Prognostic significance of NDRG2 combined with EGFR patients with lung adenocarcinoma

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Research article

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Abstract

Background: N-myc downstream-regulated gene 2 (NDRG2) plays a substantial role in lung adenocarcinoma (LUAD). Epidermal growth factor receptor (EGFR) mutation could significantly improve prognosis in patients with LUAD. In this study, we aimed to elucidate the prognostic value of NDRG2/EGFR in patients with LUAD. Methods: Immunohistochemistry, Western blotting, and reverse transcription-polymerase chain reaction (RT-PCR) were conducted to detect the expression levels of NDRG2 protein. Association between NDRG2/EGFR expression and clinicopathological characteristics of patients with LUAD was examined as well. Serum level of carcinoembryonic antigen (CEA) was tested prior to treatment of patients with LUAD. Patients' overall survival was assessed by Kaplan–Meier method. Multivariate Cox regression analysis was carried out to investigate the effects of patients' demographic characteristics on OS. Results: The expression level of NDRG2 was significantly decreased in patients with LUAD. The expression level of NDRG2 was positively correlated with levels of CEA and EGFR. Advanced stages were significantly associated with low expression level of NDRG2. We found that patients in NDRG2-high/EGFR(+) group had the best outcome, while patients in NDRG2-low/EGFR(-) group had the worst. Meanwhile, Cox regression analysis showed that NDRG2-low/EGFR(+), NDRG2-high/EGFR(+), and vascular invasion were independent prognostic factors of LUAD. Conclusion: The significant prognostic value of NDRG2/EGFR should be highly considered in patients with LUAD.

Background

Lung cancer remains the leading cause of cancer-related death worldwide, counting for 19.4% mortality of cancer among adults [1]. Small-cell lung cancer (approximately 15%) together with non-small cell lung cancer (NSCLC) (approximately 85%) constitute the lung cancer. The NSCLC includes lung adenocarcinoma (LUAD), which is the most prevalent subtype of lung neoplasms [2]. However, only approximately 20% of NSCLC patients can be potentially treated by resection and the remaining are diagnosed in advanced stages [3]. Despite advances accomplished in terms of early detection and standard treatments, such as surgery, chemotherapy, radiotherapy, and iodine-125 (^{125}I) brachytherapy, the overall survival (OS) of NSCLC has still remained poor [3,4].

Targeted therapy has recently emerged as a new therapeutic approach for patients with advanced NSCLC, especially in LUAD that harbors EGFR mutations, which showed better progression-free survival (PFS) compared with squamous cell carcinoma treated with the molecular targeted drugs, such as tyrosine kinase inhibitors (TKIs) [5,6]. However, after surgical resection, several patients eventually developed incapable survival due to inevitable drug resistance, and patients lacking driver oncogene aberrations are still treated with traditional regimens, imposing great negative consequences [7]. Hidayat et al. found that FBXW7 expression in CD133-positive cells was increased and c-MYC expression was decreased in gefitinib-resistant tumors of PC9 cells in mice and in 9 out of 14 tumor specimens from EGFR-mutant NSCLC patients with acquired resistance to gefitinib [8]. Another study reported that hyperprogressive disease (HPD) promptly leads to death in patients harboring EGFR exon 20 insertion mutation and MYC amplification [9]. Consequently, the interaction between MYC and EGFR may play a significant role in malignant tumor cells, involving cell proliferation, invasion, and metastasis.

The N-myc downstream–regulated gene (NDRG) family consists of four members: NDRG1, NDRG2, NDRG3, and NDRG4 [10]. A previous study showed that NDRG2 exerts important functions in cell differentiation and tumor suppression. Researches revealed that DNA damage, hypoxia, and glucocorticoids promoted NDRG2 expression and NDRG2 can be transcriptionally activated by p53 and HIF1-α [11]. Decreased expression level of NDRG2 has been found in several types of human cancer, such as lung cancer, bladder cancer, colon cancer, pancreatic cancer, thyroid cancer, glioblastoma, melanoma and meningioma; besides, NDRG2 was found as a candidate tumor suppressor gene [12]. Although a previous analysis revealed that NDRG2 might serve as a novel prognostic marker in human lung cancer, its prognostic value in LUAD needs to be further indicated. Additionally, the association between NDRG2 expression and EGFR mutation has still remained elusive.

