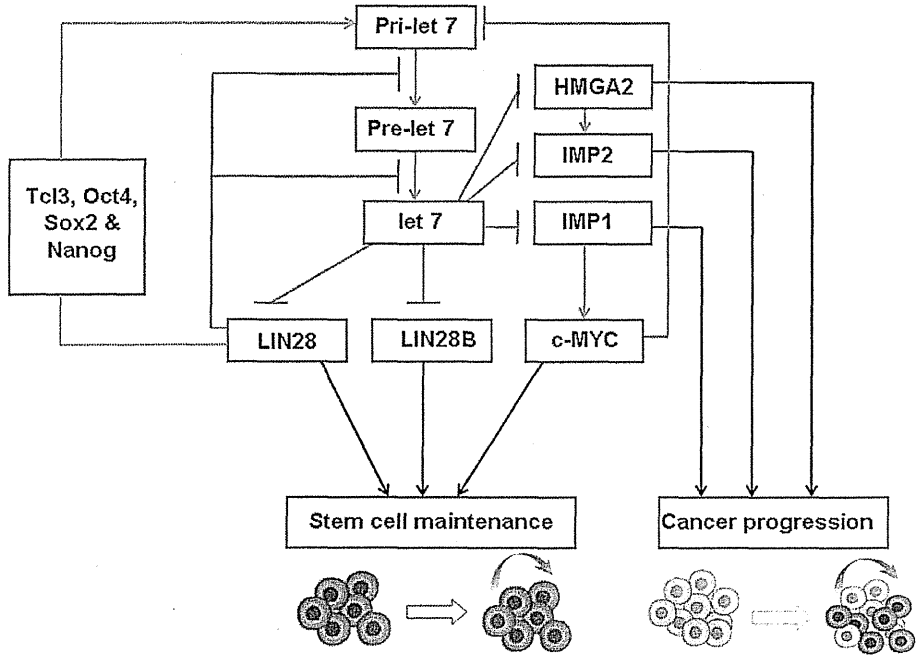

FIGURE 2 *Regulatory circuits of microRNA (miRNA) let-7. The loop consists of pluripotency promoting factors {LIN28 [lin-28 homolog* (Caenorhabditis elegans)*], OCT4 [now labelled POU5F1 (POU class 5 homeobox 1)], SOX2 [SRY (sex determining region Y)–box 2], NANOG [Nanog homeobox], and TCL3 [now labelled TLX1 (T-cell leukemia homeobox 1)]}, oncofetal genes [HMGA2 (high mobility group AT–hook 2) and IMPS (insulin-like growth factor 2 mRNA-binding proteins)], and oncogene MYC. For detail, see text. Pri-let 7 = primary transcripts of let-7; LIN28B = lin-28 homolog B* (C. elegans).

commitment [54,55]. Lin28 was recently shown to act as a posttranscriptional repressor of let-7 biogenesis, binding to the loop portion of the pri–let-7 hairpin and the stem part of pre–let-7 and thereby inhibiting its processing. Lin28 and Lin28B also inhibit processing of let-7 by mediating terminal uridylation of let-7 precursors [56]. What is unclear is whether the regulation by Lin28 occurs at the Drosha or Dicer processing step [55,57–59]. Lin28 induces pri–let-7 expression through induction of other pluripotency-promoting factors such as Pou5F1, Sox2, Nanog, and Tlx1 [60], thus regulating let-7 expression at multiple levels.

The early embryonic oncofetal gene *HMGA2* is involved in the self-renewal and maintenance of adult stem cells. It is highly expressed in hematopoietic and fetal neuronal stem cells [61,62], and the low levels of let-7 in stem cells inversely correlate with *HMGA2* expression. Thus, the undifferentiated state is maintained [63]. In differentiated tissues, *HMGA2* is downregulated because of the high expression of let-7 [61], and during induced differentiation, ectopic expression of let-7 reduces *ras* and *HMGA2* expression, leading to inhibition of cell proliferation and induction of apoptosis. Therefore, *HMGA2* is a direct target of let-7 [64].

Like normal stem cells, cancer stem cells (slowly dividing tumour-initiating cells) exhibit low levels of let-7 and possess unlimited self-renewal capability and pluripotency, allowing them to repopulate and metastasize [65,66]. It has been proposed that, during carcinogenesis, the let-7–targeted embryonic genes, which are otherwise not expressed in adult tissues, are re-expressed because of loss of let-7 control. This reprogramming promotes de-differentiation and cancer progression [67]. A good example is that of *HMGA2,* which is undetectable in most differentiated tissues, but highly expressed in various cancers, including neuroblastoma and pancreatic, lung, and thyroid cancers [68–71]. Breast cancer stem cells are also devoid of let-7, but abundantly express *HMGA2* and *ras* [36] (Figure 2).

2.2.2 Regulatory Circuit Between Myc and Let-7

IMP1 is another oncofetal gene that is expressed only during early fetal life [72,73] and is re-expressed in several cancers [74]. It is selectively expressed in young, but not in old, hematopoietic stem cells [75]. *IMP1* regulates stem cell functions by stabilizing insulin-like growth factor 2 and C-*myc* mRNAs [76,77], and the phenotype of stem cells from the *IMP1* knockout mouse resembles that of cells from the *HMGA2*-deficient mouse [73,78]. Let-7 targets *IMP1*, and therefore indirectly acts as a negative regulator of *MYC* expression [64,79,80]. It has been shown that Myc binds directly to let-7 promoter and downregulates its transcription [81]. Thus, an indirect feedback circuit exists between let-7 and Myc (Figure 2).

2.3 Let-7 Targets Multiple Oncogenes and Components of Cell Cycle, Cell Proliferation, and Apoptosis

Apart from targeting oncogenes (*ras, MYC, HMGA2*, and so on) as already discussed, let-7 regulates several key components of the cell cycle and cell proliferation. Microarray analysis of hepatocellular carcinoma (HepG2) and lung cancer (A549) cell lines revealed that let-7 inhibits multiple cell-cycle- and proliferation-associated genes, including cyclin A2 (*CCNA2*), *CDC34*, Aurora A [*AURKA* (formerly *STK6*)] and B [*AURKB* (formerly *SKT12*)] kinases, *E2F5, CDK8,* and *PLAGL2,* among others[46]. In HepG2 cells, let-7 directly represses *CCNA2, CDC25A, SKP2, AURKA, CDC16, CCND1,* and *CDK6,* among others. Let-7 also inhibits several DNA replication machinery components (*ORC1L; RRM1, 2*; and so on) and transcription factors [*E2F6, CBFB, PLAGL2, SOX9, GZF1* (formerly *ZNF336*), *YAP1, GTF2I, ARID3A,* and so on]. Surprisingly, that study also showed that let-7 represses several tumour suppressor genes (*BRCA1, BRCA2, FANCD2,* and *PLAGL1,* among others) and checkpoint regulators (*CHEK1, BUB1, BUB1B, MAD2L1,* and *CDC23,* among others). Our recent *in silico* analysis shows that let-7 may potentially target ER signalling and angiogenic pathways by targeting key molecules of these cascades[82]. Various targets of let-7 are listed in Table II and shown in Figure 3.

