

# Aromatase Expression Predicts Survival in Women with Early-Stage Non–Small Cell Lung Cancer

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## Abstract

**Estrogen signaling is critical in the progression of tumors that bear estrogen receptors. In most patients with breast cancer, inhibitors that block interactions of estrogen with its receptors or suppress the production of endogenous estrogens are important interventions in the clinic. Recent evidence now suggests that estrogen also contributes to the pathogenesis of non–small cell lung cancer (NSCLC). We used a human lung cancer xenograph model system to analyze the effect of aromatase or estradiol on tumor growth. We further examined the level of protein expression of aromatase in 422 patients with NSCLC using a high-density tissue microarray. Results were confirmed and validated on an independent patient cohort ( $n = 337$ ). Lower levels of aromatase predicted a greater chance of survival in women 65 years and older. Within this population, the prognostic value of aromatase was greatest in earlier stage lung cancer (stage I/II). In addition, for women with no history of smoking, lower aromatase levels were a strong predictor of survival. Our findings implicate aromatase as an early-stage predictor of survival in some women with NSCLC. We predict that women whose lung cancers have higher levels of aromatase might be good candidates for targeted treatment with aromatase inhibitors.** [Cancer Res 2007;67(21):10484–90]

## Introduction

Lung cancer remains the leading cause of cancer-related deaths for both men and women (1). In the United States alone, an estimated 213,380 new cases of lung cancer will be diagnosed in 2007 and ~160,390 individuals will die due to this disease (1). Very little is understood about the underlying mechanisms of development and progression of these neoplasms. Moreover, the search for effective therapeutic modalities continues to be elusive. Interestingly, the incidence of lung cancer in men has somewhat declined over the last decade whereas the reported cases in women has steadily increased. A primary factor for this is the increased level of smoking by women; however, this does not seem to be the only determinant as nonsmokers who develop lung cancer are predominantly women. Recent evidence suggests that estrogen may play a role in normal lung function as well as in the

development of lung cancer (2–11). Estrogen receptors (ER)  $\alpha$  and  $\beta$  are expressed in normal lung and many non–small cell lung cancers (NSCLC). In both *in vitro* and *in vivo* models, 17 $\beta$ -estradiol is mitogenic for NSCLC cells and stimulates gene transcription (2, 3, 5). These effects are inhibited by the ER antagonist, Faslodex (fulvestrant), as is tumor formation in a human NSCLC xenograph model *in vivo* (2).

A key enzyme in estrogen biosynthesis is aromatase (CYP19; ref. 12–14). Aromatase, a member of the cytochrome P450 family, converts the androgens androstenedione and testosterone to estrone and estradiol, respectively. In addition to its expression in the ovary and placenta, aromatase is present in male and female extragonadal tissues including breast, lung, brain, and liver (15). Aromatase expression is elevated in certain malignancies, such as breast and endometrial carcinomas, prompting the theory that the tumor-promoting properties caused by the stimulation of ER pathways could be enhanced by circulating estrogen as well as by localized autocrine or paracrine production of the steroid hormone. This rationale led to several clinical studies that confirmed the anti-tumor efficacy of aromatase inhibitors in breast cancer (13, 16–18).

Here, we examined on a population basis whether changes in the expression levels of aromatase were diagnostic for NSCLC and/or predictive of disease outcome. We observed that relatively high aromatase levels correlated with a worse prognosis of disease outcome, especially in women >65 years with early-stage NSCLC.

## Materials and Methods

**Tumor xenograph studies.** The protocol used for implantation of human lung tumor xenografts in nude mice was described previously (6, 19). Fifteen 6-week-old ovariectomized athymic female mice were injected s.c. with A549 cells ( $2 \times 10^8$  cells/mouse) and subsequently treated with daily s.c. injections with androstenedione, estradiol, or buffer control (five mice per group) as previously described (6, 19).

**Aromatase activity.** NSCLC specimens were procured under an Institutional Review Board–approved protocol from seven males and five females. Half of each specimen was snap-frozen at  $-70^\circ\text{C}$  and half was formalin-fixed, processed, and embedded in paraffin. Aromatase activity was assessed on the frozen aliquots by radioassay and immunohistochemistry was done on formalin-fixed, paraffin-embedded sections (see below). The activity of the enzyme was measured using the radiolabeled substrate [ $1\beta$ - $^3\text{H}$ ]androst-4-ene-3,17-dione (Perkin-Elmer) as previously reported (6). Aromatase activity was then correlated with protein expression by immunohistochemistry using linear regression analysis (6).