Accordingly, the identification of novel prognostic markers for LUAD is urgently required. The present study confirmed the prognostic value of the combined detection of NDRG2/EGFR for LUAD patients.

Methods

Patients

A total of 89 LUAD patients were prospectively enrolled in the Tianjin First Central Hospital of Nankai University between June, 2013 and June, 2014 (52 men and 37 women; mean age, 65.6 years old; range of age, 38-86 years old). None of the patients had received chemotherapy, radiotherapy and/or immunotherapy before sampling. The clinical and pathologic characteristics of LUAD patients are listed in Table 1. Those individuals who smoked at least 1 cigarette per day for over 1 year were defined as smokers. The criteria used for tumor staging and grading were set according to the 8th edition of the Union for International Cancer Control (UICC) TNM classification of malignant tumors. Hematoxylin and eosin (H&E) staining of tissue slides was performed and verified by two board-certified pathologists. Among patients, 34 cases underwent curative-intent surgery; in addition, 55 patients with advanced LUAD were treated with iodine-125 (^{125}I) brachytherapy. Patients' preoperative work-flow (e.g., positron-emission tomography (PET)/computed tomography (CT) and cardiac ultrasonography) was examined to exclude those cases with secondary lung cancer and those with systemic disease. Normal tissues were removed from at least 5 cm away from the edge of the tumors.

Immunohistochemistry

In the present study, formalin-fixed paraffin-embedded tissue sections (thickness, 4-μm) were used for detecting the expression level of NDRG2. Tissue sections were dewaxed, rehydrated, antigen retrieved, and cooled to room temperature. The sections were incubated with mouse monoclonal anti-NDRG2 antibody (Abcam, Cambridge, UK) at 4 °C overnight, rinsed with phosphate-buffered saline (PBS), and incubated with horseradish peroxidase (HRP)-labeled

goat anti-mouse secondary antibody for 60 min. NDRG2 expression was revealed using 3,3'-diaminobenzidine (DAB) as the chromogen. Negative control was performed by replacing the primary antibody with normal mouse serum. The brown or yellow staining was identified as positive expression. The total staining score of 0–12 was considered in a semi-quantitative manner and stratified as follows: negative (-, range of score: 0–1), weak (+, range of score: 2–4), moderate (++, range of score: 5–8), or strong (+++, range of score: 9–12). The tumor specimens were divided into low-expression group (range of score: 0–4) and the high-expression group (range of score: 5–12).

Western blot analysis

Total protein concentration was measured by a bicinchoninic acid (BCA) assay kit. Then, the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with mouse anti-human NDRG2 antibody at 4 °C overnight after being blocked with 5% non-fat milk for 1 h. Besides, β-actin was used as the internal control. Then membranes were washed and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The blots were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to manufacturer's instructions. Each experiment was performed in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted from the fresh tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The sequences of the primers were as follows: NDRG2, forward-5'-TTACAACAACCGCCGAGACCT-3', and reverse-5'- ATTACATTCCACCACGGCATC-3'; β-actin, forward-5'- GGAGATTACTGCCCTGGCTCC-3' and reverse-5'- GACTCATCGTACTCCTGCTTG-3'. The RT-PCR conditions were as follows: denaturation at 95 ℃ for 30 s, annealing at 56 ℃ for 30 s, and extension at 72 ℃ for 40 s. The relative mRNA expression of NDRG2 was calculated by $-2^{\Delta\Delta Ct}$ method. Each experiment was undertaken in triplicate.

Measurement of parameters

Blood samples were collected before initiation of treatment. Patients' blood type was examined by measuring the serum concentrations. Carcinoembryonic antigen (CEA) was evaluated as well.

