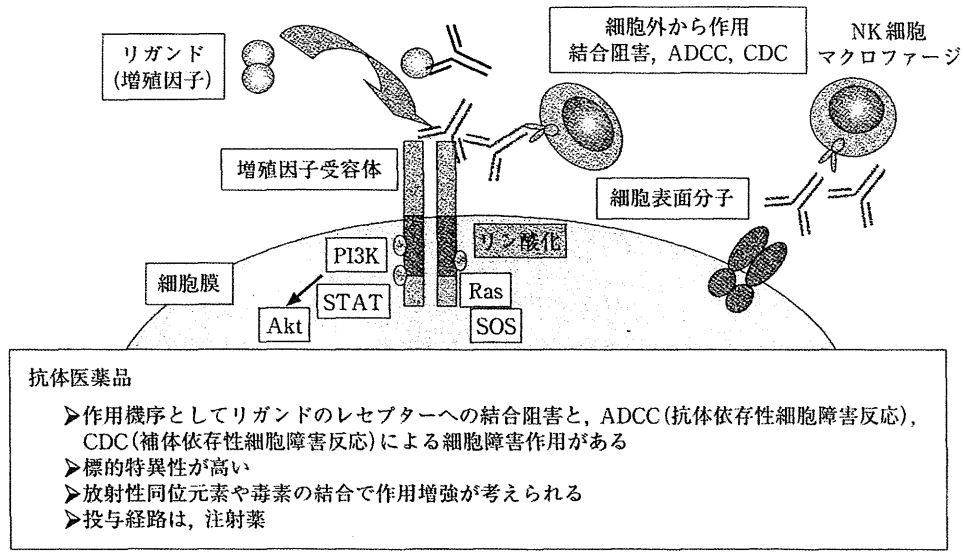

図2 分子標的治療薬：抗体と標的分子

性細胞障害反応), CDC(補体依存性細胞障害反応)による細胞障害作用が想定されている. 抗体医薬品は標的特異性が高く, 放射性同位元素や毒素を結合させることにより作用増強が図られている(**図2**).

b. 小分子阻害剤

がん細胞内で異常に発現されている分子(受容体チロシンキナーゼ, シグナル伝達因子など)を標的に制御する小分子化合物が多く開発されている. それらの作用機序は, 主にシグナル伝達分子をピンポイントに阻害する. また, 合成可能で量産が比較的容易であり, 経口薬が多い. しかし, 標的とされる分子以外にも作用する可能性があるため, 予測できないような有害事象がみられることもあり, 注意深い経過観察が必要とされる(**図3**). 代表的な標的分子として, Bcr-AblやEGF, VEGF, PDGF, FGFなどに対する受容体の細胞内ドメインにあるチロシンキナーゼのリン酸化を拮抗的に阻害する小分子化合物が注目されている(**表2-c**).

近年, 小分子阻害剤の開発は, 1つの標的分子から複数の分子を同時に標的化し, 抗腫瘍効果を増強する動向にある[13]. その代表的なものが, 慢性骨髄性白血病細胞のBcr-Ablを標的としたイマチニブ(グリベック)で, Bcr-Ablのほかにc-KitやPDGF受容体も阻害する作用をもつ. 後に, 異常なc-Kit発現をもつGIST症例にも高い抗腫瘍効果を示し, 適応疾患の拡大につながっている. ほかに, 目立つ点としては, 血管内皮成長因子VEGFや上皮成長因子EGFに対応する受容体のチロシンキナーゼを同時に阻害する分子標的薬や, 血管新生因子であるVEGF, FGF, PDGFに対する受容体のチロシンキナーゼ分子を同時標的とした阻害剤が多い.

4. 治療効果予測のためのバイオマーカー開発

1990年代に始まったがん分子標的薬の登場は, 難治がんとされていた種々の血液腫瘍や固形がんの退縮やがん増殖・進展の長期にわたる抑制(stable disease: SD)効果を可能とした点で大きなインパクトを与えている. その結果, がん分子標的治療薬の臨床効果を評価する方法も, 従来のがん縮小(奏効率)だけでなく, SD効果も評価され, 増悪(再発)までの期間をエンドポイントとする考え方も支持されている.

ピンポイントで攻撃する分子標的薬の作用機序は臨床の場で次々と明らかにされ, 更に治療効果や有害事象の発生を予測するバイオマーカーの開発へと, この10年余りで大きな展開がみ

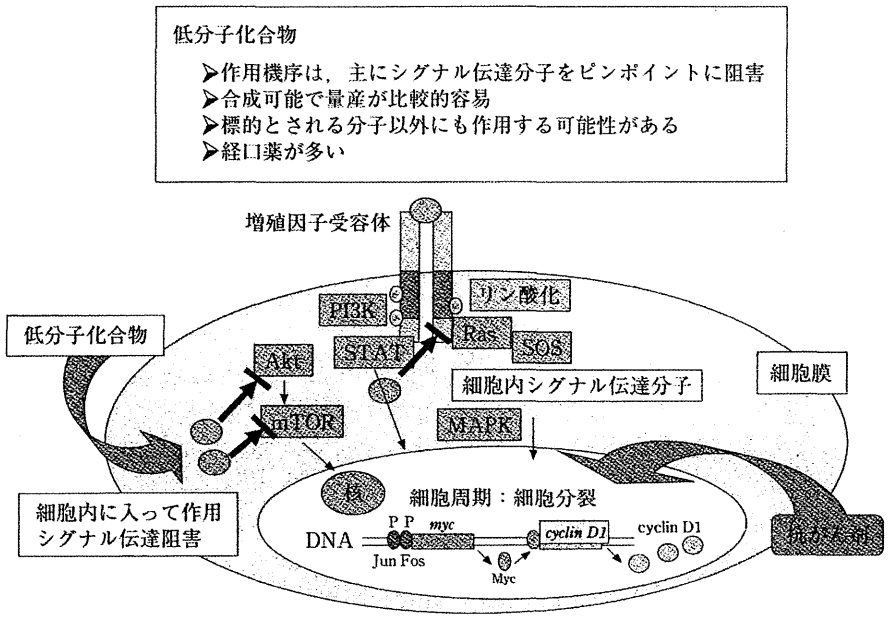

図3 分子標的治療薬の特徴

られている[14-17]．すなわち，PubMedを用いて，'cancer'と'biomarker'の2つをキーワードとして検索すると，2000年の12,654編から2009年には45,571編と，4倍の発表論文数となっている．更に，'treatment'を含めた3つのキーワードで検索すると，発表論文数は約半数になるものの20,243編が2009年に発表されている(図4)．このようなバイオマーカー研究の流れを大きくしたのは，2005年に米国FDAが提唱したDrug-Diagnostic Co-development構想である(図5)．この構想はがん治療薬物の開発において，前臨床の早い時期からその有効性を予測するための診断法(バイオマーカー)を同時開発していくことの重要性を明確にした点で評価が高い．現在，がんの進展や薬剤感受性を反映するバイオマーカーを探索する研究にはタンパクレベル，RNAレベル，DNAレベルでの，いろいろな先端的な方法が用いられている(表3)．その結果，幾つかのバイオマーカー(HER2, EGFR mutation, K-ras, ALK, BRAFなど)は特定のがん患者の個別化治療へと臨床応用されている[18]．特に，薬剤感受性を予測するためのバイオマーカーの確立は個別医療という観点から患者にとって大きな朗報であり，診断的な意義は極めて大きいといえる．臨床的により簡便で再現性や定量性に富むバイオマーカー開発は今後ともホットな領域として国際競争の中で成長していくものと思われる．

また，最近では，分子標的治療薬(Bcr-Abl阻害薬，EGFR-TK阻害薬，HER2阻害薬など)に対する耐性化誘導の分子機構も明らかにされ，その克服に向けた取り組みが精力的になされている[19-24]．このような背景の中で分子標的治療薬に対する感受性や耐性化を予測するバイオマーカーの開発は，臨床の場で大きな役割を担うことが期待されている．

5. 分子標的治療とQOL評価

がん治療におけるQOL評価はKarnofsky performance status scale(KPS)(1948)に始まる．がん患者の活動指標(performance status: PS)は予後との強い相関が知られているが，PSは第三者(主に医師)が患者の身体活動性から判定するものであり，患者の心理的・社会的な面からの評価は不十分である．1980年以後，より有効な抗がん剤が臨床に登場し，がん患者の生存

