tissues and peripheral blood serum samples were collected from 120 patients with cervical lesions who came to our hospital from May 2016 to December 2017, including 30 patients with low grade squamous intraepithelial lesions (LSIL group), age 51.21 ± 1.34 years; 35 patients with high grade squamous intraepithelial lesion (HSIL group), age 56.31 ± 2.34 years; 28 patients with atypical squamous epithelial cells (ASC group), age 49.44 ± 2.34 years old; 55 patients with cervical cancer (CC group), age 54.11 ± 3.61 years old, and 40 healthy women (normal group) with cervical biopsy specimens and peripheral blood serum were collected as control ages 49.58 ± 2.11 years old. All patients with cervical lesions and healthy examinations were diagnosed by cervical biopsy and cytology.

Histopathological examination

CIN I-III lesions were classified according to TBS criteria into mild, moderate, and severe cervical squamous epithelial atypical hyperplasia. The pathologists in our hospital read the film on the premise that they did not know the patient's condition, and each film was read by two pathologists. When there was a disagreement, it was reviewed again according to the Richart standard.

TCT cytological examination

The cytological diagnosis was divided into HSIL, LSIL, ASC, and CC using the TBST classification method.

RT-PCR detection of CircEIF4G2 expression

1) Trizol extraction of total RNA from cervical tissue and serum, followed by UV spectrophotometry to detect the concentration and purity of the extracted RNA, A260/A280 = 1.8 - 2.0 can be used; 2) By reverse transcription, the mRNA is synthesized into cDNA and stored in a refrigerator at -80°C; 3) RTPCR system: 2.5 μL 10 x Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2 x 10^6 U/L), 19 μL ddH_2O. The amplification systems for RT-PCR were identical.

Cell culture

Cervical tissue excised from benign lesions was taken, and the suspended cervical epithelial cells were obtained by repeated digestion with trypsin-EDTA and diapase II to obtain suspended cervical epithelial cells. Cervical epithelial cells were cultured in a serum-free medium at 37°C. HeLa cells were cultured in 10% FBS 1,640 medium at 37°C.

Purification and isolation of exosomes

HeLa cells in logarithmic growth phase were selected. After 24 hours of serum-free culture, 40 mL of the supernatant in the culture medium was aspirated and centrifuged at 2,000 rpm for 10 minutes. The supernatant was extracted and filtered through a sterile filter into a centrifuge tube. After centrifugation at 2,000 rpm for 1 hour at 4°C, an exosome concentrate was obtained. Subsequent operations were consistent with the RT-PCR reverse transcription steps.

Morphological observation of exosomes

After isolation, 2 μL of exosome concentrate was taken and diluted to 50 μL with PBS. An aqueous solution of uranyl acetate was added to the sample, uniformly mixed, and a drop was added to the copper mesh, dried naturally, and observed under a transmission electron microscope to take a photograph.

Table 1. Correlation between EIF4G2 expression in peripheral blood and clinicopathological features of patients with cervical cancer.

Clinicopathological features		Expression level of EIF4G2		X^2	p
		High	Low		
Case		23	32		
Age	≥ 46	10	15	0.004	0.852
	< 46	13	17		
Tumor size	≥ 4 cm	11	16	0.238	0.778
	< 4 cm	12	16		
Lymphatic metastasis	(-)	3	26	12.232	0.001
	(+)	20	6		
FIGO staging	IB - IIA	3	21	14.127	0.001
	IIB - IIIA	6	6		
	IIIB - IV	14	5		
Pathological grading	G1 + G2	10	14	1.211	0.451
	G3	13	18		
Depth of invasion	≥ 2/3	4	24	10.218	0.001
	< 2/3	19	8		

Statistical analysis

All data were analyzed using SPSS 22.0 software. Measurement data were expressed as mean ± standard deviation, and data comparison between the two groups was performed by *t*-test. $p < 0.05$ represents a statistically significant difference.

RESULTS

Expression of EIF4G2 in cervical tissues of patients with cervical lesions

First, we examined the expression levels of EIF4G2 in cervical tissues of patients with different cervical lesions. The results of RT-PCR (Figure 1) showed that there was no significant difference in the expression level of EIF4G2 when ASC group, LSIL group, and HSIL group compared with control group ($p > 0.05$). The expression level of EIF4G2 in the cancerous tissues of CC the group was significantly higher than that of the other four groups ($p < 0.05$).