Statistical analysis

Data from categorical variables were expressed as frequencies or percentages. Continuous variables were presented as mean ± standard deviation (SD). Differences between categorical groups were investigated by chi-square test or Fisher's exact test. The differences in mean values between the groups were analyzed using the Mann-Whitney U test or Student's t-test. Associations between the two variables were quantified via Spearman's rank correlation coefficient. The OS rate was evaluated using the Kaplan-Meier method, and differences between the groups were assessed by the log-rank test. Multivariate analysis was conducted using Cox's proportional hazards regression model to investigate the effects of patients' demographic characteristics on OS. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

NDRG2 was downregulated in patients with LUAD compared with that in normal tissues

To investigate whether NDRG2 was detectable and altered in LUAD patients compared with normal tissues, immunohistochemistry (Figure 1 A-C), Western blotting (Figure 1 D), and RT-PCR (Figure 1 E) were carried out to detect the expression levels of NDRG2. NDRG2 protein was mainly appeared in the cytoplasm, and a weak expression could be found in a limited number of cell nuclei. The results showed that the expression of NDRG2 at protein and mRNA levels in LUAD was significantly decreased compared with normal tissues.

Relationship between expression level of NDRG2 and clinicopathological features of patients with LUAD

As shown in Figure 2, the expression level of NDRG2 was remarkably correlated with CEA ($P < 0.001$).

To assess the correlation between the expression level of NDRG2 and clinical data, clinicopathological features of patients with LUAD are summarized in Table 2. The chi-square test revealed that the expression level of NDRG2 was notably higher in LUAD tissues in stages I–II than that in stage III–IV ($P<0.001$). In addition, the incidence of no vascular invasion and EGFR positive (+) was significantly higher in patients with high expression level of NDRG2 than that in patients with low expression level of NDRG2 ($P<0.001$ and 0.001, respectively). However, there were no associations between expression levels of NDRG2 and other clinicopathological features, including patients' age, gender, smoking history, and blood type ($P>0.05$).

Regarding 34 patients who received surgery, the expression level of NDRG2 was significantly higher in stage I–II than that in stage III–IV ($P=0.028$). The incidence of no vascular invasion and EGFR positive (+) was markedly higher in patients with high expression level of NDRG2 than that in the patients with low expression level of NDRG2 (0.008 and 0.030, respectively).

Prognostic implications of NDRG2 and EGFR expression

Based on the clinicopathologic features of LUAD patients, as well as the expression levels of NDRG2, EGFR, and CEA, the survival time was analyzed by Kaplan–Meier method (Figure 3 A-H). The results showed that iodine-125 radioactive seeds brachytherapy for advanced LUAD patients with high expression level of NDRG2 had significantly higher OS than LUAD patients with low expression level ($P = 0.0261$, Figure 3A); besides, LAUD patients with EGFR(+) and

CEA < 2.0 ng/ml had higher OS (P < 0.0001, 0.0314, Figure 3 B, C). Additionally, in operated patients with high expression level of NDRG2 (Figure 3E), EGFR(+) (Figure 3F), and CEA < 2.0 ng/ml (Figure 3G), higher OS was noted (P = 0.0022, < 0.0001 and 0.013, respectively).

According to the conjoined expressions of NDRG2/EGFR, the subjects were categorized into four groups: NDRG2-low/EGFR-negative(-), NDRG2-low/EGFR-positive(+), NDRG2-high/EGFR-negative(-), and NDRG2-high/EGFR-positive(+). The association between co-expression of NDRG2/EGFR and the OS was tested by Kaplan–Meier method. In these four groups, iodine-125 radioactive seeds brachytherapy for advanced LUAD patients in NDRG2-high/EGFR(+) group accompanied by the best prognosis during the 5-year follow-up period (P < 0.0001, Figure 3D), and the same results were observed in operated patients (P = 0.0002, Figure 3H).

Cox regression analysis

As shown in Table 3, NDRG2-low/EGFR(+) (hazard ratio (HR)=6.508; 95% confidence interval (CI), 2.619-16.174; P<0.001), NDRG2-high/EGFR(+) (HR=3.519; 95% CI, 1.384-8.949; P=0.008), and vascular invasion (HR=4.480; 95%CI, 2.291-8.760; P<0.001) were independent prognostic factors of OS.