Apoptosis regulatory functions of let-7 have recently been reported in both human and mouse. Let-7 targets Casp3 in the A431 and HepG2 cell lines, and inhibits doxorubicin- and paclitaxel-induced apoptosis[85]. In NIH3T3 mouse fibroblast cells, let-7

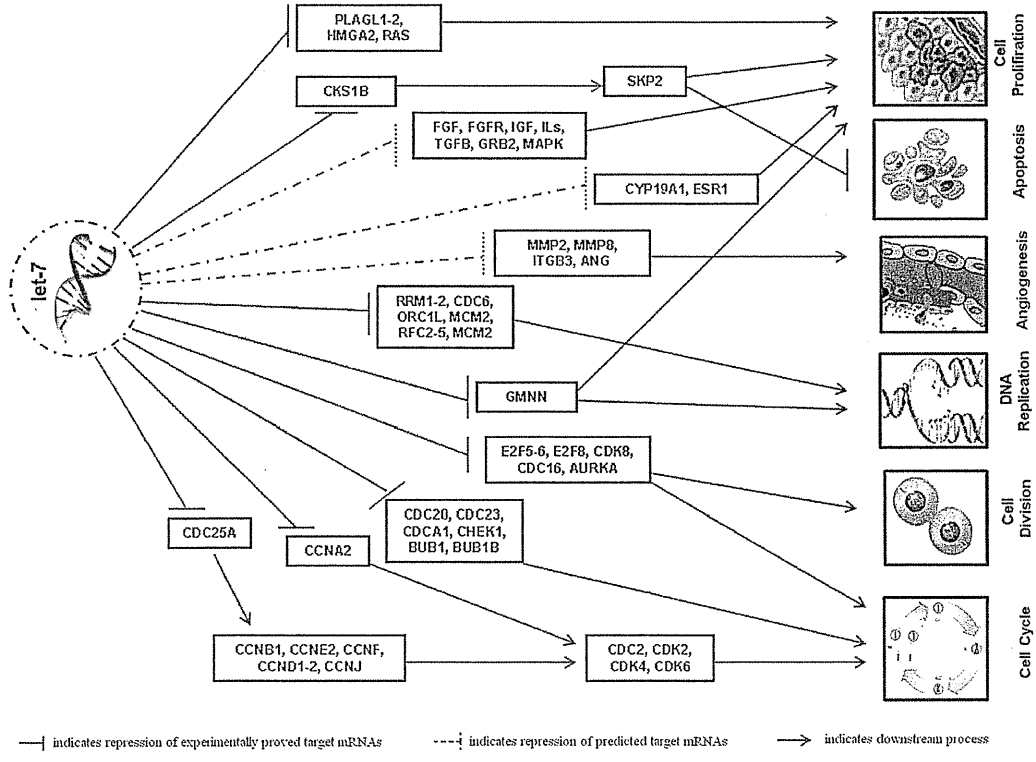

FIGURE 3 *Let-7 targets various key components of mitogenic and tumorigenic pathways to exert its tumour suppressor activity. Pathways include cell cycle, cell division, cell proliferation,* DNA *replication, angiogenesis, and apoptosis. PLAGL1, 2 = pleomorphic adenoma gene-like 1, 2; CKS1B =* CDC28 *protein kinase regulatory subunit 1B; SKP2 = S-phase kinase-associated protein 2 (p45);* FGF, FGFR *= fibroblast growth factor and fibroblast growth factor receptor;* IGF *= insulin-like growth factor;* IL-S *= interleukin S;* TGFB *= transforming growth factor β; GRB2 = growth factor receptor-bound protein 2;* MAPK *= mitogen-activated protein kinase; CYP19A1 = cytochrome P450, family 19, subfamily A, polypeptide 1; ESR1 = estrogen receptor 1; MMP2, 8 = matrix metallopeptidases 2, 8; ITGB3 = integrin β3; ANG = angiogenin; RRM1, 2 = ribonucleotide reductases M1 and M2; CDC6 = cell division cycle 6 homolog* (Saccharomyces cerevisiae)*; ORC1L = origin recognition complex, subunit 1-like (yeast); MCM2 = minichromosome maintenance complex component 2; RFC2–5 = replication factor C (activator 1) 2–5; GMNN = geminin,* DNA *replication inhibitor; E2F5, 6, 8 =* E2F *transcription factors 5, 6, 8; CDK8 = cyclin-dependent kinase 8; CDC16 = cell division cycle 16 homolog* (S. cerevisiae)*; AURKA = aurora kinase A; CDC25A = cell division cycle 25 homolog A* (Schizosaccharomyces pombe)*; CCNA2 = cyclin A2; CDC20, 23 = cell division cycle 20 and 23 homologs* (S. cerevisiae)*; CDCA1 = (now labelled NUF2) NDC80 kinetochore complex component, homolog* (S. cerevisiae)*; CHEK1 =* CHK1 *checkpoint homolog* (S. pombe)*; BUB1, 1B = budding uninhibited by benzimidazoles 1 and 1 β homologs (yeast); CCNB1, D1, D2, E2, F, J = cyclins B1, D1, D2, E2, F, J; CDC2 = cell division cycle 2, G1 to S and G2 to M; CDK2, 4, 6 = cyclin-dependent kinases 2, 4, 6;* MRNA *= messenger* RNA.

TABLE II MicroRNA let-7 targets in various cancers

Cancer	*MicroRNA let-7*			*Model used*	*References*
	Expression	*Targets*	*Effect on targets*		
Breast cancer	let-7 ↓	*ANG*; *CCND1, 2*; *CDC25A*; *CDK4, 6*;*CYP19A1*; DNA polymerases; *E2F5, 6*; *ESR1, 2*; *FGF11*; FGFR; *GRB2*; *HMGB2*; *IGF1, 1R*; *IL6*; *ITGB3*; *MAPK4, 6*; *MMP2*; *MMP8*; *MYC*; *ras*; *RB1*; *SKP2*; *TGFB1, BR1*; *TP53*	Transcription	*In silico*	Barh *et al.*, 2008 [82]
	let-7 ↓	*HMGA2*, H-*ras*	Transcription	Cell line, mouse model	Sempere *et al.*, 2007 [35] Yu *et al.*, 2h007 [36]
Burkitt lymphoma	let-7a ↓	*MYC*	Transcription/translation	Cell line	Sampson *et al.*, 2007 [38]
Colon cancer	let-7 ↓	*ras*, *MYC*	Translation	Cell line	Akao *et al.*, 2006 [40]
Hepatocellular cancer	let-7 ↓	*AURKA*; *BRCA1, 2*; *BUB1*; *CCNA2, B1, E2, F, J*; *CDC2, 6, 20, 23, 25A, 34, 45L*; *NUF2*; *CBX2*; *CDCA2, 3, 4, 5, 7, 8*; *CDK8*; *CHEK1*; *CKS1B*; *DBF4*; *DICER1*; *E2F5, 6, 8*;*FANCD2*; *GMNN*; *CDT1*; *HMGA2*; *LIN28B*; *MAD2L1*; *NRAS*; *ORC1L*; *PLAGL1, 2*; *RRM1, 2*; *SKP2*; *SOX9*; *ARUKB* (formerly STK12)	Transcription	Cell line	Johnson *et al.*, 2007 [44]
Lung cancer	let-7 ↓	*MYC*, *ras*	Transcription/translation	Cell line	Johnson *et al.*, 2005 [46] Kumar *et al.*, 2008 [52]
	let-7 ↓	*AURKA*; *CCNA2*; *CDC34*; *CDK8*; *DBF4*; *DICER1*; *E2F5*; *GMNN*; *HMGA2*; *LIN28B*; *NRAS*; *PLAGL1, 2*; *ARUKB* (formerly STK12)	Transcription	A549 lung cancer cells	Johnson *et al.*, 2007 [44]
	let-7 ↓	*HMGA2*	Transcription	Cell line	Kumar *et al.*, 2008 [52] Lee and Dutta, 2007 [83]
Malignant melanoma	let-7b ↓	*CDK4*; cyclins A, D1, D3	Translation	Cell line	Schultz *et al.*, 2008 [47]
Uterine leiomyoma	let-7 ↓	*HMGA2*	Transcription	Tumour sample, cell line	Peng *et al.*, 2008 [84]

FGFR = fibroblast growth factor receptor; ↓ = downregulation.

is involved in ultraviolet B–induced apoptosis by modulating Casp3, Bcl2, Map3k1, and Cdk5 [86].

