Original Article

Upregulation of estrogen receptor beta protein but not mRNA predicts poor prognosis and may be associated with enhanced translation in non-small cell lung cancer: a systematic review and meta-analysis

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Background: An increasing number of original studies suggest that estrogen receptor beta (ERβ) expression may be related to non-small cell lung cancer (NSCLC) prognosis; however, the evidence remains inconclusive and conflicting. We aimed to systematically evaluate the expression and prognostic value of ERβ in NSCLC, and to explain the inconsistency between ERβ protein and mRNA level.

Methods: PubMed, Embase, and Web of Science databases were searched for studies (published before October 6, 2020) reporting the prognostic value of ERβ protein expression in NSCLC. The pooled hazard ratios (HRs) with 95% confidence intervals (CIs) for overall survival (OS) were calculated. Transcriptome and survival data of lung adenocarcinoma patients were obtained from public databases for differential expression and survival analyses. Immunohistochemistry (IHC) was performed to examine the ERβ protein expression in 39 NSCLC patients. Western blotting and RT-qPCR were performed to analyze ERβ expression in two paired NSCLC and normal adjacent tissue samples. The effect of methyltransferase-like 13 (METTL3) on ERβ expression was investigated in a lung cancer cell line.

Results: Meta-analysis of 23 studies with a total of 3744 patients demonstrated that high protein expression of overall ERβ and cytoplasmic ERβ indicated poor OS (HR: 1.05, 95% CI: 1.00 to 1.10; HR: 1.48, 95% CI: 1.13 to 1.95) in NSCLC. For lung adenocarcinoma especially, high protein expression of both overall/cytoplasmic ERβ and nuclear ERβ suggested poor OS (HR: 1.54, 95% CI: 1.05 to 2.25; HR: 1.36, 95% CI: 1.03 to 1.80). Bioinformatics analysis indicated the expression of ERβ mRNA was not associated with the prognosis of lung adenocarcinoma. Analysis of public databases showed that ERβ mRNA is not highly expressed in tumor tissues, however, IHC results revealed that ERβ protein is highly expressed in NSCLC tissues. We validated this inconsistency in ERβ expression in paired tumors and normal adjacent tissues from patients. Moreover, METTL3 knockdown in the A549 cell line downregulated ERβ protein expression but not ERβ mRNA expression.

Conclusions: Our study elucidated the inconsistency between ERβ protein and mRNA expression levels and their prognostic values. The results indicated that METTL3-driven enhanced translation in NSCLC may cause this inconsistency.

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Introduction

Estrogen receptor beta (ERβ, also known as ESR2) is one of the two classical subtypes of the estrogen receptor (1) and is widely expressed in the lung. Lung cancer cells exhibit a stronger ERβ protein expression than normal lung cells (2-4). Previous studies, including ours, have reported that ERβ in the nucleus acts as a transcription factor that exerts effects on lung carcinogenesis (5,6); and ERβ in the cytosol participates in the signaling pathways that promote non-small cell lung cancer (NSCLC) progression and metastasis (7,8), leading to a poor prognosis in NSCLC patients.

Despite unraveling the putative role of ERβ in lung cancer, the prognostic value of ERβ protein expression reported in different studies is not consistent (9,10). A meta-analysis in 2015 reported a favorable effect of ERβ on NSCLC survival (11). Another meta-analysis of 11 studies reported that ERβ is not an independent predictor of NSCLC survival (12). These two meta-analyses lacked evaluation by different subcellular localizations of ERβ, and neither of these studies considered the effect of ERβ in lung adenocarcinoma. Several additional original studies were published subsequently.

Studies focusing on the expression and prognostic value of ERβ mRNA have shown negative results. ERβ mRNA expression is not upregulated in lung cancer tissues (13). In 2015, a meta-analysis showed that ERβ mRNA is not a prognostic factor for NSCLC (14). Therefore, we suppose that the expression of ERβ protein and mRNA is not consistent in NSCLC, and that this inconsistency is reflected in their prognostic values. There might be an abnormal translation control that can upregulate the protein expression of ERβ in the presence of low mRNA expression level, which further affects the prognosis. As the most abundant base modification of RNA, N6-methyladinosine (m^6A) drives translation initiation in human cells (15). METTL3 acts as a key enzyme in RNA m^6A modification. In 2016, Lin *et al.* (16) reported that METTL3 promotes the translation of abundant mRNAs in human cancer cells, revealing the important role of translation control in cancer cells.

The expression of ERβ and its relationship with lung cancer survival have not been systematically evaluated to date. Therefore, in this study, we conducted a meta-analysis with specific subgroups and a large sample size. By combining abundant transcriptome data, we illustrated the prognostic value of ERβ protein and mRNA. Expression data from clinical samples and public databases were included to explore the difference between ERβ protein and mRNA expression. Furthermore, we verified this difference in paired tissue samples from NSCLC patients. Finally, we explored the possible mechanism of METTL3-driven translation regulation in cell lines. In summary, based on our studies of estrogen-mediated NSCLC progression (7,8,17-19), we provide insights into ERβ expression profiles, and the possibility of anti-estrogen therapy in NSCLC.

We present the following article in accordance with the PRISMA reporting checklist (available at https://dx.doi.org/10.21037/jtd-21-658) (20).

Methods

Literature search

We comprehensively searched the PubMed, Web of Science, and Embase databases for articles published before October 6, 2020 (the search strategy is detailed in Table S1) that analyzed the prognostic value of ERβ protein expression in NSCLC patients. We identified studies using medical subject headings (MeSH) from PubMed to develop a controlled vocabulary where applicable. In addition to MeSH, we included relevant free-text entry terms.

Selection criteria and study selection

Original articles that achieved all the following criteria were included: (I) presented data, including different

histological types, from primary NSCLC patients; (II) detected ERβ protein expression in primary tumor tissues using immunohistochemistry (IHC); (III) provided detailed clinical and pathological features of the study population; (IV) addressed the prognostic value of tumor ERβ expression using survival analysis.

Articles that achieved any of the following criteria were excluded: (I) estimated the prognostic value for subtypes of ERβ only; (II) considered disease-free survival, recurrence-free survival, or progression-free survival as an endpoint but not overall survival (OS).

After removing duplicate studies, two investigators (W Meng and H Xiao) initially screened the titles and abstracts of the identified records for full-text review. Disagreements were resolved by consensus.

Data extraction

Using a pre-specified data collection sheet, two investigators (W Meng and H Xiao) independently extracted data from the retrieved articles, and agreement was achieved in group discussions. The extracted data included the authors, year of publication, country or region, sample size, tumor stage, composition of different histology types, median/mean and range of age, follow-up duration, epidermal growth factor receptor (EGFR) mutation status, aromatase expression status, sample type, detection method of ERβ expression, ERβ subcellular localization, positive cut-off definition, number of positive cases, antibody type, and covariates adjustment. We selected OS as the endpoint for our meta-analysis because OS is widely used as a significant prognostic indicator. Hazard ratios (HRs) for the association between three subcellular localizations of ERβ [cytoplasmic ERβ, nuclear ERβ, and overall ERβ (cytoplasmic and nuclear ERβ)] and OS were extracted.

Quality assessment

We used the Newcastle-Ottawa Scale (NOS) (21) for cohort studies to assess the methodological quality of the included studies. It is a nine-point scoring system that considers participant selection, exposure measurement, ascertainment of outcomes, covariate adjustment, and adequacy of follow-up. A high-quality study was defined as a study with at least seven points. Two reviewers (W Meng and H Xiao) independently assessed the quality of the included articles, and disagreements were resolved through group discussions.

Statistical analysis of meta-analysis

HRs and the corresponding 95% confidence intervals (CIs) were considered the effect sizes in this study. For studies in which HRs and 95%CIs were not available, we used the method described by Parmar *et al.* (22) to derive estimates from the published Kaplan-Meier survival curves. The most adjusted study-specific HRs and 95%CIs were primarily pooled using a random-effects model and the inverse variance method. Heterogeneity between studies was assessed using the I^2 index, and we considered I^2<50% as low heterogeneity, I^2 between 50% and 75% as medium heterogeneity, and I^2>75% as high heterogeneity. If the heterogeneity was low (I^2<50%), a fixed-effects model was used. Subgroup analyses were performed based on several variables, including histology type, geographical location and whether a multivariate or univariate analysis was performed. Sex-specific HRs were also pooled using data from the studies. For study groups with relatively high heterogeneity (I^2>50%), we performed sensitivity analysis (in the random-effects model) using a leave-one-out strategy to investigate the sources of heterogeneity.

Publication bias was assessed using funnel plots and the Egger's test. All statistical analyses for the meta-analysis were performed using STATA version 16.0 (Stata Corp, College Station, TX, USA); a 2-sided P value of <0.05 was considered statistically significant.

