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Prognostic relevance of estrogen receptor α, β and aromatase expression in non-small cell lung cancer

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ABSTRACT

Sex steroids and their receptors are important in the fetal development of normal lung tissue. In addition emerging evidence reveals their significance in lung cancer pathogenesis. This encourages the exploitation of hormone receptors as treatment targets in lung cancer, as it has been successfully used in breast cancer.

This study investigates the prognostic impact of estrogen receptor (ER) α and β and the aromatase (AR) enzyme in non-small cell lung cancer (NSCLC) patients.

Tumor tissue from 335 NSCLC patients was collected and tissue microarrays (TMAs) were constructed. Immunohistochemical analyses were performed to evaluate the expression of ERα, ERβ and AR in the cytoplasme and nuclei of cells in the tumor epithelial and stromal compartment. By use of survival statistics we investigated the markers impact on disease-specific survival (DSS).

Nuclear ERβ expression in tumor epithelial cells in female patients (HR 3.03; 95% CI 1.39–6.61) and tumor cell AR expression in all patients (HR 1.55; 95% CI 1.08–2.23) were significant negative prognostic markers of disease-specific survival in our cohort.

High ERβ expression correlates with worse outcome in female patients. Further, patients with high AR expression had an unfavorable prognostic outcome compared with patients expressing low AR levels. These results emphasize the importance of sex steroids role in NSCLC, and, as anti-hormonal drugs are widely available, could lead to the development of novel palliative or even adjuvant treatment strategies in this patient population.

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1. Introduction

Lung cancer remains the leading cause of cancer related deaths in the western world. In 2015, 221.200 new cases of lung cancer and 158.040 lung cancer related deaths are estimated in the US [1]. Lung cancer patients present with a 5-year survival rate of 11–16% for all stages combined. Non-small cell lung cancer (NSCLC) constitutes 80–85% of lung-cancer cases while small-cell lung cancer constitute the remaining 15–20% [2]. As the majority of these patients are diagnosed in advanced stages, responds poorly to chemotherapy, and has a dismal prognosis, new treatment strategies are surely needed.

Hormone-related cancers, such as cancers of the breast, ovaries, prostate and uterus are directly related to endogenous and exogenous steroid hormones affecting cell proliferation [3]. Nevertheless, emerging evidence has established estrogens and progesterone, the female sex steroids, as important growth promoters in other cancer types, such as NSCLC, previously thought unrelated to gender [4,5].

Binding to and activating the progesterone receptor (PR) plays a crucial role in the development and differentiation of the female reproductive organs [6]. In addition to functioning as a key regulator of normal sexual and reproductive physiology, estrogens are important in the development of normal lung tissue by regulating the proteolytic/antiproteolytic balance, hence ensuring lung elasticity [7]. Estrogen signaling is conducted through activation of the two estrogen receptors ERα and ERβ, expressed both in normal lung tissue [8] as well as in neoplastic cells in NSCLC [4,9]. Aromatase (AR), also known as CYP19, is a key regulator of the estrogen biosynthesis by converting the androgens testosterone

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and androstenedione to estradiol and estrone, respectively [10]. The enzyme, a member of the cytochrome P450 family, is localized in the membrane of the endoplasmatic reticulum and expressed in ovarian and placental tissue, as well as extragonadal tissues including lung, brain, breast and liver [11]. AR expression is shown to correlate with estrogen production in NSCLC [12,13].

While the 5-year survival rates for most cancers have increased notably over the past 3 decades, only incremental improvements can be observed for lung cancer patients. It is pivotal to conceive new therapeutic options to increase the overall survival in this patient group. However new prognostic and predictive molecular markers are required. We have previously investigated the prognostic impact of PR expression in NSCLC [14]. In the presented study we aim to 1) quantify expression of AR, ERα and ERβ in NSCLC tissue, 2) investigate the biomarkers impact on the prognosis of NSCLC patients and 3) explore the correlations between the investigated biomarkers and a number of previous markers investigated in our cohort of NSCLC patients. To strengthen our study we have investigated marker expression in both tumor epithelial and stromal compartment.

2. Experimental

2.1. Patients and clinical samples

An unselected population of 371 patients diagnosed with NSCLC stage I to IIIA at the University Hospital of North Norway (UNN) and Nordland Hospital (NLSH) from 1990 through 2004 were identified from the hospital records. Of these 371 patients, 36 patients were excluded from the study due to [1]: other malignancy within 5 years prior to the NSCLC diagnosis (n = 13) [2], radiotherapy or chemotherapy prior to surgery (n = 10) [3], inadequate paraffin-embedded tissue blocks (n = 13). In total, 335 patients with adequate paraffin-embedded tissue blocks and complete medical records were included in this study. Adjuvant chemotherapy had not yet been introduced as a therapeutic option in Norway during this time span (1990–2004). The patients were staged corresponding to the 7th edition of the UICC TNM classification [2], and the resected tumors were subtyped and histologically graded according to the World Health Organization (WHO) guidelines [15]. Staging and classification was performed by two experienced pathologists.

2.2. Tissue microarray constructions

All the lung cancer specimens were embedded in paraffin blocks. The most representative areas of viable tumor epithelial tissue and tumor-surrounding stroma were selected. Two cores from the representative epithelial neoplastic area and two cores from the tumor-surrounding stromal areas were collected using a 0.6-mm-diameter stylet. Tissue microarray (TMA) blocks were constructed using a tissue-array instrument (Beecher Instruments, Silver Springs, MD, USA) as previously reported [16,17]. In order to include all of the collected cores, 8 blocks were constructed. Multiple 4-µm sections were cut with a Micron microtome (HM355S). The cores were then stained with specific antibodies for immunohistochemical (IHC) analyses.

2.3. Immunohistochemistry

For immunohistochemistry, the tissue sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by two treatments of 10 min microwave heating at 450 W in 0.01 M citrate buffer at pH 6.0. The sections were cooled to room temperature (RT) and endogenous peroxidase activity was blocked by incubation with a solution of 0.5% hydrogen peroxide for 10 min. The sections were then incubated in 5% normal serum ABC kit (Vector Laboratories) for 1 h at RT to block nonspecific binding. Subsequently, the sections were incubated overnight at 4 °C with primary antibodies. In this study the following antibodies were used: rabbit polyclonal ERα (SC-543, Santa Cruz, 1/100), mouse monoclonal ERβ1 (clone PPG5/10, MCA1974s, AbD Serotec, 1/10) and goat polyclonal aromatase (SC-14245, Santa Cruz, 1/100). After washing, the sections were incubated with the corresponding secondary antibodies for 1 h at RT. The Vectastain ABC kit (Vector Laboratories) was used for the avidin-biotin complex method according the manufacturer's instructions. The sections were lightly counterstained with hematoxylin, dehydrated through an ethanol series, cleared in xylene and mounted. Two different controls for our staining method were applied. Firstly, staining control of the sections was with an isotype-matched control antibody without the primary antibody. Secondly, a multiple organ tissue microarray including both positive and negative controls were used to verify the specificity of the staining in every staining procedure. The positive tissue controls comprised normal ovary for ERa and ER-Beta1and placenta for aromatase; Negative tissue controls were samples of normal pancreas and liver tissue.

