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Reviewer A

Comment 1: The authors should state whether the analysis can distinguish between the 5 isoforms of ER beta. Isoform 1 is the full-length protein and the other forms (2-5) are smaller proteins that do not have intrinsic activity of their own, but can heterodimerize with full length ER beta 1 to produce activity. If the smaller isoforms are more abundant (at either the mRNA or protein level) this could contribute to lack of effect on survival, since activity would be dependent on how much full-length form is present. Excess amount of isoforms 2-5 might not produce any effect. Do the antibodies used only detect ER beta 1 in the different studies? And do the mRNA analyses distinguish different isoform transcripts?

Reply 1: We gratefully thank you for the precious time in making constructive remarks. We summarized the antibodies used in different studies of our meta-analysis in Table S4. Five studies mentioned they used antibodies which only detect ERβ 1, and the other studies did not used ERβ 1 isoform-specific antibodies. None of the included studies referred to isoforms 2-5. Therefore, according to the actual situation of included studies in this meta-analysis, we could not distinguish the between the 5 isoforms of ERβ. However, you gave us a very inspiring direction to explore.

For mRNA analyses in TCGA and GEO datasets, sequencing only targeted full-length mRNA of ERβ. In RT-qPCR, we used primer which matches the longest transcript variant of ERβ (Table S3). Therefore, we think it cannot distinguish different isoform transcripts.

Our team also paid attention to isoforms 1,2,5 of ERβ [(1)] and applied for NSFC [82072593] on this topic. However, we will further explore in the experiments for prognosis and expression patterns of other ERβ isoforms in lung cancer.

As you mentioned, if small isoforms (ERβ 2–5) are more abundant than the full-length form of ERβ (isoform 1), the negative effect of the latter on survival would only be marginally evident and the results would be biased. Therefore, we modified our text in the *Limitations* section as advised that because most of the studies did not use ERβ isoform-specific antibodies, this meta-analysis cannot distinguish between the five isoforms of ERβ. Thanks again for your valuable comment.

Table S3 Summary of primers used in this study

Gene Description	Species	PrimerBank ID	NCBI GeneID	Sequence (5'–>3')	Length	Tm	Location
ESR2	Human	333609292c1	2100	Forward Primer: AGCACGGCTCCATATACATACC	22	61.4	77-98
				Reverse Primer: TGGACCACTAAAGGAGAAAGGT	22	60.4	275-254
GAPDH	Human	378404907c1	2597	Forward Primer: GGAGCGAGATCCCTCCAAAAT	21	61.6	108-128
				Reverse Primer: GGCTGTTGTCATACTTCTCATGG	23	60.9	304-282

ESR2: ERβ, estrogen receptor beta; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Table S4 (partial content)

Author (year)	ERβ Antibody
Kawai 2005	H-150, Santa Cruz Biotechnology,1:100 dilution in PBS
Schwarz 2005	mouse anti-ERβ-1 monoclonal antibody–MCA1974S (Serotec, Oxford, United Kingdom)
Wu 2005	BioGenex, 1:100
Skov 2008	Oestrogen Receptor Clone PPG5/10, Code M7292, Dako Cytomation, Denmark
Toh 2010	Oestrogen Receptor Clone PPG5/10, Dako Cytomation, Denmark 1:100
Mauro 2010	Chicken polyclonal antibody
Nose 2011	H-150 (Biotechnology,Santa Cruz, CA) diluted 1:10
Mah 2011	mouse anti-ERβ-1 monoclonal antibody (clone PPG5/10, product #MCA1974ST, AbDSerotec, Raleigh, NC)
Stabile 2011	mouse anti-ERβ-1 monoclonal antibody MCA1974ST, AbD Serotec, Raleigh, NC
Monica 2012	mouse anti-ERβ (clone PPG5/10, Dako), dilution,1:50
Navaratnam 2012	monoclonal, 14C8, Genetex,TX, USA
Verma(1)2012	clone 14C8; GeneTex Inc., San Antonio, TX,1:50
Verma(2)2012	clone 14C8; GeneTex Inc., San Antonio, TX,1:50
He 2015	from Beijing Bioss Biosynthesis Biotech-nology Co., Ltd., (Beijing, China)
Tanaka 2016	clone14C8 GeneTex, CA, USA ,1:200
Gao 2017	ERβ (B-1) Santa Cruz sc-390243 1: 500
Ding 2018	mouse monoclonal antibody 14C8 (cat.no.ab288,Abcam, Cambridge, UK) 1: 100
Yu 2018	Abcam 288#14C8
Cheng 2018	PPG5/10 (ERβ-1 isoform specific) AbD Serotec MCA1974ST
He 2019	mouse monoclonal anti-human ERβ1 antibody PPG5/10 (cat.no. M7292, Dako) 1:50;
Lee 2020	clone 14C8, Abcam, Cambridge,UK 1:100,
Enwere	mouse monoclonal, clone PPG5/10, 1:500, Abcam, Cambridge, MA, USA)

Changes in the text: (Page 17, line 406-409): 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 S4), this meta-analysis could not distinguish between the five isoforms of ERβ.

