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Original Paper

Overexpression of Long Non-Coding RNA ZXF2 Promotes Lung Adenocarcinoma Progression Through c-Myc Pathway

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Key Words
LncRNA ZXF2 • Lung adenocarcinoma • Cell proliferation • Migration and invasion • c-Myc

Abstract
Objective: To investigate the expression of long non-coding RNA ZXF2 in lung adenocarcinoma tissues and its effect on cell proliferation, migration and invasion. ***Methods:*** Forty pairs of cancerous and adjacent non-cancerous lung adenocarcinoma specimens were collected for the studies. Quantitative real-time PCR was used to analyze the expression of ZXF2 in tumor tissues and adjacent normal tissues. The expression of ZXF2 was correlated with patients' clinico-pathological data. Molecular pathway controlled by ZXF2 was explored by using small interfering RNA (siRNA) technology. CCK-8 cell proliferation assay, flow cytometry analysis and transwell assays were used to evaluate cell proliferation, migration and invasion. ***Results:*** The expression of ZXF2 was 2 fold or higher in 27 out of 40 (67.5%) cases of lung adenocarcinoma specimens than that in non-cancerous tissues ($P<0.05$). The relative expression level of ZXF2 was positively correlated with tumor lymph node metastasis ($\chi^2=8.485$, $P<0.05$) and poor prognosis of the patients ($p=0.0217$). In order to explore the molecular mechanisms of ZXF2 mediated tumor progression, ZXF2 expression was inhibited by siRNA in A549 cells, a highly aggressive and metastatic lung adenocarcinoma cell line. We found that siRNA-ZXF2 treatment inhibited cell proliferation ($P<0.01$) leading to cell cycle arrest ($P<0.01$). The cell migration and invasion were suppressed by siRNA-ZXF2 treatment ($P<0.01$). Further biochemical studies revealed that the knockdown of ZXF2 led to down regulation of c-Myc signaling. ***Conclusion:*** ZXF2 was overexpressed in lung adenocarcinoma tissues and the high expression of ZXF was closely related to tumor progression through c-Myc related pathway. Given the fact that both ZXF2 and c-Myc are located in the same chromosome 8q24.2 loci, the potential interaction between ZXF2 and c-Myc might be a novel target for treatment of lung adenocarcinoma.

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Introduction

Long chain of non-coding RNAs (LncRNAs), a class of non-coding transcripts longer than 200 bps, are involved in regulating gene expression on a variety of levels including epigenetic, transcriptional and post-transcriptional regulations [1-3]. Recently, lncRNAs have attracted a broad attention for their function in X-chromosome silence, genomic imprinting and chromatin modification, transcription activation, transcription interference and nuclear transport, among others. Previous studies suggested an important role of lncRNAs in tumor progression [4]. For example, high expression of lncRNAs, such as BANCR, LSINCT5, HULC are closely related to the high incidence of melanoma, breast cancer and liver cancer [5-7]. However, the role of lncRNAs in lung adenocarcinoma remains largely unknown.

In our previous study, we screened human lung adenocarcinoma and non-cancerous tissues by Arraystar lncRNA gene chip and found that a group of lncRNAs are differently expressed in cancer and adjacent normal tissues [8]. In the present study, we verified our findings by comparing the expression of lncRNAs in cancer tissues and adjacent normal tissues derived from 40 patients with lung adenocarcinoma. We found a lncRNA, named ZXF2 (SEQ_ID: AA311918, located on chromosome 8, loci 8q24.2) had an average of 8.879 fold higher expression in lung adenocarcinoma tissues than that in non-cancerous tissues. Further analysis revealed that overexpression of ZXF2 was correlated with high lymph node metastasis and poor prognosis. Further study on the molecular mechanisms underlying ZXF2 meditated tumor progression, we found that siRNA mediated suppression of ZXF2 led to cell cycle arrest and inhibition of cell proliferation, migration and invasion through regulating c-Myc, a strong proto-oncogene which is located adjacent to ZXF2 in chromosome loci 8q24.2, a common location for multiple tumors.

Materials and Methods

Patients and specimens

Forty pairs of samples were obtained from patients with lung adenocarcinoma, who were treated in the Department of Cardiothoracic Surgery, Zhongnan Hospital, Wuhan University. Average length of tumor tissue was 2.4cm, average age of patients was 62 years old. All of the non-cancerous tissues were more than 5 cm away from tumor tissues. These patients underwent surgical resection of tumors and were pathologically diagnosed with lung adenocarcinoma (stage I, II and III) between June 2010 and June 2012. The clinicopathological parameters of the patients were summarized in Table 1. None of the patients received chemotherapy and radiotherapy before surgery. The study was approved by The Human Research Ethics Committee of Wuhan University.