**Lung tissue microarray.** A lung tissue microarray (TMA) was constructed under appropriate Institutional Review Board and Health Insurance Portability and Accountability Act regulations, using archival formalin-fixed, paraffin-embedded lung tissue samples from the Department of Pathology and Laboratory Medicine at the University of California at Los Angeles Medical Center as previously described (8). The TMA contained tissue from 696 surgical specimens from 671 patients. Of these samples, 422 were marker-informative cases linked to outcome information

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(survival versus death due to disease) for individuals with NSCLC. Sampled tissues included primary lung tumor, matched nonneoplastic lung parenchyma, and metastatic lung carcinoma to lymph nodes and distant sites. Sections of all blocks that were used were reviewed by a board-certified pathologist to confirm the diagnosis. At least three core tissue biopsies, each 0.6 mm in diameter, were taken from select, morphologically representative regions of each paraffin-embedded lung tumor and precisely arrayed using a custom-built instrument, as previously described (8, 20–22). Supplementary Table S1 summarizes the patient cohort used in this study. The demographics, histopathologic distribution, grade, stage, clinical variables, smoking history, and outcome (survival and death due to disease) of the population studied here were similar to those reported in the United States (ref. 23; American Cancer Society. Cancer Facts & Figures 2006; American Lung Association, Trends in Lung Cancer Morbidity and Mortality, May 2005; National Cancer Institute, SEER Cancer Statistics Review, 1975–2003). Individuals who were classified as “nonsmokers” had no history of ever smoking cigarettes. Individuals classified as “smokers” had all smoked >100 cigarettes at one point in their life.

**Immunohistochemistry.** Lung TMA blocks were sectioned immediately prior to being stained. The protocol for slide preparation and section staining has previously been described (6, 21). Goat anti-human aromatase (CYP19A) antibody C-16 was purchased from Santa Cruz Biotechnology. The entire lung TMA was stained using a standard two-step indirect immunohistochemistry protocol (6). Briefly, TMA sections were incubated in 0.01 mol/L of sodium citrate buffer (pH 6.0) at 95°C for 30 min for antigen retrieval. Endogenous peroxidases were quenched using 3% hydrogen peroxide, and nonspecific binding was blocked with Background Sniper (Biocare Medical) for 5 min. After rinsing, slides were incubated with anti-aromatase antibody (1:100 dilution) for 1 h at room temperature. Antibody binding was detected using the Dako Envision System with diaminobenzidine. The sections were counterstained with Harris’ hematoxylin. Negative controls were done on identical TMA sections using the same protocol as above except with nonimmune goat IgG replacing the primary antibody. A semiquantitative analysis of the aromatase-stained lung TMA was done on relevant malignant cells or normal lung epithelium by a board-certified pathologist (V. Mah) blinded to clinical information, and spot-checked by a second pathologist (D.B. Seligson). Cytoplasmic aromatase staining per array spot was determined based on staining intensity (0, below the level of detection; 1, weak; 2, moderate; and 3, strong) and the percentage of cells staining at each intensity level (0–100%; ref. 20). A final integrated value of intensity and frequency was derived using the following formula:  $[(3x) + (2y) + (1z)] / 100$ , where  $x$ ,  $y$ , and  $z$  are the percentages of staining at intensity 3, 2, and 1, respectively. This value was then used to compare tissue staining.

**Statistical analysis.** Analyses were done using R software<sup>6</sup> (including survival and rpart packages), which are available for free. Pooling criteria are discussed in the Supplementary Materials. Aromatase expression differences among various subgroups were determined using the Wilcoxon signed rank test or Kruskal-Wallis rank-sum test. For dichotomized (high versus low) aromatase expression, the Fisher exact test or Pearson  $\chi^2$  test was used for analysis with categorical variables such as stage, grade, and smoking history. Survival curves were calculated using the Kaplan-Meier method and comparisons were done using the log-rank test. The Cox proportional hazards model (univariate and multivariate) was used to determine the significance of various factors related to survival. The proportional hazards assumption was verified using Schoenfeld, martingale, and dfbeta residuals. Log-rank and Fisher exact  $P$  values were two-sided and a  $P < 0.05$  was considered significant.

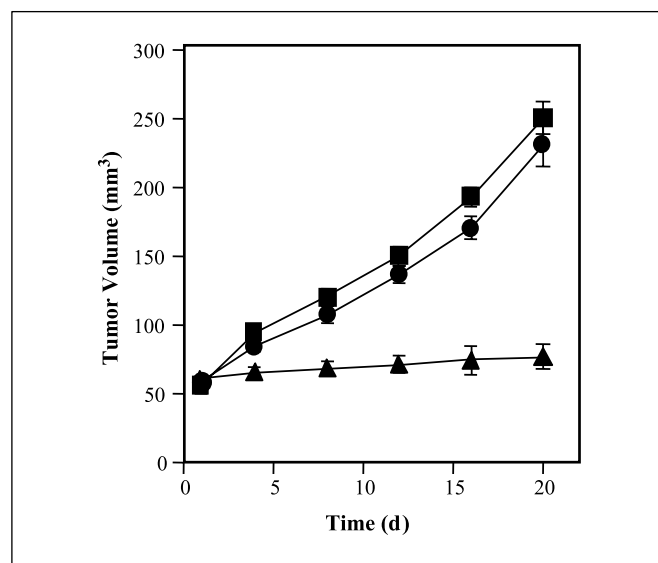
The midpoint and median intensity of 1.5 was used to define low and high aromatase expression. This represents a conservative, nonbiased decision which was used in order to prevent any artificial overfitting of the data. However, in addition to this, using recursive partitioning, regression trees (rpart package), and plotting log-rank  $P$  values versus hazard ratios, we independently determined the cut-point that gave optimal results for the

relevant subpopulations discussed here. This value (1.3) is close to the midpoint value used here; relevant results and a discussion of this cut-point determination is presented in the Supplementary Materials. Internal validation to protect against overfitting of data was done as described (24, 25).

