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# miR-20a Promotes Prostate Cancer Invasion and Migration Through Targeting ABL2

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## ABSTRACT

The aberrant expression of microRNAs (miRNAs) has been found in various types of cancer. The present study found miR-20a was significantly up-regulated in prostate cancer compared with normal prostate tissues. Patients with a higher miR-20a expression had a Gleason score of 7–10 and shorter survival time. The transwell and wound healing assays revealed that blocking expression of miR-20a by miR-20a ASO suppresses the invasion and migration of PC-3 and DU145 cells in vitro and also inhibits tumor growth in vivo. Furthermore, we identified miR-20a directly targets the ABL family non-receptor tyrosine kinases ABL2 and negatively regulates the phosphorylation of its downstream gene p190RhoGAP. Knockdown of ABL2 promoted cell invasion and migration and we identified miR-20a-induced cell invasion and migration can be rescued by ABL2. In conclusion, our findings show that miR-20a significantly contributes to the progression of prostate cancer by targeting ABL2. J. Cell. Biochem. 115: 1269–1276, 2014. 

**KEY WORDS:** miR-20a; ABL2; p190RhoGAP; PROSTATE CANCER; INVASION; MIGRATION

Prostate cancer (PCa), the second leading cause of cancer-related deaths among men in most countries, is a complex and multifactorial disease. Nevertheless, the molecular basis of PCa is insufficiently characterized with regard to incidence [Sharma et al., 2010]. Recent investigations into the molecular events involved in the development of PCa have intensified and focused not only on a better knowledge of the development of the disease but also on potential therapeutic targets as well as prognostic markers [Gandellini et al., 2009]. Examples of recent promising new candidate molecular markers and therapeutic targets are the miRNAs.

MicroRNAs (miRNAs), a recently discovered class of genes transcribing small non-coding RNAs, come into our focus as therapeutic targets or prognostic markers [Ferracin et al., 2010]. miRNAs are endogenously expressed, small non-coding RNAs, which regulate gene expression by the inhibition of the translation and/or decreasing of the stability of target mRNAs via complimentary pairing predominantly to the 3′-untranslated region (3′-UTR) [Pang et al., 2010]. The miRNAs can function both as oncogenes by down-regulating tumor-suppressor genes and as tumor-suppressor genes by down-regulating oncogenes [Esquela-Kerscher and Slack, 2006]. Recently, an increasing number of studies have demonstrated the misexpression patterns of miRNAs in many human cancers, which participate in critical pathways linked to proliferation, cell cycle progression, and metastasis.

The most often studied group of miRNAs is the mir-17-92 cluster which contains miR-20a. There is evidence that the mir-17-92 cluster is involved in many types of human cancer. The amplification of the region containing this cluster has been described in lymphoma [Mu et al., 2009] and lung cancer [Hayashita et al., 2005]. While loss of heterozygosity of the genes in this cluster was detected in breast cancer [Hossain et al., 2006], nasopharyngeal carcinoma [Cho, 2010], and hepatocellular carcinoma [Chow et al., 2010]. These results show that members of the mir-17-92 cluster could play a role both as oncogenes and tumor-suppressor genes. Studies found miR-20a is down-regulated in breast cancer. While, it is overexpressed

Xiao-Fei Qiang, Zheng-Wei Zhang, and Qian Liu contributed equally to this work.

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in the lung cancer [Volinia et al., 2006] and blocking miR-20a expression suppresses the proliferation of PCa MDA-PCa-2b cells in vitro and also inhibits tumor growth in vivo [Li et al., 2012a].

The ABL family non-receptor tyrosine kinases ABL1 (also known as c-Abl) and ABL2 (also known as Arg) are ubiquitously expressed and essential regulators of many cellular functions including cell proliferation, adhesion, and migration through remodeling of actin cytoskeleton structures in response to stimulation of cell surface receptors [Greuber et al., 2013]. ABL kinases have been implicated in actin reorganization functions mediated by the tyrosine phosphorylation of multiple actin remodeling regulator proteins including the adaptor proteins CRK and CRKL [Colicelli, 2010]. Despite their homology, ABL2 has a number of distinct cellular functions including adhesion-dependent neurite branching, epithelial cell adhesion, and migration [Bradley and Koleske, 2009]. The ABL2 has been described as a novel mediator of RhoA inactivation. ABL2 bound to p190RhoGAP, phosphorylating p190RhoGAP and activating it, which triggers p190RhoGAP localization to the cell membrane where it inhibits Rho activity [Zandy, 2008]. Inactivation of RhoA resulted in depolymerization and severing of F-actin, thereby collapsing the cytoskeleton and inhibiting cell migration [Peacock et al., 2007].

In this report, we identify the important molecular mechanism by which Blocking of miR-20a exerts negative effects on PCa cell migration and invasion in human PCa cells PC-3 and DU-145, which involves the direct targeting the 3′-UTR of ABL2 mRNA and affects the P190 pathway.

## MATERIAL AND METHODS

### HUMAN TISSUE SAMPLES

Patients who underwent a prostate biopsy between May 2010 and September 2013 Pingjing Hospital, Logistics University of the Chinese People's Armed Police Forces, were selected. We randomly chose 30 PCa patients tissues and adjacent normal tissues to detect miR-20a expression level. According to the value of the Gleason score, the patient's samples were divided into patients with BPH and Gleason score with 0–6 and 7–10, and the detect the expression level of miR-20a. Patients with low ($dCT < 35.5$) and high ($dCT > 35.5$) level of miR-20a expression were assessed using Kaplan–Meier survival curves.

