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ORIGINAL ARTICLE

Silencing *NID2* by DNA Hypermethylation Promotes Lung Cancer

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Abstract

To characterize the DNA methylation as well as exploring the relationship between *NID2* methylation and the lung cancer development. Collecting chip data of 9 lung cancer samples and 11 adjacent normal samples from the Gene Expression Omnibus database. Tissues and cells *NID2* gene methylation level was measured by methylation-specific PCR. *NID2* mRNA level and protein level were validated by Real-Time PCR and Western blot separately. Functional study of lung cancer cells was performed with Cell Counting Kit-8 assay. Colony formation assay, transwell assay, wound healing assay and low cytometry were performed. Finally, *NID2* tumorigenesis in vivo was tested in nude mice xenograft models. Microarray analysis outcome present *NID2* hypermethylation status in lung cancer tissues. High methylation and low mRNA expression levels of *NID2* were detected. After *NID2* demethylation or overexpression in cancer cells, cell viability, proliferation, migration as well as invasion ability decreased. Nevertheless, a significant enhancement in apoptosis rate were observed. Overexpressing *NID2* or demethylation in lung cancer cells inhibited the tumorigenesis of lung cancer in nude mice. The mRNA and protein level of *NID2* in tumors obtained from nude mice xenograft were unanimous with the in vitro assays' outcome, which significantly decreased after overexpressing *NID2* or demethylation. *NID2* methylation reduces its expression level and promotes the development of lung cancer.

Keywords Lung cancer · DNA methylation · *NID2* · Nude mice xenograft

Introduction

Lung cancer as one of the leading reasons cause cancer-related death world-widely, the impact of lung cancer makes identification of its in-depth mechanisms of great significance [1, 2]. In China, the number of lung cancer patients increase rapidly every year, and now, it has become the most common cancer type around the nation [3, 4]. There are multiple reasons discovered as the pathogenic factor for lung cancer, for instance, tobacco smoke, chronic pulmonary obstructive disease, and genetics [5]. Normally, non-small cell lung cancer (NSCLC) is reckoned as a common subtype of lung cancer and accounts for 85% of all lung cancers [6]. In clinical, lung cancer is a disease particularly implicated in early mortality, especially in NSCLC, it's 5-year survival rate is between 2% and 47% depending on individual's condition [7]. Thus, discovering new lung cancer treatment approaches to obtain better therapeutic effects is always an important concept in the future.

DNA methylation is a wildly discussed topic in the epigenetic study [7]. It is a type of mechanism that leading to cellular consequence by modulating gene expression as well as organizing chromatin in dynamic manner [8]. Usually, S-adenosylmethionine (SAM) is the origin of methylation, who is the product of methionine cycle [9]. SAM is always coupled with one-carbon metabolism, which is reported participating in lots of tumors cells proliferation through its role in biosynthesis as well as redox metabolism reaction [10, 11]. Moreover, the methionine cycle mediates the histone and DNA methylation,

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thereby a link is formed completely between intermediary metabolism and epigenetics [12, 13]. Nevertheless, even though the extent of the appearance of DNA methylation in tumor is unknown, but it is confirmed that it participates in tumors pathology indeed. For instance, the hypomethylation of global DNA as well as silencing tumor suppressor by aberrant DNA hypermethylation are two famous pathways that have significant effects on cancers, have been identified as cancer associate alterations [14]. In addition, it is found that methylation level was obviously higher in tumor tissue in comparison with corresponding normal tissues [15]. In recent years, the relationship between aberrant DNA methylation with cancer development has drawn vast of scientists' attention since it was discovered abnormal in early-stage tumors including lung tumor [16]. For example, WNT5A, SMN2 and HSPB1 were hypermethylated in prostate cancer, spinal muscular atrophy and breast cancer respectively [17, 18]. Above all of these evidences suggest that aberrant DNA methylation maybe a potential target in cancer therapy.

Nidogen-2 (*NID2*) is a type of protein coding gene. It secrets NID1 and *NID2* who are from nidogen protein family [19]. A study shows that those proteins exist in the basement membrane and capable of maintaining the membrane's stability and integrity [20]. However, aberrant hypermethylation of *NID2* is reported existing in different cancer types [19]. For instance, Ulazzi et al. proposed existing of *NID2* promoter hypermethylation in gastrointestinal malignancy [21]. Yegin Z. et al detected that 96.4% of *NID2* gene was methylated in bladder cancer tissues. In addition, the incident of aberrant *NID2* methylation has also reported as a biomarker in NSCLC patient's diagnosis [22]. Also, there is another research discovered an increase in lung metastasis in the *NID2*-deficient mice [23]. Therefore, we propose *NID2* may play a significant role in lung cancer therapy. In spite of it is certain that the *NID2*'s methylation status is associate to lung cancer development. However, currently the correlation between lung cancer and *NID2* methylation is still unclear. This study attempted to characterize the *NID2* methylation level in lung cancer tissues and cells, and revealed the relationship between *NID2* methylation as well as the development of the disease. In addition, research may provide a potential therapeutic approach for lung cancer treatment.

Materials and Methods

Human Tissues

Lung cancer tissues and the adjacent healthy tissues were sampling from 38 patients at China-Japan Union Hospital of Jilin University (Changchun, China). All experimental samples were stored in liquid nitrogen before use. The human specimen experiments were supported by the ethics committee of China-Japan Union Hospital of Jilin University. Patients who provided specimens had signed informed consent ahead of biopsy.

Cell Culture

Purchasing human bronchial epithelial cell line HBE and human lung cancer cell lines HCC827, H157, A549 and H1299 from BeNa Culture Collection (Chaoyang District in Beijing, China). Performed HBE cells culture under condition of high glucose DMEM (Gibco, USA) addition with 10% FBS containing penicillin (100 U/mL) and 100 mg/mL Streptomycin (Gibco). HCC827, H157, A549 and H1299 cell lines were cultured in RPMI1640 medium (Gibco) with 10% FBS supplied with penicillin (100 U/mL) and 100 mg/mL Streptomycin (Gibco). The incubator environment for cells was maintained at 37 °C with 5% CO_2.

