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Long non-coding RNA DANCR promoted non-small cell lung cancer cells metastasis *via* modulating of miR-1225-3p/ErbB2 signal

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Abstract. – OBJECTIVE: Currently, we aimed to illustrate the role of lncRNA differentiation antagonizing non-protein coding RNA (DANCR) and erb-b2 receptor tyrosine kinase 2 (ErbB2) in non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Expression of DANCR, microRNA-1225-3p (miR-1225-3p) and ErbB2 mRNA was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) assays. The clinical value of DANCR was checked by a ROC curve analysis, a Kaplan-Meier analysis and a Pearson Chi-Square test. Transwell chamber assays were performed to determine the migration and invasion ability changes of SPCA1 and A549 cells. The protein expression of ErbB2 was tested by Western blot assays. The targeted binding effect between miR-1225-3p and DANCR or ErbB2 was confirmed by a Dual-Luciferase reporter assay and an RNA pull-down assay, respectively.

RESULTS: In the current study, it was found that DANCR was upregulated and correlated with poor prognosis in patients with NSCLC. DANCR promoted NSCLC cells migration and invasion *via* upregulation of ErbB2. DANCR regulated ErbB2 at posttranscriptional level. Mechanically, it was illustrated that miR-1225-3p negatively regulated ErbbB2 and it-mediated migration and invasion *via* directly targeting in NSCLC cells. Meanwhile, it was showed that DANCR interacted with miR-1225-3p in a reciprocal suppression manner. Even further, through a RIP assay and a luciferase assay, we showed that DANCR interacted with miR-1225-3p through a microRNA response element (MRE-1225-3p) *via* directly binding. Finally, it was demonstrated that DANCR served as a miR-1225-3p sponge to promote ErbB2 expression and to facilitate ErbB2-mediated migration and invasion in NSCLC cells.

CONCLUSIONS: In the current study, it was illustrated that DNACR promoted ErbB2-mediated migration and invasion *via* working as a ceRNA of miR-1225-3p in NSCLC cells.

Key Words:
DANCR, ErbB2, Metastasis, Non-small cell lung cancer.

Introduction

As the most prominent histological subtype of lung cancer, NSCLC accounts for the leading cause of lung cancer-associated mortality and is with a high rate of metastasis[1,2]. The prognosis of NSCLC is very poor with about 15% of 5-year survival rate. The reasons for the bad prognosis of NSCLC include failure of early interventions, relapse, and the lack of effective treatment for advanced cases[3]. Therefore, seeking out new molecules and uncover their functions is still urgent for targeted treatment of NSCLC.

Long non-coding RNAs (lncRNAs), length of more than 200 nucleotides, are a great amount of transcriptions without protein coding ability. LncRNAs are widely associated with various biological progresses including chromatin dynamics, gene expression, growth, metastasis, and development in a variety of malignancies[4-7]. As a member of the lncRNAs family, DANCR is reported as an oncogene in multiple cancers. Wang et al[8] reported that DANCR promoted osteosarcoma progression via serving as miRNA sponges to facilitate osteosarcoma cells proliferation and metastasis. Wen et al[9] found

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that DANCR was upregulated in nasopharyngeal carcinoma (NPC), and promoted NPC cells metastasis through interacting with NF90/NF45 complex. Lu et al[10] reported that DANCR accelerated lung adenocarcinoma (LAD) progression by working as a competitive endogenous RNA (ceRNA) of miR-496 to modulate mTOR expression. However, the role of DANCR and its working mechanism in NSCLC remains deeply exploring.

ErbB2, also named as human epidermal growth factor receptor 2 (HER2), belongs to ErbB family and is identified as a classical oncogene in various malignant tumors, especially breast cancer[11]. Like other family members, ErbB2 is commonly overexpressed, amplified, or mutated in various cancers, making it important therapeutic targets. Zeng et al[12] reported that ErbB2 upregulated activating transcription factor 4 (ATF4) to promote breast cancer cell migration via activation of ZEB1 and downregulation of E-Cadherin. Li et al[13] found that ErbB2 was upregulated in NSCLC, and microRNA-331-3p (miR-331-3p) suppressed epithelial-mesenchymal transition (EMT) via targeting ErbB2 and VAV2. Till present, whether DANCR could regulate ErbB2 and its mediated metastasis remains uncovered.

In the present research, we found that DANCR work as an oncogene in NSCLC. Also, we displayed that DANCR promoted NSCLC cells migration and invasion via upregulation of ErbB2 by sponging of miR-1225-3p.

Patients and Methods

Patients and Tissue Samples

A total of 84 NSCLC and paired para-tumor tissue specimens were collected from patients with NSCLC at Cancer Hospital of China Medical University/Liaoning Cancer Hospital and Institute from February 2011 to March 2016. Written informed consent was provided by the patients whose tissues were used in the current research. All procedures were granted under the approval of the Institute Research Medical Ethics Committee of Cancer Hospital of China Medical University/Liaoning Cancer Hospital and Institute.

Cell Culture

A human normal bronchial epithelial cell line 16HBE, four human NSCLC cell lines A549, SPCA1, H1299 and H1975 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). 16HBE cells were cultured in Airway Epithelial Cell Basal Medium (ATCC, Manassas, VA, USA), NSCLC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, El Paso, TX, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin (Baomanbio, Shanghai, China) and 100 mg/ ml streptomycin (Baomanbio). All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Reverse Transcription and Quantitative Real-time PCR

The procedure was performed as previously described[14]. A TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNAs from tissue specimens and cell lines in accordance with the manufacturer's instructions. 1 μg total RNA was reverse transcribed using TransScript first-strand cDNA synthesis SuperMix (TransGen, Beijing, China). qRT-PCR assay was performed by SYBR green qPCR SuperMix (Applied Biosystems Life Technologies, Foster City, CA, USA). U6 and GAPDH were used to normalize the expression levels of the miRNAs and DANCR/ErbB2, respectively. Primer sequences were synthesized by RiboBio Co., Ltd. (RiboBio, Guangzhou, Guangdong, China) as listed in Table I.