Survival analysis of gene expression in lung adenocarcinoma

After the removal of 59 normal tissues, we analyzed the prognostic value of four genes [ESR2, ESR1 (estrogen receptor alpha; ERα), G-protein coupled estrogen receptor 1 (GPER1) and CYP19A1 (also known as aromatase, which is a key enzyme for estrogen synthesis)] in 526 lung adenocarcinoma tissues from The Cancer Genome Atlas (TCGA)-lung adenocarcinoma (LUAD). The "survminer" and "survival" packages were used to draw Kaplan-Meier curves. The Kaplan-Meier Plotter (http://kmplot.com/) is an online database used to assess the association of genes with survival in four types of cancer (lung, breast, gastric, and ovarian cancer). It was used to verify the prognostic value of the four target genes in lung adenocarcinoma

patients (n=719).

Differential expression analysis of the transcriptome in lung adenocarcinoma

Based on the findings of the relationship between ERβ and lung cancer at the protein level using meta-analysis, we performed bioinformatics analysis to further explore the relationship between ERβ and lung cancer at the transcriptional level.

To quantify ERβ mRNA expression in lung adenocarcinoma, RNA-seq data of a lung adenocarcinoma cohort (tumor tissues =526 *vs.* normal tissues =59) from TCGA and those of normal lung tissues (n=288) from the Genotype-Tissue Expression (GTEx) were obtained from the University of California Santa Cruz Xena platform (https://xenabrowser.net/datapages/). RNA-seq data from the GTEx (https://commonfund.nih.gov/GTEx/) project were used to reduce the bias in the data from TCGA-LUAD. ESR2, ESR1, GPER1 and CYP19A1 were selected as the target genes for differential expression analyses. The R package "limma" was used to screen the differentially expressed genes (DEGs) among the four target genes. Genes with an absolute value of logFC (the logarithm of fold change) >1 and P<0.05 were defined as the DEGs. The results of "limma" analysis are presented in the form of a heatmap. Four gene expression profile datasets (GSE10072, GSE40791, GSE32863, and GSE43458) of lung adenocarcinoma were obtained from the Gene Expression Omnibus (GEO) for further validation of the above DEG analysis results.

Patients and tissue specimens

Paired samples of primary NSCLC tumors and corresponding normal adjacent tissues from 39 Chinese patients for IHC were obtained at the time of surgical resection at the Department of Thoracic Surgery, Tongji Hospital of Huazhong University of Science and Technology Tongji Medical College (Wuhan, China).

Paired samples of primary tumors and normal adjacent tissues from two patients for western blotting and RT-qPCR were obtained at the time of surgical resection at the Department of Thoracic Surgery, Union Hospital of Huazhong University of Science and Technology Tongji Medical College (Wuhan, China). The exterior of the NSCLC tissue which is a hard white part, was selected. The study was approved by the Ethics or Institutional Review Board of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki (as revised in 2013). All of the patients had sufficient tissue blocks available for the analysis of ERβ expression.

Immunohistochemical analyses

Sample processing and IHC were performed as previously described (17). Rabbit anti-human ERβ polyclonal antibody (dilution 1:50, Proteintech 14007-1-AP) was purchased from Proteintech (Wuhan, China). Protein expression levels were independently scored by two pathologists. Immunoreactivity scores of cancer tissue samples were determined based on staining intensity and positive staining area according to the method described by Tang *et al.* (17). Proportion of the positive cells was scored as follows: 1, ≤25% positive cells; 2, 25–50% positive cells; 3, 50–75% positive cells; and 4, >75% positive cells. Staining intensity was evaluated as follows: 1, negative; 2, weakly positive; 3, moderately positive; and 4, strongly positive. A score of 1–8 was obtained by adding the staining intensity score and the proportion score. A total score ≥5 was defined as high expression, and a score ≤4 was defined as low expression. These criteria were based on the evaluations reported by Nose *et al.* and Kawai *et al.* (23,24).

Cell lines and culture conditions

The human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), grown for 2 weeks, and passaged four times before freezing aliquots for subsequent analyses. The cell lines were tested and authenticated by ATCC. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and then incubated in a humidified atmosphere with 5% CO_2 at 37 ℃.

Cell transfection

Transfection of small interfering RNA (siRNA) was performed using Lipofectamine 3000 (Invitrogen; Shanghai, China) according to the manufacturer's instructions. siRNAs targeting METTL3 (siRNA_METTL3: CTGCAAGTATGTTCACTATGA) and control siRNAs (siRNA_NC) were obtained from Ribobio Co., Ltd,

Guangzhou China.

Western blotting of cultured cells and NSCLC samples

Lung cancer tissues or cell lines were lysed in RIPA buffer, followed by homogenization and determination of protein concentration. Thereafter, 20 μg of protein was loaded for SDS-PAGE and transferred onto PVDF membranes. Immunoblotting was performed to detect protein expression. The corresponding antibodies included METTL3 (Abcam 195352), ERβ (Proteintech 14007-1-AP), and GAPDH (Proteintech 60004-1-Ig). Specifically bound HRP-conjugated secondary antibodies were detected using an ECL detection system (ChemiDocTM XRS+ machine, Bio-Rad Laboratories). Densitometric analyses were performed using ImageJ software. Relative quantification was performed after normalization to GAPDH band intensities. A Mann-Whitney test was performed to assess the difference in protein expression between the groups. Each experiment was performed in triplicates and repeated at least three times.

RNA isolation and RT-qPCR

Total RNA was extracted from NSCLC tissues (preserved in RNA Keeper Tissue Stabilizer, Vazyme Biotech Co. LTD, Nanjing, China) and cells using TRIzol (Invitrogen), following the manufacturer's instructions. 1 μg total RNA was reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBR® Green PCR Master Mix with the StepOne Real-Time PCR System (Applied Biosystems). GAPDH was used as the internal control for the normalization. The primers were purchased from Tsingke Co. LTD, Beijing, China. Primer information from PrimerBank is shown in Table S2.

Results

Literature search

Electronic searches identified 2,845 citations from PubMed, Web of Science and Embase, of which 1,764 titles and abstracts were reviewed. After the excluding 1,688 records, the full texts of 76 articles were further reviewed (*Figure 1*). A total of 23 unique studies in 22 articles met our selection criteria and were included in the meta-analysis.

Characteristics and quality of the included studies

The characteristics of the included studies are summarized in *Table 1*. The included studies reported data from a total of 3,744 patients. All studies were published between 2005 and 2020, including 22 retrospective cohort studies and one prospective cohort study. Fifteen studies from fourteen articles were on NSCLC, and eight studies were on lung adenocarcinoma (25-32).

All studies measured the protein expression of ERβ in tumor tissue using IHC. Six studies included cytoplasmic ERβ (2,10,25,33-35), 16 studies included nuclear ERβ (9,10,23,26-29,31,32,36-40), and five studies included overall ERβ, which considered cytoplasmic and nuclear ERβ expression (2,30,32,34,41). Four studies reported two HRs for these different subcellular localizations of ERβ (2,10,32,34). Navaratnam *et al.* included two distinct studies that reported two HRs for nuclear ERβ (38). Eight studies performed multivariate analyses of HRs to adjust for sex, age, stage at diagnosis, or smoking status (2,9,10,28,34,39,42). Fourteen studies were performed in East Asia (9,23,25-27,29-33,35,40,41), six in North America (2,10,28,34,38,42), two in Western Europe (37,39) and one in South America (36). The tumor stages of patients in most studies were stage I–IV or I–III. He (in 2015) *et al.* (33) and He (in 2019) *et al.* (32) studied stage IV lung cancer patients only. Monica *et al.* (37) studied stage III-IV patients, and Mauro *et al.* (36) used stage Ⅰ patients.

The quality of the studies was carefully assessed using the NOS, with scores ranging from 7 to 9 (*Table 2*). Overall, three studies had a score of 9, eight studies had a score of 8, and the other twelve studies had a score of 7, which led to an average score of 7.61. Detailed descriptions of these studies are summarized in Table S3.