Cell Transfection

First of all, purchased *NID2* expression vector pcDNA3.1-*NID2* from Invitrogen (Shanghai, China), and obtained demethylation drug 5-Aza-dC from Sigma-Aldrich (St. Louis, MO, USA). Secondly, seeding HCC857 cells into 6-well plates at an approximate density of 1×10^6 cells for each well, culturing at 37 °C until 40%-60% confluence. Transfecting pcDNA3.1-*NID2* and empty vector pcDNA3.1-vector into cells by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with manufacturers' recommendation. The transfection solution was replaced by cell medium with fresh 10% FBS after 6-h-transfection.

Genome-Wide Methylation Analysis

Gathering all GSE53051 data (illuminaga_rnaseqv2.1.0.0) of all samples from GEO database in order to conduct Illumina Infinium human-methylation 450 k BeadChip assay (Abbiotec, San Diego, CA, USA) for lung cancer (GSE53051) (illuminaga_rnaseqv2.1.0.0). Statistical analyze of DNA genome methylation profile was performed on Illumina BeadStudio software (Genetech Biotech, Taipei, Taiwan). β values were calculated during this procedure, obtained results were selected from 0 to 0.1 to represent CpG loci, and 0% to 100% on behalf of the percentage of methylation respectively.

Methylation-Specific PCR

DNA isolation was proceeded through JetFlex Genomic DNA Purification Kit (Invitrogen) following manufacturer's instruction. Then methylation-specific PCR (MSP) was applied to validate the samples' *NID2* methylation level. Briefly, modifying former isolated DNA by treated genomic DNA with bisulfite in order to convert unmethylated cytosine into uracil,

wherearea the status of methylated cytosine stay intact. In the end, the sequence of bisulfite-modified DNA could be able to use as the representative of genomic DNA's methylation status. PCR reactions were performed with the HotStar Taq polymerase (Qiagen) with bisulfite-modified DNA who was the template. Primer sequences used were listed at Table 1.

Real-Time Quantitative PCR (RT-PCR)

Tissues or cells were divided into groups and total RNA was obtained by using Trizol reagent (Invitrogen). RNA was quantified using NanoDrop (Thermo Scientific, USA). First-strand cDNA was further synthesized from miScript Reverse Transcription kit (Qiagen, Duesseldorf, Germany). Then the reverse transcriptional product mentioned ahead were added as templates in iTaq™ Universal SYBR Green supermix (Bio-Rad, USA) to continue qRT-PCR analysis. The calculation process was proceeding with adopting $2^{-\Delta\Delta Ct}$ method, moreover, GAPDH was adapted as internal reference. Each experiment was performed in triplicates and repeated for three times. Primer sequences were listed at Table 2.

Western Blotting

Extracting proteins from tissues and sample cells using RIPA lysing, and the concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific, USA). 12% SDS-PAGE gel was applied to separate 50 μg proteins. Polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) were blocked with skim milk for 2 h. After the blockage, membranes were divided into two groups subsequently and incubated with anti-*NID2* antibody (ab232883, Abcam, Cambridge, MA, USA) or anti-beta Actin antibody (ab8227, Abcam) respectively overnight at 4 °C. However, all membranes were uniformly incubated with secondary antibodies labelled with horseradish peroxidase for 1 h, the procedure were conducted under the room temperature. ECL kit (Bio-Rad, USA) was adapted to expose the target protein band and quantified the expression of relative protein by ImageJ software (NIH, USA).

Table 1 Methylation-specific primers

Gene	Sequence (5′-3′)
Methylation	
NID2 forward	5' AGGTTATCGTTTTCGTGGTC 3′
NID2 reverse	5' TCCGCTCCGTTTAAAAAATA 3′
No Methylation	
NID2 forward	5′ TTTAGGTTATTGTTTTTGTGGTT 3'
NID2 reverse	5' TCCACTCCATTTAAAAAATAAAA 3'

Table 2 qRT-PCR primers

Gene	Accession number		Sequence (5′-3′)
NID2	NM_007361	Forward	TTCCAACACCGAGGTCTTCA
		Reverse	GTCATGGGTAAAGGCAGCAC
β-actin	NM_008084	Forward	CTCCATCCTGGCCTCGCTGT
		Reverse	GCTGTCACCTTCACCGTTCC

Cell Counting Kit-8 (CCK-8)

Evaluation of HCC857 cells viability were finished by conducting CCK-8 assay (Dojindo, Kumamoto, Japan). Firstly, cell lines were treated with 5-Aza-Dc and transfected using pcDNA3.1-*NID2* as well as pcDNA3.1-vector individually. Followed with seeded cells into 96-well plates and cultured at 37 °C for 0, 12, 24 and 48 h. CCK-8 solution was added to test cells viability following the manufacturers' protocol, and measured Optical density value at 450 nm for viability quantitative purpose after incubation at 37 °C for 1 h.

Colony Formation Assay

Cells were treated as prescribed procedures mentioned before and seeded into six-well plates at 500 cells per well density. Incubation environment were maintained at 37 °C with 5% CO_2. The culture medium was replenished every 3 days, after 14 days incubation, colonies were fixed by using methanol and stained with Giemsa stain for 15 min. Then the colonies were counted under an inverted microscope and pictured.

Wound Healing Assay

Cells were prepared from the same prior procedures and seeded in 6-well plates afterward, then incubated them at 37 °C and 5% CO_2. Once the confluent reaching up to 95%, pipette tips were adapted to scratch wounds. Washing cells with PBS, and cultured with serum-free medium. Wounds were photographed after incubation for 24 h.

Transwell Assay

Cell's invasion ability was determined in transwell system where the upper chambers were planted a polyethylene terephthalate (PET) membrane with 6.5 mm in diameter and 8 μm in pore size. PET was coated with BD BioCoat Matrigel (BD Biosciences, USA). 5×10^4 HCC857 cells were plated with serum-free medium in the upper chamber, meanwhile filled the lower chamber with medium and addition 10% FBS. Culturing cells in transwell system for 24 h at 37 °C, scraped off upper chamber cells by cotton swab afterward. Fixing invaded cells with 70% ethanol and stained by crystal violet

solution immediately. Counting stained cells from five randomly selected fields under the light microscope and photographed (× 400 magnification).

