Food and Chemical Toxicology 133 (2019) 110779

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

MiR-92a antagonized the facilitation effect of extracellular matrix protein 1 in GC metastasis through targeting its 3′UTR region

Lin-Qing Liu[a,b,c,*], Lei Hu[d,1], Xi-Bei Hu[e], Jiang Xu[f], Ai-Mei Wu[g], Hao Chen[d], Peng-Yin Gu[b], Shi-Lian Hu[a,b,c,**]

[a] School of Medicine, Shandong University, Jinan, 250012, China
[b] Department of Geriatrics, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, 230036, China
[c] Anhui Provincial Key Laboratory of Tumour Immunotherapy and Nutrition Therapy, Hefei, 230001, China
[d] Department of General Surgery, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, 230001, China
[e] Department of Anesthesiology, The First Affiliated Hospital of USTC, Division of Life Science and Medicine, University of Science and Technology of China, Hefei, 230036, China
[f] Department of Endocrinology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, 230001, China
[g] Department of Neurology, Affiliated Hefei Hospital of Anhui Medical University, Hefei, 230011, China

ARTICLE INFO

Keywords:
ECM1
Gastric carcinoma
Metastasis
EMT
miR-92a

ABSTRACT

MicroRNAs were known to play very important roles in human diseases, and have attracted great interests of research scientists in medicine, toxicology and functional foods. Gastric carcinoma (GC) remains one of the most common and lethal types of malignancy worldwide. However, the molecular mechanism of GC and the role of microRNAs in GC development remain unclear. The expression of extracellular matrix protein 1 (ECM1) is up-regulated in many cancer types, but its functional role is inconstant. In the present study, we aimed to investigate the correlation between GC development and ECM1 expression, along with its regulation by microRNAs. Immunohistochemical results showed that ECM1 was up-regulated in GC tissues and ECM1 expression level was negatively correlated with the prognosis of GC. ECM1 was found to promote gastric cancer cell metastasis in cell migration assays by facilitating the expression of proteins involved in epithelial-mesenchymal transition (EMT). MiR-92a was recognized for the first time to suppress the migration of human GC cells by directly targeting to the 3′UTR of ECM1 gene in a dual-luciferase reporter assay. These results highlighted the antagonistic roles of ECM1 and miR-92a in GC development, which may serve as a new target for gastric cancer.

1. Introduction

Gastric carcinoma (GC) remains one of the most common and lethal types of malignancy worldwide (Siegel et al., 2016). An estimated 951,600 new GC cases were reported in 2012 (Ye et al., 2016). Although extensive efforts to develop tactics for the management of GC patients have been made, the 5-year survival rates remain low (Qiu and Xu, 2013), and the exploration of the underlying mechanisms driving gastric cancer progression is still required. Extracellular matrix protein 1 (ECM1) is a secreted, soluble protein and its germ line mutations may lead to lipoid proteinosis, which is characterized by skin and mucosa thickening and widespread hyaline (glycoprotein) deposition and basement membrane disruption, and high ECM1 expression is related to some types of cancers, including laryngeal carcinoma (Gu et al., 2013), hepatocellular carcinoma (Chen et al., 2016), cholangiocarcinoma cancers (Xiong et al., 2012), cervical cancer (Ye et al., 2016), thyroid cancer (Kebebew et al., 2005) and breast cancer (Gomez-Contreras et al., 2017). However, the expression and clinical significance of ECM1 in gastric cancer remain unclear. It is widely accepted that epithelial-mesenchymal transition (EMT) activation enables cancer cells to

* Corresponding author. School of Medicine, Shandong University, Jinan 250012, China.
** Corresponding author. School of Medicine, Shandong University, Jinan, 250012, China.
E-mail addresses: liulinqing2011@163.com (L.-Q. Liu), hu1169514818@126.com (L. Hu), vikihu_0815@sina.com (X.-B. Hu), 363860505@qq.com (A.-M. Wu), ch2446@126.com (H. Chen), hellengpy@163.com (P.-Y. Gu), hushilian@126.com (S.-L. Hu).
[1] Co-first author.

https://doi.org/10.1016/j.fct.2019.110779
Received 29 July 2019; Received in revised form 18 August 2019; Accepted 20 August 2019
Available online 28 August 2019

acquire the ability to invade and disseminate (Chaffer et al., 2016), and these effects are characterized by the down-regulation of epithelial markers, such as E-cadherin, and the up-regulation of mesenchymal markers, such as vimentin and Snail (Pradella et al., 2017). A variety of studies have demonstrated that reprogramming the EMT promotes tumorigenesis and invasiveness (Santamaria et al., 2017), which favors uncontrolled tumor cell growth and metastasis. Nevertheless, there are few studies on the role of ECM1 in the EMT process of GC.

MicroRNAs (miR) are a class of small non-coding RNAs with a length of about 22 nucleotides that generally function to suppress gene expression by targeting the 3' UTR of mRNAs. MiRNAs have been demonstrated to regulate multiple cellular functions such like cell proliferation, metastasis, cell cycle and metabolism, involved in many types of human diseases (Barger et al., 2016). Abnormal miR-92a expression was reported in multiple cancers such as breast cancer, cervical cancer, colorectal cancer, and osteosarcoma (Chen et al., 2018; Li et al., 2014; Xiao et al., 2017; Zhou et al., 2015). It has been demonstrated that miR-92a in most cancer was upregulated to promoted cancer cell proliferation and invasion, however, it was also found that downregulation of miR-92a was associated with aggressive breast cancer (Nilsson et al., 2012) and multiple myeloma (Yoshizawa et al., 2012). These results suggest that miR-92a may be important in cancer pathogenesis and progression by targeting different genes. But little is known about miR-92a expression and function in the gastric cancer.

MicroRNAs are identified as critical regulators in human diseases and development (Barger et al., 2016), and has attracted great interests of researchers in medicine, toxicology and functional foods (White et al., 2016). In this study, we aimed to explore the expression and functional role of ECM1 in gastric cancer and demonstrated ECM1 is a novel target gene of miR-92a and indicated their effect in the development of GC.

2. Materials and methods

2.1. Materials and tissue samples

The study was approved by the Ethics Committee of University of Science and Technology of China (USTC). Between March 2009 and July 2013, 74 gastric cancer and paired adjacent normal specimens were collected from the gastric cancer patients received curative surgical resection at The First Affiliated Hospital of USTC with an informed consent. Prior to surgery, none of them received adjuvant therapy. All patients contributed to the complete follow-up data, and they were followed up from 1 March 2013 to 10 July 2018, which resulted in an average 60-month follow-up period. The overall survival (OS) was defined as the interval between the surgery and patient death or the last follow-up.

2.2. Cell culture

The human gastric cancer cell lines BGC-823, MKN-45, MGC-803 and SGC-7901 were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-modified culture medium (HyClone, USA) containing 10% (v/v) fetal bovine serum (HyClone, USA) in a humidified incubator at 37 °C with 5% CO2 (Arathi et al., 2016).