Prognostic value of ERβ protein expression in different subcellular localizations

Because four studies reported two HRs based on the different subcellular localizations of ERβ (2,10,32,34), we performed a meta-analysis by different subcellular localizations of ERβ (overall ERβ, cytoplasmic ERβ, and nuclear ERβ) separately to ensure that the HRs of different ERβ subcellular localizations in the same study population were not pooled together (*Figure 2*). High overall ERβ expression was associated with a poorer OS (HR: 1.05; 95% CI: 1.00 to 1.10, P=0.034) than low overall ERβ expression,

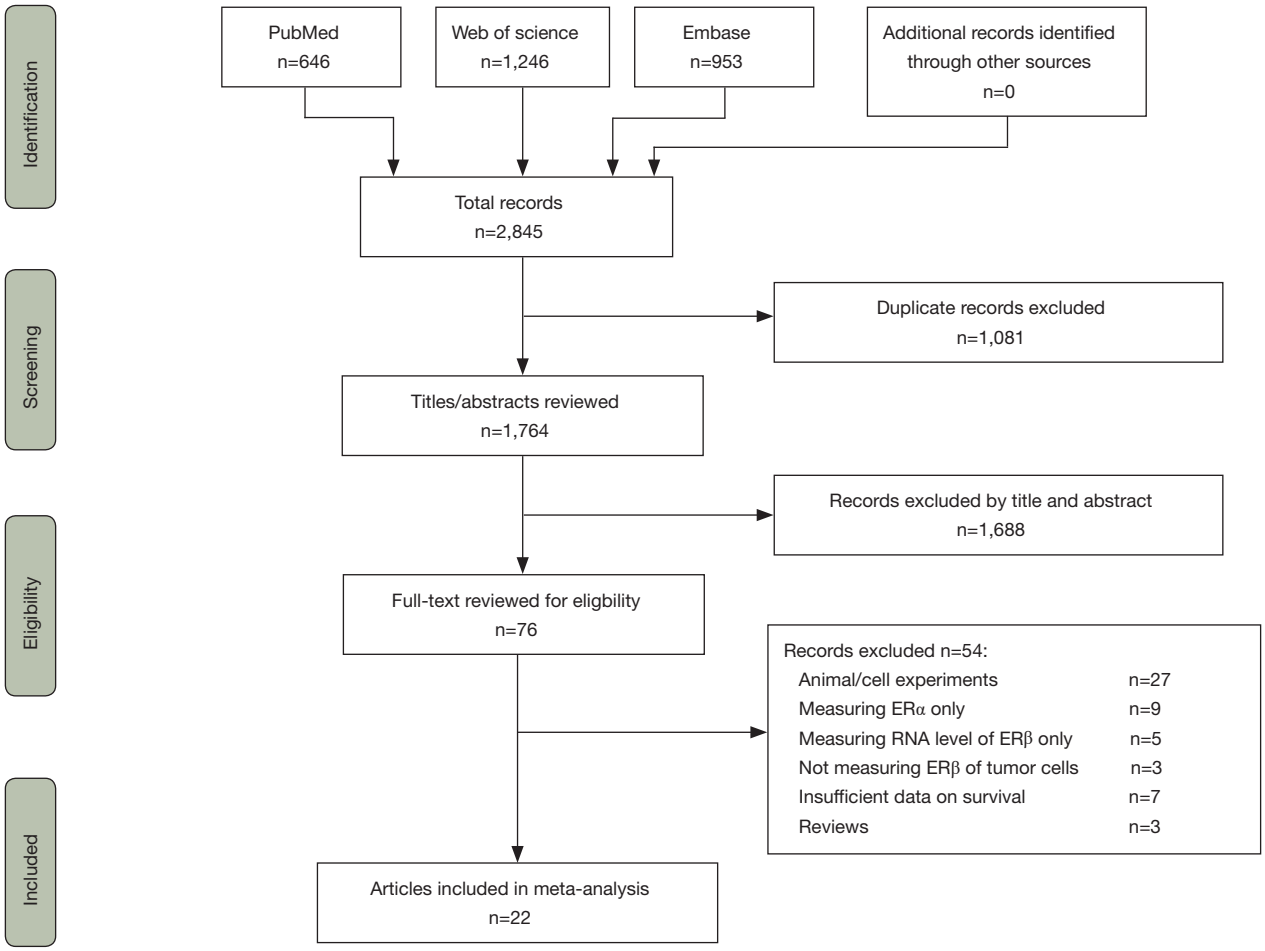

Figure 1 Search results and study selection process. ERβ, estrogen receptor beta; ERα, estrogen receptor alpha.

and there was no evidence of significant heterogeneity across the studies (I^2=0.0%, P=0.730). High cytoplasmic ERβ expression was associated with an even poorer OS than those with low expression, with a pooled HR of 1.48 (95% CI: 1.13 to 1.95, P=0.005), and moderate heterogeneity was indicated using a random-effects model (I^2=53.4%, P=0.057). Nuclear ERβ expression was not a predictor of OS, achieving a pooled HR of 1.08 (95% CI: 0.89 to 1.31, P=0.501); moderate heterogeneity was indicated using a random effects model (I^2=47.5%, P=0.018).

Subgroup analyses and sources of heterogeneity

Subgroup analyses of different histological types

ERβ is highly expressed in NSCLC and is closely related to the progression of lung adenocarcinoma; therefore, subgroup analysis was performed to investigate potential sources of heterogeneity between studies and assess the consistency of conclusions between lung adenocarcinoma patients and NSCLC patients. We divided all of the studies into two subgroups: studies specific to adenocarcinoma and studies that did not differentiate the histological types of NSCLC.

Among the eight adenocarcinoma studies, five studies included nuclear ERβ (26-29,31,32), two studies included overall ERβ (30,32), and only one study reported cytoplasmic ERβ (25). Therefore, we performed a combined analysis of the three overall ERβ and cytoplasmic ERβ studies (25,30,32) (*Figure 3A*) and concluded that patients with high expression of overall/cytoplasmic ERβ had statistically longer OS than those with low expression (HR: 1.54, 95% CI: 1.05 to 2.25). Notably, high expression of nuclear ERβ was associated with poor OS in adenocarcinoma patients, with an HR of

Table 1 Summary of included studies and patient characteristics

First author and year	Country or region	No. of patients	Male/ female	Stage and histology	Median or mean/ SD or range of age (year)	Median/range of follow to up (month)	Sample and method	ERβ subcellular localization	ERβ expression high/low
Kawai 2005	Japan	132	76/56	stage I–IV NSCLC (ADC/ SCC/other 102/28/2)	66 (38 to 81)	0.01 to 60	Tumor tissue IHC	Nuclear	67/65
Schwarz 2005	United States	216	NR	stage I–III adenocarcinoma	52.7 (±11.4)*	16 (0.01 to 60)	Tumor tissue IHC	Nuclear	128/88
Wu 2005	Taiwan	301	174/127	stage I–III NSCLC (ADC/ SCC/other 194/90/17)	63 (30 to 83)	41 (2 to 168)	Tumor tissue IHC	Nuclear	138/163
Skov 2008	Denmark	104	71/33	stage I–IIIB NSCLC (ADC/ SCC/other 40/56/8)	NR	>180	Tumor tissue IHC	Nuclear	72/32
Toh 2010	Singapore	106	63/43	stage I–IV adenocarcinoma	62 (54 to 69)	NR	Tumor tissue IHC	Overall	10/96
Mauro 2010	Argentina	57	40/18	stage IA-IB NSCLC (ADC/ SCC/other 18/33/6)	65 (47 to 83)	48 (3 to 116)	Tumor tissue IHC	Nuclear	22/35
Nose 2011	Japan	43	23/20	stage IA–IV adenocarcinoma	65.9 (45 to 85)	1 to 25	Tumor tissue IHC	Nuclear	21/22
Mah 2011	United States	60	NR	stage IA–IV NSCLC (NR)	NR	0.01 to 120	Tumor tissue IHC	Overall/ cytoplasmic	26/34
		190	NR		NR	NR	Tumor tissue IHC		NR
Stabile 2011	United States	183	92/91	stage IA–IV NSCLC (ADC/ SCC/other 103/62/18)	68 (38 to 92)	46 (0.01 to 172)	Tumor tissue IHC	Overall/ cytoplasmic	147/30
									64/105
Monica 2012	Italy	106	79/27	stage IIIA–IV NSCLC (ADC/ SCC/other 57/34/15)	66	1 to 36	Tumor tissue IHC	Nuclear	NR
Navaratnam 2012 cohort 1	Canada	79	32/47	stage I–IV NSCLC(NR)	69.2 (45.7 to 85.2)	1 to 36	Tumor tissue IHC	Nuclear	40/39
Navaratnam 2012 cohort 2	Canada	47	NR	stage I–IV NSCLC(NR)	62.8 (38.4 to 74.1)	1 to 25	Tumor tissue IHC	Nuclear	23/24
Verma[1] 2012	Japan	162	98/64	stage I–IV NSCLC (ADC/ SCC/other 120/38/4)	45 to 82)	1 to 110	Tumor tissue IHC	Cytoplasmic	62/100
Verma[2] 2012	Japan	169	103/66	stage I–IV NSCLC (ADC/ SCC/other 129/36/4)	NR	1 to 111	Tumor tissue IHC	Nuclear	148/21
He 2015	China	46	31/15	stage IV NSCLC (ADC/ SCC/other 33/13/0)	58 (40 to 80)	2 to 23	Tumor tissue IHC	Cytoplasmic	36/10
Tanaka 2016	Japan	78	40/38	stage IA–IIIB adenocarcinoma	69 (36 to 84)	66 (1 to 117)	Tumor tissue IHC	Nuclear	59/19