Oligonucleotide Transfection

Specific small interfering RNA (siRNA) oligonucleotides that targeted DANCR (si-DANCR-1 and si-DANCR-2), DANCR overexpression plasmids (oeDANCR/wt- oeDANCR and mut-oeDANCR, containing wild and mutant miR-1225-3p binding sites) and ErbB2 overexpression plasmids were synthesized by GenePharma Co., Ltd. (GenePharma, Shanghai, China). MiR-1225-3p mimics (mimic-1225-3p) and corresponding control (mimic-con) as well as miR-1225-3p inhibitors (inh-1225-3p) and inhibitors control (inh-con) were chemically synthesized by RiboBio Co., Ltd. (RiboBio) to overexpress or knockdown miR-1225-3p, respectively. All the oligonucleotides and plasmids were transfected into CRC cells according to different requirements by using a Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The sequences of small interfering RNAs were also listed in Table I.

Table I. Primer sequences and oligonucleotides used in this research.

Gene	Sequences of primers/oligonucleotides
DANCR forward	GCGCCACTATGTAGCGGGTT
DANCR reverse	TCAATGGCTTGTGCCTGTAGTT
ErbB2 forward	ACGTGCTCATCGCTCACAACCAAGTGAGG
ErbB2 reverse	CCATTGTCTAGCACGGCCAGGGCATAGTT
GAPDH forward	TGTTCGTCATGGGTGTGAAC
GAPDH reverse	ATGGCATGGACTGTGGTCAT
miR-1225-3p RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGGG
miR-1225-3p forward	GCGGCGGTGAGCCCCTGTGCCG
miR-548b-5p RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCCAA
miR-548b-5p forward	GCGGCGGAAAAGTAATTGTGG
miR-548j-5p RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCAAA
miR-548j-5p forward	GCGGCGGAAAAGTAATTGCGGTC
miR-548ak RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAAAA
miR-548ak forward	GCGGCGGAAAAGTAACTGCGG
Reverse primer	ATCCAGTGCAGGGTCCGAGG
U6 forward	CGCTTCGGCAGCACATATACTA
U6 reverse	CGCTTCACGAATTTGCGTGTCA
siDANCR-1	GCCCCUGUGCCGCCCCCAG
siDANCR-2	AGCCCCUGUGCCGCCCCCA
Mimic-1225-3p	TGAGCCCCTGTGCCGCCCCCAG
Inh-1225-3p	CTGGGGGCGGCACGGGGCTCA

Western Blot Analysis

Total proteins were extracted by applying a radio immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). 5 μl proteins samples were qualified by using a bicinchoninic acid (BCA) protein quantification Kit (Keygen, Nanjing, Jiangsu, China) according to the manufacture's protocol. 50 μg proteins samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% defatted milk, the membranes were incubated with primary antibodies (Abcam, Cambridge, MA, UK; dilution rates of 1:1000 for ErbB2 antibody and 1:500 for GAPDH antibody) overnight at 4°C. The next day, the membranes were incubated with secondary antibodies (Abcam, dilution rates of 1:2000) at room temperature for 1 h. Protein bands were detected on X-ray film using an enhanced chemiluminescence detection system.

Transwell Assay

Transwell assays were performed to detect the migration and invasion ability changes of SPCA1 and A549 cells as previously described[15]. SPCA1 and A549 cells were seeded on Matrigel-coated (for invasion assay) or uncoated (for migration assay) upper chambers (BD Bioscience, Franklin Lakes, NJ, USA) separately. Culture medium non-containing and containing 10% FBS were supplemented into the upper and lower wells respectively and incubated for 24 h. The next day, the non-invaded cells were wiped out. Then, the filters were fixed in 90 % alcohol and followed by a crystal violet staining. Five random fields were counted per chamber by using an inverted microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Assay

The procedure was carried out as previously described[16]. Wild (containing miR-1225-3p seed region) and mutant (containing mutant miR-1225-3p seed region) DANCR (wt-DANCR-luc and mut-DANCR-luc) or ErbB2 (wt-ErbB2-luc and mut-ErbB2-luc) reporter plasmids were designed and chemically synthesized by GenePharma, respectively. The constructed reporter plasmids were cotransfected with mimic-1225-3p or mimic-con and incubated for 48 h, individually. Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

RNA Pull-Down Assay

The procedure was performed as previously reported[17]. DANCR transcripts with wild and mutant miR-1225-3p binding sites were biotin-labeled with the Biotin RNA Labeling Mix (Roche, Basel, Switzerland) and T7 RNA poly-

merase (Roche), treated with RNase-free DNase I (Roche), and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One milligram of whole-cell lysates from SPCA11 cells or A549 cells was incubated with three micrograms of purified biotinylated transcripts for 1 h at 25°C. The complexes were isolated by streptavidin agarose beads (Invitrogen). A qRT-PCR assay was followed to detect the pulled-down miR-1225-3p.

Statistical Analysis

All experiments were repeated in triplicate and all data from three independent experiments were expressed as mean ± SD. GraphPad Prism V5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software and SPSS 19.0 statistical software (IBM, Armonk, NY, USA) were used for statistical analysis. Correlation between DANCR and clinicopathological features of NSCLC patients was analyzed by using the Pearson's chi-squared test. Survival analysis was performed using the log-rank test in GraphPad Prism V5.0. Differences in two groups were analyzed by the Student's *t*-test or one-way ANOVA. Differences were considered significant or very significant if p-value < 0.05 or 0.01, respectively.