Expression of EIF4G2 in serum of patients with cervical lesions

Further, we extracted total RNA from peripheral blood samples from four groups of people and measured the amount of EIF4G2 in peripheral blood. The results showed (Figure 2) that the expression of EIF4G2 in peripheral blood was consistent with its expression in cervical epithelial tissues. There was no significant difference in expression between control group, ASC group, LSIL group, and HSIL group ($p > 0.05$), but they were all significantly lower than CC group ($p < 0.05$).

Correlation between EIF4G2 expression level and clinicopathological features of patients with cervical cancer

According to the average expression level of EIF4G2 in serum of patients in the CC group (8.718), we divided 55 CC patients into EIF4G2 high-expression group (higher than 8.718, n = 23) and EIF4G2 low-expression group (lower than 8.718, n = 32) (Table 1). By analyzing the expression level of EIF4G2 and the clinicopathological features of CC patients, we found that the proportion of EIF4G2 high expression was significantly higher in patients with lymph node metastasis than in patients with low expression ($p < 0.05$). In addition, there was a significant correlation between EIF4G2 expression level and FIGO stage. The higher the expression level of EIF4G2, the higher the FIGO stage of CC patients ($p < 0.05$). The higher the expression level of EIF4G2 in CC patients, the deeper the tumor infiltration ($p < 0.05$).

Relationship between EIF4G2 expression level and progression-free survival in patients with cervical cancer

As shown in Figure 3, data from the Kaplan-Meier method showed that the progression-free survival time of patients with low expression of EIF4G2 was 28 ± 2.31 months, while the progression-free survival time of patients with high expression of EIF4G2 was approximately 13 ± 1.82 months, $\chi^2 = 32.123$, $p < 0.001$, so there is a statistical difference.

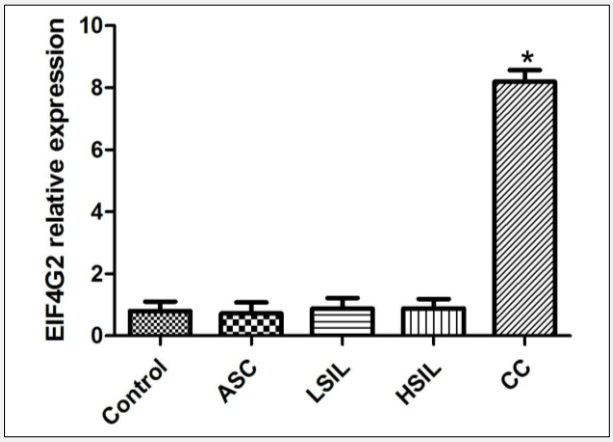

Figure 1. Expression of EIF4G2 in cervical tissues of patients with cervical lesions.

Control - represents control group, ASC - represents atypical squamous cell group, LSIL - represents low-grade squamous intraepithelial lesion group, HSIL - represents highly squamous intraepithelial lesion group, $p < 0.05$ represents difference as compared with control group.

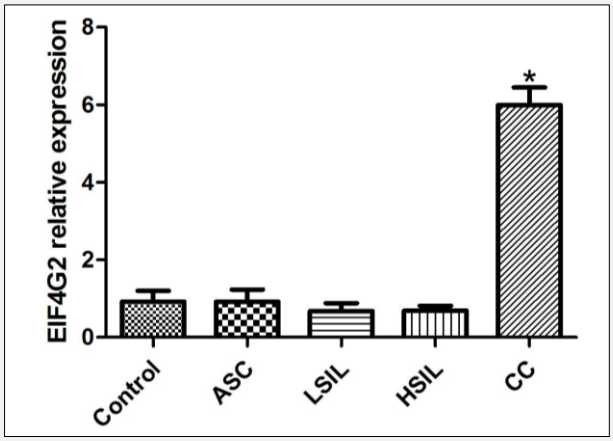

Figure 2. EIF4G2 expression in serum of patients with cervical lesions.

All abbreviations are consistent with Figure 1. $p < 0.05$ presents difference as compared with control group.

