Estrogen Receptor-β Affects the Prognosis of Human Malignant Mesothelioma

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Abstract

Malignant pleural mesothelioma is an asbestos-related neoplasm with poor prognosis, refractory to current therapies, the incidence of which is expected to increase in the next decades. Female gender was identified as a positive prognostic factor among other clinical and biological prognostic markers for malignant mesothelioma, yet a role of estrogen receptors (ERs) has not been studied. Our goal was to investigate ERs expression in malignant mesothelioma and to assess whether their expression correlates with prognosis. Immunohistochemical analysis revealed intense nuclear ERβ staining in normal pleura that was reduced in tumor tissues. Conversely, neither tumors nor normal pleura stained positive for ERα. Multivariate analysis of 78 malignant mesothelioma patients with pathologic stage, histologic type, therapy, sex, and age at diagnosis indicated that ERβ expression is an independent prognostic factor of better survival. Moreover, studies *in vitro* confirmed that treatment with 17β-estradiol led to an ERβ-mediated inhibition of malignant mesothelioma cell proliferation as well as $p21^{CIP1}$ and $p27^{KIP1}$ up-regulation. Consistently cell growth was suppressed by ERβ overexpression, causing a G_2-M-phase cell cycle arrest, paralleled by cyclin B1 and survivin down-regulation. Our data support the notion that ERβ acting as a tumor suppressor is of high potential relevance to prediction of disease progression and to therapeutic response of malignant mesothelioma patients. [Cancer Res 2009;69(11):4598–604]

Introduction

Malignant mesothelioma is an asbestos-related malignant tumor, the incidence of which will increase dramatically in the next decades (1). The mechanism of asbestos carcinogenesis has been linked to the activation of proinflammatory cytokines and nuclear factor-κB (2). Only a fraction of subjects exposed to high levels of asbestos develops malignant mesothelioma, suggesting that additional factors, such as SV40 infection (3) and genetic predisposition, may render some individuals more susceptible to asbestos carcinogenicity (4). Asbestos exposure induces cell death, whereas survival pathways rescue cells from asbestos damages and lead to transformation of human mesothelial cells (HMC). Female gender has been identified as a positive prognostic factor for malignant mesothelioma (5), although no experimental explanation of this finding has been provided thus far. This prompted us to explore the role of estrogens in determining malignant mesothelioma risk and prognosis. The role of estrogens in human tumors is still controversial (6) and different molecular mechanisms explaining estrogen pleiotropic functions in different tissues evolved rapidly during the past two decades. In particular, the mechanism by which 17β-estradiol (E2) induces cell proliferation has been the subject of extensive studies (7–12). However, recent reports showed that E2 could even decrease cell growth by significantly increasing apoptosis in breast cancer MCF-7 cell variants and several other cell types (13). Whether the E2 proliferative or apoptotic effects can be explained by the expression of different estrogen receptor (ER) isoforms is presently unknown. Two main subtypes of ERs have been described, ERα and ERβ. ERs are ligand-activated transcription factors that bind specific DNA sequences (estrogen response elements) regulating the expression of genes required for cell proliferation. However, transcriptional activity of the ER/E2 complex has not fully explained thus far. It has been assumed that E2 exerts survival and proliferative effects mainly by rapid nongenomic mechanisms originating from the hormone binding to ERα (8, 9, 14–16). The disruption of this pathway originated at the membrane level completely prevents the E2-induced DNA synthesis and cyclin D1 expression (17). These results point to the concept that ERα is the primary endogenous mediator of rapid E2 actions committed to cell proliferation. Less information is available on the role played by ERβ in E2-dependent proliferation. Data from cell cultures, gene expression, and knockout mice clearly indicate that E2-activated ERβ may function as a tumor suppressor by modulating the proliferative effects of ERα (18–21). These studies support a functional antagonism between ERα and ERβ with respect to the E2-induced cell proliferation but do not clarify either the putative role of ERβ in E2-induced apoptosis or the signal transduction pathways involved. Evidences on the ability of ERβ to activate or inactivate rapid nongenomic mechanisms have been reported (22–24), although the existence of nongenomic mechanisms underling the antiproliferative effects of ERβ is still unknown.

In the present study, we aimed at examining ERα and ERβ expression in malignant mesothelioma cells and their clinical and biological significance.