Real-time RT-PCR

Quantitative Real-time RT-PCR (qRT-PCR) was performed using FastStart Universal SYBR Green Master (Rox) kit on an ABI 7300 real time PCR System. ACTIN was used as internal control. The relative expression levels of ZXF2 were determined by normalizing expression of ZXF2 to that of ACTIN. ZXF2 primer sequence, sense: 5'-CAC CCA GGT CAG AGA AAG CA-3'; antisense: 5'-TGG AAG GGA CAC TAG AAG AAG AAT-3'. ACTIN primer sequence, sense: 5'-CAC CCA GCA CAA TGA AGA TCA AGA T-3'; antisense: 5'-CCA GTT TTT AAA TCC TGA GTC AAG C-3'. We defined positive expression of ZXF2 as a greater than 2 fold increase in carcinoma tissues than that in non-cancerous tissues.

ZXF2 siRNA transfection

Three siRNA sequences specifically against ZXF2 and control scramble siRNA (siRNA-NC, as a negative control) were synthesized from Gene-pharma (shanghai, China). SiRNA against ZXF2 and scramble siRNA were then transfected by lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The efficiency of siRNAs was determined by comparing expression of ZXF2 in siRNA treated cells to that in siRNA-NC treated cells.

Cell proliferation assay by CCK-8

To determine the effect of ZXF2 on cell proliferation, A549 cells transfected with siRNA-ZXF2 were seeded into 96well plates at a density of 3×10^3 cells/well and incubated for 24h, 48h, 72h, and 96h, respectively. SiRNA-NC treatment was used as a negative control. The effect of ZXF2 on cell proliferation was determined by using cell count kit-8 (CCK-8) (Boster,China) as described previously [9]. Briefly, at the end of incubation, 10 μl of CCK-8 solution was added to each well. Four hours later, the number of viable cells was determined by the absorbance of water-soluble formazan at 460 nm using a FlexStation 3 plate reader (Molecular Devices, CA).

In vitro migration and invasion assays

The procedure was described previously [10]. In brief, *in vitro* cell migration was examined in Boyden chamber system. A549 cells (1×10^5) treated with either siRNA against ZXF2 or siRNA-NC suspended in serum-free medium were added to the upper chamber (6.5-mm diameter, 8-μm pore size, Corning), and the chamber was placed in 24-well dishes. Migration was permitted for 24 h, and cells were then fixed with 4% formaldehyde.

In vitro cell invasion assays were conducted using a Boyden chamber (2×10^5 cells per well) with Matrigel-coated invasion inserts (BD Biosciences, 8 μm pore membranes) placed according to the manufacturer's instructions.

Twenty-four hours after incubation, trans-migrating cells on the underside of the inserts were fixed with 4% formaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images of five random fields were acquired, the average number of cells per field was calculated and presented.

Table 1. Relative ZXF2 level in tumor tissues of the patients with lung cancers

Patient No.	Relative ZXF2 levels	Patient No.	Relative ZXF2 levels
1	3.530	21	1.880
2	8.950	22	6.330
3	9.730	23	19.150
4	0.140	24	0.800
5	15.700	25	2.840
6	20.130	26	20.030
7	0.560	27	17.050
8	10.110	28	0.880
9	0.350	29	1.560
10	5.600	30	2.050
11	0.840	31	18.340
12	11.810	32	20.330
13	4.600	33	0.050
14	14.770	34	5.700
15	1.200	35	12.990
16	1.826	36	10.700
17	4.130	37	6.800
18	7.810	38	0.770
19	9.560	39	7.540
20	0.030	40	2.160

Western blot

Protein was extracted from cells by RIPA buffer. The protein lysate was separated by 12.5% SDS-PAGE, and electrophoretically transferred to a PVDF (polivinylidene difluoride) membrane (Roche). Then, the membrane was probed with antibodies against c-myc (Proteintech), e-cadherin (Proteintech) and GAPDH (as an internal control) (Proteintech); and horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL). The chemiluminescence of proteins on PVDF membranes was detected with ECL Plus kit (Pierce).