## Results

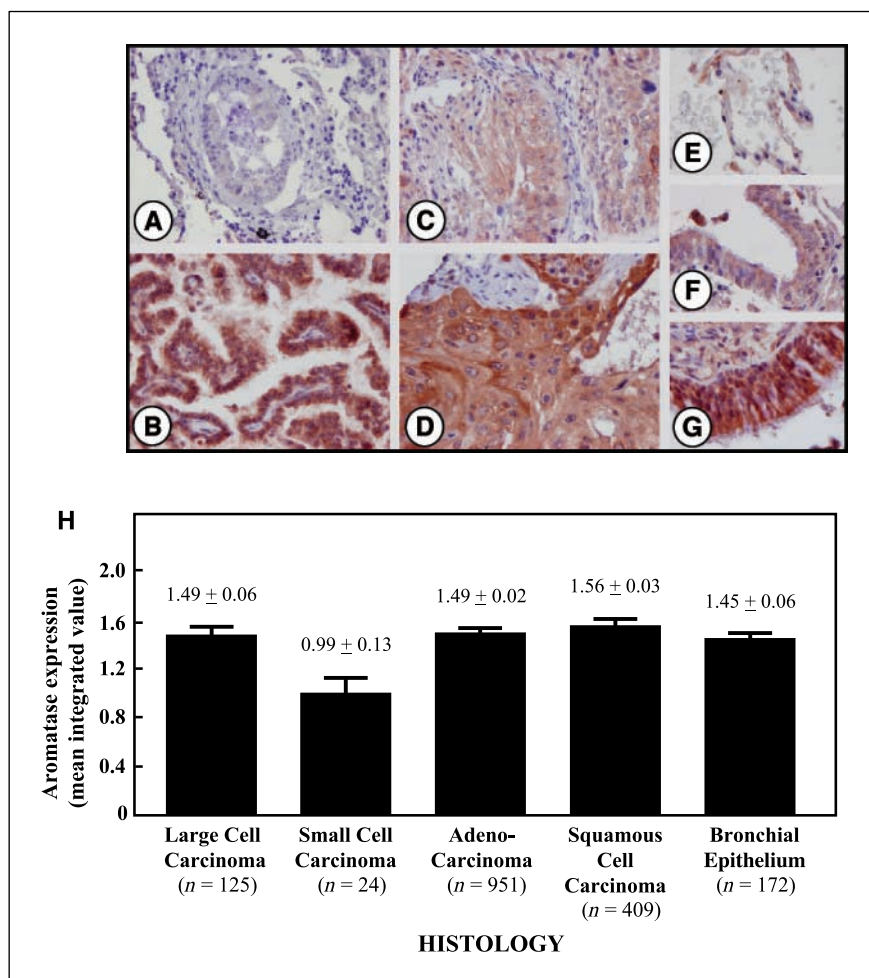
Previous results suggest that increased local estrogen levels and/or enhanced stimulation of the ER signaling pathway might play a role in lung cancer progression (2–4, 6). In this study, we examined aromatase, a key enzyme in the estrogen synthesis pathway. We hypothesized that expression of aromatase in lung cancer cells could result in a focal increase in estrogen levels and serve to promote tumor development and/or progression. Recently, we have shown that aromatase is expressed and is active in human lung cells (6). An aromatase inhibitor reduces cell growth *in vitro* and lung tumor xenograph growth in nude mice (6). To further assess estrogen signaling in NSCLC *in vivo*, we implanted A549 lung tumor cells which express endogenous aromatase in ovariectomized female athymic mice. As shown in Fig. 1, mice treated with androstenedione, an aromatase substrate, showed significantly enhanced tumor growth compared with untreated control animals ( $P < 0.001$ ). Moreover, treatment with estradiol, the ligand for ER, showed a similar effect on enhancing lung tumor growth (Fig. 1).

Based on the results above, as well as earlier studies showing enhanced levels of aromatase expression and activity in NSCLC cell lines and human tumor samples (6), we initiated a study to examine the *in situ* expression of aromatase on a population basis using a high-density lung TMA consisting of malignant and benign clinical samples (the clinical and pathologic patient categories of the TMA are shown in Supplementary Table S1). Protein expression levels were determined by immunohistochemical staining. In this study, 1,681 TMA spots were stained representing 422 surgical



**Figure 1.** Growth-promoting effects of estrogen and aromatase substrate on non-small cell lung tumor xenografts *in vivo*. A549 lung tumor cells which express endogenous aromatase were implanted as subcutaneous xenografts in ovariectomized female athymic mice. Mice were then divided into three groups (five animals per group). ■, animals were given daily s.c. treatments of the aromatase substrate androstenedione as previously described (6). ●, animals were given daily s.c. treatments of estradiol in a biodegradable binder as previously described (19). ▲, control animals were supplemented with s.c. injection of vehicle alone. Growth of A549 tumors was significantly increased in mice treated with androstenedione and estradiol as compared with control animals ( $P < 0.001$  for both).