2.4 Emerging Role of Let-7 in Cancer Diagnosis and Therapy

The facts discussed here indicate that let-7 acts as a tumour suppressor by targeting various oncogenes and key components of the cell cycle and developmental pathways. Most reports reveal that let-7 is frequently underexpressed (Table I) and that the chromosomal region of human let-7 is frequently deleted in many cancers [87]. Similarly, in more differentiated tumour cells, let-7 is expressed at higher levels, and its target oncogenes (*HMGA2* and *ras*) are downregulated. Thus, loss of let-7 expression is a marker for less differentiated cancer [88], and expression levels are also found to be effective prognostic markers in several cancers [40,46,88]. In lung cancer, reduced let-7 expression was also found to significantly correlate with shortened postoperative survival regardless of disease stage [45].

From the therapeutic viewpoint, let-7 is attractive molecule for preventing tumorigenesis and angiogenesis [89]; it is a potential therapeutic in several cancers that underexpress let-7. Let-7 replacement was found to inhibit anchorage-independent growth and cell-cycle progression in melanoma cells by repressing regulators of the cell cycle and cell proliferation such as cyclins A, D1, and D3 and *CDK4* [47]. Together with *TP53, ras* and *MYC* have been implicated as key oncogenes in lung cancer. The reduced expression of let-7 in lung cancer directly correlates with upregulation of oncogene *ras*; introduction of let-7 represses *ras* and *MYC* translation by targeting the related mRNAs [45,46]. In both lung and hepatocellular carcinomas, replacement or restoration of normal expression levels of let-7 inhibits cancer growth by repressing multiple cell-cycle and proliferation pathways, together with *ras* and *MYC* [37,44,45,52] (Table II). Intranasal let-7 administration was found effective in reducing tumour growth in a K-*ras* mutant mouse model of lung cancer [90]. Similarly, restoration of let-7 restrains the growth and proliferation of colon and hepatic cancers [40,80]. Transfection of let-7 in a Burkitt lymphoma cell line downregulates *MYC* and reverts *MYC*-induced cell growth [38]. Ectopic expression of let-7 inhibits cell proliferation by directly repressing the *HMGA2* oncogene in lung cancers [52,83] and uterine leiomyoma [84].

Induced expression of let-7 in breast cancer cells targets *HMGA2* and H-*ras* [36], and in a mouse model of breast cancer, exogenous let-7 delivery suppresses cell proliferation, mammosphere formation, and the population of undifferentiated cells by downregulating both of the foregoing oncogenes [35,36]. In our *in silico* analysis, we recently showed that, apart from repressing *MYC, ras,* and *HMGA2,* let-7 may also target *CYP19A1, ESR1,* and *ESR2,* thereby potentially blocking estrogen signalling in ER-positive breast cancers. Similarly, by repressing angiogenin, fibroblast growth factor, transforming growth factor, interleukin 6, and matrix metallopeptidase 2, let-7 may prevent growth, angiogenesis, and metastasis in breast cancer [82] (Table II).

2.5 Limitations of Let-7–Based Therapy

2.5.1 Limitations Because of Limited Knowledge of Let-7 Biology

Although restoration of normal let-7 expression proves beneficial, limited knowledge concerning its transcriptional and processing control during biogenesis and its exact role in tumorigenesis make it difficult to directly apply let-7 as a therapeutic. It is necessary to know whether downregulation of let-7 in tumours is a primary or secondary phenomenon during tumorigenesis. Supporting the CSC hypothesis, we agree with the opinion that epigenetic downregulation of let-7 in CSCs leads to upregulation of oncofetal genes (*HMGA2* and *LIN28,* among others) and, thereby, to loss of differentiation and tumorigenesis. In that scenario, downregulation of let-7 is the primary event, a view that can be supported by observation of where in ovarian cancer let-7 is hypermethylated [48].

Because miRNAs act on the 3′ UTR of target mRNAs, it is important to determine how efficiently let-7 will work as a therapeutic, because 3′ UTR truncated oncogenes may be prevalent in neoplasia. Grimm *et al.* [91] reported that delivery of adeno-associated virus (AAV)–mediated recombinant pre-miRNAs causes death in mice from severe liver cytotoxicity. Details of the immunogenic and cytotoxic effects of let-7 therefore need to be explored so that such side effects can be minimized in an effective treatment strategy. Similarly, we proposed that let-7 may be involved in an as-yet-unknown regulatory network of miRNAs that resembles the gene regulatory network involving transcription factors. Therefore, anti-miRNA oligo-based knockdown of let-7 inhibitory miRNAs is not currently possible.

2.5.2 Limitations in Delivery Methods and Systems

Lack of an appropriate, safe, and effective delivery method for let-7 is another drawback of possible therapy. Biological vectors such as AAV and lentivirus may be used for targeted delivery [92], but standardization of the method is required to prevent non-targeted site introduction. Also, brain-specific miRNA delivery is not yet successful [93], and effective neuron-specific delivery methods have to be developed to tackle brain and neuronal tumours. As discussed earlier, AAV- and lentivirus-mediated delivery of let-7 in a mouse model of lung cancer [52,90] was found to be inefficient in pre-existing tumours because of the resistance to let-7 developed by the tumour over time [52]. A strategy for let-7–mediated therapy for pre-existing tumours therefore also has to be developed.

2.6 Strategies to Overcome the Limitations

The optimal or normal level of let-7 may be restored in cancer cells either by administering exogenous

let-7 *in situ* with a vector overexpressing let-7, or by repressing let-7 repressors. Recent miRNA technologies are, in general, designed to use complementary or chemically modified single-stranded RNA analogs (or both) to repress the specific miRNAs responsible for a given disease or cancer. These analogs, including ASOs (antisense oligonucleotides), AMOs (anti-miRNA ASOs called "antagomirs"), locked nucleic acids, and antisense-technology-based small interfering RNAs, are widely and effectively used in regulation of miRNA expression [92,94–99]. But direct information is not available on the miRNAs that regulate let-7 expression; this aspect limits the scope for such a strategy. Instead, technologies are required that can effectively upregulate let-7 expression. Hence, either vector-mediated overexpression of let-7 or transient transfection of double-stranded let-7 will be the choice.

Introduction of double-stranded let-7 duplex may produce mature let-7, equivalent to the endogenous version, during Dicer processing, potentially rescuing a downregulated let-7 level. This strategy has already been successfully used [83]. Vectors containing pre–let-7–like synthetic short hairpin RNAs, driven by highly inducible Pol III promoters such as H1 and U6 [100,101] may provide high expression of let-7 from predefined transcription start and termination sites [102]. But instead of designing artificial hairpins, direct cloning of the entire natural pri–let-7 hairpin with flanking sequences into the expression vector may be a better approach—assuming that natural pre–let-7 will be a better substrate for generating mature let-7 during Dicer processing [103–107]. A pri-MIR–Pol II transgene system has been successfully used to overexpress MIR155 [104], MIR30 [108], and MIR122 [109]. This system was also found useful in expressing multiple miRNAs from a single transcript [104] and can therefore be adopted for let-7 expression too.

High-density lipoprotein conjugated siRNA has been reported to increase delivery efficacy in certain specific organs such as liver, gut, kidney, and steroid secreting organs [110]. A similar approach may therefore have the possibility to be effective in let-7 delivery as well. But the synthesis and purification of therapeutic-grade let-7 is difficult. A nanoparticle-based delivery system may prove beneficial.