Cell cycle and apoptosis assay

The cells were harvested after transfection with siRNA against ZXF2 and siRNA-NC for 48 hours. Then the cells were subjected to either cell cycle detection kit (Beyotime, China) or cell apoptosis detection kit (Sungene, China) by using a FACS Calibur instrument (Becton Dickinson, CA, USA).

Statistical analysis

Statistical analyses were conducted using SPSS17.0 software. Student's T test was used for paired data. Composition ratio was used to describe counting data. Chi-square test was used for clinical data analysis. Survival curves were plotted by Kaplan-Meier method and log-rank method. P values of <0.05 were considered statistically significant.

Results

ZXF2 overexpresses in lung adenocarcinoma and the expression is negatively correlated with patients' prognosis

We collected cancer and adjacent normal tissues from 40 patients with lung adenocarcinoma and analyzed the expression of ZXF2 in these samples by qRT-PCR. As shown in Fig. 1A and Table 1, the expression of ZXF2 in tumor tissue is significantly higher than that in adjacent normal tissues (with a mean increase of 7.23 fold, and a median increase of 5.56 fold). In addition, 27 out of 40 cancer tissues (67.5%) had a 2 fold or higher expression of ZXF2 than that in adjacent non-cancerous tissues ($p<0.05$). This result confirm our previous observation[8].

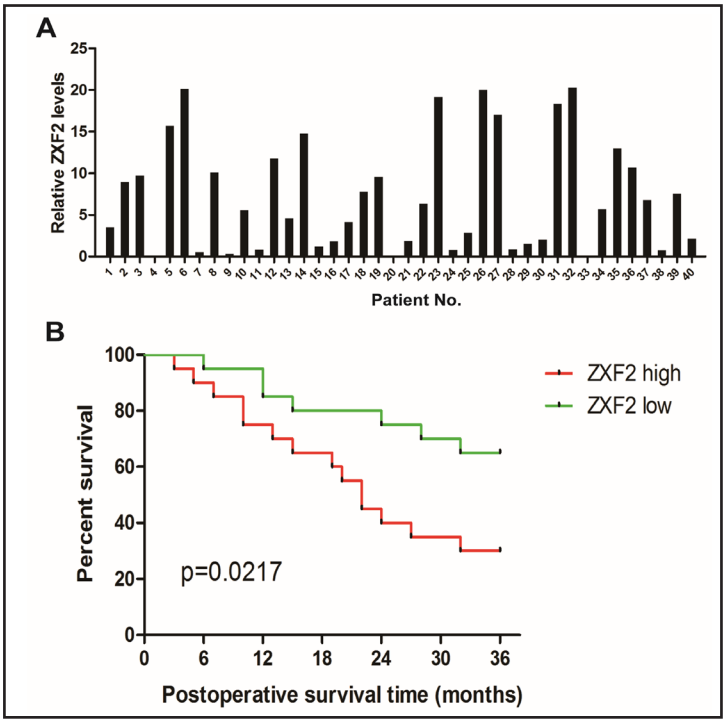

Fig. 1. Expression of LncRNA ZXF2 in lung cancer tissues and its correlation with patients' postoperative survival. (A) LncRNA ZXF2 is overexpressed in tumor tissues as compared to adjacent normal tissue. 40 pairs of specimens were detected by qRT-PCR. 27 out of 40 cancer tissues had 2 fold or more lncRNA-ZXF2 than adjacent non-cancerous tissues ($P<0.01$). (B) The post-operative survival time was determined by Kaplan-Meier survival analysis. Compared with those in low ZXF2 group, the patients in high ZXF2 group had significant shorter survival time ($P=0.0217$).