Comment 2: The authors should acknowledge and discuss that there is a considerable literature about the down regulation of ER mRNA in the presence of estrogen. Since lung tumors are known to express aromatase, there can be local estradiol in the tumor microenvironment that could be stimulating ER signaling, and this would down regulate the mRNA. (Example Read et al 1989, Molec Endocrinol). Thus the more active the ER protein is in signaling, the lower the mRNA could be, and this could explain why high mRNA levels do not correlate with poor survival.

Reply 2: We appreciate for your valuable comment. As Read et al. reported in 1989, the estrogen signaling pathway in MCF-7 cells was activated after estrogen stimulation; however, the mRNA level of ERβ was decreased [(2)], which may be a negative feedback regulation. We noticed that another study reported that patients with high E2 levels had low ERα mRNA levels and poor prognosis in astrocyte tumors [(3)]. Therefore, the downregulation of ER mRNA in the presence of active ER signaling pathway could be explained by the negative feedback regulation. We discussed the possibility of this mechanism in the *Discussion* section as advised. Thank you for your nice suggestion.

Changes in the text: (Page 16, line 384-389): 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 [(2)]. Another study reported that, in astrocyte tumors, patients with high E2 levels had low ERα mRNA levels and poor prognosis [(3)]. Therefore, it is possible that the more active the ER signaling pathway, the lower the ER mRNA level.

References:
[1]. Liu Z, Liao Y, Tang H, et al. The expression of estrogen receptors beta2, 5 identifies and is associated with prognosis in non-small cell lung cancer. Endocrine 2013;44:517-24.
[2]. Read LD, Greene GL, Katzenellenbogen BS. Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol Endocrinol 1989:3(2): 295-304.
[3]. Dueñas Jiménez JM, Candanedo Arellano A, Santerre A, et al. Aromatase and estrogen receptor alpha mRNA expression as prognostic biomarkers in patients with astrocytomas. J Neurooncol 2014:275-84.

Reviewer B

Major points:

Comment 1: The authors reported in Introduction line108-109 "also the search for therapeutic targets in NSCLC". I could not find any contents about novel therapeutic targets and therapy.

Reply 1: Thank you so much for your careful check. Our original intention was to express that this study could provide the possibility of anti-estrogen therapy for lung cancer, but we failed to express it correctly. We feel sorry for our carelessness. We deleted this sentence and replaced it with a more specific one.

Changes in the text: (Page 4, line 85-86): We provided insights into not only ERβ expression profiles, but also the possibility for anti-estrogen therapy in NSCLC.

Comment 2: Immunohistochemical analyses. How did the authors decide the criteria "A total score ≧5 was defined as high expression, and a score ≦4 was defined as low expression"? For example, it should be referred to the evaluation of ERβ in breast cancer.

Reply 2: We gratefully appreciate for your valuable suggestion. According to your suggestion, we cited two related references in the revised manuscript [(1,2)]. In the two references, the ERβ expression of the tumor was categorized into negative or weak expression when the score was ≦4, and strong expression when the score was ≧5. Thank you so much for your careful check.

Changes in the text: (Page 8, line 190-191): We added "These criteria were based on the evaluations reported by Nose et al. and Kawai et al [(1,2)]."

Comment 3: About Subgroup analyses and sources of heterogeneity. It makes no sense to compare adenocarcinoma patients with NSCLC patients without knowing the proportion of Adenocarcinoma in NSCLC. Probably it seems that there are many cases of adenocarcinoma.

Reply 3: We gratefully thanks for the precious time you spent making constructive remarks. We totally understand the reviewer's concern. It is important to know the

proportion of adenocarcinoma in NSCLC. Therefore, we collected specific information for all the studies included in the meta-analysis, including the proportion of lung adenocarcinoma in each study (Table 1, Table S4). To reduce study heterogeneity, we analyzed lung adenocarcinoma studies separately in subgroup analysis (Figure 3). Thanks again for your valuable comment.