Other delivery methods that have been found promising in both *in vitro* and *in vivo* conditions include lentivirus-mediated pre–let-7 oligonucleotides [36], adenovirus-mediated delivery of hairpin sequences of mature let-7 [90], cationic liposome–mediated delivery of pre–let-7 [40], and electroporation of synthetic let-7 [90]. Although such methods are at the bench level, they might be translated into therapeutic approaches in the near future.

2.7 Current Industry Status of Let-7 Therapy

Because of its potential as a cancer therapeutic, let-7 has been filed for patent protection (Australia: 2007/333109 A1; United States: 20090163430). While diagnostic companies are developing let-7–based tests for various diseases, including several cancers, pharma giants are working toward development of effective delivery systems. But let-7 restoration methods are not yet satisfactory. Asuragen (www.asuragen.com), the RNA-based therapeutic and diagnostics major with a core focus on miRNA through its subsidiary Mirna Therapeutics (www.mirnatherapeutics.com), is developing miRNA-based diagnostics and therapeutics for non-small-cell lung cancer, metastatic prostate cancer, and acute myeloid leukemia—all currently in preclinical trials. For lung cancer and acute myeloid leukemia, their main focus is let-7. Similarly, Regulus Therapeutics LLC (www.regulusrx.com) is using more than 60 miRNAs, including let-7, to develop miRNA therapeutics to treat several diseases (including cancers). Their main focus is on delivery systems and enhancement of treatment efficacy.

3. SUMMARY

Let-7 exerts its tumour suppressor and antiproliferative activities by repressing several oncogenes and by regulating key regulators of the cell cycle, cell differentiation, and apoptotic pathways. Downregulation of let-7 is a common phenomenon in several cancers, and restoration of normal let-7 expression has been found to prevent cancer growth. As a result, let-7 is a molecular marker in certain cancers and a potential therapeutic in cancer therapy. However, efficient delivery strategies have to be developed if this molecule is to be used as a therapeutic *in vivo*. Use of viral vectors, artificial virus-like particles, and nano materials may be a promising way to realize this goal, but optimization is needed. Also, a better understanding of let-7 biology and its regulatory networks is required to exploit the curative benefits of let-7 and to reduce off-target side effects.

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CLINICAL TRIAL

Aromatase expression and outcomes in the P024 neoadjuvant endocrine therapy trial

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Abstract *Background* Expression of aromatase by malignant breast epithelial cells and/or the surrounding stroma implies local estrogen production that could influence the outcome of endocrine therapy for breast cancer. *Methods* A validated immunohistochemical assay for aromatase was applied to samples from the P024 neoadjuvant endocrine therapy trial that compared tamoxifen and letrozole. The presence of aromatase expression by tumor or stromal cells was correlated with tumor response, treatment induced changes in proliferation index (Ki67), relapse-free survival (RFS) and breast cancer-specific survival (BCSS). *Results* Tumor and stromal aromatase expression were highly correlated ($P = 0.0001$). Tumor cell aromatase, as a semi-continuous score, also correlated with smaller tumor size at presentation ($P = 0.01$) higher baseline ER Allred score ($P = 0.006$) and lower Ki67 levels ($P = 0.003$). There was no significant relationship with clinical response or treatment-induced changes in Ki67. However, in a Cox multivariable model that incorporated a post-treatment tumor profile (pathological T stage, N stage, Ki67 and ER status of the surgical specimen), the presence of tumor aromatase expression at baseline sample remained a favorable independent prognostic biomarker for both RFS ($P = 0.01$, HR 2.3, 95% CI 1.2–4.6 for absent expression) and BCSS ($P = 0.008$, HR 3.76, 95% CI 1.4–10.0). *Conclusions* Autocrine estrogen synthesis may be most characteristic of smaller, more indolent and ER-rich breast cancers with lower baseline growth rates. However, response to endocrine treatment may not depend on whether the estrogenic stimulus has a local versus systemic source.

Keywords Aromatase · Letrozole · Tamoxifen · Neoadjuvant endocrine therapy

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Introduction

After the menopause, estrogen continues to be synthesized through peripheral conversion of androgenic precursors to estrone and estradiol by the CYP P450 enzyme aromatase (CYP19). Since this enzyme is widely expressed, sources

of estrogen for breast cancers can therefore be through the circulation (endocrine), from within the breast stroma (paracrine) or through synthesis by the tumor cell (autocrine) [1, 2]. Intra-tumoral estrogen production has been directly demonstrated by measuring the conversion of radio-labeled androgen to estrogen in breast cancer biopsy material [1–3]. However correlations between biochemical measurements of intra-tumoral estrogen synthesis and clinical outcomes have not been firmly established, largely because in vivo assays of aromatase activity are difficult to execute in a large numbers of patients [4–6]. As alternative approaches, aromatase immunohistochemistry (IHC) and measurements of aromatase mRNA levels have been explored [7, 8]. However, most investigators have not validated their IHC assays against the "gold standard" of a biochemical assay for intra-tumoral aromatase activity. Our group has recently developed and characterized a monoclonal antibody against aromatase. The antibody has been utilized in IHC studies which demonstrated positive correlations between aromatase IHC scores and intra-tumoral aromatase activity [9] and aromatase mRNA expression measurements [10] in breast cancer specimens.

In this investigation we applied the aromatase IHC assay to formalin-fixed paraffin-embedded biopsy samples accrued from patients enrolled onto the P024 neoadjuvant endocrine therapy study, a Phase III double blind randomized trial that compared four months neoadjuvant tamoxifen with an equivalent period of letrozole treatment [11–13]. The design of this study provided a valuable opportunity to evaluate simultaneously the relationship between tumor aromatase expression and response to neoadjuvant endocrine therapy as well as the long-term outcomes for patients receiving adjuvant tamoxifen treatment.

Methods

Study population and tumor bank

The P024 protocol compared four months neoadjuvant letrozole with tamoxifen in post-menopausal women with clinical stage II and III hormone receptor positive (classified as at least 10% nuclear staining for ER and/or PgR) breast cancers that were ineligible for breast conservative surgery [11]. The tumor bank characteristics, ER and Ki67 measurements have been described previously [12, 13]. Tumor grade, tumor histological subtype, pathological staging information and long-term outcomes were collated from case report forms. The long-term outcomes and the development of the preoperative endocrine prognostic index (PEPI) based on pathological stage, and the ER status and Ki67 expression level of the surgical specimen has also been published [14].

Aromatase immunohistochemistry

The aromatase monoclonal antibody #677 was raised against native recombinant human aromatase protein. Details of its characterization and utilization for IHC have been previously reported [9]. Tissue sections were immunostained by a biotin-streptavidin method using a Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). The antigen-antibody complex was visualized with 3.3′-diaminobenzidine solution (DAB) and counterstained with hematoxylin. Evaluation of aromatase IHC was performed by assessing the approximate percentage of cells staining (proportion score) and classifying the level into four groups: 0 = <1%, 1 = 1–25%, 2 = 26–50%, and 3 = >50% immuno-positive cells. The relative intensity of aromatase immune-positive cells was classified as follows: 0 = no immunoreactivity, 1 = weak, 2 = moderate and 3 = intense immunoreactivity. When aromatase immunoreactivity was evaluated as a semi-continuous variable, a total score was applied that was composed of the proportion score + relative immunointensity score (SIP score). For contingency table analysis, aromatase staining was classified as any staining present versus absent staining. Immunohistochemical staining patterns of normal ducts, stromal cells, adipose cells and carcinoma cells were evaluated separately.

