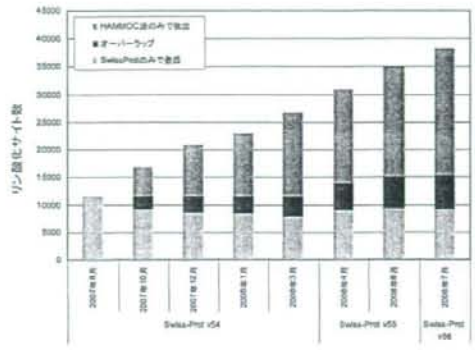

図 4　HAMMOC 法によって検出されたヒトタンパク質のリン酸化サイト数

一研究室での成果が世界中の研究成果の合計よりも 2 倍以上多い結果となっており、HAMMOC 法がいかに優れた性能を持っているかを示すデータのひとつとなっている。また、今のところリン酸化サイト数の増加割合はいっこうに鈍る気配もみせておらず、SwissProt とのオーバーラップも緩やかながら着実に進んでいることから、今後も同定サイト数はこのペースで増加するものと思われる。なお、筆者らの結果については、MS/MS スペクトルを含めて現在公表する準備を進めている（論文化されたものについてはすでに PepBase（http://pepbase.iab.keio.ac.jp）にて公開中）。

HAMMOC 法の応用例として、最近筆者らは植物のリン酸化プロテオーム解析を行った[16]。モデルとしてシロイヌナズナ培養細胞株を用いて網羅的解析を行い、チロシンリン酸化が哺乳動物と同様の割合で存在していることを明らかにした。哺乳動物タイプのチロシンキナーゼがシロイヌナズナのゲノムにコードされていなかったことからその存在が疑問視されていた植物のチロシンリン酸化であるが、今回確認されたリン酸化チロシンプロテオームにより、哺乳動物タイプのチロシンキナーゼモチーフとは異なったユニークなモチーフが明らかとなり、植物特有のチロシンキナーゼファミリーの存在が示唆された。

3. タンパク質キナーゼとリン酸化プロテオミクス

ヒトタンパク質キナーゼの全容（キノーム，kinome）は Manning らによると約 500 とされる[17]。現在、SwissProt にはヒトのタンパク質キナーゼが 488 個、リン酸化情報が得られているタンパク質すなわちタンパク質キナーゼの基質と考えられるタンパク質が 5763 個、登録されている。多くのタンパク質キナーゼは、他のタンパク質キナーゼの基質になったり、自己リン酸化を起こすことが知られているため、SwissProt 登録タンパク質キナーゼ 488 個のうち、リン酸化タンパク質として登録されているものを調べてみたところ、394 個（81%）であった。残り 94 個のキナーゼのうち HAMMOC 法によりリン酸化タンパク質であると実験的に証明されたものが現在のところ 31 個あり、合計すると約 9 割のタンパク質キナーゼはリン酸化を受けていることとなる。この 425 個のリン酸化を受けるタンパク質キナーゼのうち、現在までに HAMMOC 法で同定されているものは 311 個あり、これはタンパク質キナーゼ全体に対して 64%、リン酸化を受けるタンパク質キナーゼに対して 73%となる（表 1）。

表 1　タンパク質キナーゼのリン酸化

	タンパク質数
Swiss-Prot v55.6に登録されているタンパク質キナーゼ	488
リン酸化されているタンパク質キナーゼ*	425
HAMMOC法で同定されたタンパク質キナーゼ**	311

* Swiss-Prot登録数とHAMMOC法による実測数より算出
** リン酸化タンパク質として検出

今後、様々な実験条件下でリン酸化プロテオーム解析を行うことでタンパク質キナーゼのうちリン酸化を受けるタンパク質の割合および HAMMOC 法によるタンパク質キナーゼの同定数はさらに増加すると考えられる。HAMMOC 法によって同定されたリン酸化サイトとそのキナーゼ活性との間に相関があるとは限らないが、これらのデータは、HAMMOC 法がキノーム基質のプロテオームだけでなく、キノーム自体を含んだ形での網羅的解析法であり、シグナル伝達ネットワークを記述する上で十分な情報量を提供できることを示唆している。

タンパク質キナーゼの推定総数と、筆者らの解

析結果で示されたタンパク質キナーゼ/基質比から考慮すると最終的には少なくとも 10,000 個以上のタンパク質および 50,000 個以上のリン酸化サイトがタンパク質キナーゼによってリン酸化されていると推定される。

4. おわりに

測定技術の進歩により、リン酸化プロテオーム解析はこの2,3年で驚くべきほどの進歩を遂げている。筆者らの開発した HAMMOC 法も含めていくつかの方法はすでにキット化され市販され始めており、数年の後にはより一般的な測定法になるものと思われる。筆者らも、より少ない細胞数試料から 1 万個レベルのリン酸化サイトの同定・定量が可能な高性能 HAMMOC 法の開発を更に進めており、これが実現すれば、現在 in vitro キナーゼアッセイによってプロファイル解析がされているキナーゼに対する分子標的薬評価についても、HAMMOC 法で可能になるであろう。In vivo 反応をモニターできるだけではなく、標的キナーゼを含めたパスウェイへの影響を評価でき、さらには未知の標的キナーゼを含めたシグナル伝達ネットワーク全体への影響を評価できるものと考えている。Olsen らによる EGF 刺激による動態実験 [18] のように安定同位体標識法と組み合わせることで、定量的な時間経過も測定可能であり、薬物の濃度依存性も評価できるため、今後の展開が期待できる。

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Original Article

Prognostic Significance of Insulin-like Growth Factor Binding Protein (*IGF*BP)-4 and *IGF*BP-5 Expression in Breast Cancer

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Background: Expression of estrogen-regulated genes has been considered as potential predictive markers for endocrine therapy. We focused on two insulin-like growth factor binding proteins (IGFBPs): *IGF*BP-4, which is an early-responsive estrogen-induced gene, and *IGF*BP-5, which is an estrogen-repressed gene. Investigation of *IGF*BP-4 and *IGF*BP-5 expression would provide important information for predicting prognosis and endocrine responsiveness.

Methods: The levels of *IGF*BP-4 and *IGF*BP-5 mRNA expression in 162 human breast cancer tissues were analyzed using quantitative real-time reverse transcriptase-PCR. The association between *IGF*BP-4 and *IGF*BP-5 expression and clinicopathological factors was then analyzed.

Results: The levels of *IGF*BP-4 and *IGF*BP-5 mRNA expression were positively correlated with estrogen receptor (ER) and progesterone receptor (PgR) status and were negatively correlated with HER2 overexpression. Patients with a high level of *IGF*BP-4 mRNA expression had better disease-free and overall survival than those with a low expression. Multivariate analysis showed that *IGF*BP-4 mRNA expression is an independent prognostic factor for disease-free survival. When analyzed in 116 patients with ER-positive breast cancer, patients whose tumor expressed higher levels of *IGF*BP-4 mRNA or lower levels of *IGF*BP-5 mRNA had better disease-free survival.

Conclusion: *IGF*BP-4 mRNA expression was an independent prognostic factor in breast cancer, and patients with ER-positive breast cancer whose tumor expressed higher levels of *IGF*BP-4 and lower levels of *IGF*BP-5 had a better prognosis than those without such findings.

Key words: breast cancer – IGFBP-4 – IGFBP-5 – prognosis

INTRODUCTION

Endocrine therapy has become the most important treatment option for women with estrogen receptor (ER)-positive breast cancer. Nevertheless, many breast cancer patients with tumors expressing high levels of ER are unresponsive to endocrine therapy and all patients with advanced disease eventually develop resistance to the therapy (1)..Expression of estrogen-regulated genes has been considered to provide predictive markers for endocrine therapy, because their expression may indicate the presence of a functional estrogen-signaling pathway. Using microarray technology, we have identified more than 100 estrogen-regulated genes in MCF-7 human breast cancer cells (2). Of these

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estrogen-regulated genes, in the present study we focused on two insulin-like growth factor binding proteins (IGFBPs): *IGF*BP-4, which is an early-responsive estrogen-induced gene, and *IGF*BP-5, which is an estrogen-repressed gene.