Table 1 (*continued*)

Table 1 (*continued*)

First author and year	Country or region	No. of patients	Male/ female	Stage and histology	Median or mean/ SD or range of age (year)	Median/range of follow to up (month)	Sample and method	ERβ subcellular localization	ERβ expression high/low
Gao 2017	China	62	NR	stage II–IV NSCLC (NR)	NR	1 to 74	Tumor tissue IHC	Overall	37/25
Ding 2018	China	126	56/70	stage IV adenocarcinoma	62 (31 to 81)	1 to 98	Tumor tissue IHC	Cytoplasmic	17/109
Yu 2018	China	140	0/140	stage I–IV adenocarcinoma	56.7*	1 to 60	Tumor tissue IHC	Nuclear	109/47
Cheng 2018	United States	813	363/450	stage IA–IIIB NSCLC (ADC/ SCC/other 465/200/148)	NR	62	Tumor tissue IHC	Cytoplasmic/ nuclear	NR
He 2019	China	201	56/145	stage IV adenocarcinoma	65 (27 to 84)	1 to 32	Tumor tissue IHC	Overall/nuclear	98/103
									99/102
Lee 2020	Korea	84	43/41	stage IA-IIIB adenocarcinoma	63.9 (±8.8)*	1 to 99	Tumor tissue IHC	Nuclear	71/13
Enwere	Canada	299	150/149	stage I–IV NSCLC (ADC/ SCC/other 162/94/43)	65 (33 to 88)	0 to 90	Tumor tissue Fluorescence IHC	Nuclear	34/264

*, mean/SD of age. NR, not reported; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinomas; IHC, immunohistochemistry; ERβ, estrogen receptor beta; SD, standard deviation.

1.36 (95% CI: 1.03 to 1.80).

For the 15 studies that did not differentiate the histological types of NSCLC (*Figure 3B*), high overall ERβ expression and high cytoplasmic ERβ expression were associated with poor OS (HR: 1.05, 95% CI: 1.00 to 1.10 and HR: 1.39, 95% CI: 1.06 to 1.83), and the result indicated no significant association between nuclear ERβ and OS (HR: 0.99, 95% CI: 0.80 to 1.22).

Subgroup analyses of other study characteristics

Subgroup analyses were also performed for geographical location and based on whether a multivariate or univariate analysis was used (*Table 3*). The results indicated that high cytoplasmic ERβ expression was related with a poorer OS than low cytoplasmic ERβ expression in the multivariate analysis group (which was also the North America study group, HR: 1.56, 95% CI: 1.28 to 1.89). These two characteristics may have contributed to the heterogeneity in cytoplasmic ERβ and nuclear ERβ groups because substantial heterogeneity was observed in the univariate analysis and the Eastern Asia and Europe study groups.

We also pooled sex-specific HRs using data from studies that reported associations separately for men and women (*Table 3*; Figure S1). The results showed that high nuclear ERβ expression may be associated with a poor OS in women (HR: 1.62, 95% CI: 1.13 to 2.32), and that nuclear ERβ expression had no prognostic value in men (HR: 0.66, 95% CI: 0.32 to 1.36).

Sensitivity analyses

We performed sensitivity analyses using a leave-one-out strategy to evaluate the source of heterogeneity in the cytoplasmic ERβ group (*Figure 2B*). When we excluded, He (in 2015) *et al.* (33), the pooled HR was 1.61 (95% CI: 1.35 to 1.92) and the heterogeneity between studies was markedly reduced (I^2=0.0%, P=0.692). When we excluded any other single study in turn, the pooled HR of the remaining studies was not substantially altered and ranged from 1.39 (95% CI: 1.06 to 1.83) to 1.47 (95% CI: 1.06 to 2.03) (*Figure 2B*), indicating that He (2015) *et al.* (33), which focused on stage IV lung adenocarcinoma only and had a relatively small sample size, may be the main source of heterogeneity in the cytoplasmic ERβ group.

Publication bias of the included studies

We performed publication bias analyses for the nuclear ERβ

Table 2 Scores in each domain of the Newcastle-Ottawa tool assessing the quality of cohort studies

Author	PMID	Representativeness	Selection of unexposed	Exposure ascertainment	Free of outcome at baseline	Selection overall	Comparability	Outcome assessment	Follow up duration	Follow up adequacy	Outcome overall	Total score
Schwartz 2005	16243798	1	1	1	1	4/4	2/2	1	1	0	2/3	8/9
Nose 2011	20615575	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Lee 2020	31718122	1	1	1	1	4/4	2/2	1	1	0	2/3	8/9
Ding 2018	30013628	1	1	1	1	4/4	1/2	1	1	1	3/3	8/9
Toh 2010	19875972	1	1	1	1	4/4	2/2	1	0	0	1/3	7/9
Tanaka 2016	27069542	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Kawai 2005	16033821	1	1	1	1	4/4	1/2	1	1	0	1/3	7/9
He 2019	31289556	1	1	1	1	4/4	1/2	1	1	1	3/3	8/9
Gao 2019	31511014	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
He 2015	26137132	1	1	1	1	4/4	1/2	1	1	1	3/3	8/9
Mah 2011	21511357	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Monica 2012	22658344	1	1	1	1	4/4	1/2	1	1	1	3/3	8/9
Navaratam 2012 cohort1	22302352	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Navaratam 2012 cohort2	22302352	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Skov 2008	17905466	1	1	1	1	4/4	2/2	1	1	1	3/3	9/9
Stabile 2011	21062926	1	1	1	1	4/4	2/2	1	1	1	3/3	9/9
Verma[1] 2012	22313325	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Wu 2005	16214508	1	1	1	1	4/4	2/2	1	1	1	3/3	9/9
Yu 2018	30250297	1	1	1	1	4/4	1/2	1	1	1	3/3	8/9
Verma[2] 2012	22982181	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Cheng 2018	29346580	1	1	1	1	4/4	2/2	1	1	0	2/3	8/9
Mauro 2010	20878128	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9
Enwere	32676313	1	1	1	1	4/4	1/2	1	1	0	2/3	7/9

Symbols: 1 = good, 0 = not adequate/unclear.