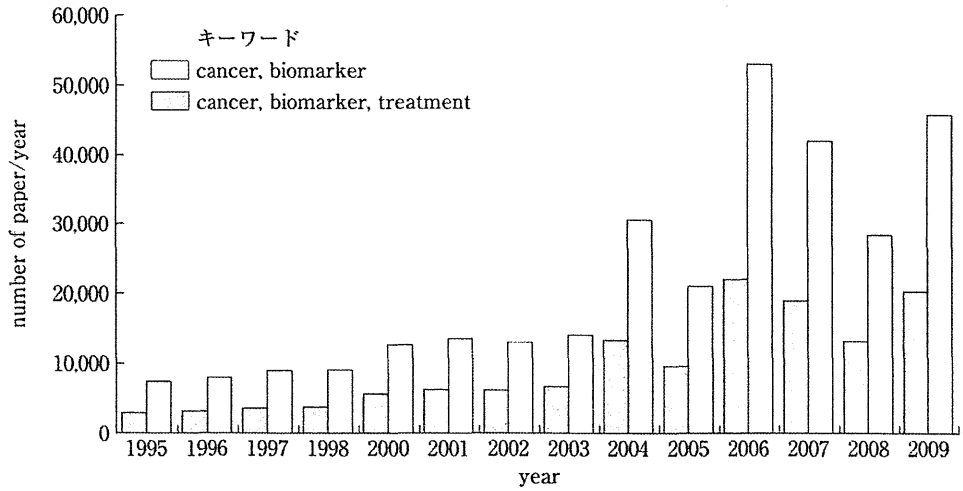

図4 がんバイオマーカー関連論文数の年度別推移

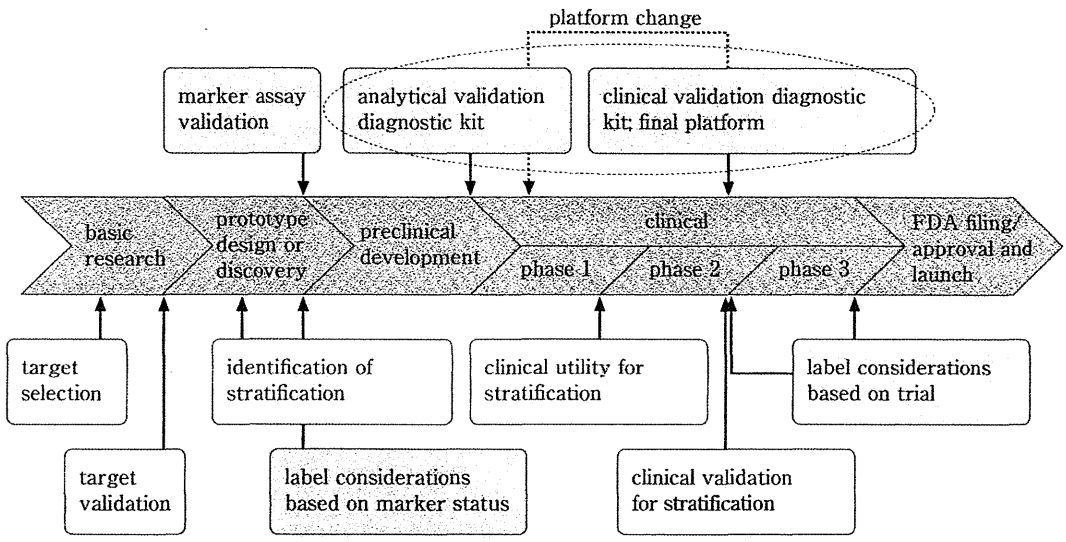

図5 米国でのがん治療効果予測マーカーの同時開発に向けた構想
(FDA Concept Paper, Drug-Diagnostic Co-development(Draft), April 2005)

期間が延長されるに伴って，多種類のQOLの調査票が考案されてきたが，どれも医師などの第三者による評価方式であった．その後，患者自身による自己記入式のQOL評価法(Linear Analogue Self-Assessment(LASA), Functional Living Index-Cancer(FLIC)など)が考案され，1985年には米国FDAが抗がん剤の認可に際して腫瘍の縮小率，生存期間延長効果に加えて，QOLに関する評価基準を提案した．これを契機に，QOL関連の臨床試験が急激に増加した．その後，米国臨床腫瘍学会もがん治療ガイドライン(1995)の中にQOL重視の考え方を明記したこともあり，身体的，心理的，社会的評価の3項目を含めたQOL調査票が汎用されつつある．

今までに開発されたQOL評価法の中で，EORTC QLQ-C30調査票は患者自ら質問事項に回答するpatient-reported outcome(PRO)方

表3 バイオマーカー探索方法

解析項目	方法
protein expression	IHC, ELISA, ELISA array
protein phosphorylation	IHC
RNA expression	RT-PCR, Taqman array, RNA microarray, ISH
miRNA	RT-PCR, miRNA array
DNA SNP	RT-PCR, sequencing, SNP microarray
DNA copy number	FISH, RT-PCR, copy number microarray
DNA methylation	RT-PCR, sequencing, methylation microarray
DNA mutation	RT-PCR, sequencing

IHC: immnohistochemistry, FISH: fluorescent *in situ* hybridization, miRNA: microRNA, SNP: single nucleotide polymorphism.

式であり，がん薬物療法を用いた第III相臨床試験で副次的評価項目の一つとして汎用されている[24,25]．この10年を振り返ると，抗がん剤治療から分子標的治療へと大きなパラダイムシフトが起こっており，がん分子標的薬を用いた第II相，第III相試験においても，奏効率，生存期間延長効果とともにPRO評価が副次的なエンドポイントとして，治療効果を検証する手段の一つに用いられるべきである．事実，がん分子標的治療で臨床効果がstable disease(SD)であってもpartial response(PR)群に近いQOL改善効果が得られるとの報告がなされている[26]．

今後，高齢者を含めたがん患者のQOL状態をPRO評価により多面的に把握できれば，適切な治療法選択や精神的ケアなどにつながる可能性が考えられる．

おわりに

近年の分子生物学的手法の進歩によりがんの進展・転移に関与する複雑な分子機構も急速に解明が進んできた．その成果は，がん分子標的薬の探索から臨床開発トランスレーショナルリサーチがこの20年の間に大きく前進し，多くのがん患者に還元されつつある．がん分子を標的とした薬のもつ特性や特色を生かした治療ががん診療の中で適切に行われるには，臨床効果や有害事象の発生を的確に予測できるバイオマーカーの開発が必須である．同時に，がん分子標的薬の臨床的な有用性が検証されるには，がんの縮小や消失ではなく，がんをもっている患者自身のQOLが改善保持され，生存期間の延長に結びつかなければ治療薬としての意義はない．現在，がん分子標的治療に使われる数多くの候補医薬品が開発の途上にあるが，それらが臨床で正当なポジションを得るには個別医療に向けたバイオマーカー研究やQOL研究の進展に依存するところが大きい．今後の発展に期待したい．

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ORIGINAL RESEARCH

Individual transcriptional activity of estrogen receptors in primary breast cancer and its clinical significance

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Breast cancer, ERE transcriptional activity, estrogen receptor α, Ki67, Luminal A

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Abstract

To predict the efficacy of hormonal therapy at the individual-level, immunohistochemical methods are used to analyze expression of classical molecular biomarkers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2. However, the current diagnostic standard is not perfect for the individualization of diverse cases. Therefore, establishment of more accurate diagnostics is required. Previously, we established a novel method that enables analysis of ER transcriptional activation potential in clinical specimens using an adenovirus estrogen response element–green fluorescence protein (ERE-GFP) assay system. Using this assay, we assessed the ERE transcriptional activity of 62 primary breast cancer samples. In 40% of samples, we observed that ER protein expression was not consistent with ERE activity. Comparison of ERE activity with clinicopathological information revealed that ERE activity was significantly correlated with the ER target gene, PgR, rather than ER in terms of both protein and mRNA expression. Moreover, subgrouping of Luminal A-type breast cancer samples according to ERE activity revealed that ERα mRNA expression correlated with ER target gene mRNA expression in the high-, but not the low-, ERE-activity group. On the other hand, the low-ERE-activity group showed significantly higher mRNA expression of the malignancy biomarker Ki67 in association with disease recurrence in 5% of patients. Thus, these data suggest that ER expression does not always correlate with ER transcriptional activity. Therefore, in addition to ER protein expression, determination of ERE activity as an ER functional marker will be helpful for analysis of a variety of diverse breast cancer cases and the subsequent course of treatment.

Introduction

To predict the efficacy of hormonal therapy for breast cancer at the level of the individual, immunohistochemical methods are used to analyze classical molecular biomarkers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2 [1–3]. Novel markers such as Ki67, FOXA1, and GATA3 are also examined and used to predict long-term outcome after neoadjuvant endocrine treatment [4–7]. However, the current diagnostic standard is not always suitable for the classification of cases. In ER-positive patients, endocrine therapy to antagonize ER signaling is ineffective in approximately 30% of cases [8]. This discrepancy could be the result of the activation of other ER-independent estrogen-related signaling pathways in these breast cancer cells, such as

insulin-like growth factor 1 (IGF-1)- or vascular endothelial growth factor (VEGF)-mediated signaling cascades [9, 10]. Therefore, reliable diagnostic techniques or tools are required for the sensitive evaluation of likely endocrine therapy efficacy for individual patients.

ER is activated by estrogen [11, 12] or protein phosphorylation by kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt [13, 14]. Activated ER induces transcription of genes containing the estrogen response element (ERE). The molecular mechanisms regulating transcriptional activity by ER have been well investigated in breast cancer cells. However, although ER protein expression has been evaluated by immunohistochemistry (IHC) [1, 2], its relationship with ERE transcriptional activity has not been reported. We have previously observed several cases in which ER protein expression and ER target gene mRNA expression do not correlate [15–18]. These results suggest that ER protein expression may not necessarily reflect the function of ER.

To explore the possibility of recategorizing breast cancers, we analyzed human breast cancer cases according to three features: ER protein expression, ERE transcriptional activity, and ER target gene mRNA expression. We have previously produced a construct in which the common ERE is ligated upstream of green fluorescence protein (GFP) cDNA, and packaged into an adenovirus vector [12, 19, 20]. Primary breast cancer cells, prepared from patients, were infected with this adenovirus vector, and the ERE transcriptional activity was measured by analyzing the GFP fluorescence, as previously described for endometrial cancer [20]. We also determined the protein and mRNA expression levels of ER and the ER target genes identified in our microarray [15–18], using formalin-fixed paraffin-embedded (FFPE) sections from the same patients. This is the first report describing the relationship between ER and its transcriptional activity using clinical samples. Our result indicates that Luminal A-type breast cancer may be classified into two or more types. These findings could be used for a novel predictive model of hormonal therapeutic effectiveness. Indeed, further subtyping of Luminal A-type breast cancer based on the functional evaluation of ER could contribute to more accurate diagnosis and the selection of more effective treatment strategies.

Materials and Methods

Tumor samples

Primary human breast cancer tissues were surgically obtained from 62 informed and consenting patients at the Saitama Cancer Center Hospital (Saitama, Japan) between 2005 and 2007 (Table 1) with approval from the Saitama Cancer Center and Tohoku University Ethics Committee (Saitama Cancer Center No. 216, Tohoku University No. 2008-442). These living cells were used for the assessment of ERE activity. FFPE sections were also prepared from these samples and used for hematoxylin and eosin staining, immunohistochemical staining, and real-time reverse transcription polymerase chain reaction (PCR). Preparation of FFPE and staining were carried out as previously [21] described.

Table 1. Patient clinicopathological information.

