**Table 4. Comparisons of pre- and post-treatment scores of biomarkers in tumors among groups with different baseline Ki67.**

| Biomarkers | Low-baseline Ki67 (n = 35) | | | | High-baseline Ki67 (n = 19) | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Mean change [95% CI] | p value‡ | Pre-treatment | Post-treatment | Mean change [95% CI] | p value‡ |
| VPR | 0.547 ± 0.046 | 0.551 ± 0.049 | -0.004 [-0.138, 0.129] | 0.884 | 0.551 ± 0.063 | 0.655 ± 0.067 | -0.104 [-0.221, 0.012] | 0.058 |
| Ki67 | 6.194 ± 0.600 | 4.681 ± 1.010 | 1.512 [-0.783, 3.807] | 0.033* | 26.294 ± 2.100 | 13.800 ± 1.998 | 12.494 [7.155, 17.833] | 0.001* |
| HER2 | 1.382 ± 0.120 | 1.206 ± 0.157 | 0.176 [-0.114, 0.467] | 0.225 | 1.737 ± 0.240 | 1.684 ± 0.230 | 0.053 [-0.200, 0.305] | 0.655 |
| ER | 7.543 ± 0.138 | 7.286 ± 0.198 | 0.257 [-0.270, 0.785] | 0.296 | 6.684 ± 0.546 | 6.658 ± 0.504 | 0.026 [-0.598, 0.651] | 0.774 |
| PgR | 6.029 ± 0.365 | 3.714 ± 0.467 | 2.314 [1.188, 3.441] | < 0.001* | 5.316 ± 0.635 | 3.474 ± 0.589 | 1.842 [0.714, 2.970] | 0.004* |

For scores of biomarkers in pre- or post-treatment specimen, data is shown as mean ± SEM; for the change of scores of biomarkers after treatment, data is shown as means with 95% CI [lower, upper values].
*A p value less than 0.05 is considered significant.
‡Wilcoxon matched-pairs signed-ranks test for the difference between groups.
ER: Estrogen receptor; HER2: Human EGFR2; PgR: Progesterone receptor; VPR: Vasohibin-1 positive ratio.

as to whether apoptosis is directly induced by estrogen withdrawal by AI or decreased cell proliferation as demonstrated by changes of Ki67 LI in carcinoma cells [29,37,45,46]. Irrespective of the underlying molecular mechanisms, a clearance phase inevitably follows so as to remove the dying/ dead carcinoma cells from the biological system to maintain homeostasis of the stromal microenvironment which is crucial to the immunological tolerance of the host [47,48], and to preserve tissue integrity and functions of the breast tissues.

Apoptosis or necrosis of the cells also initiated a programmed cascade of cellular mechanisms and signaling pathways essential for the removal of the 'corpses' from the system [47-51], prior to the release of potentially toxic or immunogenic intracellular substances into the microenvironment which otherwise will induce immune and also inflammatory response as a body self defense [50]. The removal of these dying/ dead carcinoma cells is carried out both by various neighboring viable cells such as epithelial cells, mesenchymal cells like fibroblasts, and/or by more complex mechanisms by phagocytes including macrophages and immature dendritic cells [49-51]. Phagocytosis of those dying or dead tumor cells induces a chain of changes in the stroma including upregulation of some pro-angiogenic genes while downregulating angiostatic genes [48,49]. An influx of inflammatory cells was observed in those responders in our cohort with the changes of inflammatory cells parallelling that of the change in angiogenesis [52]. When using the same criteria of pathobiological response of decrement of more than 40% in post-treatment Ki67 LI, statistically significant inverse correlation between Ki67 and inflammatory cells changes was found. Statistically significant increment in VPR after treatment is associated with a significant increase in inflammatory cells in the specimens. The influx of inflammatory cells detected in the responders to neoadjuvant AI therapy may represent stromal immune response on estrogen depletion as a normal body innate immune reaction in response to dying/ dead cells. The increase in inflammatory cells could either be causal or a result of the increase in neoangiogenesis. These changes subsequently induce the secretion of anti-inflammatory cytokines, and that of expression of certain growth and survival factors such as VEGF and TGFβ [48,49]. These may therefore contribute to inducing an anti-inflammatory environment around the dying/ dead tumor cells. Results of several studies had reported an increment of VEGF and angiogenesis stimulators in areas adjacent to the necrotic foci [48,49], which could promote proliferation of endothelial cells resulting in angiogenesis around the necrotic areas. The clearance of these dying/ dead carcinoma cells may recruit the same disposal mechanisms as in physiological settings that may account for our present findings of increased angiogenesis upon tumor cell death.

This increased neo-angiogenesis in the treatment responders may represent one of the processes in the spectrum of the complex carcinoma–stromal interaction. Angiogenesis

**Table 5. Comparisons of pre- and post-treatment imunohistochemical (IHC) scores of biomarkers in tumors among groups of different baseline vasohibin-1 positive ratio (VPR). The whole group was classified into high- and low-baseline VPR groups according to the mean of IHC scores of baseline VPR. Mean value of VPR was 0.549.**

| Biomarkers | Low VPR (n = 31) | | | | High VPR (n = 23) | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Mean change [95% CI] | p value‡ | Pre-treatment | Post-treatment | Mean change [95% CI] | p value‡ |
| Ki67 | 14.290 ± 2.355 | 7.413 ± 1.518 | 6.877 [2.911, 10.844] | < 0.001* | 11.886 ± 1.821 | 8.533 ± 1.689 | 3.353 [-0.265, 6.971] | 0.035* |
| HER2 | 1.452 ± 0.153 | 1.387 ± 0.178 | 0.065 [-0.234, 0.363] | 0.660 | 1.591 ± 0.182 | 1.364 ± 0.203 | 0.227 [-0.044, 0.499] | 0.096 |
| ER | 7.355 ± 0.252 | 7.081 ± 0.227 | 0.274 [-0.204, 0.752] | 0.130 | 7.087 ± 0.382 | 7.043 ± 0.423 | 0.043 [-0.667, 0.754] | 0.799 |
| PgR | 5.903 ± 0.428 | 4.226 ± 0.458 | 1.677 [0.528, 2.827] | 0.002* | 5.609 ± 0.510 | 2.826 ± 0.558 | 2.783 [1.631, 3.934] | < 0.001* |

For scores of biomarkers in pre- or post-treatment specimen, data is shown as mean ± SEM; for the change of scores of biomarkers after treatment, data is shown as means with 95% CI [lower, upper values].

*A p value less than 0.05 is considered significant.

‡Wilcoxon matched-pairs signed-ranks test for the difference between groups.

ER: Estrogen receptor; HER2: Human EGFR2; PgR: Progesterone receptor.

plays pivotal roles in providing nutrients and oxygen to sustain the growth of tumors and also as a carrier in metastasis [4-10,13,14]. However, it is also true that angiogenesis also plays crucial roles in the clearance of dying/ dead cells and repair processes, providing the nutrients and different cells such as macrophages or other inflammatory cells involved in these processes [9,15]. Results of this study also indicate that angiogenesis in this manner following AI treatment may represent a more physiological process/ response of the body in an attempt to remove dying/ necrotic carcinoma cells from the body in order to achieve homeostasis of the stromal microenvironment and hence the immunological tolerance of the host [47], in contrast to the pathobiological angiogenesis in sustaining the tumor growth and progression [4-6,8]. This is suggested by the observation of influx of inflammatory cells in the responders in our cohort [52]. This increased angiogenesis detected in responders to neoadjuvant AI treatment in our present study may therefore represent the tumor–stroma interaction at certain stages during the process of carcinoma cell death on estrogen depletion and was not necessarily related to tumor progression. It awaits further investigation of the analysis of cell death or apoptosis such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or caspases immunohistochemistry in these tissue specimens together with VASH-1 immunostaining in order to clarify the effects of estrogen depletion upon the turnover of endothelial cells in the breast cancer tissue microenvironment. Also, in view of the small number of cases in our cohort, further large scale study and the correlation with inflammatory cells changes would provide more insight into increased neoangiogenesis in AI responders.

In summary, estrogen depletion caused by AI treatment resulted in a significant increment of VPR in the responder group through a possible interaction of the tumor–stroma microenvironment in response to the apoptosis/ death of the tumor cells. VPR can be a potential surrogate marker in predicting the response to neoadjuvant endocrine therapy which could incorporate not only features of carcinoma cells but also of adjacent stromal cells in the tumor microenvironment following estrogen depletion, especially in the early stage of treatment prior to any detectable clinical or histopathological changes.

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## Declaration of interest

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EXPERT OPINION



# Cytoplasmic estrogen receptor β as a potential marker in human non-small cell lung carcinoma

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***Objectives:*** Estrogen has been reported to promote an increased susceptibility to lung cancer development. This study focusses on the role of cytoplasmic estrogen receptor β (c-ERβ) in NSCLC.

***Methods:*** NSCLC (n = 162) cases were analyzed using immunohistochemistry (IHC) for c-ERβ expression and its association with clinicopathological variables. Significance of c-ERβ expression was further examined using *in vitro* studies in NSCLC cell lines.