Results

DANCR Was Upregulated and Correlated with Poor Outcomes in NSCLC Patients

We first measured the expression of DANCR in 16 tissue samples (8 non-tumor adjacent tumor tissue samples and 8 lung adenocarcinoma tissue samples) according to a previous genome wide lncRNA expression profiling of lung adenocarcinoma and non-tumor adjacent tissues (GSE130779). As presented in Figure 1A, DANCR (probe ID: p12708) was stably upregulated in lung adenocarcinoma tissue samples. Further, we determined the expression of DANCR in collected 68 NSCLC tissue samples and paired adjacent tumor tissue samples. As shown in Figure 1B and 1C, DANCR was upregulated in most (57/68, 83.82%) NSCLC tissue samples. Meanwhile, we checked the expression of DANCR at cellular level. As was shown in Figure 1D, we found that DANCR was upregulated in four human NSCLC cell lines SPCA1, A549, H1299 and H1975 as compared to that in a normal human bronchial epithelial cell line 16HBE. Also, we concentrated whether the expression status of DANCR had clinical values in NSCLC. As the results of a

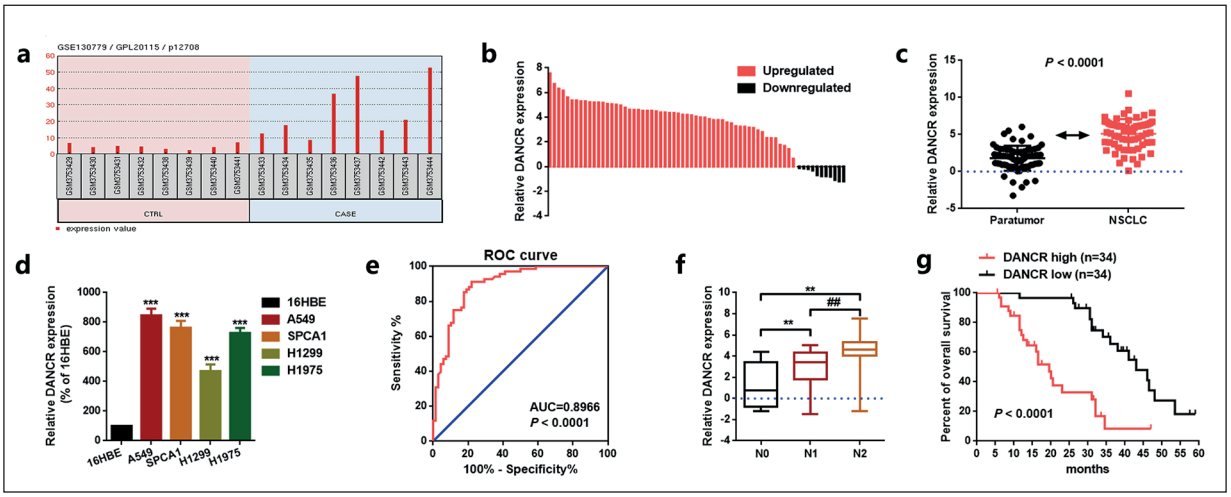

Figure 1. DANCR was upregulated and correlated with poor outcomes in NSCLC patients. **a**, Expression of DANCR was upregulated in NSCLC tissues as analyzing of a previous NSCLC lncRNA expression profiling GSE130779. **b**, **c**, The expression of DANCR was increased in NSCLC tissue specimens than that of para-tumor tissue specimens as determined by qRT-PCR assays, $p < 0.0001$ as comparing with para-tumor tissue group. **d**, The expression of DANCR in a normal human bronchial epithelial cell line 16HBE and in four NSCLC cell lines SPCA1, A549, H1299 and H1975 were measured by qRT-PCR assays, either. ***$p < 0.001$ as comparing with 16HBE group. **e**, The clinical values of DANCR in NSCLC was detected by a ROC curve analysis. **f**, DANCR was upregulated in patients with lymph node metastasis as detected by qRT-PCR assay. **$p < 0.01$ and ##$p < 0.01$ as comparing with N0 group. **g**, Overall survival (OS) in patients with high DANCR (n = 34) was significantly shorter than that of patients with low DANCR (n = 34), p <0.0001 as determined by a Kaplan-Meier analysis.

ROC curve analysis displayed in Figure 1E, the area under the curve (AUC) was 0.8966 (95% confidence interval: 0.8437-0.9496). Meanwhile, we showed that DANCR was notably higher in NSCLC patients with lymph node metastasis (N1 and N2) as comparing to that of patients without lymph node metastasis (N0) (Figure 1F). Finally, we analyzed the correlation between the elevated DANCR and the clinicopathological features in the collected 68 patients with NSCLC. As the results displayed in Figure 1G and Table II, upregulated DANCR was closely correlated with a shorter survival rate ($p < 0.0001$, determined by a Kaplan-Meier analysis), an advanced TNM stage (III + IV, p=0.010) and a significant lymph node metastasis ($p = 0.001$).

DANCR Promoted NSCLC Cells Migration and Invasion In Vitro

In this section, we tried to explore the role of DANCR in NSCLC cells migration and invasion. Specific DANCR siRNAs (siDANCR-1 and siDANCR-2) or a scramble control, and a DANCR-overexpressing plasmid (oeDANCR) or a vector control were transiently transfected into SPCA1 and A549 cells, respectively. The expression of DANCR after different intervene was confirmed by a qRT-PCR assay. As was shown in Figure 2A-D, the expression of DANCR was successfully knocked down and upregulated in SPCA1 and A549 cells, individually. Then, transwell assays with or without Matrigel were performed to evaluate the metastatic ability changes in NSCLC cells. As the representative photographs displayed in Figure 2E and Figure 2G, silencing of DANCR significantly suppressed the migration and invasion abilities in SPCA1 and A549 cells. In contrast, an ectopic upregulation of DANCR promoted migration and invasion in NSCLC cells (Figure 2F and Figure 2H).