IGFBPs are considered to bind to insulin-like growth factor (IGF)-I and IGF-II in the extracelluar space, regulating access of IGFs to IGF receptors (3), which is one of the most critical steps for proliferation of breast cancer cells. There are six IGFBPs, *IGF*BP-1 to *IGF*BP-6, which share 40–60% amino acid identity. IGFBPs bind IGF-I and IGF-II with high affinity, and are essential to transport IGFs, to prolong half-lives, and to regulate the availability of free IGFs for interaction with IGF receptors, thereby modulating the effects of IGFs on growth and differentiation. In addition, recent evidence indicates that some IGFBPs may themselves have direct receptor-mediated effects, independent of IGFs (4). *IGF*BP-3 is the most abundant IGFBP in human serum and has been shown to be a growth inhibitory, apoptosis-inducing molecule, capable of acting via IGF-dependent and IGF-independent mechanisms (5). The clinical data presented to date provide ambiguous evidence as to whether the IGFBPs, and in particular *IGF*BP-3, predict a good or poor prognosis in breast cancer (6). Recent studies indicated that high concentrations of IGF-I and *IGF*BP-3 in the circulation were associated with an increased risk of premenopausal breast cancer (7). *IGF*BP-4 appears to be a potent inhibitor of IGF function in several human cell lines (8–10). *IGF*BP-5 plays a critical role in mammary gland development, and, in particular, the removal of mammary epithelial cells by apoptosis that takes place during the involutionary stage of the lactating gland (11). However, little is known about the role of *IGF*BP-4 and *IGF*BP-5 in breast cancer.

In the present study, we examined mRNA and protein expression of *IGF*BP-4 and *IGF*BP-5 in 162 human breast cancer tissues and analyzed their significance for prognosis.

PATIENTS AND METHODS

Patients and Tumor Samples

Primary invasive breast carcinoma specimens were obtained by surgical excision from 162 female patients at Nagoya City University Hospital between 1992 and 2000. Informed consent was obtained from all patients before surgery. The study protocol was approved by the institutional review board and conformed with the guidelines of the 1975 Declaration of Helsinki. The median age of the patients was 57.9 years (range, 28–88 years). The patients' tumors were classified with the International Union Against Cancer (UICC) staging system as follows: 44 cases were classified as stage I, 98 cases as stage II, 17 cases as stage III and 3 cases as stage IV. Patients were graded histopathologically according to the modified Bloom and Richardson method proposed by Elston and Ellis (12). As post-operative adjuvant treatment, tamoxifen was given to patients with ER- and/or progesterone receptor (PgR)-positive tumors. Depending on tumor stage, the following chemotherapy regimens were given: oral 5-fluorouracil, CMF, or FEC. Since 1995, post-operative treatment has been done with reference to the recommendation of St Gullen (13). After surgery, 26 patients (16.0%) received no additional therapy. Of the remaining 136 patients, 82 (50.6%) received systemic therapy consisting of endocrine therapy alone, 10 (6.2%) received chemotherapy alone and 44 (27.2%) received combined endocrine therapy and chemotherapy. Patients were observed for disease recurrence and death at least once every 6 months for 5 years after surgery and yearly thereafter. The median follow-up period was 67 months (range, 2–128 months). Samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Isolation of Total RNA and Reverse Transcription

Total RNA from homogeneous breast cancer tissue, which was microscopically confirmed, was isolated from approximately 500 mg of frozen specimen. Total RNA was also isolated from one flask of HepG2 cells and T47D cells for use as a positive control and to generate standard curves. mRNA was isolated using the TRIZOL reagent (Life Technologies, Inc., Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription reactions were done as previously described (14).

Primers and Probes

We conducted BLAST searches (Genbank) to confirm the specificity of the nucleotide sequences chosen for the primers and probes and to confirm the absence of DNA polymorphism. To avoid detection of contaminating genomic DNA, the primers for *IGF*BP-4 were located at exon 1 and exon 2, and the primers for *IGF*BP-5 were located at exon 3 and exon 4. The specific oligonucleotide primers were synthesized according to published information as follows: *IGF*BP-4, 5′ sense TCGAGGCCATCCAGGAAA (602–619) and 3′ antisense CCCCATTGACCTTCATCTT (766–748) (165 bp); *IGF*BP-5, 5′ sense CTGTGTACCTGC CCAAT (1411–1427) and 3′ antisense CACTGAAAG TCCCCGTCAA (1561–1543) (151 bp). The donor probe for *IGF*BP-4, 5′-AGCGCCCATGACCGCAG-3′ has a fluorescein label at its 3′ end and the acceptor probe for *IGF*BP-4 5′-TGCCTGCAGAAGCACTTC GC-3′ has LC Red 640 at its 5′ end. For *IGF*BP-5, the donor probe 5′-CCGCAAACGT GGCATCTGCT-3′ and the acceptor probe 5′-GTGCGTGGA CAAGTACGGGATGA-3′ were used.

To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and normalized. *GAPDH* primers were as follows: 5′ sense AAATCAAGTGGGGCGATGCTG and 3′ antisense GCAGAGATGATGACCCTTTTG. The

sequences of the *GAPDH* probes were as follows: the donor probe, 5′-AGAAGGCTGGGGCTCATTTGCAGGG-3′ and the acceptor probe, 5′-GTCCACTGGCGTCTTCACCAC CATG-3′. All primers and probes were purchased from the Japanese Gene Institute (Saitama, Japan).

Real-time Reverse Transcription-PCR

Real-time reverse transcription-PCR was done using a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported (15). The PCR reaction for *IGF*BP-4 and *IGF*BP-5 was carried out in a final volume of 20 μl containing 2.4 μl of 25 mmol/l $MgCl_2$; 0.5 μl of 20 pmol/μl sense primer and antisense primer; 0.4 μl of 10 pmol/μl donor and acceptor probe; 2 μl of PCR master mix; 1.5 μl of cDNA and made up to 20 μl with water. After an initial denaturation step at 95°C for 60 s, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 0 s, hybridization at 57°C for 5 s, and elongation at 72°C for 6 s. The fluorescence signal was acquired at the end of the hybridization step. A total of 55 cycles were performed. Cycling conditions for *GAPDH* were as follows: initial denaturation at 95°C for 60 s, followed by 50 cycles at 95°C for 0 s, 60°C for 5 s and 72°C for 8 s.

Standard Curves and Presentation of Results

For each PCR run, a standard curve was constructed with serial dilutions of cDNA obtained each from HepG2 cells for *IGF*BP-4 and T47D cells for *IGF*BP-5. The level of expression of *IGF*BP-4 and *IGF*BP-5 mRNA were given as relative copy numbers normalized against *GAPDH* mRNA and shown as mean ± SD. Relative *IGF*BP-4 and *IGF*BP-5 mRNA expression was calculated by the formula: (*IGF*BP-4/*GAPDH*) × 1000 and (*IGF*BP-5/*GAPDH*) × 100, respectively.

A non-template negative control was included in each experiment. All of the non-template negative controls, the standard cDNA dilutions from HepG2 cells or T47D cells, and the tumor samples were assayed in duplicate. All of the patient samples with a coefficient of variation for gene mRNA copy number data >10% were retested using the method of Bieche et al. (16).

Immunohistochemical Staining of ER and PgR

Immunohistochemical staining of ER and PgR was done using monoclonal mouse antihuman ERα antibody (1D5, DAKO) at 1:100 dilution for ER and monoclonal mouse antihuman PgR antibody (636, DAKO) at 1:100 dilution for PgR as primary antibodies as previously described (17). The expression of ER and PgR was estimated in accordance with the procedure of Allred and colleagues (18). In brief, a proportion score represented the estimated proportion of tumor cells staining positive, as follows: 0 (none); 1 (<1/100); 2 (1/100 to 1/10); 3 (1/10 to 1/3); 4 (1/3 to 2/3); and 5 (>2/3). Any brown nuclear staining in invasive breast epithelium counted towards the proportion score. An intensity score represented the average intensity of the positive cells, as follows: 0 (none); 1 (weak); 2 (intermediate); and 3 (strong). The proportion and intensity scores were then added to obtain a total score, which could range from 0 to 8. Tumors with a score of 3 or greater were considered to be positive for ER or PgR expression.

Statistical Analysis

Unpaired *t* test was used for the statistical analysis of the association between *IGF*BP-4 and *IGF*BP-5 mRNA expression and clinicopathological factors. Disease-free and overall survival curves were generated by the Kaplan–Meier method and verified with the log-rank test. Cox's proportional hazards model was used for univariate and multivariate analyses of prognostic values. Differences were considered significant when a $P < 0.05$ was obtained.

RESULTS

Patient Demographics and Tumor Characteristics

Clinical characteristics are summarized in Table 1. The amount of *IGF*BP-4 mRNA in the tissue samples from 162 patients ranged from 17 to 2561 relative copy numbers (mean, 310.4), whereas the amount of *IGF*BP-5 mRNA ranged from 3 to 4060 relative copy numbers (mean, 165.6).

