ORIGINAL RESEARCH

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MicroRNA-222 promotes the proliferation and migration of cervical cancer cells

Abstract

Purpose: This study aimed to investigate the role of small non-coding RNA-222 (microRNA-222; miR-222) in the development of cervical cancer (CC).

Methods: Normal and CC specimens were obtained from 18 patients. HeLa and SiHa cells were grown in Dulbecco's modified Eagle's medium. RT-PCR, Western blot, migration assay, flow cytometry and immunofluorescence microscopy were used for analyses.

Results: When compared with normal cervical tissues, miR-222 was upregulated in human CC, and the extent of up-regulation was associated with the extent and depth of CC invasion. Expression of miR-222 was inversely related to the expression of phosphatase and tensin homolog (PTEN) and p27. The reduced the expression of PTEN and p27 by miR-222 in HeLa cells and SiHa cells was associated with increased proliferation and migration of CC cells. The expression of proteins (E-cadherin and paxillin) related to the proliferation and migration was also elevated.

Conclusion: MiR-222 plays an important role in the tumorigenesis of CC, possibly by specifically down-regulating p27^{Kip1} and PTEN. Our findings suggest that miR-222 may serve as a new therapeutic target in CC.

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Cervical cancer (CC), one of the common cancers in women worldwide, is a leading cause of cancer-related death in developing countries. Elucidation of the molecular mechanisms involved in the pathogenesis of CC are fundamental to an understanding of the occurrence and development of CC. In spite of great improvements achieved in the surgical interventions and chemoradiotherapy of CC, the prognosis for CC is still very poor. Therapeutic strategies for this disease have not changed dramatically for many years, and are mostly based on a limited understanding of the biology of CC. Although a number of genetic and molecular changes have been found to correlate with the progression of CC, an in-depth understanding of the molecular markers would be helpful to identify the targets for the treatment of CC.

p27 and phosphatase and tensin homolog (PTEN) are the biomolecular prognostic factors for cervical cancer and have negative correlation with the depth of stromal invasion, tumor size, clinical stage and survival [1,2]. Small non-coding RNAs (microRNAs; miRs) are a class of endogenous non-coding, highly conserved RNAs with about 22 nucleotides in length that are encoded in plant and animal genomes. MiRNAs have been found to be involved in the pathogenesis of most cancers [3]. They can down-regulate mRNA expression by repressing translation or by directly cleaving the targeted mRNA. In recent years, our understanding of miRNA has expanded from the initially identified functions in the development of round worms to highly expressed and ubiquitous regu-

lators implicated in a wide array of critical processes, including proliferation, cell death, differentiation, metabolism and, importantly, tumorigenesis. Over-expressed miRNAs may act as oncogenes to down-regulate tumor suppressor genes and/or genes controlling cell differentiation and apoptosis, whereas the down-regulated miRNAs act as tumor suppressor genes to negatively regulate oncogenes and/or genes controlling cell differentiation and apoptosis [4,5]. MiR-222 is composed of two highly homologous miRNAs, whose up-regulation has been recently described in several types of human cancers. MiR-222 has been considered to act as oncogenes or tumor suppressors, depending on the tumor microenvironment [6]. PCR technology has been used to investigate the expression of miRNAs in CC and adjacent normal tissues. This study was undertaken to investigate the role of miR-222 in CC. Our results may provide a new target for the treatment of CC.

Materials and Methods

Sample collection

Specimens (n = 38) were obtained from patients in the Shanghai Tenth People's Hospital, Tongji University Medical College, from 2004 to 2012. Of the 38 specimens, 19 were CC and 19 were adjacent normal tissues from the same patients (NC). Samples were immediately placed in liquid nitrogen and stored at -80°C for use.