DANCR Enhanced Migration and Invasion by Upregulation of ErbB2 in SPCA1 and A549 Cells

ErbB2 is widely involved in several cancers' metastasis including NSCLC. In this section, we tried to explore whether ErbB2 was also involved in DANCR-mediated promotion of migration and invasion in NSCLC. We firstly displayed that up- and down-regulation of DANCR positively regulated ErbB2 expression at protein level (Figure 3A-B). Further, we found that CP-724714, a selective ErbB2 inhibitor, significantly attenuated the facilitative effect of DANCR working on SPCA1 and A549 cells migration and invasion (Figure 3C-D).

Table II. Association of DANCR expression with clinicopathological features of NSCLC.

Features	No. of cases	DANCR		*p*-value†
		High expression	Low expression	
Age (yrs)				0.454
≤ 60	42	23	19	
> 60	26	11	15	
Gender				1.000
Male	51	25	26	
Female	17	9	8	
Smoking history				1.000
Yes	49	24	25	
No	19	10	9	
Pathological type				1.000
Adenocarcinoma	37	19	18	
Squamous carcinoma	31	15	16	
TNM stage				0.010
I + II	45	17	28	
III + IV	23	17	6	
Lymph node metastasis				0.001
Yes	30	22	8	
No	38	12	26	
Tumor size (cm)				0.168
≤ 3	18	6	12	
> 3	50	28	22	

†*p*-value obtained from Pearson Chi-Square test.

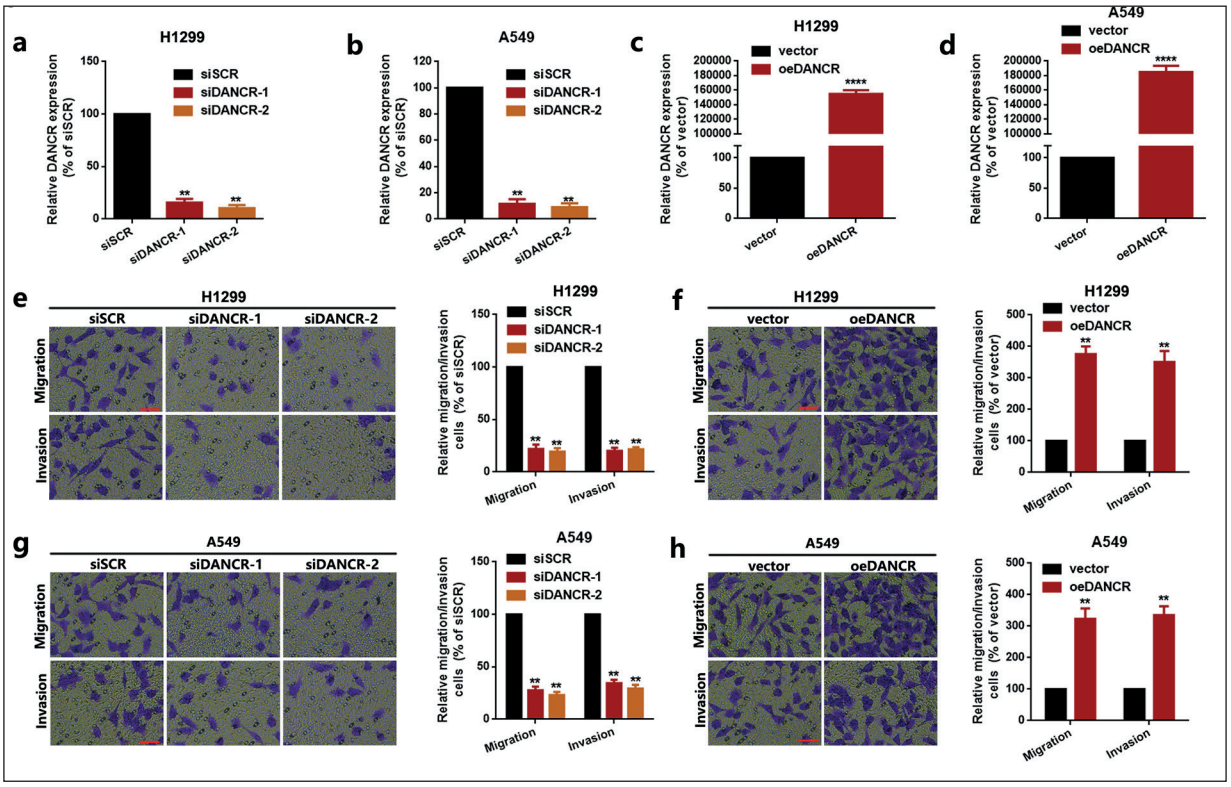

Figure 2. DANCR promoted NSCLC cells migration and invasion *in vitro*. **a-d**, Specific DANCR siRNAs (siDANCR-1 and siDANCR-2) and overexpression plasmids (oeDANCR) were transfected into SPCA1 (**a, c**) and A549 (**b, d**) cells, and the expressions of DANCR in each group were determined by qRT-PCR assays. $^{**}p < 0.01$ and $^{***}p < 0.001$ as comparing with siSCR and vector group, individually. **e, g**, Chamber-transwell migration and invasion assays were performed using SPCA1 (**e**) and A549 (**g**) cells transfected with scramble siRNA (siSCR) and specific DANCR siRNAs, $^{**}p < 0.01$ as comparing with siSCR group. Magnification, ×20; scale bar, 200 μm. **f**, **h**, Transwell migration and invasion assays were using SPCA1 (**f**) and A549 (**h**) cells transfected with blank vector (vector) and DANCR overexpression plasmids (oeDANCR), $^{**}p < 0.01$ as comparing with vector group. Magnification, ×20; scale bar, 200 μm.