### CELL CULTURE AND TRANSFECTION

PC-3, DU145, RWPE-1, VCaP, and LNCaP cells were grown at 37°C and in 5% $CO_2$ in RPMI 1640 supplemented with fetal bovine serum (FBS) plus 2 mM L-glutamine. All reagents used for cell culture media were from Gibco BRL. miR-20a mimics and miR-20a ASO were purchased from Shanghai GenePharma (Shanghai, China). Transfections were carried out using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### RNA PREPARATION AND QUANTITATIVE PCR

mirVana miRNA Isolation Kit (Ambion) was used to prepare small RNAs (smaller than 200 nt) from cells or tissue samples according to the manufacturer's instructions. cDNA was obtained using M-MLV (Promega) and 1 μg of obtained RNA. The relative level of miR-20a was detected by stem-loop RT-PCR with following conditions: denaturing the DNA at 94°C for 4 min, followed by 35 cycles of amplification: 94°C for 60 s, 56°C for 60 s, 72°C for 1 min for data collection. U6 snRNA was used as an endogenous control. Quantitative PCR was performed on an ABI 7500 thermocycler (Applied Biosystems) using SYBR® Premix Ex TaqTM (Perfect Real Time) Kits (TaKaRa, Japan) The primers used are used as followed: miR-20a: RT-qPCR stem-loop primer: 5′-GTCGTATCCAGTGCAGGGTCC-GAGGTATTCGCACTGGATACGACCTACCTG; qPCR forward primer: 5′-GCCGCGCTAAAGTGCTTATAGTG; reverse primer qPCR: 5′-CAC-CAGGGTCCGAGGT. U6: RT-qPCR stem-loop primer: 5′-GTCGTATC-CAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG; qPCR forward primer: 5′-TGCGGGTGCTCGCTTCGGCAGC; reverse primer qPCR: CCAGTGCAGGGTCCGAGGT.

### DETECTION OF CELL PHENOTYPES

For colony formation assay, cells were counted and seeded in 12-well plates (in triplicate) at 100 cells/well. Fresh culture medium was replaced every 3 days. The number of viable cell colonies was determined after 14 days and colony formation ratio was calculated. To detect cell invasion ability, A total of $5 \times 10^4$ PC-3 and $1 \times 10^5$ DU145 cells were seeded into the upper part of a Matrigel pre-coated Transwell chamber (Corning) and incubating for 28 h. Then cells invasing cells across the membrane were counted under a light microscope. For the wound healing assay, $5 \times 10^4$ PC-3 or $1 \times 10^5$ DU145 cells were seeded into 24-well plates for 24 h and a straight line simulation "wound" was made using a P200 pipette tip. The extent of wound closure was monitored at 36 or 48 h.

### FLUORESCENT REPORTER ASSAYS

The human ABL2 3′-UTR harboring four miR-20a target sequences as well as the seed-sequence mutated verson (ABL2-3UTR-mut) were synthesized by GenPharm (Shanghai, China), which were ligated after the luc ORF in the pMIR-REPORT luciferase vector (Ambion). For the fluorescent reporter assay, cells were seeded in a 48-well plate and were co-transfected with miR-20a mimics, ASO as well as ABL2-3UTR or ABL2-3UTR-mut. The cells were lyzed 48 h later and the intensity of luciferase was detected.

### KNOCKDOWN OF ABL2 BY siRNA

Transient transfection of siRNAs was performed using Lipofectamine-2000 (Invitrogen) according to the manufacturer's directions. After 48–70 h, cells were analyzed for effects of the knockdown. Silencing efficiency was confirmed by Western blot. The siRNA targeting ABL2 was purchased from SANTA CRUZ (Arg siRNA (h): sc-38945).

### IMMUNOPRECIPITATION

Anti-ABL2 was purchased from Abcam (ab134134). Anti-p190RhoGAP and -phosphotyrosine (4G10) were purchased from BD Biosciences (San Jose, CA). One milligram of PC-3 or DU145 cells lyzed in 20 mM HEPES, 1% Triton X-100, and 150 mM sodium chloride was incubated with anti-p190A antibody for 16 h. Protein G-Sepharose (GE Healthcare) was used to pull down

antibodies. Then ice-cold precipitation buffer (Boston Bioproducts, Worcester, MA) supplemented with complete protease inhibitor mixture (Roche Applied Science) was incubated with anti-phosphotyrosine antibody (4G10) and analyzed by Western Blotting.

### WESTERN BLOTTING

Western blotting was performed to determine protein expression of ABL2, p190RhoGAP, and 4G10. Total protein extracted by Trizol reagent (Invitrogen) or immunoprecipitates were transferred onto polyvinylidene fluoride membranes and immunoblotted with antibodies. Horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and chemiluminescent substrates (PerkinElmer Life Sciences) were used to detect primary antibodies. The polyclonal rabbit anti-human GAPDH (Sigma) were used.