Information on one patient was unavailable, so samples from only 18 patients were studied. The median age was 46.77 years (range: 36-61 years). There are 12 (67%) patients who were in the stage I of cancer, while 6 (33%) cases were in the stages II-IV. Among these specimens, 10 (56%) were positive for HPV and 8 (44%) negative for HPV, including 5 (28%) specimens with the invasion depth of less than 5 mm and the rest 13 (72%) with the invasion depth of deeper than 5 mm. In 8 (44%) patients, the tumor size was smaller than 5 cm². Moreover, 67% of patients were diagnosed as CC at stage I and 23% with CC in stage II-IV. Eight (44%) patients received chemotherapy and 10 (56%) had had no chemotherapy at the start of the study (Table 1).

Informed consent was obtained from each patient and the study was approved by the ethics committee of our hospital before the start of the study.

Cell lines and transfections

Human cervical cancer cell line HeLa cells and SiHa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), glutamine, and ampicillin/streptomycin in a 5% CO₂. For transfection assay, HeLa cells

TABLE 1. Characteristics of the CC tumors

Parameters	Numbers	Percents
Stage		
I	12	67%
>I	6	33%
HPV		
(+)	10	56%
(-)	8	44%
Invasion of depth		
<5 mm	5	28%
≥5 mm	13	72%
Size of tumor		
<5 cm ²	8	44%
≥5 cm ²	10	56%
Chemotherapy		
(+)	8	44%
(-)	10	56%

were plated at a density of 2.5×10^5 cells/well in 6-well plates (three wells in each group), and transfection was done with Lipofectamine 2000 (Invitrogen, San Juan, PR, USA) according to the manufacturer's instructions. MiR-222 was synthesized by the Fidelity Systems (Gaithersburg, MD, USA) and used at the final concentration of 100 nM.

RNA extraction and RT-PCR

Total RNAs (miRNA and mRNA) were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of total miRNA was performed with equal amounts of total RNA/sample (1 mg) using miScript reverse Transcription Kit (Qiagen, Milan, Italy). For reverse transcription of mRNA, SuperScript III Reverse Transcriptase (Invitrogen) was used. For cultured cells, quantitative analysis of miR-222 and U6 (as an internal reference) were performed with RT-PCR using specific primers (Qiagen) (miR-222 RT7: CTCAACTGGTGTCGTGGAGTCGGCAGTTGAGaccagt miR-222 F18: acactccagctgggAGCTACATCTGGCTA CTG), miScript SYBR Green PCR Kit (Qiagen) and iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), respectively. The reaction for detection of mRNAs was performed as follows: 95°C for 15 s, 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The reaction for detection of miRNAs was performed as follows: 95°C for 15 s, 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. The threshold cycle is defined as the cycle number at which the fluorescence passes the fixed threshold. For quantification, the $2^{-\Delta\Delta C_t}$ method was used as previously described [7] Experiments were carried out in triplicate at each time point.

Western blot assay

The cells were washed in ice-cold PBS, and subsequently lysed in ice-cold NP40 lysis buffer (0.5% NP40, 50 mM HEPES [pH=7], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4 , 0.5 mM phenylmethylsulfonyl fluoride, complete inhibitor; Roche, Basel, Switzerland). Proteins were subjected to polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad), incubated with specific primary antibodies, and visualized using enhanced chemiluminescence kit (Central Laboratory of the Shanghai Tenth People's Hospital, China). For immunoblotting, membranes were blocked for 1 h with 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween-20, and incubated at 4°C over night with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence kit (Shanghai Tenth People's Hos-

pital, China). The antibodies used in this work were: anti-p27 (sc-56338, Santa Cruz Biotechnology, Dallas, TX USA), anti-p21 (#2947s cell signaling Inc) anti-e-cadherin (sc-52327, Santa Cruz Biotechnology), anti-PTEN (sc-9145, Santa Cruz Biotechnology) and anti-paxillin (610051, BD Transduction Laboratories, Chestnut Hill, MA, USA).