Materials and Methods

Reagents and antibodies. Monoclonal antibody to α-tubulin and polyclonal antibodies to ERα ERβ, $p21^{CIP1}$, and $p27^{KIP1}$ were from Santa Cruz Biotechnology. Monoclonal antibodies to survivin and cyclin B1 were

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Table 1. Main subjects' characteristics at diagnosis by sex (n = 78)

	Male (n = 59)	Female (n = 19)	All (n = 78)
Age at diagnosis, y, median (range)	64 (45-81)	62 (32-80)	63 (32-81)
Histologic type, n (%)			
Epithelioid	42 (71.2)	15 (78.9)	57 (73.1)
Biphasic	11 (18.6)	3 (15.8)	14 (17.9)
Sarcomatoid	6 (10.2)	1 (5.3)	7 (9.0)
Stage, n (%)			
T_2	15 (25.4)	7 (36.8)	22 (28.6)
T_3	30 (50.8)	4 (21.1)	34 (43.6)
T_4	14 (23.7)	8 (42.1)	22 (28.2)
Chemotherapy, n (%)			
No	17 (28.8)	10 (52.6)	27 (34.6)
Yes	42 (71.2)	9 (47.4)	51 (65.4)
ERβ expression, n (%)			
Negative/low	51 (86.4)	15 (78.9)	66 (84.6)
High	8 (13.6)	4 (21.1)	12 (15.4)

from Cell Signalling Technology. ECL was from Amersham Pharmacia Biotech. Nitrocellulose membranes and protein assay kits were from Bio-Rad. Anti-mouse and anti-rabbit IgG peroxidase-conjugated antibodies, E2, and chemical reagents were from Sigma-Aldrich. All reagents were of analytic grade. Culture medium, sera, antibiotics, and Lipofectamine were from Invitrogen.

Patients and tissue specimens. Seventy-eight cases of malignant mesothelioma and 21 controls were selected from the archival pathology files of the Pathology Unit of the Regional Hospital of Mestre-Venice, Italy. All diagnoses of mesothelioma were based on WHO criteria (25) and confirmed in all instances by clinical, morphologic, and immunohistochemical data. The tissue samples consisted in videothoracoscopy biopsy or surgical specimens, fixed in neutral formalin, and embedded in paraffin.

Immunohistochemistry. Immunohistochemical stain was done on 3-μm-thick paraffin sections with the monoclonal antibody recognizing ERβ clone EMR02 (Novocastra). Tissue sections were deparaffinized according to established procedures and quenched with 3% hydrogen peroxidase for 5 min. They were then washed in running water and TBS consisting of 50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.05% Tween 20. Heat-induced antigen retrieval was done using a microwave oven and citrate buffer (0.01 mol/L; pH 7.0) for 40 min at 98°C. Sections were incubated with mouse monoclonal antibody anti-ERβ diluted 1:50 overnight at 4°C followed by testing with a sensitive avidin-streptavidin-peroxidase technique (Biogenex). Diaminobenzidine tetrahydrocloride was used as the chromogen and sections were counterstained with hematoxylin. Distribution and intensity were considered in the semiquantitative assessment of nuclear staining pattern according the modified Allred Score system used in breast cancer. For each case, staining intensity (0, no staining; 1+, weak; 2+, moderate; and 3+, strong) together with the percentage in 33% increments (1, 0-33%; 2, 33-67%; and 3, 67-100%) of cells expressing the receptor was recorded (26). Immunoreactive scores (range, 0-9) were calculated by multiplying percentage score of positive cells with staining intensity score. Slides were independently scored by three different pathologists without knowledge of clinicopathologic or disease outcome variables.

Cell culture treatments and transfection. We used human breast epithelial carcinoma T47D cells purchased from the American Type Culture Collection, established malignant mesothelioma cell lines MPP89 and MSTO-211H from IST Cell Bank of Genoa, MMCA, MMP, and MMB stabilized from pleural effusions of malignant mesothelioma patients (27), and primary HMC-TERT cultures obtained from patients with congestive heart failure and immortalized by expression of a human telomerase subunit (28). In particular, for *in vitro* experiments, we used the epithelioid malignant mesothelioma-derived REN cell line kindly provided by Dr. Albelda. Cells were cultured in RPMI supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere. Before the experiments, cells were maintained in the same medium lacking phenol red and containing charcoal-stripped FCS prepared as described previously (29). E2 was dissolved in ethanol and added to cell culture at the indicated concentrations; the final ethanol vehicle concentration was maintained at 0.1%. Samples indicated as controls received vehicle (0.1% ethanol).

Cells grown to 80% confluence in tissue culture dishes were transiently transfected with the pCXN2 plasmid coding for human wild-type ERβ or with the Vp16-ERα from Addgene by the Lipofectamine reagent as described by the manufacturer. Gene silencing was done using small interfering RNA (siRNA) from Qiagen targeting the CTGGTCGTGTGAAGGATGTAA sequence. Nonsilencing siRNA (Qiagen) was used as a control.

