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Overexpression of Alteration/Deficiency in Activation 3 correlates with poor prognosis in non-small cell lung cancer

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ABSTRACT

Alteration/Deficiency in Activation 3 (ADA3), the human homologue of yeast ADA3, is involved in a variety of cell biological processes and plays an important role in tumorigenesis. Here, western blotting and reverse-transcription quantitative PCR (RT-qPCR) were conducted to explore the expression pattern of ADA3 in non-small cell lung cancer (NSCLC) patients. It was found that ADA3 protein expression in cancerous tissues was significantly higher than that in adjacent normal lung tissues, but there were no differences in mRNA levels. Tissue microarray immunohistochemical assay (TMA-IHC) was performed and we investigated the prognostic significance of ADA3 expression in 84 cases of NSCLC. Survival analyses showed that high expression of ADA3 was an independent prognostic factor for unfavorable overall survival (OS) in patients with NSCLC. In summary, the ADA3 expression level elevates in NSCLC and correlates with poor OS in NSCLC patients.

1. Introduction

Lung cancer is the leading cause of cancer-related death in the world [1]. Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer, making it one of the most common histological types and malignant diseases [2]. Because of the lack of early symptoms, more than half of NSCLC patients have had distant metastases at the time of diagnosis. Although imaging technology, chemotherapy, radiation therapy, targeted therapies and immunotherapies have greatly improved, the five-year overall survival (OS) rate is less than 15% [3–5]. Currently, the identification of biomarkers for diverse cancers is the subject of intense research, and such biomarkers are crucial for predicting the potential risk of disease recurrence and the prognosis of cancer patients. Thus, it is necessary to discover new and effective biomarkers for the prognosis of NSCLC.

Alteration/Deficiency in Activation 3 (ADA3) is the human homologue of yeast ADA3, which was originally reported as a transcriptional coactivator in 1992 [6]. ADA3 is a multifunctional protein that participates in a series of biological processes, including cellular proliferation, senescence, apoptosis, chromatin remodeling, embryonic development and the DNA damage response (DDR) [7].

Histone acetyltransferases (HATs) are enzymes that acetylate conserved lysine amino acids on histone proteins, which are linked to chromatin remodeling and transcriptional activation [8–10]. A number of studies found that ADA3 is a pivotal member of HAT complexes in mammals [11–13]. Some coactivators, such as GCN5, p300 and CBP, have innate HAT activity and can directly participate in chromatin remodeling. Because ADA3 lacks a HAT domain, the ternary complex of GCN5, ADA2 and ADA3 forms a catalytic core and may form various larger complexes with many other protein molecules [14,15].

The study of ADA3 in breast cancer is relatively detailed. The HPV E6 protein, identified as an interacting protein of ADA3, inhibits ERα- and ERβ-mediated transactivation and the expression of ER target genes by targeting ADA3 for degradation in osteosarcoma and breast cancer cell lines [16]. ADA3 has an important role in the recruitment of p300, PCAF and GCN5 to ER-responsive pS2 promoters. Repression of ADA3 inhibits cell proliferation and induces a reversal of the malignant phenotype of ER-positive breast cancer cell lines in matrigel growth medium [13]. It is noteworthy that predominant expression of ADA3 in the cytoplasm positively correlates with ErbB2, EGFR and Ki67, and

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Abbreviations: ADA3, Alteration/Deficiency in Activation 3; NSCLC, non-small cell lung cancer; DDR, DNA damage response; HATs, histone acetyltransferases; HPV, human papilloma virus; ER, estrogen receptor; EGFR, epidermal growth factor receptor; CENP-B, centromere protein B

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together these may serve as a better molecular marker to predict patient prognosis than the proteins individually [17]. Analysis of a group of breast cancer tissue specimens displayed a significant association of ADA3 nuclear expression with c-MYC expression and patients outcome [18]. The oncoprotein HPV16E6 targets ADA3 and makes it unstable by inducing SUMOylation, and promotes malignancies by interfering with proliferation and apoptosis-related regulators in cervical cancer cells [19]. Additionally, ADA3 interacts with β-catenin, a classic tumor-associated factor, through its Armadillo repeat sequences 6–10 and C-terminal domain, enhancing the transcriptional activity of the β-catenin response element LEF/TCF [20].