***Results:*** Among ERβ and aromatase positive NSCLC females, c-ERβ was significantly associated with greater tumor diameter and tended to be associated with worse overall survival. A549 and LCAM1 cells expressed aromatase, as well as c-ERβ and nuclear ERβ (n-ERβ). U0126 (MAPK/extracellular-signal-regulated kinase (ERK) inhibitor) abrogated MAPK phosphorylation, caused by estradiol via c-ERβ, more effectively than ICI 182780 (ER blocker) in either cell line. However, ICI 182780 completely abrogated the estrogen responsive elements (ERE)-luciferase activity caused by estradiol. Combination therapy with ICI 182780 and U0126 turned out to be far more effective than either treatment alone in either A549 or LCAM1 cells.

***Conclusion:*** The results indicated that ERβ may contribute to NSCLC via non-genomic action of estrogen through its cytoplasmic form, in addition to the genomic actions via n-ERβ. These actions of estrogen in NSCLCs may be abrogated by combination therapy with ICI 182780 and U0126.

**Keywords:** aromatase, estrogen receptor, lung carcinoma, MAPK inhibitor, non-genomic

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## 1. Introduction

One quarter of lung cancer patients are life time never smokers [1]. The types of lung cancer demonstrating weaker association with tobacco smoke were detected predominantly in women and thereby suggest a possible role for estrogenic actions in the development of the disease [2]. Expression patterns of estrogen receptors (ERs) in NSCLCs tissues using immunohistochemistry were highly inconsistent varying from 0 to 100% for ERα and 30 to 100% for ERβ [3-5]. However, among NSCLC cell lines, ERβ was then demonstrated to be expressed frequently whereas ERα expression was either absent or inconsistent [6,7]. Results of earlier studies suggested that ERβ inhibits tumorigenesis [8]. However, results of relatively recent studies did demonstrate that ERβ can function as a tumor promoter [9,10], including studies in NSCLC cell lines lacking ERα [6,7]. These studies all suggest an intriguing hypothesis that ERβ may functions as a tumor promoter in some NSCLC cases.

ERs can regulate gene expression through direct DNA binding on estrogen responsive elements (ERE) following their binding to ligands [11]. In NSCLC cells,

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these genomic actions were demonstrated to be induced only by ligands specific to ERβ [12]. Many previous studies demonstrated the presence of estrogenic actions at the cell surface in NSCLC cells [6,7,12-15]. In breast carcinoma cells, ERs utilize the membrane EGFR to rapidly signal through various kinase cascades [16]. Similar investigations are required for further clarification of these non-genomic actions of estrogen in NSCLCs.

Aromatase, the enzyme that catalyzes the conversion from androgens to estrogens, was reported to be expressed frequently in NSCLC patients [17-21]. Aromatase in NSCLC patients was indeed associated with intratumoral estrogen concentration, ERβ expression and tumor stage in different reported studies [17-19]. In addition, a lower expression of aromatase was associated with better prognosis in post-menopausal female NSCLC patients [20]. These reports indicated the importance of elevated *in situ* estrogen concentrations through aromatase in NSCLC patients.

These findings all suggest a potential role of intratumoral aromatase along with ERs especially ERβ in biological behavior ofNSCLC. Therefore, we investigated the expression of cytoplasmic and nuclear ERs along with aromatase enzyme in 162 NSCLC cases via immunohistochemistry (IHC) analysis. The significance of IHC analysis was further examined in NSCLC cell lines, that is A549 and LCAM1 cells expressing both nuclear (n)-ERβ and cytoplasmic (c)-ERβ but not ERα. Our results suggested that c-ERβ expression in addition to n-ERβ may result in more aggressive tumor progression in NSCLC patients especially those expressing aromatase as well.

## 2. Materials and methods

### 2.1 Patients

A total of 162 NSCLC cases were retrieved from surgical pathology files of the Department of Pathology, Tohoku University. Neither anti-EGFR nor anti-hormonal therapy was given to the patients prior to surgery. Informed consent was obtained from each patient before surgery and research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (Approval Number 2009-500).

### 2.2 Immunohistochemistry

The specimens for immunohistochemistry had been fixed in 10% formaldehyde solution and embedded in paraffin. The primary antibodies used were anti-ERβ (1:50, clone 14C8; GeneTex Inc), anti-Aromatase (1:3000; clone #677/H7) [22], anti-ERα (1:50; clone 6F11; Novocastra) and anti-$K_i$-67 (1:100; clone MIB1, Dako). Immunoreactivity for ERβ and $K_i$-67/MIB1 was counted among 1000 cells per case and was recorded as 'positive' if immunereactivity was detected in more than 10% of cells [23,24]. Based on the localization of 'positive' immunoreactivity for ERβ in either cytoplasm or nucleus the patients were grouped as either c-ERβ- and/or n-ERβ-positive. Staining for aromatase in over 10% of the cells of a cancer lesion was recorded as 'positive' [25]. The evaluations of immunohistochemical stains were made independently by two different investigators (Mohit Kumar Verma and Keiko Abe).

### 2.3 Cell culture and chemicals

Human NSCLC cell lines A549, LK87 and LCAM1 were provided by the Institute of Development, Aging and Cancer, Tohoku University. The cells were cultured in RPMI 1640 (Sigma-Aldrich) with 10% fetal bovine serum (JRH Biosciences). Cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Estradiol (E2) and testosterone were commercially obtained from Sigma-Aldrich. ER blocker, Fulvestrant, was obtained from Tocris Cookson Ltd. and aromatase inhibitor, Letrozole, was provided by Novartis.

### 2.4 RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was carried out using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics). Ribosomal protein L 13a (RPL13A) was also used as an internal standard. The primer sequences used in this study were as follows: aromatase (X13589; forward: cDNA position 691 – 711 and reverse: cDNA position 766 – 786), ERα (NM_000125; forward: 1811 – 1830 and reverse: 2080 – 2099), G-protein coupled estrogen receptor (GPER) (NM_0010339966; forward: 1883 – 1902 and reverse: 1986 – 2005) and ERβ (AB006590; forward: 14600 – 1480 and reverse: 1608 – 1627), and RPL13A (NM_012423; forward: 487 – 509 and reverse: 588 – 612).

### 2.5 Immunoblotting

The protein was extracted from the cells using PhosphoSafe Extraction Reagent (Novagen) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). Nuclear and cytoplasmic extract from cells were extracted using a CelLytic NuCLEAR extraction kit (Sigma-Aldrich) according to the manufacturer's protocol. Ten micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare). The primary antibodies used were ERβ (Santacruz), Aromatase (clone #677/H7) [22], β-actin (AC-15) (Sigma–Aldrich), phosphor-p42/44 EGFR (tyr1068 and tyr845) and p42/44 EGFR (1F4) (Cell Signaling Technology), phospho-MAPK (20G11) and MAPK (Cell Signaling Technology), Phospho-Akt (D9E) and Akt (Cell Signaling Technology), phospho-c-Src (tyr416); (Cell Signaling Technology), c-Src (17AT28) (Santacruz) and transcription termination factor 1 (TTF1) (Dako). TTF1 was used as marker of nuclear extract isolation efficiency.

**Table 1. Clinical and pathological features of 162 NSCLC patients.**

| Clinicopathologic characteristics | Number of cases (%) |
|---|---|
| Age | 45 – 82 years |
| Sex | |
| Male | 98 (60.4%) |
| Female | 64 (39.5%) |
| Histology | |
| Adenocarcinoma | 120 (74.1%) |
| Squamous cell carcinoma | 38 (23.4%) |
| Adenosquamous cell carcinoma | 4 (2.5%) |
| Tumor size | |
| T1 | 73 (45.1%) |
| T2 | 69 (42.6%) |
| T3 | 10 (6.2%) |
| T4 | 10 (6.2%) |
| Lymph node metastases | |
| N0 | 115 (70.9%) |
| N1 | 15 (9.2%) |
| N2 | 32 (19.7%) |
| Distance metastasis | |
| M0 | 156 (96.3%) |
| M1 | 6 (3.7%) |
| Estrogen receptor | |
| ERα | 26 (16.0%) |
| ERβ | 143 (88.3%) |
| Aromatase | |
| Aromatase positive | 140 (86.4%) |
| Aromatase + ERβ positive | 127 (78.4%) |
| ERβ localization | |
| Nuclear only ERβ | 81 (50%) |
| Cytoplasmic only ERβ | 2 (1.2%) |
| Cytoplasmic + Nuclear ERβ | 60 (37.0%) |

### 2.6 Cell proliferation assay

Cells were serum-starved for 48 h in a 96-well plate and then treated with test compounds for either 48 or 72 h. Then cells were harvested and evaluated for cell proliferation using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) method (Cell Counting Kit-8; DOJINDO Laboratories). Optical densities (at 450 nm) were obtained with a microplate spectrophotometry (Model680, Bio-Rad Laboratories).