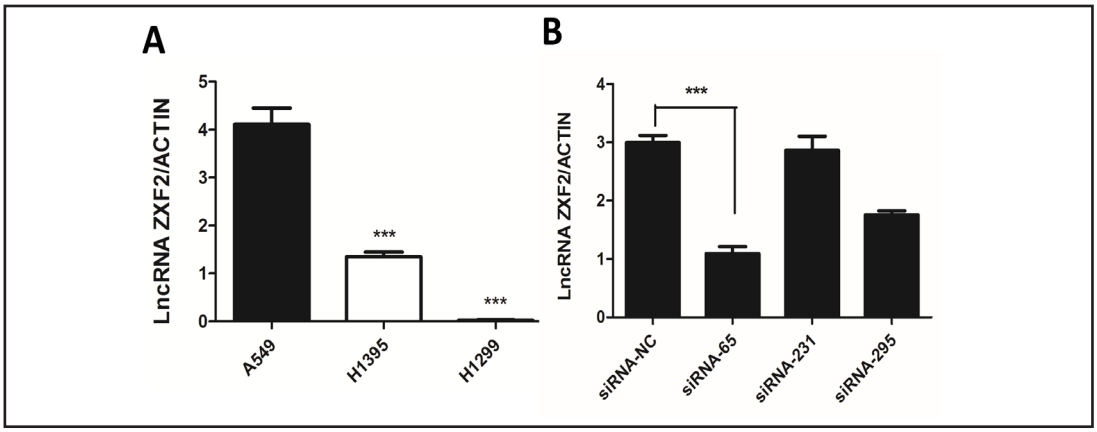

Fig. 2. LncRNA ZXF2 is overexpressed in A549, a high invasive and metastatic cell line. (A) LncRNA ZXF2 expression in different lung adenocarcinoma cell lines was determined by Western blot analysis. A549 cells had significantly higher expression compared with other two cell lines ($P<0.01$). (B) Three different interference sequences were originally designed to knockdown ZXF2 expression. Compared to siRNA-NC (negative control), transfection of siRNA-65 led to about 70% reduction of ZXF2 expression, which showed the strongest inhibitory efficiency among three siRNAs probes.

To correlate the expression level of ZXF2 in tumor tissues with patients' clinicopathological parameters, we first equally divided patients into two groups using a cut-off of median using a relative ZXF2 expression value: the average level of ZXF2 in one group is 12.67 ±5.09 (ZXF2 high group) and that in the other group is 1.79 ±1.60 (ZXF2 low group). Next, the association between ZXF2 expression in tumor tissues and clinicopathological features of patients with lung cancers was examined. The clinicopathological parameters of the two groups were summarized in Table 2. Multivariate analysis (Table 2) revealed that high expression of ZXF2 was positively correlated with histological grade (p=0.024), T stage classification (p=0.016), lymph node metastasis (p=0.014) and advanced clinical staging (p=0.006). No correlation was observed between ZXF2 expression and other factors such as age, gender, smoking history, tumor size and location (P>0.05). Furthermore, Kaplan-Meier survival curves revealed correlations between high ZXF2 expression and poor overall survival and recurrence-free survival (Fig. 1B, p<0.01). Taken together, this analysis suggested that high expression of ZXF2 was closely correlated with tumor progression in human lung adenocarcinoma.

Table 2. The relationship between lncRNA ZXF2 expression and clinicopathological parameters of 40 patients with lung adenocarcinoma

Clinicopathological features	ZXF2 high levels (n=20）	ZXF2 low levels (n=20)	Chi-square	P value
Age			1.290	0.256
＜60	6	3		
≥60	14	17		
Gender			0.417	0.519
Male	9	7		
Female	11	13		
Smoking history			0.476	0.490
Yes	13	15		
No	7	5		
Location			1.129	0.288
Central	7	4		
Peripheral	13	16		
Histological grade			7.444	0.024
Low	11	3		
Middle	6	9		
High	3	8		
Size(cm)			0.125	0.723
＜3	5	6		
≥3	15	14		
T stage classification			8.307	0.016
T1	5	6		
T2	5	12		
T3	10	2		
Lymph node metastasis			8.485	0.014
N0	8	16		
N1	7	4		
N2	5	0		
Advanced clinical staging			7.619	0.006
Ⅰ, Ⅱ	10	18		
Ⅲ	10	2		

ZXF2 regulates cell cycle, proliferation, migration and invasion of lung cancer cells

In order to elucidate molecular mechanism of ZXF2 in promoting lung cancer development, *in vitro* cell culture experiments were employed. We first tested the expression ZXF2 in A549, H1299, and H1395 lung adenocarcinoma cell lines by qRT-PCR. As shown in Fig. 2, A549 had highest level of ZXF2 among these cell lines ($P<0.01$, Fig.2A). Because A549 was reported to be a high invasive and metastatic lung adenocarcinoma cell line [11], we chose it in the following experiments. We used siRNA technology to specifically knockdown the endogenous ZXF2 in A549 cells.