<sup>6</sup> <http://www.R-project.org>

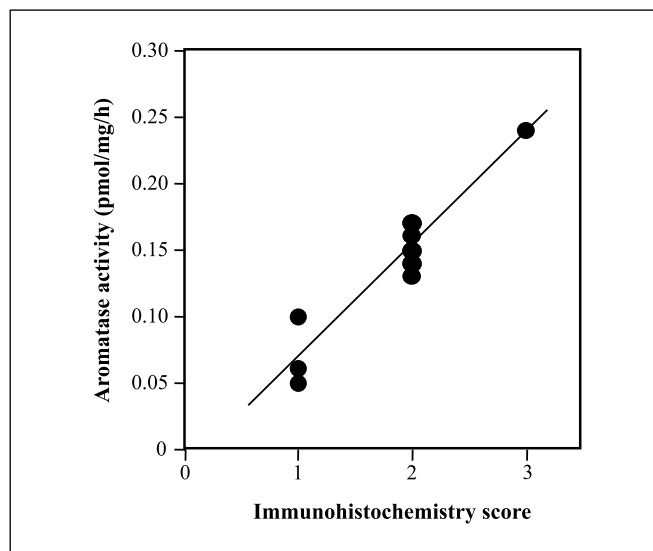


**Figure 2.** Aromatase expression in lung tissue samples. A–G, immunohistochemical staining of aromatase. Expression of aromatase in the cytoplasm of representative cases of lung adenocarcinomas showing weak (A) and strong (B) staining. Expression of aromatase in the cytoplasm of representative cases of lung squamous cell carcinomas showing weak (C) and strong (D) staining. Aromatase expression in histologically normal alveolar lining (E) and bronchial epithelial cells with low (F) and high (G) expression. In all experiments, substitution of anti-aromatase antibody with species-matched nonimmune antibody gave no staining. H, aromatase expression distribution as classified by TMA spot-level histologic category. Columns, means; bars, SE. Only small cell carcinoma showed significant differences from other categories ( $P < 0.001$  compared with large cell carcinoma, adenocarcinoma, and squamous cell carcinoma;  $P = 0.005$  compared with bronchial epithelial cells).

cases. Aromatase displayed a cytoplasmic localization with patterns ranging from below the level of detection (score of 0.0) to abundant (score of 3.0; Fig. 2A–G). Moreover, aromatase staining intensity as determined by immunohistochemistry, correlated well with aromatase enzyme activity as assessed by a radioassay (Fig. 3). Aromatase staining was observed in all tumor subtypes as well as nonneoplastic bronchial/bronchiolar epithelium. On a per-spot basis, mean integrated staining intensities (a measure of intensity and cellular frequency; see Materials and Methods) were similar among adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and nonneoplastic bronchial epithelium (Fig. 2H).

#### Aromatase expression predicts disease outcome in women.

Despite the lack of significant differences of aromatase staining on a per-spot basis, there was patient-to-patient diversity in the staining pattern. Therefore, we examined whether aromatase expression levels offered any predictive value for patient survival from disease-related death. We first examined aromatase expression as a continuous variable. Using the univariate Cox proportional hazards model, aromatase expression approached significance as a predictor of survival ( $P = 0.056$ ; hazard ratio, 1.28; 95% confidence level, 0.994–1.65). To consider whether aromatase expression was predictive for a subset of the population, we next studied a dichotomized population. We initially chose the midpoint-integrated intensity (1.5 on a scale of 0.0–3.0) to define “higher” and “lower” expression of aromatase (see Materials and Methods). Coincidentally, 1.5 was also the median value for aromatase-integrated