Migration assay

Transwells Permeable Supports with 6.5-mm diameter inserts, 8.0 μm pore size, and polycarbonate membrane (Corning Incorporate, Corning, NY, USA) were used to perform migration assay. HeLa cells and SiHa cells were grown as indicated above, and harvested by TrypLE Express (Invitrogen). Then, 10^5 cells were washed three times and re-suspended in 1% FBS containing Dulbecco's modified Eagle's medium and seeded in the upper chamber. The lower chamber was filled with 500 μl of culture medium containing 10% FBS and 5 $\mu\text{g/ml}$ fibronectin. Cells were incubated at 37°C for 24 h. The transwells were then removed from the 24-well plates and stained with 0.1% crystal violet in 25% methanol. Non-migrated cells were removed from the top of transwell with a cotton swab. The percentage of migrated cells was determined by eluting crystal violet in 1% SDS. Representative images were captured under a microscope.

Flow cytometry

For cell cycle detection, 1×10^6 cells were collected and fixed in 70% ice-cold ethanol at 4°C overnight. Before staining, all samples were washed twice with PBS, treated with 1 mg/ml ribonuclease A and stained with 20 $\mu\text{g/ml}$ propidium iodide (Sigma-Aldrich, St. Louis, MO, USA), followed by flow cytometry (FACScan). For apoptosis analysis, cells were harvested and stained in duplicate with Allophycocyanin-annexin V and propidium iodide (Sigma-Aldrich) according to the manufacturer's instructions. The percent of apoptotic cells was determined as the apoptosis index (AI). Each experiment was performed in triplicate.

Immunofluorescence microscopy

Cells were grown on coverslips and fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in 1% BSA. Primary anti-bodies were used at 1:1000 in blocking buffer. After incubation for 1-24 h at room temperature (depending on the antibodies), cells were washed in blocking buffer and then incubated with secondary antibody at 1:500 in blocking buffer (plus fluorescently-labeled phalloidin if required) for 1 h. Coverslips were washed and then mounted on

FIGURE 1. MiR-222 expression in the cervical cancer (CC). Quantitative RT-PCR showed about 2.48-fold increase in miR-222 expression in CC. ($P < 0.05$, Wilcoxon test) (A). Results showed the miR-222 expression increased as the invasion depth increased (median, 1.22 and 2.97, $P < 0.05$; Mann-Whitney test) (B) as well as the volume of the tumor (median, 1.2134 and 3.1152, $P < 0.05$, Mann-Whitney test) (C). MiR-222 expression was not associated with chemotherapy (D), stage of cancers (E) or HPV-infection (F). NC: no cancer, CC: cervical cancer

FIGURE 2. Relationship between p27, PTEN and miR-222. The miR-222 expression was increased in transfected HeLa and SiHa cells (A). Spearman's correlation analysis showed significantly negative correlation between p27 and miR-222(B) as well as between PTEN and miR-222(C) ($R=-0.556$, $P=0.038$; $R=-0.552$, $P=0.049$, respectively).

a slide with Vectashield (with or without DAPI) or ProLong Gold antifade and sealed with clear nail varnish. The slides were imaged using the confocal microscope and LCS software (Leica, Solms, Germany).

Statistical analysis

All of the experiments were performed for three times. Continuous variables were expressed as means $\pm$ standard deviation. One-tailed Student's t-test was used for comparisons. A value of $P<0.05$ was considered statistically significant. Data were expressed as means from experiments performed in triplicate. Statistical analysis of the tissues samples was performed using the Mann-Whitney test to evaluate the significance of differences between groups.

Results

MiR-222 expression in CC

To evaluate the miR-222 expression *in vivo*, the miR-222 expression was detected in CC tissues and adjacent normal tissues. Quantitative RT-PCR showed a 2.48-fold increase in miR-222 expression in CC as compared with that in adjacent normal tissues ($P<0.01$; Figure 1A). In addition, the miR-222 expression increased as the invasion depth (median, 1.22 and 2.97, $P<0.05$, Mann-Whitney) and the tumor volume (median, 1.21 and 3.11, $P<0.05$, Mann-Whitney) increased (Figure 1B and C). miR-222 expression was not associated with chemotherapy (Figure 1D), stage of CC (Figure 1E) or HPV infection (Figure 1F).