2.3. Immunohistochemical staining

Tissue slides were boiled in citrate buffer (pH 6.0) for 20 min for antigen retrieval and then cooled at room temperature. Subsequently, the slides were incubated in a 10% hydrogen peroxide solution for 15 min to eliminate endogenous peroxidase activity. The sections were then immunostained with a rabbit anti-ECM1 antibody (11521-1-AP, Proteintech Co., Rosemont, USA) at 4 °C overnight. After rinsing with PBS for 5 min, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (PV-6000, ZSGB-BIO Co., Beijing, China) for 20 min. After further washing, the peroxidase activity was visualized using freshly prepared DAB (ZLI-9017, ZBGB-BIO Co., Beijing, China); then the slides were counterstained lightly with Harris hematoxylin. The negative controls were processed in a similar manner but primary antibody was with replaced PBS. The expression levels of ECM1 was quantified by a combination scoring system of positive staining cell percentage and cell staining intensity. A, the scoring standard for positive staining cell percentage was as follows: < 5% was scored as 0, 6%~25% as 1, 26%~50% as 2 and > 51% as 3; B, the scoring standard for cell staining intensity was as follows: absent/weak was 0, pale yellow was 1, claybank was 2 and sepia was 3. Five fields were selected randomly using a low power (× 100) microscope and the mean of A × B in each field was used as the final score for each tissue section. Sections with a final score of ≤3 were classified as having low ECM1 expression, and those with a final score of > 3 were classified as high ECM1 expression.

2.4. Real-time RT-PCR

For RNA isolation and quantitative real-time PCR, total RNA was extracted from the cells using the TRIzol method and evaluated by a One Drop OD-1000 spectrophotometer. Total RNA was then reverse transcribed (TaKaRa, Dalian, China) into cDNA and analyzed by real-time PCR using SYBR Green PCR Master Mix (TaKaRa, Dalian, China) and a StepOne platform (Applied Biosystems, Shanghai, China) (Wang et al., 2018). Q-PCR was performed in 20 μl volumes for 40 cycles (15 s at 95 °C and 1 min at 60 °C). The gene expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method (Qi et al., 2014). The following primers used for miR-92a: GGGGCAGTTATTGCACTTGTC. ECM1 forward 5′- CATGGATCACCCTGACTCCT -3′; reverse 5′- GGAGAGAGGGC AGCTCTTT-3′; β-actin forward 5′- AACTGGGACGACATGGAGAAA-3′; reverse 5′- ATAGCACAGCCTGGATAGCAAC-3′;

2.5. Wound healing assay

Cells were cultivated into a confluent monolayer in six-well tissue culture plates. Then, a wound was created in the cell monolayer using a sterile 10 μl pipette tip. The cells were washed with PBS and then incubated for 24 h. Images of the wound area were captured at 0 h and 24 h with an inverted phase contrast microscope (Rodríguez-Ribera et al., 2017).

2.6. miRNA transfection and Lentivrius infection

MiR-92a mimic or its inhibitor (miR-92a-in) or the respective control were synthesized by GenePharma (Shanghai, China) and transfected into the cells according to the manufacture's instruction (Brooks and Fry. 2017). For 12-well plate, cells were seeded at a density of 4×10^5 per well. 24 h later, 20 pmol miRNA and 1.5 μl miRNA mate plus reagent (GenePharma, Shanghai, China) were both diluted with 50 μl Opti-MEMI medium followed by a gentle mix together for 5 min at room temperature and adding into the culture medium from a single well. Lentivirus expressing ECM1 shRNAs (targeting sequences: shRNA1, ACTGCTTCAACATCAATTA; shRNA2, CTGCTGTGACCTGCCA TTT); or ECM1 cDNA (NM_004425), or the respective control were produced form GenePharma (Shanghai, China). Lentivirus infection was performed according to the manufacturer's instructions and selected in medium with blasticidin for two weeks to establish stable transduced cells. Real-time RT-PCR and Western blotting analysis were performed to detect ECM1 expression after lentivirus infection.

2.7. Western blotting analysis

Whole cell lysates were separated on 10% denaturing polyacrylamide gels before electro-transference onto PVDF membranes. A

