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Differential Role of Estrogen Receptor Beta in Early Versus Metastatic Non-small Cell Lung Cancer

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Abstract Although women have an increased susceptibility to lung cancer, they also have a favorable clinical outcome. This may in part be due to female specific genetic and hormonal factors. In the present study, expression of ER-beta was investigated by immunohistochemistry using tissue samples from two cohorts: non-small cell lung cancer (NSCLC) diagnosed in 1999 in Manitoba and advanced NSCLC patients from the NCIC-CTG BR.18 trial. In the Manitoba cohort assessable tissue samples available in 79 patients (32 females and 47 males) and the majority (75%) had early stage disease. Fifty-one percent of patients expressed high levels of ER-beta (defined by ≥60, the median immunohistochemistry score) and its expression was comparable in males and females. The 3-year overall survival of the group was 53% and males had significantly worse survival compared to females (HR=2.37, 95%CI 1.15–4.91, P=0.02). Higher ER-beta 1 expression was associated with better survival in both univariate (HR=0.41, 95%CI 0.21–0.80, P=0.009) and in multivariate (HR=0.37, 95%CI 0.18–0.77, P=0.008) analysis. In the NCIC-CTG cohort that were more often later stage, assessable tissue samples from 48 cases were available however higher ER-beta 1 expression correlated with poorer survival (HR= 1.94, 95%CI 1.01–3.75 P=0.047). These results suggest a differential impact of ER-beta 1 expression on clinical outcome by disease stage, that needs to be explored further and may explain contradictory observations reported in the literature.

Keywords Estrogen receptor beta variants · Sex · Lung cancer · Tissue micro-arrays · Immunohistochemistry · Clinical outcome

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Introduction

Emerging evidence suggests there are sex differences in non-small cell lung cancer (NSCLC) incidence, histology, and survival [1–5]. Variations in smoking patterns alone do not fully account for the disparities in lung cancer risk, biology, and outcomes between sexes. Complex interactions between genetic and hormonal factors likely underlie these observed differences [6]. The involvement of estrogen signaling in lung cancer is controversial. But the finding that ERβ null (ERβ−/−) mice have an altered lung phenotype [7] supports the idea that ERβ may have a role in normal and possibly in neoplastic lung biology. Several studies have evaluated expression of estrogen (ER) and progesterone receptors (PR) in both normal and neoplastic human lung tissues with variable conclusions. Interestingly, although detection of ERβ is variable, detection of ERβ is a consistent finding [8–14]. However, correlations of ERβ expression with histopathological parameters and clinical outcome in lung cancer are variable [8–14]. Similar to breast cancer [15] this could be due to how ERβ expression was determined (RNA or protein) and/or the antibody used [15]. The latter is important as variant non-ligand binding ERβ proteins have been detected in breast and other tissues [16, 17] and variant function may differ from full-length ligand binding ERβ1 [18, 19]. Some antibodies specifically detect, full-length ERβ1 only, while others will detect total (full-length plus variants) ERβ. Previously published studies in lung cancer have only determined either total ERβ or ERβ1 expression, while no other ERβ variant isoforms have been investigated.

Recently, aromatase expression was detected in lung cancer tissues and adjacent normal lung tissue [20, 21]. Aromatase converts androgenic substrates into active estrogens and inhibiting this enzyme is efficacious in breast cancer treatment [22]. If such data are supported by other studies, co-expression of aromatase and ERβ1 in lung cancer would support a role of estrogen regulated signaling pathways in lung cancer, potentially contributing to sex differences in lung cancer biology and providing the rationale for targeting this pathway for treatment in some lung cancer patients [23, 24]. However, expression of other ERβ variants could alter responsiveness to estrogens [18, 19] and other agents that target estrogen signaling pathways. To obtain more precise information as to the nature and role of ERβ-regulated pathways in lung cancer, we have investigated the type and frequency of ERβ isoform expression, in a cohort of Manitoba patients with mainly early-stage lung cancer, as well as in a cohort of cases derived from the NCIC-CTG BR18 lung cancer clinical trial [25] representing a cohort of late-stage metastatic NSCLC.