Characteristic	*n*
Age	
<50	27
≥50	35
Menopausal	
Pre	28
Post	33
No (men)	1
Tumor size (mm)	
<20	27
≥2	30
Unknown	5
Stage	
0	3
I	13
II	33
III	5
Unknown	8
Grade	
1	7
2	9
3	33
Unknown	13
ER	
Positive	46
Negative	13
Unknown	3
PgR	
Positive	46
Negative	13
Unknown	3
HER2	
Positive	10
Negative	47
Unknown	5

Reagents

ICI 182,780 (Fulvestrant, pure antiestrogen) and 4-hydroxytamoxifen (Tamoxifen) were purchased from Sigma-Aldrich (St. Louis, MO).

IHC of the ER, PgR, and HER2

We analyzed the expression of ER and PgR by IHC. ER was detected using monoclonal anti-ERα antibody 1D5

(M7047; Dako, Glostrup, Denmark), and PgR using monoclonal antibody PgR 636 (M3569; Dako). Immunointensity was graded on the basis of Allred scoring [22] (ER: Fig. 1A and B; PgR: Fig. 1C and D). We also assessed HER2 positivity using the HercepTest™ (Dako) and scored the results as 0, 1, 2, and 3, according to the ASCO/CAP guidelines [1, 2] (Fig. 1E and F). A HER2-positive status was defined as HER2 protein 3 or 2 and FISH ratio of more than 2.2. Histologic grading was evaluated according to the Elston and Ellis grading scheme [23] with slight modification.

ERE transcriptional activity assay in primary tumor cells: Ad-ERE-GFP assay

To assess ERE transcriptional activity in primary tumor cells, we used the Ad-ERE-GFP assay [12, 19, 20]. The isolation of tumor cells was performed as previously described by Ackerman [24] with slight modifications. Briefly, cancer tissue specimens were minced to ~1 mm^3 in size after being rinsed with phosphate-buffered saline (PBS), and digested with collagenase solution (1 mg/mL collagenase, 40 mg/mL bovine serum albumin, 2 mg/mL glucose, 1× antibiotic-antimycotic, and 50 μg/mL gentamicin in HBSS [Hank's balanced salt solution]) for 20–30 min at 37°C. The cells, including tumor cells, were washed several times with PBS, and incubated in 24-well plates with 400 μL of PRF-RPMI (phenol red-free RPMI) 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA). The cells were then infected with 2×10^9 PFU (plaque forming unit) (in 293A cells) Ad-ERE-GFP, and incubated for a further 3 days at 37°C in 5% CO_2–95% air. To examine the infectivity of the adenovirus in primary tumor cells, the cells were infected with 2×10^9 PFU Ad-ERE-GFP or Ad-CMV-DsRed. Approximately 80% of cells were confirmed to be infected. To evaluate drug sensitivity, the cells were simultaneously treated with or without ICI 182,780 or 4-hydroxytamoxifen at a final concentration of 1 μmol/L at

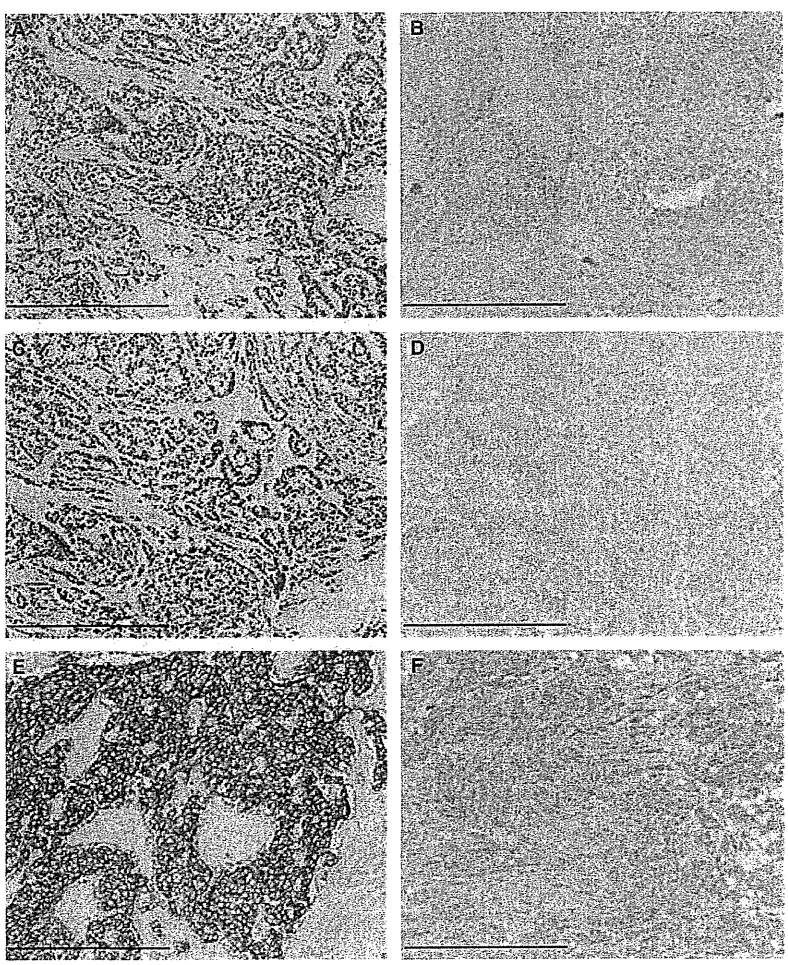

Figure 1. Representative images of IHC labeling of ER (A: positive; B: negative), PgR (C: positive; D: negative), and HER2 (E: positive; F: negative). Scale bars, 500 μm.

the time of infection. To quantify the GFP expression level, the number of cancer cells expressing GFP was counted under a fluorescence microscope after harvesting by treatment with trypsin. The pathologist checked that only cancer cells expressed GFP. All experiments were done in duplicate, and the ERE activity was determined by the percentage of cells expressing GFP.

Total RNA preparation and real-time reverse transcription PCR

RNA was extracted from 40 μm FFPE sections containing a large tumor site using RecoverAll™ Total Nucleic Acid Isolation (Ambion, Austin, TX) according to the manufacturer's instructions after paraffin removal with xylene. The RNA concentration from FFPE samples was determined using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Total RNA (0.5 or 1 μg) was converted to first-strand cDNA primed with a random hexamer in a 20 μL reaction volume using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc., Otsu, Japan). An aliquot of this solution (2 or 4 μL) was used as a template for real-time reverse transcription PCR to quantify the mRNA expression levels of ER and several ER target genes that were identified in our previous study [15–18] (Table 2) using the StepOne™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). The PCR thermal settings were as follows: initial denaturation at 95°C for 10 min followed by 40 amplification cycles of 95°C for 15 sec, and annealing and elongation at 60°C for 1 min. The primer sequences used in this study are listed in Table 2.

Table 2. Primers used for real-time PCR.

Gene	Sequence
RPL13A	F: 5′-CCT GGA GGA GAA GAG GAA AG-3′
	R: 5′-TTG AGG ACC TCT GTG TAT TT-3′
Bcl-2	F: 5′-GTG GAT GAC TGA GTA CCT GAA C-3′
	R: 5′-GCC AGG AGA AAT CAA ACA-3′
Efp	F: 5′-CAT CTC TCA AGG CCA AGG-3′
	R: 5′-GCT ACT GTA TAG CAC TCT GAG A-3′
EGR3	F: 5′-GAG CAG TTT GCT AAA CCA AC-3′
	R: 5′-AGA CCG ATG TCC ATT ACA TT-3′
ERα	F: 5′-CTC CCA CAT CAG GCA CAT-3′
	R: 5′-CTC CAG CAG CAG GTC ATA-3′
HDAC6	F: 5′-GTC TAC TGT GGT CGT TAC ATC-3′
	R: 5′-GGC CTG ACA GTA GTA ACA C-3′
IGFBP4	F: 5′-CCA CGA GGA CCT CTA CAT CAT AC-3′
	R: 5′-ACA CAC CAG CAC TTG CCA C-3′
IGFBP5	F: 5′-TCT CTG CAC CTG AGA TGA GA-3′
	R: 5′-GTC ACA ATT GGG CAG GTA-3′
Ki67	F: 5′-GTC TCT GGT AAT GCA CAC TC-3′
	R: 5′-TCC ACA TGG ATT TCT GAA C-3′
PgR	F: 5′-AGC TCA CAG CGT TTC TAT CA-3′
	R: 5′-CGG GAC TGG ATA AAT GTA TTC-3′

Statistical analysis

Statistical analysis for comparison of two independent groups was performed with the Mann–Whitney U test and the StatFlex 6.0 software program (Artech Co., Ltd., Osaka, Japan). For comparison among three groups or more, the Kruskal–Wallis test was used. Correlation coefficients were also calculated with StatFlex 6.0. Data are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Results

Human breast cancer clinical samples exhibit varying ERE transcriptional activity and drug sensitivity

We have previously established an adenovirus-mediated ERE-GFP assay, named Ad-ERE-GFP assay, which enables the quantitative evaluation of endogenous ER transcriptional activity in clinical specimens [12, 19, 20]. Using this assay system, we investigated the ERE transcriptional activity of breast cancer cells isolated from surgical specimens. These clinical samples showed various levels of GFP expression representative of ERE activity, which was not associated with the status of ER (Fig. 2A). The range of the GFP positivity measured for all samples was 0–57%, where the average and median were 23.8% and 20%, respectively. In the ER-positive group alone, the range of GFP positivity was 2–57% (0–55%), and the average and median were 26.2% (17.1%) and 28.5% (18%), respectively. In drug sensitivity tests (Fig. 2B), Tamoxifen (Tam) and Fulvestrant (Ful) treatments effectively reduced ERE transcriptional activity to 75% and 85% of ER-positive samples, respectively; however, some samples were insensitive to either one (representative samples 340, 341, and 453, Fig. 2B) or both drugs (representative samples 493, 467, and 379, Fig. 2B). Notably, some ER-negative samples showed high GFP positivity that was reduced by antiestrogen treatment (representative samples 363, 342, 361, and 385, Fig. 2B). Furthermore, local recurrence was reported for two patients: ER-positive 467 and ER-negative 385. While ER-positive 467 showed low drug sensitivity in our test, ER-negative 385 showed high drug sensitivity. These data reiterate that sensitivity to endocrine therapy is not solely dependent on the status of ER. Thus, these results suggest that IHC to determine the ER status combined with Ad-ERE-GFP assay as an auxiliary diagnostic might more accurately predict the sensitivity of breast cancers