TargetScan analysis showed that p27 and PTEN were the targets of miR-222, but the clinicopathological findings did

not demonstrate a relationship between miR-222 and p27 or PTEN in CC. To further investigate the association of miR-222 with p27 and PTEN, the expression levels of p27 and PTEN were determined in 10 pairs of samples by RT-PCR. Spearman's correlation analysis showed significantly negative correlation between p27 and miR-222, as well as between PTEN and miR-222 ($R=-0.556$, $P=0.038$; $R=-0.552$, $P=0.049$, respectively, Figure 2 B,C).

Impact of miR-222 over-expression in HeLa cells and SiHa cells

MiR-222 promotes the growth of HeLa cells and SiHa cells

To test whether miR-222 functions as an oncogene, the effect of miR-222 on CC cell proliferation was investigated. Firstly, the increase in miR-222 expression was determined in transfected HeLa and SiHa cells using RT-PCR (Figure-2A) (256- and 512-fold increase, respectively). At 48 h post-transfection, the effect of miR-222 over-expression on cell proliferation was evaluated by MTT assay and colony-formation test. MiR-222 significantly increased the proliferation of CC cells when compared with control group (Figure 3A and C, Figure-3B and D, Figure 3E). Moreover, the protein expression of p27 was also measured in HeLa and SiHa cells at 48 h after transfection with miR-222 and compared with the control group. Over-expression of miR-222 resulted in the down-regulation of p27 expression (Figure 3F). A reduction was consistently observed in cell growth after treatment with antisense oligonucleotides against miR-222.

FIGURE 3. MiR-222 up-regulates the proliferation of HeLa and SiHa cells.

MiR-222 significantly increased the proliferation of CC cells when compared with control group (MTT assay: A and C, colony-formation test: B, D, E). The protein expression of p27 was also measured in HeLa and SiHa cells at 48 h after transfection with miR-222 and compared with control group. Over-expression of miR-222 resulted in the down-regulation of p27 expression (F).

MiR-222 elevates the number of HeLa cells and SiHa cells in S phase

After transfection with miR-222 in HeLa cells, the cell cycle was examined by flow cytometry. Results showed the number of HeLa and SiHa cells in S phase increased when compared

with control group (Figure 4A, Figure 4B, Figure 4D). There was no significant difference in apoptosis between the miR-222 transfection group and the control group in either cell line. The up-regulation of miR-222 in HeLa cells did not induce an increase in cell apoptosis, suggesting miR-222 may not affect the

FIGURE 4. Ectopic expression of miR-222 affects cell cycle of HeLa and SiHa cells. After transfection with miR-222 in HeLa (A) and SiHa (B) cells, cell cycle was examined by flow cytometry. There was no significant difference in the extent of apoptosis of HeLa cells transfected with scrambled RNA or miR-222(C). The number of HeLa and SiHa cells in S phase increased when compared with the control group (D).

apoptosis of HeLa cells (Figure 4C). Similar findings were observed in SiHa cells (data not shown).

MiR-222 enhances the migration and invasion of HeLa and SiHa cells

Previous work has shown that miR-222 may increase the invasiveness of some cancers, such as hepatocarcinoma, glioma and gastric cancer [8]. To demonstrate the role of miR-222 in the migration of HeLa cells, the scratch wound healing assay was performed to investigate the influence of miR-222 over-expression on the migration of HeLa and SiHa cells. At 48 h after transfection, the wound width in the treated group was smaller than that in control group (Figure 5-A, Figure 5-B). To further demonstrate the effects of miR-222 on the invasion of HeLa and SiHa cells, the Transwell invasion assay was done. Similar findings were acquired (Figure 5C, Figure 5D). Furthermore, over-expression of miR-222 in HeLa cells and SiHa cells reduced the CDH1 and PTEN expression with an increase in Paxillin expression in HeLa cells and MMP1 expression in SiHa cells: two proteins related to the invasion ability (8) (Figure 6, Figure 7).

The above findings demonstrate that the transfection of miR-222 increases the invasion of HeLa and SiHa cells, suggesting the elevated invasion of CC.