Methods

Patient Selection and Tissue Micro-array Construction

All patients with NSCLC who were diagnosed with NSCLC in 1999 were identified from the Manitoba Cancer Registry. Formalin-fixed paraffin-embedded (FFPE) blocks of tissue sample from 85 cases were available from the Department of Pathology at the Health Sciences Center site. The blocks were assessed for suitability to generate tissue micro-arrays (TMA) using the facilities and expertise of the Manitoba Breast Tumor Bank (MBTB, http://www.umanitoba.ca/institutes/manitoba_institute_cell_biology/MBTB/MBTB_Homepage.htm). The MBTB embraces the policies and operating protocols of the Canadian Tumor Repository Network (ref: www.CTRNet.ca) and operates with approval from the Research Ethics Board of the Faculty of Medicine, University of Manitoba.

The FFPE blocks were assessed by our study pathologist and three cores (3×0.6 mm diameter) from cancerous tissue were collected for TMA construction. In some cases there was no tumor in the block to core and also due to cellular heterogeneity of tumor cores, the case numbers (*n*) analyzed for some markers were less than 85 (as indicated in the "Results" section).

Ethics approval to undertake this project was obtained from the Research Ethics Board of the Faculty of Medicine, University of Manitoba. Clinical information was collected on these patients using the CancerCare Manitoba electronic charts. The clinical information available includes: age at diagnosis, sex, stage, preliminary treatment, and length of survival (from pathological diagnosis to date of death).

TMA sections were also obtained from a subset of the NCIC-CTG BR.18 clinical trials patients. The trial was a randomized phase III study, which looked at the effect of the broad-spectrum matrix metalloproteinase inhibitor BMS-275291 in combination with paclitaxel and carboplatin in advanced NSCLC [25]. From the 774 patients enrolled in the trial only 58 cases were available on the TMA. As well, due to cellular heterogeneity of tumor cores, the case numbers (*n*) analyzed for some markers were less than 58 (as indicated in the results section).

Immunohistochemistry

Immunohistochemistry (IHC) for TMA sections was performed as described previously [26]. Serial sections (5 um) of the TMAs were stained with ERβ1 (polyclonal, GC17/385P, Biogenex, CA, USA, raised against a peptide containing amino acids 449–465) at 1:100 dilution; total ERβ (monoclonal, 14C8, Genetex, TX, USA), raised to peptide derived from the N terminus (amino acids 1–153) at 1:100; ERβ2/cx (mouse monoclonal, clone 57/3, raised to synthetic peptide

derived from the specific C terminus of hERβ2/cx isoform; Serotec, UK) used at 1:50; PR (monoclonal Ncl-PgR Novocastra labs, USA) at 1:50; ERβ, p53, Ki67, and cleaved caspase 3 antibodies were used as previously described [26, 27].

Briefly, sections were dewaxed in three xylene baths (5 min each), taken through a series of alcohols (100%, 95%, and 70%), rehydrated in distilled water, and then submitted to heat-induced antigen retrieval in the presence of a tris/borate/EDTA buffer (CC1, pH 8.0; Ventana Medical Systems, AZ, USA), using an automated tissue immunostainer (Discovery Staining Module, Ventana Medical Systems, AZ, USA). Antigen retrieval was set to the "Mild and Standard Cell Conditioning" procedure for all antibodies as previously described. Initial dilutions quoted above applied to the Discovery module were diluted further 1:3 with buffer dispensed onto the slides with the primary antibody. Total ERβ IHC was performed manually as previously described using 14C8 antibody [28]. Total ERβ and ERβ1 antibodies have been validated for IHC previously [28]. ERβ2/cx antibodies were validated for specificity in lung tissues as shown in Fig. 1.

Quantification and Cutoff Selection

Slides were viewed and scored using standard light microscopy. IHC scores derive from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100%). These two scores when multiplied generate an overall IHC score of 0–300. Only specific nuclear staining was evaluated for the ER isoforms. TMAs were evaluated independently by two investigators (GPS, GQ) and where discordance was found, cases were re-evaluated to reach consensus. Since no relevant clinical cutoff points are presently reported for any ER isoforms in NSCLC, positivity reported in this study was solely based on IHC scores equivalent to or greater than the median. Overall survival (OS) was defined as time from diagnosis to death at 3 years of follow-up for the Manitoba cohort and as previously described for the NCIC-CTG BR.18 cohort [25]. In the latter cohort the pathology blocks were from tissue taken at the time of diagnosis, and the follow-up was from randomization and trial entry (i.e., the disease was metastatic) until death.

Statistical Methodology

Survival analysis was undertaken using Cox regression analyses. Each model was tested and all complied with the assumption of proportional hazard. Statistical analyses were performed using SAS™version 9.1.