In this study, we analyzed the protein and mRNA expression of ADA3 in clinical NSCLC specimens and evaluated the correlation of ADA3 staining with tumor progression and patients outcome by immunohistochemical analysis. Our results highlighted a role of ADA3 in predicting the prognosis of NSCLC patients.

2. Materials and methods

2.1. Human tissue specimens and tissue microarray (TMA)

Matched tumorous and adjacent normal tissues used for western blot and RT-qPCR were collected from NSCLC patients at the Affiliated Hospital of Nantong University, Jiangsu Province, China, frozen in liquid nitrogen and stored at −80 °C. All the 84 cancerous tissues and 58 matched adjacent normal tissues for construction of tissue microarrays (TMA) were obtained from the human clinical biobank at the Affiliated Hospital of Nantong University. The ages of all patients ranged from 44 to 83 years, with a median of 63 years, including 43 women and 41 men. No patients received chemotherapy, radiotherapy or immunotherapy before surgery. The clinical information can be queried from the patients' medical records. The study protocol was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University, Jiangsu Province, China. Written informed consent was provided by all participants.

2.2. Tissue microarray immunohistochemical assay (TMA-IHC)

TMA sections were cut to a thickness of 4 μm, and immunohistochemical staining for ADA3 expression was performed. TMA was generated by UNITMA Quick-Ray manual staining system (UT06, UNITMA, Korea). The monoclonal rabbit anti-TADA3L antibody (ab181984, Abcam, Cambridge, UK) was diluted 1:200. Two individuals assessed and recorded the IHC staining independently, who were blinded to each other's scores and the clinicopathological parameters of the samples.

For evaluation of ADA3 expression, we applied a classic scoring method. IHC Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), while the grading for marking frequency ranged from 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%) and 4 (76%–100%), depending on the percentage of positive cells. Then the X-tile software (Rimm Laboratory at Yale University; http://www.tissuearray.org/rimmlab) was used to find a suitable cut-off value (here it is set to 3) and to divide the protein levels into two categories (no/low expression and high expression).

2.3. Western blot analysis

Tumor tissues were resuspended in RIPA Lysis Buffer (P0013B, Beyotime Institute of Biotechnology, Shanghai, China) containing 1% protease inhibitor (ST506, PMSF, Beyotime Institute of Biotechnology). Protein concentrations were measured with the BCA Protein Assay Kit (BL521A, Biosharp, China). Equal amounts of total protein per sample were resolved by SDS-PAGE, then transferred to PVDF membranes (IPVH00010, Millipore Corporation, USA). The membranes were blocked in 5% fat-free milk and incubated overnight at 4 °C with the primary antibodies: anti-TADA3L (dilution 1:5000, ab181984, Abcam, Cambridge, UK) and anti-GAPDH (dilution 1:2000, AB0037, Abways, Shanghai, China). After washing, the membranes with proteins were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000, ab205718, Abcam) for 2 h at room temperature. The membranes were washed again, and protein signals were visualized using an ECL system (Bio-Rad, Hercules, CA, USA).

2.4. RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) according to the instructions. The primers for RT-PCR, ADA3 forward: 5′-AGATGGATGCCCCTTTGGTG-3′, ADA3 reverse: 5′-AGGGCTTGTTCTGATTGCGA-3′; GAPDH forward: 5′-GGTAGACAAGTTTCCCTT-3′ and GAPDH reverse: 5′-ATATGTTCTGGATGATTCT-3′, were purchased from Sangon (Shanghai, China). RT-qPCR was performed using the ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and SYBR Green Master Mix (Vazyme, Nanjing, China). The mRNA levels were quantified with the $2^{-\Delta\Delta Ct}$ method after normalization to GAPDH mRNA.

2.5. Statistical analyses

All statistical analyses were performed by SPSS ver. 20.0 software (IBM, USA) and GraphPad Prism 6.01 (GraphPad, USA). The data were expressed as the mean ± standard deviation (SD). Correlations between clinicopathological variables of NSCLC patients and ADA3 expression in tissue sections were determined with Pearson's χ^2 test for categoric variables. Univariate analyses were performed with the log-rank test and multivariate analyses were performed with the Cox proportional hazard model. Survival curves were completed by the Kaplan–Meier method, and the significance of differences between groups was tested using the log-rank test. The data were expressed as the mean ± standard deviation (SD), and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression level of ADA3 in NSCLC tissues

To test the difference in expression of ADA3 between NSCLC and adjacent normal tissues, we examined the protein and mRNA levels of ADA3. Western blot analysis and quantification of protein levels confirmed that the protein expression of ADA3 in NSCLC tissues is higher than that in adjacent tissues (Fig. 1A, B). Interestingly, however, RT-qPCR analysis was conducted and the result showed no difference in ADA3 mRNA levels between cancer and adjacent tissues (Fig. 1C). Taken together, the difference in expression of ADA3 protein in cancer and adjacent tissues is not reflected in mRNA levels.