### 2.7 Luciferase assay

Cells were serum starved for 48 h in a 24-well plate and then transient transfections were carried using TransIT-LT Transfection Reagents (TaKaRa Bio Co). Transfections contained ERE-tk-luc reporter plasmid and pRL-TK plasmid along with either CMV-ERβ expression vector or CMV-ER control expression vector. After 6 h of incubation, the media was replaced and treated with steroids and test compounds for 24 h. Cells were harvested and lysed in 100 µl of 1 × Reporter Lysis Buffer (Promega). Luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega).

### 2.8 Statistical analysis

Statistical analyses used Fisher's protected least significant difference (PLSD) test, Scheffe's F test, Bonferroni/ Dunn test, Kaplan-Meier test, Logrank (Mantel-Cox) test and Student's t test. A p-value less than 0.05 was considered to be significant. The statistical calculations used STATVIEW version 5.

## 3. Results

### 3.1 Expression of estrogen receptors and aromatase in NSCLC patients

Clinicopathological characteristics of the patients examined are summarized in Tables 1 and 2. ERβ and ERα expression was detected in 88 and 16% of the patients, respectively. ERβ immunoreactivity was present with different intracellular distribution patterns; only nuclear ERβ (n-ERβ), both cytoplasmic and nuclear ERβ (c + n-ERβ) and only cytoplasmic ERβ (c-ERβ) in 81, 60 and 2 cases respectively, (Table 1, Figure 1A). c-ERβ status was significantly associated with N factor of tumor nodes metastases (TMN) classification (Table 2). However, neither aromatase only nor n-ERβ only was associated with any of the clinicopathological factors of the patients examined in this study (Table 2).

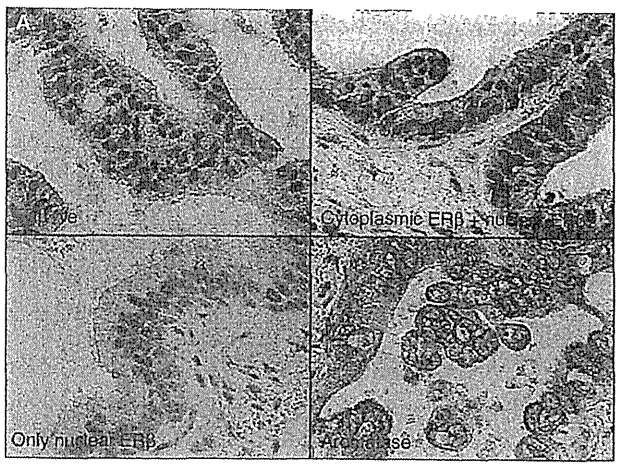
### 3.2 Association between cytoplasmic ERβ and survival in NSCLC cases

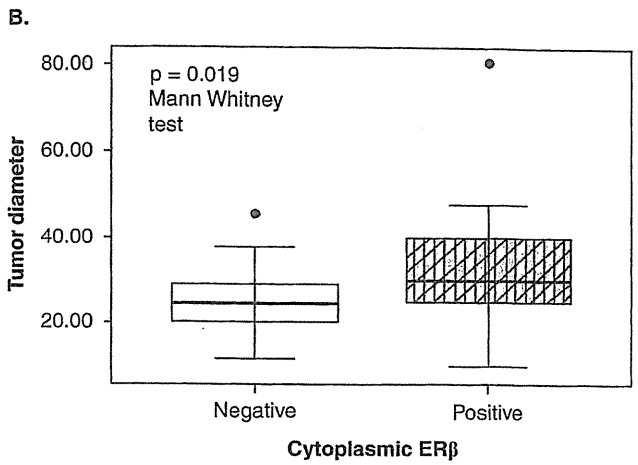
Among the patients, 42.5% (54 cases) demonstrated c-ERβ staining among aromatase and ERβ double positive patients (127 cases), (Table 3). We tentatively classified these 127 NSCLC cases into two groups, that is i) c-ERβ positive cases (including patients with only c-ERβ and both c + n-ERβ immunoreactivity in more than 10% of cells) and ii) c-ERβ negative cases (patients with only n-ERβ immunoreactivity in more than 10% of cells). Associations between c-ERβ immunoreactivity and clinicopathological features are summarized in Table 3. C-ERβ was associated with greater tumor diameter among ERβ and aromatase double positive female NSCLC patients (48 cases), ($p = 0.037$) (Table 3) and ($p = 0.019$) (Figure 1B). Moreover, among ERβ and aromatase double positive female NSCLC patients c-ERβ tended to be associated with worse overall survival (c-ERβ positive = 60% versus c-ERβ negative = 71.4%) (Figure 1C). In addition, among all the patients (162 cases) examined, the presence of c-ERβ was associated with poorer 5 year survival and median survival rates (c-ERβ positive = 54.8% and 1992 days versus c-ERβ negative = 62.0% and 2805 days, respectively) but the differences did not reach statistical significance (Figure 1D). Following a multivariate analysis tumor size turned out an independent prognostic factor in this study, (Hazard ratio (HR) = 2.5; $p = 0.016$) (Table 4).

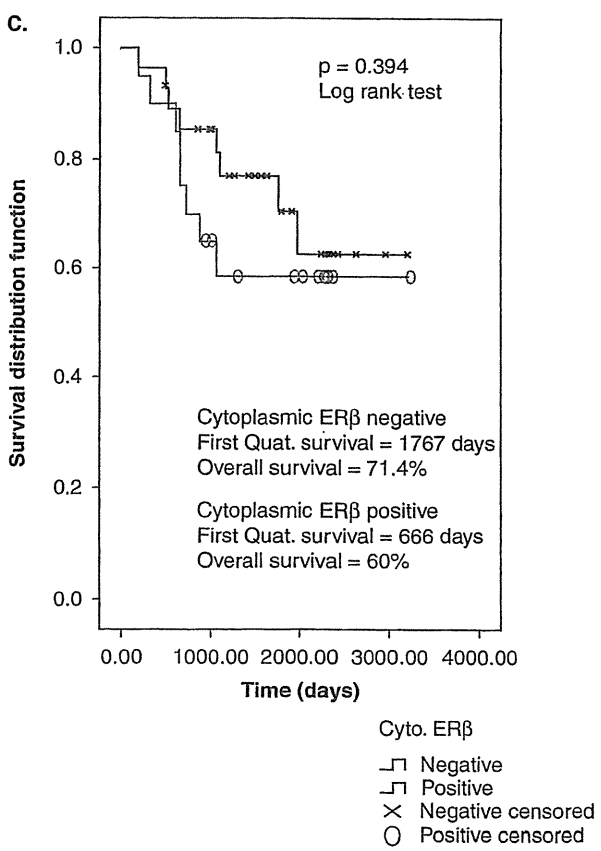
**Table 2. Association between clinicopathological variables and c-ERβ/n-ERβ/aromatase status in 162 NSCLC patients.**

| 162 cases | | c + n-ERβ/c-ERβ only | | | n-ERβ only | | | Aromatase | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Positive n = 62 | Negative n = 100 | p | Positive n = 81 | Others n = 81 | p | Positive n = 140 | Negative n = 22 | p |
| Sex | Male | 39 | 59 | | 48 | 50 | | 87 | 11 | |
| | Female | 23 | 41 | 0.741 | 33 | 31 | 0.747 | 53 | 11 | 0.288 |
| Histology | Adenocarcinoma | 48 | 72 | | 59 | 61 | | 104 | 16 | |
| | SCC | 12 | 26 | | 20 | 18 | | 32 | 6 | |
| | Adenosquamous cell carcinoma | 2 | 2 | 0.575 | 2 | 2 | 0.933 | 4 | 0 | 0.672 |
| Stage | I | 38 | 60 | | 49 | 49 | | 82 | 16 | |
| | II | 9 | 7 | | 7 | 9 | | 15 | 1 | |
| | III | 15 | 29 | | 22 | 22 | | 39 | 5 | |
| | IV | 0 | 4 | 0.171 | 3 | 1 | 0.741 | 4 | 0 | 0.536 |
| pT | pT1 | 28 | 45 | | 39 | 34 | | 62 | 11 | |
| | pT2 | 26 | 43 | | 33 | 36 | | 61 | 8 | |
| | pT3 | 1 | 9 | | 6 | 4 | | 8 | 2 | 0.842 |
| | pT4 | 7 | 3 | 0.053 | 3 | 7 | 0.480 | 9 | 1 | |
| pN | pN0 | 44 | 71 | | 58 | 57 | | 94 | 17 | |
| | pN1 | 10 | 5 | | 5 | 10 | | 14 | 1 | |
| | pN2 | 8 | 24 | 0.023 | 18 | 14 | 0.337 | 28 | 4 | 0.675 |
| pM | pM0 | 61 | 95 | | 77 | 79 | | 134 | 22 | |
| | pM1 | 1 | 5 | 0.239 | 4 | 2 | 0.401 | 6 | 0 | 0.322 |
| Diameter* (mm) | | 10 – 80 (30) | 8 – 90 (30) | 0.479 | 8 – 90 (30) | 10 – 90 (30) | 0.166 | 8 – 90 (30) | 10 – 90 (30) | 0.479 |
| $K_i$-67* (%) | | 2.1 – 54.9 (18.2) | 0 – 54.2 (18.2) | 0.222 | 0 – 54.2 (18.2) | 0 – 50.9 (18.2) | 0.494 | 0 – 54.2 (18.2) | 0 – 48.9 (18.2) | 0.667 |

Statistical analysis was conducted by Fisher exact test, Wilcoxon rank sum test and Pearson $\chi 2$ test.
*Data were continuous variables and the median with minimum–maximum values are presented.