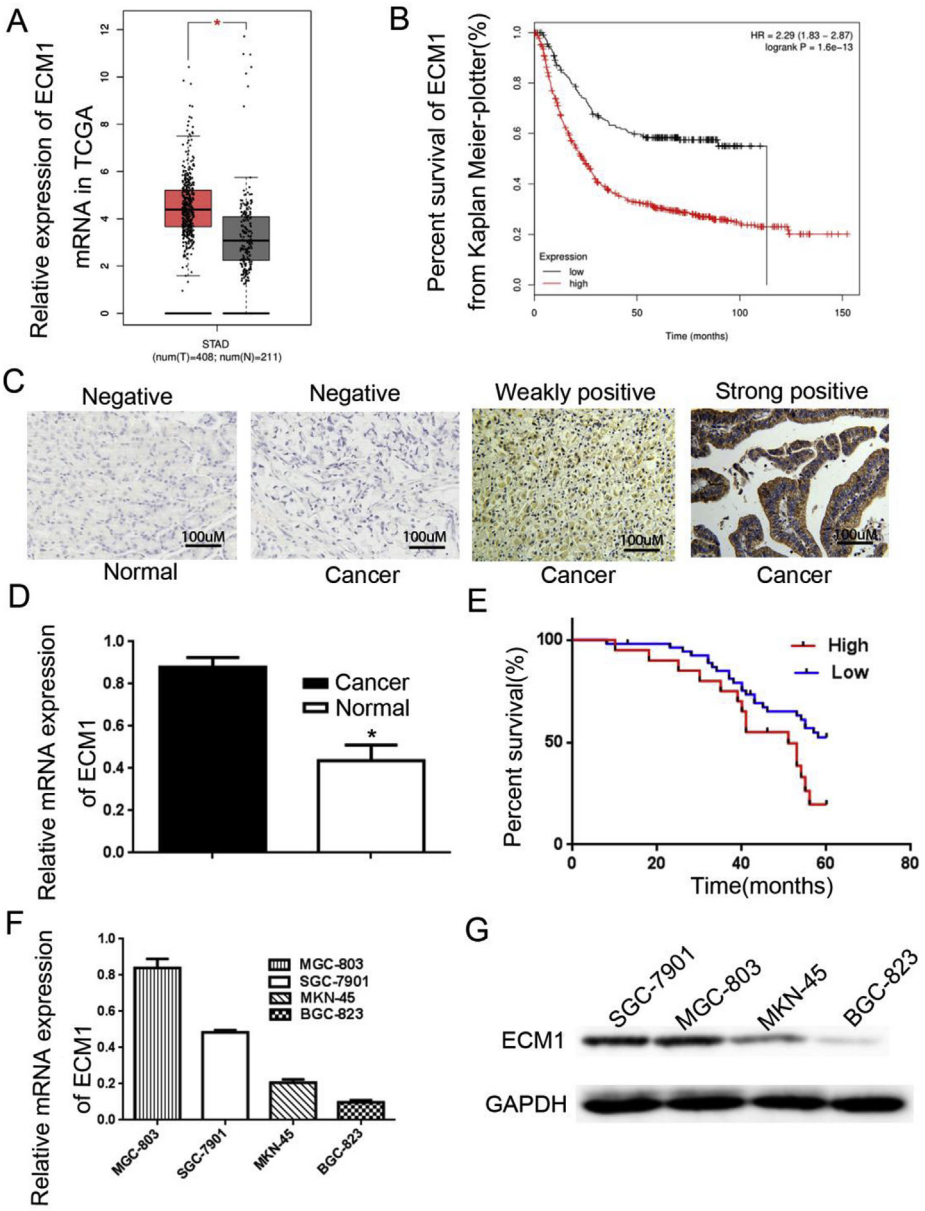

Fig. 1. The expression of ECM1 in GC tissues and the negative correlation with the prognosis of GC. (A) Relative ECM1 mRNA expression levels in tumor samples (N = 408) and control samples (N = 211) from the TCGA database. (B) Kaplan–Meier survival curves showed better overall survival with low expression of ECM1 compared with high ECM1 expression group (p = 1.6e-13). (C) The representative histochemical staining of ECM1 in the normal tissue, the para-carcinoma tissue and tumor tissues. (D) Relative ECM1 mRNA expression in normal and tumor tissues. (E) The Kaplan-Meier survival curves showed patients with low ECM1 expression had a better prognosis than those with high ECM1 expression. (F, G) Relative ECM1 mRNA levels and protein levels in the four types of gastric cell lines. The data are represented as the mean ± sd. of three independent experiments. *P < 0.05.

TBST solution containing 5% (m/v) fat-free dry milk was used to block the membranes before their incubation with primary antibodies against human ECM1, E-cadherin, vimentin, GAPDH and Snail (3879T, CST) at 4 °C overnight. The membranes were washed three times and then incubated with a horseradish peroxidase-conjugated secondary antibody. The proteins were detected using ECL reagent (Pierce, USA), and the band intensities were normalized to β-actin or GAPDH. Each experiment was performed three times (Zhang et al., 2018; Wang et al., 2019).

2.8. Transwell assay

These experiments were performed using 6.5 mm transwell chambers with a pore size of 8 μm (Corning, USA). RPMI plus 10% FBS medium was added to the bottom chamber. Cells were washed three times with PBS and suspended in FBS-free RPMI medium. A total of 2×10^4 cells were added to the upper well of each transwell chamber and incubated for 24 h. Cells in the lower chamber were fixed in a 4% (v/v) paraformaldehyde solution for 15 min, stained with a crystal violet solution for 15 min at room temperature, and counted under a light microscope (at 200 × magnification). Four random visual fields were counted for each well (Brooks and Fry. 2017).

2.9. Anoikis assay

Human GC cells undergoing anoikis were identified and quantified with a Cytoselect 24-well Anoikis Assay kit (Cell Biolabs, USA) according to the manufacturer's instructions.

2.10. Adhesion assay

The adhesion of GC cells to each ECM component was evaluated using a CytoSelect 48-Well Cell Adhesion assay kit (Cell Biolabs, USA) according to the manufacturer's instructions. Matrigel was pre-coated in a 24-well plate, and GC cells were overlaid on the plate bottom. After incubation at 37 °C for 60 min and removing non-adherent cells with three gentle washes with PBS, the cells adherent to each component were quantified in MTT assays or counted under a microscope, respectively.

Table 1
The association between ECM1 and clinicopathological factors in 74 GC patients.

Clinical parameter	Number of cases	ECM1 expression		p
		Low	High	
Gender				
Male	55	14	41	0.604
Female	19	6	13	
Age (years)				
< 60	41	12	29	0.628
≥60	33	8	25	
Differentiation				
Poor	33	9	24	0.966
Well to moderate	41	11	30	
Tumor size (cm)				
> 5	38	15	23	0.013
≤5	36	5	31	
TNM stage				
I + II	41	15	26	0.039
III + IV	33	5	28	
Lymphatic invasion				
Yes	48	8	40	0.006
No	26	12	14	

2.11. In vivo tumorigenesis assay

All animal experiments were approved by the First Affiliated Hospital of University of Science and Technology of China and performed in accordance with the Chinese guidelines for animal experiments. Four-week-old male BALB/C nude mice were purchased from the Institute of Zoology at the Chinese Academy of Sciences, Shanghai. To establish the peritoneal metastasis model, 1×10^6 cells were resuspended with 0.1 ml of PBS and injected into the abdominal cavity. After 80 days, the mice were sacrificed, and the number of nodules presented in the abdominal cavity and the volume of ascites from each mouse were measured. The survival time of each mice was recorded to generate the survival curves.

2.12. Soft agar colony formation assay

GC cells were resuspended with 0.3% soft agar (Sigma-Aldrich, USA) in RPMI 1640 medium containing 10% FBS and layered on 0.6% solidified agar in six-well plates. After two-week incubation, colony sizes were photographed under a microscope at 10 × magnification.