Cell lysis and immunoblot. Cells were extracted with 1% NP-40 lysis buffer [1% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8), 5 mmol/L EDTA, 10 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 0.4 mmol/L Na_3VO_4, 10 μg/mL leupeptin, 4 μg/mL pepstatin, and 0.1 unit/mL aprotinin]. Cell lysates were centrifuged at 13,000 × g for 10 min and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad). Proteins were separated by SDS-PAGE under reducing conditions. Following SDS-PAGE, proteins were transferred to nitrocellulose, reacted with specific antibodies, and then detected with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent. Densitometric analysis was done using the GS 250 Molecular Imager (Bio-Rad). For cyclin D1 expression, cells were extracted in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7), 0.4 mmol/L Na_3VO_4, 10 μg/mL leupeptin, 4 μg/mL pepstatin, and 0.1 unit/mL aprotinin] and analyzed as indicated above.

Proliferation assay by cell count. REN or MSTO-211H cells were seeded at a density of 10×10^4 per well on 6-well plates in growth medium with FCS and incubated overnight at 37°C in a humidified environment containing 5% CO_2 to allow adherence. Cells were alternatively transiently transfected with the pCXN2 ERβ or Vp16-ERα plasmids, with empty vector, ERβ siRNA, or nonsilencing siRNA, by the Lipofectamine reagent as described by the manufacturer. After 24 h, cells were treated 24, 48, and 96 h with 10 nmol/L E2 in 2% charcoal-stripped FCS growth medium. Cells were then trypsinized and stained with trypan blue. The number of cells considered viable was counted in a Burker chamber within 5 min after staining.

Cell cycle analysis. For cell cycle/apoptosis analysis, 5×10^5 cells per well were seeded on tissue culture plates and treated with 10 nmol/L E2 for 24 h at 37°C in a 5% CO_2 atmosphere. After incubation, adherent cells were detached with trypsin (0.5% trypsin/0.1% EDTA in PBS). Detached and suspended cells were harvested in complete DMEM and centrifuged at 500 × g for 10 min. Pellets were washed with PBS and fixed with ice-cold 75% ethanol overnight at 4°C, treated with 100 μg/mL RNase A (Sigma), and

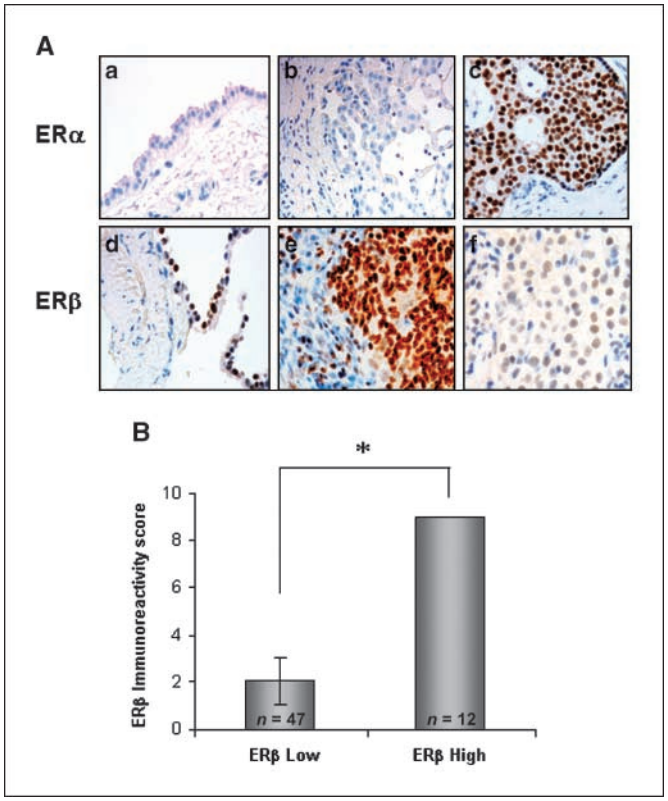


Figure 1. *A,* representative ERα (*top*) and ERβ (*bottom*) immunohistochemical staining in normal pleura and in a malignant mesothelioma tissue (×200). ERα: no immunoreactivity is evidenced either in normal pleura (*a*) or in tumor tissue (*b*) but is strongly expressed in case control (human breast cancer; *c*). ERβ: an intense nuclear staining (3+) is evidenced in normal pleura (*d*) and in the epithelial component of a biphasic malignant mesothelioma (*e*); the spindle-cell sarcomatoid component is negative. *f,* an example of epithelioid malignant mesothelioma with weak ERβ nuclear staining (1+). *B,* comparison of ERβ immunoreactive scores (mean ± SD) calculated in high and low expresser malignant mesothelioma. *, $P < 0.05$, significant statistical differences.

subsequently stained with 25 μg/mL propidium iodide (Sigma) and then were analyzed by using a flow cytometer FACS (Becton Dickinson) and Modfit software (Verity Software House).