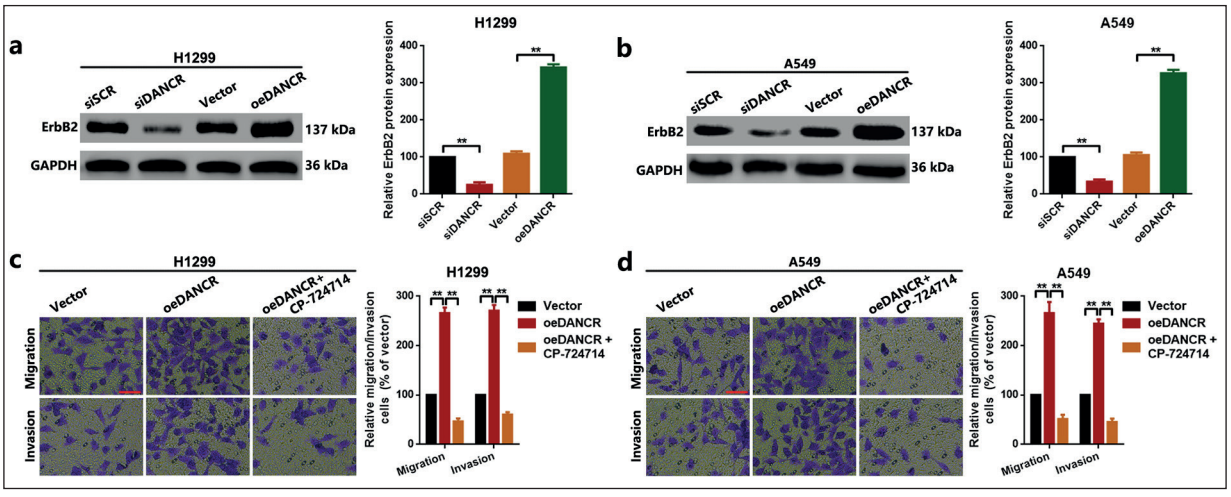

Figure 3. DANCR enhanced migration and invasion by upregulation of ErbB2 in SPCA1 and A549 cells. **a**, **b**, The expression of ErbB2 protein in SPCA1 (**a**) and A549 (**b**) cells was detected by western blot assays. $^{**}p < 0.01$ as comparing with siSCR or vector group, respectively. **c**, **d**, Transwell migration and invasion assays were performed applying of SPCA1 (**c**) and A549 (**d**) cells transfected with vector, oeDANCR and oeDANCR + CP724714, separately. $^{**}p < 0.01$ as comparing with vector group. Magnification, ×20; scale bar, 200 μm.

MiR-1225-3p Was Downregulated and Regulated Migration and Invasion Through ErbB2 Targeting in SPCA1 and A549 Cells

In this section, we firstly found that up- and down-regulation of DANCR had no effect on ErbB2 mRNA (Figure 4A-B, $p > 0.05$). Combined with the results of last section, this phenomenon indicated that DANCR regulated ErbB2 expression post-transcriptionally but not directly. It is well known that miRNAs are frequently involved in lncRNAs actions. Therefore, we wondered whether any miRNAs participated in the interacted network between DANCR and ErbB2. Through online screening (Diana-LncBase, Targetscan, miRWalk and miRDB), we locked 4 miRNAs (miR-548b-5p, miR-548j-5p, miR-1225-3p and miR-548ak) for further detection (Figure 4C). By using GEO Datasets (GSE102286) and a further qRT-PCR detection, we found that miR-1225-3p was stably downregulated in NSCLC (Figure 4D-E) and then be selected in the subsequent research. Next, we illustrated that an increasing or a decreasing of miR-1225-3p negatively regulated ErbB2 at protein level, correspondingly (Figure 4F). Further, we observed that miR-1225-3p targeted ErbB2 directly (Figure 4G-I). Lastly, we showed that miR-1225-3p inhibited SPCA1 and A549 cells' migration and invasion, and the suppressive effect was reversed by a functional ErbB2 cDNA (oeErbB2) (Figure 4J-K). In brief, the findings of this section indicated that the function of ErbB2 on migration and invasion was partially controlled by miR-1225-3p in SPCA1 and A549 cells.

DANCR Promoted ErbB2-Mediated Migration and Invasion Via MiR-1225-3p Sponging in SPCA1 and A549 Cells

In this part, we tried to explore the relationship between DANCR, miR-1225-3p and ErbB2. We firstly illustrated that DANCR interacted with miR-1225-3p in a reciprocal suppressive manner (Figure 5A-D). Secondly, a RNA-pull down assay was performed to illustrate the binding effect between miR-1225-3p and DANCR. Wild and mutant DANCR transcriptions were labeled with biotin (Bio-wt-DANCR and Bio-mut-DANCR) and incubated with cell lysates from SPCA1 and A549 cells. A qRT-PCR assay was applied to analyze the relative expression of miR-1225-3p being pulled-down. As the result displayed in Figure 5E-F, the enrichment of miR-1225-3p was significantly abundant by Bio-wt-DANCR than that by Bio-mut- DANCR. Even more, we displayed that DANCR was a direct target of miR-1225-3p through a luciferase assay (Figure 5G-H). Further, we illustrated that a wt-oeDANCR (a wild DANCR overexpressing plasmid containing wild miR-1225-3p binding sites) promoted ErbB2 protein expression. When the binding sites were mutated (mut-oeDANCR, a mutant DANCR overexpressing plasmid containing mutant miR-1225-3p binding sites), the facilitating effect was dismissed. More interestingly, the promotive effect of wt-oeDANCR on ErbB2 was remarkably attenuated by an up-regulation of miR-1225-3p (cotransfection of wt-oeDANCR + mimic-1225-3p) (Figure 5I-J). Lastly, we demonstrated that SPCA1 and A549 cells migration and invasion ability was enhanced by wt-oeDANCR but not mut-oeDANCR, and the facilitative was significantly reversed by mimic-1225-3p (Figure 5K-L). Taking all, as the mechanism diagram displayed in Figure 6, all the findings above indicated that DANCR promoted NSCLC cells migration and invasion via modulating of miR-1225-3p/ErbB2 signal.