Flow Cytometry

After harvested HCC857 cells used in planned treatment, they were suspended again by treating with 500 μL Annexin V binding buffer. Staining cells in suspension with 5 μL FITC-Annexin V and 5 μL Propidium Iodide (PI) dye solution for 15 min in dark situation. Apoptotic cells were detected and analyzed by FACSCalibur Flow Cytometer (BD Biosciences).

Xenograft Mice Assay

Raised 4 weeks old male BALB/c nude mice in pathogen-free conditions, the whole program was supported by China-Japan Union Hospital of Jilin University. In the beginning, divided nude mice into groups randomly. Then, injected HCC857 cells who were transfected with pcDNA3–1-*NID2*, empty vector, and 5-A-za-Dc treatment into the mice's posterior flank subcutaneously. Measuring and calculating tumor volume every week using equation: volume = length×width^2/2. Finally, the mice were killed after continuous implanting tumor for 21 days, in order to remove and measure tumor's weight.

Statistical Analysis

All the experiments in this study were essentially repeated for three times. The data were presented as mean ± SD. GraphPad Prism 7 were used to exhibit the statistical results. The differences among groups were determined by variance analysis or Student's *t* test. $P < 0.05$ was adopted in this study to indicate the significance statistically.

Results

Genome Methylation Profile for Lung Cancer

The public microarray GSE53051 of all samples were analyzed in GEO database. Also, considering β-values acting as CpG sites methylation level. The different distribution of β-values in the probe assay curves of type-I and type-II probe were normalized by procedures, in order to obtain a proper quality control. Here in Fig. 1, zero was on behalf of unmethylated site and 1 indicated fully methylated site (Fig. 1a). The poor performances of both tumor group and normal group were discovered and revealed from analyzing β-values in their density plot (Fig. 1b). Furthermore, the Multidimensional scaling plot (MDSplot) confirmed that they were different in clustering indeed. These results indicating their methylation patterns are not the same. (Fig. 1c). In addition, the dendrogram analysis of 485,512 probes in lung and healthy tissues was showed in Fig. 1d. In the end, a significant distinction was observed from the heatmaps of top 1000 differentially methylated CpG sites between lung cancer and healthy tissues (Fig. 1e). Collectively, these results suggesting that there may existing a general hypermethylation in lung cancer tissue.

The Distributional Characteristics of Methylated CpGs in Different Regions

The top 1000 CpG sites mentioned above as well as its neighborhoods (shelves, shores and open sea) on CpG island presented various methylation level among them intuitively. For details, the methylation level in CpG open sea was obviously higher than those in shelves, shores and islands (Fig. 2a). As to the position on genes and promoter sequences, the Body position was most hypermethylated, followed by IGR, TSS1500, 5'-UTRs, 3'-UTRs, TSS200 and 1st Exon, among the top 1000 hypermethylation CpG sites (Fig. 2b). Overall, learning from the outcomes of Fig. A and B, it revealed a highly methylated status of body and open sea positions in CpGs island (Fig. 2c).

The Hypermethylation of *NID2* in Lung Cancer

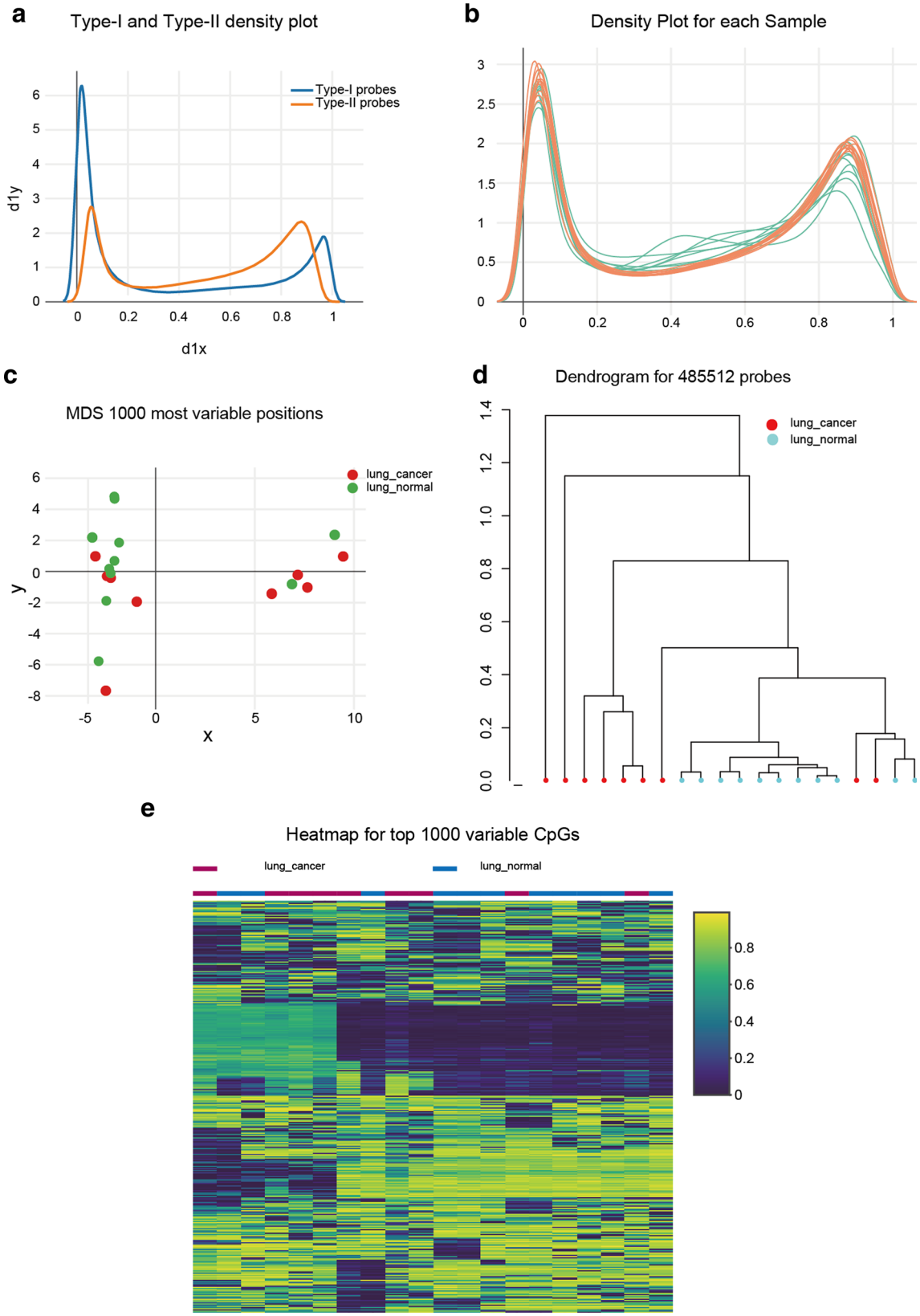
Top 10 highly methylated genes in both cancer and normal tissues were screened out and the methylation level of *NID2* was found higher in lung cancer tissues than in others (Fig. 3a). Use DMP analysis, the beta values of *NID2* CpG sites (cg25685519, cg11087503, cg10843707, cg26085721, cg26923084, cg26394244, cg21125441, cg04701034, cg25277187, cg14321412) were exhibited possessed a relatively higher methylated level in comparison with normal group. These findings revealed the existing of *NID2* hypermethylation in lung cancer tissues (Fig. 3b-d). Together, the overall results suggested that the *NID2* is highly methylated in lung cancer tissues in comparison with the normal lung tissues.