Statistical analyses. Pearson's χ^2 test, Fisher's exact test (for categorical variables), and independent-samples *t* test (for continuous variables) were used to compare all baseline factors between male and female patients. Kaplan-Meier estimates (30) of the cumulative probability of death, defined as the time from diagnosis to the time of death from any cause, were obtained for all baseline factors, including age, sex, tumor stage, histologic type, and ERβ expression. A multivariate Cox proportional hazards model (31) was used to assess the independent prognostic effect of all factors under investigation. All calculations were done using SPSS (version 15; SPSS) and Stata (version 9; Stata) software.

Results

Normal pleura and malignant pleural mesothelioma tissues express ERβ. We examined the expression of ERα and ERβ in a well-defined cohort of malignant mesothelioma patients with >5 years of follow-up. Paraffin-embedded, histologic materials were collected from 78 patients (clinicopathologic characteristics are reported in Table 1) and 21 normal controls and ERα and ERβ were analyzed by immunohistochemistry. Intense nuclear ERβ staining was observed in all normal pleural specimens and at a lesser intensity and extent in 59 of 78 (76%) tumor samples. Twelve of positive tumors had >75% stained cells with high intensity, thus comparable with normal tissues, whereas the remaining had <75% stained cells with weak or moderate intensity (Fig. 1*A, d-f*). Immunoreactive scores (range 0-9) were calculated, as described in Materials and Methods, for these two categories; mean ± SD is reported in Fig. 1*B*. Moreover, in biphasic tumors, ERβ positivity was restricted to the epithelial component. Neither tumors nor normal pleura stained positive for ERα (Fig. 1*A, a-c*).

ERβ represents a new independent prognostic factor for malignant mesothelioma. Table 1 summarizes the distribution of gender according to established clinicopathologic factors and ERβ expression at diagnosis. Overall, 59 of 78 (76%) patients were males and only 19 (24%) were females. Median age was 63 years, nearly the same in men as in women; histologic tumor types were well balanced between males and females, with a great predominance of epithelioid tumor (73%) on biphasic (18%) and sarcomatoid (9%) types. Moreover, chemotherapy was done more frequently in males (71%) than in females (47%) even if differences were not statistically significant. ERβ expression was evenly distributed between men and women. After a median follow-up time of 1.2 years (interquartile range, 0.5-2.2 years), a total of 56 (72%) subjects died. Overall survival curves according to sex and ERβ expression are shown in Fig. 2*A* and *B*. Median survival times for females were 2.1 versus 1.1 years for males ($P = 0.01$, log-rank test). The cumulative probability of survival after 2 years of follow-up was 80% [95% confidence interval (95% CI), 41-95%] for subjects with high ERβ expression versus 31% (95% CI, 20-43%) for subjects with negative of low ERβ expression ($P = 0.02$, log-rank test). Table 2 shows Cox multivariate analysis of overall survival adjusted for age at diagnosis and chemotherapy. Histologic sarcomatoid and biphasic tumor type (versus epithelial type) were associated with a significant increased risk of death [hazard ratio (HR), 6.8; 95% CI, 2.6-17.4 and HR, 5.1; 95% CI, 2.5-10.8, respectively]. Moreover, high ERβ expression and, to a lesser extent, female gender were independent protective factors of all-cause mortality (HR, 0.2; 95% CI, 0.05-0.6 and HR, 0.5; 95% CI, 0.2-1.0, respectively). To clarify if

Table 2. Cox proportional hazard model of overall survival ($n = 78$)

	n	HR (95% CI)	*P**
Sex			
Male	59	1.00	
Female	19	0.49 (0.24-1.01)	0.05
Histologic type			
Epithelioid	57	1.00	
Biphasic	14	5.14 (2.46-10.77)	<0.001
Sarcomatoid	7	6.75 (2.62-17.39)	<0.001
Stage			
T_2	22	1.00	
T_3	34	1.90 (0.91-3.95)	0.09
T_4	22	1.88 (0.91-3.90)	0.09
ERβ expression			
Negative/low	66	1.00	
High	12	0.18 (0.05-0.61)	0.006

NOTE: Risk estimates are adjusted for age at diagnosis and chemotherapy.
*Two-sided Wald test.