Discussion

Accumulating researches have indicated that lncRNAs are extensively associated with multiple cell biological behaviors and processes[18]. As one of the lncRNAs members, DANCR, also named as ANCR, is located at chromosome 4q12 and contains 5 exons. DANCR was primary identified as a noncoding RNA which is necessary to enforce the undifferentiated cell state within epidermis[19]. Till present, related researches showed that DANCR is widely involved in various cell biological behaviors including cell differentiation, proliferation, apoptosis, migration and invasion[20-23]. Wang et al[16] found that DANCR was upregulated in colorectal cancer (CRC), and DANCR promoted CRC cells proliferation and metastasis *via* modulating of heat shock protein 27 (HSP27)/miR-577 axis. Guo et al[24] reported that DANCR enhanced the progression of NSCLC by inhibiting p21 expression. In the current study, we demonstrated that DANCR was upregulated in NSCLC tissues and cell lines. Meanwhile, we illustrated that DANCR promoted NSCLC cells migration and invasion through gain and loss of functional transwell assays. Also, we showed that ErbB2 was positively regulated by DANCR, and was involved in DANCR induced migration and invasion in NSCLC cells. When we

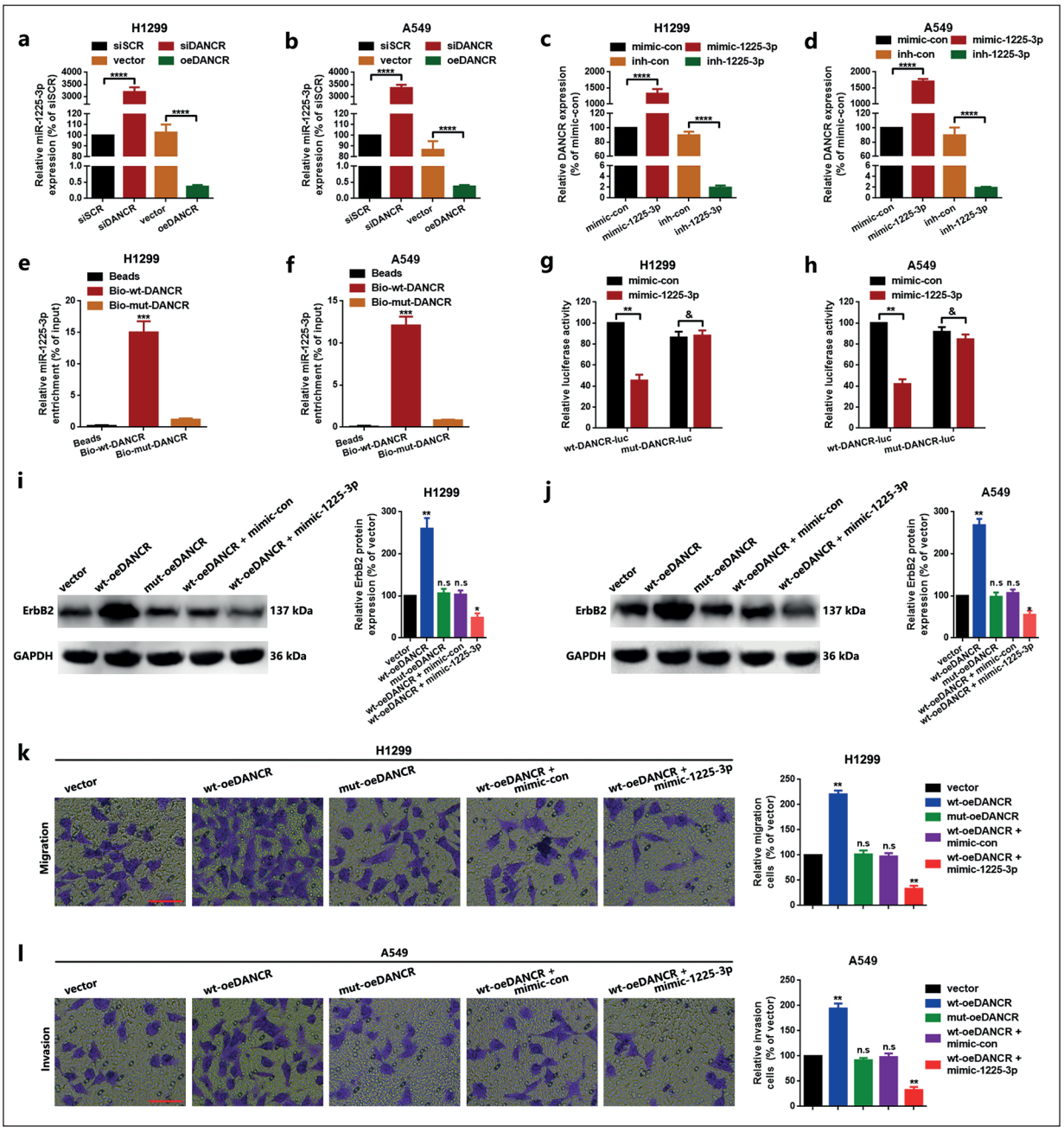

Figure 4. MiR-1225-3p was downregulated and regulated migration and invasion through ErbB2 targeting in SPCA1 and A549 cells. **a**, **b**, The mRNA changes in SPCA1 (**a**) and A549 (**b**) cells of ErbB2 after different DANCR intervene were measured by qRT-PCR assay. n.s $p > 0.05$. **c**, Schematic illustration exhibiting overlapping of the target miRNAs of DANCR and ErbB2 predicted by TargetScan (http://www.targetscan.org/vert_71/), Diana-Lncbase (http://carolina.imis.athena-innovation.gr/), MiRwalk (http://mirwalk.umm.uni-heidelberg.de/), and miRDB (http://mirdb.org/). **d**, Expression of miR-1225-3p in NSCLC was analyzed by using a previous miRNA expression profiling GSE102286. **e**, Expression of miRNAs in collected 4 NSCLC tissue samples were determined via using a qRT-PCR assay. **f**, ErbB2 protein expression in NSCLC cells was measured by application of western blot assay, $^{**}p < 0.01$ as comparing with mimic-con and inh-con group, respectively. **g**, Sequence alignment of ErbB2 reported plasmids (wt-ErbB2 and mut-ErbB2) containing wild and mutant miR-1225-3p seed region. **h**, **i**, Luciferase assays were performed in SPCA1 (**h**) and A549 (**i**) cells. Comparing with mimic-con, cotransfection of wild ErbB2 reporter plasmids (wt-ErbB2-luc) and mimic-1225-3p induced a significant decrease of luminance, while cotransfection of mutant ErbB2 reporter plasmids (wt-ErbB2-luc) and mimic-1225-3p reinforced the intensity of luminance. $^{**}p < 0.01$ and $\&p > 0.05$ as comparing with mimic-con group, respectively. **j**, **k**, Transwell migration and invasion assays were performed applying of SPCA1 (**j**) and A549 (**k**) cells transfected with mimic-con, mimic-1225-3p, mimic-1225-3p + vector and mimic-1225-3p + oeErbB2, individually. $^{**}p < 0.01$ as comparing with mimic-con group. Magnification, ×20; scale bar, 200 μm.