Discussion

Studies have been undertaken to investigate the correlation between several human cancers and expression of miRNAs [3]. It has been found that the locations of miRNAs in the genome seem to be non-random, and a significant number of miRNA are located at the fragile sites (unstable regions that have been shown to promote DNA instability in cancer cells) or genomic regions linked to cancer [9]. Deregulation (e.g., over-expression or loss of expression) of these “cancerous” miRNAs contributes to the occurrence and progression of cancers by favoring uncontrolled proliferation and promoting invasive behaviors, and by regulating apoptosis [10,11]. Another major issue in clinics is clearly represented by the need of biomarkers for an early diagnosis, extremely important considering that the survival and prognosis of patients depend on the stage

of cancer at the time of diagnosis: diagnosis of cancers at early stage is usually associated with a better prognosis. MiRNAs have a great potential as novel biomarkers for the early diagnosis of cancers [12]. It has been shown that miR-222 is

FIGURE 5. Effects of miR-222 on cell migration and invasion. HeLa cells transfected with miR-222 exhibited increased migration when compared with cells transfected with scrambled RNA. Scratch wound healing assay showed, at 48 h after transfection, the wound width in the treated group was smaller than that in control group in HeLa (A) and SiHa cells (B). Transwell invasion assay was used to confirm the effects of miR-222 on the invasion of HeLa and SiHa cells. Similar findings were acquired (C, D).

over-expressed in a majority of epithelium derived cancers [13], but plays a tumor-suppressive role in erythroleukemic cells by inhibiting erythropoiesis through down-regulating c-Kit receptor. Galardi *et al.* identified the cell cycle regulator, p27^{Kip1}, as a target of miR-222 [14,15], which was confirmed in thyroid papillary carcinoma [16], breast cancer [17], hepatocellular carcinoma and lung cancer [18]. It is evident that a comprehensive understanding of the functional role of miRNAs in oncogenesis will be achieved only by elucidating their mechanisms of action in each type of tumors. Although there are no reported studies concerning the relationship between miR-222 and CC; however, our study did show a correlation between miR-222 and CC.

In addition to microarray-based studies on the expression profiling, other methods have been developed for the detection

of miRNAs, such as quantitative real-time PCR [19]. In order to investigate the involvement of miR-222 in tumorigenesis of CC, RT-PCR was done to detect the miR-222 expression in normal cervical tissues and in CC. Results showed miR-222 was up-regulated in CC in comparison with normal tissues, suggesting a role of miR-222 in the development of CC. This might also imply that there is dysfunction of miR-222 in the CC, which may be an important factor in the development of CC. MiR-222 over-expression has important consequences in the tumorigenesis of CC and may be considered a marker of increased tumorigenesis.

The cell cycle inhibitor, p27^{Kip1}, is a member of the family of tumor suppressors. Recent reports have described it as a target gene of miR-222 [14,15]. The cell cycle regulatory IDP p27^{Kip1} (p27) can switch the activity of cyclin-dependent ki-

FIGURE 6. Transfection of miR-222 in HeLa and SiHa cells decreased the e-cadherin1 and PTEN protein expression, but increased MMP1 protein expression in SiHa cells.

FIGURE 7. MiR-222 regulates paxillin protein expression in HeLa cells. Confocal microscopy showed paxillin was highly expressed on the membrane of HeLa cells transfected with miR-222.

nases (Cdks) from an inhibited to an activated state through tyrosine phosphorylation (20). Deregulation of p27 has been implicated as a reliable prognostic indicator of many human malignancies [21]. Mice deficient in p27 exhibit increased susceptibility to tumor progression, and tumorigenicity is accelerated in $Rb^{+/-}p27^{-/-}$ and $p18INK4c^{-/-}p27^{-/-}$ mice [22], suggesting that $p27^{Kip1}$ is a rate-limiting factor for tumorigenesis. Lee *et al.* found the direct evidence related to the effect of p27 on hTERT expression in both human CC cells and primary MEFs deficient in p27 [23]. In the present study, the HeLa and SiHa cells were independently transfected with miR-222 to interfere