3.2. Expression pattern of ADA3 in human NSCLC tissue microarrays

We further detected the expression pattern and subcellular localization of ADA3 protein in NSCLC and normal tissues by TMA-IHC analysis. Representative images of ADA3 staining are shown in Fig. 2. In the TMA cohort, NSCLC samples showed predominantly positive cytoplasmic and nuclear staining. The protein level of ADA3 in normal tissues (Fig. 2A1 and A2) was significantly lower than that in adenocarcinoma (Fig. 2B1 and B2) and squamous cell carcinoma tissues (Fig. 2, C1 and C2).

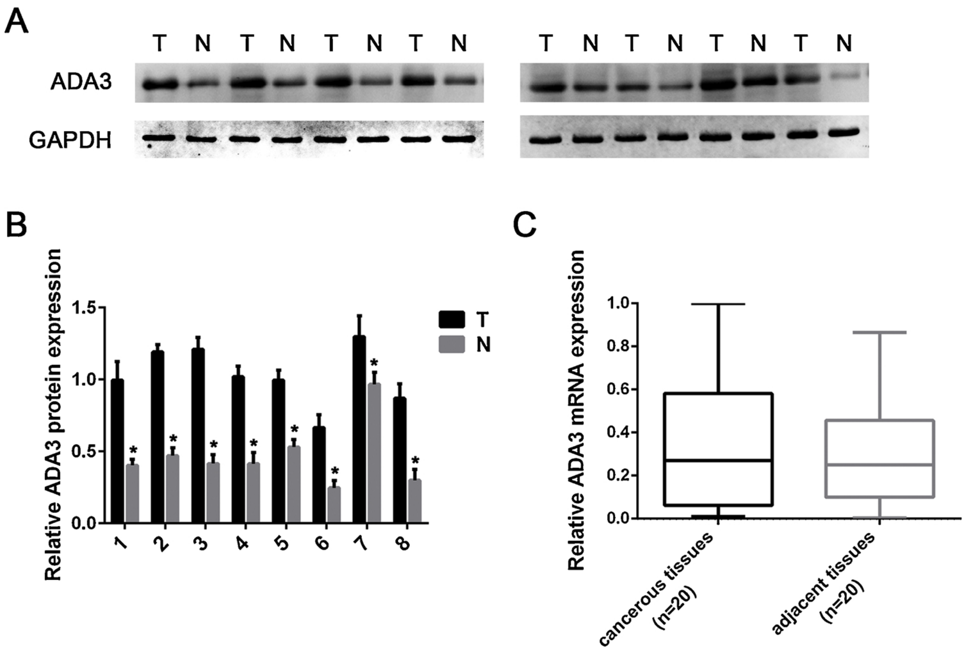

Fig. 1. Protein and mRNA expression of ADA3 in NSCLC tissues.

A and B. Western blot analysis (A) and quantification (B) of ADA3 protein expression in eight pairs of NSCLC tissues (lanes labeled with 'T') and matched normal human lung tissues (lanes labeled with 'N'). GAPDH was probed as a loading control. *P < 0.05 vs. the normal tissues. C. The RT-qPCR analysis of relative expression of ADA3 in 20 pairs of NSCLC tissues and matched normal lung tissues. *P < 0.05. Data are presented as the means ± SD from three independent experiments.

3.3. Association between ADA3 expression and clinicopathological characteristics

The relationship between ADA3 expression and clinicopathological parameters of NSCLC patients (n = 84) is summarized in Table 1. A total of 52 (61.90%) patients showed high expression of ADA3, while 32 (38.10%) patients showed low or no expression of ADA3. High ADA3 expression was obviously associated with the degree of differentiation (P = 0.004), lymph node metastasis (P = 0.005) and the tumor node metastasis (TNM) stage (P = 0.008). On the contrary, no significant connection (P > 0.05) was observed between ADA3 level and other clinicopathological parameters, such as sex, age at diagnosis, smoking, histological typing and primary tumor. In summary, we can conclude that elevated ADA3 expression in NSCLC may accompany lymph node metastasis, poor tumor differentiation and advanced TNM staging.