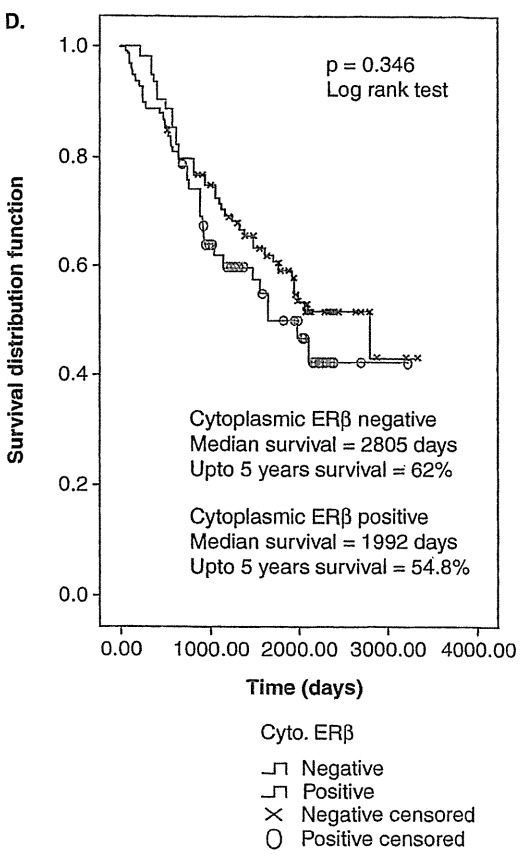




**Figure 1.** **(A)** Expression of ERβ and aromatase in NSCLC patients. **(B)** Association between c-ERβ and tumor diameter among ERβ and aromatase double positive female NSCLC cases. Kaplan–Meier survival curves for cytoplasmic ERβ **(C)** c-ERβ positive versus negative survival curve for aromatase and ERβ double-positive females. **(D)** c-ERβ positive versus negative survival curve for all patients.

First Quat.: The first quartile (or the 25th percentile). The first quartile of the survival time is the time beyond which 75% of the patients in the patient cohort under study are expected to survive.

**Table 3. Association between cytoplasmic ERβ and clinicopathological variables in both ERβ and aromatase positive patients.**

| 129 cases | | Cytoplasmic ERβ | | |
|---|---|---|---|---|
| | | Positive (n = 54) | negative (n = 73) | p |
| Sex | Male | 34 | 45 | |
| | Female | 20 | 28 | 0.879 |
| Histology | Adenocarcinoma | 43 | 53 | |
| | Squamous cell carcinoma | 9 | 18 | |
| | Adenosquamous cell carcinoma | 2 | 2 | 0.541 |
| Stage | I | 34 | 42 | |
| | II | 8 | 7 | |
| | III | 12 | 21 | |
| | IV | 0 | 3 | 0.307 |
| pT | pT1 | 24 | 34 | |
| | pT2 | 23 | 31 | |
| | pT3 | 1 | 5 | |
| | pT4 | 6 | 3 | 0.281 |
| pN | pN0 | 38 | 59 | |
| | pN1 | 9 | 5 | |
| | pN2 | 7 | 19 | 0.107 |
| pM | pM0 | 53 | 69 | |
| | pM1 | 1 | 4 | 0.298 |
| Diameter (mm)* | Male + Female | 10 – 80 (30) | 8 – 90 (30) | 0.95 |
| | Female only | 10 – 80 (26.5) | 12 – 45 (25.5) | 0.037 |
| $K_i$-67 LI %* | | 2.1 – 50.9 (18.2) | 0 – 54.2 (18.2) | 0.449 |

Statistical analysis was conducted by Fisher exact test, Wilcoxon rank sum test and Pearson $\chi 2$ test.
*Data were continuous variables and the median with minimum-maximum values were presented.

**Table 4. Univariate and multivariate analyses for clinical outcome in 162 NSCLC patients.**

| Variables | Overall survival | | Hazard ratio |
|---|---|---|---|
| | Univariate | Multivariate | (95% CI) |
| Sex | 0.016* | 0.420 | |
| Histology | 0.564 | – | |
| Stage | < 0.0001* | 0.610 | |
| pT | 0.0039* | 0.310 | |
| pN | < 0.0001* | 0.160 | |
| pM | 0.834 | – | |
| Diameter (mm) | < 0.0009* | 0.016 | 2.5 (1.2 – 5.2) |
| $K_i$-67 percentage | 0.0016* | 0.152 | |
| Aromatase | 0.512 | – | |
| c-ERβ | 0.346 | – | |
| n-ERβ only | 0.337 | – | |

*Data were considered significant in the univariate analyses and were examined in the multivariate analyses.

### 3.3 Expression of ERβ and aromatase in NSCLC cell lines

Western blot analysis demonstrated that ERβ was present both in cytoplasm as well as nucleus, but aromatase is expressed only in the cytoplasm of all three cell lines examined, A549, LK87 and LCAM1 (Figure 2A). Moreover, in addition to ERβ and aromatase, GPER was also expressed in all three NSCLC cell lines but ERα expression was undetected (Figure 2B).

### 3.4 Non-genomic and genomic actions of estrogen in NCSLC cell lines

Estradiol (E2) via c-ERβ rapidly phosphorylated MAPK within 5 min of its treatment in A549 and LCAM1 cells. This effect was effectively abrogated by a 30 min pretreatment with MAPK/ERK kinase inhibitor U0126, but not by ER blocker ICI 182780 (Figure 2C). However,

a 24 h pretreatment with ICI 182780 significantly abrogated the MAPK activation caused by E2 treatment in both A549 and LCAM1 cells, (Figure 2D and E). E2 treatment in A549 cells was unable to transactivate other protein kinases involved in EGFR signalling, that is EGFR, Akt and c-Src, (Figure 2F). On the other hand endogenous ERβ (either c-ERβ or n-ERβ) was unable to cause ERE–luciferase activity on either exogenous E2 or testosterone treatment in both A549 and LCAM1 cells, Figure 3A – C. In addition, ERα- and ERβ-transfected A549 cells on treatment with E2 but not with EGF produced enhanced ERE-luciferase activity ($p = < 0.0001$) when compared to control vector transfected cell, Figure 3D.

### 3.5 Targeting non-genomic actions of estrogen in NSCLC cell lines

E2 treatment triggered enhanced proliferation in A549 and LCAM1 cells, ($p = <.0001$), (Figure 3E). E2 triggered proliferation was inhibited more significantly on treatment with MAPK inhibitor, U0126 than ER blocker, fulvestrant in both A549 and LCAM1 cells, ($p = < 0.0001$), (Figure 3E). However, the combination therapy with both U0126 and fulvestrant resulted in an enhanced anti-proliferative effects in both A549 and LCAM1 cells, ($p = < 0.0001$), (Figure 3E). In addition, the combination therapy with fulvestrant and Iressa was two times more effective than that with fulvestrant and U0126, within 48 h of treatment, ($p = < 0.0001$), (Figure 3F).

## 4. Discussion

The potential roles of ERs in the pathogenesis of many hormone-dependent diseases led to the widespread use of ER modulators for their treatment [26]. The presence of ERs has been also reported in NSCLCs [3-7]. Therefore, there is an emerging interest in the role of ERs in lung cancer development. In this study we particularly focused on c-ERβ in NSCLC tissues and cell lines.

Previously, only ERα was considered as a tumor promoter [8]. However, many NSCLC cell lines which lacked ERα but expressed ERβ demonstrated tumor promoting features [6,7]. NSCLC cell lines used in our study also lacked ERα, (Figure 2B) but expressed ERβ both in nucleus and the cytoplasm (Figure 2A). Earlier reports on ERα and ERβ expression in NSCLCs using IHC were highly inconsistent and varied from 0 to 100% for ERα and 30 to 100% for ERβ [27]. However, it is important to note that several different criteria and antibodies were employed to define ERα and/or ERβ positivity in these studies and a comparison using a standard IHC method has to be performed to determine status and clinical significance of ERs in NSCLC patients. However, there is currently no standardized IHC assay for the measurement of ERs in lung cancer [28]. The three antibodies used in this study i.e., aromatase antibody (#677), ERα antibody (6F11) and ERβ antibody (14C8) have been used effectively for staining paraffin-embedded lung cancer tissues [17,18]. In the present study we found that ERβ expression was significantly higher than ERα expression among NSCLC patients, (Table 1). We also detected the co-expression of ERβ and aromatase ($p = 0.028$) (data not shown) with different ERβ intracellular distribution patterns; only n-ERβ, both c + n-ERβ and only c-ERβ, (Figure 1A). c-ERβ staining was detected in 42.5% of the cases with nuclear immunoreactivity and in 9.5% of the cases without nuclear immunoreactivity which was consistent with the result of previous study which demonstrated a similar trend in c-ERβ expression in NSCLCs (Table 1) [5]. In addition to carcinoma cells, normal bronchial epithelial cells were also occasionally weakly positive for ERβ, suggesting a possible upregulation of ERβ in cancer cells.