2.4. Antibody validation

2.4.1. Cell lines

Six human lung, prostate and breast cancer cell lines A549 (CCL-185), NCI-H460 (HTB-177), DU145 (HTB-81), PC3 (CRL-1435), MCF7 (HTB-22) and MDA-MB-231 (HTB-26), all from ATCC (Manassas, VA), were cultured in RPMI 1640 media (R8758, Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum (S0415, Biochrom) and 1× penicillin-streptomycin antibiotic mixture (P0781, Sigma-Aldrich). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. STR-profiling was performed to verify the cell lines authenticity by the department of Forensic Medicine at UiT-The Arctic University of Norway.

2.4.2. Western blot analysis

Western blot was used to verify the specificity of the primary antibodies (Fig. 1). Cells were washed in ice-cold phosphate-buffered saline, and lysate was added directly in NuPAGE LDS Sample Buffer (NP0007, Life Technologies) with dithiothreitol. For further evaluation, HEK 293 over-expressed cell lysates were utilized also from OriGene for ERα (LY400046), ERβ1 (LY425704) and CYP-19 (LY400031) and were incubated with 2xSDS Sample Buffer for 10 min at 100 °C. Equal amounts of protein lysates were resolved onto a 4–12% Bis-Tris gel (NP0322; Life Technologies). The resolved proteins were transferred onto an Odyssey nitrocellulose membrane (926-31092, LI-COR), and the membranes were subsequently blocked for 1 h at room temperature using the Odyssey blocking buffer (927-40000, LI-COR). For all three Primary antibodies 1/500 dilution was applied and the membrane incubated for over night at 4 °C. The following IRDye 800CW secondary antibodies for ERα (#926-32213, LI-COR), ERβ1 (#926-32212) and CYP19 (#935-32214) with 1/10,000 dilution incubated 1 h at RT. Rabbit anti-actin, 1:1000 (A2066, Sigma-Aldrich) was used as internal control and all lanes shows 42 kDa molecular weight protein load. Between antibody incubations, the membrane was washed three times for 5 min each time in tris-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich). Molecular weight markers used were the MagicMark XP Western Protein Standard (LC5603, Invitrogen) and SeeBlue Plus2 Pre-stained Standard (LC5925, Invitrogen).

2.5. IHC scoring

Light microscopy was used to determine the degree of cytoplasmic and nuclear expression of AR and ERβ in the tissue cores. The

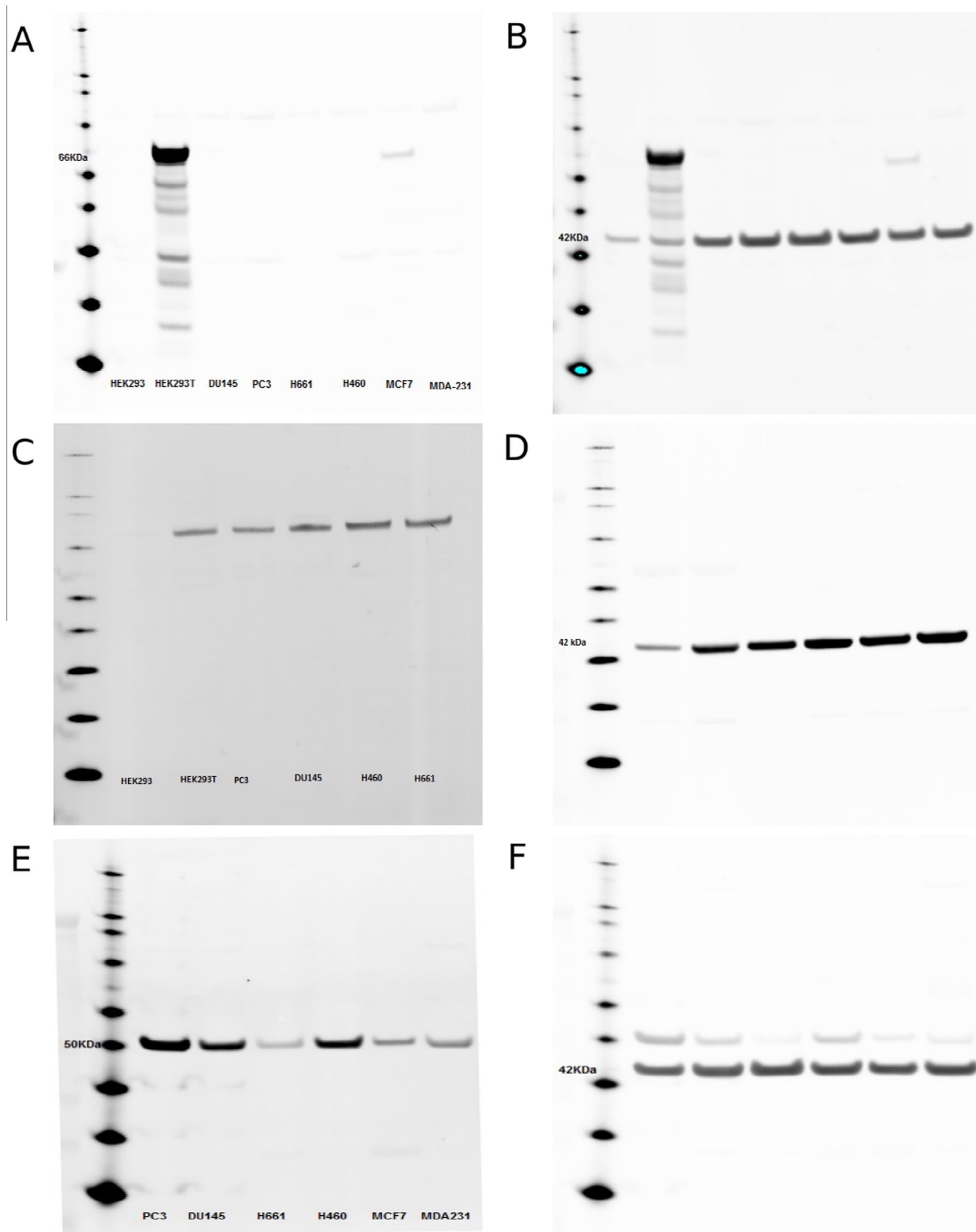

Fig. 1. Validation of antibody specificity by Western blotting. (A) Displaying protein bands corresponding to ERα, (C) ERβ and (E) AR. Rabbit anti-actin was used as internal control for each antibody, presented in (B), (D) and (F).