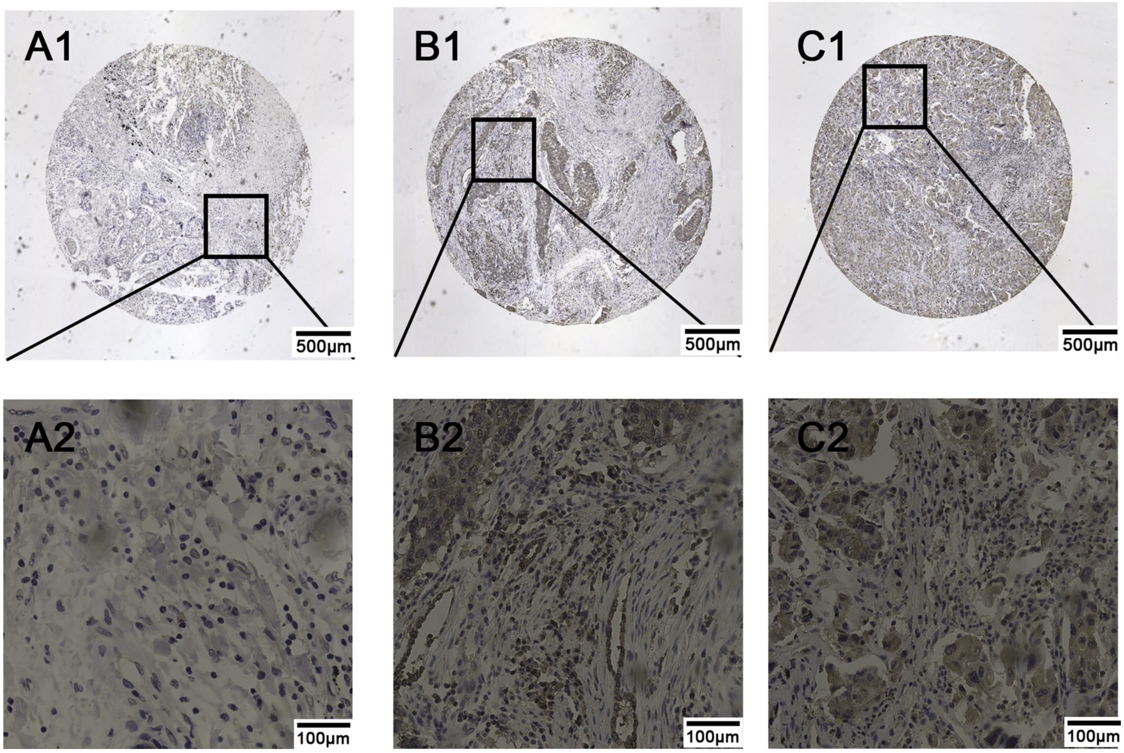

Fig. 2. Expression pattern of ADA3 in human NSCLC tissue microarrays.

A1 and A2. Negative for ADA3 protein expression in normal lung tissues adjacent to cancerous tissue specimens. B1 and B2. Positive for ADA3 protein expression in adenocarcinoma tissues. C1 and C2. Positive for ADA3 protein expression in squamous cell carcinoma tissues. A1, B1, and C1 were 40× magnification, A2, B2, and C2 were 200× magnification.

Table 1

Correlation of expression of ADA3 protein with clinicopathologic parameters in NSCLC patients.

Clinicopathologic parameters	ADA3				
	n	Low or no expression	High expression	Pearsonχ^2	P value
Total	84	32	52		
Sex				0.385	0.654
Male	41	17	24		
Female	43	15	28		
Age at diagnosis(years)				3.054	0.099
≤60	28	7	21		
> 60	56	25	31		
Smoking				0.932	0.235
No smoking	45	15	30		
Smoking	39	17	22		
Histopathology grading				3.222	0.200
Adenocarcinoma	33	9	24		
Squamous cell carcinoma	46	20	26		
Adenosquamous carcinoma	5	3	2		
Differentiation				10.045	0.004*
Low grade	26	4	22		
Middle grade	51	23	28		
High grade	7	5	2		
Primary tumor				1.430	0.151
T1	24	11	13		
T2	36	14	22		
T3 + T4	24	7	17		
Lymph node metastasis				8.168	0.005*
Present	29	5	24		
Absent	55	27	28		
TNM stage				9.733	0.008*
I	23	14	9		
II	37	14	23		
III + IV	24	4	20		

* P < 0.05.