The status of n-ERβ was reported to be associated with better clinical outcome especially among male NSCLC patients [23,29]. This is in contrast with both *in vitro* and *in vivo* studies in NSCLC cell lines demonstrating tumor promoting features of ERβ particularly through a non-genomic pathway via c-ERβ [12,14]. In this study among all patients tumor size was found to be an independent prognostic factor, where a higher tumor size was associated with poorer survival (Table 4). In addition, no significant association was detected between clinicopathological factors and n-ERβ staining (Table 2). However, c-ERβ was found to be significantly associated with the N factor of the TMN classification (Table 2). Results of a recently published study demonstrated that elevated c-ERβ was an important predictor of poorer survival in both men and women with lung cancer, whereas n-ERβ status could not demonstrate such a correlation [30]. In the same report, an addition of high aromatase status to c-ERβ status also provided cumulative effects upon the poorer prognosis of patients whereas aromatase alone failed to show any prognostic significance for the patients. In addition, we could not detect any association between clinicopathogical factors and aromatase status of the patients. However, it is also true that results of previous reports indicated a potential role of the elevated *in situ* estrogen concentrations through aromatase in NSCLC patients [17-20]. Therefore, it became important for us to focus on both c-ERβ and aromatase in our patient cohort as well.

All the patients in our study were postmenopausal. Therefore, we divided aromatase and ERβ double-positive patients (127 cases) into two groups according to c-ERβ status, (Table 3). Results of our present study demonstrated that among ERβ and aromatase double-positive NSCLC females (48 cases), the presence of c-ERβ was significantly associated with greater tumor diameter, ($p = 0.019$), (Figure 1B); and tended to be associated with worse overall survival (c-ERβ positive = 60% versus c-ERβ negative = 71.4%), although the correlation did not reach statistical significance (Figure 1C). The upward trend of lung cancer deaths among

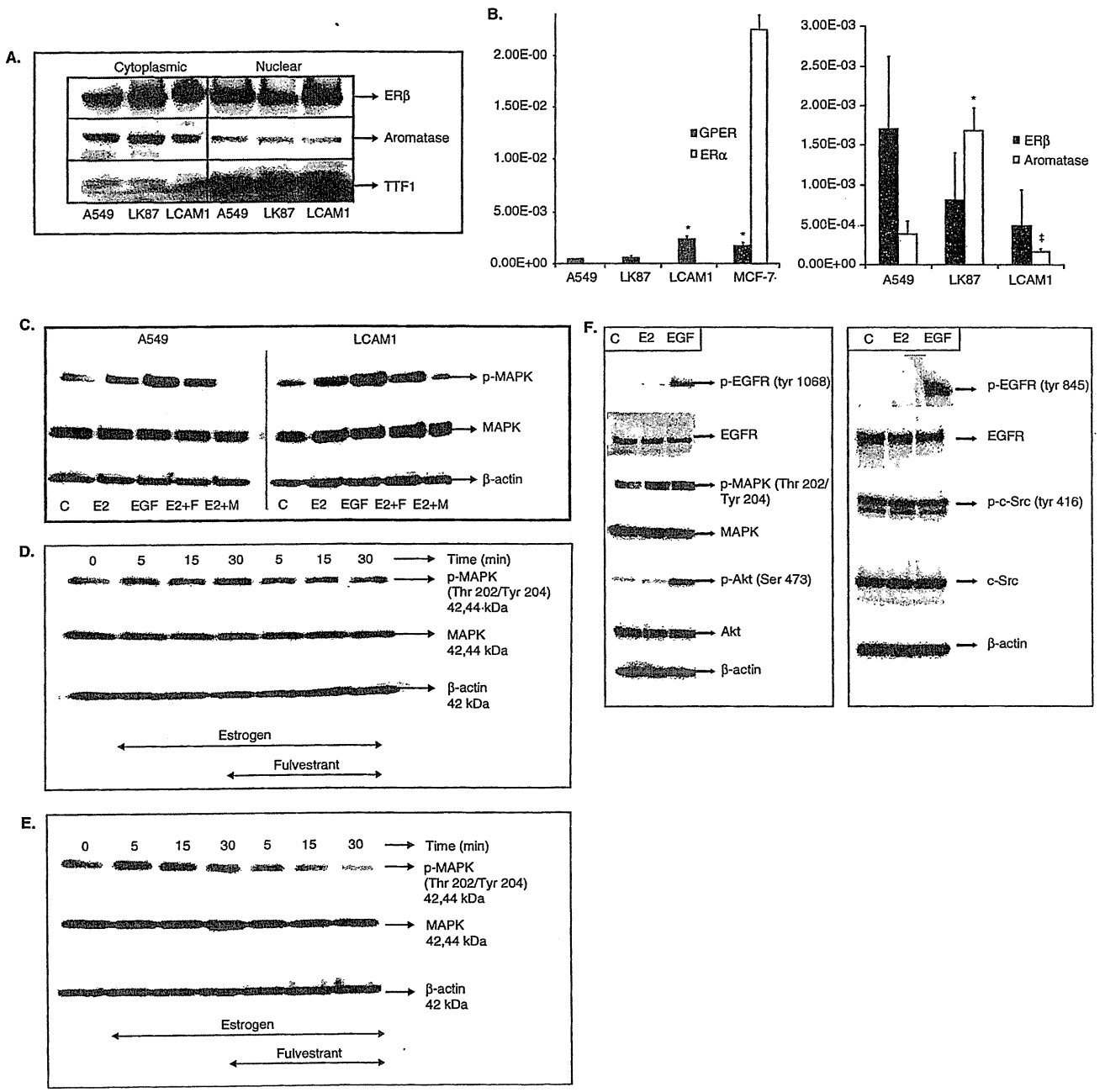



**Figure 2. All immunoblots are representative of three independent experiments. A.** Expression and localization of ERβ and aromatase in NSCLC cell lines. **B.** Expression of ERα, GPER, ERβ and aromatase in NSCLC cells. **C.** MAPK activation in A549 and LCAM1 cells within 5 min of either estrogen (E2) or EGF treatment and its abrogation by U0126. Abrogation of the MAPK activation caused by E2 treatment on pretreatment with ER blocker, ICI 182780 for 24 h Letrozole (Letro). in **D.** A549 cells and **E.** LCAM1 cells. **F.** Activation of various protein kinases within 5 min of treatment with either E2 or EGF in A549 cells.

F: ER blocker Fulvestrant; M: MAPK inhibitor U0126.

*represents $p < 0.0001$ vs. control.

†represents $p < 0.0001$ vs. either testosterone treatment or E2 and/or EGF treatments.

female nonsmokers was also confined to these elderly women [31]. However, among NSCLC patients, intratumoral concentration of estradiol was significantly higher in men than postmenopausal women [17] and males frequently co-express ERs and aromatase [18]. Therefore, ER pathway is postulated to be also active in males with NSCLC, although it awaits further investigations for clarification of its significance. In addition, among all the patients examined,