### STATISTICAL ANALYSIS

A Student's test was performed to analyze the significance of differences between the sample means obtained from three independent experiments. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### miR-20a IS UP-REGULATED IN PCa TISSUES AND AFFECTS PATIENTS SURVIVAL TIME

To explore the potential role of miR-20a in the progression of PCa, 30 freshly frozen PCa samples and their corresponding adjacent non-cancer tissues were obtained for miR-20a expression analysis by qRT-PCR. The vast majority of cancer specimens exhibited increased miR-20a expression levels compared with adjacent non-cancer tissues (Fig. 1A). Interestingly, we found that miR-20a expression up-regulation was accompanying with the Gleason score, which is an important variable describing the behavior of PCa and has been correlated with pathologic stage, metastasis, and outcome. We observed a higher expression of miR-20a in the group with a Gleason score of 7–10 than in the group with a Gleason score of 0–6 (Fig. 1B), which may explain the result that miR-20a was significantly overexpressed in plasma with higher stage of PCa from PCa patients. Patients with low level of miR-20a (dCT $< 35.5$) showed longer mean survival time with 62 months; whereas patients expressing high level of miR-20a exhibited a relatively shorter survival time, 49 months (Fig. 1C). Endogenous expression levels of miR-20a were measured in the normal prostate cell RWPE-1 and PCa cell lines LNCaP, PC-3,

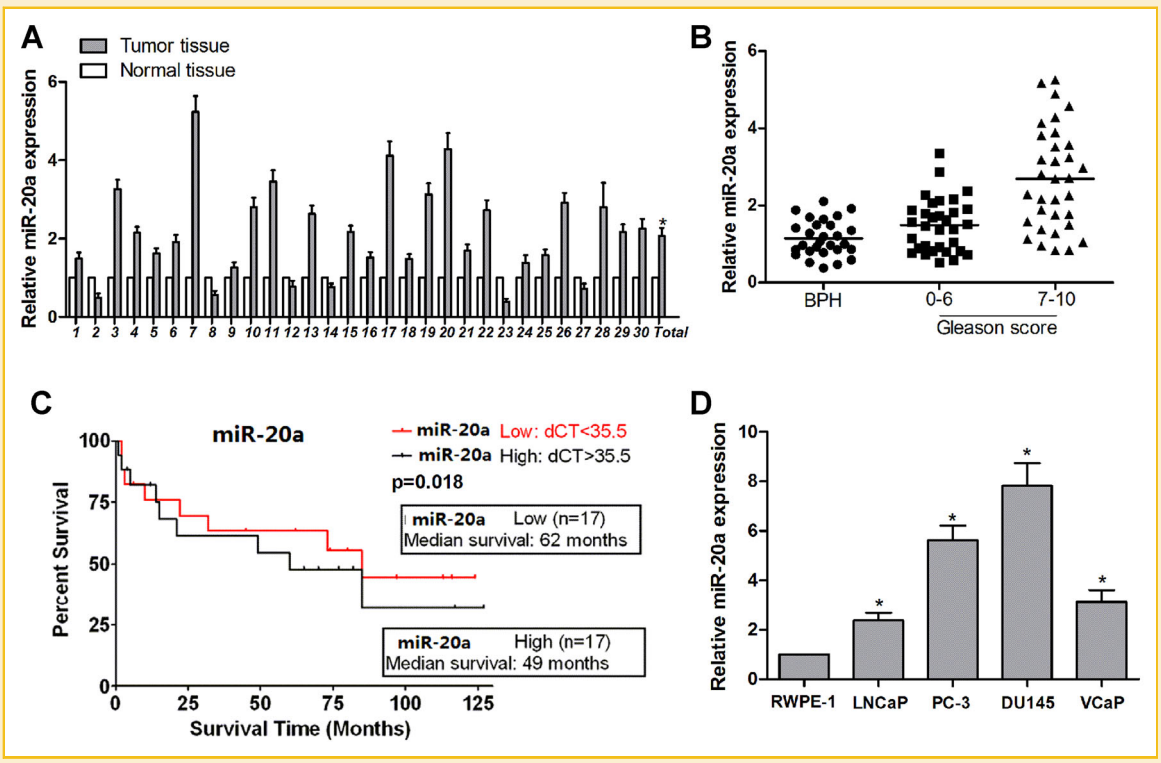


Fig. 1. miR-20a expression in human PCa and its association with survival time. A: The expression level of miR-20a in 30 pairs of PCa cancer tissues and its adjacent normal tissues. B: Expression of miR-20a in PCa samples with BPH or Gleason score 0–6 and Gleason score 7–10. There was a significantly higher expression of miR-20a in Gleason score 7–10 group in comparison with Gleason score 0–6 group, $P < 0.05$ (BPH vs. 0–6, BPH vs. 7–10, 0–6 vs. 7–10). C: Kaplan–Meier survival curves for miR-20a. Two groups were formed according to the median value (dCT $< 35.5$ and dCT $\geq 35.5$) of the miR-20a expression levels and analyzed to determine its association with survival rate in PCa. D: miR-20a was isolated from PCa cell lines (RWPE-1, LNCaP, PC3, DU145, and VCaP). The expression level of miR-20a was confirmed by qRT-PCR and normalized to the expression U6 RNA. Data are shown as the ratio of miR-20a expression in LNCaP, PC3, DU145, and VCaP cells to that of RWPE-1 (*$P < 0.05$).