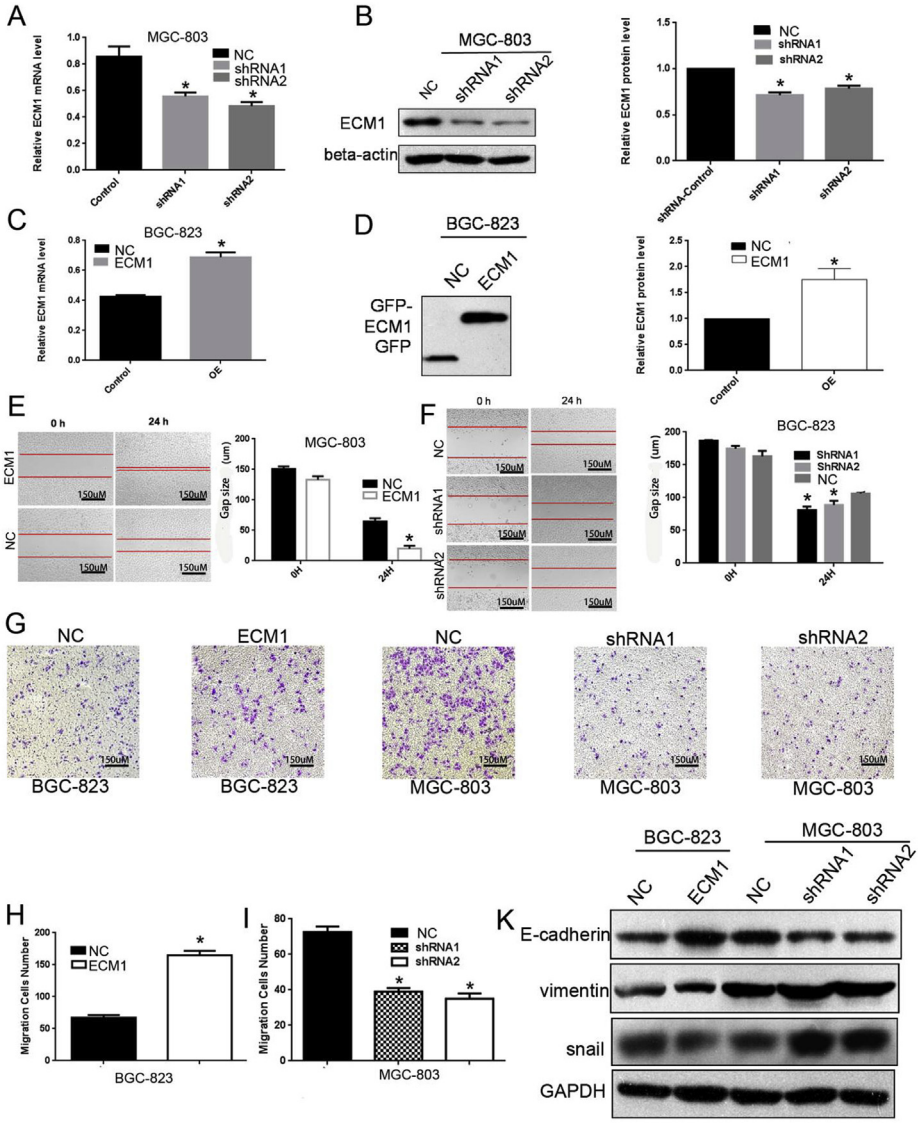

Fig. 2. ECM1 promoted the proliferation and migration of GC cells *in vitro*. (A, B) The relative mRNA and protein levels of ECM1 in MGC-803 cells transduced with shRNA or NC lentivirus. (C, D) The relative mRNA and protein levels of ECM1 in BGC-823 cells transduced with ECM1 or NC lentivirus. (E, F) Representative images (left) from ECM1-overexpressed MGC-803 cells, ECM1-knockdown BGC-823 cells and their control groups were recorded 0 and 24 h after scratching of the cell surface and their statistics results (right). (G) The migration behavior were evaluated by using a transwell chamber after GC cells infected with ECM1-overexpression, ECM1 shRNA lentivirus and their respective controls. (H) Histograms show the number of migrating cells from G. (K) Representative Immunoblotting image for EMT-related proteins in GC cells. The data are represented as the mean ± SD of three independent experiments (*$P < 0.05$).

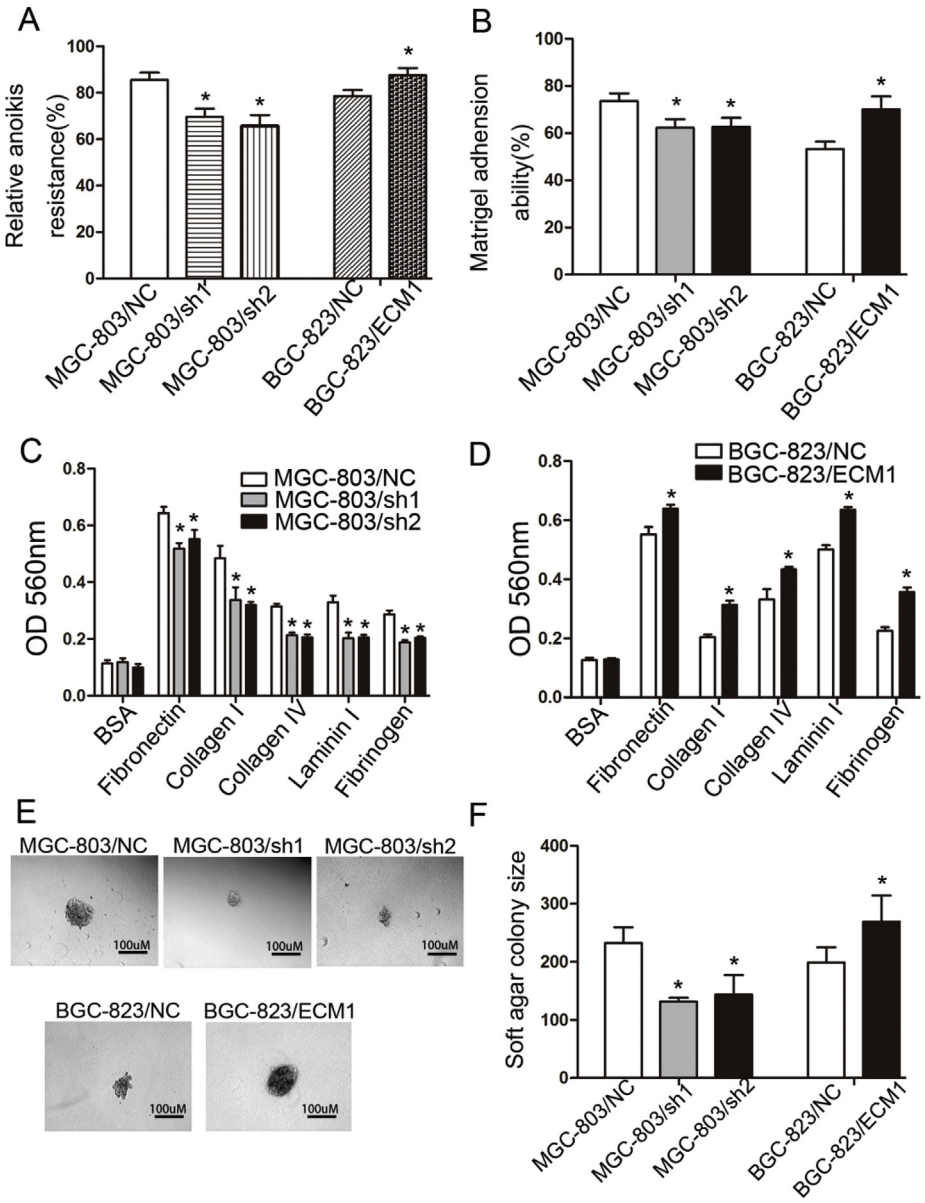

Fig. 3. ECM1 promoted the peritoneal migration of GC cells *in vitro*. (A) Anoikis assays of BGC-823 and MGC-803 cells infected with ECM1 overexpression lentivirus or a specific ECM1 shRNA lentivirus and the respective controls, as indicated. (B) Adhesion of ECM1 downregulated or overexpressed GC cells to Matrigel coated surfaces as indicated. (C, D) The percentage of GC cells in which ECM1 was knocked down (C) and overexpressed (D) that adhered to plates coated with different ECM components after 30 min of incubation were quantified by $OD_{560\ nm}$. (E, F) Representative images (E) and colony size of the indicated cells (F) in soft agar assay after 14 days of incubation. Error bars represent mean ± SD from three independent experiments. * represent $P < 0.05$ comparison with the control.