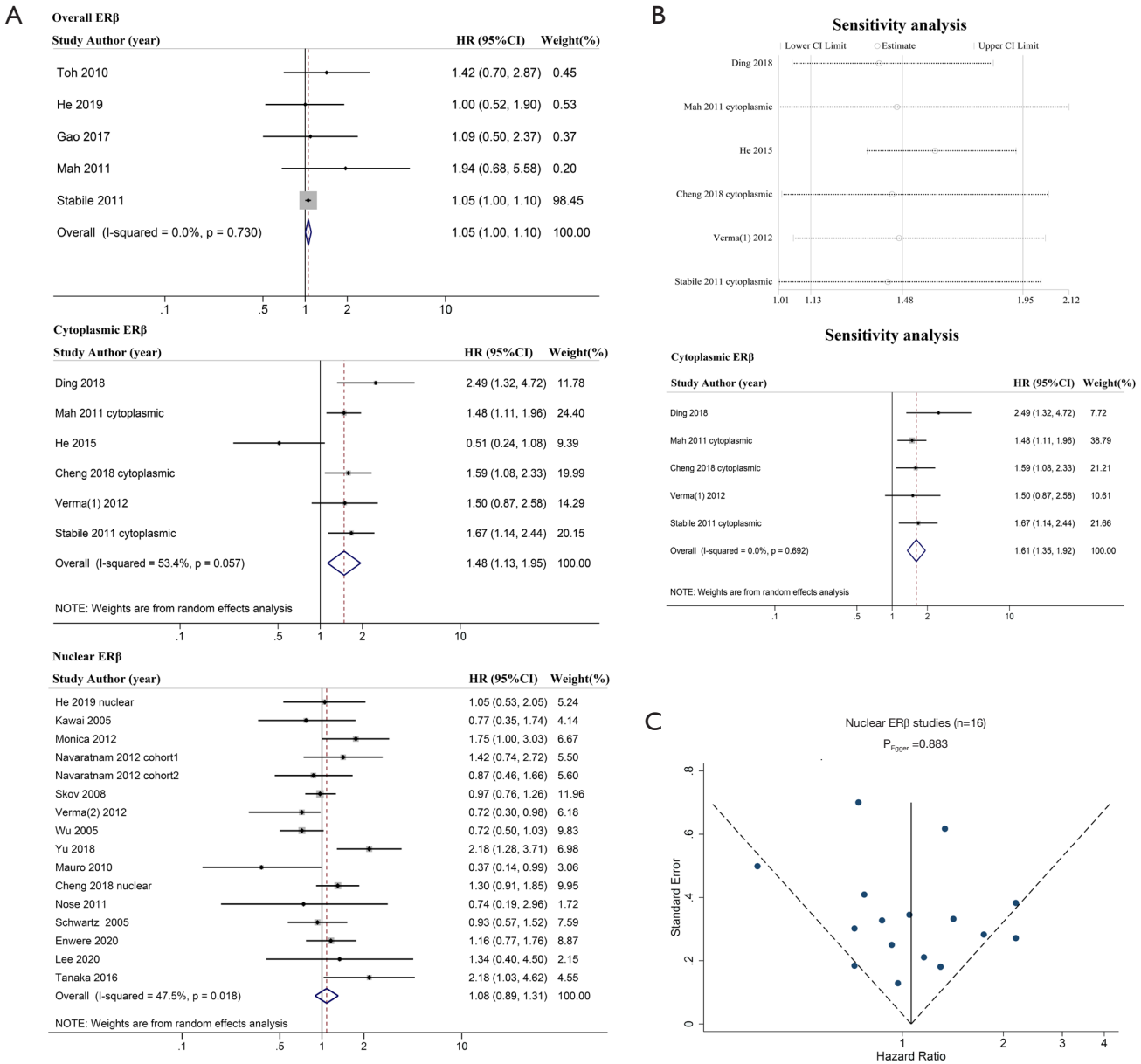

Figure 2 Forest plots of associations between ERβ protein expression and OS. (A) Effect of overall ERβ, cytoplasmic ERβ, and nuclear ERβ on OS of NSCLC (included lung adenocarcinoma-specific studies). (B) Sensitivity analyses for cytoplasmic ERβ using a leave-one-out strategy and forest plot for cytoplasmic ERβ after exclusion of He *et al.*'s (in 2015) study. (C) Funnel plot of Publication bias using Egger's test for nuclear ERβ group. HR, hazard ratio; CI, confidence interval; ERβ, estrogen receptor beta. The size of the blocks or diamonds represents the weight, and the length of the straight line represents the width of 95% CI. Each dot represents a single study in the funnel plot.

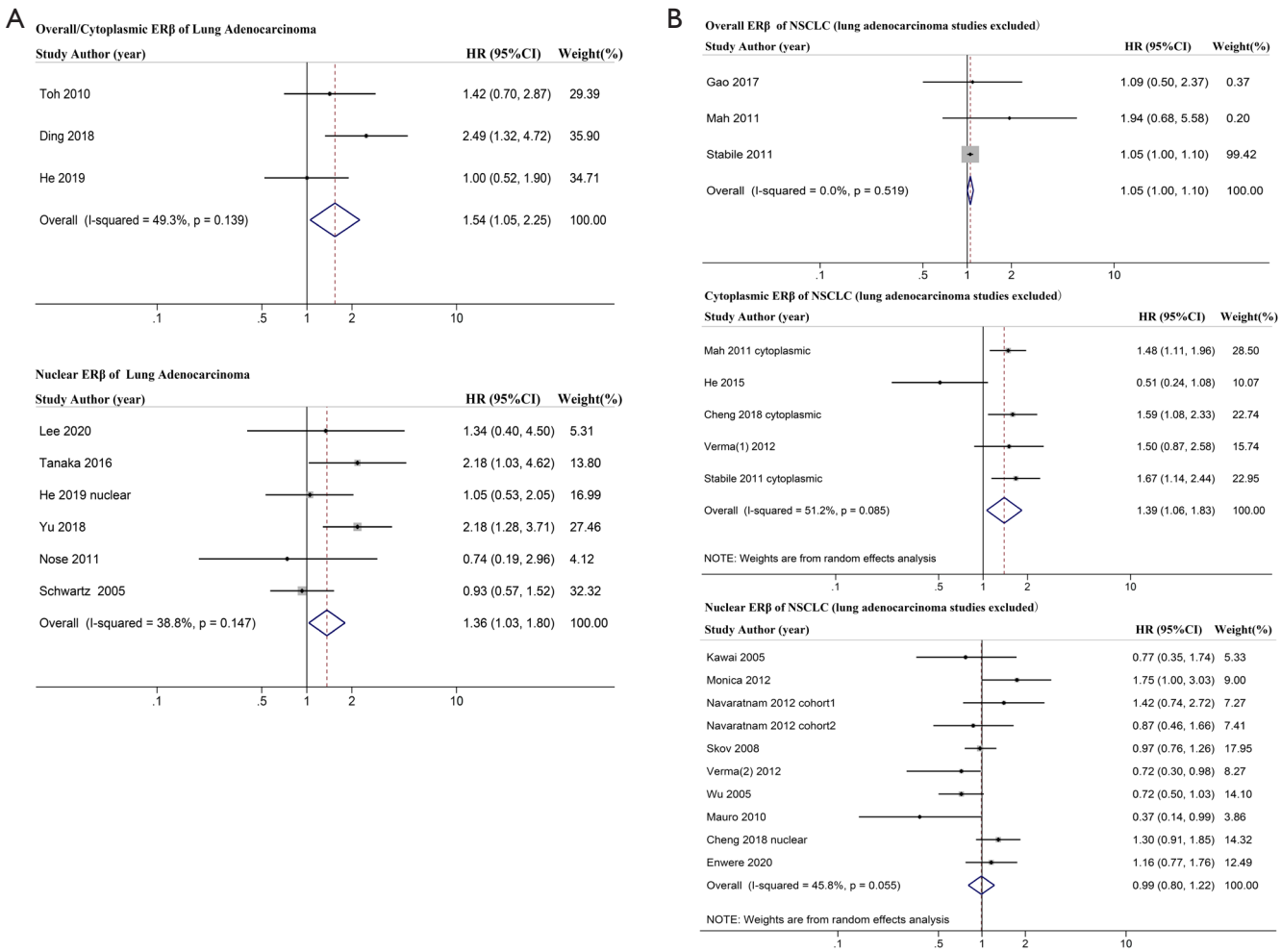

Figure 3 Subgroup analysis of associations between ERβ protein expression and OS. (A) Effect of overall/cytoplasmic ERβ and nuclear ERβ on OS of lung adenocarcinoma. (B) Effect of overall ERβ, cytoplasmic ERβ, and nuclear ERβ on OS of NSCLC (excluded lung adenocarcinoma-specific studies). The size of the blocks or diamonds represents the weight, and the length of the straight line represents the width of 95% CI. HR, hazard ratio; CI, confidence interval; ERβ, estrogen receptor beta.

group (n=16, *Figure 2C*) and NSCLC nuclear ERβ group (n=10, Figure S2). There was some evidence of publication bias in the inspection of the funnel plot, with two studies reporting standard errors (SEs) greater than those of other studies, however, the Egger's test was not significant (P=0.883 for nuclear ERβ group and P=0.616 for NSCLC nuclear ERβ group).

Survival analysis of the ERβ mRNA expression in lung adenocarcinoma

Survival analyses were performed with prognostic information from the tumor samples of TCGA-LUAD (n=526), and the mRNA expression of none of the four genes showed statistical prognostic value (*Figure 4*, Figure S3). The results from the Kaplan-Meier Plotter analysis of 719 patients with lung adenocarcinoma patients indicated a non-significant prognostic value of ERβ mRNA expression (HR: 1.23, 95% CI: 0.98 to 1.55) (*Figure 4B*). These results indicate that the ERβ mRNA expression may not be a prognostic predictor of lung adenocarcinoma.