Relationship between EIF4G2 expression level and total survival time of patients with cervical cancer

As shown in Figure 4, Kaplan-Meier results showed that the overall survival of patients with low expression of EIF4G2 was 46 ± 1.45 months and that of the higher expression group 11 ± 2.51 months, $\chi^2 = 25.713$, $p < 0.001$.

Expression of EIF4G2 in primary cervical epithelial cells and HeLa cells

To further investigate the mechanism of EIF4G2 expression in the development of cervical cancer, we examined the expression levels of EIF4G2 in primary cervical epithelial cells (PCEC) and HeLa cells cultured for 24 hours. The results showed that the expression level of EIF4G2 in Hela cells and the level of EIF4G2 in the

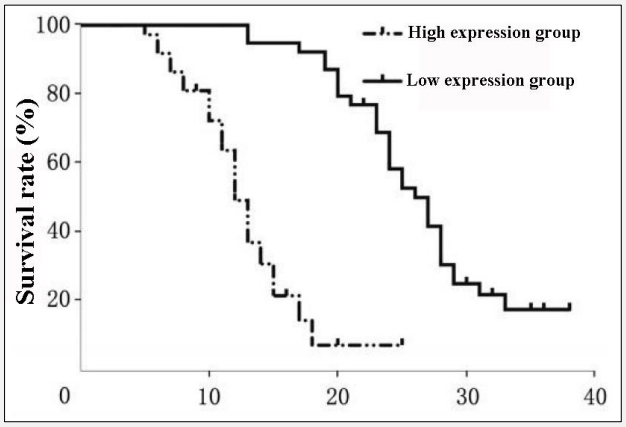

Figure 3. Relationship between EIF4G2 expression level and progression-free survival in patients with cervical cancer.

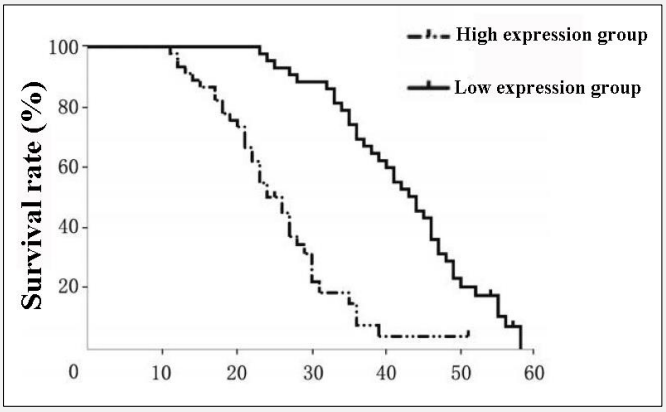

Figure 4. Relationship between EIF4G2 expression level and total survival time of patients with cervical cancer.

medium after 24 hours were significantly higher than those in PCEC cells ($p < 0.05$) (Figure 5).

Internalization of exosomes derived CircEIF4G2 in primary cervical epithelial cells

Considering that HeLa cells are capable of secreting exosomes containing CircEIF4G2, we investigated whether exosomes affect normal primary cervical epithelium. After adding the isolated exosomes to the culture medium of the primary cervical epithelium, we observed the dynamic changes of the exosomes at different time points. The results are shown in Figure 6. At 2 hours, the exosomes began to fuse in the primary cervical epithelial membrane; and at 4 hours, the exosomes had completely entered the epithelial cells. These results confirm that exosome-derived CircEIF4G2 secreted by cervical cancer can enter normal epithelial cells.

DISCUSSION

Cervical cancer is one of the most serious malignant tumors of the female reproductive system, especially in developing countries, and its incidence is very high due