2.13. Luciferase reporter assay

3′-UTR of ECM1 gene or its mutant for potential miR-92a target site were amplified and subcloned into pMIR-Report luciferase reporter vector. MGC-803 and BGC-823 cells were seeded in a 24-well plate with a density of 1×10^5 cells per well and co-transfected with wild-type 3′-UTR or mutant 3′-UTR and miR-92a lentivirus or miR-92a inhibitors. After 48 h, luciferase activity was determined using a dual-luciferase reporter assay (Promega, USA) according to the manufacturer's protocol, in which Renilla luciferase activity was normalized to that of firefly luciferase (Gong et al., 2019).

2.14. Statistical analyses

Statistical analyses such as the $\chi 2$ test, Kaplan-Meier survival analysis and one-way ANOVA method were performed with SPSS 16.0 and GraphPad Prism 6. Data was expressed as the mean ± standard deviation (SD), and $P < 0.05$ and $P < 0.01$ were used to indicate statistically significant differences.

3. Results and discussion

3.1. ECM1 is upregulated in GC tissues and its connection to the survival and prognosis

ECM1 is a glycoprotein highly distributed in epithelial organs and throughout the intestine, which interacts with the basal membrane. Previous studies revealed that increased ECM1 expression was found in many types of malignant epithelial tumors (Kebebew et al., 2005; Wang et al., 2003). ECM1 was considered to promote cancer progression and invasion and also acts as an indicator of poor prognosis for cancer patients (Chen et al., 2011; Lal et al., 2009). To evaluate the expression of ECM1 in GC, ECM1 mRNA expression data was retrieved from the TCGA database (408 tumor cases and 211 normal control cases) and it showed that ECM1 mRNA level was significantly higher in tumor tissues compared to normal tissues (Fig. 1A). The Kaplan Meier analysis and the log-rank test results ($p = 1.6e\text{-}13$) revealed the overall survival of patients with low ECM1 expression level was significantly higher than that of high ECM1 expression (Fig. 1B, $p < 0.001$). Further the expression study of ECM1 in gastric tissues showed weak or strong positive signal in tumor tissues, but negative signal in paracarcinoma

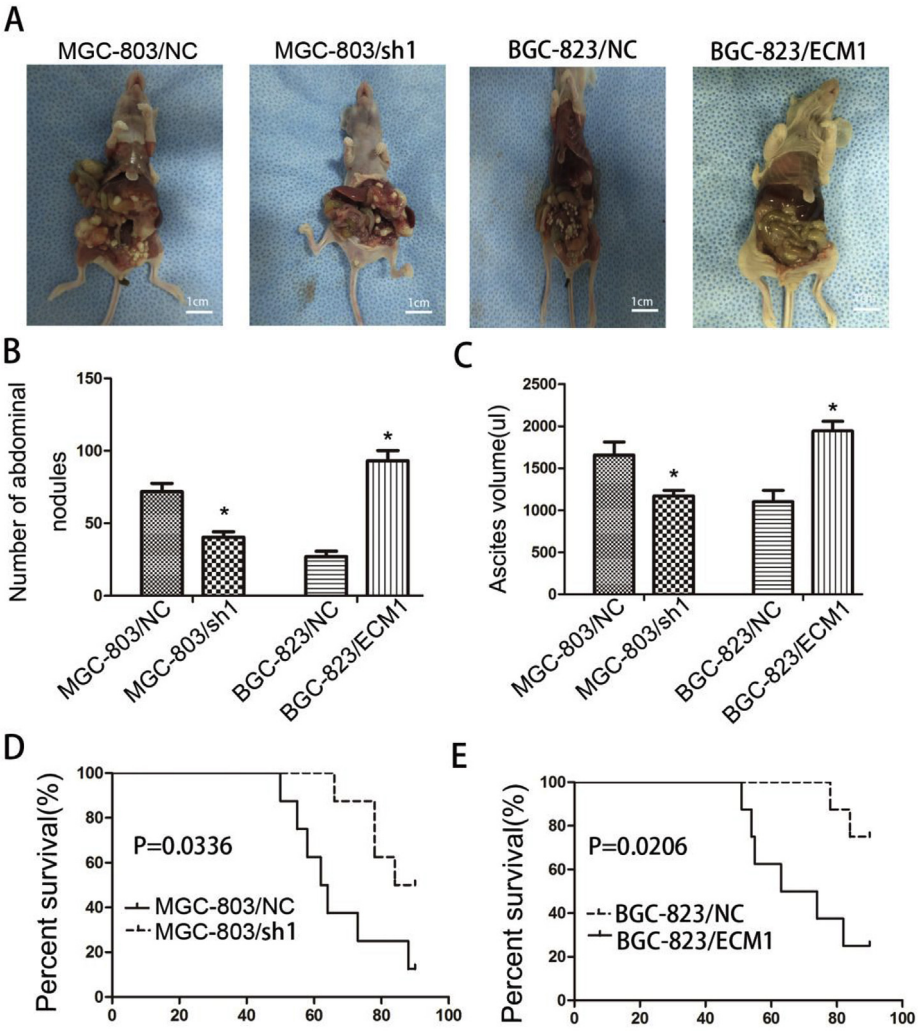

Fig. 4. ECM1 promoted GC cell metastasis *in vivo*. (A–C) *In vivo* tumorigenesis examined by subcutaneous tumor growth from mice injected with cells expressing either ECM1 cDNA or shRNA and the respective controls. (D, E) Kaplan-Meier analysis of overall survival of mice with high or low expression of ECM1. Error bars represent mean ± SD from three independent experiments (*$P < 0.05$).

tissues (Fig. 1C). ECM1 expression levels in tissues from 74 gastric cancer patients were investigated to further confirm its association with clinicopathologic and prognostic value (showed in Table 1). It was found a significant association between ECM1 expression and lymphatic vessel invasion, tumor size, tumor (T) stage, node (N) stage and metastasis (M) stage. ECM1 mRNA level was significant higher in tumor tissues than normal tissues as well (Fig. 1D), and patients with low expression of ECM1 showed a better prognosis than that of high ECM1 expression (Fig. 1E). These findings suggested increased ECM1 expression was correlated with carcinogenesis and lymphatic metastasis in human gastric cancer, but the mechanism remains elusive.