DU145, and VCaP using RT-PCR assay (Fig. 1D). We measured about 2.5- and 3-fold increase of miR-20a level in LNCaP and VCaP cells as compared with that of RWPE-1 cells. While in PC-3 and DU145 cells, we detected relatively higher level of miR-20a, six- and eightfold increase compared with RWPE-1 cells.

### INHIBITION OF miR-20a SUPPRESSES PCa CELL INVASION AND MIGRATION IN VITRO

Given that miR-20a was up-regulated in PCa tissue with a tendency that expressed increasingly in higher Gleason score of the PCa tissue, we next try to explore the functional role of miR-20a in PCa cell pathogenesis. The transfection efficiency was confirmed by qRT-PCR and indicated a 86/77% reduction in PC-3 and DU145 cells (Fig. 2A). Cell proliferation and invasion are important characteristics of cancer cells, and the ability of cell growth was monitored using colony formation assays in cells transfected with miR-20a ASO, which indicated a minor inhibition effect on PC-3 and DU145 cells growth (Fig. 2B). The invasion and migration ability was determined in PC-3 and DU145 cells by transwell and wound healing assay. We found that blockade of miR-20a significantly suppressed the cell invasion and migration in PC-3 and DU145 cells (Fig. 2C,D). The results demonstrate that miR-20a mainly plays an important role in the invasion and migration of PCa cells.

### ABL2 SERVES AS A TARGET OF miR-20a FOR POST-TRANSCRIPTIONAL REPRESSION

Bioinformatics searches identified several putative miR-20a binding site in the 3′-UTR of ABL2 (Fig. 3A). In particular, ABL2 is a well-known oncogene that plays an important role in regulating many cellular functions including invasion and migration. However, the underlying mechanism of ABL2 regulation by miR-20a is not understood. To determine whether miR-20a-mediated silencing of ABL2 is another mechanism responsible for miR-20a-induced cell metastasis, we ectopically expressed miR-20a in four PCa cells