3.4. *Impact on prognosis of ADA3 expression in NSCLC patients*

The prognostic value of high expression of ADA3 and the clinicopathological characteristics were assessed by univariate analyses. The results indicated that the poor prognosis of NSCLC patients correlated to overexpression of ADA3 (P < 0.001, Table 2). Multivariate Cox regression analysis further confirmed that ADA3 overexpression was an independent prognostic marker for patients with NSCLC by five-year OS (P = 0.002). Advanced TNM stage was also an independent risk factor for patient survival (P = 0.028, Table 2).

Finally, survival analysis using the Kaplan–Meier method showed a significant association between ADA3 protein expression and the OS of NSCLC patients (log-rank test, P < 0.001). The survival curve perfectly displayed the significant discrimination in the five-year OS corresponding to ADA3 protein levels (P < 0.001, Fig. 3A). In addition, the survival time of NSCLC patient was negatively associated with the occurrence of poor differentiation (P < 0.001, Fig. 3B), lymph node metastasis (P = 0.001, Fig. 3C) and advanced TNM stage (P < 0.001, Fig. 3D).

4. Discussion

Lung cancer is a heterogeneous disease related to multiple factors, stages and genes. Although existing predictive markers have greatly improved our capacity to manage NSCLC patients, this suggests that our exploration of biomarkers is markedly meaningful and more effective markers are needed to improve patient outcomes.

Considerable investigations in varied fields have identified the biological functions of ADA3, a transcriptional coactivator. The discovery journey of ADA3 began with identification of its significance in transcriptional co-activator systems, mainly involving various HATs [12,21–24]. In the recent past, more research has focused on the impact of ADA3 on cancers. Studies confirmed that ADA3 closely associates with centromere protein B (CENP-B), a highly conserved centromeric protein [25]. ADA3 knockdown decreases CENP-B binding to centromeres, suggesting a significant role for ADA3 in maintaining genomic stability and regulating cell proliferation [26,27]. Abnormal activation of β-catenin contributes to diverse human cancers, and ADA3 is required for β-catenin-mediated proliferation [20]. ADA3 deletion in MEFs delays transition from G1 to S phase and G2/M to G1 phase, and induces mitotic defects [28]. ADA3, identified as a novel interacting protein of p53, is indispensable for enhancing the transcriptional activity of p53 as well as p53-mediated apoptosis [29,30]. Depletion of ADA3 causes a disorder in human lymphocyte toxins granzyme B (hGrzB)/perforin and induces Bid cleavage, resulting in reduced apoptosis [31].

Table 2

Univariate and multivariate analyses of predictive factors for prognosis of NSCLC patients.

Characteristic	Univariate analysis			Multivariate analysis		
	OR	P value	95%CI	HR	P value	95%CI
ADA3 expression High vs Low	5.529	< 0.001*	2.879-10.963	3.246	0.002*	1.546-6.812
Sex Male vs Female	1.542	0.103	0.916-2.594			
Age at diagnosis(years) ≤60 vs > 60	0.840	0.523	0.492-1.435			
Smoking No smoking vs smoking	0.912	0.723	0.546-1.522			
Histopathology grading Ad vs Sq vs ASC	0.937	1.049	0.319-3.452			
Differentiation Low vs middle vs high grade	9.861	0.001*	2.716-35.800			
Primary tumor T1 vs T2 vs T3 + T4	0.732	0.382	0.364-1.472			
Lymph node metastasis N0 vs N1 vs N2	11.914	< 0.001*	5.791-24.514			
TNM stage I vs IIvs III + IV	0.085	< 0.001*	0.036-0.199	0.340	0.028*	0.130-0.891

Ad: Adenocarcinoma; Sq: Squamous cell carcinoma; ASC: Adenosquamous carcinoma.

* P < 0.05.