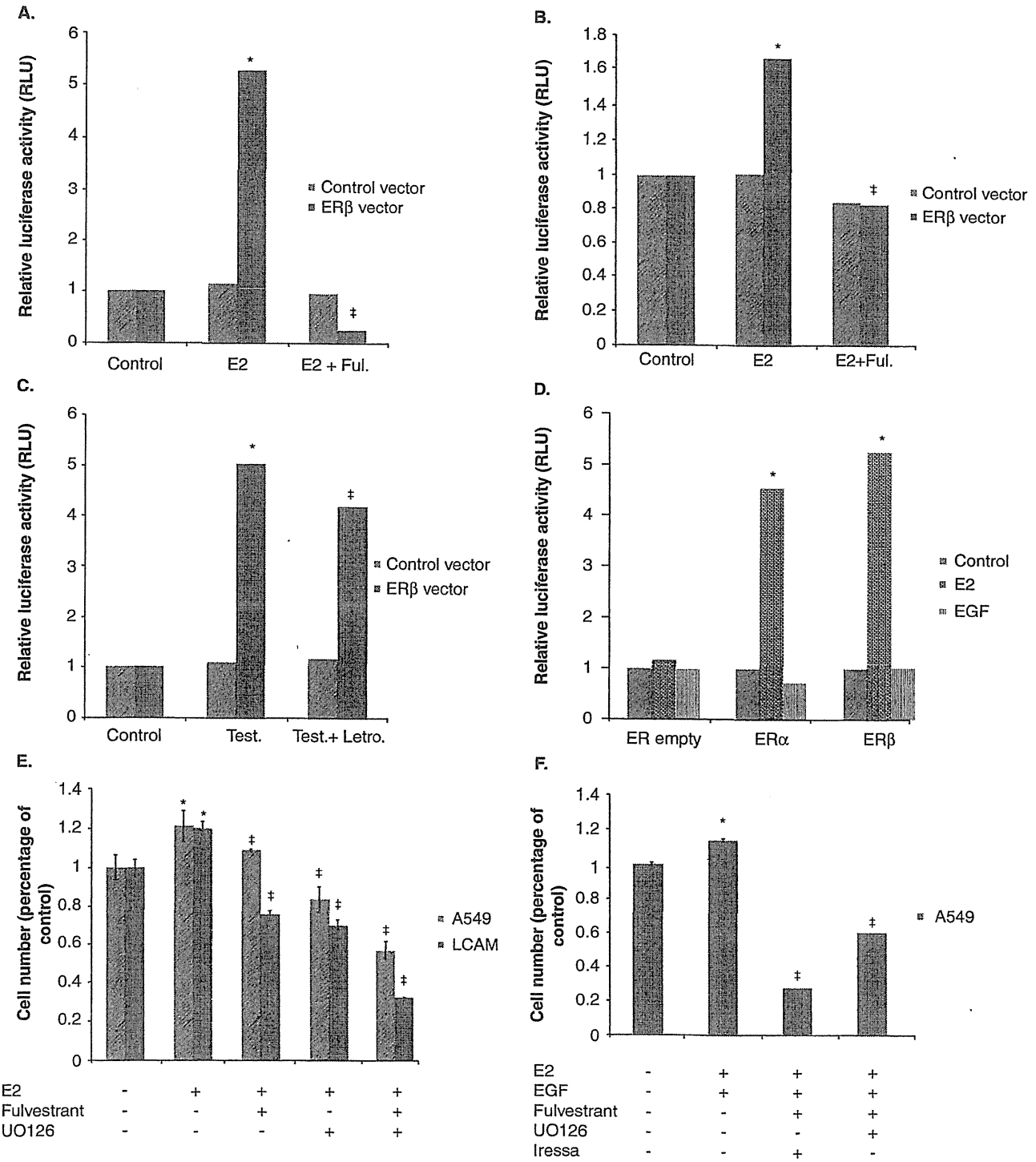



**Figure 3. ERE-Luciferase activity (relative light units (RLU)) on treatment with E2 A.** A549 cells. **B.** LCAM1 cells (Ful: fuvestrant). **C.** ERE-Luciferase activity on treatment with testosterone in A549 cells. **D.** ERE-luciferase activity in ER empty, ERα and ERβ vector transfected A549 cell on treatment with either estrogen or EGF. **E.** Proliferative effects after 72 h of treatment in A549 and LCAM1 cells. **F.** Anti-proliferative effects of combination therapy with Iressa and fulvestrant versus combination therapy with U0126 and fulvestrant after 48 h of treatment in A549 cells. Data represent three independent experiments, each performed in triplicate. Results are represented as mean ± SD.

*Represents $p < 0.0001$ vs. control.

†Represents $p < 0.0001$ vs. either testosterone treatment or E2 and/or EGF treatments.

the presence of c-ERβ was associated with poorer 5 year survival and median survival rates (c-ERβ positive = 54.8% and 1992 days versus c-ERβ negative = 62.0% and 2805 days, respectively), although the correlation did not reach statistical significance (Figure 1D, Table 4). A similar non-significant tendency was detected when ERβ expression was demonstrated either as a continuous variable or as a dichotomized variable (high versus low). These findings suggest that neither c-ERα, nor n-ERβ 1s a predictor of survival in NSCLC patients [32]. However, c-ERβ was very recently demonstrated to be an important predictor of poorer survival in both men and women with lung cancer [30]. Results of our study also suggested that there were subgroups in which c-ERβ may enhance the estrogenic signaling in NSCLC patients in addition to the genomic actions via n-ERβ. However, the relatively small sample size examined in our present study limited our ability to clearly detect significant prognostic effects of c-ERβ in these patients. Therefore, further studies such as an examination of a large patient cohort are definitely required to obtain better understanding of the potential roles of c-ERβ as predictive biomarker NSCLC patients.

Endogenous levels of c-ERβ and/or n-ERβ could not bring any genomic activity via ERE segments on DNA in the presence of either exogenous E2 or endogenous E2 produced via aromatase activity in A549 and LCAM1 cells, (Figure 3A - C). This may be due to insufficient n-ERβ expression level [12] and/or inability of c-ERβ to translocate into the nucleus after ligand binding [14]. In addition, both ERβ- and ERα-transfected A549 cells demonstrated much higher luciferase activity following E2 treatment but not on EGF treatment, (Figure 3D) which also ruled out any ligand-independent activation of ERs and once again suggested more pronounced roles of ERβ in NSCLC cells. However, ERs can regulate gene expression via non-ERE segments by interacting with the DNA-bound transcription factors [33,34]. Therefore, further investigations are required for better clarification of genomic effects on estrogen in NSCLCs.

In breast carcinoma cells, ERs transactivate membrane EGFR via non-receptor kinases, such as c-Src to rapidly signal through various kinase cascades [16]. Similar transactivation of the EGFR via ERs on ligand binding was reported in 201T cells, a NSCLC cell line [6] even though other investigators did not demonstrate a similar transactivation of EGFR in NSCLC cells [12,14]. We detected only MAPK activation on E2 treatment within 5 min with no transactivation of either EGFR or Akt or c-Src in A549 cells, which was successfully abrogated by 30 min pretreatment with an MAPK inhibitor, (Figure 2C). In addition, pretreatment of NSCLC cells with ER blocker, fulvestrant, for 24 h successfully abrogated this MAPK activation in either A549 and LCAM1 cells, (Figure 1D and E). The fact that a pure ER antagonist, fulvestrant, cannot behave as an antagonist for GPER suggest that estrogen triggered MAPK activation was in fact caused via c-ERβ after ligand binding and not via GPER [35]. Previous reported studies did not necessarily use any ER blocker(s) to confirm whether MAPK or Akt activation on E2 treatment involves mainly membrane/cytoplasmic pools of ERs or GPER. Results of our present study also suggest that c-ERβ on ligand binding may transactivate MAPK without any transactivation of EGFR, (Figure 2F) and MAPK inhibitor could be used very effectively for abrogation of these non-genomic actions of estrogen in NSCLCs.

In addition, the combination therapy with U0126 resulted in enhanced synergistic and additive anti-proliferative effects in A549 and LCAM1 cells respectively than either treatment alone, (Figure 3E). Furthermore, fulvestarnt alone was able to abrogate proliferative effects of estrogen suggesting the possible presence of genomic estrogen signaling via non-ERE segments. However, it is also true that the EGFR pathway was reported to be activated when estrogen was depleted in NSCLC cells and thus simultaneous therapies targeting both pathways are reasonably considered most beneficial in the patients with NSCLC [6]. It was recently demonstrated that estrogen downregulates EGFR levels and tamoxifen upregulates EGFR level in NSCLC cells [36]. These results all suggested that at any given time either the E2 pathway or EGFR is activated in NSCLC cells. In addition, a high c-ERβ along with a high EGFR and low progesterone receptor status in NSCLC patients were reported to predict poorer survival suggesting a close association between ER and EGFR [30]. Therefore, systematic examination of c-ERβ along with EGFR in NSCLC patients could ultimately identify the patients who may benefit most from anti-hormonal therapy, anti-EGFR therapy or combination of both but further investigations are required for clarification. However NSCLC patients invariably develop resistance to EGFR tyrosine kinase inhibitors and the MAPK inhibitor, U0126, has been used by investigators in order to overcome this resistance, in NSCLC cells [37]. Therefore, the combination therapy with both U0126 and fulvestrant may also confer therapeutic benefits to ER expressing NSCLC patients who develop resistance to EGFR-TKI, such as Iressa.

## 5. Conclusion

The results of our present study suggest that c-ERβ and n-ERβ may result in more aggressive tumor progression in NSCLC patients expressing aromatase. Therefore, targeting both genomic or/and non-genomic estrogen signaling may confer therapeutic benefits to patients expressing both c-ERβ and n-ERβ. However, these findings are also limited by unavailability of a standardized IHC assay for the measurement of ERs in lung cancer and the relatively small number of specimens available for our present study. Therefore, a standard IHC method must be explored in order to understand the clinical significance of ERs in a large cohort of NSCLC patients.