Patient characteristics

Among the 39 patients for IHC, 23 (59.0%) were men and 16 (41.0%) were women. The clinicopathological factors of

Table 3 Analyses of hazard ratios of overall survival according to ERβ protein expression

Comparison	ERβ location	No. of studies	No. of patients	HR (95% CI)	Overall effects P value	I^2
Univariate analysis	Overall ERβ	4	460	1.23 (0.84, 1.80)	0.282	0%
	Cytoplasmic ERβ	5	683	1.35 (0.90, 2.03)	0.144	60.9%
	Nuclear ERβ	12	1427	1.06 (0.78, 1.46)	0.695	61.8%
Multivariate analysis	Overall ERβ	1	183	1.10 (1.00, 1.10)	0.039	NA
	Cytoplasmic ERβ	3	1172	1.56 (1.28, 1.89)	<0.001	0%
	Nuclear ERβ	5	1733	0.99 (0.82, 1.21)	0.207	32.2%
Eastern Asia	Overall ERβ	3	369	1.15 (0.77, 1.73)	0.498	0%
	Cytoplasmic ERβ	3	334	1.27 (0.55, 2.92)	0.572	80.2%
	Nuclear ERβ	8	1148	1.09 (0.74, 1.60)	0.655	60.1%
North America	Overall ERβ	2	243	1.05 (1.00, 1.10)	0.039	23.3%
	Cytoplasmic ERβ	3	1172	1.56 (1.28, 1.89)	<0.001	0%
	Nuclear ERβ	5	1454	1.15 (0.93, 1.42)	0.679	0%
Europe	Nuclear ERβ	2	210	1.23 (0.70, 2.18)	0.467	72.3%
Female	Nuclear ERβ	4	>239	1.62 (1.13, 2.32)	0.009	49.6%
Male	Nuclear ERβ	3	>174	0.66 (0.32, 1.36)	0.258	70.6%

I^2 represents the heterogeneity in each group. ERβ, estrogen receptor beta; HR, hazard ratio; CI, confidence interval.

the patients are shown in *Table 4*. Most of the patients were diagnosed with lung adenocarcinoma (79.5%) and eight patients were diagnosed with squamous cell lung carcinoma (20.5%). All of the patients had stage III disease, of which 27 (69.2%) had stage IIIA disease and 12 (30.8%) had stage IIIB disease.

Tissue samples for western blotting and RT-qPCR were obtained from a female patient diagnosed with adenocarcinoma and a male patient diagnosed with squamous cell carcinoma.

ERβ expression in tumor and non-tumor tissue

IHC staining showed that 32 patients (82.1%) had high ERβ expression and seven patients (17.9%) had low ERβ expression in the tumor tissues, whereas only nine patients (23.1%) had high ERβ expression in the normal adjacent tissues. The mean ERβ score in the tumor and normal adjacent tissues is 5.72 and 3.69, respectively. Overall, the IHC score of ERβ protein expression was significantly higher in the tumor tissues than in the normal adjacent tissues (P<0.0001) (*Figure 5*).

In the lung adenocarcinoma gene expression profile dataset, 526 cases were tumor tissues (all from TCGA-LUAD) and 347 cases were normal tissues (288 from GTEx-LUNG and 59 from TCGA-LUAD). ERβ mRNA expression was not significantly different between tumor tissues and normal tissues (*Figure 4C,D*; Table S4). To verify the differential expression in the above dataset, four GEO datasets (Table S5) of lung adenocarcinoma were selected. The heatmap drawn from the logFC and p-values showed that the relatively stable ERβ mRNA expression (*Figure 4E*).

To further validate the inconsistency between ERβ protein and mRNA expression, we analyzed the protein and mRNA expression in paired clinical tissues from two patients (*Figure 5*). Consistent with the IHC results, Western blotting of paired tissues from the two patients (*Figure 6*) also showed that the protein expression of ERβ was more prominent in the NSCLC tissues than in the normal adjacent tissues (*Figure 6A,C*). However, the corresponding ERβ mRNA expression of one of the two patients showed no significant difference between the cancerous tissues and the normal adjacent tissues; the other patient showed even lower ERβ mRNA expression in the cancerous tissues than in the normal adjacent tissues (*Figure 6B,D*).

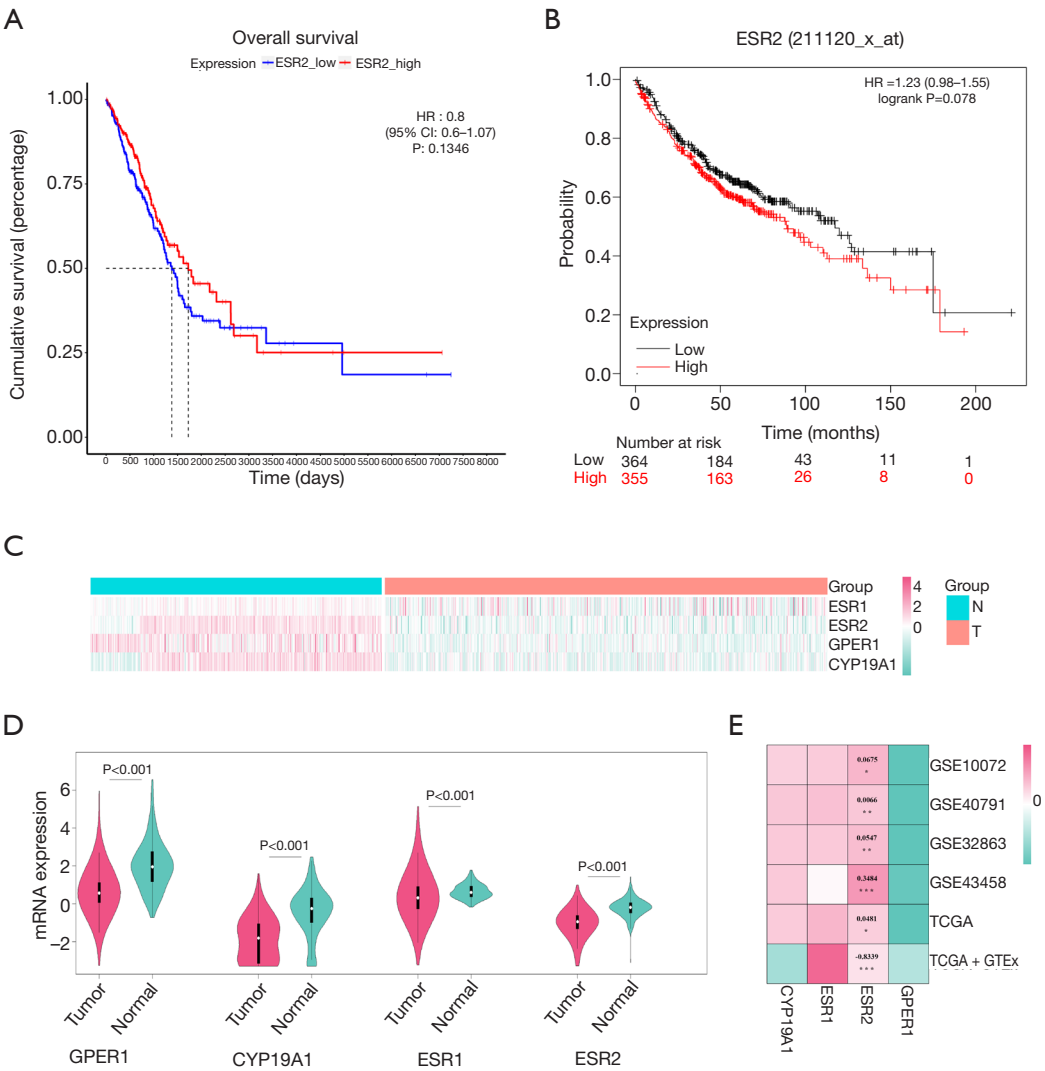

Figure 4 Survival analysis and differential expression analysis from public databases. (A,B) The Kaplan-Meier curves showing associations between ERβ mRNA expression and overall survival in lung adenocarcinoma. (A) Survival analysis from TCGA-LUAD. (B) Survival analysis from Kaplan-Meier Plotter. (C,D,E) The identification of differentially expressed genes from TCGA-LUAD, GTEx-LUNG, and 4 GEO datasets. (C) Heatmap of differential expression analysis for 4 target genes in the training dataset. (D) Violin plot indicating the expression of 4 target genes between tumor and normal tissues in the training dataset. (E) Heatmap of differential expression analysis for 4 target genes in 4 GEO validation datasets. Every cell representing differentially expressed genes in each dataset is labeled with logFC value and P value (*P<0.05, **P<0.01, ***P<0.001). ESR2, ERβ, estrogen receptor beta; HR, hazard ratio.

Effect of METTL3 knockdown on ERβ expression

To investigate the possible role of METTL3 in the regulation of ERβ expression, we measured the protein and mRNA levels of ERβ after knockdown of METTL3 in the A549 cell line. The validation of the efficiency of siRNA_METTL3 by RT-qPCR and western blotting is shown in *Figure* 7. Interestingly, we found that knockdown of METTL3 had a minor effect on ERβ mRNA levels, whereas it resulted in a significant decrease in ERβ protein levels. These results indicated that METTL3 might regulate the translation process of ERβ and cause high ERβ protein expression level in the presence of low ERβ mRNA expression level.

Table 4 Clinicopathological characteristics of NSCLC cases (n=39)

Clinicopathological variables	Patients (n=39), n (%)
ERβ expression in tumor tissue	
High	32 (82.1)
Low	7 (17.9)
ERβ expression in normal adjacent tissue	
High	9 (23.1)
Low	30 (76.9)
Gender	
Male	23 (59.0)
Female	16 (41.0)
Histology	
Adenocarcinoma	31 (79.5)
Squamous cell	8 (20.5)
Stage	
IIIA	27 (69.2)
IIIB	12 (30.8)

NSCLC, non-small cell lung cancer; ERβ, estrogen receptor beta.