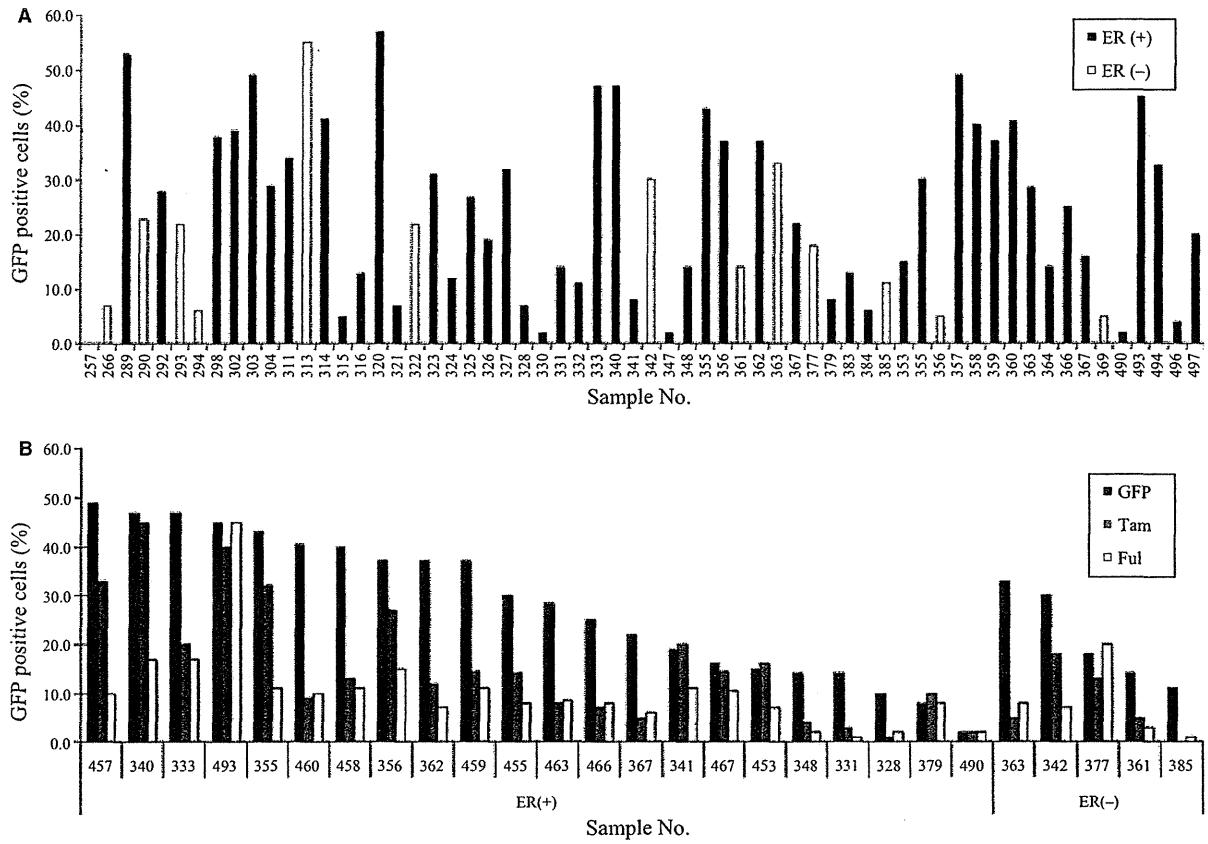

Figure 2. ERE transcriptional activity of primary breast tumor cells. (A) Primary breast tumor cells were infected with Ad-ERE-GFP and incubated for 3 days. Cells expressing GFP were then counted. Black bars represent ER-positive samples and white bars represent ER-negative samples. (B) Ad-ERE-GFP infected cells simultaneously received ethanol (EtOH; black bars), 4-hydroxytamoxifen (Tam; gray bars), and ICI 182,780 (Ful; white bars) at a final concentration of 1 μmol/L to determine drug sensitivity.

to hormonal therapy. Furthermore, some patients defined as ER negative may still be candidates for endocrine therapy.

ERE transcriptional activity significantly correlates with PgR protein expression

We next assessed the relationship between ERE transcriptional activity and clinicopathological information including ER, PgR, and HER2 protein expression as assessed by IHC (Fig. 3). ER protein expression appeared to correlate with ERE transcriptional activity, but this was not statistically significant (Fig. 3A). In contrast, ERE transcriptional activity was significantly correlated with the protein expression of PgR, an ER target gene (Fig. 3B). HER2 protein expression, on the other hand, did not correlate with ERE transcriptional activity (Fig. 3C). We also examined whether ERE transcriptional activity might be associated with other clinical information including age and tumor grade and whether patients were pre- or postmenopausal. In this analysis, ERE transcriptional activity was only correlated with postmenopausal status (Fig. 3D); age and tumor grade did not associate with ERE transcriptional activity. The malignant phenotype, however, such as tumor size or higher clinical stage, tended to show low-ERE transcriptional activity (data not shown). The positive correlation of ERE transcriptional activity with PgR protein suggests that our Ad-ERE-GFP assay reliably reflects ERE transcriptional activity and tumor malignancy as ER functional target. Additionally, because Ad-ERE-GFP uses only ERE as readout of ER-driven transcriptional activity, it is more specific than PgR, which is influenced by many transcriptional cofactors.

ER target gene expression does not correlate with ERE transcriptional activity

Next, we focused on the relationship between ER protein expression and ERE transcriptional activity. According to our previous studies [25, 26], samples with no less than

20% GFP positivity were designated as having high-ERE transcriptional activity. Using this threshold, samples were divided into two groups of high- and low-ERE transcriptional activity. We then compared ERE transcriptional activity, from the high and low groups, with mRNA expression levels of ER and three ER target genes, FOXA1, GATA3, and PgR, in ER-positive cases (Fig. 4). Statistical analysis uncovered significant intergroup differences in ER mRNA expression. ER mRNA expression was significantly higher in the low-ERE group than in the high-ERE group (Fig. 4A). Although PgR mRNA expression was not significantly different between low- and high-ERE groups, there was a tendency for mRNA expression to be higher in the high-ERE-activity group than in the low-ERE-activity group that was in agreement with protein expression analysis (Figs. 3B and 4D). For the other ER target genes examined (Efp, EGR3, HDAC6, IGFBP4, and IGFBP5), mRNA expression levels were not significantly different between low- and high-ERE transcriptional activity groups (data not shown). FOXA1 (Fig. 4B) and GATA3 (Fig. 4C), two genes recently proposed to be related to Luminal-type breast cancer [5–7], also showed no significant difference in mRNA expression regardless of the level of ERE transcriptional activity (FOXA1, $P = 0.786$; GATA3, $P = 0.689$). Therefore, our data suggest that ER target gene expression is not correlated with

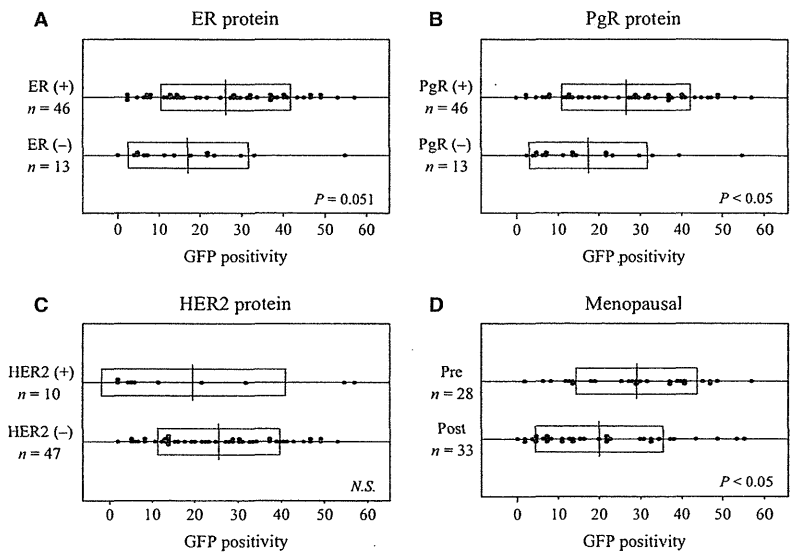

Figure 3. Comparative analysis of GFP positivity in 62 primary breast tumor samples by clinicopathological information. These box plots show the intergroup comparison of (A) ER protein expression, (B) PgR protein expression, (C) HER2 protein expression, and (D) menopausal status.

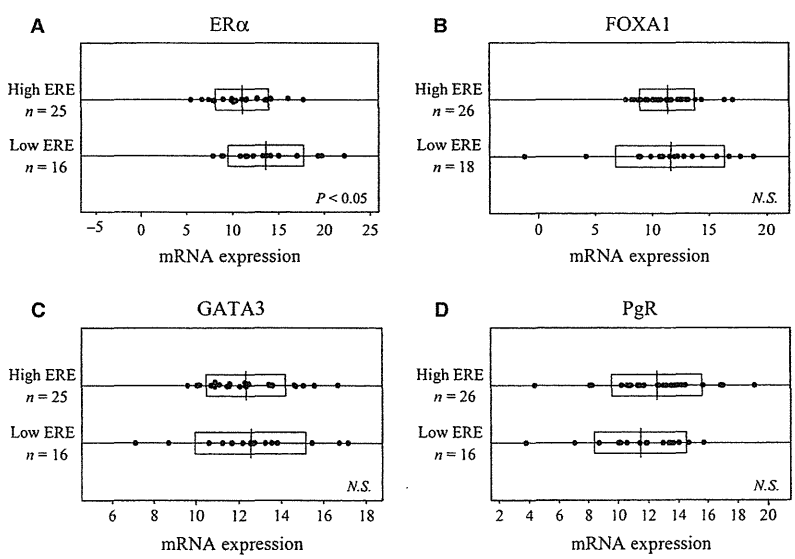

Figure 4. The intergroup difference of ERα and its related or target gene mRNA expression in 46 ER-positive breast tumor samples divided into high- or low-ERE transcriptional activity groups. These box plots show the intergroup differences of (A) ERα; (B and C) ER-related genes: (B) FOXA1, (C) GATA3; and (D) ER target gene: PgR.