3.2. ECM1 promoted the migration of human GC cells in vitro

To investigate the role of ECM1 in human GC, western blotting and qRT-PCR were performed to determine ECM1 expression level in four human GC cell lines (SGC-7901, MGC-803, MKN-45, and BGC-823). As illustrated in Fig. 1F and G, BGC-823 cells showed the lowest ECM1 expression level, and MGC-803 cells had the highest ECM1 expression level among the four GC cell lines. MGC-803 and BGC-823 cells were chosen to use in the following functional experiments. To manipulate ECM1 expression in GC cells, two lentivirus-based short hairpin RNAs (shRNAs) against ECM1 were employed to efficiently knockdown its expression in MGC-803 cells ($p < 0.05$, Fig. 2A and B) or a lentivirus encoded GFP-ECM1 in BGC-823 cells to overexpress the fusion protein ($p < 0.05$, Fig. 2C–E). To investigate the effect of ECM1 on GC cell motility, a wound-healing assay was performed and it showed that the wound-healing ability was significantly enhanced in ECM1-overexpressed MGC-803 cells ($p < 0.05$, Fig. 2E), while suppressed in ECM1-knockdown BGC-823 cells ($p < 0.01$, Fig. 2F). Likewise, ECM1 overexpression induced a significant increase in the number of migration cells, while ECM1 knockdown resulted in fewer migration cells in the transwell assays compared to the control ($p < 0.01$, Fig. 2G–I). These results suggested that ECM1 can promote the migration of gastric cancer cells. ECM1 has been indicated by increasing evidences to exert its functions on cancer cell metastasis. To determine whether ECM1 affect EMT, the protein expression levels of EMT-related proteins including E-cadherin, vimentin and Snail were investigated and it was found a correlation between ECM1 protein and EMT-related proteins. Specifically, vimentin and Snail were increased, but E-cadherin was decreased in ECM1-overexpressed BGC-823 cells. On the contrary, vimentin and Snail were decreased but E-cadherin was increased in the MGC-803 cells infected with ECM1 shRNA lentiviruses (Fig. 2K). These results indicated ECM1 plays a key role in GC metastasis and progression, facilitating the expression of EMT-related proteins.

In consideration of a detached microenvironment where GC resides after passing through the serous layer into the abdominal cavity and the importance of adhesion ability in peritoneal metastasis, we investigated

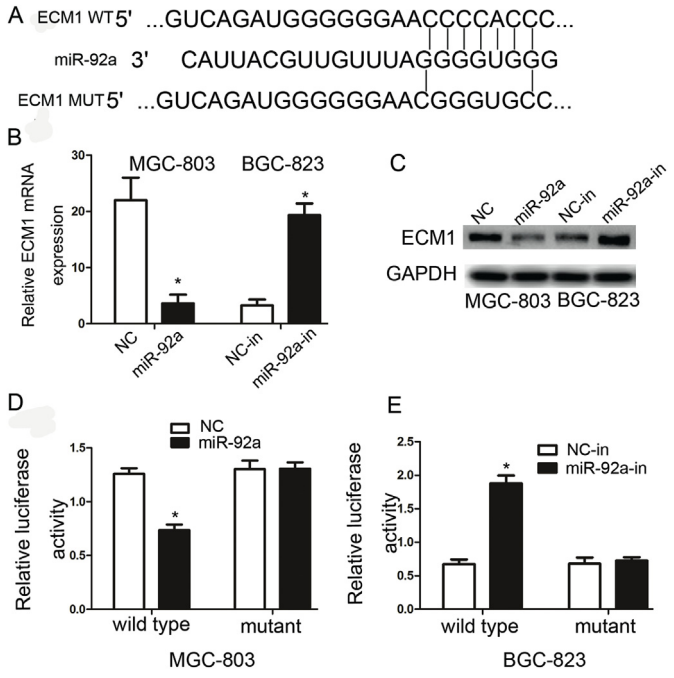

Fig. 5. ECM1 is a target of miR-92a. (A) Schematic graph of the putative binding sites of miR-92a in the ECM1 3′UTR. ECM1-mut indicates the ECM1-3′UTR with mutation in miR-92a binding sites. (B–C) ECM1 mRNA (B) and protein level (C) after GC cells infected with miR-92a lentivirus or transfected with miR-92a inhibitors and their respective controls. (D–E) Luciferase reporter activity after Luciferase reporter activity after infected with miR-92a lentivirus (D) or transfected with miR-92a inhibitors (E) and their respective controls in wild-type ECM1 3′UTR or mutant ECM1 3′UTR reporter. Error bars represent mean ± SD from three independent experiments (*$p < 0.05$).

the effect of ECM1 expression on the relative anoikis resistance and adhesion ability to Matrigel coated surfaces. As shown in Fig. 3A and B, overexpression of ECM1 significantly increased the relative anoikis resistance and adhesion ability to Matrigel coated surfaces compared with the control. Conversely, knockdown ECM1 expression led to the decrease in anoikis resistance compared with the control, and adhesion ability as well ($p < 0.05$, Fig. 3A and B). To further confirm the effect of ECM1 on GC cell adhesion, adhesion ability to different extracellular matrix (ECM) components was examined, including Fibronectin, Collagen I, Collagen IV, Lamin I and Fibrinogen. As expected, the adhesion ability to different ECM component was significantly attenuated in ECM1-knockdown cells, while enhanced in ECM1-overexpressed cells ($p < 0.05$, Fig. 3C and D). To simulate the anchorage independent growth environment of GC cells in the abdominal cavity, a soft agar colony-formation assay was conducted and we also observed a remarkable increase in the size of soft agar colony was observed in cells transplanted with ECM1-overexpressed cells, while a significant decrease was noticed in ECM1 knockdown cells (Fig. 3E and F). These results indicated that ECM1 can promote the peritoneal metastasis-related traits of GC cells *in vitro*.

3.3. ECM1 promoted the migration of GC cells in vivo

To investigate whether ECM1 affect GC development *in vivo*, ECM1-knockdown MGC-803 and ECM1-overexpressed BGC-823 cells and their respective control cells were subcutaneously injected into the nude mice. As shown in Fig. 4 A-C, compared with the corresponding control, the number of abdominal nodules and the ascites volume were significantly increased in the ECM1-overexpressed mice, while decreased in the ECM1 knockdown mice ($p < 0.05$). The overall survival percentage for the xenograft mice was significantly increased by ECM1 knockdown ($p = 0.0336$) and decreased by the overexpression of ECM1 ($p = 0.0206$) (Fig. 4 D, E). Collectively, these results highlighted the metastasis promotion role of ECM1 in human GC both *in vitro* and *in vivo*.