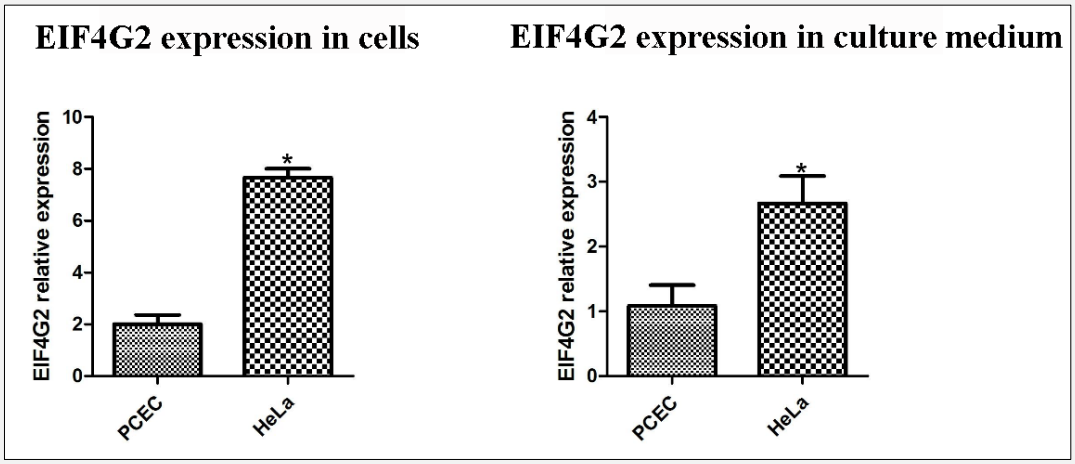

Figure 5. Expression of EIF4G2 in cervical epithelial cells and HeLa cells.

PCEC represents primary cervical epithelial cells, $p < 0.05$ represents difference as compared with PCEC group.

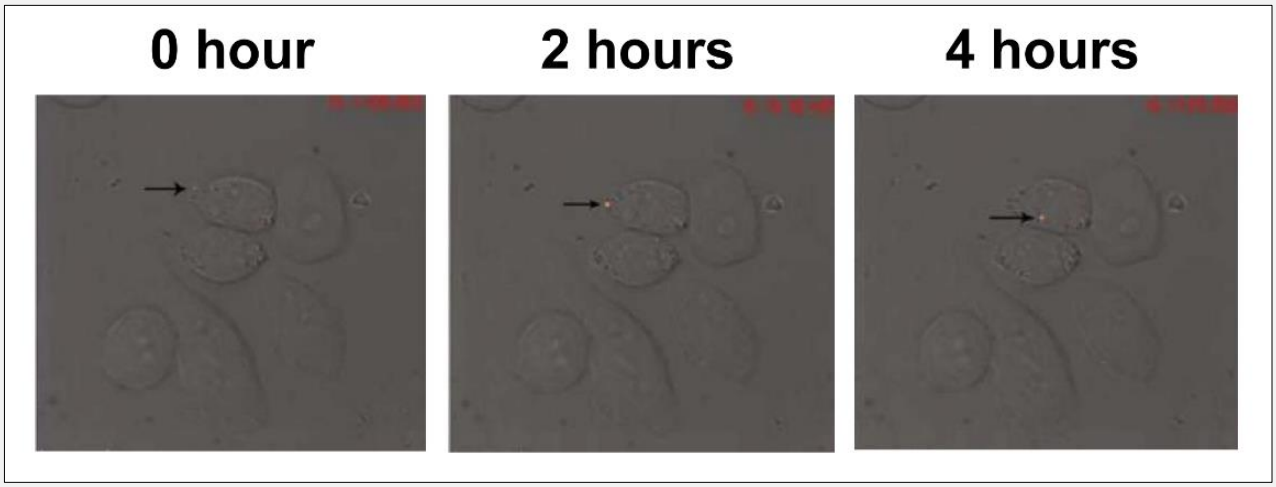

Figure 6. Internalization of exosomes-derived CircEIF4G2 in primary cervical epithelial cells.

to the incomplete popularization of vaccines. According to the National Cancer Center, the incidence and mortality of cervical cancer in China are $10.4/10^5$ and $2.59/10^5$, respectively. At present, the prevention of cervical cancer mainly includes the vaccination of HPV vaccine and cervical cancer screening, but the HPV vaccine has not yet been popularized in China [10-13]. On the other hand, because the treatment strategy for cervical cancer mainly includes surgical resection plus radiotherapy and chemotherapy, the effect is not satisfactory [14-16]. Therefore, aiming at the signal transduction process in the development of cervical cancer, various targeted molecular drugs are designed to inhibit the proliferation, apoptosis, invasion and metastasis and angiogenesis of cervical cancer cells, and to find sensitive markers that are of great significance for the treatment and diagnosis of cervical cancer.