ERE transcriptional activity. Thus, the regulation of ER target genes is likely not solely dependent on ER, but could instead involve the convergence of other signaling pathways.

ERE transcriptional activity suggests there are two distinct classes of Luminal A-type breast cancer

Because no significant difference in FOXA1 and GATA3 mRNA expression was observed in the ER-positive group, we decided to explore a more specific breast cancer subtype. Therefore, we conducted correlation analysis of ER and its target genes in Luminal A group breast cancer (Fig. 5). Analysis of this subset of ER-positive breast cancer specimens unveiled that ERα mRNA expression levels significantly correlated with Efp, IGFBP4, IGFBP5, FOXA1, and GATA3 in the high-ERE group, but not in the low-ERE-group, with the exception of GATA3. Moreover, FOXA1 and GATA3 mRNA levels correlated not only with ERα but also the other ER target genes: Efp, EGR3, HDAC6, IGFBP4, and IGFBP5, in the high-ERE group alone. On the other hand, some ER target genes, HDAC6, IGFBP4, and IGFBP5, significantly correlated with each other in the low-ERE group (data not shown). This result supports the hypothesis that some ER target genes are activated through signal pathways other than ER. These data also suggest that ERE activity can further distinguish Luminal-type breast cancer into two classes. Although there was large variation in the mRNA expression profiles of ER target genes between tumor cases, the determination of ERE transcriptional activity appears to be worthwhile for distinguishing ER function-dependent and -independent cases among Luminal A-type breast cancer.

Ki67 is strongly inversely correlated with ERE transcriptional activity

Ki67 [4] and Bcl-2 [27] have been reported to correlate with the malignancy of breast cancer. Therefore, we determined the correlation between ERE transcriptional activ-

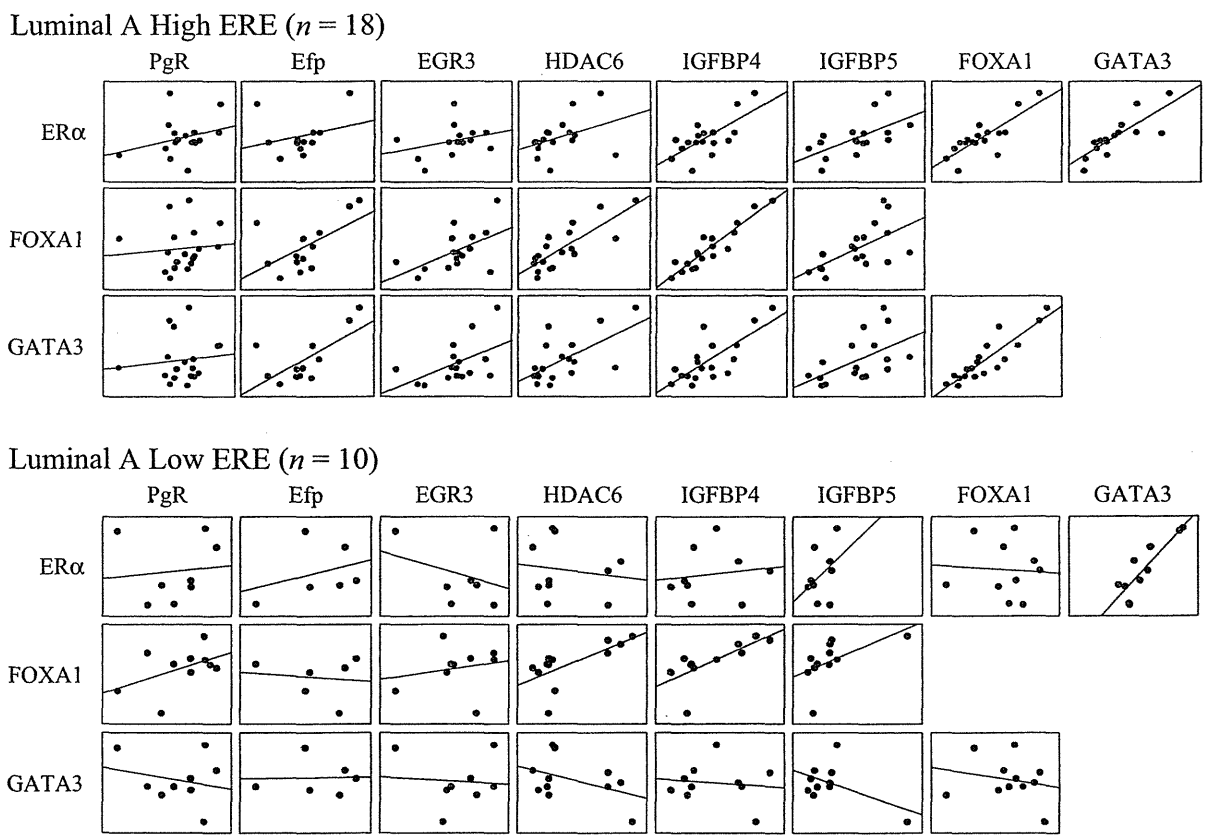

Figure 5. Correlation diagrams of ERα and ER target genes in 28 Luminal A-type breast tumor samples divided into high- or low-ERE transcriptional activity groups. The dots in each square represent the mRNA expression of each gene, and the straight lines show the correlation graphs. The gray squares represent significant correlation ($P < 0.05$), and the white squares reflect no significant correlation.

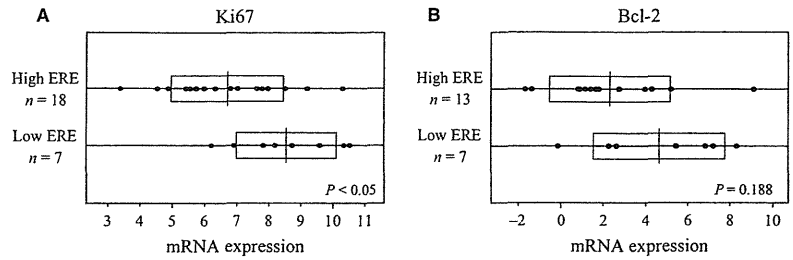

Figure 6. The intergroup difference of Ki67 and Bcl-2 mRNA expression in 28 Luminal A-type breast tumor samples divided into high- or low-ERE transcriptional activity groups. These box plots show the intergroup difference of (A) Ki67 and (B) Bcl-2 mRNA expression levels in each group.

ity and mRNA expression levels of Ki67 and Bcl-2 in Luminal A breast cancer samples. Interestingly, Ki67 mRNA expression was significantly higher in the low-ERE-activity group than in the high-ERE-activity group (Fig. 6A). Bcl-2 mRNA expression also tended to be higher in the low-ERE-group than in the high-ERE group (Fig. 6B). These genes are recognized as poor prognosis factors, but their mechanisms of action for breast cancer are not well defined. Therefore, further exploration of the relationship between ERE transcriptional activity, Ki67 and Bcl-2 may lead to mechanistic insights and explain why the latter two are higher in the group with low-ERE activity.

Discussion

ER is one of the most important transcription factors related to malignancy and proliferation in breast cancer. In this study, we focused on the function of ER as a transcription factor and analyzed human-derived breast cancer specimens according to three features: ER protein expression, mRNA expression profiles of ER target genes, and ERE transcriptional activity as an index for ER function. First, we analyzed ERE transcriptional activity in human breast cancer clinical samples by Ad-ERE-GFP assay. Ad-ERE-GFP assay is highly sensitive, even more than luciferase assays. In contrast to FACS, the Ad-ERE-GFP assay requires fewer cells and can measure the ERE activity of living cells in culture. Therefore, this assay is suitable for measuring transcriptional activity of heterogeneous clinical samples. Indeed, using the Ad-ERE-GFP assay, we demonstrated that primary breast cancer tumor cells exhibit various levels of ERE transcriptional activity in spite of ER positivity (Fig. 2A). The GFP fluorescence, an index of ERE transcriptional activity, was reduced by antiestrogen treatment with either Tamoxifen or Fulvestrant in almost all samples (Fig. 2B). However, several samples did not show drug sensitivity, especially to tamoxifen, suggesting that ER antagonism does not always correlate with inhibition of ER target gene transcription. ER genomic effects are activated not only by estrogen but also by its phosphorylation mediated by signaling pathways such as MAPK or PI3K/AKT pathway [12, 13]. The breast cancer cells in which GFP (ERE transcriptional activity) was not reduced in response to antiestrogenic drugs may have adopted these pathways.

Next, we compared ERE transcriptional activity with general clinicopathological information. These analyses revealed that ERE transcriptional activity had a tendency to correlate with ER protein expression levels (Fig. 3A) as well as menopausal status, but these data were not statistically significant. In contrast, a significant correlation was observed between ERE transcriptional activity and PgR protein expression levels (Fig. 3B). PgR protein expression has been clinically used for evaluating the function of ER activity [1], as confirmed by the present result with Ad-ERE-GFP assay. However, ERE transcriptional activity remains a better readout of ER function as PgR is just one many ER target genes and is regulated by many other transcription factors such as Sp1 or AP-1 [28, 29]. Additionally, the Ad-ERE-GFP assay excludes the influence of other transcription factors and therefore more directly reflects the function of the ER protein than PgR. Our results also demonstrated that ERE transcriptional activity does not correlate with ER protein expression. Together with the results of the drug sensitivity tests mentioned above, our data suggest that not only ER protein expression but also its functional evaluation should be determined to more accurately decide the treatment with most likely efficacy for ER-positive breast cancers.