3.4. ECM1 as a direct target of miR-92a

miRNA has been demonstrated to regulate multiple cellular functions and especially abnormal miR-92a expression has been reported in multiple cancers with a conflicting role (Chen et al., 2018; Li et al., 2014; Nilsson et al., 2012; Xiao et al., 2017; Yoshizawa et al., 2012; Zhou et al., 2015). To explore the role of miR-92a in gastric cancer and its potential regulation on ECM1 expression, we performed a miRNA

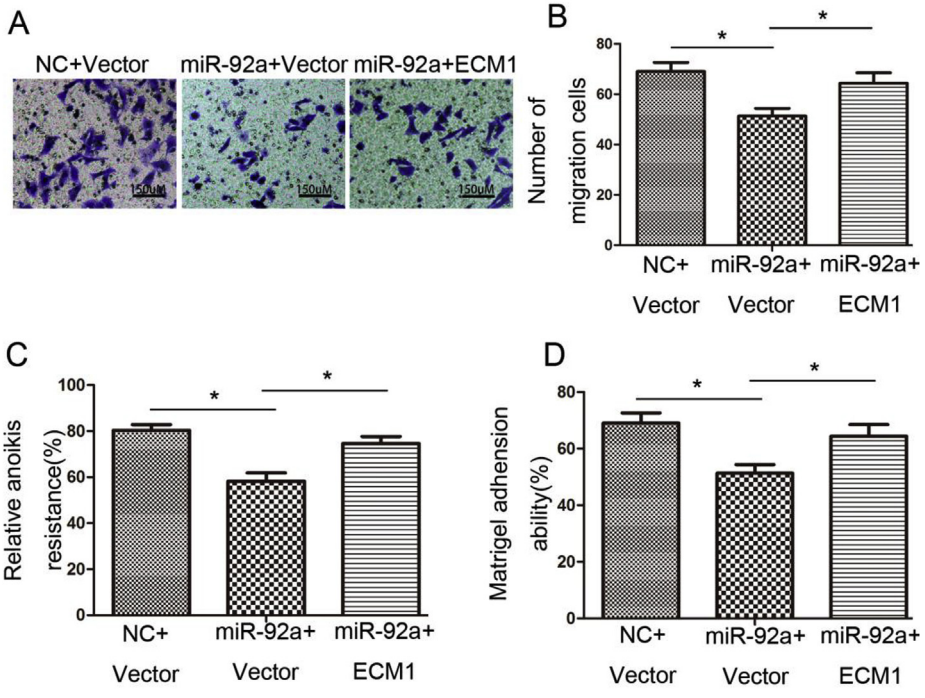

Fig. 6. miR-92a affects the peritoneal migration of GC cells by targeting ECM1. (A, B) Representative images (A) of MGC-803 cell migration in transwell assay and number of migration cells (B) after the indicated treatment. (C, D) Anoikis assays (C) and adhesion to Matrigel coated surfaces (D) of MGC-803 cells infected with miR-92a lentivirus or miR-92a inhibitor lentivirus or the control plus ECM1 or the control, as indicated. All the data are expressed as means ± SD of three independent experiments ($p < 0.05$).

binding site prediction with the STarMir tool and found a putative binding site of miR-92a within the 3′UTR of ECM1 gene. The matched sequences were illustrated in Fig. 5A and a mutant construct of ECM1 3′UTR was generated by site-directed mutagenesis to disrupt the miR-92a binding site (Fig. 5A). In MGC-803 cells, infection with miR-92a lentivirus induced a significant decrease in the mRNA and protein levels of ECM1, while the induction of miR-92a inhibitor into BGC-823 cells caused a significant increase expression of ECM1 at both mRNA and protein levels ($p < 0.05$, Fig. 5B and C). To further dissect the role of miR-92a on ECM1 expression and confirm the binding site of miR-92a within the 3′UTR of ECM1 gene, we conducted a luciferase reporter assay, in which the relative luciferase activity reflects the ECM1 mRNA level regulated by miR-92a. As shown in Fig. 5D, MGC-803 cells infected with miR-92a virus exhibited a significant decrease in the relative luciferase activity and disruption of the putative binding site of miR-92a diminished the effect ($p < 0.05$). In BGC-823 cells, induction of miR-92a inhibitor significantly increased the relative luciferase activity and it was also reversed by the mutation within the putative binding site ($p < 0.05$, Fig. 5E). To further confirm the role of miR-92a in gastric cancer, we employed MGC-803 cells to conduct the migration assay. As expected, miR-92a showed an inhibitory effect on the migration capacity of MGC-803 cells and this effect could be blocked by ECM1 overexpression ($p < 0.05$, Fig. 6A and B). It was also observed the relative anoikis resistance and adhension ability of MGC-803 cells to Matrigel coated surfaces were significantly reduced by miR-92a, which could be restored by ECM1 overexpression ($p < 0.05$, Fig. 6C and D).

4. Conclusion

In present study, GC patients with higher ECM1 expression exhibited significantly poor overall survival and ECM1 promoted human GC cell migration through enhancing the expression of EMT-related proteins. We demonstrated for the first time that miR-92a can suppress GC metastasis by directly targeting 3′UTR of ECM1 gene. Nonetheless, the expression profile of miR-92a in gastric cancer need to be further investigated and it should be more convincing to repeat the experiments in the genetic manipulated mouse models. These findings provide a novel understanding about the antagonistic roles of ECM1 and miR-92a in GC development and a new insight into developing potential therapeutic target to combat the human GC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by grants from the Anhui Science and Technology Research Project (1501041142), Anhui Provincial Key Laboratory Performance Project (1606c08236, 2017070503B041, 2018080503B0031), National Natural Science Foundation of China (81802329) and the Fundamental Research Funds for the Central Universities (WK911000038).

References

Arathi, B.P., Sowmya, P.R., Kuriakose, G.C., Vijay, K., Baskaran, V., Jayabaskaran, C., Lakshminarayana, R., 2016. Enhanced cytotoxic and apoptosis inducing activity of lycopene oxidation products in different cancer cell lines. Food Chem. Toxicol. 97, 265–276.

Barger, J.F., Rahman, M.A., Jackson, D., Acunzo, M., Nana-Sinkam, S.P., 2016. Extracellular miRNAs as biomarkers in cancer. Food Chem. Toxicol. 98, 66–72.

Brooks, S.A., Fry, R.C., 2017. Cadmium inhibits placental trophoblast cell migration via miRNA regulation of the transforming growth factor beta (TGF-β) pathway. Food Chem. Toxicol. 109, 721–726.

Chaffer, C.L., San Juan, B.P., Lim, E., Weinberg, R.A., 2016. EMT, cell plasticity and metastasis. Cancer Metastasis Rev. 35, 645–654.

Chen, E.F., Li, Q.Q., Wang, H., Yang, F.F., Min, L.L., Yang, J., 2018. MiR-92a promotes tumorigenesis of colorectal cancer, a transcriptomic and functional based study. Biomed. Pharmacother. 106, 1370–1377.

Chen, H., Jia, W., Li, J., 2016. ECM1 promotes migration and invasion of hepatocellular carcinoma by inducing epithelial-mesenchymal transition. World J. Surg. Oncol. 14, 195.