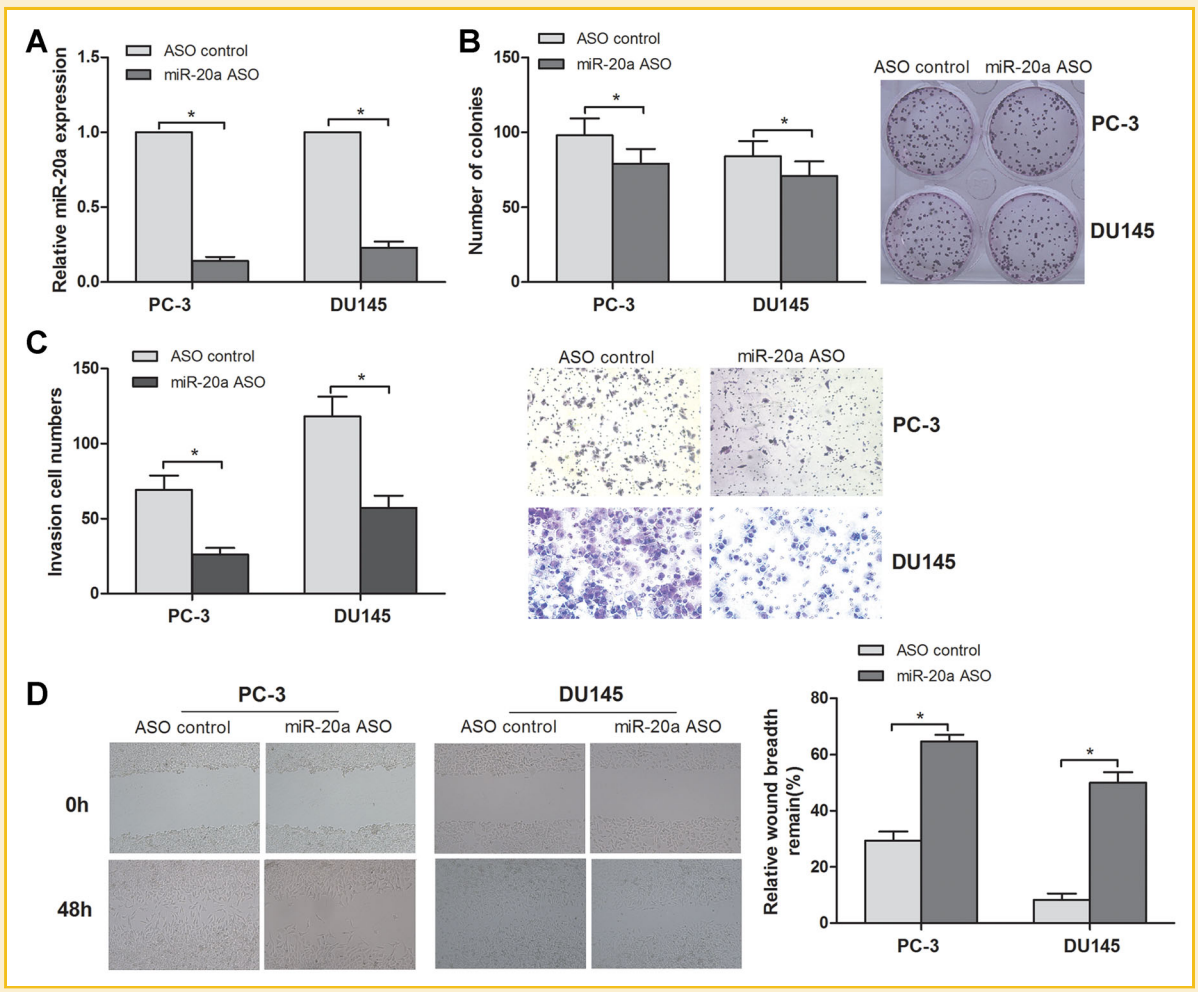


Fig. 2. Influences of miR-20a blocking on prostate cancer cells. A: PC-3 and DU145 cells were transfected with ASO control or miR-20a ASO. The expression level of miR-20a was detected by qRT-PCR. B: Colony formation assay of PC-3 and DU145 cells transfected with miR-20a ASO to investigate the effect of miR-20a on the cell proliferation. C: ASO control or miR-20a ASO transfected cells were plated on Matrigel-coated membranes and allowed to invade for 24 h. D: Wound healing assay was used to evaluate the effect of reduced miR-20a expression on PC-3 and DU145 cells migration capacity. At 24 h post-transfection, a "scratch" was generated in a confluent monolayer of the cells and then measured the degree of "wound closure" after another 48 h. Dashed lines indicate width of "wound" (*$P < 0.05$).

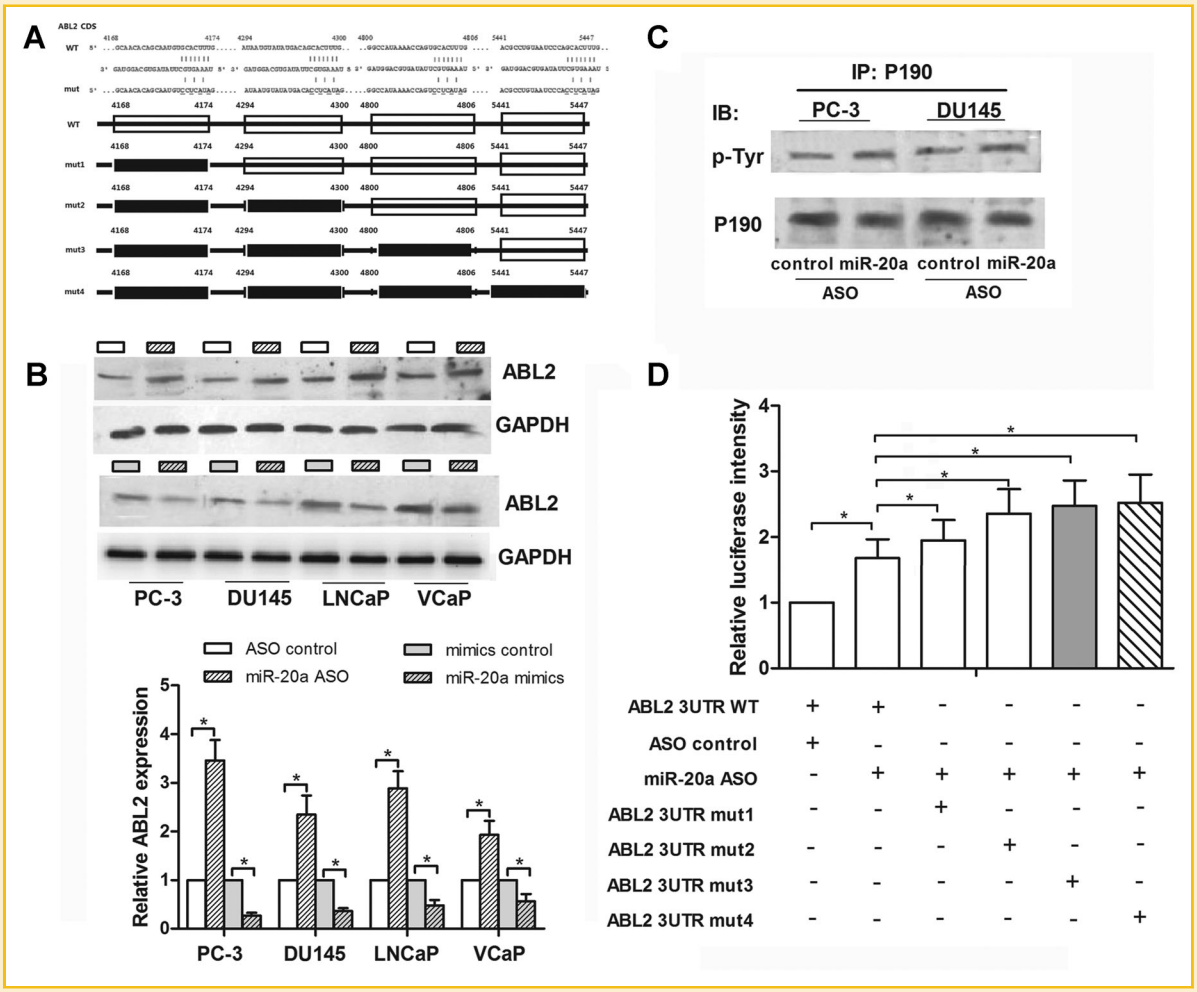


**Fig. 3.** miR-20a directly targets ABL2 and inhibits its expression. A: A schematic of the bioinformatics predicted seed region in the 3′-UTR of ABL2 as well as mutated 3′-UTR used in this study. B: Effect of miR-20a ASO on the luciferase activity of ABL2-3′UTR and ABL2-3′UTR-mut. C: Western blot was used to detect the ABL2 protein when cells transfected with miR-20a ASO or miR-20a mimics. D: Immunoprecipitation (IP) of p190RhoGAP and immunoblot (IB) of phosphotyrosine (p-Tyr) (4G10). miR-20a was blocked with ASO in PC-3 and DU145 cells and induced phosphorylation of p190RhoGAP.