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## Declaration of interest

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## Affiliation

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# Runt-related transcription factor 2 in human colon carcinoma: a potent prognostic factor associated with estrogen receptor

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**Runt-related transcription factor 2 (RUNX2) belongs to the RUNX family of heterodimeric transcription factors, and is mainly associated with osteogenesis. Previous *in vitro* studies demonstrated that RUNX2 increased the cell proliferation of mouse and rat colon carcinoma cells but the status of RUNX2 has remained unknown in human colon carcinoma. Therefore, we examined clinical significance and biological functions of RUNX2 in colon carcinoma. RUNX2 immunoreactivity was examined in 157 colon carcinoma tissues using immunohistochemistry. RUNX2 immunoreactivity was evaluated as percentage of positive carcinoma cells [*i.e.*, labeling index (LI)]. We used SW480 and DLD-1 human colon carcinoma cells, expressing estrogen receptor-β (ER) in subsequent *in vitro* studies. RUNX2 immunoreactivity was detected in colon carcinoma cells, and the median value of RUNX2 LI was 67%. RUNX2 LI was significantly associated with Dukes' stage, liver metastasis and ERβ status. In addition, RUNX2 LI was significantly associated with adverse clinical outcome of the colon carcinoma patients, and turned out an independent prognostic factor following multivariate analysis. Results of *in vitro* studies demonstrated that both SW480 and DLD-1 cells transfected with small interfering RNA against RUNX2 significantly decreased their cell proliferation, migration and invasive properties. In addition, RUNX2 mRNA level was significantly decreased by ER antagonist in these two cells. These findings all suggest that RUNX2 is a potent prognostic factor in human colon carcinoma patients through the promotion of cell proliferation and invasion properties, and is at least partly upregulated by estrogen signals through ERβ of carcinoma cells.**



Colon cancer is the third leading cause of cancer-related deaths in both men and women in the United States.[1] It is true that the recent advances in chemotherapy prolonged the survival of the patients with advanced clinical stages[2] but these results are still unsatisfactory and further studies are being required to understand the disease process and to improve the clinical outcome of the patients.

**Key words:** colon carcinoma, Runt-related transcription factor 2, immunohistochemistry, prognosis, estrogen receptor

**Abbreviations:** BSP: bone sialoprotein; ER: estrogen receptor; MMP: Matrix metalloproteinase; RUNX2: Runt-related transcription factor 2; VEGF: vascular endothelial growth factor

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The Runt-related transcription factor 2 (RUNX2) belongs to the RUNX family of heterodimeric transcription factors and shares a common sequence termed, the Runt domain, with other members involved in DNA binding and transactivation.[3] RUNX2 is a well-known transcription factor required in the process of bone formation or osteogenesis.[4–7] For instance, an altered chondrocyte morphology was reported in RUNX2 heterogenous mice,[8] and its deregulation was also associated with the development of osteosarcoma.[9,10]

Recently, expression of RUNX2 has been reported in several human malignancies such as prostate,[11] pancreatic,[12] thyroid[13] and breast[14] cancers, all of which highlighted the carcinogenic properties of RUNX2 in these studies. Results of previous *in vitro* studies did demonstrate that RUNX2 increased the cell proliferative activity of mouse[15] and rat[16] colon carcinoma cells, which suggests a possible role for RUNX2 also in colon carcinoma. In addition, very recently, Edvardsson *et al.*[17] conducted genome-wide expression studies in combination with gene-pathway analyses and cross-correlation to estrogen receptor-β (ERβ)-chromatin-binding sites, and demonstrated that RUNX2 expression was induced by ERβ in human colorectal carcinoma cells. However, the

status of RUNX2 has not been examined in human colon carcinoma to the best of our knowledge, and its biological and clinical significance has remained unknown. Therefore, in this study, we examined clinical significance and biological functions of RUNX2 in colon carcinoma using immunohistochemistry and *in vitro* studies.

## Material and Methods

### Patients and tissues

Colon carcinoma surgical pathology specimens were obtained from 157 consecutive patients (86 men and 71 women) operated at Miyagi Cancer Center (Natori, Japan) from 1994 to 2000. A mean age of these patients was 67.0 years (range 35–85 years). The mean follow-up time was 100 months (range 1–149 months), and overall survival data were available in all the patients examined. The specimens had been fixed in 10% formalin and embedded in paraffin-wax.

Review of the patients' charts revealed that neither of the patients received irradiation nor chemotherapy before the surgery. Informed consent was obtained from all the patients above and research protocol for this study was approved by the Ethics Committees at the Miyagi Cancer Center (2007–2006).

### Immunohistochemistry

Mouse monoclonal antibody for human RUNX2 was purchased from Abnova Corporation (Taipei, Taiwan). The characterization of this antibody has been reported using both immunoblotting and immunohistochemistry.[18] Monoclonal antibodies for ERβ (MS-ERB13-PX1) and Ki-67 (MIB1) were purchased from GeneTex (Irvine, CA) and DAKO (Carpinteria, CA), respectively. A Histofine kit (Nichirei, Tokyo, Japan), using the streptavidin–biotin amplification method, was used in this study. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution [1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6) and 0.006% $H_2O_2$], and counterstained with hematoxylin. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibodies, and no immunoreactivity was detected in these tissue sections.

Immunoreactivity of RUNX2 and Ki67 was detected in the nuclei. Their immunoreactivity was evaluated in more than 1,000 carcinoma cells for each case, and subsequently, the percentage of immunoreactivity, that is, labeling index (LI), was determined. The status of ERβ immunoreactivity was evaluated using Allred score.[19] Briefly, following an evaluation of the proportion (0: none, 1: <1/100, 2: 1/100–1/10, 3: 1/10–1/3, 4: 1/3–2/3, and 5: >2/3) and immunointensity (0: none, 1: weak, 2: moderate, and 3: strong) in the carcinoma cells, the total score more than 3 was considered ERβ-positive case. An association between RUNX2 LI and clinicopathological factors of colon carcinoma patients was statistically evaluated using a correlation coefficient ($r$) and regression equation, Student's $t$ test, or a one-way ANOVA and Bonferroni test. Overall survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the log-rank test. Both univariate and multivariate analyses were performed by a proportional hazard model (COX) using StatView 5.0 software (SAS Institute, Cary, NC), and differences with $p < 0.05$ were considered significant.

### Cell lines and chemicals

Two human colon carcinoma cell lines (SW480 and DLD-1) were provided from the American Type Culture Collection (Manassas, VA). The cells were cultured in the recommended medium [L-15 Medium Leibovitz (Sigma-Aldrich, St. Louis, MO) for SW480 or RPMI1640 (Sigma-Aldrich) for DLD-1, containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA)]. A pure ER antagonist ICI 182,780[20] was purchased from Tocris Cookson (Ellisville, MO) in this study.

### Real-time PCR

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using a SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction Kit (Invitrogen) from 5 μg of total RNA.

The LightCycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semiquantify the mRNA expression levels by real-time PCR.[21] The primer sequences used in our study are as follows: RUNX2 (NM_004348 forward: 782–800 and reverse: 943–961),[22] ERβ (AB006590; forward: 1,460–1,480 and reverse: 1,608–1,627), and ribosomal protein L 13a (RPL13A) (NM_012423; forward: 487–509 and reverse: 588–612).[23] The mRNA level in each sample was represented as a ratio of RPL13A and was evaluated as a ratio (%) compared with that of each control.

### Small interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotide for RUNX2 used in this study was Stealth RNAi siRNA Duplex Oligoribonucleotides (Invitrogen), and Stealth RNAi Negative Control Duplexes (Invitrogen) was also used as the negative control. The sequence of siRNA against RUNX2 (RUNX2-HSS189448) was as follow: sense 5′-AUCUACUGUAACUU UAAUUGCUCUG-3′ and antisense 5′-CAGAGCAAUUAAA GUUACAGUAGAU-3′. siRNA against ERβ (forward 5′-GUGUGAAGCAAGAUCGCUA-3′ and reverse 5′-UAGCGA UCUUGUUUCACAC-3′) was also used in this study. siRNAs were transfected (10 nmol/L) using HiperFect transfection reagent (Qiagen GmbH, Hilden, Germany) according to the instruction manual.

### Immunoblotting

The protein of SW480 and DLD1 cells was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). 20 μg of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel).