with the miR-222 expression. In the control group, transfection of scrambled miR-222 was performed. Results showed that miR-222 over-expression resulted in a phenotypic change in HeLa and SiHa cells that was characterized by more and denser colonies, and down-regulation of miR-222 inhibited the colony formation (data not shown). The cell proliferation assay showed similar results to those from the colony formation assay. In addition, miR-222 increased the number of HeLa and SiHa cells in the S phase and had no influence on the apoptosis. Moreover, decreased expression of p27 was noted in HeLa and SiHa cells after miR-222 transfection. The above findings

suggest that miR-222 affects p27^{Kip1} to regulate the proliferation and cell cycle of HeLa cells. MiR-222-induced p27^{Kip1} down-regulation may promote the growth of HeLa cells, but this relationship needs further study. The mechanism(s) by which miR-222 up-regulates the proliferation of HeLa cells will be investigated in future studies.

The cell migration assay showed that HeLa and SiHa cells that were transfected with miR-222 showed increased migration. Comparable results were obtained from the scratch wound healing assay of SiHa and HeLa cells. As miRNAs may affect the expression of many proteins, our findings showed that the miR-222 expression in HeLa cells transfected with miR-222 was inversely related to the expression of E-cadherin, PTEN and paxillin. E-cadherin has been demonstrated to directly affect the migration of cancer cells [24]. Loss of expression of E-cadherin and cadherin 2 may be associated with the loss of epithelial cell polarity and alterations in the intercellular adhesion [25]. It has been reported that miR-221 and 222 may regulate TRAIL resistance and enhance tumorigenicity through down-regulating PTEN and TIMP3 [8]. PTEN has been proposed as a promising biological marker for early diagnosis and prognostic evaluation of CC [1]. Our results showed miR-222 can down-regulate PTEN protein expression, which up-regulates the protein expression of MMP1 and paxillin to promote the migration of CC; therefore, miR-222 expression may be used to predict the aggressive behaviors of CC. The mechanisms by which miR-222 works are under investigation.

In summary, our studies revealed that miR-222 expression is positively related to the progression of CC. Furthermore, miR-222 may function as a tumor promotor through down-regulating p27^{Kip1} and PTEN in CC. These findings suggest that miR-222 may serve as a potential novel target for the clinical therapy of CC.

Challenges

In the present study, only 19 patients were recruited and only 18 sets of samples analysed fully. Future studies with large sample sizes are required to validate our findings. In addition, the role of miR-222 in CC was investigated in only two CC cell lines, and whether miR-222 affects the aggressive behavior of other types of CC cell lines (such as Caski cells) is still unknown.

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References

1. Lu D, Qian J, Yin X, Xiao Q, Wang C, Zeng Y. Expression of PTEN and survivin in cervical cancer: promising biological markers for early diagnosis and prognostic evaluation. *British Journal of Biomedical Science*. 2012;69(4):143-146.
2. Moukova L, Nenutil R, Fabian P, Chovanec J. [Prognostic factors for cervical cancer]. *Klinicka Onkologie : Casopis Ceske a Slovenske Onkologicke Spolecnosti*. 2013;26(2):83-90.
3. Sevignani C, Calin GA, Siracusa LD, Croce CM. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mammalian genome : official journal of the International Mammalian Genome Society*. 2006;17(3):189-202. doi: 10.1007/s00335-005-0066-3.
4. Naeini MM, Ardekani AM. Noncoding RNAs and cancer. *Avicenna Journal of Medical Biotechnology*. 2009;1(2):55-70.
5. Su X, Xing J, Wang Z, Chen L, Cui M, Jiang B. microRNAs and ceRNAs: RNA networks in pathogenesis of cancer. *Chinese Journal of Cancer Research = Chung-kuo yen cheng yen chiu*. 2013;25(2):235-239. doi: 10.3978/j.issn.1000-9604.2013.03.08.
6. Garofalo M, Quintavalle C, Romano G, Croce CM, Condorelli G. miR221/222 in cancer: their role in tumor progression and response to therapy. *Current Molecular Medicine*. 2012;12(1):27-33.
7. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods (San Diego, Calif)*. 2001;25(4):402-408. doi: 10.1006/meth.2001.1262.
8. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankee A, Taccioli C, Pichiorri F, Alder H, Secchiero P, Gasparini P, Gonelli A, Costinean S, Acunzo M, Condorelli G, Croce CM. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell*. 2009;16(6):498-509. doi: 10.1016/j.ccr.2009.10.014.
9. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(9):2999-3004. doi: 10.1073/pnas.0307323101.
10. Garofalo M, Condorelli G, Croce CM. MicroRNAs in diseases and drug response. *Current Opinion in Pharmacology*. 2008;8(5):661-667. doi: 10.1016/j.coph.2008.06.005.