Three different RNA interference sequences were originally designed in this study: siRNA-65: 5'-GCU GUU GGA UGU CAC ACA CTT-3'; siRNA-231: 5'-CCC AAA UCU UCC UUC AAA UTT-3'; siRNA-295: 5'-GCC UGU CCU CAA UCC CUA UTT-3'. Compared to negative control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3'), transfection of siRNA-65 resulted in highest inhibitory efficiency among these three siRNAs, which led to about 70% reduction of ZXF2 expression, (Fig. 2B). Based on this result, we selected siRNA-65 in the following experiments to test the functions of ZXF2 in lung cancer cells.

We first tested the effect of siRNA on cell proliferation. We found that siRNA-ZXF2 treatment significantly suppressed proliferation at different time points compared to A549 cells treated with siRNA-NC ($P<0.01$, Fig. 3A). In order to elucidate the mechanisms underlying siRNA-ZXF2 induced proliferation suppression, we investigated whether siRNA-ZXF2 treatment can induce cell death by flow cytometry. Interestingly, we did not observe obvious apoptosis in A549 cells treated with siRNA-ZXF2 (Fig. 3B-3D).

Then, we tested whether knockdown of ZXF2 can affect cell cycle. Flow cytometry analyses of cell cycle distribution revealed that reduction of ZXF2 resulted in a significant increase of cell number in the G_0/G_1 phase and a slight reduction in S and G_2/M phases compared with cells transfected with siRNA-NC as a control. ($P<0.01$, Fig.4A-C, Table 3), indicating G_0/G_1 arrest might contribute to proliferation inhibition by siRNA-ZXF2. We further examined the changes of several cell cycle related proteins that are known involved in lung cancers. The results shown that knockdown of ZXF2 did not change the expression of p21, Cyclin B1, but resulted in down regulation of Cyclin D1, whereas transfection of siRNA-NC had no effects on these proteins (Fig. 4D). Given the fact that Cyclin D1 plays an important role in the proliferation and progression of lung cancers [12], the Western blot results were consistent with the cell cycle distribution.

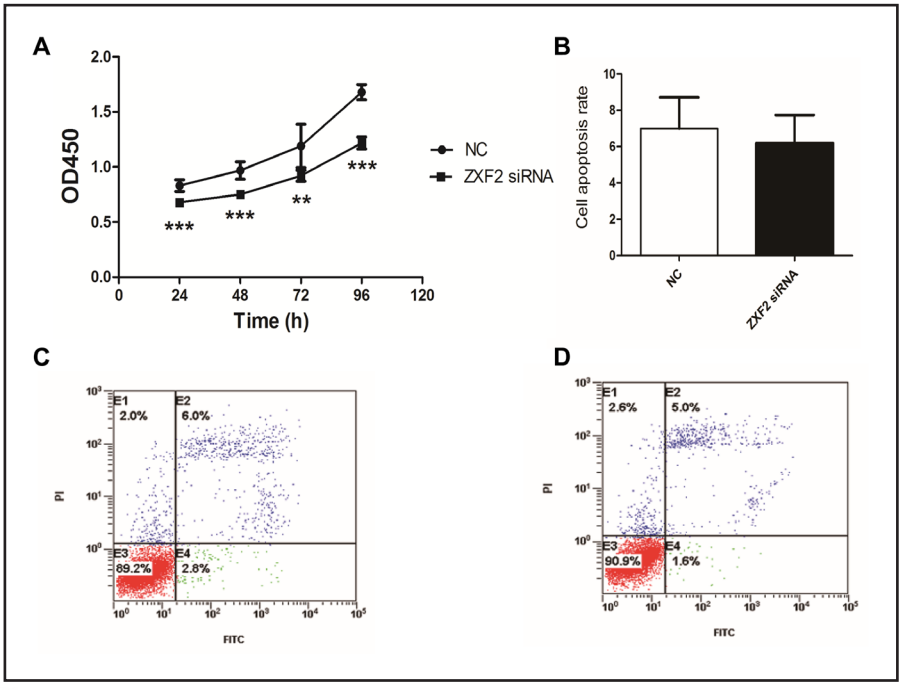

Fig. 3. Knockdown of ZXF2 inhibits cell proliferation. (A) A549 cells transfected with siRNA-ZXF2 or siRNA-NC and incubated for 24h, 48h, 72h, 96h, respectively. The cells were detected by CCK-8 assay, the bars indicate means±SDs (n=8). (B-D) Flow cytometry analysis revealed that knockdown of ZXF2 does not induce apoptosis of A549 cells.