(PC-3, DU-145, LNCaP, and VCaP). As expected, ABL2 expression was significantly lower in the miR-20a mimics transfected cells than in control cells (Fig. 3B, upper panel). In the cell lines, the level of suppression was more than 55% (Fig. 3B, lower panel). To further determine the specificity of the miR-20a targeting of ABL2, we used miR-20a ASO to knockdown the miR-20a level. As shown in Figure 4C, miR-20a ASO was able to enhance ABL2 expression by more than twofold (Fig. 3B). To further determine the effect of miR-20a on ABL2, we then examined ABL2 downstream target gene, p190RhoGAP (p190). miR-20a ASO suppressed the phosphorylation of p190RhoGAP. As shown in Figure 3C, p-Tyr expression was reduced in cells transfected with miR-20a ASO. These results suggest that miR-20a can suppress ABL2 and its downstream gene p190RhoGAP phosphorylation.

To determine whether miR-20a directly targets ABL2, we constructed a luciferase reporter carrying ABL2 3′-UTR with a putative miR-20a binding site (Fig. 3A). We detected a increase of luciferase activity by twofold in the miR-20a transfected cells as compared with ASO control. This alteration of the luciferase activity was specific to miR-20a because the mutated miR-20a binding site in the ABL2 3′-UTR resulted in obvious increase of the luciferase activity compared with cells co-transfected with ABL2 3′-UTR WT and ASO control (Fig. 3D). Thus, these results demonstrated that miR-20a directly targets ABL2 through interacting with this binding site, affecting the phosphorylation of p190RhoGAP.

## KNOCKDOWN OF ABL2 PROMOTES INVASION AND MIGRATION OF PCa CELLS

To investigate the functional role of ABL2 in PC-3 and DU145 cells, we used ABL2 siRNA to transfect PC-3 and DU145 cells with significant knockdown of ABL2 analyzed by Western blot (Fig. 4A). Consistent with our predication, knockdown of ABL2 abrogated phosphorylation of p190RhoGAP with reduced p-Tyr expression (Fig. 2B). Additionally, Cells with reduced levels of ABL2 had a