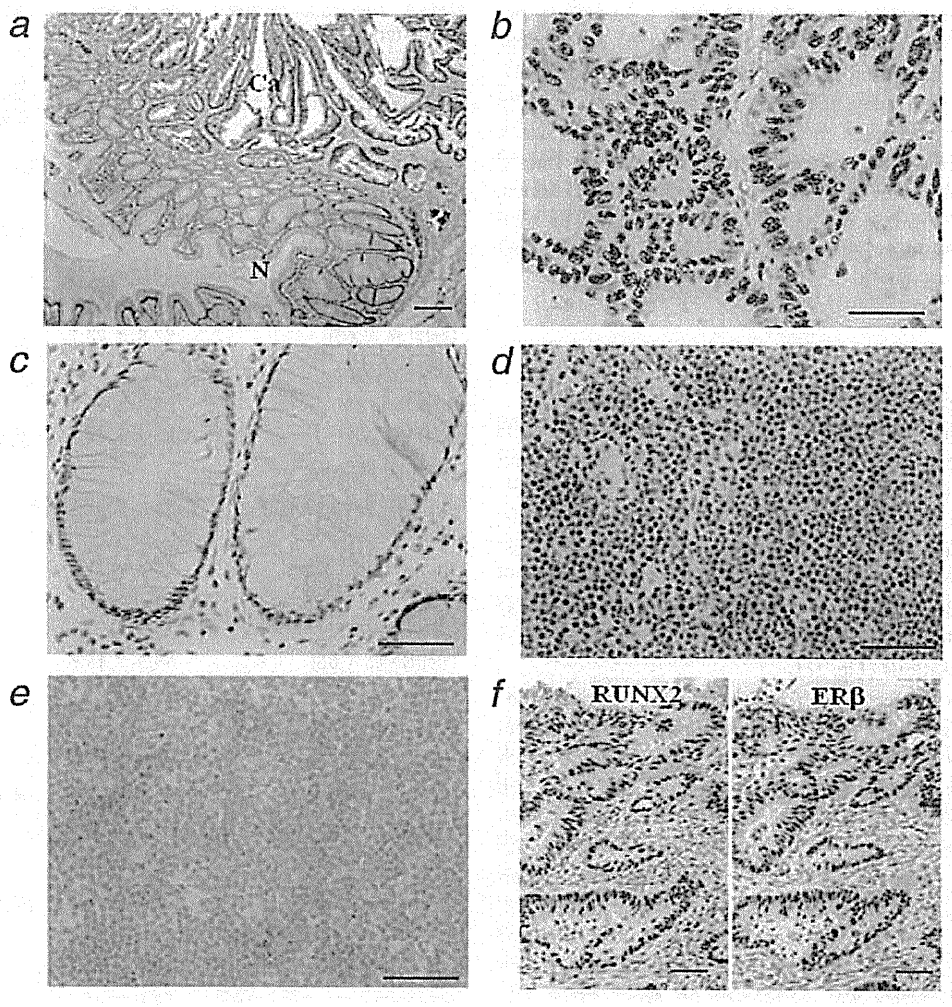


**Figure 1.** Representative illustrations of RUNX2 immunohistochemistry in colon carcinoma cases. (*a*) RUNX2 immunoreactivity was detected in the carcinoma cells (Ca), while almost negligible in the non-neoplastic colonic epithelium (N). Lower magnification and scale bar = 200 μm. (*c* and *d*) RUNX2 was immunolocalized in the nuclei of carcinoma cells (*b*), while focally and weakly positive in non-neoplastic colonic epithelium cells (*c*). Higher magnification and scale bar = 100 μm, respectively. (*d* and *e*) Positive (*d*) and negative (*e*) control sections of RUNX2 immunohistochemistry (same area of the lymph node). Scale bar = 100 μm, respectively. (*f*) Immunohistochemistry for RUNX2 and ERβ in the colon carcinoma on serial tissue sections (same area). Scale bar = 100 μm, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). Primary antibodies used were anti-RUNX2 antibody used for immunohistochemistry (Abnova Corporation) and anti-β-actin antibody (AC-15, Sigma-Aldrich). Antibody–protein complexes on the blots were detected using ECL-plus Western blotting detection reagents (GE Healthcare), and the protein bands were visualized with LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

### Cell proliferation, migration, and invasion assays

SW480 and DLD-1 cells were transfected with RUNX2-specific siRNA or control siRNA in a six-well culture plate. In one day after transfection, these cells (approximately $1 \times 10^4$ cells/well) were transferred to 96-well culture plate and cultured in medium containing 10% FBS. Three days after the transfection, the status of cell proliferation was measured by a cell counting kit-8 (Dojindo, Kumamoto, Japan).

The migration assay was performed according to the procedure reported by Fang *et al.*[24] Briefly, 4 days after the transfection, colon carcinoma cells cultured in a six-well culture plate were carefully scratched by sterile 20-μl pipette tips, and then incubated for 24 hr. The scratched edges were imaged by phase-contrast microscopy, and the migration area was calculated using the NIH image software (http://rsb.info.nih.gov/nih-image/Default.html).[24]

Cell invasion assay was performed using a 24-well (8 μm pore size) BD Matrigel™ Invasion Chamber (BD Biosciences, Bedford, MA), according to the manufacturer's protocol. After incubation for 1 day, the noninvading cells are removed from the upper surface of the membrane using cotton swabs. The cells on the lower surface of the membrane are stained with Diff-Quik stain™ (Sysmex International Reagents, Kobe, Japan) according to the instruction manual. The total number of invading cells of the membrane was counted under the microscope.

## Results

### RUNX2 immunolocalization in human colon cancer

RUNX2 immunoreactivity was detected in the nuclei of the colon carcinoma cells (Figs. 1*a* and 1*b*), and the median value of RUNX2 LI was 67.0% (range 0–100%). RUNX2 immunoreactivity was weakly and focally detected in some non-neoplastic colonic epithelial cells (Fig. 1*a* and 1*c*). In the positive control, RUNX2 was mainly positive in lymphocyte of the lymph node (Fig. 1*d*), as previously reported,[25] whereas no significant immunoreactivity was detected in the negative control tissue sections (Fig. 1*e*).

Associations between RUNX2 LI and various clinicopathological parameters in colon carcinoma patients were summarized in Table 1. RUNX2 LI was significantly associated with Dukes' stage ($p = 0.04$), liver metastasis ($p = 0.02$) and ERβ status ($p = 0.02$) of the cases examined. However, no significant association was detected in other factors examined, such as patient age, gender, tumor site, depth of invasion, lymph node metastasis, peritoneal metastasis, histological differentiation and Ki67 LI. Co-localization of RUNX2 and ERβ was detected in the majority of colon carcinoma cells, when their immunohistochemistry was performed on serial tissue sections (Fig. 1*f*).

### Association between RUNX2 LI and clinical outcome of the patients

As demonstrated in Figure 2*a*, higher RUNX2-LI group was significantly ($p < 0.0001$) associated with adverse clinical outcome of the patients when the 157 cases were tentatively classified into two groups according to the median value of RUNX2 LI. Similar tendencies were detected regardless of the Dukes' stage (Figs. 2*b* and 2*c*) or in the ERβ-positive cases ($n = 131$; Fig. 2*d*). Results of the univariate analysis studied by COX analysis (Table 2) demonstrated that RUNX2 LI ($p < 0.0001$), liver metastasis ($p < 0.0001$), Ki-67 LI ($p = 0.01$), lymph node metastasis ($p = 0.03$) and ERβ ($p = 0.04$) turned out significant prognostic variables for overall survival of the patients in this study. Results of the following multivariate analysis, however, showed that only RUNX2 LI ($p = 0.003$) and Ki67 LI ($p = 0.02$)[26] were independent prognostic factors. RUNX2 LI was also detected as an independent prognostic factor, when evaluated as a continuous variable in the multivariate analysis ($p = 0.0002$; data not shown).

**Table 1.** Association between RUNX2 LI and clinicopathological parameters in 157 colon carcinoma patients

| Value | RUNX2 LI ($n = 157$) | $p$-value |
|---|---|---|
| Patient age[1] | | 0.91 ($r = -0.18$) |
| **Gender** | | |
| Men ($n = 86$) | 57.7 ± 26.6 | |
| Women ($n = 71$) | 58.7 ± 27.2 | 0.46 |
| **Tumor site**[2] | | |
| Proximal ($n = 94$) | 58.2 ± 26.4 | |
| Distal ($n = 63$) | 57.8 ± 24.7 | 0.91 |
| **Dukes' stage** | | |
| A+B ($n = 83$) | 52.5 ± 23.1 | |
| C+D ($n = 74$) | 64.3 ± 19.4 | **0.04** |
| **Depth of invasion** | | |
| Submucosa-muscularis propria ($n = 27$) | 47.0 ± 28,6 | |
| Through muscularis propria ($n = 130$) | 60.4 ± 27.5 | 0.14 |
| **Lymph node metastasis** | | |
| Positive ($n = 66$) | 63.9 ± 19.8 | |
| Negative ($n = 91$) | 53.8 ± 28.4 | 0.15 |
| **Liver metastasis** | | |
| Positive ($n = 20$) | 70.9 ± 13.7 | |
| Negative ($n = 137$) | 56.0 ± 26.4 | **0.02** |
| **Peritoneal metastasis** | | |
| Positive ($n = 4$) | 69.0 ± 10.8 | |
| Negative ($n = 153$) | 57.6 ± 25.8 | 0.38 |
| **Histological differentiation**[3] | | |
| Well ($n = 44$) | 60.4 ± 25.5 | |
| Moderate + poor ($n = 103$) | 58.0 ± 25.2 | 0.61 |
| **ERβ status** | | |
| Positive ($n = 131$) | 60.1 ± 25.1 | |
| Negative ($n = 26$) | 47.0 ± 25.9 | **0.02** |
| Ki67 LI[1,4] | | 0.17 ($r = 0.26$) |

[1]The association was statistically evaluated using a correlation coefficient ($r$) and regression equation. The data were presented as mean ± SD. $p$-values of less than 0.05 were considered significant, and are shown in bold. [2]Proximal colon included in ascending and transverse colon. [3]Cases of mucinous adenocarcinoma were excluded in this study. [4]The mean Ki67 LI of these patients was 49.8% (range 2–96%).

### Effects of RUNX2 on cell proliferation, migration and invasion in colon carcinoma cells

In this study, we transfected two human colon carcinoma cells (SW480 and DLD-1) with specific siRNA against RUNX2 to examine biological functions of RUNX2 in these cells. RUNX2 mRNA level compared to RPL13A examined by real-time PCR analysis was 1.8% in SW480 cells and 1.1% in DLD-1 cells (data not shown), and RUNX2 LI evaluated