anonymized tissue samples were independently and semi-quantitatively scored by one experienced pathologist (SAS) and one medical student (KS, trained by a pathologist). The ARIOL imaging system (Applied Imaging Corp., San Jose, CA, USA) was used to scan and digitalize the IHC stained ERα TMA slides. The slides were loaded in the SL 50 automated slide loader and scanned at a low resolution (1.25×) and high resolution (20×) using an Olympus BX61 microscope with an automated platform (Prior Scientific, Cambridge, UK). Images of the cores were uploaded into the Ariol Software and scored manually on computer screen (SAS, KS) according to ERα expression in the cytoplasmic and nuclear compartment. In case of disagreement ⩾2, the slides were re-examined and consensus was reached between the observers. When evaluating the marker expression, the observers were blinded to patient outcome. The staining intensity in tumor and stromal cells was scored as: 0 = negative, 1 = weak, 2 = intermediate, 3 = strong. For stromal AR and ERβ staining, density was also scored in the following matter: 0 = no cells showing positivity, 1 = less than 5% positivity, 2 = 5–50% positivity, and 3 = 51–100% positivity. For each patient, the mean intensity score of duplicate cores was calculated. For stromal AR and ERβ, the intensity and density score was combined. Finally, biomarker expression was dichotomized into high and low cytoplasmic and nuclear staining using mean cut-off values. A high AR score of tumor epithelial and stromal cells was defined as an expression ⩾ mean value (1.69, 1.52 respectively). High tumor epithelial and stromal ERβ expression was defined as

scores above the mean values, 2.3 and 1.67 respectively. Finally, ERα expression was regarded as high with a score ⩾1.80, which was the mean value of ERα expression in tumor epithelial cells. Staining in fibroblasts, fibrocytes and endothelial cells in the blood and lymph vessels were included in the AR and ERβ stromal score. A weak staining was also observed in plasma cells and macrophages, but they were not included in the stromal score.

2.6. Statistical analysis

All statistical analyses were performed using the statistical package IBM SPSS, version 21 (SPSS Inc., Chicago, IL, USA). The Kaplan-Meier method was used for drawing univariate survival curves illustrating the association between marker expression and disease-specific survival (DSS). DSS was defined from the date of surgery until the time of lung cancer death. Statistical significance between the survival curves was assessed utilizing the log-rank test. The survival curves were terminated at 120 months, due to less than 10% of patients at risk after this point. Only variables with significant P-values from the univariate analyses were entered into the multivariate analysis, applying the Cox proportional hazards model. The data was run in a backward stepwise Cox regression, probability for stepwise entry and removal set at 0.05 and 0.10. To investigate the difference in biomarker expression between lung tumor and normal lung tissue, the Wilcoxon non-parametrical test was used. Chi-square and Fisher's exact test were used to examine the correlation among biomarker expression, clinicopathological factors and other molecular markers our group has previously investigated. The r-values represent Spearman's rank correlation coefficients. P-values less than 0.05 were considered statistically significant for all analyses.

3. Results

3.1. Scoring agreement

There was a significant scoring agreement between the scorers, with intra-class correlation coefficients of 0.754 ($p < 0.001$) for ERα, 0.798 ($p < 0.001$) for ERβ and 0.921 ($p < 0.001$) for AR.

3.2. Patient characteristics

The clinical, demographic and histopathological variables of the patient cohort are presented in Table 1. Of the 335 NSCLC patients, 76% were males and 96% were previous or present smokers. The median age was 67 years (range 28–85) and the median follow-up of survivors was 105 months (range 73–234 months). Histologically subtypes comprised 191 squamous cell carcinomas (SCCs), 113 adenocarcinomas (ACs) and 31 large cell carcinomas (LCCs).

3.3. Biomarker expression in NSCLC cells

ERα was primarily observed in the nucleus of tumor epithelial cells. ERβ was expressed in both nucleus and the cytoplasmic compartment in tumor epithelial and stromal cells, however most evident as nuclear staining. AR expression was solely cytoplasmic (Fig. 2). Table 2 presents biomarker expression according to cell type, cell compartment and gender. When comparing biomarker expression in malignant tissue with normal lung tissue samples collected distant from the tumor, we observed no significant differences in ERβ and AR expression. However, ERα displayed a significantly higher prevalence of both nuclear and cytoplasmic expression in tumor epithelial cells compared with non-malignant epithelial lung cells (cytoplasmic, $p = 0.001$: nucler, $p = 0.010$).

3.4. Correlation

We found a significant correlation between AR expression in the cytoplasmic compartment of tumor epithelial cells and tumor surrounding stromal cells ($r = 0.46$; $p < 0.001$). Following gender stratification, we observed this correlation for both females ($r = 0.46$, $p < 0.001$) and male patients ($r = 0.46$, $p < 0.001$). Further, we discovered a significant correlation between expression of ERβ ($r = 0.21$; $p < 0.001$) in tumor epithelial and stromal cell nuclei. This correlation was also observed when separating the genders, (females: $r = 0.23$, $p < 0.001$, males: $r = 0.21$, $p < 0.001$). A significant correlation was also found between ERα and ERβ expression in tumor epithelial cell nuclei ($r = 0.26$; $p < 0.001$). Stratification revealed similar results for both females ($r = 0.34$; $p < 0.001$) and males ($r = 0.23$; $p < 0.001$).

For all markers the correlations with other clinicopathological variables were weak or non-significant ($r < 0.2$, not presented).

3.5. Correlation between AR, ERα and ERβ expression with other molecular markers

Significant correlations between the three investigated markers and previously analyzed markers in the cohort were found. AR expression in tumor epithelial cells was significantly correlated with tumor epithelial cell expression of the angiogenic markers vascular endothelial growth factor (VEGFR; $r = 0.277$; $p < 0.001$) and platelet derived growth factor (PDGFA; $r = 0.239$; $p < 0.001$). Similar correlations were observed between tumor cell ERβ expression and VEGFR ($r = 0.238$; $p < 0.001$) as well as PDGFD ($r = 0.280$; $p < 0.001$). Stromal AR expression correlated significantly with expression of PDGFR in the tumor associated stromal cells ($r = 0.283$; $p < 0.001$). ERα did not correlate significantly with any of our previous markers.

3.6. Univariate analyses

Table 1 presents our results from the univariate analyses regarding the clinical variables and their impact on DSS. tStage ($p < 0.001$), nStage ($p < 0.001$), pStage ($p < 0.001$), WHO performance status ($p = 0.016$), histology ($p = 0.028$), vascular infiltration ($p = 0.001$), differentiation ($p < 0.001$) and surgical procedure ($p = 0.007$) were significant prognosticators. Bold indicates P-value < 0.05 in Tables 1–3.

The biomarkers impact on median and 5-year DSS is presented in Table 2. ERα expression showed no significant impact on survival in univariate analyses. Neither nuclear nor cytoplasmic ERβ expression was found to correlate with survival ($p = 0.093$). Although, gender stratification revealed high ERβ expression in tumor epithelial cell nuclei of female patients to be associated with poor DSS ($p = 0.010$).