Discussion

Summary of the main results

The present systematic review and meta-analysis summarized data from 23 independent studies to clarify the association between ERβ protein expression and lung cancer survival. We demonstrated that high overall ERβ and cytoplasmic ERβ were negatively associated with the OS of NSCLC, and this association was consistent in lung adenocarcinoma. High nuclear ERβ expression had no effect on NSCLC OS, however, it had a negative effect on OS in lung adenocarcinoma and the female population. Our results conflict with those of two previous meta-analyses (11,12). This conflict may be due to the following reasons. First, we included lung adenocarcinoma-specific studies additional newly published NSCLC studies via a systematic literature search. Second, we excluded studies of ERβ at the mRNA level to ensure that all of the retrieved studies evaluated ERβ protein levels. Third, we extracted additional and detailed prognosis information from each study and performed comprehensive analyses using different subcellular localizations. In addition to survival analysis based on protein level, we performed bioinformatics analysis and found that ERβ mRNA was not predictive of lung adenocarcinoma survival, which is consistent with the conclusion of a meta-analysis published in 2015 (14). Therefore, our results revealed that the prognostic value of ERβ protein and mRNA expression is different.

Public database analysis revealed that ERβ mRNA was not highly expressed in lung adenocarcinoma tissues, when compared with that in normal lung tissues. Our validation in paired tissues of patients who underwent surgical resection also showed that ERβ mRNA was not highly expressed in NSCLC. All this evidence from mRNA level is inconsistent with the high ERβ protein level in tumor tissues proved by our IHC results and western blotting results from paired tissues of patients. To further explore the possible mechanism of this inconsistency, we knocked down METTL3 in an NSCLC cell line and found that protein—but not mRNA—expression of ERβ was downregulated.

We found that ERβ protein was highly expressed in tumor tissues, when compared with that in non-tumor tissues; however, ERβ mRNA was undifferentiated. We propose that ERβ mRNA is regulated by post-transcriptional modifications. Post-transcriptional modification of mRNA regulates protein expression in many tumors (43). For example, Choe *et al.* reported that mRNA circularization by METTL3-eIF3h enhances translation and promotes oncogenesis (44). Liu *et al.* reported that m^6A methylation regulated CTNNB1 (which encodes β-catenin) to promote the proliferation of hepatoblastoma (45). Arango *et al.* reported that N4-acetylcytidine (ac4C) is an mRNA modification that improves translation efficiency (46). We propose that the aberrant post-translational modification of m^6A mediated by METTL3 in NSCLC tissues enhanced the subsequent translation process, which in turn resulted in the inconsistency between ERβ protein and mRNA levels. Therefore, further studies are required for us to explore the specific mechanism by which METTL3 regulates the expression of ERβ in NSCLC progression.

The downregulation of ERβ mRNA in tumor tissues was reported by Read *et al.* in 1989, in which the estrogen signaling pathway in MCF-7 cells was activated after estrogen stimulation; however, the mRNA level of ERβ was decreased, which may be a negative feedback regulation (47). Another study reported that, in astrocyte tumors, patients with high E2 levels had low ERα mRNA levels and poor prognosis (48). Therefore, it is possible that the more active the ER signaling pathway, the lower the ER mRNA level.

Further studies are also required for the different effects of nuclear and cytoplasmic ERβ in NSCLC, particularly

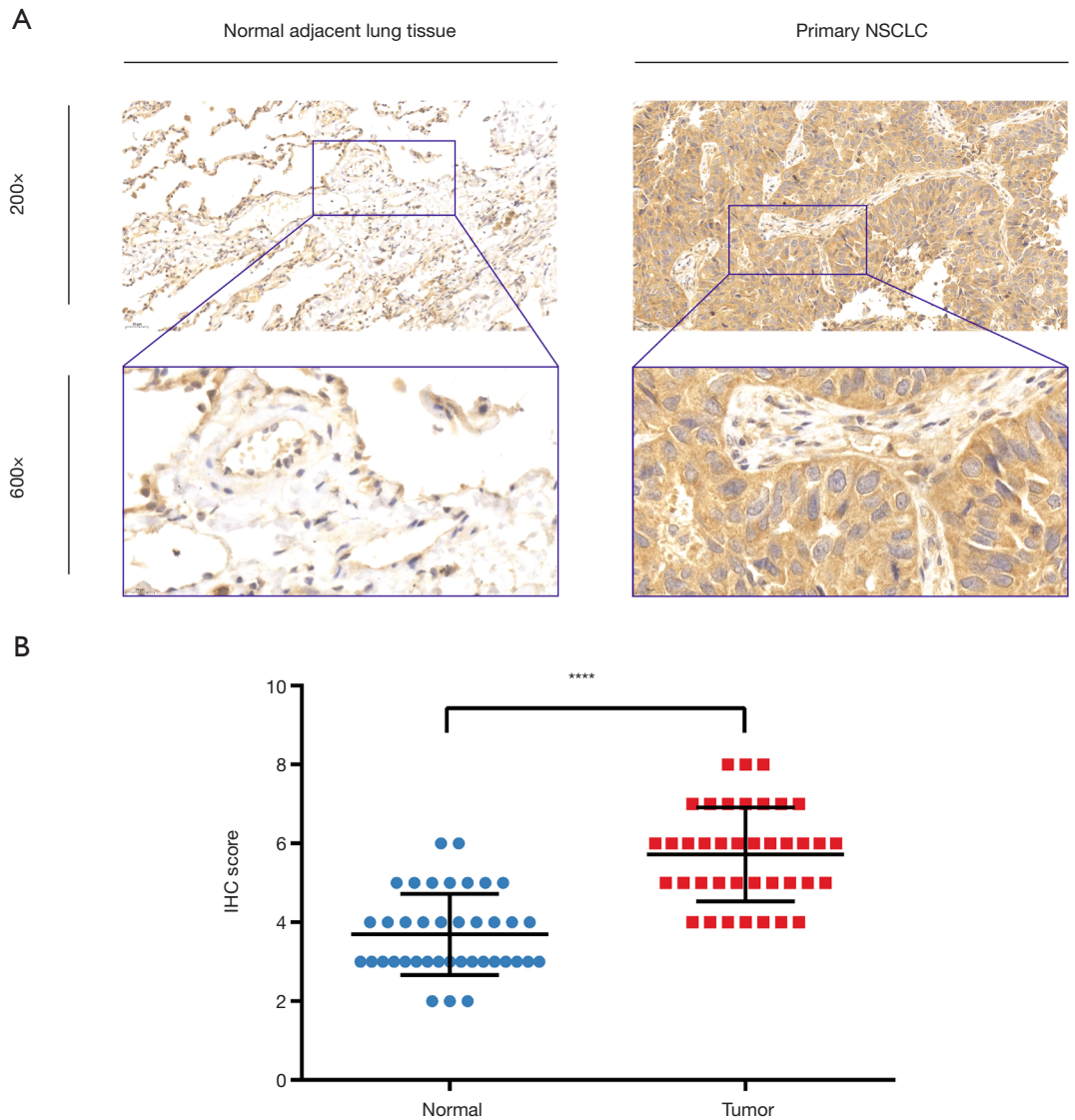

Figure 5 Expression of ERβ and evaluated via immunohistochemical analyses of primary NSCLC tissue and normal adjacent lung tissue. (A) Representative IHC staining images from paired human primary NSCLC tissue and their normal adjacent lung tissue for ERβ. (B) Quantification data of IHC score for 39 paired primary NSCLC tissues and normal adjacent lung tissues. ****P<0.0001, *t*-test. ERβ, estrogen receptor beta; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

lung adenocarcinoma. ERβ of different subcellular localizations exerts its influence via genomic and non-genomic signaling. Zhang *et al.* reported that mitochondrial ERβ may be involved in the inhibition of apoptosis by disrupting the interactions of Bad-Bcl-XL and Bad-Bcl2 in NSCLC (49). Our team reported that ERβ up-regulates the expression of IL-6 to promote the progression of lung adenocarcinoma (7). ERβ and insulin-like growth factor 1 synergistically promote the development of lung adenocarcinoma (17). Nuclear ERβ is a transcription factor in the genomic pathways (50), and it may increase lncRNA-MALAT1 (MALAT1) expression by directly binding to the estrogen response elements located on the promoter of MALAT1 (31), leading to enhanced tumor cell proliferation, invasion, and metastasis and poor prognosis in lung adenocarcinoma patients. However, further research is needed to explain why cytoplasmic ERβ leads to a poorer prognosis than nuclear ERβ.