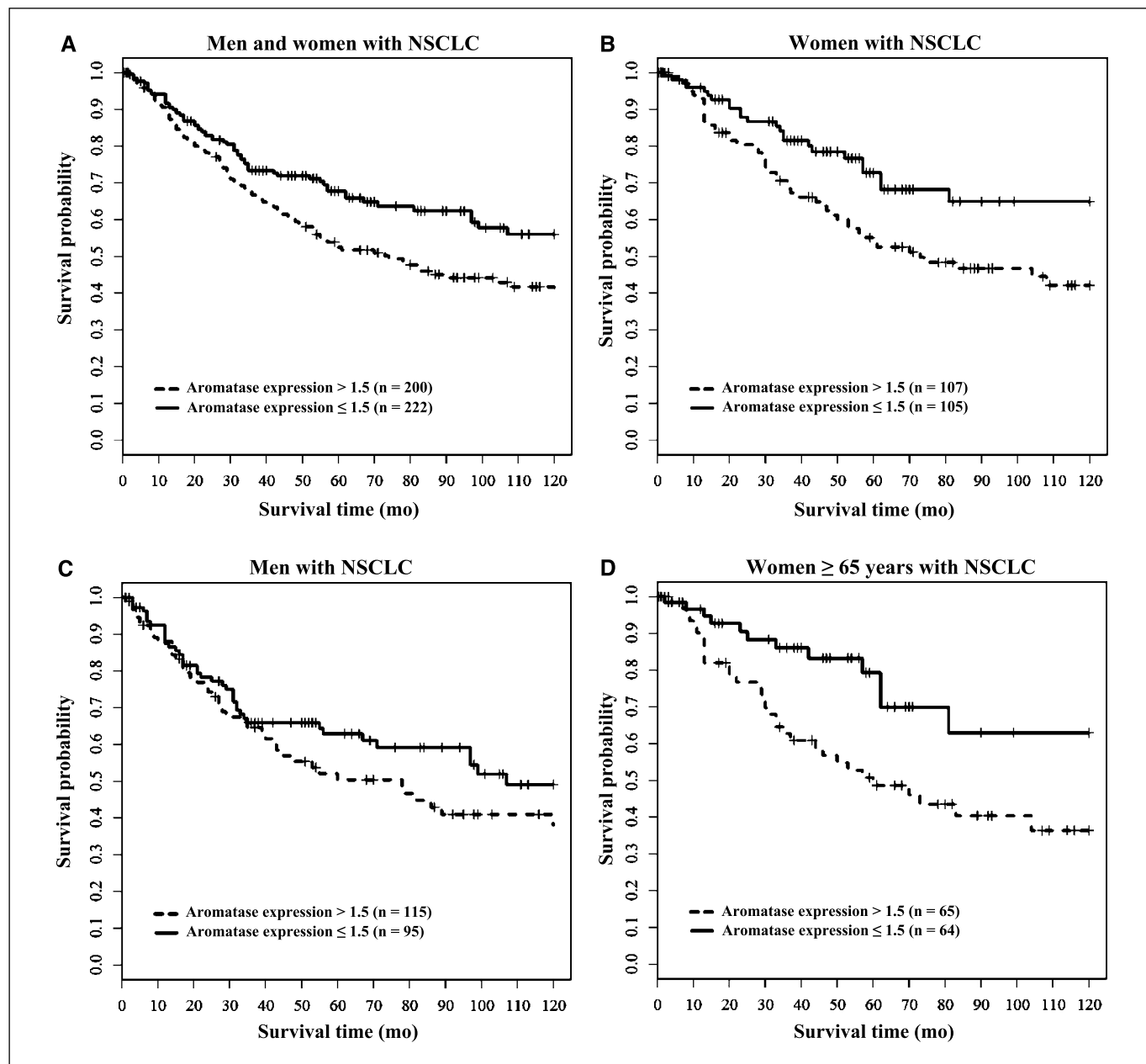
**Figure 3.** Levels of NSCLC aromatase protein expression correlate well with tumor enzyme activity. Frozen archival NSCLC specimens from seven males and five females were examined for aromatase enzyme activity and for protein expression. These specimens correlated with cases on the TMA. Enzyme activity was determined by radioassay and protein expression was assessed by immunohistochemistry as described in Materials and Methods. The correlation between immunohistochemistry and aromatase activity was then assessed by linear regression analysis with the correlation coefficient  $r = 0.95$ .

expression in the patient population. As shown in Fig. 4A, there was a slight survival advantage for individuals with lower levels of aromatase expression compared with individuals with higher levels ( $P = 0.008$ ). Significantly, upon dividing the population by gender, a predictive value for aromatase expression was observed for women (Fig. 4B;  $P = 0.009$ ) but not for men (Fig. 4C;  $P = 0.182$ ). This observation did not seem to be due to differences in the cellular expression or activity of aromatase in the lungs in women compared with men (ref. 6; Supplementary Table S2).