High AR expression in malignant epithelial cells emerged as a significant negative prognosticator in our patient cohort ($p = 0.017$). Following gender stratification, high AR expression in tumor epithelial cells was a significant detrimental prognosticator for DSS in male patients ($p = 0.010$). This was not seen in the female patient group (Fig. 3).

3.7. Multivariate analysis

Only significant clinicopathological and biomarker variables from univariate analyses were included in the multivariate analysis. The results are presented in Table 3. High ERβ expression in tumor epithelial cells was a significant prognosticator for female patients in both univariate ($p = 0.010$) and multivariate analyses (HR: 3.03; 95% CI: 1.39–6.61; $p = 0.005$).

Table 1
Clinicopathological variables as predictors of disease-specific survival all NSCLC patients and differentiated into female and male subgroups (N = 335, 82 and 253 respectively, univariate analyses; log rank test) in 335 NSCLC patients.

Characteristic	Patients N (%)			Median survival (months)			5-Year survival (%)			p-Value		
	Combined	Female	Male	Combined	Female	Male	Combined	Female	Male	Combined	Female	Male
Age										0.42	0.49	0.56
⩽65 years	156 (47)	39 (48)	117 (46)	98	127	83	56	61	54			
⩾65 years	179 (53)	43 (52)	136 (54)	NR	NR	122	60	67	58			
Sex										0.22		
Female	82 (24)	82 (24)	253 (76)	190	190	98	64	64	56			
Male	253 (76)			98			56					
Smoking status										0.26	0.27	0.15
Never	15 (5)	6 (7)	9 (4)	19	21	18	43	50	38			
Previous	105 (31)	21 (26)	84 (33)	84	NR	NR	55	71	51			
Present	215 (64)	55 (67)	160 (63)	NR	NR	NR	60	63	60			
WHO Performance status										**0.016**	0.053	0.096
ECOG 0	197 (59)	53 (65)	144 (57)	NR	NR	NR	63	67	62			
ECOG 1	120 (36)	27 (33)	93 (37)	64	127	51	52	63	49			
ECOG 2	18 (5)	2 (2)	16 (6)	25	19	36	33	0	40			
Histology										**0.028**	0.26	**0.043**
Squamous cell carcinoma	191 (57)	36 (44)	155 (61)	NR	NR	NR	66	77	63			
Adenocarcinoma[*]	113 (34)	38 (46)	75 (30)	54	69	43	46	56	41			
Large cell carcinoma	31 (9)	8 (10)	23 (9)	98	47	98	56	43	61			
Weight loss										0.76	0.61	0.97
<10%	303 (90)	74 (90)	229 (91)	190	190	84	58	65	56			
>10%	32 (10)	8 (10)	24 (9)	98	47	98	57	50	61			
Differentiation										**<0.001**	0.734	**<0.001**
Poor	138 (41)	28 (34)	110 (43)	47	NR	32	47	61	43			
Moderate	144 (43)	36 (44)	108 (43)	190	190	NR	65	63	66			
Well	53 (16)	18 (22)	35 (14)	NR	NR	NR	68	71	67			
Surgical procedure										**0.007**	0.493	**0.011**
Wedge + Lobectomy	243 (73)	64 (78)	179 (71)	190	190	NR	62	67	60			
Pneumectomy	92 (27)	18 (22)	74 (29)	37	NR	30	47	50	47			
Pathological stage										**<0.001**	**0**	**<0.001**
I	157 (47)	41 (50)	116 (46)	NR	190	NR	72	80	69			
II	136 (41)	29 (35)	107 (42)	62	NR	42	51	57	50			
IIIA	42 (12)	12 (15)	20 (12)	17	19	16	24	25	25			
tStage										**<0.001**	0.153	**<0.001**
1	85 (25)	23 (28)	62 (24)	190	190	NR	75	77	74			
2	188 (56)	42 (51)	146 (58)	84	NR	71	57	66	55			
3	62 (19)	17 (21)	45 (18)	25	190	19	37	41	36			
nStage										**<0.001**	**0.001**	**<0.001**
0	232 (69)	61 (75)	171 (67)	NR	190	NR	67	73	65			
1	76 (23)	11 (13)	65 (26)	35	47	29	43	40	44			
2	27 (8)	10 (12)	17 (7)	18	21	16	18	30	9			
Surgical margins										0.374	**0.008**	0.687
Free	307 (92)	74 (90)	233 (92)	190	190	84	59	67	56			
Not free	28 (8)	8 (10)	20 (8)	47	23	NR	48	38	53			
Vascular infiltration										**0.001**	0.352	**<0.001**
No	284 (85)	62 (76)	222 (88)	190	190	NR	62	68	60			
Yes	51 (15)	20 (24)	31 (12)	27	NR	25	33	52	24			

[*] 18 of these patients had bronchioalveolar carcinomas; NR, not reached.

A high AR expression in tumor epithelial cells was significantly and independently associated with a poor prognosis for both genders combined (HR: 1.55; 95% CI: 1.08–2.23; p = 0.017). Though, the marker did not reach statistical significance for male patients (HR: 1.42; 95% CI: 0.93–2.15; p = 0.103).

4. Discussion

Applying TMA methodology on an unselective cohort of NSCLC patients, we show that female patients with high ERβ expression in malignant epithelial cells have an unfavorable prognosis compared with low-expressing ERβ females. We have further documented that a high AR expression is a significant and independent prognosticator for poor survival. A major strength of our study is the long follow-up time and the use of reliable antibodies, in routine clinical use for detection of hormone receptor-expressing cancer cells. Reports on ERα, -β and aromatase expression in NSCLC tissue have been diverging, probably caused by the use of various and/or unspecific antibodies. We have performed western blot analyses to confirm antibody specificity, further supporting the validity of our marker staining. In addition we use the median value as cut off point when evaluating marker expression, thus excluding potential biases accompanying the "optimal cutoff value". This is a huge strength with regard to representability and reproducibility. Our analyses explore marker expression in both tumor epithelial cells as well as adjoining stromal tissue, giving a more adequate optimal account of hormone receptor expression in tumor environment.

An emerging body of evidence confirms estrogen and activation of ER to be of utmost importance in lung cancer pathogenesis [18] and several studies have reported a correlation between AR expression and impact on NSCLC survival and progression [19–21].