Table 1. Adjuvant systemic treatments for patients after surgery

Adjuvant therapy	No. (%)
Total patients	162
None	26 (16.0)
Endocrine therapy	82 (50.6)
Tamoxifen	72
LHRH agonist	2
LHRH agonist + tamoxifen	7
Aromatase inhibitors	1
Chemotherapy	10 (6.2)
Oral 5-fluorouracil	6
CMF	4
Combined	44 (27.2)
Tamoxifen + oral 5-fluorouracil	37
Tamoxifen + CMF	1
Tamoxifen + CAF	2
LHRH agonist + tamoxifen + CMF	3
LHRH agonist + tamoxifen + paclitaxel	1

LHRH, luteinising hormone-releasing hormone;
CMF, cyclophosphamide methotrexate 5-fluorouracil;
CAF, cyclophosphamide adriamycin 5-fluorouracil.

Correlation between *IGF*BP-4 and *IGF*BP-5 mRNA Expression and Clinicopathological Factors

The level of *IGF*BP-4 mRNA expression was significantly correlated with histological grade ($P = 0.0032$). Positive associations were observed between *IGF*BP-4 mRNA expression and ER ($P = 0.0031$) and PgR ($P = 0.0045$) expression. An inverse correlation was found between *IGF*BP-4 mRNA expression and HER2 overexpression ($P = 0.0007$). No association was found between *IGF*BP-5 mRNA expression and histological grade, ER, PgR and HER2 expression (Table 2).

There was no association between *IGF*BP-4 and *IGF*BP-5 mRNA expression and age, menopausal status, tumor size, or lymph node status. Interestingly, *IGF*BP-4 mRNA expression was strongly correlated with *IGF*BP-5 mRNA expression (Fig. 1).

Patients whose Tumor Expressed Higher Levels of *IGF*BP-4 mRNA had Better Disease-free and Overall Survival

To identify a clinically meaningful cutoff point for levels of *IGF*BP-4 and *IGF*BP-5 mRNA expression that could be used in disease prognosis analysis, various levels of *IGF*BP-4 and *IGF*BP-5 mRNA expression were tested using the Kaplan–Meier method and verified by the log-rank test. When analyzing disease-free and overall survival, the cutoff points for the levels of *IGF*BP-4 and *IGF*BP-5 mRNA were set at 205 and 38, respectively. Patients with a high level of *IGF*BP-4 mRNA expression (470.2 ± 366.2; $n = 86$) had better disease-free survival than those with a low expression (102.9 ± 54.0; $n = 72$) ($P = 0.0002$, Fig. 2a). Similarly, patients with a high level of *IGF*BP-4 mRNA expression had better overall survival than those with a low level of expression ($P = 0.022$, Fig. 2b). However, *IGF*BP-5 mRNA expression status did not affect disease-free or overall survival (Fig. 2c and d).

*IGF*BP-4 mRNA Expression is an Independent Prognostic Factor of Disease-free Survival in Breast Cancer

Univariate analysis demonstrated that *IGF*BP-4 mRNA expression ($P = 0.0044$), as well as tumor size ($P = 0.016$), lymph node status ($P < 0.0001$), ER ($P = 0.0016$), PgR ($P = 0.031$), HER2 ($P = 0.031$), and the type of adjuvant therapy ($P = 0.028$) was strongly able to predict disease-free survival (Table 3). In multivariate analysis, patients with tumors with high *IGF*BP-4 mRNA expression ($P = 0.049$), negative lymph node status ($P = 0.012$), and the type of adjuvant therapy ($P = 0.031$) had significantly increased disease-free survival (Table 3). For overall survival, univariate analysis (Table 4) showed significant associations between overall survival and *IGF*BP-4 mRNA expression ($P = 0.027$), lymph node status ($P = 0.0001$), histological grade ($P = 0.010$), ER ($P = 0.0002$), PgR ($P = 0.0027$) and HER2 ($P = 0.023$). There was no significant relation between overall survival and *IGF*BP-4 mRNA expression in multivariate analysis (Table 4). We concluded from these

Table 2. Correlation between clinicopathological factors and *IGF*BP-4 and *IGF*BP-5 of 162 breast cancer patients

		No. of patients	IGFBP-4 mRNA	*P*	IGFBP-5 mRNA	*P*
Age (years)	50	55	285.3 ± 376.9	0.48	97.5 ± 123.0	0.13
	>50	107	323.6 ± 299.2		200.5 ± 496.4	
Menopausal status	Pre	67	281.2 ± 346.7	0.34	135.8 ± 354.9	0.43
	Post	95	331.6 ± 312.9		187.2 ± 449.4	
Tumor size (cm)	<2.0	31	362.4 ± 322.8	0.12	133.3 ± 148.1	0.67
	2.0	126	275.5 ± 256.7		168.9 ± 458.3	
Lymph node status	negative	90	347.4 ± 398.2	0.15	110.7 ± 160.9	0.07
	positive	61	265.0 ± 212.9		195.3 ± 395.5	
Histological grade	1,2	114	317.7 ± 280.1	0.0032*	188.2 ± 482.2	0.22
	3	36	165.0 ± 197.5		87.5 ± 91.4	
ER	negative	37	174.0 ± 222.3	0.0031*	78.8 ± 123.0	0.17
	positive	119	323.5 ± 271.5		187.4 ± 469.8	
PgR	negative	48	195.3 ± 245.4	0.0045*	90.0 ± 146.2	0.15
	positive	108	328.4 ± 268.0		193.5 ± 488.8	
HER2	negative	119	327.3 ± 283.3	0.0007*	192.0 ± 472.2	0.10
	positive	34	149.6 ± 151.7		59.0 ± 61.7	

IGFBP, insulin-like growth factor binding proteins; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor type 2.
P, unpaired *t*-test.
*$P < 0.05$.

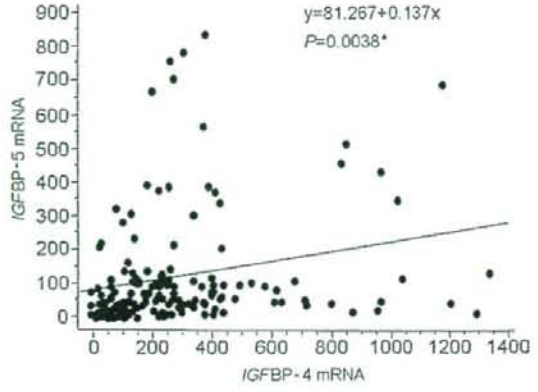

Figure 1. Correlation between *IGF*BP-4 and *IGF*BP-5 mRNA expression in human breast carcinomas. Expression is shown as relative copy numbers normalized against *GAPDH* mRNA. *IGF*BP-4, insulin-like growth factor binding protein-4; *IGF*BP-5, insulin-like growth factor binding protein-5.

analyses that *IGF*BP-4 mRNA expression is an independent prognostic factor of disease-free survival in breast cancer.

Patients with ER-positive Breast Cancer whose Tumor Expressed Higher Levels of *IGF*BP-4 mRNA or Lower Levels of *IGF*BP-5 mRNA had Better Disease-free Survival

We then analyzed disease-free and overall survival in 119 patients with ER-positive breast cancer. Kaplan–Meier analysis of disease-free survival showed that a high level of *IGF*BP-4 mRNA expression (449.1 ± 272.9; $n = 72$) was significantly associated with a reduced risk of recurrence than a low level of *IGF*BP-4 mRNA expression (118.3 ± 52.7; $n = 43$) ($P = 0.018$, Fig. 3a); however, there was no correlation between *IGF*BP-4 mRNA expression and overall survival in these patients (Fig. 3b). Furthermore, only one patient with a tumor that expressed a low level of *IGF*BP-5 mRNA (16.9 ± 9.8; $n = 28$) relapsed ($P = 0.046$, Fig. 3c) and all patients were alive during the follow-up periods (Fig. 3d), whereas 19 patients relapsed who had tumors that expressed a high level of *IGF*BP-5 mRNA (239.9 ± 526.9;

Table 3. Prognostic factors in 158 breast cancers compared with disease-free survival

	Univariate	Multivariate		
	P	*P*	Relative risk	95% confidence interval
Age	0.27	–	–	–
Menopausal status	0.68	–	–	–
Tumor size	0.016*	0.36	0.665	0.280–1.579
Lymph node status	<0.0001*	0.012*	0.419	0.213–0.824
Histological grade	0.087	–	–	–
ER	0.0016*	0.052	2.369	0.991–5.663
PgR	0.031*	0.42	0.699	0.292–1.675
HER2	0.031*	0.47	0.764	0.367–1.591
IGFBP-4 mRNA	0.0044*	0.049*	0.480	0.232–0.996
IGFBP-5 mRNA	0.50	–	–	–
Adjuvant therapy	0.028*	0.031*	0.111	0.015–0.817

*$P < 0.05$.