Table 1 (partial content)

Author and Year	Stage and Histology
Kawai 2005	stage I-IV NSCLC (ADC/SCC/other 102/28/2)
Schwarz 2005	stage I-III adenocarcinoma
Wu 2005	stage I-III NSCLC (ADC/SCC/other 194/90/17)
Skov 2008	stage I-IIIB NSCLC (ADC/SCC/other 40/56/8)
Toh 2010	stage I-IV adenocarcinoma
Mauro 2010	stage IA-IB NSCLC (ADC/SCC/other 18/33/6)
Nose 2011	stage IA-IV adenocarcinoma
Mah 2011	stage IA-IV NSCLC (NR)
Stabile 2011	stage IA-IV NSCLC (ADC/SCC/other 103/62/18)
Monica 2012	stage IIIA-IV NSCLC (ADC/SCC/other 57/34/15)
Navaratnam 2012 cohort 1	stage I-IV NSCLC(NR)
Navaratnam 2012 cohort 2	stage I-IV NSCLC(NR)
Verma(1)2012	stage I-IV NSCLC (ADC/SCC/other 120/38/4)
Verma(2)2012	stage I-IV NSCLC (ADC/SCC/other 129/36/4)
He 2015	stage IV NSCLC (ADC/SCC/other 33/13/0)
Tanaka 2016	stage IA-IIIB adenocarcinoma
Gao 2017	stage II-IV NSCLC (NR)
Ding 2018	stage IV adenocarcinoma
Yu 2018	stage I-IV adenocarcinoma
Cheng 2018	stage IA-IIIB NSCLC (ADC/SCC/other 465/200/148)
He 2019	stage IV adenocarcinoma
Lee 2020	stage IA-IIIB adenocarcinoma
Enwere	stage I-IV NSCLC (ADC/SCC/other 162/94/43)

Table S4 (partial content)

Author (year)	Adenocarcinoma (%)
Kawai 2005	77.3
Schwarz 2005	100
Wu 2005	64.5
Skov 2008	38.5
Toh 2010	100
Mauro 2010	31.6
Nose 2011	100
Mah 2011	NR
Stabile 2011	59
Monica 2012	53.8
Navaratnam 2012	NR
Verma(1)2012	74.1
Verma(2)2012	76.3
He 2015	71.7
Tanaka 2016	100
Gao 2017	NR
Ding 2018	100
Yu 2018	100
Cheng 2018	57.2
He 2019	100
Lee 2020	100
Enwere	54.2

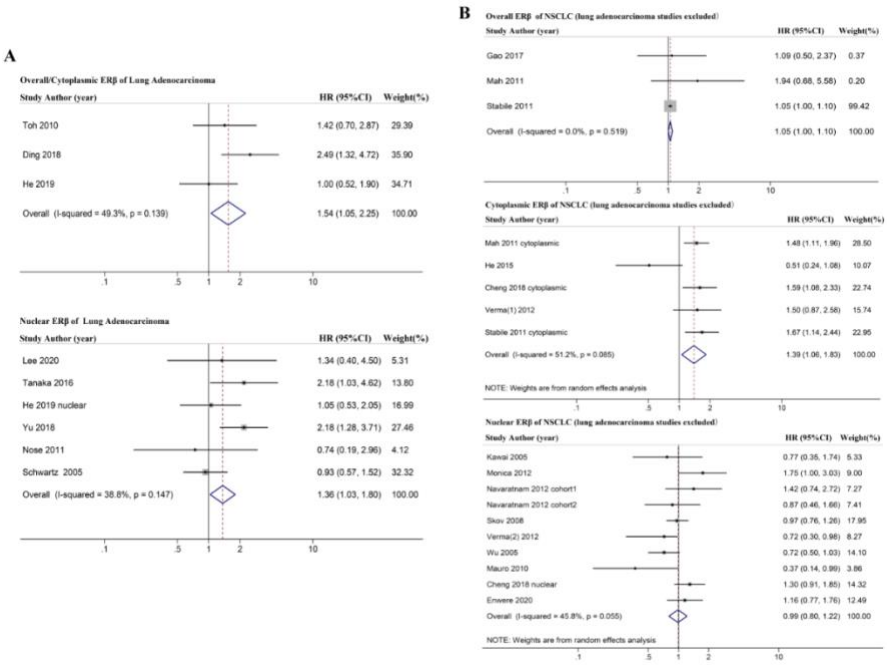

Figure 3 Subgroup analysis of associations between ERβ protein expression and OS. **(A)** Effect of overall/cytoplasmic ERβ and nuclear ERβ on OS of lung adenocarcinoma. **(B)** Effect of overall ERβ, cytoplasmic ERβ, and nuclear ERβ on OS of NSCLC (excluded lung adenocarcinoma-specific studies). HR: 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

Changes in the text: We checked the contents in Table 1 and Table S4 to ensure that they provided specific information on the proportion of adenocarcinoma in NSCLC.

Comment 4: About 4.2 Limitations

Regarding antibodies and cut-off points, it is most important problem to be solved. If the author is doing a systematic review and meta-analysis, should clarify this point.

Reply 4: We totally understand the reviewer's concern. The antibodies used in different studies are important factors. As a result, we made detailed statistics on the types of

antibodies and cut-off points used in each included literature in Table S4. Thanks again for your valuable comment.