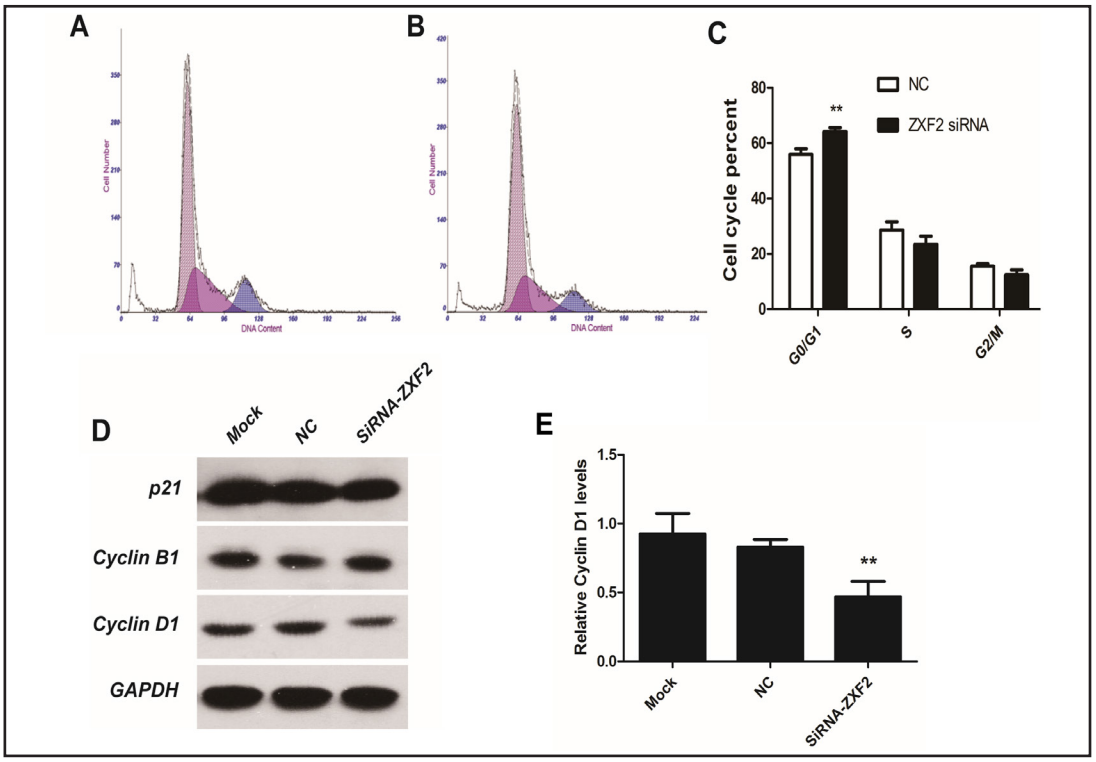

Fig. 4. Knockdown of ZXF2 leads to G_0/G_1 cell cycle arrest through down regulation of Cyclin D1 in A549 cells. Representative images of cell cycle distribution in siRNA-NC treatment group (A) and siRNA-ZXF2 treatment group (B). The data from (A) and (B) were summarized in (C), which showed G_0/G_1 cell cycle arrest after siRNA-ZXF2 treatment. The data indicate means±SD (n=3, **: p,0.01). (D) The protein expression levels of p21, Cyclin B1, Cyclin D1 were detected by Western blot after transfected with siRNA-ZXF2. The expression of Cyclin D1 was decreased after siRNA-ZXF2 treatment, while the expression of p21 and Cyclin B1 did not change.

Table 3. Summary of cell cycle distribution of A549 cells treated with siRNA-ZXF2 and siRNA-NC control. **: p<0.01

Group	Phase		
	G_0/G_1**	S	G_2/M
SiRNA-NC	(55.87±1.16)%	(28.62±1.68)%	(15.51±0.53)%
SiRNA-ZXF2	(64.18±0.79)%	(23.41±1.72)%	(12.41±1.07)%

We tested the potential function of ZXF2 on tumor cell migration and invasion. Boyden chamber transwell assay was used to determine cell migration and invasion capacities. After transfected with siRNA-ZXF2, both migration and invasion capacities were significantly inhibited as compared to those of cells treated with siRNA-NC. Specifically, the mean number of migrated cells per view in siRNA-ZXF2 treated group was 214.60 ± 11.89, while that in siRNA-NC treated group was 690.40 ± 11.54(more than 3-fold increase) (P<0.01). In addition, we observed a more than 10-fold reduction in invasive ability of the cells treated with siRNA-ZXF2 (57.80 ± 4.73 cells/view in siRNA-ZXF2 treated group vs 628.20 ± 7.08 cells/view in siRNA-NC treated group; P<0.01, Fig. 5A-F).