Statistics

All P values reported were two sided; $P \leq 0.05$ were considered to be statistically significant. There was no adjustment for multiple testing. The median and interquartile range of the aromatase SIP score was calculated to show the distribution of scores. Kendall's rank correlation coefficients were used to assess relationship between aromatase SIP values and Ki67, ER, and tumor size since aromatase SIP values were ordinal variables and not normal distributed. Fisher's exact and Chi squared tests were used to define associations between aromatase expression status and clinical and cell cycle responses. The nonparametric Mann–Whitney test was applied to compare differences in Ki67 changes between aromatase expression positive and aromatase expression negative tumors. The 95% confidence interval of the geometric Ki67 mean was calculated to show the size of effects in pair-wise comparisons. Relapse-free survival (RFS) was defined as the interval between randomization and the earliest subsequent breast cancer event (all local or systemic recurrences, there were no new breast primaries recorded in this data set). Breast cancer-specific survival (BCSS) was defined as the

interval between randomization and the date of death after breast cancer relapse. For univariable analysis, survival curves were estimated by the Kaplan–Meier product-limit method, with a two-sided log-rank to assess statistically significant differences. We subsequently applied a multivariate Cox proportional hazards regression model to evaluate the independent prognostic relevance of aromatase expression within the context of other independently prognostic variables that were obtained upon analysis of the surgical specimen obtained after completion of neoadjuvant endocrine therapy: i.e. pathological tumor size, lymph node status, ER and Ki67 levels [14]. The REMARK analysis for the multivariable analysis has also been reported [14]. All statistical analyses were performed using SAS 9.1.2 (SAS Institute Inc., Cary NC USA).

Results

Aromatase expression and correlation with baseline pathological and clinical variables

Initially four cellular components were scored for aromatase expression (fibroblast cells, adipose cells, benign breast duct cells and invasive cancer cells). However benign ducts and adipose tissue were very inconsistently present in the slides available. Thus, only stromal cell scores and invasive cancer cell scores could be adequately studied in terms of correlations with clinical parameters. Ultimately aromatase analysis was conducted on 197 cases in which central analysis confirmed ER+ status and 23 cases in which the ER status was known to be ER negative in the central laboratory (with a cut point of Allred score of 0 or 2 as the definition of negative). Of these 197 ER+ cases, 192 (96 on letrozole, 96 on tamoxifen) had sufficient tumor cells on specimens to qualify for the analysis presented in this report. Aromatase expression SIP score in the stomal cell and tumor cell compartments were highly correlated (Kendall's Tau 0.46, $P = 0.0001$, Fig. 1a) Tumor cell aromatase SIP score was positively correlated with ER levels as a continuous score (Kendall's Tau $P = 0.006$, Fig. 1b), however there was no significant correlation with progesterone receptor (PgR) level (data not shown). Finally the aromatase SIP score in the cancer compartment was inversely associated with Ki67 level (Kendall's Tau $P = 0.003$ Fig. 1c). To examine correlations between aromatase expression and dichotomized clinical variables the aromatase staining score was reduced to simple present or absent categories. Of the variables examined, both stroma and tumor epithelial aromatase expression were associated with smaller clinical tumor size at baseline and ER positive status as a dichotomous variable (Allred 0–2 vs. Allred 3–8) but aromatase status (present vs. absent) did not interact with the other factors examined (patient age, tumor grade, lymph node status, PgR and HER2 status) (Table 1).

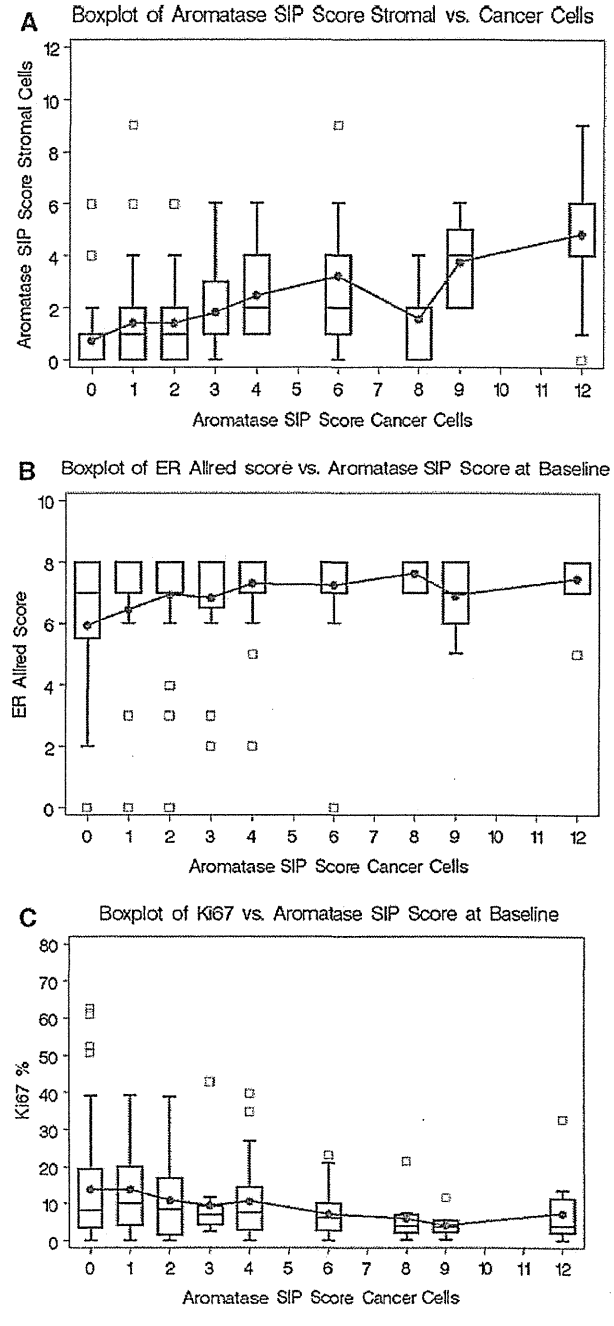

Fig. 1 Correlations between the site of aromatase expression, ER and Ki67 as semi-continuous variables. Box plots comparing the distributions of aromatase SIP scores in stromal cells and cancer cells (**a**), aromatase SIP scores in cancer cells and ER Allred scores (**b**) and Ki67 percentage and aromatase SIP scores in cancer cells (**c**) at baseline. The large boxes stretch from the 25th to 75th percentile, the lines crossing the boxes are medians, the dots are means and the small boxes are outliers.

Table 1 Patients and tumors characteristics by location of aromatase protein expression status at baseline

Characteristics	Aromatase protein expression [*n* (%)][b]			
	Tumor epithelial aromatase		Stromal aromatase	
	Negative	Positive	Negative	Positive
Treatment				
Tamoxifen	24 (53%)	72 (49%)	29 (54)	63 (48)
Letrozole	21 (47%)	75 (51%)	25 (46)	69 (52)
P-value	0.6102		0.4605	
Age (year)	66.8	67.6	67.6	67
P-value[c]	0.6214		0.6969	
Clinical tumor size (cm)	5.7	4.8	5.5	4.7
P-value[c]	0.0144		0.0398	
Pre treatment grade				
I	4 (10%)	16 (13%)	4 (8%)	16 (15%)
II/III	38 (90%)	103 (87%)	47 (92%)	90 (85%)
P-value	0.5971		0.3064	
Pathological tumor size				
≤20 mm	11 (27%)	44 (32%)	11 (27%)	44 (32%)
>20 mm	30 (73%)	95 (68%)	30 (73%)	95 (68%)
P-value	0.5567		0.5567	
Pathological node status				
Negative	16 (41%)	55 (43%)	21 (47%)	50 (43%)
Positive	23 (59%)	72 (57%)	24 (53%)	65 (57%)
P-value	0.8017		0.7160	
HER2 status[a]				
Negative	40 (91%)	140 (95%)	50 (93%)	125 (95%)
Positive	4 (9%)	7 (5%)	4 (7%)	6 (5%)
P-value	0.2806		0.4810	
ER status[c]				
Negative	11 (20%)	12 (8%)	14 (20%)	9 (6%)
Positive	44 (80%)	148 (93%)	55 (80%)	131 (94%)
P-value	0.0098		0.0027	
PgR status[c]				
Negative	16 (36%)	48 (33%)	20 (37%)	43 (33%)
Positive	28 (64%)	98 (67%)	34 (63%)	87 (67%)
P-value	0.6688		0.6072	