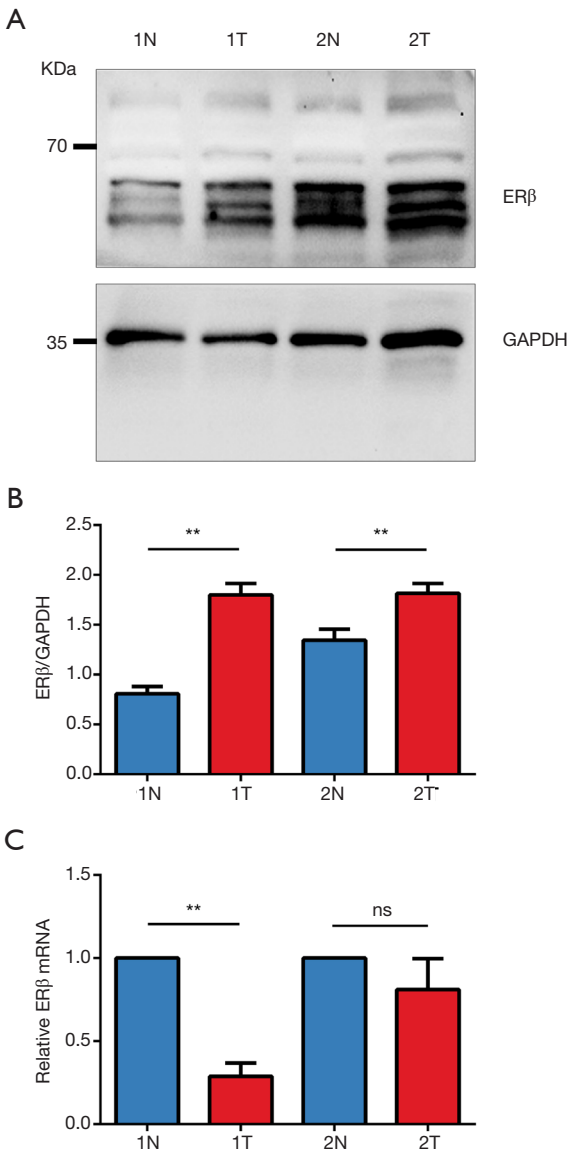

Figure 6 Protein and mRNA expression of ERβ validated in 2 pairs of NSCLC and normal adjacent samples. (A) ERβ protein expression examined by western blot. (B) ERβ mRNA expression examined by RT-qPCR. N, normal adjacent tissue; T, tumor tissue. **P<0.01, ns indicates no significant differences; *t*-test.

Limitations

Several limitations of our meta-analysis were noted. The effect sizes in some of the retrieved studies were not available and were estimated from the Kaplan-Meier survival curve. Heterogeneity existed between the study populations of retrieved studies because some studies focused on a female population, high aromatase population or EGFR wild-type population only (29,31,34). Because only five studies mentioned that they used antibodies that only detect ERβ1 and the other studies did not use ERβ isoform-specific antibodies (Table S3), this meta-analysis could not distinguish between the five isoforms of ERβ. Finally, the semi-quantitative IHC method relies on the experience of technicians and presents discrepancies between antibodies and cut-off points (Table S3) (51). These limitations may create doubt on the reliability of the summary estimates; however, our results were generally stable.

Considering the differentiation of histological types for the first time, our meta-analysis provides the latest evidence to systematically evaluate the prognostic value of cytoplasmic and nuclear ERβ in NSCLC. Our bioinformatics analysis provides additional evidence for the limited prognosis data of ERβ mRNA and proposes a new contradiction that deserves further investigation of the expression of ERβ at the protein and mRNA levels. Our experiments confirmed the protein and mRNA expression in clinical samples and improved the reliability of this inconsistency. We placed an objective hypothesis on this inconsistency, and conducted a preliminary investigation in lung cancer cell lines, which made the research highly comprehensive. In future studies, we will explore the relationship between the m6A modification and the estrogen signaling pathway in NSCLC. In addition, the question of whether METTL3 modification can enhance the translational efficiency of ERβ in promoting the progression of NSCLC will be elucidated further.

Conclusions

In conclusion, this study indicated that the high expression of ERβ protein was associated with poor outcomes in NSCLC, particularly lung adenocarcinoma, and that ERβ mRNA expression had no evident effect on lung adenocarcinoma survival. ERβ protein is highly expressed in NSCLC tissues, whereas ERβ mRNA is not. The METTL3-m^{6}A module might regulate the process of ERβ translation and cause NSCLC progression. Our results contribute to the evaluation of prognosis and clinical decisions for NSCLC. Further biological experiments are required to elucidate the specific mechanism underlying the role of m^{6}A modification of ERβ in NSCLC.

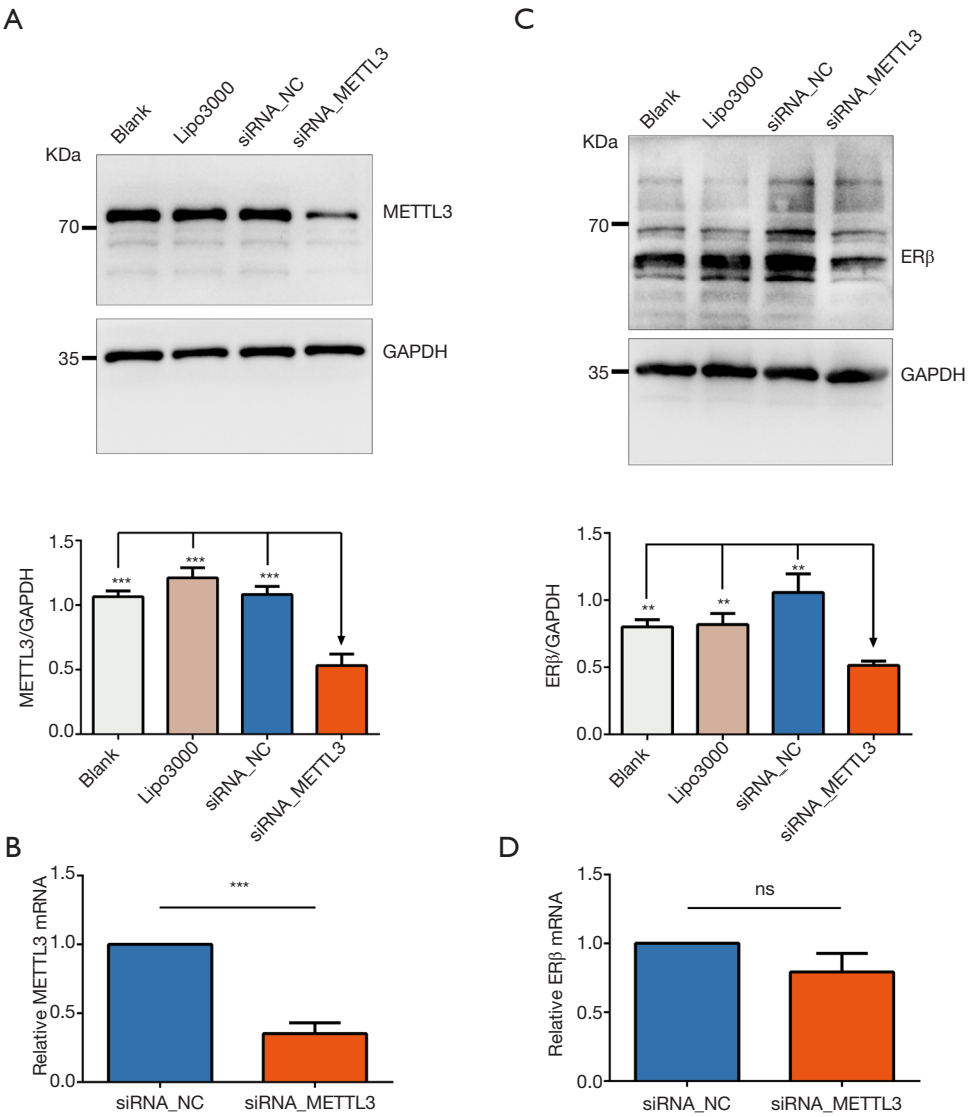

Figure 7 Expression of METTL3 and ERβ after METTL3 knockdown in A549 cells. (A) Verification of METTL3 knockdown by western blot. (B) Verification of METTL3 knockdown by RT-qPCR (C) Western blot was used to detect ERβ protein level after METTL3 knockdown. (D) RT-qPCR was used to detect ERβ mRNA level after METTL3 knockdown. **P<0.01, ***P<0.001, ns indicates no significant differences; *t*-test.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics or Institutional Review Board of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki (as revised in 2013).

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