To more fully investigate the relationship of ERE transcriptional activity to ER status and ER target gene expression, we classified ER-positive primary breast cancer samples into two groups of high- and low-ERE transcriptional activity as evaluated by Ad-ERE-GFP assay. Of note, the low-ERE-activity group had significantly higher ER mRNA expression levels than the high-ERE-activity group. In terms of expression levels of the six ER target genes examined, there were no significant intergroup dif-

ferences between high- and low-ERE-activity groups. These results suggest that there is a group in which ER does not effectively transmit estrogen signaling, in spite of high-ER protein expression. This may be because the ERE transcriptional activity is intercepted downstream, or different feedback mechanisms may exist for each target gene. Therefore, analyzing ERE transcriptional activity may help determine whether and how much the breast cancer depends on ER signaling.

Because many Luminal A-type breast cancers were contained in ER-positive samples, we extracted the Luminal A group from the ER-positive group and investigated its mRNA expression profiles (Fig. 5). FOXA1 and GATA3 have recently been reported to be associated with the Luminal type [5, 6, 26], and ER protein expression level clearly reflected their mRNA expression levels, especially for GATA3. Although the mRNA expression of both genes was not significantly different regardless of ERE transcriptional activity when all ER-protein-positive tumors were examined (Fig. 4B and C), subclassification of Luminal-type breast tumors into low- and high-ERE-activity revealed that these two groups had different correlation tendencies between ERα, FOXA1, and GATA3 mRNA expression levels and ER target genes. These results suggest that ERE activity can classify the Luminal A-type into two distinctions, whereby determination of ERE transcriptional activity may support the assessment of endocrine therapy efficacy. More interestingly, Ki67 and Bcl-2 tended to be higher in the low-ERE-activity group in ER-positive breast cancer (Fig. 6). Ki67 expression is a validated index of malignancy in breast cancer [3]. At the time of this research, local recurrence was found in two patients included in the Luminal A group. Both patients were also from the low-ERE-group, with measured GFP positivity of 7% and 16%, respectively. Although further work is required, the discrepancy in Ki67 and ERE transcriptional activity may help to explain the relationship between Ki67 and breast cancer.

It is widely known that there are individual differences in endocrine therapy efficacy despite ER positivity [2]. In this study, recategorization of breast cancer by ERE transcriptional activity suggests the possibility of distinguishing groups for whom endocrine therapy would be effective and ineffective. The range of treatment choices could also be expanded, especially in Luminal A-type breast cancer patients. We expect that ERE transcriptional activity could become an additional or surrogate marker for analysis of ER protein function and subsequently the improved treatment of breast cancer.

Acknowledgments

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Conflict of Interest

None declared.

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Long-term outcome following imatinib therapy for chronic myelogenous leukemia, with assessment of dosage and blood levels: the JALSG CML202 study*

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A prospective multicenter Phase II study was performed to examine the efficacy and safety of imatinib therapy in newly diagnosed Japanese patients with chronic-phase CML. Patients were scheduled to receive imatinib 400 mg daily. Plasma imatinib concentrations were measured by liquid chromatography–tandem mass spectrometry. In 481 evaluable patients, estimated 7-year overall survival (OS) and event-free survival (EFS) at a median follow-up of 65 months were 93% and 87%, respectively. Because imatinib dosage was reduced in many patients due mainly to adverse events, subgroup analysis was performed according to the mean daily dose during the first 24 months of treatment: ≥360 mg (400-mg group; *n* = 294), 270–359 mg (300-mg group; *n* = 90) and <270 mg (200-mg group; *n* = 67). There were no significant differences in OS and EFS between the 300- and 400-mg groups; however, cumulative rates of complete cytogenetic and major molecular responses differed significantly between the two groups. There were no significant differences in mean imatinib trough levels between these two groups for the patients in whom trough levels had been measured. Survival and efficacy in the 200-mg group were markedly inferior to the former two groups. These results suggest that, although a daily dose of 400 mg imatinib is associated with better outcomes, 300 mg imatinib may be adequate for a considerable number of Japanese patients who are intolerant to 400 mg imatinib. Blood level monitoring would be useful to determine the optimal dose of imatinib. (*Cancer Sci* 2012; 103: 1071–1078)

Imatinib mesylate, a selective BCR-ABL1 kinase inhibitor, has demonstrated remarkable long-term efficacy in the treatment of chronic-phase (CP) CML[1] and now is the standard therapy for this disease.[2] An 8-year follow-up during the International Randomized Study of Interferon and STI571 (IRIS) on newly diagnosed CP CML demonstrated that continuous imatinib therapy exhibited superior efficacy and improved survival.[3] In Japan, imatinib was approved for the treatment of CML in 2001, and a multicenter prospective Phase II study of imatinib therapy (CML202 study) for newly diagnosed CP CML was immediately initiated by the Japan Adult Leukemia Study Group (JALSG). Herein, we report on the results of this study after a median follow-up period of 65 months.

In the present study, although the daily dose of imatinib was set at 400 mg, because of adverse events in many patients the dosage was reduced to less than 400 mg. Nevertheless, the overall efficacy and outcomes were excellent compared with that reported in other studies.[1,4,5] The relatively smaller body size of Japanese patients may explain why a daily dose of < 400 mg imatinib was adequate in some patients.[6] To confirm this assumption, we measured plasma trough levels of imatinib in patients receiving 400 or 300 mg imatinib daily and evaluated the association between plasma concentrations of imatinib and the efficacy, as well as long-term outcome, in these patients.

Materials and Methods

Study design and treatment. The present study was a prospective multicenter Phase II study on previously untreated, newly diagnosed patients with CP CML, with patients receiving a daily dose of 400 mg imatinib. The primary endpoint was overall survival (OS). Secondary endpoints included the rate of a complete hematologic response (CHR), the rate of a cytogenetic response, progression-free survival (PFS), event-free survival (EFS), and safety. The study was registered with the UMIN Clinical Trials Registry (http://www.umin.ac.jp/ctr/index/htm, accessed 10 Sep 2005; registration no. C000000153, the JALSG CML202 study).

Patients. Patients were eligible for inclusion in the study if they were 15 years or older, had de novo Philadelphia (Ph)-chromosome positive CP CML and had not received interferon-α treatment for CML. Further eligibility criteria were adequate liver function (serum bilirubin level ≤2.0 mg/dL and serum liver aminotransferase less than threefold the upper limit of normal), kidney function (serum creatinine ≤2.0 mg/dL), heart and lung function, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–3, and no prior

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*Name of trial register: JALSG CML202. Registration no. C000000153, UMIN Clinical Trials Registry.

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or concurrent malignancy. Written informed consent was obtained from all patients prior to registration. The study protocol was reviewed and approved by the institutional review board of all the participating centers and the study was conducted in accordance with the Declaration of Helsinki.

Dose modification of imatinib. Patients were scheduled to receive imatinib at an oral daily dose of 400 mg. Lower dose of < 400 mg daily were permitted at the start of imatinib therapy in patients who were old and/or had a small body size, but it was planned to increase the dose of imatinib to 400 mg within the first month if patients tolerated the reduced dose. Dose escalation to 600 mg was implemented if patients failed to achieve a complete hematologic response (CHR) at 3 months or a major cytogenetic response at 6 months in the absence of dose-limiting adverse events. If patients did not exhibit a CHR at 6 months, they were switched to alternative therapy. If patients achieved a major cytogenetic response within 9 months, imatinib at 400 mg or the adjusted dose was maintained until disease progression.

If Grade 2 non-hematologic toxicities occurred and did not resolve spontaneously, imatinib was interrupted until the toxicities had been ameliorated to Grade 1 or less, and then resumed at the preceding dose. If Grade 3 or 4 non-hematologic or hematologic toxicities occurred, imatinib was interrupted until the toxicities had been ameliorated to Grade 1 or less, and then resumed at a reduced daily dose of 300 mg. Imatinib therapy was discontinued in the event of failure to achieve a CHR at 6 months, intolerance to imatinib, or disease progression to an accelerated phase (AP) or blast crisis (BC).

Definitions. The phases of CML (i.e. CP, AP, or BC) were defined as described previously in the IRIS study.[7] A CHR was defined as a reduction in the leukocyte count to $<10 \times 10^9$/L and a reduction in the platelet count to $<450 \times 10^9$/L that persisted for at least 4 weeks. Cytogenetic responses were evaluated by G-banding of at least 20 marrow cells in metaphase and were categorized as complete (CCyR; no cells positive for the Ph chromosome) and partial (PCyR; 1–35% of cells positive for the Ph chromosome). A major cytogenetic response (MCyR) was defined as complete or partial responses.[2] A major molecular response (MMR) was defined as a 3-log reduction or more in *BCR-ABL1* transcripts compared with median baseline levels, as measured by reverse-transcription real-time quantitative polymerase chain reaction (RQ-PCR)[8,9] or the transcription-mediated amplification and hybridization protection assay (TMA-HPA)[10,11] (For details, refer to Fig. S1 and Data S1, which are available as online Supplementary Material for this paper).

Event-free survival was defined as the time between registration and the earliest occurrence of any of the following events: death due to any cause, progression to AP or BC, and/or loss of MCyR or CHR. Progression-free survival was defined as the time between registration and the earliest occurrence of any of the following events: death due to any cause or progression to AP or BC. Overall survival was defined as the time between the date of registration and death due to any cause. Hematopoietic stem cell transplantation (HSCT) was not censored. Adverse events were assessed according to the National Cancer Institute–Common Toxicity Criteria version 2.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm, accessed 15 Mar 2012). The mean daily dose of imatinib in a designated period was defined as the total of the doses administered divided by the total number of days on which it was administered.