Discussion

To improve the prediction of lung cancer survival, several tumor markers (e.g., CEA) have been assessed and extensively used [13,14]. However, each marker has its own specificity and sensitivity, which might lead to limitation in judging the prognosis. The combined detection of tumor markers maybe of great for improving the prediction of lung cancer survival.

It is noteworthy that MYC influences growth, proliferation, differentiation, and apoptosis of cancer cells through regulating the expression levels of numerous genes [15]. In addition, MYC governs events associated with tumor progression, including genetic stability, migration, and angiogenesis [16]. Two human cDNAs, encoding NDRG3 and NDRG4, are homologous to NDRG1. These two genes, together with NDRG1 and a previously deposited cDNA (designated NDRG2), constitute the NDRG gene family [17]. Previous studies reported that NDRG2 was associated with human lung cancer, and the decreased expression of NDRG2 was correlated with a worse outcome of lung cancer patients [12,18]. Similarly, the results of the present study showed that the high expression level of NDRG2 in LUAD patients was significantly associated with early TNM stage and negative vascular invasion (Table 2). Low expression level of NDRG2 showed a lower OS than high expression level of NDRG2 (Figure 3). The above-mentioned findings indicate that NDRG2 may play a pivotal role in the development of LUAD.

CEA was first described in 1965 by Gold and Freedman as an antigen present in gastrointestinal carcinoma cells [14,19]. A number of researches demonstrated the prognostic value of the preoperative CEA level, as a classical marker for LUAD [20,21]. In the current study, we, for the first time, showed that CEA level > 2.0 ng/ml was associated with low expression level of NDRG2 (Table 2, Figure 2), and a lower OS was found compared with CEA level < 2.0 ng/ml (Figure 3). The study indicate that CEA level < 2.0 ng/ml might have a good prognosis for LUAD.

A research revealed that EGFR mutations in circulating tumor DNA (ctDNA) predicted a better PFS, in particular in advanced NSCLC patients treated by EGFR-TKIs. KRAS mutations in ctDNA indicated a worse PFS and OS in patients treated by chemotherapy [22]. Another study demonstrated that blood, in particular serum, is an appropriate substitute when tumor tissue is absent or insufficient for testing EGFR mutations to guide EGFR-TKIs treatment in patients with NSCLC [23]. In the present research, we reported the EGFR mutation status in patients with adenocarcinoma and its correlation with expression level of NDRG2. Mutant EGFR expression was positively correlated with higher OS in presence of high expression level of NDRG2 (Table 2, Figure 3).

MYC and EGFR have been identified as potential biomarkers that can predict the efficacy of the targeted therapy [24,25]. We, herein, analyzed the prognostic value of the combined detection of NDRG2 and EGFR for LUAD. We found that patients in NDRG2-high/EGFR(+) group had the best outcome, while patients in NDRG2-low/EGFR(-) group had the worst. Cox regression analysis revealed that NDRG2-low/EGFR(+), NDRG2-high/EGFR(+), and vascular invasion were independent prognostic factors of OS (Table 3).

This study contains several limitations. Firstly, it was a retrospective study and performed in a single center. Secondly, the sample size was very limited. Hence, further study is required to explore the putative association between NDRG2/EGFR level and OS in patients with NSCLS.

Conclusions

In summary, this study reported the different expression levels of NDRG2 in LUAD patients. In addition, for the first time, the relationship between NDRG2/EGFR expression and clinicopathological characteristics of LUAD patients, especially prognosis status, was investigated. NDRG2/EGFR can be used as a novel prognostic biomarker for LUAD patients.