11. Garofalo M, Condorelli GL, Croce CM, Condorelli G. MicroRNAs as regulators of death receptors signaling. *Cell Death and Differentiation*. 2010;17(2):200-208. doi: 10.1038/cdd.2009.105.
12. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Molecular Medicine*. 2012;4(3):143-159. doi: 10.1002/emmm.201100209.
13. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(7):2257-2261. doi: 10.1073/pnas.0510565103.
14. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafre SA, Farace MG. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *The Journal of Biological Chemistry*. 2007;282(32):23716-23724. doi: 10.1074/jbc.M701805200.
15. le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, Anile C, Maira G, Mercatelli N, Ciafre SA, Farace MG, Agami R. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *The EMBO Journal*. 2007;26(15):3699-3708. doi: 10.1038/sj.emboj.7601790.
16. Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, Borbone E, Petrocca F, Alder H, Croce CM, Fusco A. MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. *Endocrine-Related Cancer*. 2007;14(3):791-798. doi: 10.1677/erc-07-0129.
17. Di Leva G, Gasparini P, Piovan C, Nganheu A, Garofalo M, Taccioli C, Iorio MV, Li M, Volinia S, Alder H, Nakamura T, Nuovo G, Liu Y, Nephew KP, Croce CM. MicroRNA cluster 221-222 and estrogen receptor alpha interactions in breast cancer. *Journal of the National Cancer Institute*. 2010;102(10):706-721. doi: 10.1093/jnci/djq102.
18. Garofalo M, Quintavalle C, Di Leva G, Zanca C, Romano G, Taccioli C, Liu CG, Croce CM, Condorelli G. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene*. 2008;27(27):3845-3855. doi: 10.1038/onc.2008.6.
19. Perkins JR, Dawes JM, McMahon SB, Bennett DL, Orenco C, Kohl M. ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. *BMC Genomics*. 2012;13:296. doi: 10.1186/1471-2164-13-296.
20. Sivakolundu SG, Bashford D, Kriwacki RW. Disordered p27Kip1 exhibits intrinsic structure resembling the Cdk2/cyclin A-bound conformation. *Journal of Molecular Biology*. 2005;353(5):1118-1128. doi: 10.1016/j.jmb.2005.08.074.
21. Piva R, Cancelli I, Cavalla P, Bortolotto S, Dominguez J, Draetta GF, Schiffer D. Proteasome-dependent degradation of p27/kip1 in gliomas. *Journal of Neuropathology and Experimental Neurology*. 1999;58(7):691-696.
22. Park MS, Rosai J, Nguyen HT, Capodiceci P, Cordon-Cardo C, Koff A. p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(11):6382-6387.
23. Lee SH, Kim JW, Oh SH, Kim YJ, Rho SB, Park K, Park KL, Lee JH. IFN-gamma/IRF-1-induced p27kip1 down-regulates telomerase activity and human telomerase reverse transcriptase expression in human cervical cancer. *FEBS Letters*. 2005;579(5):1027-1033. doi: 10.1016/j.febslet.2005.01.005.
24. Kawauchi T. Cell adhesion and its endocytic regulation in cell migration during neural development and cancer metastasis. *International Journal of Molecular Sciences*. 2012;13(4):4564-4590. doi: 10.3390/ijms13044564.
25. Cunliffe C, Ryan F, Lambkin H, Brankin B. Expression of tight and adherens junction proteins in cervical neoplasia. *British Journal of Biomedical Science*. 2012;69(4):147-153.