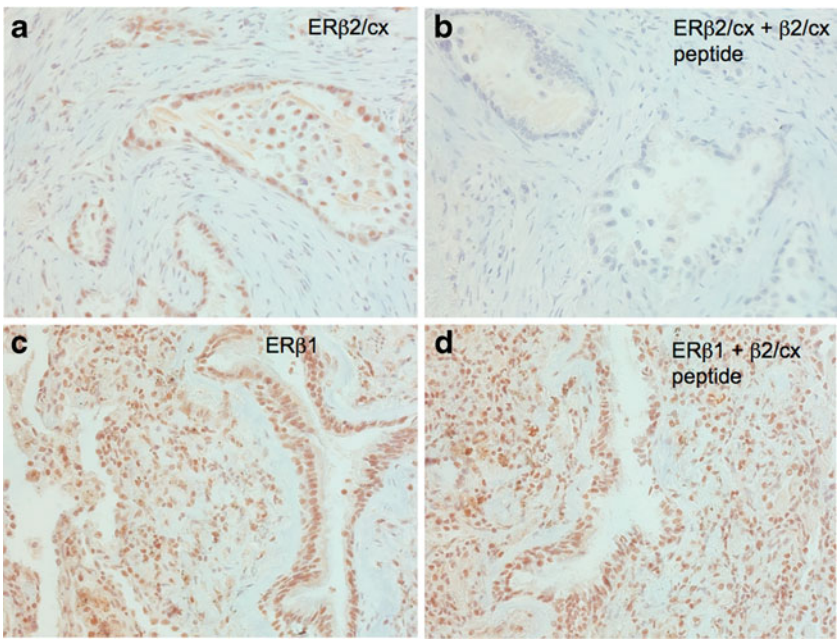

Fig. 1 IHC was performed as described in "Methods". A lung tumor section stained with the ERβ2/cx-specific antibody (mouse monoclonal, clone 57/3, raised to synthetic peptide derived from the specific C terminus of hERβ2/cx isoform; Serotec, UK) with strong, nuclear expression (**a**); elimination of staining in an adjacent section of the same tumor using ERβ2/cx-specific antibody pre-absorbed with a 30-fold excess of the C-terminal hERβ2/cx synthetic peptide (**b**); a lung tumor section stained with ERβ1-specific antibody (GC17/385P, Biogenex, CA, USA, raised to C-terminal h ERβ1 synthetic peptide) (**c**); no elimination of staining is seen in an adjacent section of the same tumor using ERβ1-specific antibody pre-absorbed with a 30-fold excess of the C-terminal hERβ2/cx synthetic peptide (**d**). All magnifications ×500

Validation of Specificity of ERβ/2cx Antibody in Lung Tissue

The antibodies used to detect ERβ1 and total ERβ were validated for IHC previously, mainly in breast cancer and their specificity reported by different groups including our own [28–30]. We had not previously optimized and validated the ERβ2/cx antibody for IHC. To undertake this, Western blots were performed on extracts from Cos1 cells that were transiently transfected with either an expression plasmid for ERβ1, ERβ2/cx, and/or empty vector. Only extracts from ERβ2/cx transfected cells showed a positive signal (data not shown). We then screened this antibody for its ability to detect nuclear staining in FFPE lung tissue (Fig. 1) and breast tissue (not shown). A NSCLC (Fig. 1a) and breast cancer (not shown) case showing strong nuclear staining were then chosen for further analysis. One section was stained with the ERβ2/cx antibody (Fig. 1a), while an adjacent section was stained with antibody that had been immuno-absorbed with ~30× excess of the peptide previously used to generate the antibody (Fig. 1b). This resulted in loss of nuclear staining supporting the specificity of the ERβ2/cx antibody. In contrast when the ERβ1 antibody was pre-absorbed with excess ERβ2/cx peptide (Fig. 1d), ERβ1 nuclear staining was not diminished (compare Fig. 1c with d). These antibodies were then used to stain TMA sections from the two cohorts of NSCLC cases as described above.