Abbreviations

NDRG2: N-Myc downstream-regulated gene2

LUAD: Lung adenocarcinoma

EGFR: Epidermal growth factor receptor

CEA: Carcinoembryonic antigen

NSCLC: Non-small cell lung cancer

TKIs: Tyrosine kinase inhibitors

HPD: Hyperprogressive disease

HIF1-α: Hypoxia-inducible factor (HIF)1-α

PBS: Phosphate-buffered saline

DAB: 3,3'-Diaminobenzidine

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF: Polyvinylidene fluoride

RT-PCR: Reverse transcription-polymerase chain reaction

HRP: Horseradish peroxidase

SD: Standard deviation

ctDNA: circulating tumor DNA

TNM: Tumor lymph node metastasis

OS: Overall survival

PFS: Progression-free survival

Declarations

Acknowledgment

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Authors' Contributions

BY, XPL, TJ, and WDZ conceived of the study. HGZ, TX, and XHL performed data analysis for experiments. BY, HGZ, TX, and LL drafted the final version of the manuscript and figure legends. BY, XPL, XHL, and LZ revised the figures, added critical content to the discussion and were responsible in revising all portions of the submitted portion of the manuscript. TX and XHL performed experiment using lung adenocarcinoma and control tissue. All contributors meet the criteria for authorship. All of the authors read and approved the final manuscript.

Ethical approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki, and was confirmed by the Ethics Committee of Tianjin First Central Hospital of Nankai University (Tianjin, China; approval no. 2018N054KY). All patients signed the written informed consent forms.

Consent for publication

Not applicable

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Availability of data and materials

The data of the current research are available from the corresponding author on a reasonable request.

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Tables

Table 1. Patient characteristics.

Clinical characteristics	Total n	%
Age, years		
≤65	42	47.2
>65	47	52.8
Sex		
Male	52	58.4
Female	37	41.6
Smoking status		
Non-smoker	41	46.1
Smoker	48	53.9
Blood type		
A	29	32.6
B	27	30.3
O	24	27.0
AB	9	10.1
Lobe location		
Right	52	58.4
Upper lobe of right lung	30	57.7
Middle lobe of right lung	5	9.6
Inferior lobe of right lung	12	23.1
Center-type of right lung	5	9.6
Left	37	41.6
Upper lobe of left lung	21	56.8
Inferior lobe of left lung	13	35.1
Center-type of left lung	3	8.1
T		
1a	9	10.1
1b	11	12.4
1c	18	20.2
2a	21	23.6
2b	13	14.6
3	7	7.9
4	10	11.2
N		
0	29	32.6
1	20	22.5
2	27	30.3
3	13	14.6
M		
0	49	55.1
1a	20	22.5
1b	6	6.7
1c	14	15.7
Stage		
I	24	27.0
II	15	16.9
III	10	11.2
IV	40	44.9
Vascular invasion		
No	38	42.7
Yes	51	57.3
EGFR		
positive	31	34.8
negative	58	65.2

T, tumor; N, node; M, metastasis; EGFR, epidermal growth factor receptor

Table 2. Patient characteristics according to NDRG2 level.

Parameters	Total patients (n=89)			Operated patients (n=34)		
	NDRG2 low group	NDRG2 high group	P-value	NDRG2 low group	NDRG2 high group	P-value
Gender			0.988			0.710
Male	31	21		6	15	
Female	22	15		5	8	
Age (years)			0.996			0.705
<65	25	17		3	9	
≥65	28	19		8	14	
Smoking status			0.771			0.717
Non-smoker	19	14		5	13	
Smoker	34	22		6	10	
Blood type			0.265			0.905
A	15	14		5	9	
B	20	7		1	4	
AB	4	5		2	5	
O	14	10		3	5	
Stage			<0.001			0.072
I	6	18		5	16	
II	6	9		3	7	
III	8	2		2	0	
IV	33	7		1	0	
			<0.001			0.028
I+II	12	27		8	23	
III+IV	41	9		3	0	
CEA			<0.001			<0.001
<2.0	4	24		0	14	
≥2.0	49	12		11	9	
Vascular invasion			<0.001			0.008
No	19	32		6	22	
Yes	34	4		5	1	
EGFR			0.001			0.030
Negative(-)	42	16		9	9	
Positive(+)	11	20		2	14	

NDRG2, N-Myc downstream-regulated gene2; CEA, carcinoembryonic antigen (ng/ml); EGFR, epidermal growth factor receptor.