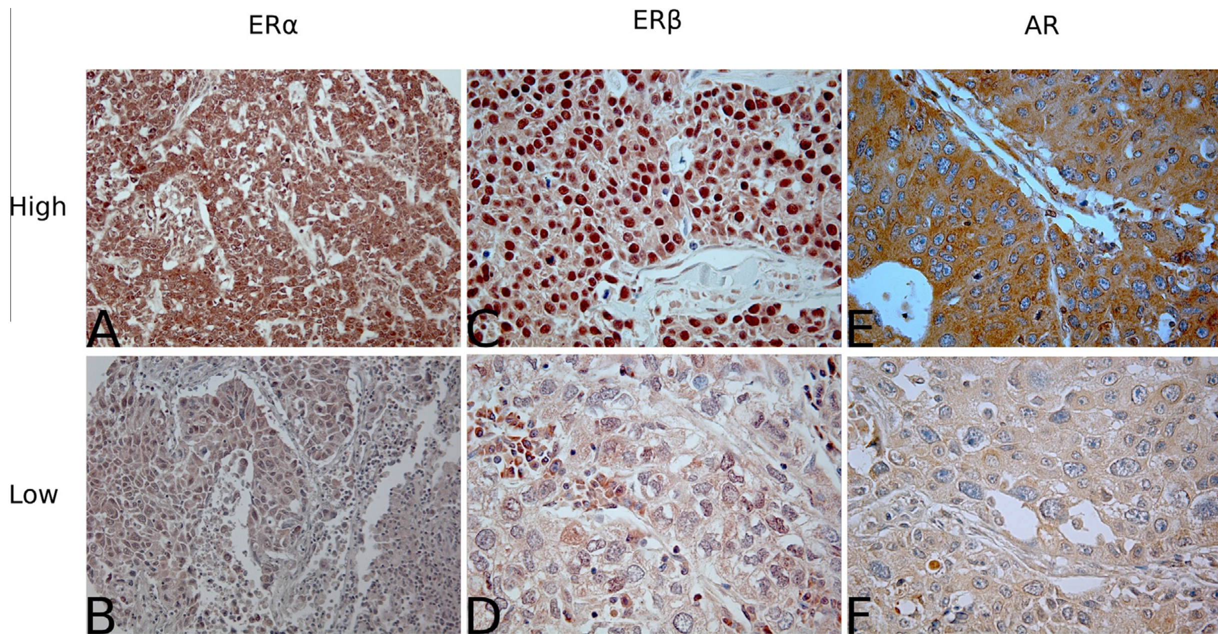

Fig. 2. Immunohistochemical analysis of ERα, ERβ and AR expression in NSCLC. Microscopic pictures of TMAs at 400× magnification, representing immunohistochemical staining of ERα, ERβ and AR expression. (A) Tumor epithelial cell ERα high expression; (B) Tumor epithelial cell ERα low expression; (C) Tumor epithelial cell ERβ high expression; (D) Tumor epithelial cell ERβ low expression; (E) Tumor epithelial cell AR high expression; (F) Tumor epithelial cell AR low expression.

Table 2
Erα, ERβ and AR expression as predictors of disease-specific survival in all patients and differentiated into female and male subgroups (N = 335, 82 and 253 resepctively, univariate analyses; log-rank test).

Characteristics	Patients, N (%)			Median survival (months)			5-Year survival (%)			p-Value		
	Combined	Female	Male	Combined	Female	Male	Combined	Female	Male	Combined	Female	Male
ERα												
Tumor epithelial cells (nucleus)										0.428	0.245	0.128
High	137 (41)	36 (44)	101 (40)	NR	64	NR	58	51	61			
Low	146 (44)	31 (38)	115 (45)	122	NR	74	56	70	53			
Missing	52 (15)	15 (18)	37 (15)									
ERβ												
Tumor epithelial cells (cytoplasm)										0.797	0.932	0.774
High	172 (51)	38 (46)	134 (53)	190	190	98	59	69	56			
Low	142 (43)	39 (48)	103 (41)	NR	NR	122	58	60	57			
Missing	21 (6)	5 (6)	16 (6)									
Tumor epithelial cells (nucleus)										0.093	**0.010**	0.625
High	144 (43)	34 (42)	110 (44)	69	64	98	53	51	53			
Low	170 (51)	43 (52)	127 (50)	190	190	122	64	74	60			
Missing	21 (6)	5 (6)	16 (6)									
Stromal cells										0.537	0.537	0.766
High	178 (53)	43 (52)	135 (53)	NR	NR	122	61	68	58			
Low	142 (42)	34 (42)	108 (43)	190	190	98	54	59	53			
Missing	15 (5)	5 (6)	10 (4)									
AR												
Tumor epithelial cells (cytoplasm)										**0.017**	0.762	**0.010**
High	146 (44)	37 (45)	109 (43)	64	190	54	51	62	47			
Low	161 (48)	41 (50)	120 (47)	NR	NR	NR	67	71	66			
Missing	28 (8)	4 (5)	24 (10)									
Stromal cells										0.766	0.188	0.635
High	143 (43)	34 (41)	109 (43)	NR	NR	71	60	73	56			
Low	176 (52)	44 (54)	132 (52)	127	190	122	58	60	57			
Missing	16 (5)	4 (5)	12 (5)									

Abbrevations: NR, not reached; NSCLC, non-small cell lung cancer.

A mapping of ER expression in normal as well as malignant tissue provides information that may be utilized when exploring the mechanisms of action for pharmacological mediators such as the selective estrogen receptor modulators (SERMs) and estrogen receptor (ER) antagonists in different tissues. The anti-estrogens tamoxifen and fulvestrant have substantially increased breast cancer survival [22]. In the breast, ERα is responsible for mediating the mitogenic effect of the most potent estrogen, 17β-estradiol [23].

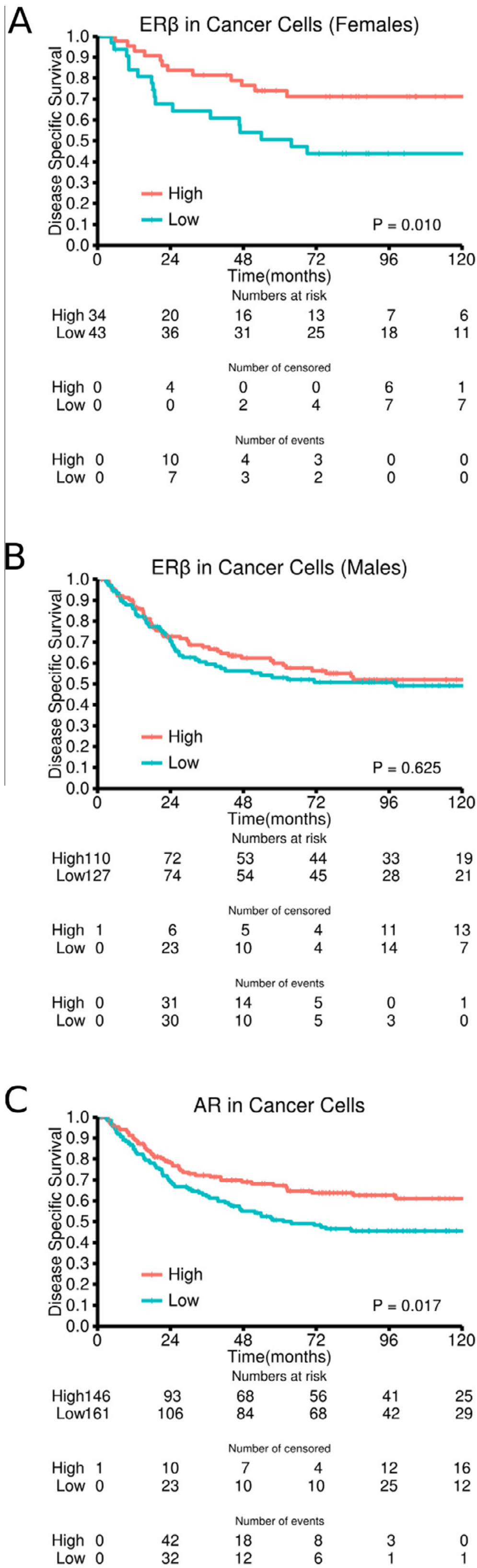

Fig. 3. Kaplan-Meier curves displaying disease-specific survival in relation to high or low biomarker expression ERβ expression in tumor epithelial cells from (A) females, (B) males, and (C) AR expression in tumor epithelial cells.