Results

Expression of ERβ1 and ERβ2/cx in Human Lung Cancer

Clinical-pathological characteristics of the final study cohort are shown in Table 1. In both NSCLC cohorts used in this study no IHC detection of ERα or PR was found under identical experimental conditions where ERα and PR were detected in human breast cancer cases (data not shown). In contrast positive nuclear staining for both ERβ1 and ERβ2/cx was found in multiple NSCLC cases. In the Manitoba cohort, using the median IHC score (Table 2) as the cut point, 51% of the cases expressed high levels of ERβ as determined using an antibody that would detect both ERβ1 and ERβ2/cx (total ERβ), 51% of cases expressed high levels of ERβ1, and 41% of cases expressed high levels of ERβ2/cx. In the NCIC-CTG BR.18 cohort using the median IHC score (Table 2) as cut point, 49% of cases expressed high levels of total ERβ 48% of cases expressed high levels of ERβ1 and 44% of cases expressed high levels of ERβ2/cx. Therefore, similar frequency of detection of ERβ isoforms was found in both cohorts, with a tendency for higher frequency in the Manitoba cohort.

If IHC scores were dichotomized into positive and negative based on the median IHC score of each cohort, then in the Manitoba cohort 26% of the tumors co-expressed ERβ1 and ERβ2/cx and 29% of the NCIC-CTG BR.18 cohort co-expressed ERβ1 and ERβ2/cx. If expression was defined as any detectable staining then the frequency of co-expression of ERβ1 and ERβ2/cx was 39% in the Manitoba cohort and 38% in the NCIC-CTG BR.18 cohort. Furthermore, a similar percentage of tumors in each cohort, was found when those only expressing ERβ1 or only expressing ERβ2/cx or those with no expression of either isoform was considered (data not shown). Therefore the two cohorts are similar with respect to the distribution of ERβ isoform expression and co-expression.

Table 1 Lung cancer cohort characteristics

Parameter	Manitoba Lung Cancer Cohort *N* (%)	NCIC-CTG BR.18 *N* (%)
Sex		
Female	32 (40.5)	35 (60)
Male	47 (59.5)	23 (40)
Age (median, range)	69.2 (45.7–85.2) years	62.8 (38.4–74.1) years
Grouped pathological stage[a]		
1 (1a,1b,2a,2b)	54 (75)	23 (48)
2 (3a,3b,4)	18 (25)	25 (52)

[a] The numbers shown for stage in this table reflect only the cases that had assessable ERβ staining

The Manitoba Cohort—Early Stage Disease

Tissue samples were available in 79 patients (32 females and 47 males) and the majority (75%) had early stage disease. There are no clinically relevant cutoff values for ERβ expression in human lung cancer, therefore median IHC scores were used to define high and low expression of the various factors determined by IHC (Table 2).

Using the median score as a cutoff for high versus low expression, the molecular markers and other clinical-pathological parameters were analyzed for relationship to clinical outcome as defined by survival at 3 years from the time of diagnosis (Table 3). In univariate analysis (Table 3), sex (males versus females, HR=2.37, 95%CI 1.15–4.91, P=0.02, n=79), ERβ1 (high versus low, HR=0.41, 95%CI 0.21–0.80, P=0.009, n=79), and Ki67, a marker of proliferation (high versus low, HR=2.19, 95% CI 1.13–4.24, P=0.019, n=79), were found to be significantly associated with survival. In multivariate analysis, sex (male versus female, HR=2.48, 95%CI 1.19–5.15, P=0.015, n=79), and ERβ1 high versus low, HR=0.40, 95% CI 0.20–0.79, P=0.008, n=79 remained significantly associated with survival.

Table 2 Immunohistochemical analysis

Epitope measured	Manitoba Lung Cancer Cohort		NCIC-CTG BR.18	
	Median IHC score (range)	% high[a]	Median IHC score (range)	% high[a]
ERα	ND	ND	ND	ND
PR	ND	ND	ND	ND
ERβ1	60 (0–285)	51	55 (0–270)	48
ERβ2/cx	0 (0–270)	41	0 (0–285)	44
Total ERβ	15 (0–270)	51	20 (0–240)	49
p53	90 (0–270)	53	0 (0–270)	43
Ki67	20 (0–90)	47	10 (0–90)	35
Cleaved caspase 3	2.5 (0–30)	48	5 (0–20)	10

ND not detected

[a]Median IHC score was the cut point for high versus low expression

NCIC-CTG BR.18 Lung Cancer Cohort

Only 48 of the 58 cases of NSCLC arrayed from this cohort had assessable tissue staining. The NCIC-CTG BR18 clinical trial eligibility criteria required all patients to have stage IIIB or IV NSCLC at the time of entry to the clinical trial. Fifty-two percent (52%) of those patients were diagnosed with stage IIIB or IV disease, the remainder had earlier stage disease that subsequently relapsed. In this cohort the median IHC score values were as follows: ERβ1 55; ERβ2/cx, 0; total ERβ 20; p53, 0; Ki67, 10; caspase 3, 5 (Table 2). These median IHC scores were used to define high or low expression and were used for the analysis of each factor with survival as shown in Table 4. Higher ERβ1 expression correlated with poorer survival (HR=1.94, 95%CI 1.01–3.75, P=0.047, n=48) and remained significant on the multivariate analysis.