Table 3. Prognostic value of NDRG2/EGFR conjoined expression in multivariate analysis by Cox regression.

	B	SE	Wald	Df	P-value	HR	95.0 % CI for Exp(B)	
							Lower	Upper
Age	0.080	0.261	0.094	1	0.760	0.923	0.554	1.539
Sex	-0.171	0.317	0.290	1	0.590	1.186	0.637	2.209
Smoking status	0.066	0.309	0.046	1	0.831	0.936	0.511	1.716
Lobe location	0.186	0.262	0.502	1	0.479	0.831	0.497	1.388
Vascular invasion	1.500	0.342	19.202	1	<0.001	4.480	2.291	8.760
NDRG2-low/EGFR(-)			19.842	3	<0.001			
NDRG2-low/EGFR(+)	1.873	0.464	16.261	1	<0.001	6.508	2.619	16.174
NDRG2-high/EGFR(-)	0.576	0.556	1.073	1	0.300	1.779	0.598	5.292
NDRG2-high/EGFR(+)	1.258	0.476	6.984	1	0.008	3.519	1.384	8.949

Figures

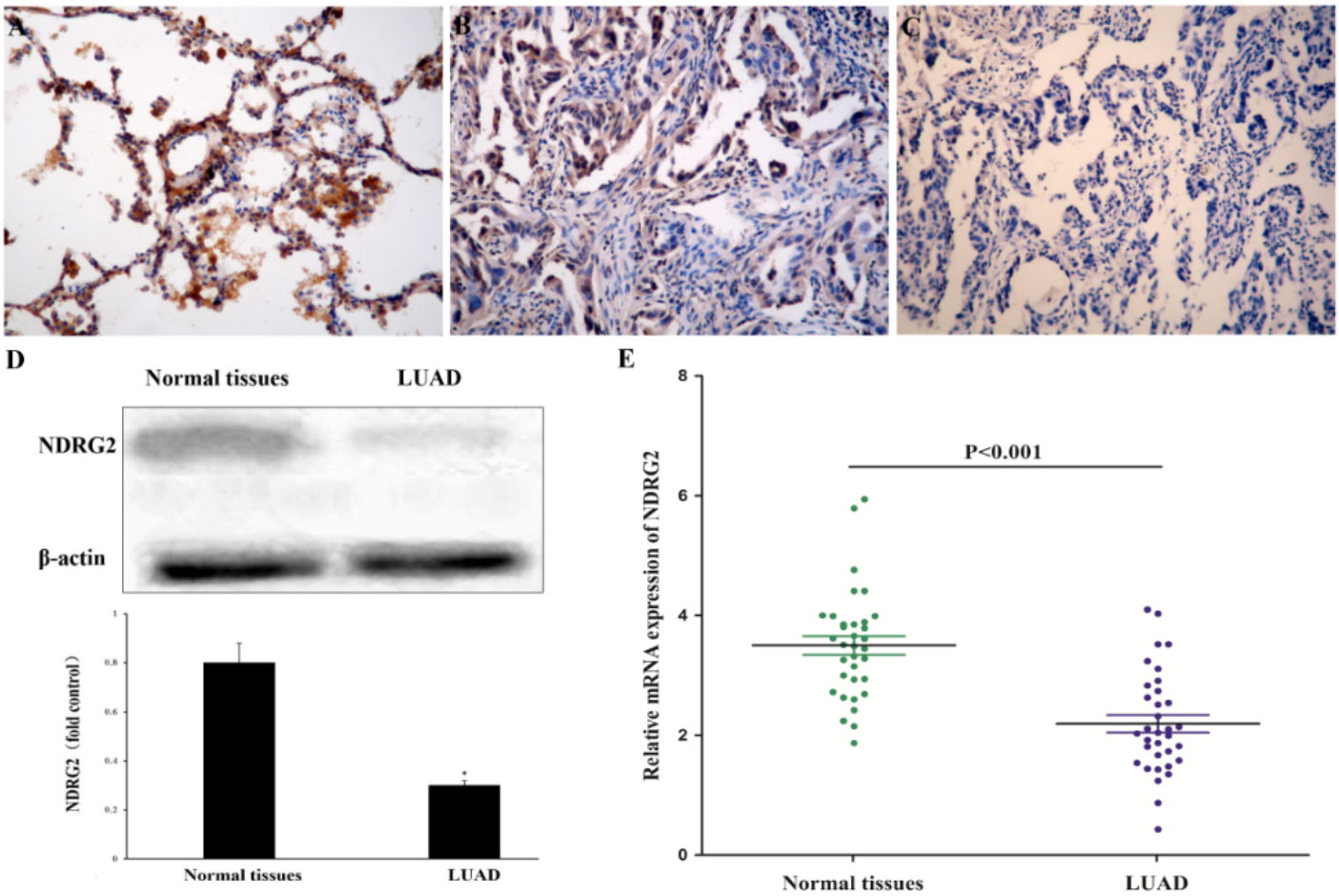

Figure 1