Hence tamoxifen, an ERα antagonist, is effective in the prevention of breast cancer growth and metastasis. But anti-estrogens appear to have possible therapeutic value in treatment and prevention of lung cancer as well. Stabile et al. reported already in 2005 inhibitory effect of the ER antagonist fulvestrant on lung tumor growth in mice [13].

Malignant breast cancer cells have been shown to produce large amounts of estrogens due to elevated levels of aromatase [24]. This contributes significantly to the malignant epithelial cells growth. Consequently, aromatase inhibitors have been successful as breast cancer treatment [25]. Reports suggest that the majority of intratumoral estradiol in lung cancer is produced locally by the tumor cells, and the aromatase-mediated conversion of androstenedion to estradiol may have an important impact on cancer cell proliferation and growth in lung [12,26,27]. This provides a possible rationale for targeting this pathway in treatment of ER-expressing lung cancer tissue, in the same way as done for breast cancer.

In fact, the nonsteroidal aromatase inhibitor anastrozole has proven to reduce lung tumor growth both *in vitro* and *in vivo* [19]. Exemestane, a steroidal aromatase inhibitor, has also proven to suppress NSCLC growth [28] and reduce cell migration and invasion [29], emphasizing the potential success of integrating aromatase inhibitors in lung cancer therapy.

Our results, showing ERβ as a negative prognosticator when expressed in NSCLC cells, have been confirmed by previous publications [30,31]. We found ERβ to be an independent prognosticator when expressed in the NSCLC nuclei in female patients. Besides, Stabile et al. [31] reported that cytoplasmic ERβ expression in male patients was an independent prognosticator in NSCLC, while Mah et al. [30] reported that both nuclear and cytoplasmic ERβ expression in NSCLC were prognosticators for poor survival regardless of gender. In contrast, an older report by Kawai et al. [32] stated that an absence of ERβ expression was associated with poor survival.

An explanation for ERβs association with a poor prognostic impact in NSCLC can be found in functional studies. Hershberger et al. reported that ERβ expression increased transcription, mitogen activation and growth in NSCLC cells [33]. This is in contrast with ERβ's role in breast cancer, where ERβ expression is considered a protective factor, repressing ERα's proliferative effect [8]. This suggests that ERβ expression and activation plays a different role in lung cancer, indicating that different signaling pathways may be activated in lung cancer tissue, compared to breast cancer tissue.

The expression of various sex steroids in relation to development of NSCLC has been presented in several studies [19,30,31]. Herein, we present a significantly increased ERα expression in tumor epithelial cells compared with the corresponding non-neoplastic cells. This is in concordance with previous findings from Niikawa et al. [12], Stabile et al. [31] and Mah et al. [30]. The two latter research groups also reported a higher prevalence of ERβ in NSCLC cells. This was not observed in our cohort. Márquez-Garban et al. [18] reported a high prevalence of positive ERα and ERβ cases (45% and 52%) in NSCLC, which was concistant with our findings of 41% and 43%. Sixty-nine percent presented with high extranuclear ERβ staining in their cohort, while 51% expressed high levels of ERβ in the cytoplasmic compartment in our patient group. High prevalence of ERβ expression have been reported [32,34], while others reported low ERα expression [35]. Regarding AR, 44% of our patients showed high AR expression in NSCLC cells, which is in concordance with Mah et al. [20] but lower when compared to Verma et al. [34] who reported 86%.

The differences in expression frequency and localization between the studies may be explained by several factors, including specificity and sensitivity of antibodies, scoring and interpretation of the stained tissue samples and differences between the patient cohorts.

We report strong correlations between our hormonal biomarkers and growth factor receptors (VEGFR, PDGFA) which have emerged as important biomarkers and targets in NSCLC therapy [28,36]. The best established molecular targets for treatment of

Table 3
Results of Cox regression analyses for clinicopathological variables, AR and ERβ expression in tumor cells.

Factor	All patients, N = 335			Female patients, N = 82			Male patients, N = 253		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Tumor stage			**<0.001**	NE	NE	NE			**0.001**
T1	1 (ref)						1 (ref)		
T2	1.73	(1.05–2.85)	**0.032**				1.93	(1.05–3.53)	**0.034**
T3	3.09	(1.75–5.44)	**<0.001**				3.7	(1.85–7.40)	**<0.001**
N-stage			**<0.001**			**0.001**			**0.007**
N0	1 (ref)			1 (ref)			1 (ref)		
N1	2.01	(1.31–3.08)	**0.001**	4.63	(1.70–12.57)	**0.003**	1.42	(0.87–2.33)	0.158
N2	2.98	(1.69–5.25)	**<0.001**	3.79	(1.60–8.97)	**0.002**	3.15	(1.54–6.47)	**0.002**
Differentiation			**0.004**	NE	NE	NE			**0.014**
Well	1 (ref)						1 (ref)		
Moderate	1.1	(0.57–2.11)	0.78				2.26	(1.02–4.98)	**0.044**
Poor	2.06	(1.10–3.87)	**0.025**				1.14	(0.51–2.58)	0.749
ECOG			**0.004**	NE	NE	NE	NE	NE	NE
Normal	1 (ref)								
Slightly reduced	1.82	(1.24–2.68)	**0.002**						
In bed < 50%	2.15	(0.95–4.91)	0.068						
Vascular infiltration				NE	NE	NE			
No	1 (ref)						1 (ref)		
Yes	1.83	(1.13–2.98)	**0.015**				2.77	(1.59–4.85)	**<0.001**
Histology			**0.003**	NE	NE	NE			**0.032**
SCC	1 (ref)						1 (ref)		
ACC	1.86	(1.25–2.76)	**0.002**				1.77	(1.12–2.80)	**0.015**
LCC	0.84	(0.42–1.68)	0.617				0.9	0.43–1.86)	0.768
ERβ tumor epithelial cells	NE	NE	NE				NE	NE	NE
Low				1 (ref)					
High				3.03	(1.39–6.61)	**0.005**			
AR tumor epithelial cells				NE	NE	NE			
Low	1 (ref)						1 (ref)		
High	1.55	(1.08–2.23)	**0.017**				1.42	(0.93–2.15)	0.103

Abbrevations: HR, hazard ratio; CI, confidence interval; NE, not entered (not significant in univariate analysis); NS, not significant. Significant clinicopathological factors and molecular markers from univariate analysis were included in this analysis.

advanced NSCLC today are mutations in EGFR [37]. EGFR receptors are involved in ligand independent estrogen signaling [9,18], providing evidence for a functional interaction between the signaling pathways. Marquez-Garban et al. [28] reported that EGF signaling increased expression and activity of aromatase in NSCLC cells, providing yet another way of bidirectional crosstalk between EGFR and ER in NSCLC. Several groups have shown that combined targeting of ER and EGFR enhances tumor regress and anti-proliferative effects in NSCLC [13,28,38].