The Hypermethylation and Low Expression of *NID2* in Lung Cancer

To further validate the chip analysis results, MSP and Q-PCR were used to test the methylation level of *NID2* as well as its' expression level respectively. Consistent with the chip results,

Fig. 1 Genome-wide methylation analyzed data of overall 38 lung cancer patients. **a** The density of the DNA methylated level. The quality control was achieved by establishing the density plot. **b** The density of DNA methylation level in individual samples. **c** Multi-dimensional scaling (MDS) plot exhibited a distinction in clustering between normal group and cancer group. **d** the dendrogram analysis of 485,512 probes in normal and cancer lung. **e** The heatmap of top 1000 characterized differentially methylated CpG sites of both cancer and normal tissues, shows a difference between them

NID2 was highly methylated and a corresponding relative low expression in lung tissues compared with adjacent normal tissues (Fig. 4a, b). Moreover, the methylation levels of *NID2* in HCC857, H157, A549 and H1299 cell lines were



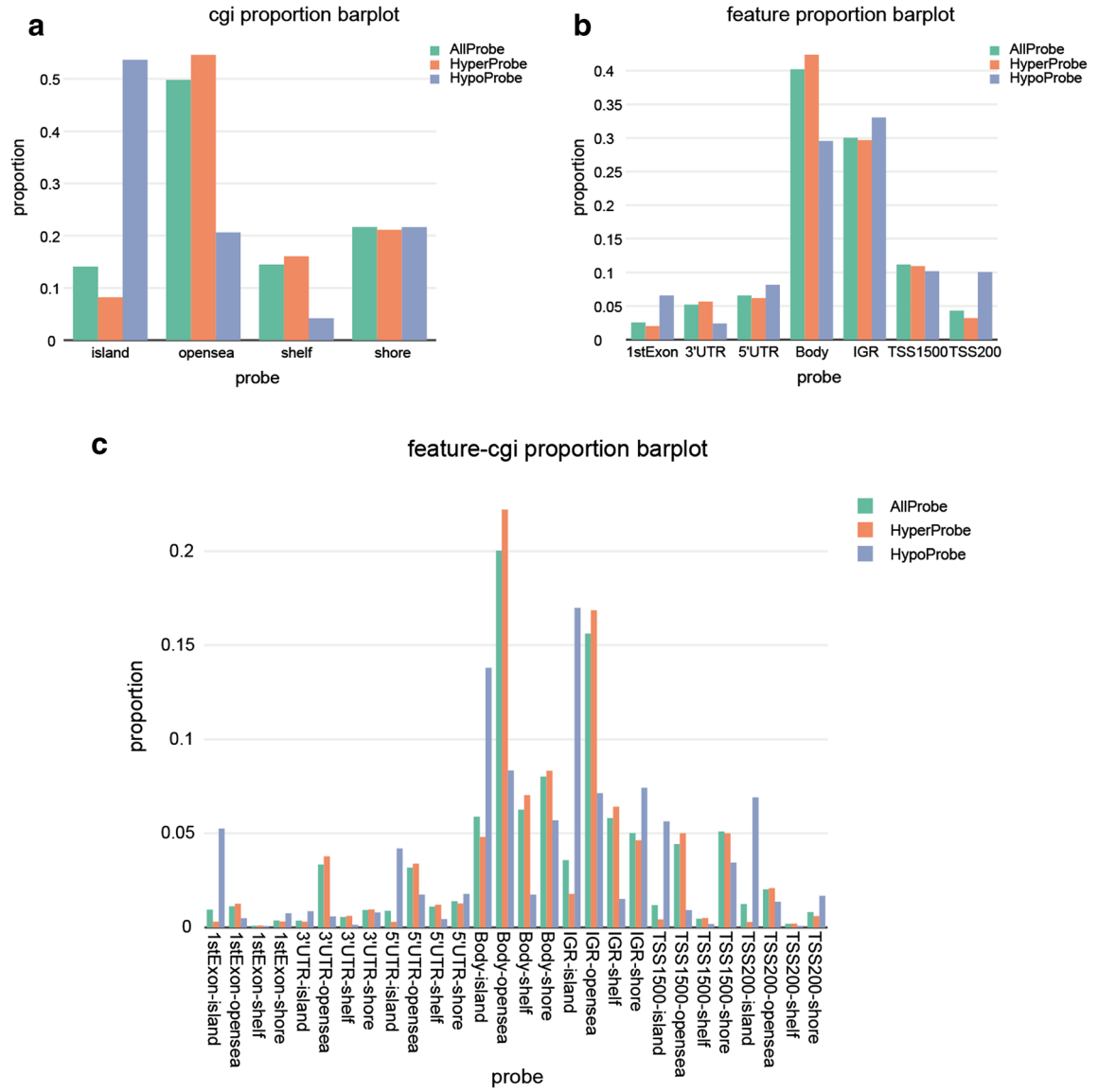


Fig. 2 Distribution of top 1000 characterized differentially methylated CpG sites. **a** The distribution of top 1000 characterized differentially methylated CpG sites in differentiate regions (island, opensea, shelf and shore). **b** Distribution of top 1000 characterized differentially methylated CpG sites in line with the position relative to genes (1stExon, 3' UTRs or 5' UTRs, body, IGR, TSS1500 and TSS200). **c** The comprehensive combination of genetic and epigenetic outcomes of the distribution of top 1000 characterized differentially methylated CpG sites