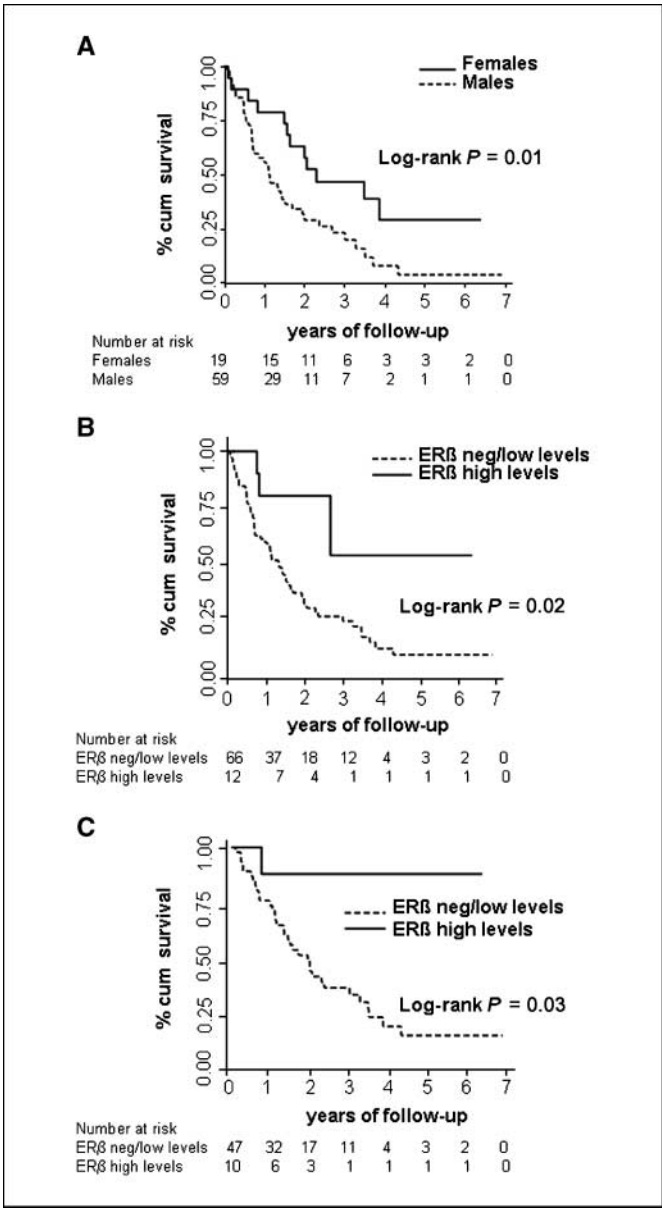


Figure 2. Kaplan-Meier survival curves by gender (*A*) and ERβ staining using cut-off of high positive tumor cells in the whole group of malignant mesothelioma patients (*B*) or restricted to patients with epithelioid malignant mesothelioma (*C*).

the effects that we attributed to the ERβ could have been an artifact caused by the fact that sarcomatoid and biphasic mesotheliomas do not express ERβ, we carried out a separate analysis among the 57 epithelial tumors (Fig. 2*C*) and the results obtained were very similar to those reported for the whole group (HR, 0.1; 95% CI, 0.01-0.9).

ERβ overexpression causes malignant mesothelioma cell growth arrest, whereas E2 inhibits malignant mesothelioma cell proliferation via ERβ. We hypothesized that the observed decreased expression in mesothelioma samples could reflect tumor suppressor properties for ERβ. To test this hypothesis, we performed studies *in vitro*. When we evaluated ER positivity in HMC (HMC-TERT) and in six different human malignant mesothelioma-derived cell lines (MMCA, MMP, REN, MMB, MPP89, and MSTO-211H) by Western blot analysis, we obtained results comparable with *in vivo* data (Fig. 3*A*). HMC-TERT cells showed the highest levels of ERβ expression, the malignant mesothelioma cell lines showed different levels of expression, and one, the MSTO-211H, resulted negative. Neither HMC-TERT nor malignant mesothelioma cells stained positive for ERα. The following experiments were done on REN cells, derived from an epithelioid malignant mesothelioma, expressing moderate levels of ERβ. *In vitro* treatment with 10 nmol/L E2 of REN cells slowed progression through all phases of the cell cycle, thereby reducing the rate of cell division at longer times (Fig. 3*B*) while maintaining a static DNA profile with the same percentage of cells in each phase of the cycle even at 96 h (Fig. 4*A*; data not shown). The precise mechanism by which E2 in mesothelioma cells leads to proportionately delayed progression throughout the cell cycle remains to be determined. E2 treatment led to the inhibition of REN cell proliferation through a ERβ-mediated mechanism because it was reverted by receptor silencing (Fig. 3*B*). Differently from E2 treatment, ERβ overexpression reduced >50% REN cell growth (Fig. 3*B*) and caused a G_2-M-phase cell cycle arrest (Fig. 4*A*), only weakly influenced by the presence of the ligand. Moreover, ERα overexpression, as shown in Fig. 3*C*, did not influence REN cell growth. Similar results were obtained when proliferation was assayed in ER-negative MSTO-211H cells transfected with expression vectors encoding ERβ or ERα (Fig. 3*D*).