These findings emphasize the importance of clinical studies elucidating the potential effect of combining multitargeted therapies in the treatment of NSCLC.

5. Conclusion

We present high ERβ expression as a negative prognosticator in female patients. Further, we show that patients expressing high levels of AR have an unfavorable prognostic outcome compared with low AR expressing patients. Our study highlights the complexity of hormone receptor expression and signaling in different malignancies, emphasizing the importance of further elucidation of the role of sex steroids in the development and progression of NSCLC. Our findings stress the significance of sex steroids in NSCLC, and we discuss the potential of integrating hormone related therapy in this patient group.

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Disclosure statement

The authors disclose no potential conflicts of interest.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2015, CA Cancer J. Clin. 65 (1) (2015) 5–29.
[2] R. Rami-Porta, J.J. Crowley, P. Goldstraw, Review the revised TNM staging system for lung cancer, Ann. Thorac. Cardiovasc. Surg. 15 (1) (2009) 5.
[3] B.E. Henderson, H.S. Feigelson, Hormonal carcinogenesis, Carcinogenesis 21 (3) (2000) 427–433.
[4] L.P. Stabile, A.L.G. Davis, C.T. Gubish, T.M. Hopkins, J.D. Luketich, N. Christie, et al., Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen, Cancer Res. 62 (7) (2002) 2141–2150.
[5] H. Ishibashi, T. Suzuki, S. Suzuki, H. Niikawa, L. Lu, Y. Miki, et al., Progesterone receptor in non-small cell lung cancer—a potent prognostic factor and possible target for endocrine therapy, Cancer Res. 65 (14) (2005) 6450–6458.
[6] V. Boonyaratanakornkit, Y. Bi, M. Rudd, D.P. Edwards, The role and mechanism of progesterone receptor activation of extra-nuclear signaling pathways in regulating gene transcription and cell cycle progression, Steroids 73 (9) (2008) 922–928.
[7] N. Heldring, A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, et al., Estrogen receptors: how do they signal and what are their targets, Physiol. Rev. 87 (3) (2007) 905–931.
[8] J.F. Couse, J. Lindzey, K. Grandien, J.-Ak. Gustafsson, K.S. Korach, Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse, Endocrinology 138 (11) (1997) 4613–4621.
[9] R.J. Pietras, D.C. Márquez, H.-W. Chen, E. Tsai, O. Weinberg, M. Fishbein, Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells, Steroids 70 (5) (2005) 372–381.
[10] S.E. Bulun, S. Sebastian, K. Takayama, T. Suzuki, H. Sasano, M. Shozu, The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters, J. Steroid Biochem. Mol. Biol. 86 (3) (2003) 219–224.
[11] J. Lo, G. Di Nardo, J. Griswold, C. Egbuta, W. Jiang, G. Gilardi, et al., Structural basis for the functional roles of critical residues in human cytochrome P450 aromatase, Biochemistry 52 (34) (2013) 5821–5829.