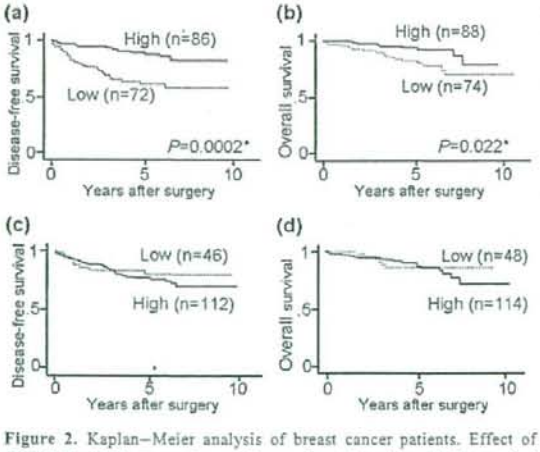

Figure 2. Kaplan–Meier analysis of breast cancer patients. Effect of *IGF*BP-4 mRNA expression on disease-free (a) and overall (b) survival among 162 patients with invasive carcinoma and effect of *IGF*BP-5 mRNA expression on disease-free (c) and overall (d) survival among 162 patients with invasive carcinoma.

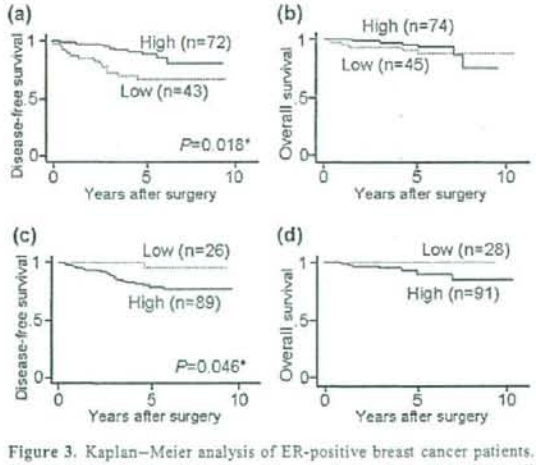

Figure 3. Kaplan–Meier analysis of ER-positive breast cancer patients. Effect of *IGF*BP-4 mRNA expression on disease-free (a) and overall (b) survival among 119 patients with ER-positive breast cancer and effect of *IGF*BP-5 mRNA expression on disease-free (c) and overall (d) survival among 119 patients with ER-positive breast cancer.

$n = 89$). Univariate analysis (Table 5) demonstrated that *IGF*BP-4 mRNA expression ($P = 0.022$) as well as lymph node status ($P = 0.0001$) was strongly able to predict risk of recurrence in ER-positive breast cancer. In multivariate analysis (Table 5), patients with tumors with high *IGF*BP-4 mRNA expression had a significantly increased disease-free survival ($P = 0.029$), indicating that *IGF*BP-4 mRNA expression is an independent prognostic factor of disease-free survival in ER-positive breast cancer. There was no significant relation between overall survival and *IGF*BP-4 and *IGF*BP-5 mRNA expression in ER-positive breast cancer (Table 6).

DISCUSSION

In the present study, we examined mRNA expression of *IGF*BP-4 and *IGF*BP-5 in 162 human breast cancer tissues, and demonstrated that *IGF*BP-4 mRNA expression was an independent prognostic factor in breast cancer, and that patients with ER-positive breast cancer whose tumor expressed higher levels of *IGF*BP-4 mRNA and lower levels of *IGF*BP-5 mRNA had a better prognosis that those without such findings.

Although the roles of *IGF*BP-4 and *IGF*BP-5 in breast cancer are not well established, it is well known that the pattern of *IGF*BP expression and secretion relates to the ER status of breast cancer cells (19). It was also reported that *IGF*BP-4 and *IGF*BP-5 mRNA concentrations were greater in ER-positive cancer tissues than in ER-negative tumors (19,20), and *IGF*BP-4 and *IGF*BP-5 protein expression was correlated positively with ER and PgR (21). Our results also showed that *IGF*BP-4 mRNA expression was positively associated with ER and PgR expression. On the contrary, it was reported that *IGF*BP-3 mRNA and protein levels were found to be inversely correlated with ER and PgR levels (22,23).

Although the role of *IGF*BP-4 in the mammary gland and breast cancer has not been fully elucidated, *IGF*BP-4 has been reported by several laboratories as one of the early-responsive estrogen-induced genes through studies using microarray technology (2,24,25). It was also reported

Table 4. Prognostic factors in 162 breast cancers compared with overall survival

	Univariate	Multivariate		
	P	*P*	Relative risk	95% confidence interval
Age	0.25	–	–	–
Menopausal status	0.82	–	–	–
Tumor size	0.089	–	–	–
Lymph node status	0.0001*	0.0019*	0.19	0.066–0.54
Histological grade	0.010*	0.99	1.01	0.37–2.75
ER	0.0002*	0.091	3.39	0.82–14.00
PgR	0.0027*	0.98	1.02	0.26–4.05
HER2	0.023*	0.85	0.91	0.34–2.45
IGFBP-4 mRNA	0.027*	0.56	1.37	0.47–3.87
IGFBP-5 mRNA	0.24	–	–	–
Adjuvant therapy	0.11			

*$P < 0.05$.

Table 5. Prognostic factors in 115 ER-positive breast cancers compared with disease-free survival

	Univariate	Multivariate		
	P	*P*	Relative risk	95% confidence interval
Age	0.92	–	–	–
Menopausal status	0.99	–	–	–
Tumor size	0.054	–	–	–
Lymph node status	0.0001*	<0.0001*	0.15	0.061–0.39
Histological grade	0.49	–	–	–
PgR	0.90	–	–	–
HER2	0.55	–	–	–
IGFBP-4 mRNA	0.022*	0.029*	0.380	0.159–0.906
IGFBP-5 mRNA	0.08	–	–	–
Adjuvant therapy	0.16			

*$P < 0.05$.

Table 6. Prognostic factors in 119 ER-positive breast cancers compared with overall survival

	Univariate	Multivariate		
	P	*P*	Relative risk	95% confidence interval
Age	0.46	–	–	–
Menopausal status	0.21	–	–	–
Tumor size	0.17	–	–	–
Lymph node status	0.020*	0.036*	0.18	0.038–0.90
Histological grade	0.63	–	–	–
PgR	0.52	–	–	–
HER2	0.59	–	–	–
IGFBP-4 mRNA	0.68	–	–	–
IGFBP-5 mRNA	–	–	–	–
Adjuvant therapy	0.68			

*$P < 0.05$.

that *IGF*BP-4 was up-regulated by estradiol on which ICI182780 acted as an antagonist, whereas tamoxifen and raloxifen acted as partial antagonists (26). We previously reported that expression of histone deacetylase (*HDAC*) 6, which is a late responsive estrogen-induced gene, is correlated with a better prognosis in breast cancer and that expression of higher levels of *HDAC*6 tended to be predictive for response to endocrine therapy (14). Our present study showed that *IGF*BP-4 mRNA expression was an independent prognostic factor in breast cancer. Because the number of patients available for evaluating responsiveness to endocrine therapy in this study was limited, further study is needed to analyze whether *IGF*BP-4 is a predictive factor for endocrine therapy. Furthermore, a recent study showed that *IGF*BP-4 is one of the key genes to correlate with tamoxifen resistance by gene expression array and immunohistochemistry tissue micro arrays (27). Because 96 of the 119 patients with ER-positive breast cancer received tamoxifen as an adjuvant therapy in our present study, *IGF*BP-4 expression levels might have affected the tamoxifen response.