Chen, H., Jia, W.D., Li, J.S., Wang, W., Xu, G.L., Ma, J.L., Ren, W.H., Ge, Y.S., Yu, J.H., Liu, W.B., Zhang, C.H., Wang, Y.C., 2011. Extracellular matrix protein 1, a novel prognostic factor, is associated with metastatic potential of hepatocellular carcinoma. Med. Oncol. 28, S318–S325.

Gomez-Contreras, P., Ramiro-Diaz, J.M., Sierra, A., Stipp, C., Domann, F.E., Weigel, R.J., Lal, G., 2017. Extracellular matrix 1 (ECM1) regulates the actin cytoskeletal architecture of aggressive breast cancer cells in part via S100A4 and Rho-family GTPases. Clin. Exp. Metastasis 34, 37–49.

Gong, L., Zhu, H., Li, T., Ming, G., Duan, X., Wang, J., Jiang, Y., 2019. Molecular signatures of cytotoxic effects in human embryonic kidney 293 cells treated with single and mixture of ochratoxin A and citrinin. Food Chem. Toxicol. 123, 374–384.

Gu, M., Guan, J., Zhao, L., Ni, K., Li, X., Han, Z., 2013. Correlation of ECM1 expression level with the pathogenesis and metastasis of laryngeal carcinoma. Int. J. Clin. Exp. Pathol. 6, 1132–1137.

Kebebew, E., Peng, M., Reiff, E., Duh, Q.Y., Clark, O.H., McMillan, A., 2005. ECM1 and TMPRSS4 are diagnostic markers of malignant thyroid neoplasms and improve the accuracy of fine needle aspiration biopsy. Ann. Surg. 242, 353–361.

Lal, G., Hashimi, S., Smith, B.J., Lynch, C.F., Zhang, L.R., Robinson, R.A., Weigel, R.J., 2009. Extracellular Matrix 1 (ECM1) Expression is a novel prognostic marker for poor long-term survival in breast cancer: a hospital-based cohort study in Iowa. Ann. Surg. Oncol. 16, 2280–2287.

Li, M.L., Guan, X.F., Sun, Y.Q., Mi, J., Shu, X.H., Liu, F., Li, C.G., 2014. miR-92a family and their target genes in tumorigenesis and metastasis. Exp. Cell Res. 323, 1–6.

Nilsson, S., Moller, C., Jirstrom, K., Lee, A., Busch, S., Lamb, R., Landberg, G., 2012. Downregulation of miR-92a is associated with aggressive breast cancer Features and increased tumour macrophage infiltration. PLoS One 7, e36051.

Pradella, D., Naro, C., Sette, C., Ghigna, C., 2017. EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression. Mol. Cancer 16, 8.

Qi, C.C., Zhang, Z., Fang, H., Liu, J., Zhou, N., Ge, J.F., Chen, F.H., Xiang, C.B., Zhou, J.N., 2014. Antidepressant effects of abscisic acid mediated by the downregulation of corticotrophin-releasing hormone gene expression in rats. Int. J. Neuropsychopharmacol. 18.

Qiu, M.Z., Xu, R.H., 2013. The progress of targeted therapy in advanced gastric cancer. Biomark. Res. 1, 32.

Rodríguez-Ribera, L., Slattery, C., Mc Morrow, T., Marcos, R., Pastor, S., 2017. Reactive carbonyl compounds impair wound healing by vimentin collapse and loss of the primary cilium. Food Chem. Toxicol. 108, 128–138.

Santamaria, P.G., Moreno-Bueno, G., Portillo, F., Cano, A., 2017. EMT: present and future in clinical oncology. Mol. Oncol. 11, 718–738.

Siegel, R.L., Miller, K.D., Jemal, A., 2016. Cancer statistics, 2016. CA A Cancer J. Clin. 66, 7–30.

Wang, J., Liao, A.M., Thakur, K., Zhang, J.G., Huang, J.H., Wei, Z.J., 2019. Licochalcone B extracted from *Glycyrrhiza uralensis* Fisch induces apoptotic effects in human hepatoma cell HepG2. J. Agric. Food Chem. 67, 3341–3353.

Wang, J., Zhang, Y.S., Thakur, K., Hussaina, S.S., Zhang, J.G., Xiao, G.R., Wei, Z.J., 2018. Licochalcone A from licorice root, an inhibitor of human hepatoma cell growth via induction of cell apoptosis and cell cycle arrest. Food Chem. Toxicol. 120, 407–417.

Wang, L.P., Yu, J.Y., Jian, N., Xu, X.M., Wang, J.J., Ning, H.Y., Pei, X.F., Chen, J.G., Yang, S.M., Underhill, C.B., Lei, L., Liekens, J., Merregaert, J., Zhang, L.R., 2003. Extracellular matrix protein 1 (ECM1) is over-expressed in malignant epithelial tumors. Cancer Lett. 200, 57–67.

White, M.C., Pang, L., Yang, X., 2016. MicroRNA-mediated maturation of human pluripotent stem cell-derived cardiomyocytes: towards a better model for cardiotoxicity? Food Chem. Toxicol. 98, 17–24.

Xiao, J., Yu, W.F., Hu, K.Z., Li, M.Q., Chen, J.W., Li, Z.C., 2017. miR-92a promotes tumor growth of osteosarcoma by targeting PTEN/AKT signaling pathway. Oncol. Rep. 37, 2513–2521.

Xiong, G.P., Zhang, J.X., Gu, S.P., Wu, Y.B., Liu, J.F., 2012. Overexpression of ECM1 contributes to migration and invasion in cholangiocarcinoma cell. Neoplasma 59, 409–415.

Ye, H., Yu, X., Xia, J., Tang, X., Tang, L., Chen, F., 2016. MiR-486-3p targeting ECM1 represses cell proliferation and metastasis in cervical cancer. Biomed. Pharmacother. 80, 109–114.

Yoshizawa, S., Ohyashiki, J.H., Ohyashiki, M., Umezu, T., Suzuki, K., Inagaki, A., Iida, S., Ohyashiki, K., 2012. Downregulated plasma miR-92a levels have clinical impact on multiple myeloma and related disorders. Blood Canc. J. 2, e53.

Zhang, Y.S., Ma, Y.L., Thakur, K., Hussain, S.S., Wang, J., Zhang, Q., Zhang, J.G., Wei, Z.J., 2018. Molecular mechanism and inhibitory targets of dioscin in HepG2 cells. Food Chem. Toxicol. 120, 143–154.

Zhou, C.Y., Shen, L.F., Mao, L., Wang, B., Li, Y., Yu, H.Z., 2015. miR-92a is upregulated in cervical cancer and promotes cell proliferation and invasion by targeting FBXW7. Biochem. Bioph. Res. Co. 458, 63–69.