significantly increased in comparison with HBE cells. In addition, the expression level of *NID2* showed a reverse trend (Fig. 4c, d). These results proposed that the hypermethylation of *NID2* in lung cancer could lead to a reduce in *NID2* expression. HCC857 cells, HCC857 cells were selected for the subsequent experiments since they equip the highest methylation level and lowest expression simultaneously.

NID2 Suppresses the Development of Lung Cancer

In order to clarify the role of *NID2* plays in lung cancer development, 5-Aza-dC treatment was applied to reduce the methylation level on *NID2* and overexpression of *NID2* in HCC857 cells was performed by plasmid transfection. In comparison with control group's data, a significant increase was obtained in *NID2* expression level of HCC857 cells in the present of demethylation treatment or overexpression of *NID2* (Fig. 5a). At the same time, western blot experiment results also showed that the protein level of *NID2* was significantly increased as well (Fig. 5b, c). Next, CCK8 assay and the wound healing assay were conducted to detect whether the *NID2* can influence tumor phenotype. The conclusion has been made that that the overexpression or demethylation of *NID2* apparently could result in

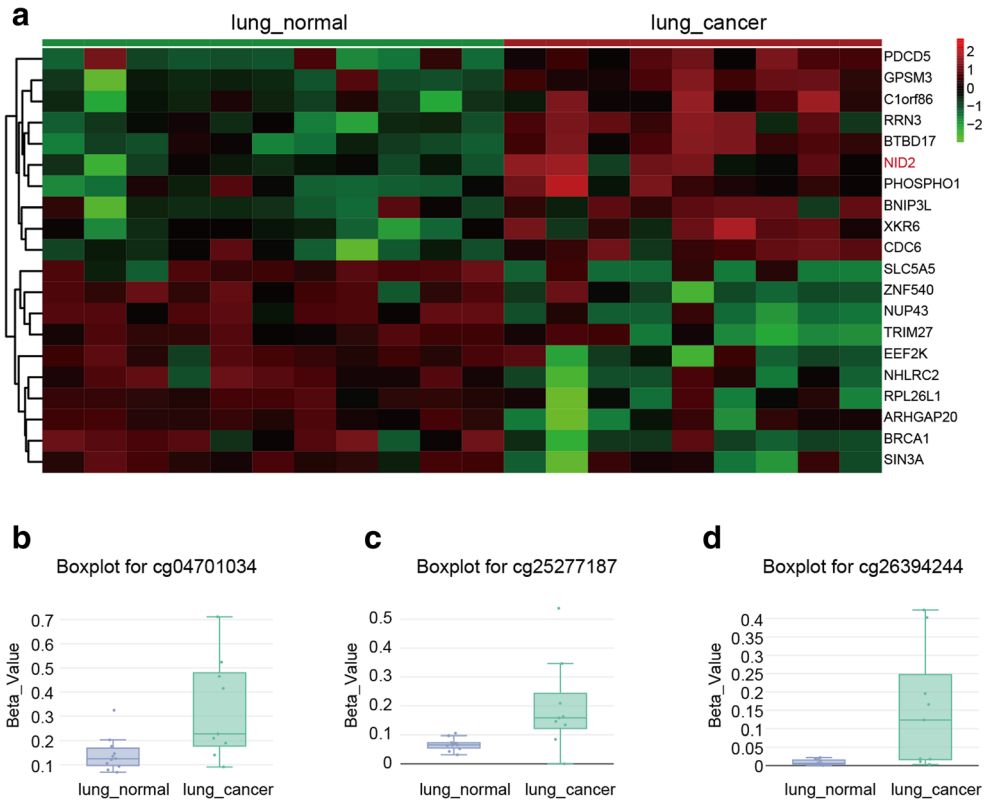


Fig. 3 Discovery of *NID2* hypermethylated in lung cancer tissues. **a** Top 10 highly methylated gene screened out from heatmap, and *NID2* was hypermethylated in lung cancer. **b** The genetic and epigenetic DMP analysis results for *NID2* CpG sites methylation level between normal and cancer tissue. The Boxplot for cg25685519 (**c**), cg11087503 (**d**), cg26923087 (**e**) presented methylation level decline in lung cancer tissue in comparison with normal lung tissues

a decrease in cell's proliferation and migration ability (Fig. 5d, e). Besides, the observation of subdued colony formation ability and invasion ability in 5-Aza-dC-treated cells or *NID2* overexpressed cells indicating a significantly increase in apoptosis rate compared control group (Fig. 6a-c). All these results implied that reducing the expression of *NID2* which is induced by methylation is able to promote the lung cancer development.