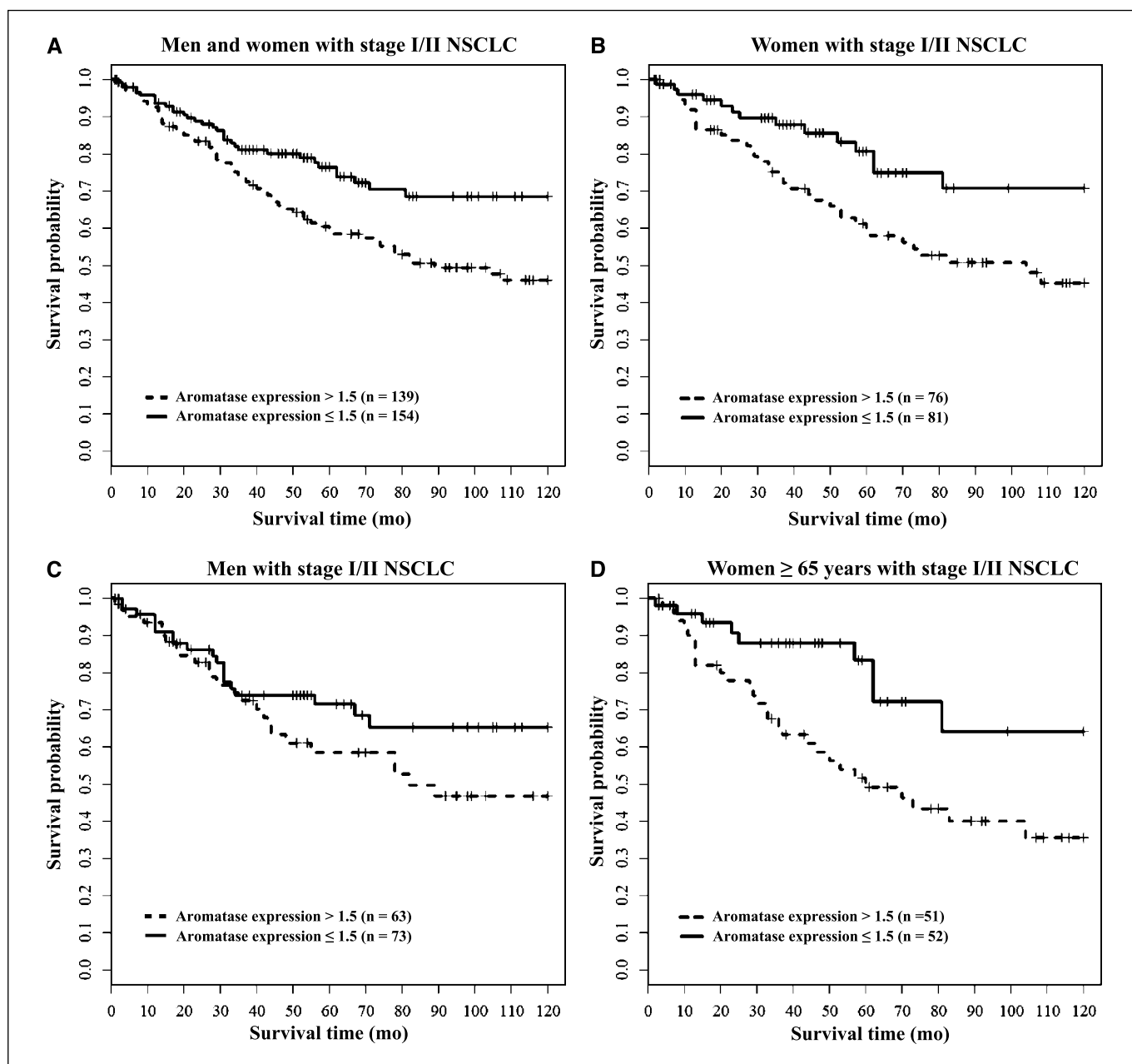
We further stratified the female population according to age (e.g., age cutoffs of 50, 55, 60, 65, and 70 years) and examined the

predictive capability of aromatase expression in each age group. Aromatase levels were not predictive of disease outcome in women younger than 65 years of age with lung cancer. However, as shown in Fig. 4D, women 65 years and older had a striking survival advantage if they expressed lower levels of aromatase ( $P = 0.006$ ), with 79% of these women surviving 5 years post-surgery compared with only 49% survival in the population of women with higher aromatase.

**Aromatase expression is an early-stage predictive tumor marker.** We next subdivided the population based on disease stage. For individuals with stage III or IV NSCLC, aromatase



**Figure 4.** Aromatase expression levels predicted the probability of survival in women with NSCLC. Kaplan-Meier survival plots for individuals with NSCLC. *Solid lines*, lower aromatase expression ( $\leq 1.5$  mean integrated intensity); *dashed lines*, higher aromatase expression ( $> 1.5$  mean integrated intensity). *N*, number of individuals in each category. *A*, individuals (men plus women) with lower aromatase expression had an increased probability of survival compared with those with higher aromatase expression ( $P = 0.0080$ ). *B*, women with lower aromatase expression had an increased probability of survival compared with those with higher aromatase expression ( $P = 0.0092$ ). *C*, aromatase expression provided no predictive power for survival for men with NSCLC ( $P = 0.182$ ). *D*, women who were ages 65 years and older and had lower aromatase expression were found to have an increased probability of survival compared with those with higher tumor aromatase expression ( $P = 0.0061$ ).