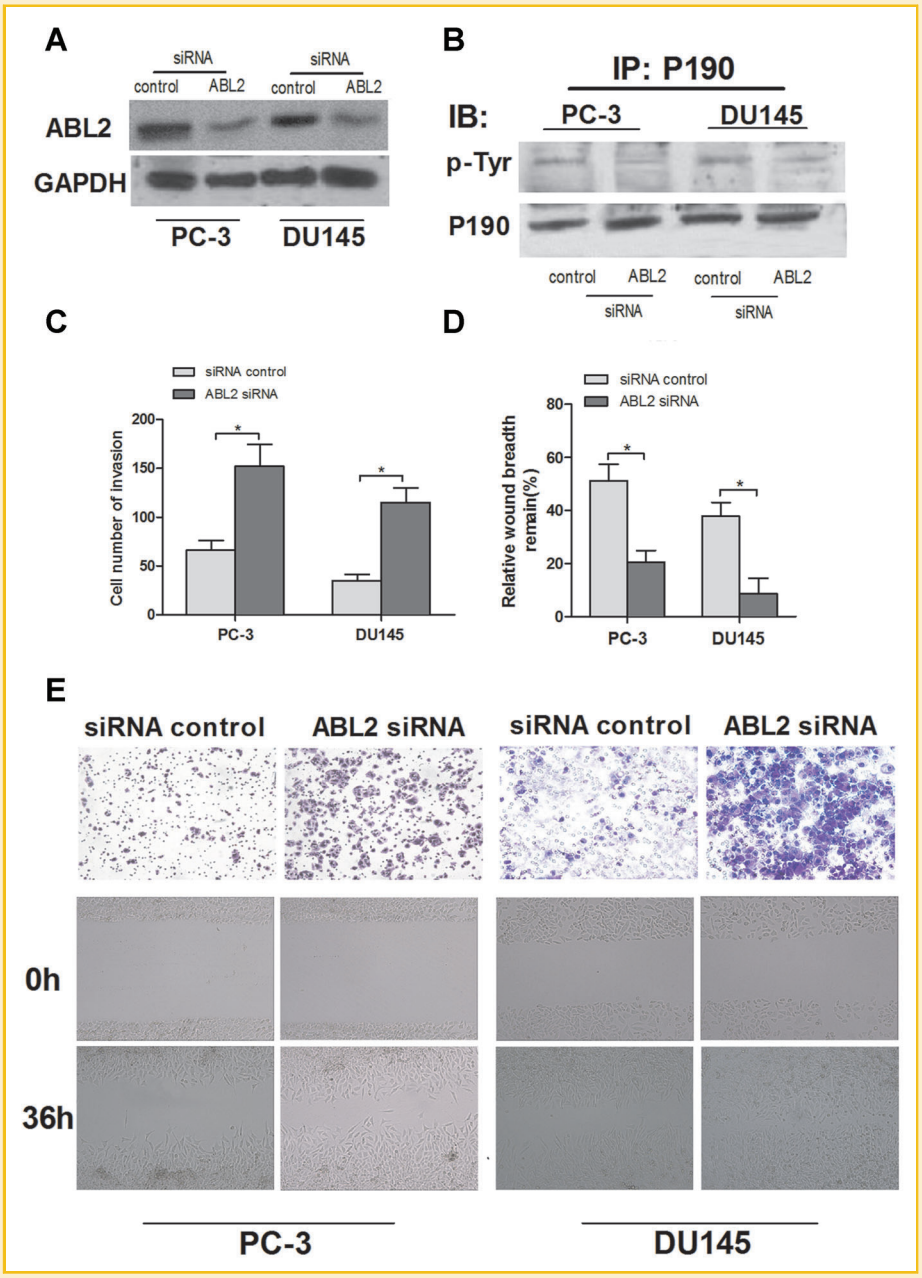


**Fig. 4.** Knockdown of ABL2 on invasion and migration of PCa cells. A: Plexin-B1 protein level was measured 48 h after PC-3 and DU145 cells were transfected by ABL2 siRNA using Western blot. GAPDH was used as loading/transfer controls and for normalization of values. B: miR-20a ASO was transfected in PC-3 and DU145 cells. Cells were lysed and immunoprecipitation (IP) of p190RhoGAP and immunoblot (IB) of phosphotyrosine (p-Tyr) (4G10). The effect of ABL2 knockdown on invasion was detected by transwell assay (C, E upper panel). D,E (down panel): Wound healing assay was used to evaluate the effect of reduced ABL2 expression on cell migration capacity.

increased invasion ability compared to the negative control cells (Fig. 4C,E upper panel) and resulted in a increased rate of migration cells into a denuded area of confluent monolayer (Fig. 4C,E lower panel).

### ROLE OF miR-20a IN ABL2-MEDIATED PCa CELL GROWTH

Given that miR-20a targets ABL2 affecting its expression, we next try to explore whether miR-20a promoted cell invasion was mediated by ABL2. As shown in Figure 5A, miR-20a ASO significantly inhibited miR-20a expression, while the expression of miR-20a in PC-3 and DU145 cells co-transfected miR-20a ASO and ABL2 siRNA or siRNA control remained the same level. Importantly, ABL2 siRNA rescued miR-20a ASO-induced increasing expression of ABL2 (Fig. 5B). To determine whether ABL2 siRNA affects miR-20a ASO repressed cell invasion, we co-transfected ABL2 siRNA and miR-20a ASO in PC-3 and DU145 cells. It was