[a] HER2 IHC with fluorescence in situ hybridization confirmation and IHC for ER and PgR were performed as previously described [12]

[b] Aromatase protein expression considered positive if any aromatase IHC staining was present

[c] For age and clinical tumor size the student's *t* test was used to compare the aromatase positive and negative groups. For binary variables the X^2 test was applied with Fisher's exact test if a count in any cell was less than 5

Aromatase expression and clinical or radiological response to neoadjuvant letrozole or tamoxifen

A series of contingency tables were examined to identify interactions between aromatase expression status and response (Table 2). In the P024 study, response was recorded according to clinical measurements, ultrasound and mammography. There was no evidence of interactions with any of the response definitions, whether the stroma or the tumor cell aromatase status was examined as the interacting factor or whether letrozole or tamoxifen treated cases were considered separately. Consistent with a lack of an influence on endocrine therapy responsiveness, there was no interaction with treatment-induced changes in Ki67 or absolute post-treatment Ki67 levels in either tamoxifen or letrozole-treated tumor samples (Table 3).

Aromatase expression and relapse-free survival and breast cancer-specific survival

Although there was no association with neoadjuvant response or Ki67 changes, the baseline interactions between aromatase expression, higher ER levels and lower Ki67 levels suggested the possibility that aromatase expression could be a favorable prognostic biomarker for patients undergoing adjuvant endocrine therapy. We therefore examined the impact of aromatase expression on RFS and BCSS (Fig. 2). Tumor aromatase expression was

Table 2 Analysis of clinical, mammogram and ultrasound response data according to aromatase protein expression status in tamoxifen or letrozole treated patients

Responses	Aromatase protein expression [*n* (%)]			
	Tumor epithelial aromatase		Stromal aromatase	
	Negative	Positive	Negative	Positive
Clinical response[a]				
Letrozole only				
No	6 (29%)	24 (32%)	11 (44%)	18(26)
Yes	15 (71%)	51 (68%)	14 (56%)	51(74)
P-value	0.7657		0.0984	
Tamoxifen only				
No	12 (50%)	37 (51%)	13 (45%)	34 (54%)
Yes	12 (50%)	35 (49%)	16 (55%)	29 (46%)
P-value	0.9067		0.4177	
Fused				
No	18 (40%)	61 (41%)	24 (44%)	52 (39%)
Yes	27 (60%)	86 (59%)	30 (56%)	80 (61%)
P-value	0.8587		0.5259	
Mammo response[a]				
Letrozole only				
No	13 (62%)	47 (63%)	17 (68%)	42 (61%)
Yes	8 (38%)	28 (37%)	8 (32%)	27 (39%)
P-value	0.9494		0.5297	
Tamoxifen only				
No	19 (79%)	54 (75%)	23 (79%)	48 (76%)
Yes	5 (21%)	18 (25%)	6 (21%)	15 (24%)
P-value	0.6803		0.7418	
Fused				
No	32 (71%)	101 (69%)	40 (74%)	90 (68%)
Yes	13 (29%)	46 (31%)	14 (26%)	42 (32%)
P-value	0.7604		0.4278	
Ultrasound response[a]				
Letrozole only				
No	12 (67%)	39 (56%)	16 (73%)	34 (52%)
Yes	6 (33%)	31 (44%)	6 (27%)	31 (48%)
P-value	0.4038		0.0959	
Tamoxifen only				
No	13 (68%)	44 (64%)	15 (60%)	41 (69%)
Yes	6 (32%)	25 (36%)	10 (60%)	18 (31%)
P-value	0.7085		0.4016	
Fused				
No	25 (68%)	83 (60%)	31 (66%)	75 (60%)
Yes	12 (32%)	56 (40%)	16 (34%)	49 (40%)
P-value	0.3845		0.5116	

Response rate refers to the percentage of patients with a complete or partial response

[a] Response definitions by WHO criteria have been previously reported [11]. The X^2 test was applied with Fisher's exact test if a count in any cell was less than 5

confirmed to have a modest association with a more favorable disease course, with fewer relapse events over time and a significant univariable log rank test $P = 0.04$ (Fig. 2a) and more prolonged breast cancer survival (Fig. 2b $P = 0.01$). To determine the independence of baseline aromatase expression as a prognostic marker in our established multivariable models based on the post-treatment surgical sample, the baseline aromatase status was analyzed in the context of the preoperative endocrine relapse index (PEPI) (Table 4) [14]. In the PEPI model pathologic tumor size (T1/2 vs. T3/4), pathological nodal status (negative vs. positive), Ki67 per natural log interval and ER status post therapy (Allred 0–2 vs. Allred 3–8) have been found to be independent factors for RFS and BCSS [14]. When tumor aromatase status was entered into a multivariable Cox model containing these four factors, the presence of aromatase expression in the baseline specimen behaved as an independent favorable prognostic biomarker for both RFS ($P = 0.01$, HR 2.3 95% 1.2–4.6 for absent expression) (Table 4A) and BCSS ($P = 0.008$, HR 3.76 95% CI 1.4–10.0 for absent expression) (Table 4B).

Discussion

The clinical significance of intra-tumoral estrogen production has been debated ever since the phenomenon was first documented by Miller et al., in 1974 [15] through the detection of the conversion of radio-labeled androgen to estradiol within breast cancers in vitro. This potential exists in about 60–70% of breast cancers [1–3]. Subsequently infusion studies with radioactive androgens showed that estrogen biosynthesis occurred in situ within the breast [16, 17] and the presence of mRNA for aromatase, the key enzyme in estrogen production, was also demonstrated in breast cancers and adipose tissue [8]. Because aromatase is the last step in the biosynthetic pathway for estradiol, the enzyme has become a critical target for pharmacological inhibitors that achieve endocrine deprivation for post-menopausal patients requiring endocrine treatment for ER+ breast cancer. Consequently third-generation aromatase inhibitors have evolved as the new standard of care for breast cancer treatment for all stages of the disease. It was therefore logical to address the possibility that the presence of aromatase within breast cancers is associated with a particular requirement for estrogen for growth and therefore whether aromatase expressing tumors are more likely to respond to endocrine therapy in general, and to aromatase inhibitors in particular.

The number of studies examining these relationships is few, have utilized small numbers of tumors and come to limited (often conflicting) conclusions [4, 18, 19]. The

Table 3 Paired Ki67 data before and after letrozole/tamoxifen therapy according to aromatase protein expression status in breast cancer cells/ stromal cells

Ki67 [Geometric mean (95% CI)][a]	Aromatase protein expression			
	Cancer cells		Stromal cells	
	Negative	Positive	Negative	Positive
Letrozole only				
Pre	5.54 (2.54–12.08)	3.56 (2.47–5.14)	3.64 (1.70–7.82)	3.92 (2.69–5.72)
Post	0.70 (0.33–1.49)	0.49 (0.31–0.75)	0.88 (0.38–2.04)	0.44 (0.29–0.68)
P-value[b]	0.0037	0.0001	0.0083	0.0001
Tamoxifen only				
Pre	5.97 (3.17–11.24)	5.63 (4.18–7.58)	7.75 (4.46–13.47)	4.67 (3.39–6.44)
Post	1.72 (0.75–3.97)	1.36 (0.88–2.09)	1.61 (0.76–3.39)	1.23 (0.77–1.95)
P-value[b]	0.0117	0.0001	0.0007	0.0001

[a] 95% CI: confidence Interval

[b] Wilcoxon signed rank test was used to compare paired Ki67 data within each group defined by aromatase expression status

major reason for this is that the low abundance of aromatase in the breast requires sophisticated, time-consuming and labor intensive methodology and relatively large amounts of fresh tissue. This has precluded routine use in large clinical trials. However the availability of an antibody which can specifically detect aromatase in fixed archival breast cancers has changed this. We can now report results on the presence (and semi-quantitative levels) of aromatase in tumor material obtained from a randomized trial of neoadjuvant endocrine therapy (P024).