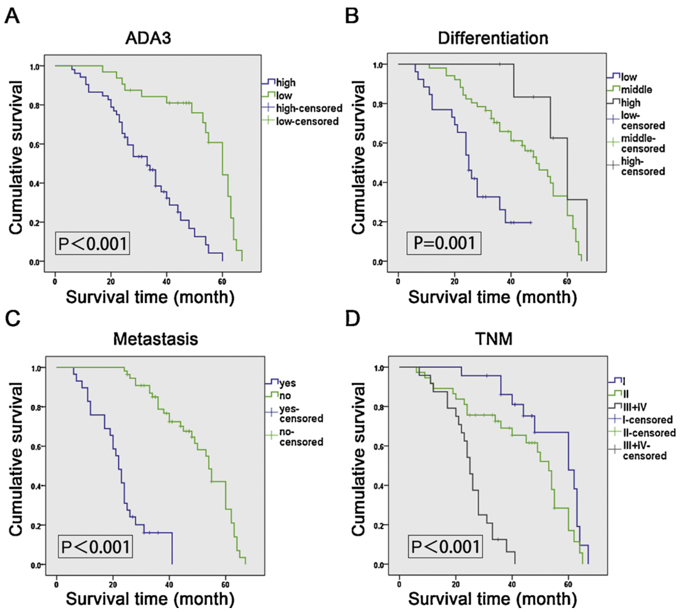

Fig. 3. Kaplan–Meier overall survival (OS) analysis of ADA3 expression and clinicopathological parameters in NSCLC patients.
A, B, C and D. Kaplan–Meier survival curves indicating that the ADA3 protein expression (A), degree of differentiation (B), the presence or absence of lymph node metastasis (C) and TNM stage (D) are associated with the OS of NSCLC patients.

Owing to the ability of ADA3 to regulate cell proliferation and apoptosis, as well as certain cancer studies, malfunction of ADA3 can be speculated to cause various human diseases, including cancer, and its level may correlate with disease prognosis. Therefore, we verified the difference in expression levels of ADA3 in NSCLC tissues and adjacent normal tissues, and further explored the relationship between ADA3 expression and NSCLC patient prognosis. The results indicated that upregulation of ADA3 is an independent factor for poor prognosis in patients with NSCLC. Interestingly, our results showed that ADA3 protein overexpressed in cancerous tissues compared with adjacent normal tissues, despite no difference in mRNA levels. Therefore, the mechanism involved is worth future investigation.

Considerable research verified the interaction between ADA3 and various HATs, and ADA3 induced multiple types of modification of its interacting proteins. However, ADA3 acetylation is dynamically regulated by its associated HATs and essential for histone acetylation by ADA3-containing HAT complexes [32]. ADA3 is acetylated in different cell types and the acetylation of ADA3 plays a key role in cell cycle progression. ADA3 acetylation mediated by the AKT-p300 pathway promotes epidermal growth factor receptor (EGFR)-dependent cell proliferation [33]. Ubiquitination of ADA3 is counteracted by p300-mediated acetylation, thereby decreasing ADA3 protein degradation to promote its function. E6 is a binding protein of ADA3, which promotes disease by acting on the degradation of ADA3 [34–36]. There have been data to confirm phosphorylation of ADA3 in response to DNA damage [26]. Overall, functions and expression of ADA3 are regulated by these post-transcriptional modifications (PTM) due to alteration in their stability, subcellular localization and interaction with other proteins. Thence, we can speculate that the difference between protein and mRNA expression is related to the modification site of ADA3 and the reduction of protein degradation.

Apart from increasing the basic understanding of ADA3 biology, investigations will reveal the therapeutic potential of human ADA3 in future clinical treatments. Our study shows that the role of ADA3 in NSCLC is worth studying, and there are still many issues guiding us to conduct further in-depth research. In future studies, it will be of considerable interest to explore the role of ADA3 protein modification in the development of NSCLC.

5. Conclusions

This study shows overexpression of ADA3 protein in NSCLC tissues and no difference of mRNA expression between cancerous and paracancerous tissues. The difference between protein and mRNA expression is of interest and worthy of future study to explore the role of ADA3 in NSCLC. Survival analyses showed that the upregulation of ADA3 was an independent prognostic biomarker for poor overall survival (OS) in NSCLC patients. However, there are still many unknowns about the value of ADA3 in tumors that are worthy of further exploration.

Declarations of interest

None.

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