Measurement of trough plasma levels of imatinib. Blood samples were obtained within 24 ± 2 h after the last imatinib administration from patients who had been receiving 300 or 400 mg imatinib daily without any dose modification for at least 2 years. Plasma was immediately separated at 4°C and at 5000*g* for 10 min by centrifugation and stored at −80°C until measurement. Plasma imatinib concentrations were measured at the Toray Research Center (Tokyo, Japan), as reported previously.[12] Briefly, sample extracts were analyzed using reverse-phase chromatography with a Waters Symmetry column (Waters, Milford, MA, USA), followed by detection with a Sciex API 3000 mass spectrometer (PE Biosystems, Foster City, CA, USA). The lower limit of quantification was 4 ng/mL imatinib mesylate and the assay was fully validated. The precision from validation ranged from 99 ± 5% to 108 ± 5% over the concentration range 4–10 000 ng/mL.[13] The internal standard, imatinib mesylate, was provided by Novartis Pharma (Basel, Switzerland) and the assay system was approved by Novartis Pharma.

Statistical analysis. The Kaplan–Meier method and 95% confidential intervals (CI) were used to analyze OS, PFS, and EFS. Differences between subgroups of patients were evaluated using the log-rank test. Cumulative rates of CHR and cytogenetic responses were estimated according to the competing risk method, in which discontinuation of imatinib was evaluated as competing risk. Comparisons of baseline characteristics in the subgroups were made using the chi square test or Fisher's exact test for categorical variables, and with the Mann–Whitney *U*-test for continuous variables. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA) and R software (http://www.r-project.org, accessed 15 Feb 2011). Two-sided $P < 0.05$ was considered significant.

Results

Patients. Between April 2002 and April 2006, 489 patients from 86 hospitals belonging to the JALSG were enrolled in the CML202 study. Of these patients, three were deemed to be ineligible for inclusion because they were in AP, and a further five were excluded because of insufficient data. The characteristics of the remaining 481 evaluable patients at the time of registration are given in Table 1. The median follow-up time was 65.2 months (range 0.4–95.1 months). Eighty-two of 481 patients (17%) discontinued imatinib therapy or were switched to other therapy (Table 2).

Efficacy. For all 481 evaluable patients, the estimated cumulative rate of CHR was 96% at 7 years, whereas the rates for MCyR and CCyR were 94% and 90%, respectively (Fig. 1a). The *BCR-ABL1* transcript was measured in 428 patients using TMA-HPA and/or RQ-PCR. Levels of the *BCR-ABL1* transcript decreased to <100 copies/μg mRNA (i.e. MMR) in 39% of patients at 18 months and in 79% of patients after 7 years from the start of imatinib (Fig. 1b). According to the Sokal scoring system,[14] the cumulative rates of CCyR were 93%, 84%, and 82% in the low-, intermediate-, and high-risk groups, respectively. There was a significant difference in the rates of CCyR between the low- and intermediate/high-risk groups ($P = 0.006$).

Long-term outcomes. The estimated 7-year rates (with 95% CI) of OS, PFS, and EFS were 93% (90–96%), 93% (90–95%), and 87% (84–91%), respectively (Fig. 1c). The estimated rate of freedom from progression to AP/BC was 97% (95% CI 96–99%) and the estimated 7-year rates of OS according to the Sokal scoring system for patients in the low-, intermediate-, and high-risk groups were 95%, 90%, and 91%, respectively. Patients in the low-risk group exhibited significantly better OS ($P = 0.016$) and EFS ($P = 0.022$) than those in the intermediate- or high-risk groups. In the landmark analysis, patients who had achieved a CCyR at 12 months or an MMR at 18 months exhibited significantly better PFS than

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Table 1. Patient characteristics

Total no. patients	489
No. evaluable patients	481
Age (years)	52 (15–88)
No. patients ≥60 years of age (%)	141 (29)
Sex (M/F, %)	310/171 (64/36)
ECOG PS	
0	441 (92)
1	36 (8)
2	4 (1)
3	0 (0)
Duration from diagnosis (months)	0.4 (0–8.3)
Sokal risk group (%)	
Low	253 (53)
Intermediate	163 (34)
High	65 (14)
Hasford risk group (%)	
Low	202 (42)
Intermediate	227 (47)
High	39 (8)
Unknown	13 (3)
Additional chromosomal abnormalities (%)	
Yes†	51 (11)
Trisomy 8	4 (0.8)
Double Ph	3 (0.6)
Loss of sex chromosome	3 (0.6)
Others	41 (8.5)
Splenomegaly (%)	
Yes	127 (27)
≥10 cm below the costal margin	29 (6)
WBC ($\times 10^9$/L)	36.7 (4.5–634.7)
Hb (g/dL)	12.9 (4.8–19.1)
Platelets ($\times 10^9$/L)	473 (96–2916)
PB blast (%)	0 (0–13.0)
PB basophils (%)	5.0 (0–19.0)
Body weight (kg)	
All patients	61.8 ± 12.1
Men	66.9 ± 10.9
Women	52.6 ± 8.2
BSA (m^2)	
All patients	1.621 ± 0.187
Men	1.714 ± 0.148
Women	1.453 ± 0.121

Data are presented as the mean ± SD, as the median with the range given in parentheses, or as the number of patients in each group with percentages given in parentheses, as appropriate. †The presence of additional chromosomal abnormalities was not an exclusion criterion for the present study. BSA, body surface area; ECOG PS, Eastern Cooperative Oncology Group performance status; Hb, hemoglobin; PB, peripheral blood; WBC, white blood cells.

Table 2. Patients' treatment status

	No. patients (%)
Continued imatinib treatment	399 (83.0)
Discontinued imatinib treatment	82 (17.0)
Reasons for discontinuation and/or change in therapy	
Adverse events	34 (7.1)
Disease progression	11 (2.3)
Unsatisfactory therapeutic effect	12 (2.5)
HSCT	6 (1.2)
Death	2 (0.4)
Lost to follow-up	7 (1.5)
Withdrawal of consent	8 (1.7)
Unknown	2 (0.4)

HSCT, hematopoietic stem cell transplantation.

those without CCyR or MMR ($P = 0.0005$ and $P = 0.012$, respectively).

Safety. The adverse events observed in all patients are listed in Table 3. Grade 3 or 4 hematologic adverse events were neutropenia (18%), thrombocytopenia (12%), and anemia (6%). Grade 3 or 4 non-hematologic adverse events included skin eruption (8%) and peripheral edema (0.6%). Grade 3 or 4 liver dysfunction was reported in 4% of patients. Congestive heart failure (Grade 3) developed in one patient and interstitial pneumonitis (Grade 3) developed in another patient. Grade 3 or 4 thrombocytopenia and skin eruptions occurred more frequently in the present study than in the IRIS study.[7]

Efficacy and outcomes in relation to imatinib dosage. Although it was planned to administer imatinib to patients at a dose of 400 mg daily, 82 patients (17%) discontinued imatinib or were switched to other treatment mainly because of adverse events or unsatisfactory efficacy (Tables 2, 3). Dose reduction or interruption were required in 223 (46%) patients, with escalated doses given to 10 patients (2%) during the first 24 months. Among all 481 patients, the initial dose of imatinib was 400 mg in 458 patients (95.2%), 300 mg in 10 patients (2.1%), 200 mg in 11 patients (2.3%), 100 mg on one patient, and 600 mg in one patient. The mean daily dose during the first 24 months of treatment was ≥360 mg in 294 patients (61%; designated the "400-mg group"), 270–359 mg in 90 patients (19%; designated the "300-mg group"), and < 270 mg in 67 patients (14%; designated the "200-mg group"). Thirty patients (6%) discontinued imatinib during the first 24 months. Regarding the safety profile, Grade 3 or 4 neutropenia, thrombocytopenia, liver dysfunction, and skin eruptions tended to be observed more frequently in the 300- and 200-mg groups because dose reductions from the scheduled dose of 400 mg imatinib daily were mostly made for patients in these groups because of adverse events (Table 3). The patients in the 300-mg group were significantly more likely to be female, older, have a lower body weight (BW), and a smaller body surface area (BSA) than patients in the 400-mg group (Table 4). Patients in the 300- and 200-mg groups had significantly higher Sokal risk than patients in the 400-mg group ($P = 0.001$). Of the patients in the 400- and 300-mg groups, age ($P = 0.0024$) and sex ($P = 0.0077$) were significant independent predictors for OS, as determined by multivariate analysis; however, dosage was not a significant predictor of OS ($P = 0.64$).

Efficacy and survival were analyzed according to the mean daily dose during the first 6, 12, and 24 months. During each period, the estimated cumulative rate of CCyR or MMR was significantly higher for patients in the 400- and 300-mg groups than for patients in the 200-mg group ($P < 0.001$ and $P < 0.0001$, respectively). There was a significant difference in achieving CCyR or MMR between the 400- and 300-mg groups ($P = 0.018$ and $P = 0.017$, respectively; Fig. 2a,b). There were no significant differences in OS and EFS between the 400- and 300-mg groups during the first 24 months ($P = 0.77$ and $P = 0.49$, respectively). However, the OS and EFS of the 200-mg group were significantly inferior to those of the 400- and 300-mg groups during the same periods ($P = 0.009$ and $P = 0.002$, respectively; Fig. 3a,b). Survival was analyzed according to the mean daily dosage of imatinib during the first 24 months per BW (Table 5). Patients who received a mean dose of imatinib per BW that was >5.0 mg/day/kg showed significantly superior OS and EFS than those receiving ≤5.0 mg/day/kg ($P = 0.0012$ and $P = 0.0016$, respectively; Fig. 4). These results indicate that patients who had relatively high daily dosage per BW had better OS and EFS, although the actual daily dose had been lower than 400 mg imatinib.