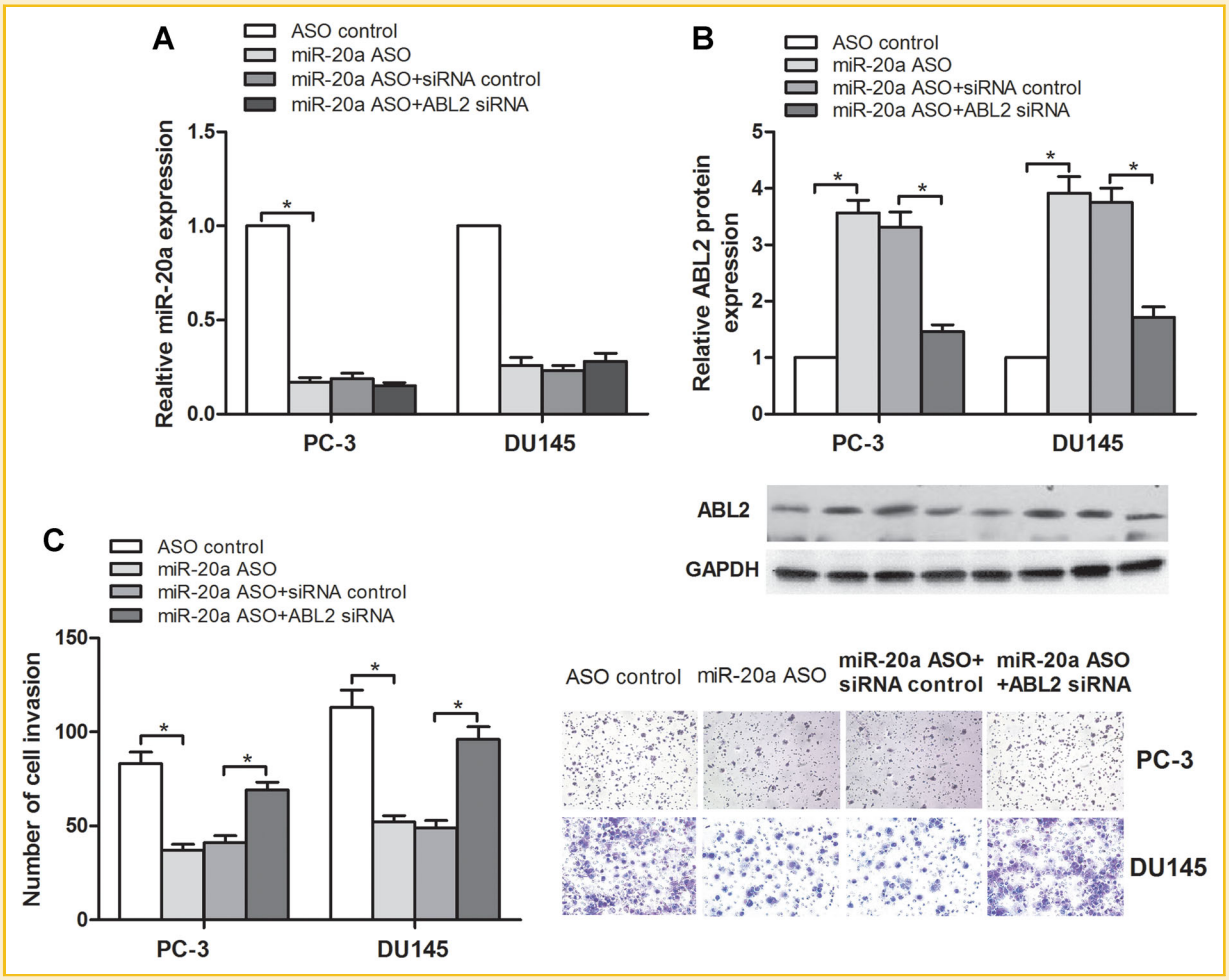


**Fig. 5.** miR-20a-induced PCa cell invasion and migration was mediated by ABL2. PC-3 and DU145 cells were transfected with ASO control and miR-20a ASO, or co-transfected with ABL2 siRNA. A: Detection of miR-20a expression. B: ABL2 protein level was detected by Western blot analysis. C: Transwell assay was used to detected the cells invasion ability (*$P < 0.05$).

found that ABL2 inhibition leaded to the increase of cell invasion repressed by miR-20a ASO. Together, miR-20a-induced cell invasion of PCa cells is mediated through directly targeting and inhibition of ABL2.

## DISCUSSION

Over the past decades, researchers have indentified numerous genes contribute to PCa growth, invasion, and migration. The dysregulated expression of miRNAs have been found to correlate with these phenotypes, thus reinforcing the importance of miRNA biology in PCa-associated tumorigenesis [Hudson et al., 2013]. Researchers found the overexpression of miR-128 in PC-3 cells can increase cell number, proliferation, with a remarkable up-regulation of Bcl2, an important anti-apoptosis protein that inhibits caspase activity [Guidi et al., 2010]. miR-34a/c could induce cell cycle arrest and subsequent apoptosis activation in PCa cells [Hagman et al., 2010]. Metastasis is a major cause of PCa-related death and patients with PCa have a tendency to develop malignant lesions outside the primary tumor. Researchers have identified that miR-29a was overexpressed in metastatic PCa targeting integrins, the absence of which has been implicated in PCa metastasis in vivo [Gerson et al., 2012; Li et al., 2012b]. Other related miRNAs associated with PCa metastasis include mir-10b, miR-143, and miR-145, etc [Ullah and Aatif, 2009; Peng et al., 2011].

To date, the mechanism of miR-20a function in PCa has not been investigated in detail, although several targets have been identified including E2F2, E2F3, and CX43. A report found that miR-20a exerts an anti-apoptotic role in a PCa cell line via modulating the translation of the E2F2 and E2F3 mRNAs via binding sites in their 3′-UTR [Sylvestre et al., 2007]. In this study, we identified compared with adjacent normal tissues, miR-20a expressed higher in PCa tissues and patients with higher expression of miR-20a had a shorter survival time. In vitro cell analysis demonstrated that blocking miR-20a could suppress PCa cells (PC-3 and DU145) invasion and migration using transwell and wound healing assays.

We identified ABL2 as a novel target of miR-20a. MiR-20a suppressed ABL2 protein level via directly binding to the ABL2-3UTR, resulting in the reduced phosphorylation of p190RhoGAP, which has been shown to be a substrate for ABL2 and is tyrosine-phosphorylated on Tyr. P190RhoGAP is a GTPase-activating protein (GAP) which inactivates RhoA to a GDP-bound state. Dephosphorylation of p190RhoGAP inhibited SEMA3F-induced cytoskeleton collapse and abrogated the SEMA3F-induced inhibition of migration. Genetic studies indicate that Abl family kinases translate signals from cell surface receptors into changes in cell shape and movement, but it is unclear which cellular processes are controlled by these kinases. We report here that knockdown of ABL2 induces PCa cell invasion and migration through ABL2 regulates p190RhoGAP phosphorylation [Peacock et al., 2007].

miRNAs have been detected associated with cancer diagnosis and prognosis as promising biomarkers to distinguish cancer patients with varied aggressiveness. miR-145 was previously found to be down-regulated in many solid tumors compared with adjacent non-tumor tissue, including PCa. A recent study found miR-145 was able to correctly classify 71% of PCa tumor tissues from non-tumor tissues. By examining 273 detectable miRNAs, researchers identified a significant association between miR-145 down-regulation and PCa grade using lymphoblastoid cell lines derived from PCa patients with tumors of different aggressiveness. miR-221 alone also displayed ability to significantly differentiate PCa tumor and non-tumor tissues [Shen et al., 2012]. In our present study, we observed miR-20a expression was higher in the majority of cancer specimens with a Gleason score of 7–10 and high level of miR-20a exhibited a relatively shorter survival time, suggesting that the miR-20a may be a specific biomarker in prediction PCa aggressiveness, but the sensitivity needs our further investigation.

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