Table S4 (partial content)

Author (year)	ERβ Antibody	ERβ Positive Cut-off Definition
Kawai 2005	H-150, Santa Cruz Biotechnology, 1:100 dilution in PBS	The proportion and intensity scores for total score, score 2-8
Schwarz 2005	mouse anti-ERβ-1 monoclonal antibody–MCA1974S (Serotec, Oxford, United Kingdom)	Samples with at least weak (1+) staining in ⩾ 10% of tumor cells
Wu 2005	BioGenex, 1:100	Moderate-to-strong nuclear staining of more than 50% of the neoplastic cells
Skov 2008	Oestrogen Receptor Clone PPG5/10, Code M7292, Dako Cytomation, Denmark	At least weak staining in more than 10% tumor cells
Toh 2010	Oestrogen Receptor Clone PPG5/10, Dako Cytomation, Denmark 1:100	At least one + staining in ⩾ 10% of tumor cells
Mauro 2010	Chicken polyclonal antibody	⩾ 5% tumor cells positive
Nose 2011	H-150 (Biotechnology, Santa Cruz, CA) diluted 1:10	5~8 score
Mah 2011	mouse anti-ERβ-1 monoclonal antibody (clone PPG5/10, product #MCA1974ST, AbDSerotec, Raleigh, NC)	[(3x)+(2y)+(1z)]//100 where x, y, and z are % staining at intensity 3, 2, and 1, respectively, ····57th percentile for overall ERβ; higher than median levels for cytoplasmic ERβ
Stabile 2011	mouse anti-ERβ-1 monoclonal antibody MCA1974ST, AbD Serotec, Raleigh, NC	Score > 7 for cytoplasmic ERβ and total ERβ
Monica 2012	mouse anti-ERβ (clone PPG5/10, Dako), dilution, 1:50	8-12 score
Navaratnam 2012	monoclonal, 14C8, Genetex, TX, USA	⩾ median IHC score
Verma(1)2012	clone 14C8, GeneTex Inc, San Antonio, TX, 1:50	⩾ 10% tumour cells positive
Verma(2)2012	clone 14C8, GeneTex Inc, San Antonio, TX, 1:50	⩾ 10% positive results
He 2015	from Beijing Bioss Biosynthesis Biotech-nology Co., Ltd., (Beijing, China)	NR
Tanaka 2016	clone14C8 GeneTex, CA, USA, 1:200	Score 1+/2+/3+
Gao 2017	ERβ (B-1) Santa Cruz sc-390243 1:500	⩾ median value of score
Ding 2018	mouse monoclonal antibody 14C8 (cat.no ab288, Abcam, Cambridge, UK) 1: 100	>10% of tumor cells exhibited specific, positive staining in the nucleus or cytoplasm with at least 1+ staining
Yu 2018	Abcam 288#14C8	NR
Cheng 2018	PPG5/10 (ERβ-1 isoform specific) AbD Serotec MCA1974ST	Quartile 4 vs 1 of formula 1*(% cells 1+)+2*(% cells 2+)+3*(% cells 3+) with the weighted average of percent positivity values
He 2019	mouse monoclonal anti-human ERβ1 antibody PPG5/10 (cat.no. M7292, Dako) 1:50;	Total ERβ score > 9, nuclear ERβ score > 6
Lee 2020	clone 14C8, Abcam, Cambridge, UK 1:100	Score 3-8
Enwere	mouse monoclonal, clone PPG5/10, 1:500, Abcam, Cambridge, MA, USA)	HALO score

Changes in the text:

(Page 17, line 409-410): We cited **Table S4** after the sentence "Finally, the semi-quantitative IHC method relies on the experience of technicians and presents discrepancies between antibodies and cut-off points."

Minor points:

Comment 5: Line 82, Please correct "ERβin".

Please check line 148 "overall survival" as the endpoint for our meta-analysis because "OS" is widely used ---

Line 409-410, Please correct "tissue tissues"

Reply 5: Thank you so much for your careful check. The mistakes have been corrected in the revised manuscript. We feel sorry for our carelessness.

Changes in the text:

(1) (Page 3, line 67): "ERβin" was replaced with "ERβ in".

(2) (Page 5, line 115-116): The sentence "We chose overall survival as the endpoint for our meta-analysis because OS is widely used as a significant prognostic indicator" was replaced with "We selected OS as the endpoint for our meta-analysis because OS is widely used as a significant prognostic indicator."

(3) (Page 13, line 325): The extra "tissue" was deleted.

References:

[1]. Kawai H, Ishii A, Washiya K, et al. Estrogen receptor alpha and beta are prognostic

factors in non-small cell lung cancer. Clin Cancer Res 2005;11:5084-9.
[2]. Nose N, Sugio K, Oyama T, et al. Association between estrogen receptor-beta expression and epidermal growth factor receptor mutation in the postoperative prognosis of adenocarcinoma of the lung. J Clin Oncol 2009;27:411-7.