ERβ overexpression induces G_2-M arrest by acting on cell cycle modulator proteins. The cell cycle is governed by a family of cyclins, cyclin-dependent kinases, and their inhibitors through activating and inactivating phosphorylation events. We observed that the treatment with 10 nmol/L E2 for 24 h of REN cells, with normal receptor levels or overexpressing ERβ, led to the up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$, whereas ERβ overexpression caused the ligand-independent down-regulation of cyclin B1 and survivin (Fig. 4*B*). Survivin is one of the few proliferation/antiapoptosis signature genes, implying that survivin down-regulation on ERβ induction might in part account for the more favorable clinical outcome in ERβ-containing cancers. Moreover, survivin was indicated as an ERβ-regulated gene in a recent microarray analysis done on breast cancer cells (32). Data concerning the modulation of proteins involved in cell cycle progression could in part explain the observed ligand-dependent and ligand-independent malignant mesothelioma cell growth modulation.

Discussion

In large retrospective series of pleural mesothelioma patients, important prognostic factors were found to be stage, age, performance status, histology, and gender (33).

In this article, we report clinical and biomolecular evidences supporting a role of ERβ expression as a further prognostic factor for malignant mesothelioma. Estrogens are key regulators of growth and differentiation in a broad range of target tissues, including the reproductive tract, mammary gland, and the central nervous and skeletal systems (34), but are also known to be involved in many pathologic processes such as breast and endometrial cancer (35). The cDNA encoding an E2 receptor protein was cloned in 1986 (36) and this receptor was long believed to be the only existing ER. However, 10 years later, an additional ER was cloned from rat prostate (37). This novel receptor was designated ERβ; consequently, the originally cloned