[12] H. Niikawa, T. Suzuki, Y. Miki, S. Suzuki, S. Nagasaki, J. Akahira, et al., Intratumoral estrogens and estrogen receptors in human non-small cell lung carcinoma, Clin. Cancer Res. 14 (14) (2008) 4417–4426.
[13] L.P. Stabile, J.S. Lyker, C.T. Gubish, W. Zhang, J.R. Grandis, J.M. Siegfried, Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced antiproliferative effects, Cancer Res. 65 (4) (2005) 1459–1470.
[14] K. Skjefstad, E. Richardsen, T. Donnem, S. Andersen, Y. Kiselev, T. Grindstad, et al., The prognostic role of progesterone receptor expression in non-small cell lung cancer patients: gender-related impacts and correlation with disease-specific survival, Steroids 98 (2015) 29–36.
[15] W.D. Travis, WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart, IARC, 2015.
[16] T. Donnem, S. Al-Saad, K. Al-Shibli, M.P. Delghandi, M. Persson, M.N. Nilsen, et al., Inverse prognostic impact of angiogenic marker expression in tumor cells versus stromal cells in non-small cell lung cancer, Clin. Cancer Res. 13 (22) (2007) 6649–6657.
[17] R. Bremnes, R. Veve, E. Gabrielson, F. Hirsch, A. Baron, L. Bemis, et al., High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer, J. Clin. Oncol. 20 (10) (2002) 2417–2428.
[18] D.C. Márquez-Garbán, H.-W. Chen, M.C. Fishbein, L. Goodglick, R.J. Pietras, Estrogen receptor signaling pathways in human non-small cell lung cancer, Steroids 72 (2) (2007) 135–143.
[19] O.K. Weinberg, D.C. Marquez-Garban, M.C. Fishbein, L. Goodglick, H.J. Garban, S.M. Dubinett, et al., Aromatase inhibitors in human lung cancer therapy, Cancer Res. 65 (24) (2005) 11287–11291.
[20] V. Mah, D.B. Seligson, A. Li, D.C. Márquez, I.I. Wistuba, Y. Elshimali, et al., Aromatase expression predicts survival in women with early-stage non-small cell lung cancer, Cancer Res. 67 (21) (2007) 10484–10490.
[21] Y. Miki, T. Suzuki, K. Abe, S. Suzuki, H. Niikawa, S. Iida, et al., Intratumoral localization of aromatase and interaction between stromal and parenchymal cells in the non-small cell lung carcinoma microenvironment, Cancer Res. 70 (16) (2010) 6659–6669.
[22] Group EBCTC, Tamoxifen for early breast cancer: an overview of the randomised trials, Lancet 351 (9114) (1998) 1451–1467.
[23] L.A. Helguero, M.H. Faulds, J.-Å. Gustafsson, L.-A. Haldosén, Estrogen receptors alfa (ERα) and beta (ERβ) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11, Oncogene 24 (44) (2005) 6605–6616.
[24] S.E. Bulun, Z. Lin, G. Imir, S. Amin, M. Demura, B. Yilmaz, et al., Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment, Pharmacol. Rev. 57 (3) (2005) 359–383.
[25] P. Lønning, The potency and clinical efficacy of aromatase inhibitors across the breast cancer continuum, Ann. Oncol. 22 (3) (2011) 503–514.
[26] Z. Hammoud, B. Tan, S. Badve, R.M. Bigsby, Estrogen promotes tumor progression in a genetically defined mouse model of lung adenocarcinoma, Endocr. Relat. Cancer 15 (2) (2008) 475–483.
[27] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, et al., Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, Endocr. Rev. 15 (3) (1994) 342–355.
[28] D.C. Márquez-Garbán, H.W. Chen, L. Goodglick, M.C. Fishbein, R.J. Pietras, Targeting aromatase and estrogen signaling in human non-small cell lung cancer, Ann. N. Y. Acad. Sci. 1155 (1) (2009) 194–205.
[29] E. Giannopoulou, K. Siatis, D. Metsiou, I. Kritikou, D. Papachristou, M. Kalofonou, et al., The inhibition of aromatase alters the mechanical and rheological properties of non-small-cell lung cancer cell lines affecting cell migration, Biochim. Biophys. Acta, Mol. Cell. Biol. Res. 1853 (2) (2015) 328–337.
[30] V. Mah, D. Marquez, M. Alavi, E.L. Maresh, L. Zhang, N. Yoon, et al., Expression levels of estrogen receptor beta in conjunction with aromatase predict survival in non-small cell lung cancer, Lung Cancer 74 (2) (2011) 318–325.
[31] L.P. Stabile, S. Dacic, S.R. Land, D.E. Lenzner, R. Dhir, M. Acquafondata, et al., Combined analysis of estrogen receptor β-1 and progesterone receptor expression identifies lung cancer patients with poor outcome, Clin. Cancer Res. 17 (1) (2011) 154–164.
[32] H. Kawai, A. Ishii, K. Washiya, T. Konno, H. Kon, C. Yamaya, et al., Estrogen receptor α and β are prognostic factors in non-small cell lung cancer, Clin. Cancer Res. 11 (14) (2005) 5084–5089.
[33] P.A. Hershberger, L.P. Stabile, B. Kanterewicz, M.E. Rothstein, C.T. Gubish, S. Land, et al., Estrogen receptor beta (ERβ) subtype-specific ligands increase transcription, p44/p42 mitogen activated protein kinase (MAPK) activation and growth in human non-small cell lung cancer cells, J. Steroid Biochem. Mol. Biol. 116 (1) (2009) 102–109.
[34] M.K. Verma, Y. Miki, K. Abe, S. Nagasaki, H. Niikawa, S. Suzuki, et al., Co-expression of estrogen receptor beta and aromatase in Japanese lung cancer patients: gender-dependent clinical outcome, Life Sci. 91 (15) (2012) 800–808.
[35] A.G. Schwartz, G.M. Prysak, V. Murphy, F. Lonardo, H. Pass, J. Schwartz, et al., Nuclear estrogen receptor β in lung cancer: expression and survival differences by sex, Clin. Cancer Res. 11 (20) (2005) 7280–7287.
[36] A. Sandler, R. Gray, M.C. Perry, J. Brahmer, J.H. Schiller, A. Dowlati, et al., Paclitaxel–carboplatin alone or with bevacizumab for non-small-cell lung cancer, N. Engl. J. Med. 355 (24) (2006) 2542–2550.
[37] J.G. Paez, P.A. Jänne, J.C. Lee, S. Tracy, H. Greulich, S. Gabriel, et al., EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy, Science 304 (5676) (2004) 1497–1500.
[38] E.B. Garon, R.J. Pietras, R.S. Finn, N. Kamranpour, S. Pitts, D.C. Márquez-Garbán, et al., Antiestrogen fulvestrant enhances the antiproliferative effects of epidermal growth factor receptor inhibitors in human non-small cell lung cancer, J. Thorac. Oncol. 8 (3) (2013) 270.

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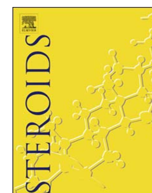
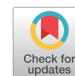


Corrigendum

Corrigendum to "Prognostic relevance of estrogen receptor α, β and aromatase expression in non-small cell lung cancer" [Steroids 113 (2016) 5–13]



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After publication of the original article, the authors have unfortunately detected an error.

Fig.3 is not correct and does not reflect the results presented in the manuscript. The designations "High" and "Low" were interchanged, causing misinterpretation of the results. Please find the correct figure attached. The authors apologize for any inconvenience caused.

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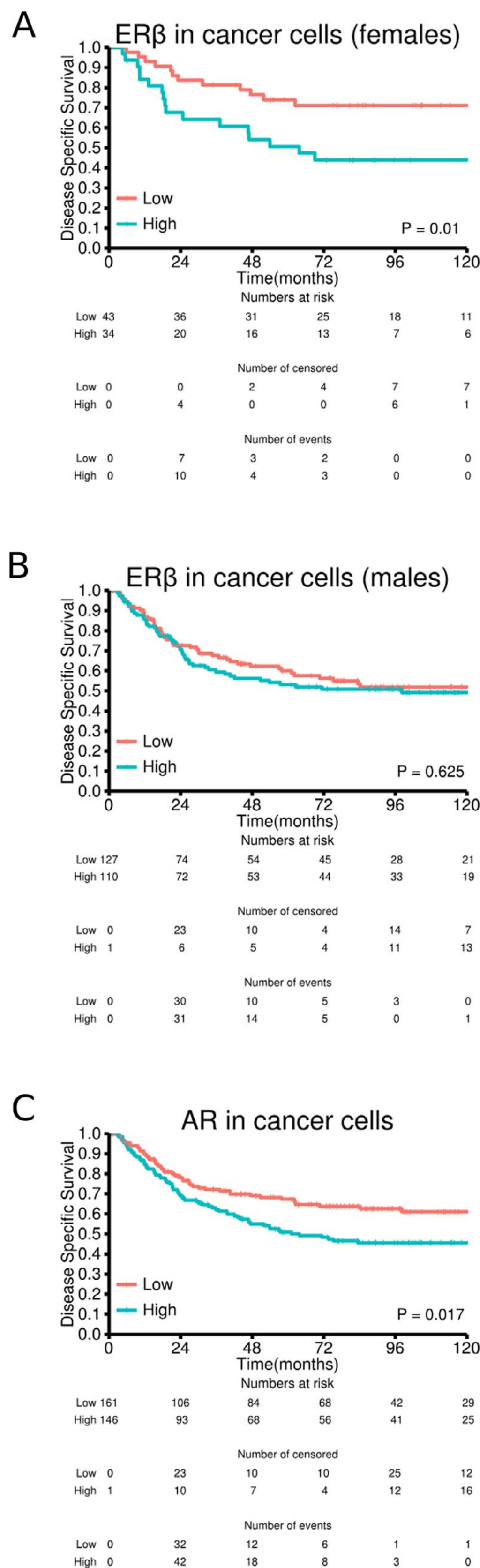

Fig. 3. Kaplan-Meier curves displaying disease-specific survival in relation to high or low biomarker expression. ERβ expression in tumor epithelial cells from (A) females, (B) males and (C) AR expression in tumor epithelial cells.