However, *IGF*BP-5 is an estrogen-repressed gene and our results indicated that only one patient with a tumor that expressed a low level of *IGF*BP-5 mRNA relapsed and all such patients were alive during the follow-up periods in ER-positive breast cancer. Patients with hormone receptor-positive tumors were given tamoxifen as adjuvant therapy and received endocrine therapy as initial treatment after relapse. Therefore, *IGF*BP-5 expression might be predictive of response to endocrine therapy. Furthermore, studies using a gene expression profile demonstrated that *IGF*BP-5 was a gene signature of a poor prognosis (28), and that *IGF*BP-5 protein expression was elevated in samples of lymph node metastasis (29). Although there was no difference between *IGF*BP-5 expression and lymph node status or survival in any of the patients in our study, *IGF*BP-5 expression might be a poor prognostic factor in breast cancer.

We cannot readily explain why *IGF*BP-4 and *IGF*BP-5 mRNA expressions were shown to be strongly and positively correlated in our study, although higher levels of *IGF*BP-4 and lower levels of *IGF*BP-5 had a better prognosis. Our preliminary immunohistochemical study for *IGF*BP-4 and *IGF*BP-5 protein expression of human breast cancer tissues showed that these proteins were present both in the cytoplasm and the nuclei. Moreover, some *IGF*BP-4 or *IGF*BP-5-positive cells were noted in the stroma of normal breast and carcinoma tissues, and the *IGF*BP-4 or *IGF*BP-5-positive cells in the stroma were considered lymphocytes or macrophages. Further studies are needed to clarify the function of *IGF*BP-4 and *IGF*BP-5 in the cytoplasm and the nuclei of cancer cells and also of stromal cells in order to understand the role of *IGF*BP-4 and *IGF*BP-5 in breast cancer.

The present study demonstrated that *IGF*BP-4 mRNA expression was an independent prognostic factor in breast cancer, and that patients with ER-positive breast cancer whose tumor expressed higher levels of *IGF*BP-4 mRNA and lower levels of *IGF*BP-5 mRNA had a better prognosis that those without such findings.

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Conflict of interest statement

None declared.

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Demethylation of promoter C region of estrogen receptor α gene is correlated with its enhanced expression in estrogen-ablation resistant MCF-7 cells

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Abstract

Long-term estrogen deprivation (LTED) MCF-7 cells showing estrogen-independent growth, express estrogen receptor (ER) α at a much higher level than wild-type MCF-7 cells. Enhanced expression of ERα associated with partial localization of ERα to the plasma membranes in LTED cells is thought to be an important step for acquisition of estrogen-ablation resistance. In this study, we compared the regulation of ERα gene expression between wild type and LTED cells, examining the usage of the promoters A and C as well as their methylation status. We found that transcription from the promoter C was drastically enhanced in LTED cells, compared with that in wild-type cells. Furthermore, the promoter C region was highly unmethylated in LTED cells, but partially methylated in wild-type cells. Our findings imply that demethylation of promoter C region in the ERα gene is in part responsible for the enhanced expression of ERα gene in LTED cells.

Keywords: Estrogen receptor α; Breast cancer; Methylation

1. Introduction

Experimental, clinical, and epidemiologic data suggest that estrogens contribute to the development of breast cancer. Estrogens bind to ERα or β and stimulate the transcription of target genes involved in cell proliferation. Thus, the anti-estrogen therapy such as tamoxifen has been generally used for ERα-positive breast cancer for several decades. Recently, clinical trials in the adjuvant, neoadjuvant and advanced disease setting have demonstrated a greater clinical efficacy of the aromatase inhibitors aiming to decrease the concentration of estrogen, compared with selective estrogen receptor modulators represented by tamoxifen [1,2]. On the other hand, clinical observations suggested that some human breast cancers adapted to hormone-ablative therapy involving surgically deprivation of estrogen production. Hormone-dependent breast cancers often regress in response to surgical removal of the ovaries, a treatment which lower circulating plasma estradiol (E2) from approximately 200–15 pg/ml [3]. In response to this acute deprivation of E2, tumors regress for 12–18 months on average before they begin to regrow. Second-line therapy with surgical oophorectomy or with aromatase inhibitors can then induce additional tumor regression by lowering E2 concentrations further to 1–5 pg/ml [4]. These

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observations for the first time demonstrated enhanced sensitivity to circulating E2.

In order to demonstrate the phenomenon of adaptive hypersensitivity and to determine the mechanisms involved, we have established a model system involving MCF-7 human breast cancer cells *in vitro*. Wild-type MCF-7 cells were cultured over a prolonged period in estrogen-free medium to mimic the effect of ablative endocrine therapy such as induced by surgical oophorectomy [5]. This process involves long-term E2 deprivation; the adapted cells are called LTED cells. When LTED cells were cultured in the presence of E2 for 4 months, the cells showed estrogen-dependent growth as was observed for wild-type MCF-7 cells [5].

Importantly, ERα is expressed at a much higher level in LTED cells than in wild-type MCF-7 cells [6]. In concert with this observation, an elevated basal ER transactivation activity (mean increase; 5-fold) was measured in LTED cells compared with the wild-type cells using pERE-tk-CAT, a reporter gene driven by estrogen responsive element-thymidine kinase promoter [6].

In addition to the established role as a nuclear receptor, ERα may have another function on the plasma membrane. In LTED cells, ERα localizes predominantly to the nuclei and some also present on the plasma membranes [7]. The sub-cellular localization of ERα to the plasma membranes in LTED cells may, at least in part, be due to the enhanced expression of ERα in LTED cells, since plasma membrane-associated ERα can only be observed in ERα-enriched MCF-7 sub-cell lines (mER^{high}) but not ERα-depleted ones ($mER\alpha^{low}$) [8]. Constitutively activated MAP kinase activity was observed in LTED cells independent of serum factors [9]. A rapid physical interaction of the plasma membrane-associated ERα and an adaptor protein Shc has been observed upon addition of E2 [7]; Shc is subsequently phosphorylated and triggers the MAP kinase signaling pathway [10,11].

Thus, when ERα works as a transcription factor in the nuclei and also as a signal transducer on the plasma membranes, typically in LTED cells, enhanced expression of ERα may be an obligatory step for acquisition of estrogen-ablation resistance. Though, the mechanisms how ERα expresses at high level in LTED cells still remain unknown.

Several human ERα gene promoters (A–F) are identified so far [12]. These promoters are differently utilized in a tissue- and cell-dependent manner [13–15]. Among these promoters, we previously demonstrated that the transcript from promoter A was constitutively used in both normal and cancerous mammary tissue, while the transcript from promoter C (formerly called promoter B) showed remarkable correlation to the ERα protein levels in ERα-positive breast cancer [16]. Furthermore, we have identified a *cis*-acting element, ERBF-1, that plays an important role in the expression of the ERα gene transcribed from promoter C in breast cancer cells [17]. On the other hand, a transcription factor ERF-1, a member of AP2 transcription factor, is important for the transcriptional regulation of promoter A [18,19]. In addition, methylation of the promoter A and C regions was critical for the repression of gene transcription from these promoters [20]. Collectively, methylation of these promoter regions as well as alteration of critical transcription factors are thought to be important for ERα gene expression in breast cancer cells.

In this study, we examined regulation of ERα gene expression in wild-type MCF-7 and LTED cells. We first found that transcription from the promoter C was drastically enhanced in LTED cells, compared with that in the wild-type cells. Transient transfection with a reporter gene driven by the promoter A or promoter C of ERα gene revealed that transcription factors are equally available in these cells. Second, differences in epigenetic alterations of promoter C were found between LTED and wild-type cells: The promoter C region was highly unmethylated in LTED cells, while that in wild-type cells was partially methylated. Our findings imply that demethylation of promoter C region in the ERα gene is in part responsible for the enhanced expression of ERα gene in LTED cells.

2. Materials and methods

2.1. Tissue culture

Human breast cancer cells wild-type MCF-7 were maintained in improved MEM (IMEM) containing 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) and 10 nM E2. LTED cells were established by long-term culture of wild-type MCF-7 cells in IMEM containing 5% DCC-FBS. The established LTED cells were stored in liquid nitrogen until use. LTED cells were maintained in IMEM containing 5% DCC-FBS.

2.2. Plasmid

Reporter plasmids pGL3-ProA 1.3K and pGL3-ProC (formerly called pGL3-ProB1.4K) were described previously [20]. An internal control pRL-TK was purchased from Promega (Madison, WI).

2.3. RNA extraction and cDNA synthesis

Total RNA was prepared from wild type and LTED cells using RNeasy Mini kit (QIAGEN, Hilden, Germany). One μg of total RNA was reverse transcribed with Quantitect Reverse Transcription (QIAGEN) using RT primer mix as primers in a final volume of 20 μl at 42 °C for 15 min.