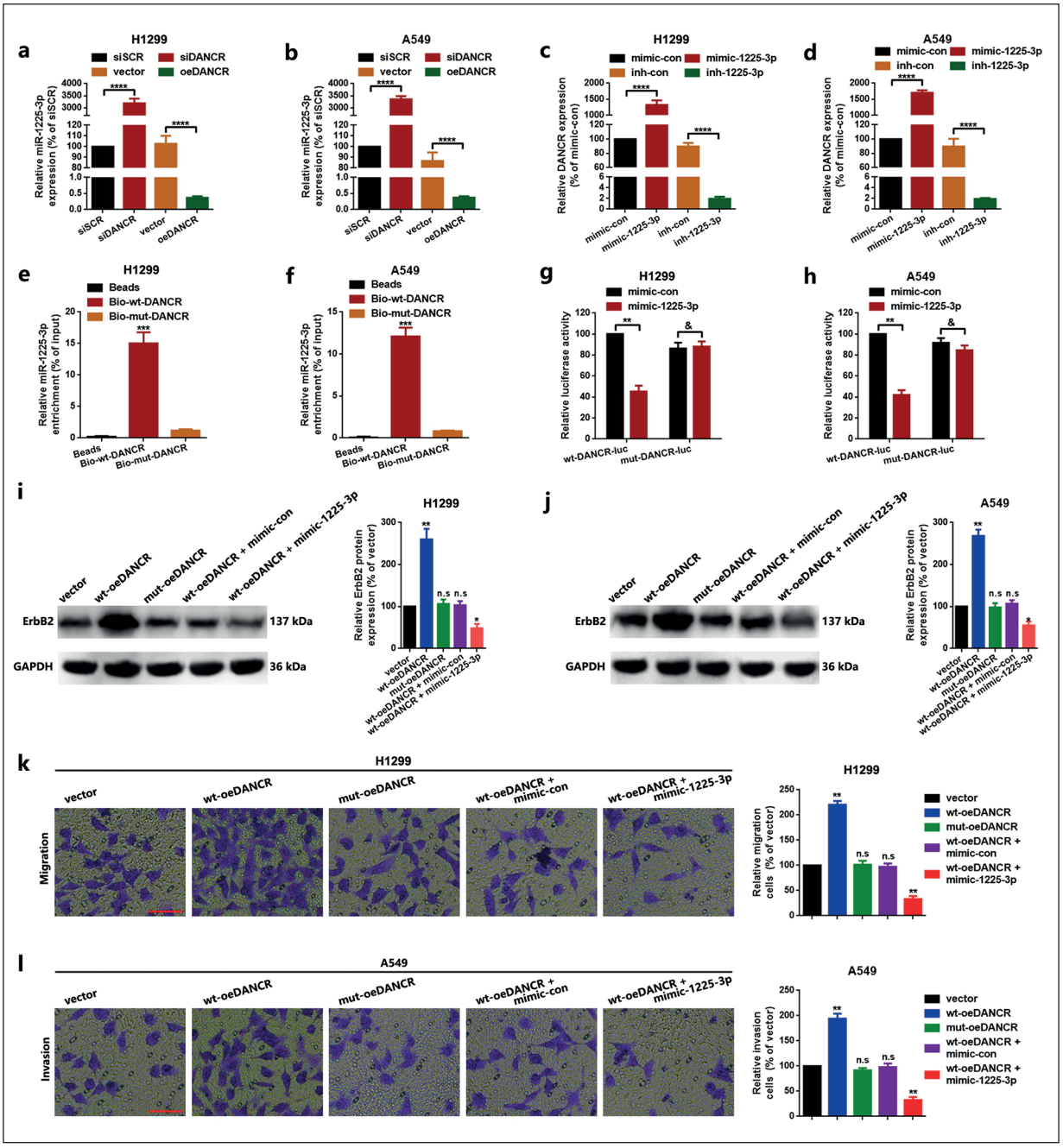

Figure 5. DANCR promoted ErbB2-mediated migration and invasion via sponging of miR-1225-3p in SPCA1 and A549 cells. **a, b**, The mRNA changes in SPCA1 (**a**) and A549 (**b**) cells of ErbB2 after different DANCR intervene was measured by a qRT-PCR assay. ****$p < 0.0001$. **c**, **d**, The expression of DANCR in SPCA1 (**c**) and A549 (**d**) cells was determined by a qRT-PCR assay, either. ****$p < 0.0001$. **e**, **f**, SPCA1 (**e**) and A549 (**f**) cell lysates were incubated with biotinlabeled wild (Bio-wt-DANCR) or mutant (Bio-mut-DANCR) DANCR transcriptions. A qRT-PCR assay was performed to check the expression of miR-1225-3p that was pulled down. G and H. Luciferase assays were performed in SPCA1 (**g**) and A549 (**h**) cells. Comparing with mimic-con, cotransfection of wild DANCR reporter plasmids (wt-DANCR-luc) and mimic-1225-3p induced a significant decrease of luminance, while cotransfection of mutant ErbB2 reporter plasmids (wt-DANCR-luc) and mimic-1225-3p reinforced the intensity of luminance. ** $p < 0.01$ and &$p > 0.05$ as comparing with mimic-con group, respectively. **i**, **j**, ErbB2 protein expression in SPCA1 (**i**) and A549 (**j**) cells was measured by a Western blot assay, n.s $p > 0.05$, * $p < 0.05$ and **$p < 0.01$ as comparing with vector group, individually. **k**, **l**, Transwell migration (**k**) and invasion (**l**) assays were performed in SPCA1 (**k**) and A549 (**l**) cells transfected with vector, wt-oeDANCR, mut-oeDANCR, wt-oeDANCR + mimic-con and wt-oeDANCR + mimic-1225-3p, separately. n.s $p > 0.05$, *$p < 0.05$ and **$p < 0.01$ as comparing with vector group, respectively. Magnification, ×20; scale bar, 200 μm.

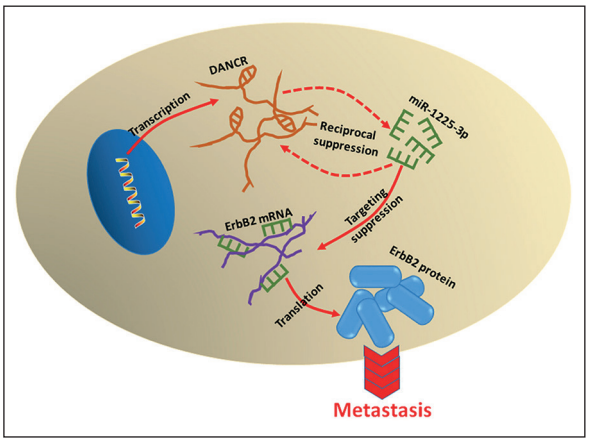

Figure 6. Schematic diagram of mechanism on this research. DANCR promoted NSCLC cells metastasis via modulating of miR-1225-3p/ErbB2 signal.

applied a selective ErbB2 inhibitor, CP-724714, to suppress the activity of ErbB2, the promotive effect of DANCR on NSCLC cells migration and invasion was also inhibited. And this phenomenon indicated that the role of DANCR on NSCLC cells migration and invasion was partially dependent on ErbB2.

LncRNAs act their functions via multiple mechanisms, like working as decoys to bind miRNAs or proteins, acting as scaffolds or guides to regulate interactions between protein and genes, and as enhancers to modulate transcription of their targets after being transcribed from enhancer regions or their neighboring loci[25-27]. A popular theory of lncRNAs working is ceRNA hypothesis. CeRNA hypothesizes that all types of RNA transcripts communicate through a new ‘‘language’’ mediated by micro-RNA-binding sites (‘‘microRNA response elements,’’ or ‘‘MREs’’) and that recent advances in experimental techniques are finally allowing us to hear and translate this language[28]. In this investigation, we found that an up- and downregulation of DANCR only affected ErbB2 at protein level, but not at mRNA level. This phenomenon indicated that the regulation occurred at posttranscriptional level. Through online informatics filtration and qRT-PCR, we assumed that miR-1225-3p was the key bridge connecting DANCR and ErbB2 at RNA level. MiR-1225-3p is located at chromosome 16p13.3. Till present, related researcher on miR-1225-3p were few. Cheng et al[29] reported that overexpression of miR-1225-3p impaired the antiviral effect of interferon (IFNs) and facilitated viral infection. In the current study, we found that miR-1225-3p was downregulated in NSCLC. Meanwhile, we displayed that an up- and downregulation of miR-1225-3p positively regulated ErbB2 protein expression. Further, through the subsequent luciferase assays and RIP binding assay, we confirmed the targeted binding effect between miR-1225-3p and DANCR as well as miR-1225-3p and ErbB2. Meanwhile, we illustrated that miR-1225-3p suppressed NSCLC cells migration and invasion via inhibiting ErbB2. Even more, we showed that only the wild DANCR overexpression plasmid (wt-oeDANCR) which containing miR-1225-3p response elements (MRE-1225-3p) promoted ErbB2 protein expression. When the MRE-1225-3p in DANCR overexpression plasmid was mutated (transfection of mut-oeDANCR), the facilitative effect was dismissed. More convincingly, an upregulation of miR-1225-3p (cotransfection of wt-oeDANCR and mimic-1225-3p) significantly attenuated the promotive effect of wt-oeDANCR did on ErbB2 protein expression. These findings strongly indicated that DANCR promoted ErbB2 expression via sponging of miR-1225-3p. Lastly, through a transwell assay, we demonstrated that DANCR enhanced ErbB2-mediated migration and invasion by absorbing miR-1225-3p.

Conclusions

To sum up, we elucidated that long noncoding RNA, DANCR, working as an oncogene, promoted NSCLC cells migration and invasion by upregulation of ErbB2 via miR-1225-3p sponging. DANCR/miR-1225-3p/ErbB2 axis may be a novel target in molecular treatment of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical statements

Written informed consent was provided by the patients whose tissues were used in the present study. The Institute Research Medical Ethics Committee of Central Hospital Affiliated to Shenyang Medical College granted approval for this study.

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