The expression level of NDRG2 in LUAD patients and normal tissues (Figure 1A-E). Immunohistochemistry showed the expression level of NDRG2 in normal lung tissues (Figure 1A), LUAD patients (Fig. 1B), and negative control (Figure 1C) (200x magnification). The expression level of NDRG2 protein was determined by Western blot assay (Fig. 1D) and RT-PCR (Figure 1E). It was significantly downregulated in patients with LUAD compared with that in normal tissues at both protein and mRNA levels (*P < 0.001).

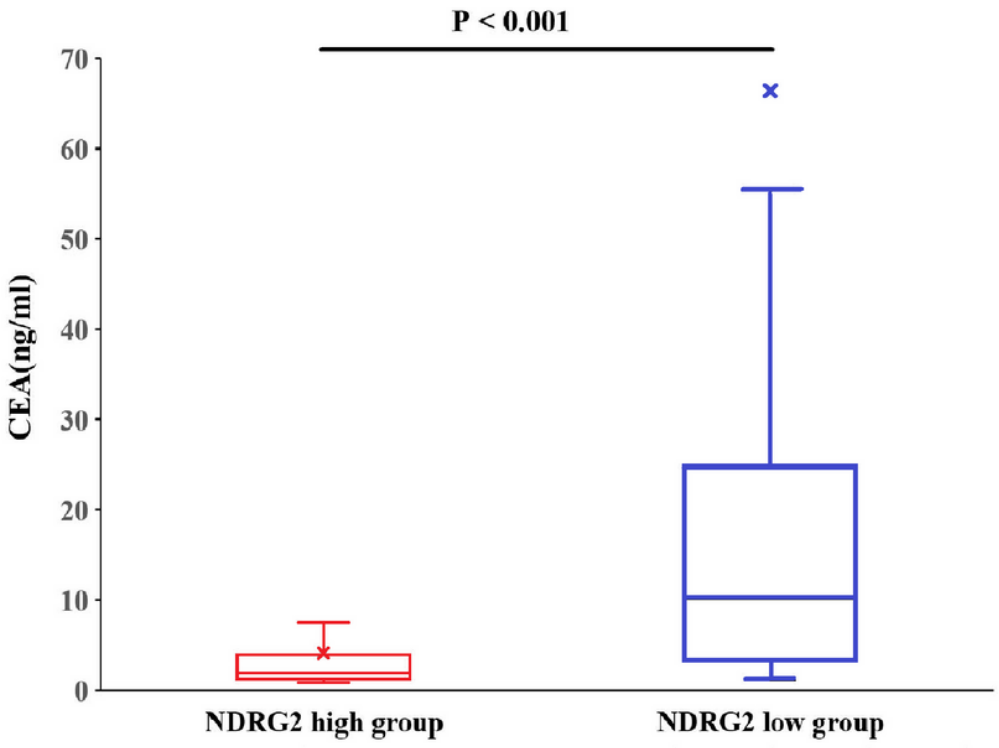

Figure 2

Associations between the expression levels of NDRG2 and clinicopathological features of patients with LUAD. The level of CEA was notably lower in patients with high expression level of NDRG2 than that in patients with low expression level of NDRG2 (P < 0.001). CEA, carcinoembryonic antigen; NDRG2, N-Myc downstream-regulated gene 2.