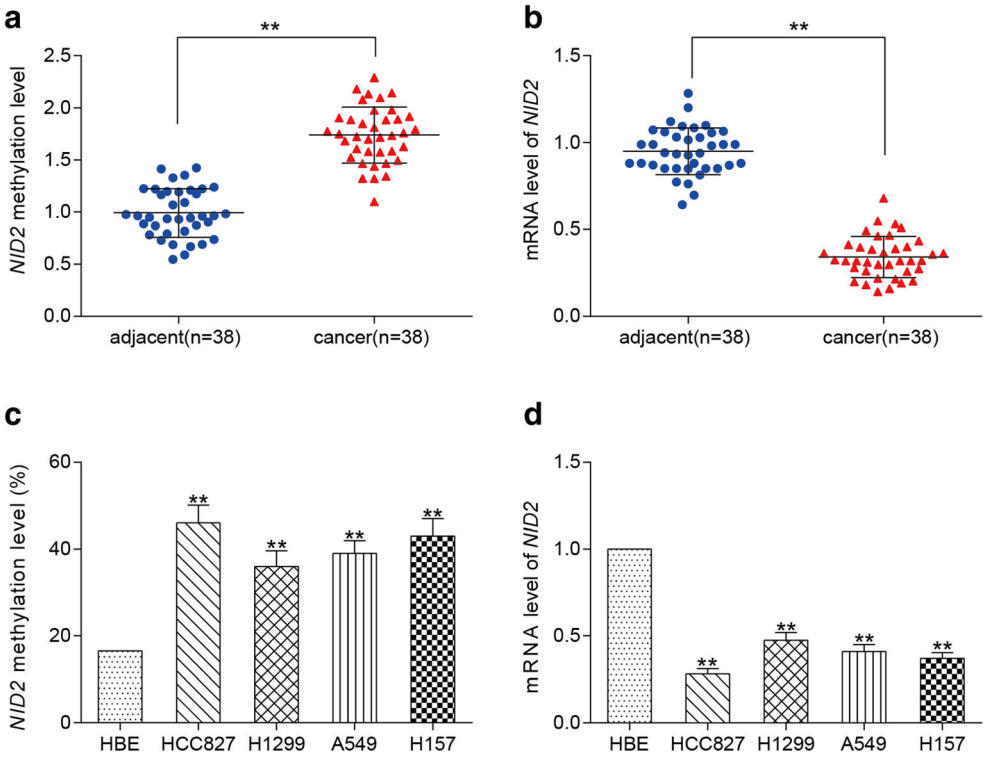


Fig. 4 Validation of NID2 hypermethylated in lung cancer. **a** Methylation-specific PCR outcome for cancer tissues and adjacent normal tissues, indicated a significant increase in cancer tissues *NID2* methylation level. **b** q-PCR result for cancer tissues as well as adjacent normal tissues, a significant reduce was occurred in *NID2* expression in cancer tissues. **c** Methylation-specific PCR graph data for *NID2* methylation level in cells (HBE, HCC827, H1299, A549, H157), the significant increases were observed in all cancer cell lines. **d** The *NID2* expression level in cells (HBE, HCC827, H1299, A549, H157), the significant decreases were observed in cancer cells which is consistent with MSP result. All the above differences are significant (**$p < 0.01$)

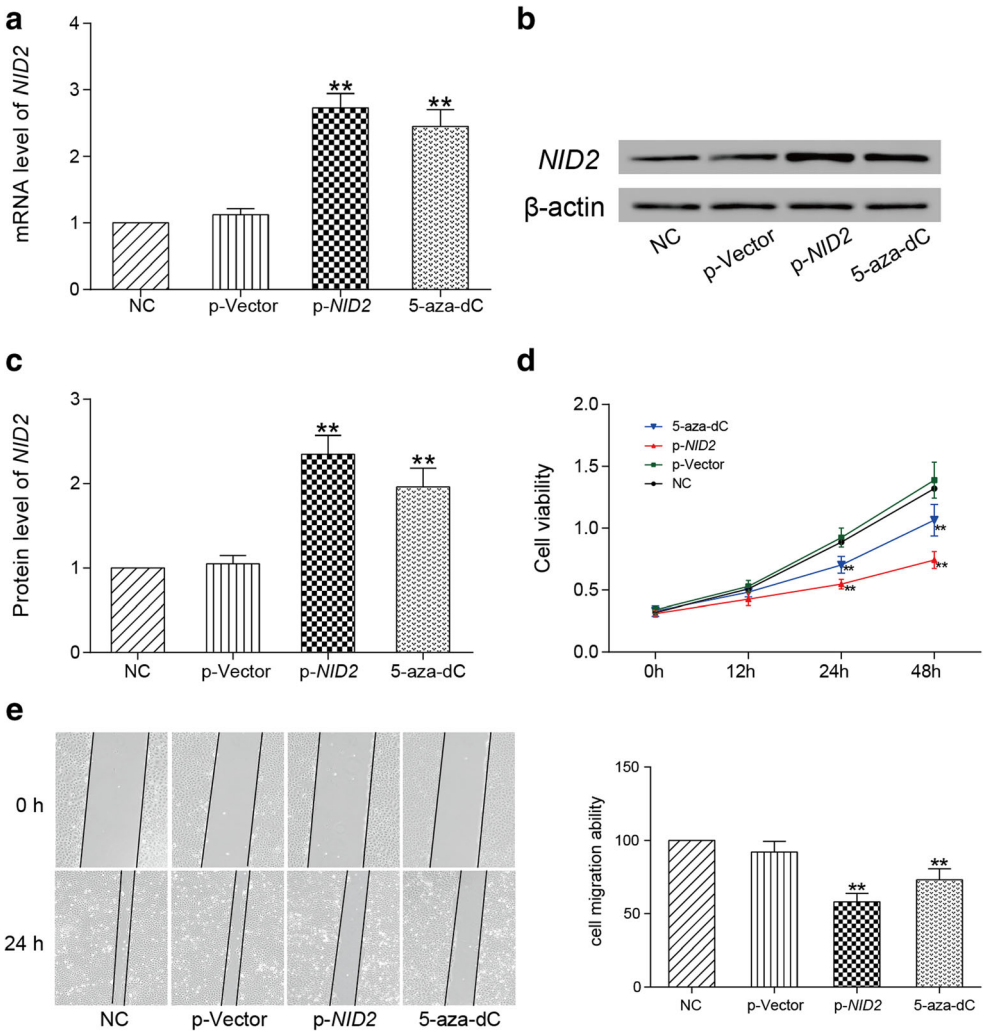


Fig. 5 Effects on lung cancer cells after overexpression and demethylation of *NID2* gene. **a** The *NID2* gene expression level in HCC857 cells of *p-NID2* group and 5-aza-dC group were significantly higher than control group. **b**–**c** *NID2* protein expression level visualizing showed that *p-NID2* group and 5-aza-dC group in HCC857 cells had significant higher protein expression in comparison with control group. **d** HCC857 cells' CCK8 assay data diagram presented a decrease observed in cells' viability from *p-NID2* group and 5-aza-dC group compared with control group. **e** Wound healing data's images and graph exhibited that HCC857 cell's migration ability reduced in *p-NID2* group as well as 5-aza-dC group compared with control group. All the above differences are significant (**$p < 0.01$)