Knockdown of ZXF2 leads to inhibition of c-Myc signaling

In order to elucidate the molecular mechanisms of siRNA-ZXF2 mediated suppression of migration and invasion, two key proteins were selected. One was c-Myc which is a strong

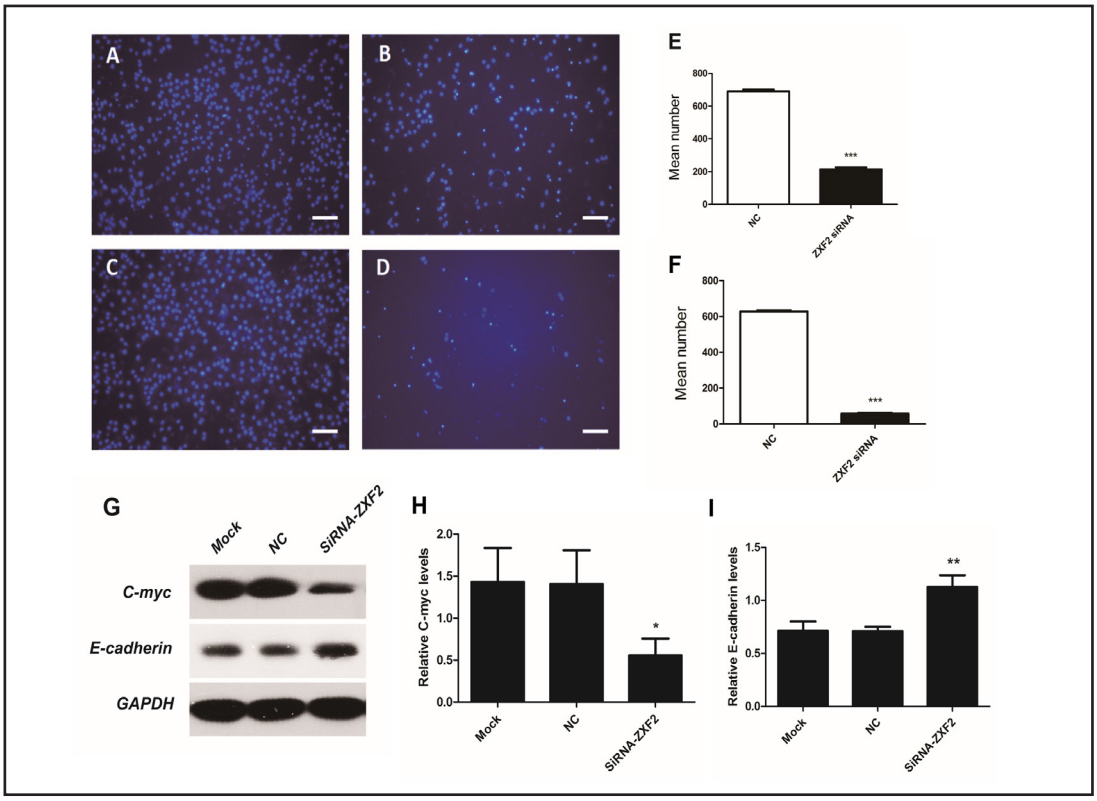

Fig. 5. Knockdown of ZXF2 inhibits migration and invasion through c-Myc/e-Cadherin signaling pathway. Transwell assay was used to detect migration and invasion of transfected A549 cells. The representative image showed siRNA-ZXF2 treatment (B) inhibited A549 cell migration as compared to those treated with siRNA-NC (A). Similarly, the representative images showed siRNA-ZXF2 treatment (D) inhibited A549 cell invasion as compared to those treated with siRNA-NC (C). (E) The number of migrated cells through filter was quantified by counting DAPI nuclear staining. Scale bar: 100 μm. The data represented means±SD (n=5). (F) The number of invaded cells was quantified by counting DAPI nuclear staining. Scale bar: 100 μm. The data represented means±SD(n=5). (G) The protein expression levels of c-Myc and E-cadherin were detected by Western blot after transfected with siRNA-ZXF2 and control. The results showed that treatment of siRNA-ZXF2 led to down regulation of c-Myc and up regulation of E-cadherin. (H-I) Band densities of Western blot were quantified by densitometry. The results show a significant changes of expression of c-Myc and E-cadherin after siRNA-ZXF2 treatment. *: $p<0.05$; **: $p<0.01$.