Exosomes are special vesicles containing proteins or

RNA secreted by cells [17]. These proteins or RNAs are "injected" into target cells by cell membrane fusion of target cells, which exert various biological effects regulating genes or proteins in cells, including anti-oxidative stress, regulation of tumor cell invasiveness, and maintenance of tumor cell tumorigenicity [18-20]. In addition, studies have shown that RNA in exosomes is more stable than other free RNA and therefore has potential as a diagnostic marker for disease. For example, in the gastric cancer cell line AZ-P7a, gastric cancer cells secrete a rich amount of let-7 miRNAs, thereby reducing the expression of let-7 miRNAs in gastric cancer cells, and ultimately maintaining the high tumorigenicity and invasiveness of gastric cancer cells [21,22]. EIF4G2 is a eukaryotic translation initiation factor that plays a crucial role in protein synthesis. Overexpression of EIF4G2 can promote normal cell transformation, causing normal cells to lose contact inhibition and immortalize, forming the phenotype of cancer cells. Animal experiments have shown that the injection of adenovirus overexpressing EIF4G2 into the skin of mice can induce the formation of subcutaneous tumors in mice. The expression level of EIF4G2 in paracancerous tissues of patients with nasopharyngeal carcinoma was lower than that of cancer tissues, and the expression level of EIF4G2 was positively correlated with tumor grade, clinical stage, and lymph node metastasis of nasopharyngeal carcinoma [23]. The results of cell experiments also showed that the expression of EIF4G2 in nasopharyngeal carcinoma cells was significantly higher than that in normal nasal mucosa epithelial cells. However, after shRNA was used to knock out EIF4G2 in nasopharyngeal carcinoma cells, apoptosis of nasopharyngeal carcinoma cells increased significantly, while angiogenesis, migration, and proliferation were inhibited. These results also indicate that EIF4G2 may play a role as a cancer-promoting gene. Our previous studies also confirmed that EIF4G2 can act as a miR-218 adsorption sponge that regulates the biological behavior of cervical cancer cells by affecting the expression of HOXA1. In this study, EIF4G2 was detected in cervical tissue and peripheral serum of different cervical lesions, and it was found that EIF4G2 was significantly higher in cancer tissues and serum of patients with cervical cancer than in normal population and cervical intraepithelial neoplasia. In addition, by analyzing the relationship between the expression level of EIF4G2 and the clinicopathological features of cervical cancer patients, we found that the expression level of EIF4G2 was positively correlated with FIGO stage, depth of invasion, and lymph node metastasis and negatively correlated with progression-free survival time and overall survival time of cervical cancer patients. The results of *in vitro* experiments further confirmed that EIF4G2 is secreted by cervical cancer cells instead of normal cervical cells, and EIF4G2 secreted by cervical cancer cells can enter normal cervical cells. However, the biological effects on normal cervical cells are yet to be further studied.

CONCLUSION

This study demonstrates for the first time that the eukaryotic translation initiation factor EIF4G2 can be used as a marker for cervical cancer screening and diagnosis, and is also a key intervention target for the treatment of cervical cancer.

Author Contributions:
All authors participated in the interpretation of collected literature and in the drafting, critical revision, and approval of the final version of the manuscript.

Declaration of Interest:
All the authors declare that they have no conflicts of interest.

References:

1. Cai P, Jin X, Zhu H, Zhu S, Wang L, Jin J. OGT-mediated O-GlcNAcylation on GLDC promotes metastasis in cervical cancer. Oncotarget 2018 (https://doi.org/10.18632/oncotarget.24094).
2. Zhang Z, Wang J, Li J, Wang X, Song W. MicroRNA-150 promotes cell proliferation, migration, and invasion of cervical cancer through targeting PDCD4. Biomed Pharmacother 2018;97: 511-7 (PMID: 29091902).
3. Wei L, Cui L, Zhao X. miR-186 down-regulates EGFR expression and effect on proliferation and invasion of cervical cancer. Progress Obstet Gynecol 2018;30:155-8 (http://en.cnki.com.cn/Article_en/CJFDTotal-XDFC201803008.htm).
4. Chaichian S, Khateri S, Moradi Y, et al. Trends in Cervical Cancer Incidence in Iran from 2003 to 2009. Middle East J Cancer 2018;9:57-63 (http://mejc.sums.ac.ir/article_42093.html).
5. Tamura R, Yoshihara K, Saito T, et al. Novel therapeutic strategy for cervical cancer harboring FGFR3-TACC3 fusions. Oncogenesis 2018;7:4 (PMID: 29358619).
6. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 2018;75:193-208 (PMID: 287 33901).
7. Zhang H, Deng T, Liu R, et al. Exosome-delivered EGFR regulates liver microenvironment to promote gastric cancer liver metastasis. Nat Commun 2017;8:15016 (PMID: 28393839).
8. Xie X, Li YS, Xiao WF, et al. MicroRNA-379 inhibits the proliferation, migration and invasion of human osteosarcoma cells by targetting EIF4G2. Biosci Rep 2017;37(3). (PMID: 28381518).
9. Hao G, Hao H, Ding Y, et al. Suppression of EIF4G2 by miR-379 potentiates the cisplatin chemosensitivity in nonsmall cell lung cancer cells. FEBS Lett. 2017;591:636-45 (PMID: 28117895).
10. Behtash N, Mehrdad N. Cervical cancer screening and prevention. Asian Pac J Cancer Prev 2006;7:683-6 (PMID: 17250453).
11. Biswas A. Human Papillomavirus (HPV) and cervical cancer. J Indian Med Assoc 2016;98:53-5 (PMID: 11016151).

12. Chen W, Zheng R, Zeng H, Zhang S, He J. Annual report on status of cancer in China, 2011. Chin J Cancer Res 2015;27:2-12 (PMID: 25717220).

13. Mwanahamuntu MH, Sahasrabuddhe VV, Parham GP. HPV screening for cervical cancer in rural India. N Engl J Med 2009; 361:305 (PMID: 19610158).

14. Santos C, Muñoz N, Klug S, et al. HPV types and cofactors causing cervical cancer in Peru. Br J Cancer 2001;85:966-71 (PMID: 11592767).

15. Kidd EA, Siegel BA, Dehdashti F, Grigsby PW. The standardized uptake value for F-18 fluorodeoxyglucose is a sensitive predictive biomarker for cervical cancer treatment response and survival. Cancer 2010;110(8):1738-44 (PMID: 17786947).

16. Marth C, Landoni F, Mahner S, et al. Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2018;29:iv262 (PMID: 29741577).

17. de Rivero Vaccari JP, Brand F 3rd, Adamczak S, et al. Exosome-mediated inflammasome signaling after central nervous system injury. J Neurochem 2016;136:39-48 (PMID: 25628216).

18. Felicetti F, De Feo A, Coscia C, et al. Exosome-mediated transfer of miR-222 is sufficient to increase tumor malignancy in melanoma. J Transl Med 2016;14:56 (PMID: 26912358).

19. Syn N, Wang L, Sethi G, Thiery JP, Goh BC. Exosome-Mediated Metastasis: From Epithelial-Mesenchymal Transition to Escape from Immunosurveillance. Trends Pharmacol Sci 2016;37(7): 606-17 (PMID: 27157716).

20. Jia Y, Chen Y, Wang Q, et al. Exosome: emerging biomarker in breast cancer. Oncotarget 2017;8:41717-33 (PMID: 28402944).

21. Hu H, Zhao X, Jin Z, Hou M. Hsa-let-7g miRNA regulates the anti-tumor effects of gastric cancer cells under oxidative stress through the expression of DDR genes. J Toxicol Sci 2015;40: 329-38 (PMID: 25972194).

22. de Freitas RCC, Bortolin RH, Lopes MB, et al. Modulation of miR-26a-5p and miR-15b-5p Exosomal Expression Associated with Clopidogrel-Induced Hepatotoxicity in HepG2 Cells. Front Pharmacol 2017;8:906 (PMID: 29311920).

23. Tu L, Liu Z, He X, et al. Over-expression of eukaryotic translation initiation factor 4 gamma 1 correlates with tumor progression and poor prognosis in nasopharyngeal carcinoma. Mol Cancer 2010;9:78 (PMID: 20398343).