Before discussing the findings it is worth considering methodological issues and potential limitations of the study. Firstly, IHC estimation of protein provides no information on activity and protein may be present that is deactivated or inhibited [20]. This certainly will be the case in patients treated with aromatase inhibitors. For this reason we have excluded outcome correlations with aromatase status in "on treatment" samples because we have not validated relationships between aromatase activity and expression in the presence of an endocrine agent. Secondly, because aromatase is present in different compartments of the breast (and at different levels) complete assessment requires quantification of multiple tissue types and an estimate of the relative amounts of each compartment. In this study, to simplify these confounders, we have not used assessments in adipose and benign tissue of tissue sections which were generally low in staining score and proportion. Aromatase scores were highest in the malignant and stromal compartments of breast cancers. However, these were highly related in breast cancers suggesting a field effect of trophic factors regulating aromatase. We have therefore restricted our correlations to the status of the cancer cells which were reliably present in all the samples eligible for analysis and therefore more consistent to score.

In terms of demographics we have combined the two arms of the P024 trial for long term outcome analysis, not discriminating between patients subsequently treated with tamoxifen or letrozole since all patients received tamoxifen as adjuvant therapy. This has formed a database which represents the largest published series of breast cancers assessed by aromatase IHC. The results show that tumor aromatase was positively and significantly related to smaller tumor size and ER level/status. These findings would be consistent with data published by members of the group on aromatase activity [4] but not with others using IHC with a different antibody [7]. A significant inverse correlation was observed with the proliferation marker, Ki67. To the best of our knowledge there have been no other published studies relating tumor aromatase to proliferation.

In terms of endocrine responsiveness, no significant association was detected between tumor aromatase and clinical response to either letrozole or tamoxifen. While positive correlations have been reported between the presence of in vitro and in vivo aromatase activity and response to aromatase inhibitors, these relationships were not strong and were observed in advanced disease, not in the neoadjuvant setting [18, 19] Other studies on response to tamoxifen have been negative. Thus, the response to endocrine therapy does not appear to be strongly modulated by whether the source of estrogen is autocrine or endocrine.

Despite a failure to observe significant relationships between aromatase expression and clinical or biomarker response to treatment in the neoadjuvant phase of the study, significant associations were found between the presence of tumor aromatase expression and long-term outcome following neoadjuvant treatment. Thus, tumors with positive aromatase scores had significantly greater

Table 4 Univariate and multivariate analysis of pathological tumor size, node status, post-treatment Ki67, post-treatment ER and pre treatment aromatase status[a]

A

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Relapse-free survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Tumor size[a] (T1/2 vs. T3/4)	138/33	47/171	2.7 (1.4–5.0)	0.002	2.82 (1.36–5.85)	0.006
Node status (Yes vs. No)	90/69	44/159	3.9 (1.8–8.4)	0.0005	3.44 (1.58–7.48)	0.002
Ki67 level, per 2.7 fold increase[b]		48/174	1.4 (1.2–1.6)	0.0002	1.1 (1.02–1.09)	0.003
ER Allred[c] (0.2 vs. 3–8)	16/157	48/173	2.4 (1.0–5.3)	0.04	2.74 (1.1–6.67)	0.03
Aromatase status[d] (not present versus present)	37/132	48/169	1.88 (1.01–3.47)	0.04	2.34 (1.2–4.58)	0.01

B

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Breast cancer-specific survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	*P*	HR (95% CI)	*P*
Tumor size (T1/2 vs. T3/4)	138/33	24/171	3.5 (1.5–8.3)	0.004	3.42 (1.21–9.66)	0.02
Node status (Yes vs. No)	90/69	22/159	4.6 (1.4–15.8)	0.01	4.05 (1.14–14.38)	0.03
Ki67 level, per 2.7 fold increase		25/174	1.4 (1.1–1.7)	0.009	1.05 (1.0–1.11)	0.06
ER Allred (0.2 vs. 3–8)	16/157	25/173	4.3 (1.6–11.7)	0.005	7.98 (2.58–24.7)	0.0003
Aromatase status (not present versus present)	37/132	24/169	2.82 (1.2–6.63)	0.02	3.76 (1.42–9.98)	0.008

[a] The four elements of the preoperative endocrine relapse index (PEPI) score (pathological T and N stage, surgical specimen ER and Ki67 status has been previously described [14]

[b] Surgical specimen Ki67 was analyzed as the natural log interval, or per 2.7 fold increase according to the original scale of percentage values [14]

[c] The ER analysis refers to the post-treatment values, before treatment all the tumors in this data set were ER positive. In the PEPI model, an Allred cut off of 0 or 2 is used to define ER negative

[d] The aromatase expression status was defined as present or positive if any positive staining presented in invasive breast cancer cells. Table 4A and B shows the RFS data and BCSS data, respectively

RFS and BCSS. It is not possible to ascertain whether this is directly caused by increased sensitivity to endocrine therapy in the adjuvant setting. However, the lack of association of response in the neoadjuvant situation would not be compatible with this. Furthermore the positive correlations with small clinical size and ER status levels (favorable prognostic biomarkers) and the inverse correlation with Ki67 (a poor prognosis biomarker) suggest that aromatase positive tumors may be inherently less aggressive. This is supported by data from multivariable analyses in which tumor aromatase scores predicted for long-term outcome independently of other factors that have been shown to be predictive for outcome in the post neoadjuvant endocrine therapy setting. This finding also implies that the most accurate models for the prediction of outcomes for patients with ER+ disease may combine baseline prognostic biomarker analysis, in combination with the "on-treatment" predictive biomarker analysis derived from an analysis of the tumor after several months of endocrine treatment [14].

It is therefore suggested that routine IHC measurements of aromatase in breast cancer will not generally aid

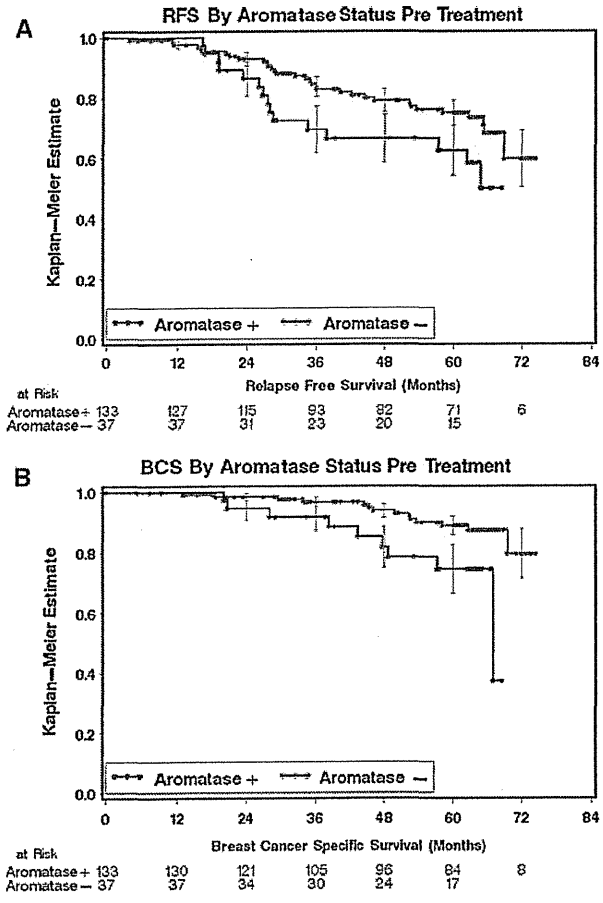

Fig. 2 Kaplan–Meier curves for relapse-free and breast cancer-specific survival by aromatase protein expression status in cancer cells. (**a**) Relapse-free survival (RFS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; (**b**) Breast cancer-specific survival (BCSS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; Censorship observations are marked with open circles; log rank tests were used to estimate the difference between Kaplan–Meier curves for RFS and BCSS. 95% confidence intervals are provided on each curve