2.4. Real-time PCR analysis of ERα mRNA expression

The real-time PCR was performed in triplicate using iCycler iQ (Bio-Rad Laboratories, Hercules, CA). Reaction mixture consisted of 1 μl of cDNA products, 0.2 μM of each primers and 12.5 μl of SYBR Green ROX Mix (ABgene, Epsom, UK) in a total volume of 25 μl. PCR thermal conditions were as following: 95 °C for 15 min for 1 cycle and 95 °C for 20 s, 60 °C for 15 s, 72 °C for 10 s, and 86 °C for

15 s (fluorescent signal collection) for 50 cycles for detection of ERα mRNA from promoters A and C; 95 °C for 15 min for 1 cycle and 94 °C for 15 s, 68 °C for 30 s, and 86 °C for 15 s (fluorescent signal collection) for 50 cycles for detection of total ERα mRNA. The following primers were used: PROA1, 5′-ACC TCG GGC TGT GCT CTT-3′ and PRODW, 5′-GAG GGT CAT GGT CAT GGT-3′ for ERα mRNA from promoter A; PROB3, 5′-GCC CAG GAA CAT TTC TGG AA-3′ and PRODW for ERα mRNA from promoter C; EREX1, 5′-AGA ACG AGC CCA GCG GCT AC-3′ and EREX2-R, 5′-CCT TGC AGC CCT CAC AGG AC-3′ for total ERα mRNA. For construction of standard curves, serially diluted plasmids harboring fragment of target gene sequences were used after digestion with *Not* I restriction enzyme to release the insert fragments. As a control, β-actin mRNA was also measured as described previously [21].

2.5. Transient transfection assays

A transient transfection of the plasmids was performed in triplicate using SuperFect Transfection Reagent (QIAGEN) under manufacturer's instruction. Briefly, 1×10^5 cells were plated onto 24 wells plastic dish. After over night culture, 1 μg of pGL3-ProA1.3K or pGL3-ProC and 0.1 μg of pRL-TK were mixed with 5 μl of the SuperFect Transfection Reagent in 350 μl of the medium with 5% DCC-FBS and subjected to transfection. After 2 h incubation, the medium was replaced with a fresh medium and cells were incubated for 48 h. Then, cells were collected and lysed using Passive Lysis Buffer (Promega). Luciferase assays were performed using Dual-Luciferase Reporter Assay System (Promega) and TD-20/20 Luminometer (TURNER DESIGNS, CA).

2.6. Bisulfite modification and methylation-specific PCR

Total DNA was extracted from wild type and LTED cells using NucleoSpin Tissue (MACHEREY-NAGEL, Düren, Germany). DNA was subjected to bisulfite conversion using EZ DNA Methylation kit (ZYMO Research, Orange, CA) according to the manufacture's instruction, essentially based on the report by Herman et al. [22]. Briefly, after denaturation with NaOH, 500 ng of DNA was incubated in a buffer containing sodium bisulfite at 50 °C for 16 h, followed by purification using ZYMO-Spin I column. Bisulfite-converted DNA was eluted in 10 μl of M-Elution buffer. First PCR amplification aiming to amplify bisulfite-converted DNA fragments was performed in 20 μl of reaction mixture containing 1 μl of bisulfite-treated genomic DNA, 0.5 μM each primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystem, Foster, CA) under the following conditions: 95 °C for 5 min for 1 cycle and 95 °C for 15 s, 54 °C for 15 s, and 72 °C for 1 min for 35 cycles. Second PCR amplification using methylation- or unmethylation-specific primers was performed in 20 μl of reaction mixture containing 1 μl of first PCR product (1/100 diluted with MilliQ water), 0.5 μM each primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1.25 units of AmpliTaq Gold DNA polymerase under the following conditions: 95 °C for 5 min for 1 cycle and 95 °C for 15 s, 57 °C (methylation) or 54 °C (unmethylation) for 30 s, and 72 °C for 20 s for 35 cycles. The amplified fragments were electrophoresed to 8% polyacrylamide gel. All the primers used for this methylation analysis were designed using MethPrimer [23]. Primers used for the first PCR and second PCR are summarized in Table 1.

For quantification, real-time PCR amplification was performed in 25 μl of reaction mixture containing 1 μl of the first PCR product (1/100 diluted with MilliQ water), 0.2 μM each primers and 12.5 μl of SYBR Green Rox Mix according to the manufacturer's protocol using iCycler iQ under the following conditions: 95 °C for 15 min for 1 cycle and 95 °C for 15 s, 57 °C (methyl) or 54 °C (unmethyl) for 15 s, 72 °C for 15 s, and fluorescent signal collection at 77 °C (methyl) or 76 °C (unmethyl) for 20 s for 40 cycles. The assay was conducted in triplicate and repeated for three times. For construction of a standard curve, serially diluted control PCR fragments were used.

Table 1
Primers used for the methylation-specific PCR

Promoter	PCR	Specification	Primer name	Primer sequence	Annealing temperature (°C)
A	1st	Bisulfite conversion specific	ERPROABSF	5′-TTAATGTTAGGGTAAGGTAATAGTTTT-3′	54
			ERPROABSR	5′-AACCACCTAAAAAAAAAACACAA-3′	
	2nd	Methylation specific	ERPROAM1F	5′-AGTTTAGGAGTTGGCGGAGGGC-3′	60
			ERPROAM1R	5′-CCGAAATTAAAAACGACGCAACG-3′	
		Unmethylation specific	ERPROAU1F	5′-GGGAGTTTAGGAGTTGGTGGAGGGT-3′	60
			ERPROAU1R	5′-ACCCAAAATTAAAAACAACACAACACA-3′	
C	1st	Bisulfite conversion specific	ERPROCBSF	5′-AGTAGATAGTAAGTTTTTTTTTATTTTTT-3′	54
			ERPROCBSR	5′-AAAAACAACCAATAAACAAAA-3′	
	2nd	Methylation specific	ERPROCM1F	5′-TTTTTTATTGTTATTTATTTAGCGT-3′	57
			ERPROCM1R	5′-AAAACACTTAACAACCCCTCCCGAC-3′	
		Unmethylation specific	ERPROCU1F	5′-TATTTTTTATTGTTATTTATTTAGTGT-3′	54
			ERPROCU1R	5′-AAACACTTAACAACCCCTCCCAAC-3′	

2.7. Bisulfite-sequencing and combined bisulfite restriction analysis (COBRA)

PCR amplification of ERα promoter C region using bisulfite-converted DNA fragments was performed as described above except for the cycle numbers to be 50. The amplified fragments were purified using NucleoSpin Extract II kit (MACHEREY-NAGEL). Direct sequencing of the fragments was conducted using ERPROCBSF as a primer, BigDye Terminators v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). For COBRA, 5 μl of purified DNA fragment was digested in a total volume of 20 μl using 5 units of *Hpy*188III restriction enzyme (New England BioLabs, Ipswich, MA) that cleave CpG sites retained because of methylation at 37 °C for 4 h. The resultant DNA fragments together with undigested ones were electrophoresed onto 8% polyacrylamide gel. After staining with ethidium bromide, the image was visualized under UV illumination. Intensity of the fluorescence of each band was quantified using ChemImager 5500 (Alpha Innotech, San Leandro, CA).

2.8. Statistical analysis

Student's *t*-test was conducted for statistical analysis. When necessary, the *t*-test was modified to all for unequal variances. *P*-values less than 0.05 were considered as significant.

3. Results

3.1. Expression levels of ERα mRNA in wild-type and LTED cells

We previously demonstrated that ERα protein and mRNA were expressed at higher levels in LTED cells than in wild-type cells using Western and Northern blot analyses [6]. As a first step, we conducted real-time PCR analysis to quantitatively evaluate ERα mRNA expression in wild-type and LTED cells: LTED cells showed 27-fold total ERα mRNA expression levels as compared with wild-type cells did ($P = 0.004$) (Fig. 1).

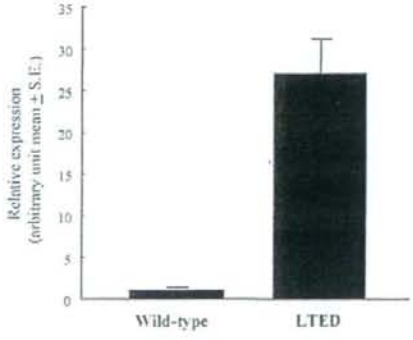

Fig. 1. Relative expression of total ERα mRNA in wild type and LTED cells. Real-time RT-PCR was conducted for quantification of total ERα and β-actin mRNA as described in Section 2. Expression levels of total ERα mRNA were normalized using β-actin mRNA.