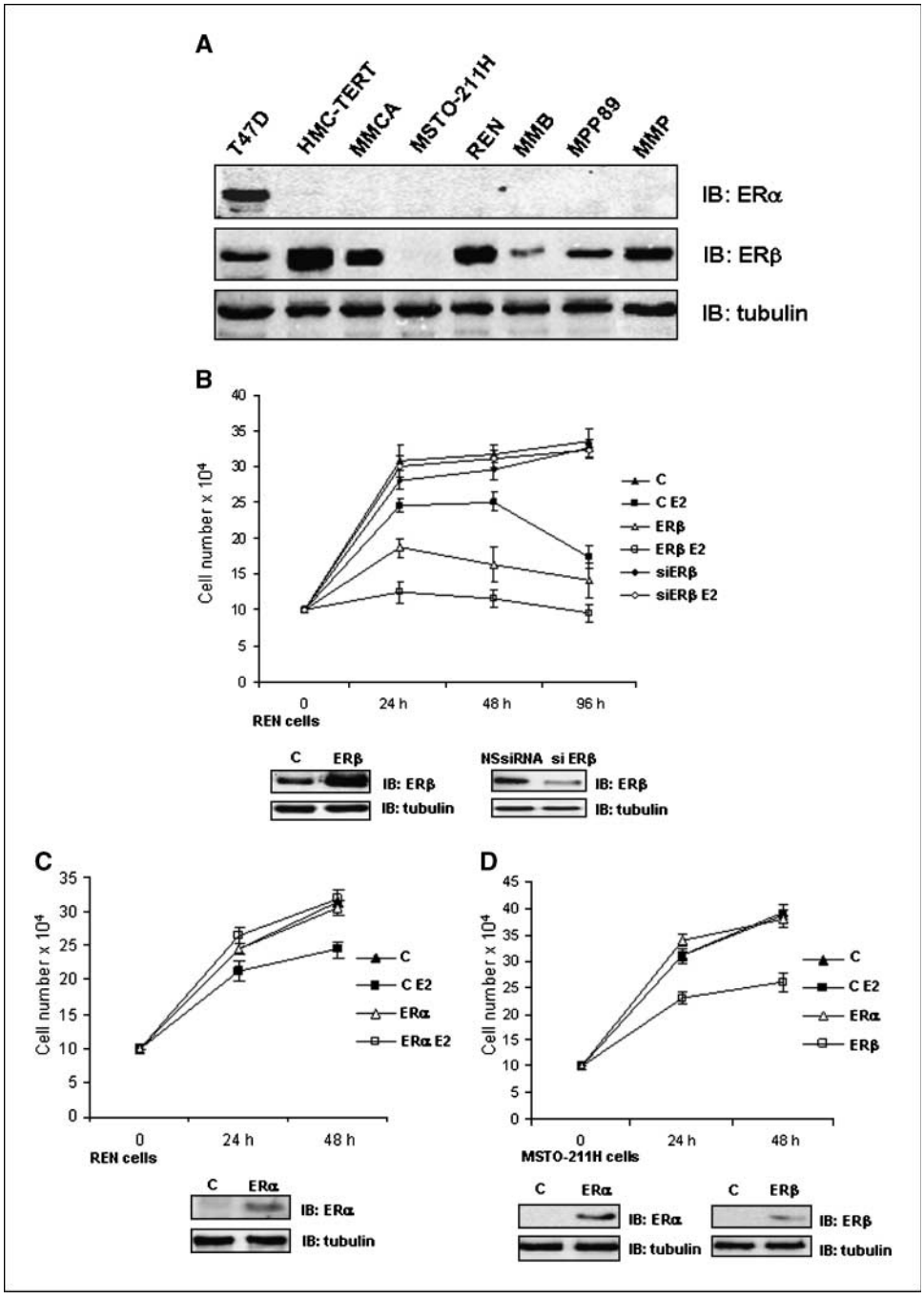


Figure 3. *A,* ERα and ERβ positivity was assayed by Western blot analysis in HMC (HMC-TERT) and in six different malignant mesothelioma cell lines (MMCA, MMP, REN, MMB, MPP89, and MSTO-211H). As a control, we used ER-positive T47D human breast cancer cells. The membrane was reblotted with antibodies to tubulin to show equal loading. *B* and *C,* growth curves of control (cell transfected with empty vector also representative of nonsilencing siRNA), ERβ and ERβ siRNA-transfected REN cells treated or not with 10 nmol/L E2 for 24, 48, and 96 h, and ERα-transfected REN cells treated or not with 10 nmol/L E2 for 24 and 48 h. Mean ± SD (n = 3). *D,* growth curves of control and ERβ- or ERα-transfected MSTO-211H. The same number of cells was seeded and cultured for 24 and 48 h. At each time point, the cells were assayed for proliferation. Mean ± SD (n = 3). Adjacent to each graph is a representative Western blot analysis that documents ERs expression in transfected cells. Tubulin staining indicates equal loading of the proteins.

ER was renamed ERα. ERα and ERβ belong to the superfamily of nuclear receptors and specifically to the family of steroid receptors that act as ligand-regulated transcription factors (38). Models of action involving cooperation, as well as competition, between the two proteins have been proposed (39). The tissue distribution of these two receptors varies and includes several estrogen "target" and "non-target" tissues. ER expression has been detected in normal lung and lung tumor tissues. No data are available instead on the expression of hormone receptors in normal pleura and malignant mesothelioma. Only two recent reports deal with ERs in malignant mesothelioma. One classifies malignant mesothelioma as ER negative (perhaps ERα), as a result of a survey made on a battery of immunohistochemical markers (40), whereas another reports a statistically significant increase of ESR1 methylation in malignant mesothelioma versus non tumor lung samples (41).

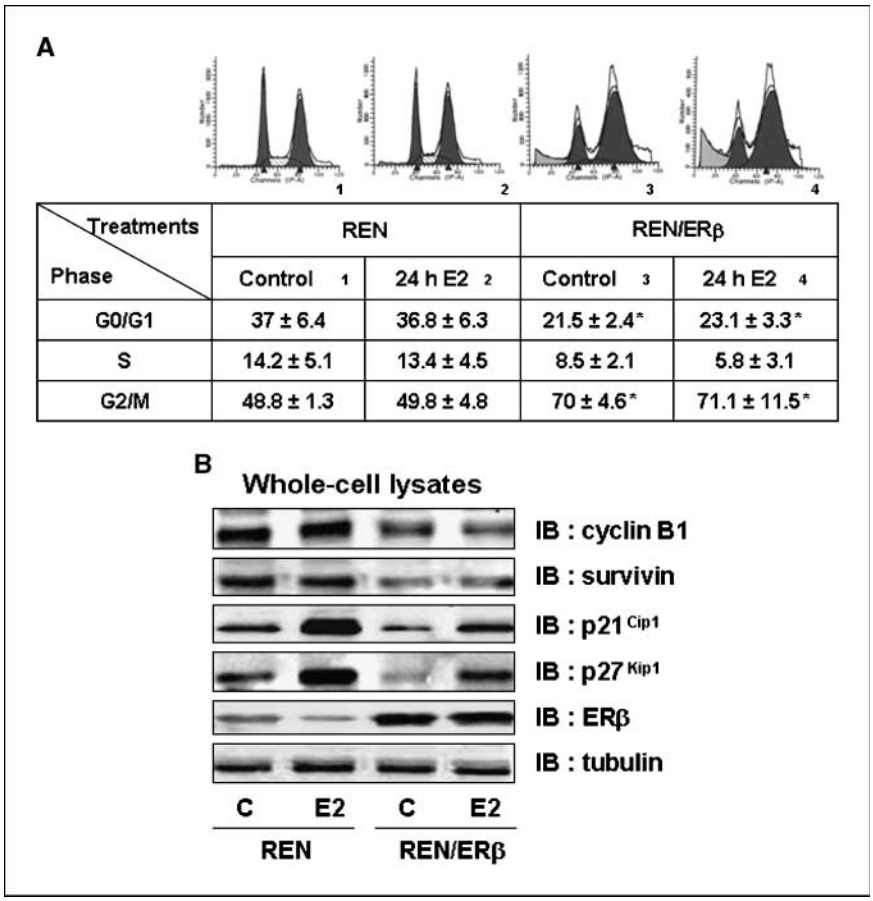
We first analyzed the expression of these two hormone receptors in normal pleura and malignant mesothelioma tissues. Nuclear ERβ immunoreactivity was detected in human normal pleura and in a fraction of the 78 malignant mesothelioma samples, even if with reduced extension and intensity, compared with controls. Differently from other lung cancers, none of 78 malignant mesothelioma biopsies or normal pleura stained positive with ERα antibody.