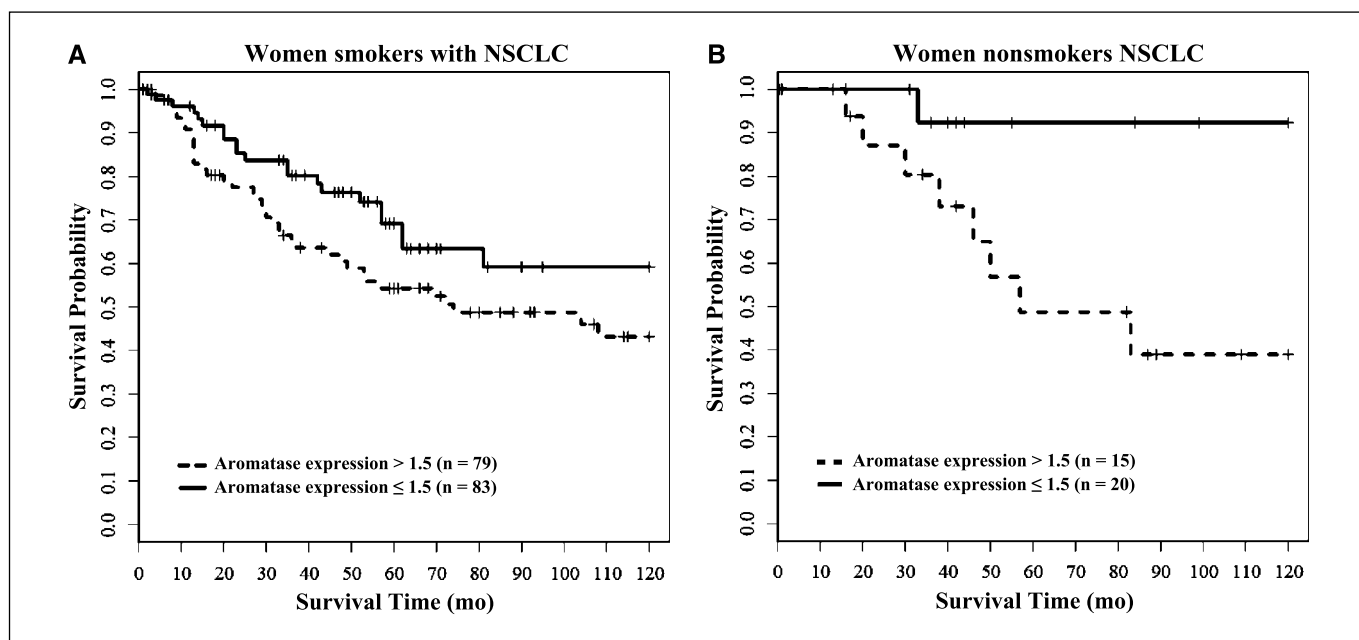


**Figure 5.** Aromatase expression levels predict the probability of survival in women with earlier stage NSCLC (stages I and II). Kaplan-Meier survival plots for individuals with NSCLC. *Solid lines*, lower aromatase expression ( $\leq 1.5$  mean integrated intensity); *dashed lines*, higher aromatase expression ( $> 1.5$  mean integrated intensity). *N*, number of individuals in each category. *A*, individuals (men plus women) with stage I/II NSCLC and lower aromatase expression had an increased probability of survival compared with those with higher tumor aromatase expression ( $P = 0.0048$ ). *B*, women with stage I/II NSCLC and lower aromatase expression had an increased probability of survival as compared with those with higher tumor aromatase expression ( $P = 0.0102$ ). *C*, aromatase expression provided no predictive power for survival for men with stage I/II NSCLC ( $P = 0.152$ ). *D*, women with stage I/II NSCLC who were ages 65 years and older and had lower aromatase expressions had an increased probability of survival compared with those with higher tumor aromatase expression ( $P = 0.0080$ ).

expression levels did not display any significant associations with disease outcome (data not shown). In contrast, for patients of all genders with earlier stage lung cancer (stage I and II), lower levels of aromatase predicted greater survival (Fig. 5A;  $P = 0.005$ ). Furthermore, this predictive value was gender-specific with low aromatase predicting greater survival probability in women (Fig. 5B;  $P = 0.010$ ) but not men (Fig. 5C;  $P = 0.152$ ) with stage I/II disease. Of the 157 cases of women who were afflicted with stage I and II NSCLC, the 5-year survival among the population with lower aromatase ( $n = 81$ ) was 81%, whereas the survival

of women with higher aromatase expression ( $n = 76$ ) was 60% (Fig. 5B). Again, the predictive value of aromatase expression was far more pronounced in women 65 years and older (Fig. 5D;  $P = 0.008$ ) than in younger women (data not shown). Internal validation of survival predictions, done as described using 400 bootstrapped samples (24, 25), guaranteed that data was not overfit.

To further validate the findings, an additional independent set of patients with NSCLC was examined. We examined aromatase expression using a TMA constructed from patients at the University



**Figure 6.** Kaplan-Meier survival plots for women with and without a history of smoking. Kaplan-Meier survival plots for women with NSCLC. *Solid lines*, lower aromatase expression ( $\leq 1.5$  mean integrated intensity); *dashed lines*, higher aromatase expression ( $> 1.5$  mean integrated intensity). *N*, number of women in each category. *A*, women (all ages and stages) with NSCLC who had a smoking history and lower aromatase expression had a slight, but not statistically significant, increased probability of survival compared with those with higher aromatase expression ( $P = 0.063$ ). *B*, women (all ages and stages) with NSCLC who had no history of ever smoking and had lower aromatase expression levels had an increased probability of survival compared with those with higher tumor aromatase expression ( $P = 0.022$ ). Note that although the population size was relatively small, 92% of the female nonsmokers with lower tumor aromatase expressions survived beyond 5 y.

of Texas M.D. Anderson Cancer Center (Supplementary Table S3). This set consisted of 337 patients with a breakdown of 183 women (104 women ages 65 or over, 79 women under the age of 65) and 154 men. The median staining intensity was used to divide the patient groups as described above. Significantly, an identical pattern was identified for the predictive power of aromatase levels and survival in women  $\geq 65$  years old with early-stage NSCLC completely consistent with results from the University of California at Los Angeles patient cohort ( $P = 0.005$ ; Supplementary Fig. S2).