Since an increase of ERα mRNA from promoter C was responsible for the enhanced expression of ERα protein in ERα-positive primary breast cancer and since promoter A was constitutively utilized in both normal and cancerous mammary tissue [16], we next compared ERα mRNA expression levels transcribed from these promoters A and C between wild-type and LTED cells using real-time PCR (Fig. 2). ERα mRNA from promoter A in LTED cells was 35-fold higher than that in wild-type cells ($P = 0.0007$) (Fig. 2). Furthermore, ERα mRNA expression level from promoter C in LTED was 149-fold higher than that in wild-type cells ($P = 0.01$) (Fig. 2). In wild-type cells, promoter A was dominant while promoters A and C were equally utilized in LTED cells (Fig. 2). These results indicate that reinforced utilization of promoter C in LTED cells as compared with wild-type cells may be important for the enhanced expression of ERα in LTED cells.

3.2. Transient transfection of reporter gene constructs with ERα gene promoters

The *cis*- and *trans*-acting factors are thought to generate differences in transcription activity on various promoters. We first tested the possibility that alterations of transcription factors may generate an increased level of ERα expression in LTED cells, compared with wild-type cells. We then measured the promoter activities in wild type and LTED cells using reporter gene constructs driven by ERα promoters A and C. No significant differences in promoter activities between wild-type and LTED cells were observed for these two promoters ($P = 0.3$ and 0.1 for promoters A and C, respectively, Fig. 3), suggesting that *trans*-acting factors specific to promoters A and C are not responsible for the different utilization of ERα promoters A and C between wild-type and LTED cells.

3.3. Different methylation status of ERα gene promoters in wild-type and LTED cells

We next hypothesized that alternations of higher order chromatin structure caused by DNA methylation may contribute to the expression level of ERα in these cells. Then, we compared methylation status of ERα gene promoters A and C in wild-type and LTED cells by methylation-specific PCR. Promoter A of ERα gene was unmethylated in both wild-type and LTED cells (Fig. 4). On the other hand, promoter C of ERα gene showed partial methylation in wild-type cells (Fig. 4), in good agreement with our previous report [20]. In LTED cells, the unmethylated band for promoter C was clearly observed, while the methylated one was considerably weaker (Fig. 4), in accordance with our

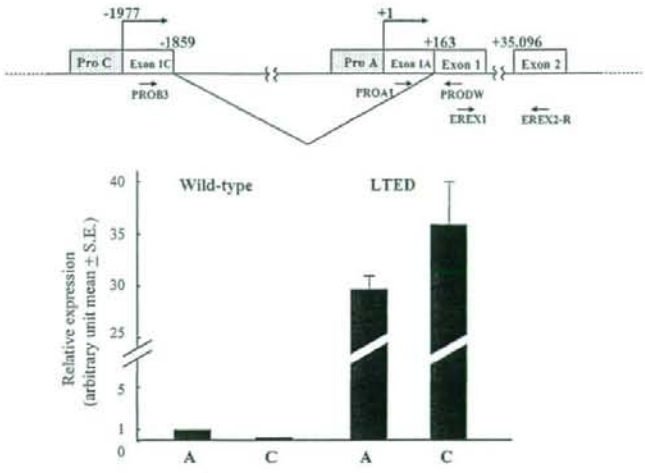

Fig. 2. Expression of ERα mRNA transcribed from promoters A and C in wild-type and LTED cells. Schematic representation of a part of ERα gene organization is shown above. The transcription start site of promoter A is defined as +1. Relative expression of ERα mRNA from promoters A and C in wild-type and LTED cells is shown. Expression levels of ERα mRNA from promoters A and C were quantified by real-time RT-PCR as described in Section 2 being normalized by β-actin mRNA.

observation that promoter C was actively utilized in LTED cells.

In order to confirm the difference of methylation status of the promoter C between wild-type and LTED cells, we next conducted direct sequencing of the PCR fragment amplified from bisulfite-converted DNA. Cytosines were predominantly observed at nucleotides −2103, −2082, and −2073 within CpG dinucleotides, while thymines are faintly detected at these sites in wild-type cells (Fig. 5). On the other hand, both thymines and cytosines were clearly observed at these sites in LTED cells (Fig. 5), confirming the difference of methylation status between wild-type and LTED cells.

3.4. Quantitative analysis of methylation status of ERα gene promoter C in wild-type and LTED cells

In order to quantitatively analyze the methylation of the promoter C region, we next performed two different analyses, i.e. COBRA assay and bisulfite-real-time PCR. In COBRA assay, the fragment amplified from methylated

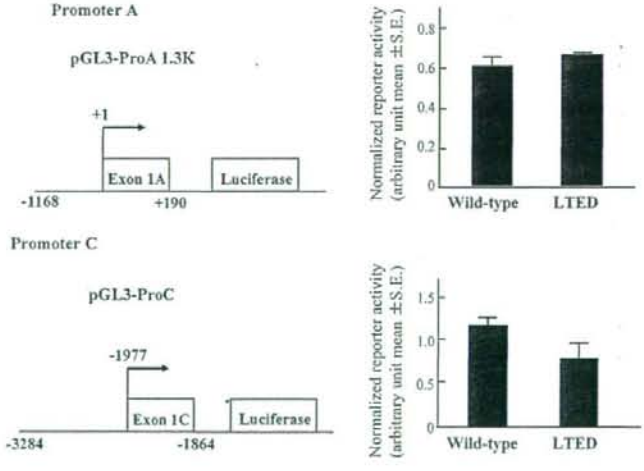

Fig. 3. Reporter activities of promoters A and C in wild-type and LTED cells. Wild-type and LTED cells were transiently transfected with a reporter gene construct pGL3-ProA 1.3K or pGL3-ProC together with control vector pRL-TK as described in Section 2. The measured luciferase activities were normalized using the control Renilla luciferase activity.

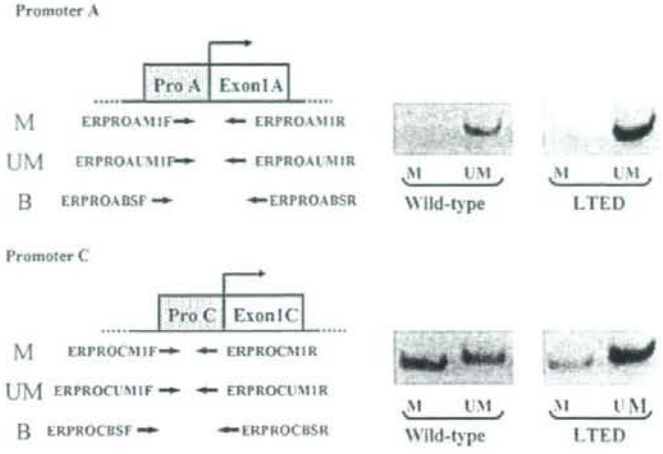

Fig. 4. Methylation-specific PCR analysis of ERα gene promoters A and C in wild-type and LTED cells. Location of primers used in methylation-specific PCR for promoters A and C is shown in the left. Photos of electrophoresis of PCR products of methylated (M) or unmethylated (UM) DNA fragments are shown in the right.

DNA can be identified as digestible bands with restriction enzyme *Hpy*188III, because of the retention of methylcytosine residue at −2073 even after bisulfite-treatment (Fig. 6). Quantification of the fragments revealed that 54% of the fragments were methylated in wild-type cells, while only 7.7% were methylated in LTED cells (Fig. 6).