NID2 Suppresses the Development of Tumor in Mice

To further validate the role of *NID2* in vivo, HCC857 cells were treated with 5-aza-dC, transfected with pcDNA3.1-*NID2* and empty vector were subcutaneously injected to nude mice. As we expected, cells treated with 5-aza-dC or *NID2* overexpression obviously decreased in tumor volume and weight (Fig. 7a-c). In addition, *NID2* mRNA expression as well as its protein level in tumor were increased after the demethylation treatment of 5-aza-dC, the uniform outcome was obtained from *NID2* overexpression group (Fig. 7d-e). Collectively, the methylation of *NID2* could down-regulate its' expression, and result in the tumorigenesis of lung cancer.

Discussion

Generally, this study revealed the relationship between *NID2* gene and the development of lung cancer. *NID2* was hypermethylated in lung cancer tissues in comparison with normal and adjunct healthy tissues. Moreover, *NID2* methylation level was in an inverse proportional relationship with its mRNA expression level in cells. Also, this research found that improving the expression of *NID2* is able to increase cells apoptosis rate, leading to a decrease in cells viability and colony formation ability respectively. Additionally, the consistent result obtained from nude mice xenograft models which is tumors volume and weight reduced after increasing the normal *NID2* expression. In conclusion, our research suggested a direct promotional relationship between *NID2* methylation with lung cancer development, may provide a useful lung cancer therapeutic strategy.

Abnormal methylated gene is found existing in vast of the tumor sites in human [24]. DNA methylation as part of epigenetic changes, always exist on cytosine bases from 5′ of a guanosine in a CpG dinucleotide site. More importantly, the scientific research confirmed that the gene site is usually unmethylated in normal cells [24]. These evidences point out new therapeutic approaches in lung cancer treatment. For instance, Geng J et al. found that *NID2* gene is highly

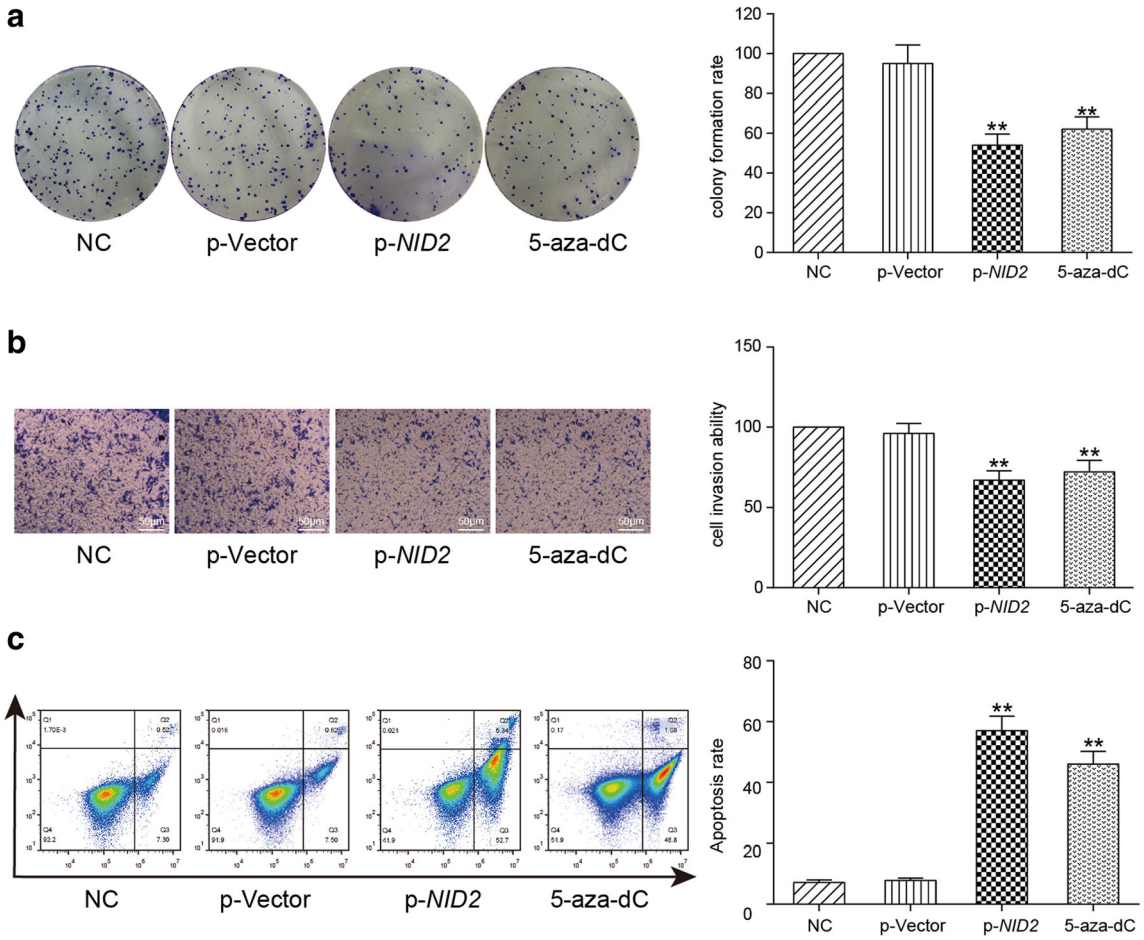


Fig. 6 The influences on migration and invasiveness capability in lung cancer cells after treatmented with p-*NID2* and 5-aza-dC. **a–c** The apoptosis rate in p-*NID2* group and 5-aza-dC group lung cancer HCC857 cells were significantly increased compared with the control group. All the above differences are significant (**$p < 0.01$)