**Aromatase expression and NSCLC in women with no smoking history.** The population of women with NSCLC was further analyzed in order to identify any associations based on smoking history. Women were dichotomized into those who had smoked  $> 100$  cigarettes at any point in their lifetime (smokers), and those who had never smoked (nonsmokers; see Materials and Methods). Within the population of women (all ages) who had smoked during their lifetime, there was a slightly reduced—albeit not statistically significant—predicted survival advantage with lower levels of aromatase (Fig. 6A;  $P = 0.063$ ). Although in this particular patient cohort, the subset of women who had no smoking history, yet developed NSCLC, was relatively small, there was still a highly significant predictive value for survival with lower tumor aromatase levels (Fig. 6B;  $P = 0.022$ ) with a 5-year survival percentage of 92% ( $n = 20$ ) compared with a 49% survival percentage for women with higher tumor aromatase ( $n = 15$ ).

Finally, we addressed whether aromatase expression had predictive information beyond other known NSCLC prognostic indicators such as tumor stage, tumor grade, and patient age. In multivariate Cox proportional hazards models, aromatase as either a continuous ( $P = 0.0015$ ; Table 1) or dichotomized ( $P = 0.0042$ ) variable was a significant independent predictor of survival for women ages 65 and older.

## Discussion

In this study, we have identified aromatase expression levels as an early-stage predictive biomarker of lung cancer survival. Specifically, lower relative levels of aromatase predict a higher probability of survival in women with NSCLC who are 65 years of age and older. The predictive power of aromatase expression was particularly informative at earlier stages of lung cancer (stage I and II). In contrast, women with higher aromatase expression have a worse prognosis.

For malignancies such as breast cancer, stimulation of the ER pathway contributes to the pathogenesis of this disease (26). The pathogenic effect of estrogen is thought to be a by-product of estrogen metabolism and/or estrogen-induced gene transcription promoting the proliferation and inhibition of apoptosis (26). It is now appreciated that the normal lung is also responsive to estrogen for regulating differentiation and development (2). We and others have hypothesized that increased local estrogen levels and/or enhanced stimulation of the ER signaling pathway also plays a role in lung cancer progression (2–4, 6). As part of this

**Table 1.** Multivariate Cox proportional hazards analysis for women 65 years and older ( $n = 129$ )

Variable	Hazard ratio (95% confidence interval)	<i>P</i>
Tumor stage	1.82 (1.30–2.56)	0.0005
Tumor grade	1.02 (0.70–1.47)	0.9300
Age	1.08 (1.02–1.14)	0.0054
Aromatase mean intensity	2.37 (1.39–4.04)	0.0015

concept, we predict that lung cancer cells may adopt one or more strategies to maintain or enhance the ER signaling pathway, including the local production of estrogens via aromatase-catalyzed biosynthesis.

It is intriguing that aromatase expression levels are most predictive in a later age in women. That this is purely due to diminishing levels of circulating estrogen in postmenopausal women is unlikely because the median age for menopause is ~51 years. However, circulating levels of androgens (e.g., androstenedione and testosterone), the substrate for aromatase, remain relatively constant in women throughout menopausal changes, and gradually decrease after age 65 (27–31). Therefore, we predict that in such situations in which systemic levels of estrogen are relatively decreased due to declined estrogen produced by the ovaries (32) and androgen levels are decreasing, tumor cells and/or neighboring host cells might compensate by increasing the endogenous production of estrogen through aromatase. Growth factor receptor signaling pathways may also contribute to the increased expression and activity of aromatase, thereby further stimulating estrogen biosynthesis in NSCLC cells (33). In contrast to these results, we observed no predictive value for aromatase expression levels in men of any age. Of note, estrogen levels in men stay relatively constant with age (34). Thus, it is possible that in men with lung cancer, an alternate strategy is employed to enhance ER pathway signaling.

An interesting yet surprising finding was the association between aromatase levels and survival differences in women with NSCLC and no smoking history. In this subpopulation, >90% of women who had lower aromatase expression survived beyond 5 years. As a cautionary note, although these latter results are

statistically significant, the population size was very small ( $n = 20$ ); it will be important to verify this finding in a larger study.

It is intriguing to consider that in addition to allowing a predictive stratification of NSCLC patients, these results may also suggest novel therapeutic approaches to treat NSCLC using currently available aromatase inhibitors. Aromatase inhibitors are showing promising results in malignancies such as breast cancer (16–18). Of note, a recent trial was conducted in which postmenopausal women with primary breast cancer either remained on tamoxifen or were switched to a third-generation aromatase inhibitor, exemestane (17). Significantly, there was a reduction in the subsequent development of primary lung cancer in the cohort on exemestane compared with those women maintained on tamoxifen (17). Thus, a compelling prediction of the current study is that aromatase inhibitors may be a beneficial addition to the armamentarium of compounds currently being used to treat and possibly prevent lung cancer.

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