Table 3 Manitoba cohort

	No.	Hazard ratio	95%CI	*P* value
Single predictor models				
Males vs females[a]	79	2.37	1.15–4.91	0.020*
High path stage[b]	72	1.76	0.86–3.62	0.123
ERβ1≥60	79	0.41	0.21–0.80	0.009*
ERβ2/cx>0	79	0.69	0.35–1.35	0.280
Total ERβ>15	79	1.42	0.74–2.72	0.291
p53>90	79	1.83	0.95–3.52	0.072
Ki67>20	79	2.19	1.13–4.24	0.019*
Caspase 3>2.5	79	1.26	0.66–2.40	0.484
Multi-predictor model 1[c]	79			
Males vs females[a]		2.48	1.19–5.15	0.015*
ERβ1≥60		0.40	0.20–0.79	0.008*
Ki67>20		1.81	0.93–3.53	0.079

Cox proportional hazard regression: data censored at 3 years

HR hazard ratio, *CI* confidence interval, *P P* value

*P<0.05

[a] Gender: 0 = female, 1 = male

[b] Pathological stage: 1 = ia, ib, iia, and iib; 2 = iiia, iiib, and iv

[c] Model satisfied the proportional hazards assumption

Discussion

Several studies have now identified the expression of ERβ in NSCLC and from these studies it is concluded that 45–69% of lung cancers are positive for or have high levels of ERβ expression [31]. The predominant sub-cellular localization of ERβ in NSCLC is the nucleus, although variably cytoplasmic staining has been reported [31]. In the cohorts examined in this study, little cytoplasmic staining was detected and no plasma-membrane-associated staining was detected. Immuno-neutralization of cytoplasmic staining tended to be variable among cases suggesting some may be specific and some non-specific therefore only nuclear staining was considered in this study. There is no clinically relevant cutoff point presently reported for any ER isoform expression in NSCLC and similar to studies of ERβ

Table 4 NCIC-CTG BR18 advanced lung cancer cohort

	No.	Hazard ratio	95%CI	*P* value
Single predictor models				
Males vs females	58	1.59	0.86–2.94	0.14
High path stage[a]	58	1.35	0.97–1.89	0.08
ERβ1>55	48	1.94	1.01–3.75	0.047*
ERβ2/cx>0	48	1.21	0.64–2.30	0.56
Total ERβ>20	47	0.87	0.46–1.66	0.68
p53>0	47	0.92	0.48–1.78	0.81
Ki67>10	48	1.31	0.68–2.52	0.41
Caspase 3>5	48	1.92	0.67–5.50	0.22
Multi-predictor model				
ERβ1>55	47	2.32	1.16–4.65	0.02*

*P<0.05

[a] Stage (I, II) vs stage (III, IV)

expression in breast cancer, different antibodies, and different definitions of ERβ-positive and ERβ-negative expression have been used [10, 15, 28, 32, 33] [32]. Nuclear staining however, is most often used to define ERβ expression in lung cancer with different criteria being used to define positive expression [10, 32, 33]. Our approach was to be more quantitative by using an IHC score (H-score) that is derived by multiplying nuclear staining intensity (0, 1, 2, 3) with the % of tumor cells displaying detectable nuclear staining. Using the median IHC score as a cut point 51% of the Manitoba lung cancer cohort and 48% of the NCIC-CTG BR.18 cohort were found to express high levels of ERβ1. The frequencies are within the range previously published by other groups [10, 31–33]. As well, using this approach the Manitoba cohort showed that women had a significantly increased 3-year survival advantage than men which is consistent with other reports [32] as well as overall lung cancer statistics (Canadian Cancer Statistic 2009, http://www.cancer.ca/canada-wide/about%20cancer/cancer%20statistics/canadian%20cancer%20statistics.aspx) (NCI-SEERS cancer statistics, http://seer.cancer.gov/statistics/). The lack of this finding in the NCIC-CTG BR.18 cohort may be explained by the more advance stage of disease, smaller number of cases analyzed, and perhaps differences in treatment. In particular drawing conclusions is problematic from such small numbers of patients. Furthermore, since the IHC analysis of this cohort was from specimens obtained at the initial time of diagnosis, we cannot discount the possibility that the expression of ERβ isoforms may have changed during the interval from initial diagnosis to relapse and this remains a limitation of the current study.