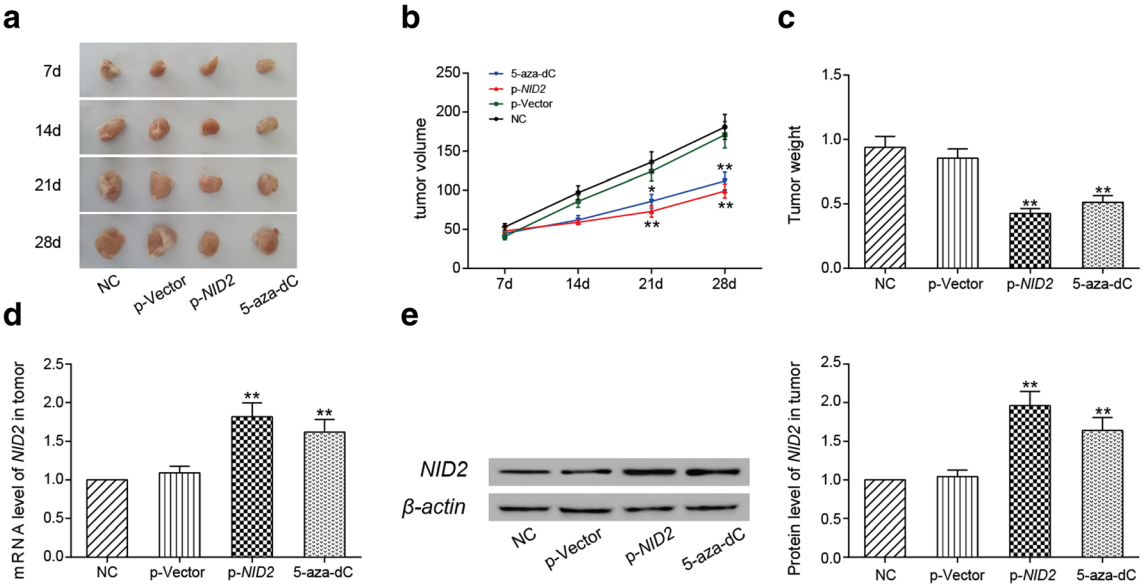


Fig. 7 The effects on tumor in mouse after injected HCC857 cells which treatment with p-*NID2* and 5-aza-dC. **a–c** The mouse's tumor volume and weight from p-*NID2* group and 5-aza-dC group were smaller than in control group. (D-E) The *NID2* gene expression level as well as protein expression level in p-*NID2* group and 5-aza-dC group were both significant higher than those in control group. All the above differences are significant (*$p < 0.05$, **$p < 0.01$)

methylated in lung cancer tumor tissues, meanwhile, they also proposed *NID2* as a biomarker for lung cancer diagnosis [16]. Moreover, there is also a research found that there was 29% methylated *NID2* gene in colon cancer tissues, and 95% in gastric cancer tissues. Instead, it was completely unmethylated in corresponding normal tissues [25]. Similarly, our investigation discovered the aberrant hypermethylated *NID2* gene is occurring in the patient's lung and cells. It is observed that *NID2* hypermethylation was corresponding to its low mRNA expression in both cells and tissues. *B.LI* et al. conducted a similar research in regard with DNA methylation analysis in lung cancer tissues earlier. They reported that the DNA methylation participates in lung cancer's pathology at transcriptional and post-modification level [22].

Furthermore, they elucidated the aberrant methylated DNA inhibits its mRNA expression by inhibiting the binding with upstream regulatory protein and subsequently suppress the expression of downstream mRNA [24]. Thereby, assuming that the abnormal methylated *NID2* gene prevents the connection from upstream target proteins, dysfunction the specific downstream genes and consequence in its mRNA expression reduced. *NID2* protein as a member of nidogen protein family. It has the function of constituting the three-dimension structure of the basement membrane with collagen IV and laminins [22, 26]. It serves as a surface receptor who responsible for cell differentiation, cell migration, and invasion process [22, 27, 28]. Moreover, Linda Ulazzi et al. proposed an idea of losing the expression of *NID2* would contribute to cancer cells invasion and metastasis abilities by reducing the interactions between basement membrane [29]. They also discovered that aberrant gene methylation was the main factor leading to nidogen expression decreased in gastrointestinal cancer [21]. Consistently, in our research, cells invasion and migration ability were significantly declined by demethylation and overexpression of *NID2* in cells.

However, tumor metastasis and formation is a complicated process which require multiple cell-matrix interactions, such as adhesion of cancer cells to basement membrane components, location the proteolysis in basement membrane, and migration into the other tissues. *Sharada Mokkapati* et al. reported in their research that the absence of nidogen 2 resulted in a significantly increase in lung colony [22]. Accordantly, our research shows a reduction in tumor weight and volume in nude mice xenograft models due to in presence of increased in normal *NID2* gene. Therefore, it is reasonable for us to summary that increasing the expression of *NID2* would likely to decrease lung colony formation ability. These results all implicate that *NID2* is directly correlated with lung cancer.

Nevertheless, the further work for this study is to investigate and validate the in-depth mechanism of the *NID2* action in lung cancer, such as how it intervenes the specific signaling network. Furthermore, there are still some limits in present study, such as the specific signal pathway for *NID2* methylation. In addition, there might be some other genes methylation or demethylation exist leading to influences on experiment results.

In conclusion, the research proved that *NID2* is useful in inhibiting and controlling the development of lung cancer. Providing a clear understanding of *NID2* methylation involving in lung cancer progress. Above all, aberrant methylated *NID2* plays a provital role in lung cancers development. Improving *NID2* gene expression have positive effect on lung cancer's therapy.

Author Contributions Substantial contribution to the conception and design of the work: Jianfeng Wang, Yan Zhao and Hongyan Xu; Acquisition, analysis, and interpretation of the data: Jianfeng Wang, Jun Ma, Feihai Liang and Qingxu Zou; Drafting the manuscript: Jianfeng Wang; Revising the work critically: Fengwu Lin; Final approval of the the version to be published: All authors.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the China-Japan Union Hospital of Jilin University committee. Informed consent was obtained from all individual participants included in the study.

All procedures involving animals were performed in compliance with guidelines of Q China-Japan Union Hospital of Jilin University.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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