proto-oncogene promoting cancer cell migration and invasion [13]. The other was E-cadherin which is frequently lost during tumor progression and transition to a more motile and invasive phenotype [14]. Interestingly, bioinformatics analysis revealed that both ZXF2 and c-Myc are adjacently located on 8q24.2 loci of human chromosome 8, which is a "hot spot" for multiple human cancers [15-17]. The Western blot analysis showed that transfection of siRNA-ZXF2 suppressed c-myc and enhanced e-cadherin expression, indicating a potential role of ZXF2 on controlling lung cancer progression through c-myc/e-cadherin pathway (Fig. 5G-5I).

Discussion

It has been reported that 4%-9% of the transcription in mammalian genome are lncRNAs, whereas the rate of protein-coding RNA is only 1%. Over a long period of time, lncRNA was considered as a "noise" in genome transcription without any biological

functions. However, recent studies have showed that lncRNAs participate in a lot of critical biological regulations, such as X-chromosome silence, genomic imprinting and chromatin modification, transcription activation, transcription interference and nuclear transport et al. [4]. In recent years, increasing evidences have linked lncRNA to various biological processes and diseases [18]. For example, HOTAIR, one of the first lncRNAs, was reported to be involved in the progression of breast cancer [6]. Our group previously identified a group of lncRNAs that were differentially expressed between lung cancer tissues and adjacent normal tissues derived from patients with lung adenocarcinomas [8].

In the present study, we tested the functions of one of the novel lncRNAs, ZXF2 (located on chromosome 8, SEQ_ID:AA311918) in lung cancer progression. We found that expression of ZXF2 was increased in human lung cancer tissues, negatively correlated with post-surgery survival and positively correlated with lymph node metastasis of patients with lung cancers. We knockdowned ZXF2 in A549 cells, a metastatic lung cancer cell line, in order to explore the cellular and molecular mechanisms of potential tumor promoting function of ZXF2. We found that siRNA-ZXF2 treatment led to suppression of cell proliferation, migration and invasion, and G_0/G_1 arrest through regulating c-myc/e-cadherin pathway.

As development of cancer is a result of orchestration of a multiple genes network, some traditional approaches that targets single gene might only have limited effect on controlling tumor growth. Given this background, LncRNAs, which play an important role in regulation of transcription and post-transcription processes, might be a better approach for developing effective means to treat cancers. In the present study, we observed a very interesting phenomenon that ZXF2 can regulate tumor progression by targeting c-myc/e-cadherin pathway. C-myc has been shown to negatively regulate e-cadherin through activating the transcription of microRNA-9 in breast cancer cells [19], which was consistent with our observation. In addition, c-Myc can induce expression of Cyclin D1 to promote cell cycle progression in multiple systems [20, 21], and functional interactions between c-Myc and cylclin D1 play a critical role in tumor initiation, progression and response to chemotherapy [22]. Therefore, we suspect that our observation that knockdown of ZXF2 led to down regulation of Cyclin D1 could also be c-Myc dependent. The potential molecular mechanisms underlying ZXF2 mediated regulation of c-myc could be summarized as following: First, given the fact that both human c-myc and ZXF2 are adjacently located on chromosome 8 (8q24.2), and some lncRNAs have been demonstrated to control myc expression through regulate long-range interactions between the MYC promoter and its enhancers in cancer cells [23, 24], it is possible that ZXF2 functions as a transcriptional enhancer of MYC gene. Second, a recent elegant study show that lncRNA can directly bind and serve as a coactivator of c-Myc to activate transactivation functions of c-Myc [25]. Finally, lncRNA has been shown to regulate mRNA stability of c-Myc [26]. Further studies will confirm the mechanism involved in ZXF2 mediated regulation of c-Myc.

Conclusion

In conclusion, lncRNA ZXF2 was overexpressed in human lung adenocarcinoma. The high level of lncRNA ZXF2 was associated with lymph node metastasis and poor post-operative survival time. The overexpression of ZXF2 promoted proliferation, migration and invasion of cancer cell. The study revealed that ZXF2 might be an important marker in lung adenocarcinoma metastasis and might also be a therapeutic target in the treatment of lung adenocarcinoma.

Acknowledgement

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Disclosure Statement

The authors confirm that there is no conflict of interest.

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