The main issue addressed in this study was whether expression of ERβ represents a prognostic factor in malignant mesothelioma. In our study, high levels of nuclear ERβ positivity in tumors were associated with significantly longer patient survival. Recently, Fasco et al. reported that the expression of ERs in lung cancer is gender-dependent and that ERα expression occurs more often in the lungs of women than of men (42). We did not found such distinction, although the number of women in our sample was small. Moreover, in multivariate analysis of overall survival, we showed the prognostic significance of ERβ staining even if the presence of ERβ polymorphisms, the expression of other ER isoforms, or the expression of hormones need further investigations. Estrogens levels may vary by sex and, in women, by the menopausal status. Therefore, different functions may be attributed to ERs in gender-specific fashion, as well as in women age-dependent manner, according to menopausal status. However, local estrogen production may be of even greater significance in the lung, where aromatase and 17β-hydroxysteroid dehydrogenase are expressed (43). Estrogens and ERs play important roles in regulating growth and differentiation of various tissues by acting through two potentially different ligand-activated mechanisms. One leads to an alteration of transcription, by binding of ERs to the estrogen response elements or to transcription factors in the promoter regions of target genes. Another mechanism requires very rapid and non-genomic actions of E2, dependent on the ability of ERα to activate proliferative pathways. The present article reports strong evidence that ERβ plays a role in the control of malignant mesothelioma cell proliferation. We show that *in vitro* treatment with E2 led to an inhibition of malignant mesothelioma cell proliferation via a receptor-mediated mechanism. Moreover, ERβ overexpression caused a G_2-M-phase cell cycle arrest of malignant mesothelioma REN cells in both a ligand-dependent and a ligand-independent manner. We observed that E2 treatment of REN cells resulted in an up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$ levels, whereas ERβ overexpression determined a down-regulation of cyclin B1 and survivin.

The results of the modulation of proteins involved in cell cycle progression could in part explain the observed modulation of ligand-dependent and ligand-independent malignant mesothelioma cell growth.

At present, we cannot discriminate between genomic and nongenomic actions exerted by ERβ; modulation of cell cycle regulating proteins is compatible with rapid signaling, but proliferation assays require longer hormone treatment and also genomic actions could act. An additional possible mechanism may be the crosstalk between ERs and growth factor receptor-mediated pathways at the plasma membrane (44, 45), such as the functional interactions described for ERβ and epidermal growth factor receptor (46).

In summary, we have identified differential nuclear ERβ expression in normal and malignant mesothelioma tissues and survival differences by ERβ status. Both ERβ overexpression and E2 ligand/ERβ interaction negatively affect malignant mesothelioma cell proliferation although presumably through different mechanisms. Despite that, our findings clearly address a suppressive role



Treatments / Phase	REN		REN/ERβ	
	Control 1	24 h E2 2	Control 3	24 h E2 4
G0/G1	37 ± 6.4	36.8 ± 6.3	21.5 ± 2.4*	23.1 ± 3.3*
S	14.2 ± 5.1	13.4 ± 4.5	8.5 ± 2.1	5.8 ± 3.1
G2/M	48.8 ± 1.3	49.8 ± 4.8	70 ± 4.6*	71.1 ± 11.5*

Figure 4. *A,* cell cycle analysis was done on control and ERβ-transfected REN cells treated with 10 nmol/L E2 for 24 h. After treatments, cells were stained with propidium iodide as described in Materials and Methods and analyzed for cellular DNA content by flow cytometry. Data reported in the bottom table represent mean ± SD ($n = 3$) of the percentage of cells in each phase of the cell cycle. Exemplificative histograms that plot cell count versus DNA content are reported for each treatment. *, $P < 0.05$ compared with controls. *B,* proliferation rate of REN cells treated with 10 nmol/L E2 for 24, 72, and 96 h versus untreated cells. Mean ± SD ($n = 3$). *C,* Western blot analysis of cyclin B1, survivin, $p21^{CIP1}$, $p27^{KIP1}$, and ERβ expression in control and ERβ-transfected REN cells treated 24 h with 10 nmol/L E2. Tubulin staining indicates equal loading of the proteins. Representative experiment of three distinct experiments.

of ERβ in this tumor and provide the rationale to consider ERβ status in mesothelioma as a prognostic marker with potential implications for therapeutical manipulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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