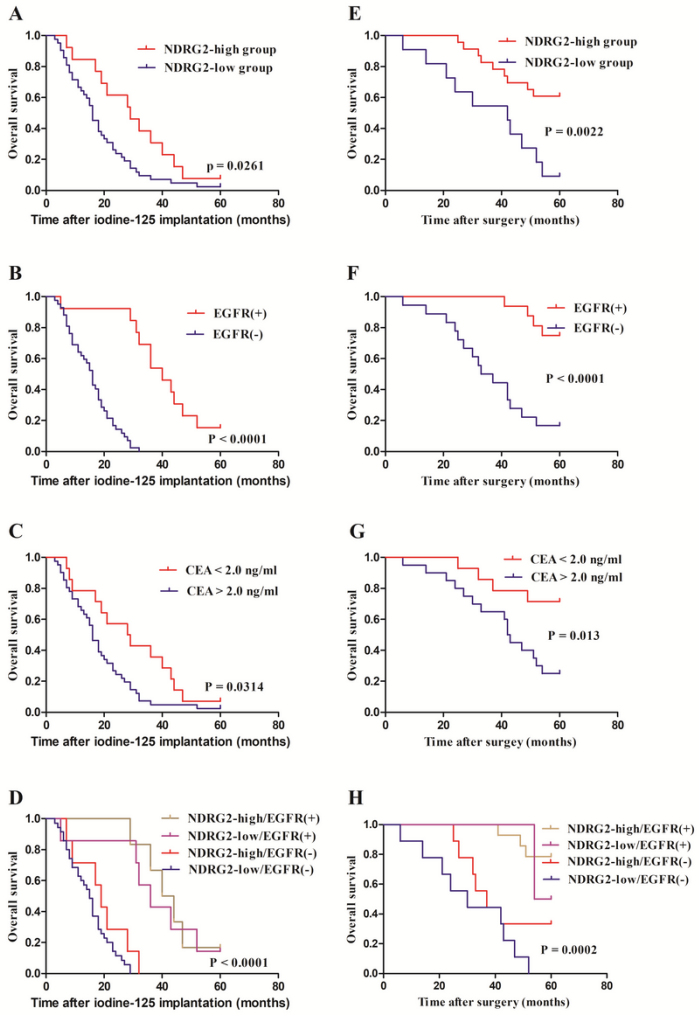

Figure 3

Overall survival of LUAD patients (Figure 3A-H). Overall survival of the iodine-125 radioactive seeds brachytherapy for advanced LUAD patients (Figure 3A-D). The patients with low expression level of NDRG2 (P = 0.0261, Figure 3A), negative EGFR expression (P < 0.0001, Figure 3B), and CEA > 2.0 ng/ml (P = 0.0314, Figure 3C) exhibited significantly lower overall survival rates. According to the conjoined expressions of NDRG2/EGFR, the subjects were categorized into four groups: NDRG2-low/EGFR-negative(-), NDRG2-low/EGFR-positive(+), NDRG2-high/EGFR-negative(-), and NDRG2-high/EGFR-positive(+). Patients with co-expression of NDRG2-low/EGFR-negative(-) had the worst outcome of overall survival among the 4 groups (P < 0.0001, Figure 3D). Overall survival of operated patients (Figure 3E-H). The patients with low expression level of NDRG2 (P = 0.0022, Figure 3E), negative EGFR expression (P < 0.0001, Figure 3F), and CEA > 2.0 ng/ml (P = 0.013, Figure 3G) exhibited remarkably lower overall survival rates. Patients with co-expression of NDRG2-low/EGFR-negative had the worst outcome for overall survival among the 4 groups (P = 0.0002, Figure 3H).