Previous studies of ERβ expression in NSCLC only analyzed either total ERβ expression or ERβ1 expression, with the latter being the most frequent analysis [10, 32–34]. However, other isoforms of ERβ have been detected in breast, prostate, and ovarian cancer tissues [30, 35–37]. In particular an abundant and frequently expressed isoform is ERβ2/cx [35, 36, 38]. ERβ2/cx is generated by alternative splicing and the protein formed is C-terminally truncated and in contrast to ERβ1 does not bind ligand [18]. In addition, ERβ2/cx can heterodimerize with other isoforms of ERβ as well as ERα and modulate activity of the ligand binding forms of both ERs [37, 39]. We therefore determined the expression of ERβ1, ERβ2/cx, and total ERβ in two NSCLC cohorts. This is the first study to detect not only expression of ERβ2/cx in two different NSCLC cohorts but also to demonstrate co-expression with ERβ1.

Interestingly, in the current study, high levels of expression of ERβ1 but not ERβ2/cx were found to be associated with OS in both cohorts of NSCLC. This relationship was maintained in multivariate analysis for both cohorts. However, differences were noted, since in the Manitoba cohort high ERβ1 was associated with better outcome while in the NCIC-CTG BR.18 cohort high ERβ1 was associated with worse outcome. Although both cohorts had small numbers of cases, major differences between the cohorts were disease stage and treatment received. The Manitoba cohort was dominated by early stage disease. The NCIC-CTG BR.18 cohort consisted of cases from patients with advanced disease and the clinical outcome analyzed reflects the survival of metastatic NSCLC, even though the tissue samples may have been obtained at the time of initial diagnosis with earlier stage disease. Interestingly, Wu et al., based on a subgroup analysis suggested that over expression of ERβ1 was associated with better clinical prognosis only in patients with stage II and III disease [10]. In a study by Kawai et al. [14], where ERβ1 positivity was associated with better outcome in NSCLC in multivariate analysis, the study cohort was predominately stages 1 and 2 (68%) disease. Furthermore, another study found that higher levels of ERβ1 expression were associated with well and moderately differentiated lung tumors [40]. Our results add to and are consistent with these previous observations, and support the hypothesis that there may be a differential effect of ERβ1 expression in NSCLC due to stage of the disease and/or the type of treatment they received.

Differential activity of signaling factors and pathways at different stages of cancer progression is not unusual, for example, TGFβ [41]. Indeed, ERβ1 can regulate expression of several members of the TGFβ family [42] in breast cancer cells, suggesting the possibility that differential effects of ERβ during cancer progression could, in part, be mediated by TGFβ. In addition, ERβ expression in ERα-positive breast cancer is associated with parameters of good prognosis but in contrast is associated with poor prognostic factors in the more aggressive ERα-negative disease [26, 43]. In prostate cancer progression there is a general decrease in ERβ1 expression in primary tumors compared to normal tissue with the reappearance of expression in prostate cancer metastases [30] again suggesting differential activity at different stages of the disease.

Another difference between the two studies is treatment received. The aim of the BR.18 trial was to determine if any clinical benefit could be realized by adding the broad-spectrum matrix metalloproteinase inhibitor (MMPI), BMS-275291, to paclitaxel and carboplatin in advanced NSCLC, while the local cohort would have received a range of different treatment regimens without MMPIs. Recently, it was shown that ERβ1 can be cleaved at its N terminus by MMP26 [44], which may alter the regulation and function of ERβ1 [45]. Therefore, it could be speculated that the BR.18 patients treated with MMPI may have altered the regulation and therefore function of ERβ1 due to inhibition of MMP26.

In conclusion, the results obtained from this study suggest a differential impact of ERβ1 expression on clinical outcome in NSCLC based on the disease stage and/or

treatment, which could contribute, at least in part, to the contradictory observations reported in the literature and needs to be explored further. It should be noted that the current study was focused on hypothesis generation and the limitations regarding small case numbers, arbitrary cutoff values for the definition of high, and low expression of ERβ1 suggest caution when comparing the cohorts since other unknown factor(s) may be driving the differences observed in this study.

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