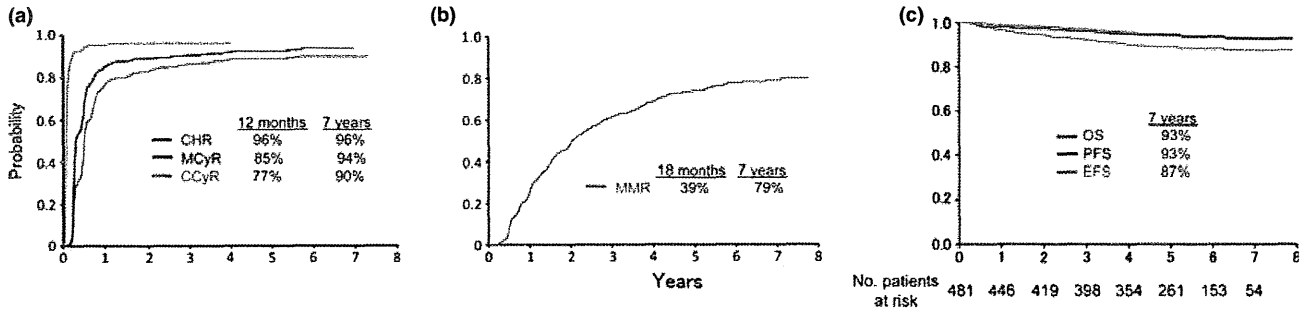

Fig. 1. Cumulative best (a) cytogenetic and (b) molecular responses and (c) survival of patients on imatinib therapy for chronic phase CML. Cumulative rates of responses were estimated according to the competing risk method. Discontinuation of imatinib was evaluated as a competing risk. CHR, complete hematologic response; MCyR, major cytogenetic response; CCyR, complete cytogenetic response; MMR, major molecular response; OS, overall survival; PFS, progression-free survival; EFS, event-free survival.

Table 3. Adverse events associated with imatinib therapy

Adverse event†	No. patients (%)				
	All patients (n = 481)		400-mg group‡ (n = 294)	300-mg group‡ (n = 90)	200-mg group‡ (n = 67)
	All grades	Grade 3 or 4	Grade 3 or 4	Grade 3 or 4	Grade 3 or 4
Non-hematologic					
Superficial edema	234 (48.6)	3 (0.6)	0	3 (3.3)	0
Nausea/vomiting	106 (22.0)	4 (0.8)	2 (0.7)	1 (1.1)	1 (1.5)
Anorexia	94 (19.5)	5 (1.0)	2 (0.7)	2 (2.2)	1 (1.5)
Muscle cramps	81 (16.8)	1 (0.2)	0	1 (1.1)	0
Musculoskeletal pain (myalgia)	100 (20.8)	5 (1.0)	2 (0.7)	0	2 (3.0)
Arthralgia	47 (9.8)	1 (0.2)	0	0	0
Rash	192 (39.9)	37 (7.7)	7 (2.4)	10 (11.1)	14 (20.9)
Fatigue	114 (23.7)	0 (0)	0	0	0
Diarrhea	75 (15.6)	2 (0.4)	1 (0.3)	0	0
Headache	36 (7.5)	1 (0.2)	0	0	0
Hemorrhage	24 (5.0)	3 (0.6)	2 (0.7)	0	1 (1.5)
Pyrexia	49 (10.0)	1 (0.2)	1 (0.3)	0	0
Depression	25 (5.2)	0 (0)	0	0	0
Infection	35 (7.3)	8 (1.7)	5 (1.7)	0	2 (3.0)
Interstitial pneumonitis	3 (0.6)	1 (0.2)	0	0	1 (1.5)
Hematologic					
Anemia	197 (41.0)	28 (5.8)	12 (4.1)	4 (4.4)	10 (14.9)
Neutropenia	188 (39.1)	85 (17.7)	36 (12.2)	25 (27.8)	18 (26.9)
Thrombocytopenia	199 (41.4)	59 (12.3)	19 (6.5)	20 (22.5)	16 (23.9)
Biochemical					
Elevated ALT/AST	99 (20.6)	18 (3.7)	3 (1.0)	6 (6.7)	7 (10.4)
Renal dysfunction	37 (7.7)	1 (0.2)	1 (0.3)	0	0

†Adverse events were assessed according to the National Cancer Institute–Common Toxicity Criteria version 2.0. ‡Mean daily doses in the 400-, 300-, and 200-mg groups were ≥360, 270–359, and < 270 mg imatinib, respectively. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Plasma trough levels of imatinib according to the daily dose. Plasma trough levels (C_{min}) of imatinib were determined in 50 patients who continuously received imatinib at a daily dose of 300 mg (n = 24) or 400 mg (n = 26) without any dose modification (Table 6). The patients receiving 300 mg imatinib tended to be older and to have a smaller BSA than patients in the 400-mg group. These tendencies did not different from those of the entire study population (Tables 4 and 6). There was no significant difference in mean C_{min} between the two groups (P = 0.673). The C_{min} in 15 of 24 patients (63%) receiving 300 mg imatinib and in 15 of 26 patients (58%) receiving 400 mg imatinib were distributed above 1000 ng/mL, and the ratio of patients >1000 ng/mL C_{min} did not differ significantly between the two groups (P = 0.10). However, the C_{min} in patients receiving 300 mg imatinib was distributed towards lower concentrations compared with those receiving 400 mg imatinib. There was a significant correlation between C_{min} and age only in the 400-mg group (P = 0.034), with weak correlations between C_{min} and BW or BSA. These results indicate that small, elderly, and/or female patients receiving 300 mg imatinib daily had almost the same C_{min} as patients receiving 400 mg daily.

Discussion

In the present study (CML202), the best cumulative rates of MCyR and CCyR 7 years after the start of imatinib were 94% and 90%, respectively, and the estimated 7-year OS and EFS

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Table 4. Patient characteristics in each of the mean daily dose groups during the first 24 months of treatment

	Imatinib daily dose group†				*P*-value
	400 mg	300 mg	200 mg	Discontinued	
No. patients	294	90	67	30	
Daily dose (mg)	398 ± 17	310 ± 23	187 ± 68	NA	
No. men/women	212/82	46/44	30/37	22/8	<0.0001
Age (years)	48 (16–81)	57 (19–79)	63 (19–87)	52.5 (15–88)	<0.0001
Body weight (kg)	64.6 ± 11.8	57.6 ± 10.5	55.3 ± 10.0	61.8 ± 15.3	<0.0001
BSA (m^2)	1.67 ± 0.18	1.55 ± 0.16	1.51 ± 0.17	1.61 ± 0.22	<0.0001
Sokal risk group (*n*)					
Low	180	39	23	11	<0.0001
Intermediate	84	30	32	13	
High	30	21	12	6	
Dose reduction (*n*)	1	69	59	NA	
Interruption (*n*)	65	21	8	NA	
Dose escalation (*n*)	10	0	0	NA	

Unless indicated otherwise, data are given as the mean ± SD or as the median with the range given in parentheses. †Mean daily doses in the 400-, 300-, and 200-mg groups were ≥360, 270–359, and <270 mg imatinib, respectively. BSA, body surface area; NA, not applicable.

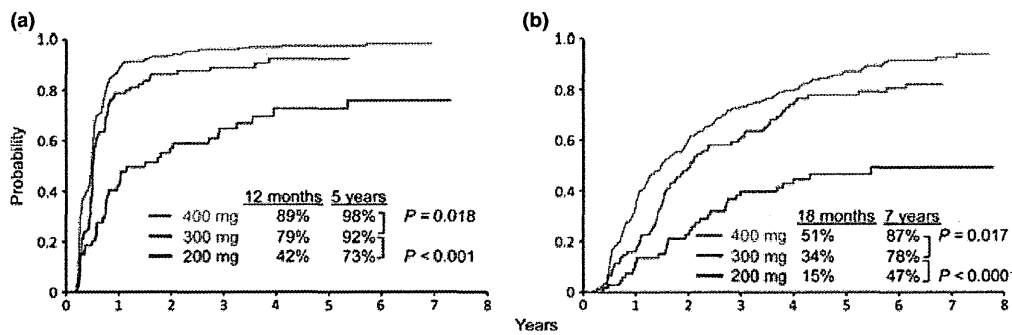

Fig. 2. Cumulative rates of best responses according to the mean daily dose during the first 24 months of treatment with imatinib. (a) Cumulative rates for complete cytogenetic responses (CCyR). (b) Cumulative rates of major molecular responses (MMR). Mean daily doses in the 400- (*n* = 294), 300- (*n* = 90), and 200-mg (*n* = 67) groups were ≥360, 270–359, and <270 mg imatinib, respectively.

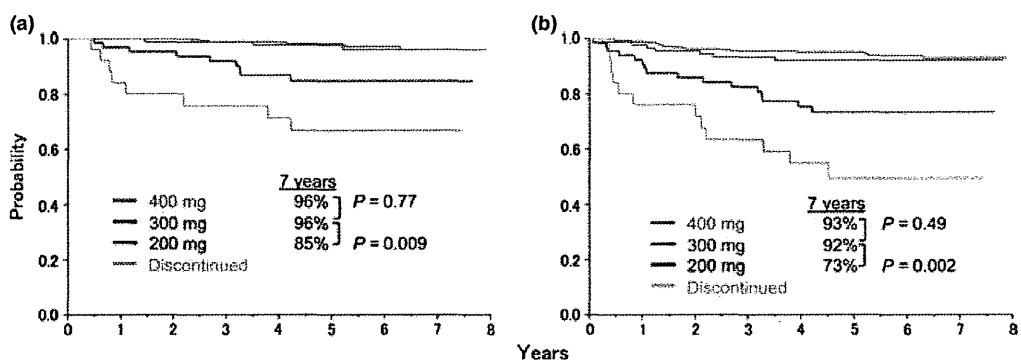

Fig. 3. (a) Overall and (b) event-free survival according to the mean daily dose during the first 24 months. Mean daily doses in the 400- (*n* = 294), 300- (*n* = 90), and 200-mg (*n* = 67) groups were ≥360, 270–359, and < 270 mg imatinib, respectively.

rates were 93% and 87%, respectively. The Sokal risk showed favorable prognostic significance in low-risk patients compared with intermediate- or high-risk patients. These results are comparable to those reported in the IRIS trial and others studies in Western countries.[3–5] In terms of baseline characteristics, there was a tendency for fewer patients with a high-risk Sokal score in the present study compared with the IRIS study. We believe this is due to the Japanese medical system, in which a considerable number of people undergo annual medical check-ups.

Imatinib is currently established as the first-line therapy for patients with CP CML. Nevertheless, several controversial issues remain,[15] with the dose of imatinib as one of the most important.[6,16–21] In the present study, many patients received a lower dose of imatinib than the planned initial dose of 400 mg. Therefore, we performed subgroup analysis according