This was also examined with bisulfite-real-time PCR analysis using methylation- or unmethylation-specific primers as

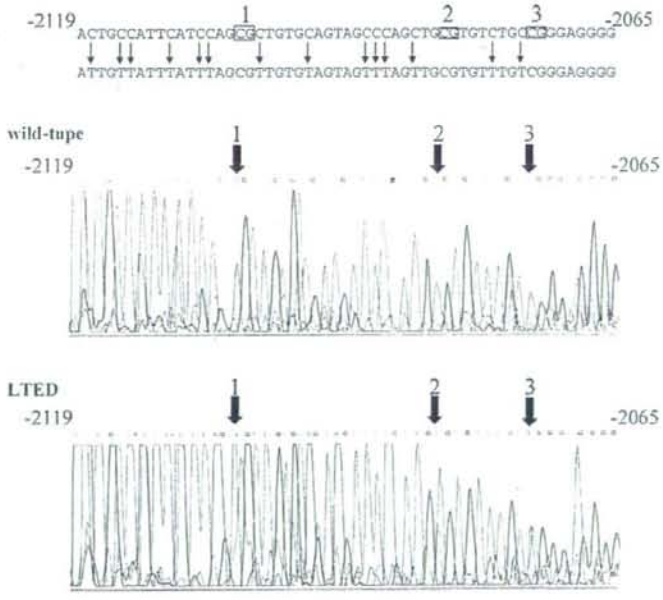

Fig. 5. Direct sequencing of PCR products of ERα gene promoter C using bisulfite-converted DNA from wild-type and LTED cells. First PCR products of bisulfite-converted DNA from wild-type or LTED cells using primers ERPROCBSF and ERPROCBSR were subjected to direct sequencing with ERPROCBSF primer as described in Section 2. Three CpG sites within the amplified region are indicated by rectangles with numbering over the rectangle. Cytosine residues that will be shown as thymine in the sequence of PCR product of bisulfite converted DNA are marked with vertical arrows in black. Thick arrows indicate the positions of three CpG sites.

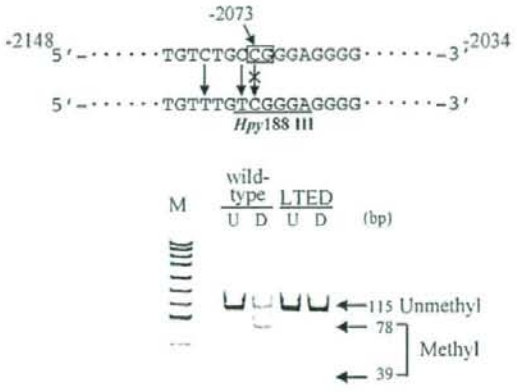

Fig. 6. COBRA assay of ERα gene promoter C in wild-type and LTED cells. First PCR products of bisulfite converted DNA from wild-type or LTED cells using primers ERPROCBSF and ERPROCBSR were digested with restriction enzyme *Hpy*188III as described in Section 2. Rectangle indicates the position of the CpG site to be tested. Underline indicates the recognition site of *Hpy*188III that will be generated by bisulfite conversion of methylated DNA, but not unmethylated one. An image of the polyacrylamide gel electrophoresis is shown below. U, untreated. D, digested with *Hpy*188III.

described above. Amount of methylated DNA in the tested sample from LTED cells was 42% of that from wild-type cells, though this was not statistically significant ($P = 0.063$) (Fig. 7). On the other hand, unmethylated DNA amount was 14.2-fold in LTED cells as compared with wild-type cells ($P < 0.001$) (Fig. 7). These results demonstrated that the promoter C region was highly unmethylated in LTED cells, but partially methylated in wild-type cells.

4. Discussion

Clinical observations suggest that human breast tumors can adapt to endocrine therapy by developing hypersensitivity to estrogen. To understand the mechanisms underlying this, we have previously examined estrogenic stimulation of cell proliferation in a model system and provided *in vitro* and *in vivo* evidence that long-term E2 ablation causes adaptive hypersensitivity. Importantly, LTED cells express ERα at a much higher level than wild cells and consequently show sub-cellular localization of ERα to the plasma membrane in addition to the nucleus. LTED cells showed higher basal estrogen responsive transcription activity as compared with wild-type cells. In addition, LTED cells activate MAP kinase signaling pathway mediated by plasma membrane-bound ERα. In both roles of ERα in the nuclei and on the plasma membranes of LTED cells, enhanced expression of

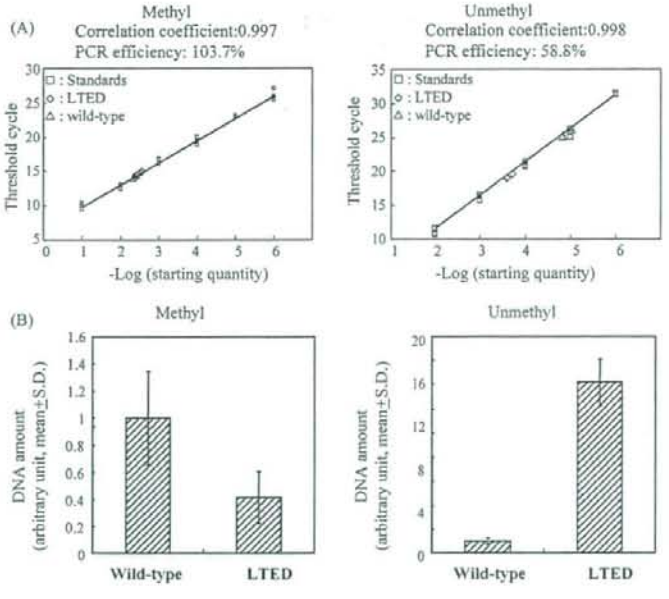

Fig. 7. Bisulfite-real-time PCR analysis of ERα gene promoter C in wild-type and LTED cells. Bisulfite-real-time PCR analysis based on SYBR-Green chemistry was conducted using methylation-specific primers ERPROCM1F and ERPROCM1R or unmethylation-specific primers ERPROCUM1F and ERPROCUM1R as described in Section 2. Representative results of bisulfite-real-time PCR are shown above. Comparison of methylated or unmethylated DNA amounts in tested samples from wild-type and LTED cells are shown below. S.D.: standard deviation.

ERα protein may be an obligatory step for acquisition of estrogen-ablation resistance.

Previously, the expression level of ERα transcript from promoter A was reported to be several folds higher than that from promoter C in MCF-7 cells [24,25], in good agreement with our current study for wild-type MCF-7 cells (Fig. 2). On the other hand, in this study, we for the first time demonstrated that ERα transcripts from promoters A and C expressed at high levels in LTED cells as compared with wild-type cells using real-time PCR (Fig. 2). Specifically, transcripts from promoter C in LTED cells were 149-fold higher than those in wild-type cells and the transcripts from promoter A and C were equally expressed in LTED cells (Fig. 2), indicating that enhanced transcription of ERα gene from promoter C may in part be responsible for the enhanced expression of ERα protein in LTED cells.

Our transient transfection experiments indicated that alterations of transcription factors may not be a major cause for the enhanced expression of ERα mRNA from promoter C in LTED cells (Fig. 3). On the other hand, methylation status of the promoter C region of ERα gene was drastically changed in LTED cells as compared with wild-type cells (Figs. 4–7). We have previously reported that specific *in vitro* methylation of promoter C region of ERα gene significantly reduced its promoter activity in transient transfection experiment [20]. In addition, methylation of promoter C was inversely associated with expression of transcripts from this promoter as well as ERα protein in primary breast cancer [20]. Taken together, hypomethylation of promoter C region in LTED cells can be one of the mechanisms responsible for the enhanced transcription of ERα gene from promoter C.

In this study, we demonstrated that ERα transcript from promoter A also expressed at high levels in LTED cells as compared with wild-type cells (Fig. 2). Since the promoter A was highly unmethylated in both LTED and wild-type MCF-7 cells (Fig. 4), some mechanisms other than DNA methylation should be involved in the regulation of promoter A in LTED cells. Our transient transfection experiments did not show any difference of the reporter gene activity of promoter A constructs in between LTED and wild-type MCF-7 cells (Fig. 3). One possible explanation for the enhanced expression of the transcript from promoter A in LTED cells could be alteration of transcription factors that bind to downstream sequences including intron of the ERα gene that are not present in our tested construct; AP2 transcription factor that bind to the ERF-1 *cis*-acting element located downstream of the transcription start site of promoter A may be one of the candidates [18,19].

Transcripts from promoter A and C share an identical coding sequence for ERα protein. Then, is there any functional difference between these transcripts in relation to the protein level of ERα? Importantly, it has recently been shown that 5′ upstream open reading frame (ORF) that is specific to the transcript A has inhibitory effects on the translation of ERα protein, while the 5′ upstream ORF of transcript C did not show the effect [26]. Notably, the inhibitory effect of the ORF of transcript A was most remarkable in MCF-7 cells among the tested cell lines, indicating that there are differences in the translational potential of transcripts from these two major promoters [26]. It would be interesting to test whether such regulatory effects of upstream ORF specific to transcript A is also present in MCF-7 LTED cells.

In conclusion, we found that transcription from promoters A and C were highly activated in LTED cells. Specifically, hypomethylation of promoter C correlated well with its drastically enhanced expression in LTED cells, suggesting that epigenetic alterations may play some roles in enhanced expression of ERα important for the acquisition of estrogen-ablation resistance of breast cancer cells.

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