prediction of neoadjuvant response to endocrine therapy, but may help identify ER positive tumors with favorable long-term outcomes.

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Runx2 in human breast carcinoma: its potential roles in cancer progression

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Runx2 has been proposed as one of the pivotal factors in the process of osteogenesis and metastasis in human malignancies including breast cancer, but its details have not been evaluated. Therefore, in this study, we evaluated its expression in human breast cancer using immunohistochemistry. One hundred and thirty-seven formalin-fixed and paraffin-embedded breast cancer specimens were used in this analysis of immunohistochemical study. Immunoreactivity was evaluated using the labeling index (LI). Runx2 immunoreactivity was detected in both carcinoma and stromal cells, as well as non-pathological ductal cells. The nuclear LI of Runx2 in carcinoma cells was associated with the clinical stage, histological grade and HER2 status of the patients examined. In addition, among the patients not associated with distant metastasis, those with high Runx2 LI demonstrated a significantly worse clinical outcome than those with a low LI. This was more pronounced in the group of estrogen receptor (ER)-negative cases. In addition, both univariate and multivariate analyses demonstrated that the Runx2 LI in breast carcinoma cells turned out an independent prognostic factor. Results of our present study demonstrated that Runx2 plays very important roles in the progression of breast cancer, especially in those of ER-negative cases. (*Cancer Sci* 2010; 101: 2670–2675)

Breast cancer is one of the most common malignancies in women worldwide. Recently, the potential association of breast cancer with its bone metastasis has been evaluated from different perspectives and, in particular, the process of osteolysis itself in its metastatic sites has been proposed to facilitate breast cancer progression.[1] It is also well known that breast carcinoma cells themselves secrete parathyroid-hormone-related peptide (PTHrP), which stimulates osteoblasts in the microenvironment of bone metastatis.[2] Osteoblasts at the sites of metastasis are also considered to secrete a receptor activator of NFκB ligand (RANKL) to facilitate the process of transition from mesenchymal cells into functional osteoclasts, which subsequently resorb bone.[3–7] In normal human adult skeleton, bone is constantly renewed or maintained through the coordinated activities of both osteoclasts and osteoblasts.[8] Metastatic breast carcinoma cells are seeded into the bone microenvironment, which results in the maturation of osteoclasts.[9] These subsequently formed osteolytic foci are associated with bone resorption, which eventually leads to the release of growth factors including transforming growth factor- β (TGF-β) and several insulin-like growth factors (IGF) from the collapsed bone matrix.[10,11] These factors are considered to subsequently mediate tumor cell proliferation at the sites of bone metastasis.

The Runt-related transcription factors 1-3 (Runxs1-3) have been shown to be required for the process of organogenesis, and mutations in these genes have been reported to be linked to several types of cancer development.[12] For instance, Runx1 and Runx3 mutations were reported to promote leukemia[13,14] and gastric cancers,[15] respectively. Among these Runx families, Runx2 plays a pivotal role in the process of bone formation or osteogenesis[16–19] and deregulation of Runx2 itself is associated with the development of osteosarcoma.[20,21] Runx2 was also reported to be highly expressed in both prostate and breast carcinoma cell lines, which can metastasize to bone in various transplanted models.[22–24] Loss of function of the Runx2 gene in the mouse was also reported to result in increased cell proliferation of *ex vivo* skeletal lineage cells.[25,26] Expression of Runx2 was also reported in mammary epithelial cells of the mouse.[27,28] In addition, aberrant Runx2 expression has been reported in breast and prostate primary tumors.[22,25] Runx2 was reported to be involved in the regulation of a mammary-gland-specific β-casein gene and osteopontin.[22,28,29] In regard to its potential roles at the sites of breast carcinoma metastasis to the bone, Runx2 was reported to regulate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone metastasis and the cell cycle of carcinoma cells themselves.[30] Runx2 was also shown to modulate several factors, which can contribute to facilitating the process of metastasis including vascular endothelial growth factor (VEGF),[31] several matrix metalloproteinases (MMP)[24,32] and bone sialoprotein.[33] However, to the best of our knowledge, its roles in the early stage of breast cancer patients have not been studied at all. In addition, the correlation of Runx2 nuclear immunoreactivity in breast carcinoma cells and histopathological features of breast cancer were reported,[34] but the correlation between Runx2 expression and prognosis has still remained unknown.

Among the anti-estrogen therapies available in cases with estrogen receptor (ER)-positive breast carcinoma, the administration of selective estrogen receptor modulator (SERM) or aromatase inhibitor (AI) has been considered the gold standard.[35,36] However, it is well known that ovarian suppression and administration of AI frequently results in osteoporosis.[36–39] The suppression of estrogenic actions in osteoclasts results in inhibition of their apoptosis and enhancement of their maturation.[36–39] Therefore, both suppression of estrogenic actions and elevated Runx2 expression in metastatic breast carcinoma cells might enhance the development of osteoporosis in these patients.

Therefore, in the present study, we evaluated the status of nuclear Runx2 immunoreactivity in breast carcinoma cells and correlated the findings with stage, histological grade, ER status and HER2 expression of the patients in order to study its clinicopathological significance.

Materials and Methods

Breast carcinoma cases. One hundred and thirty-seven cases of invasive ductal carcinoma of the breast were retrieved from

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the surgical pathology files of the Department of Pathology, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from Japanese female patients who underwent a mastectomy during 1988–1999 in the Department of Surgery, Tohoku University Hospital, Sendai city, Japan. The mean age was 52.9 years (range, 22–81 years). None of the patients examined in the present study received chemotherapy, administration of trastuzumab or irradiation prior to surgery. The mean follow-up time was 81 months (range, 1–151 months). All of the specimens had been fixed in 10% formalin at room temperature and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (approval number 2005-178).

Antibodies. Mouse monoclonal antibody for human Runx2 was purchased from Abnova Corporation (Taipei, Taiwan). The characterization of this antibody has been previously reported using both immunoblotting and immunohistochemistry.[40] Monoclonal antibodies for estrogen receptor α (ER1D5), progesterone receptor (PR; MAB429) and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA) and DAKO (Carpinteria, CA, USA), respectively. We used a standardized immunohistochemistry kit (HercepTest for Immunoenzymatic Staining; DAKO).

Immunohistochemistry. A Histofine kit (Nichirei, Tokyo, Japan), which uses the streptavidin–biotin amplification method, was used in this study. Antigen retrieval was carried out by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for Runx2, ER, PR, HER2 and Ki-67 immunostaining. The dilutions of the primary antibodies used in this study are as follows: Runx2, 1/1000; ER, 1/50; PR, 1/30; HER2, 1/200; and Ki-67, 1/50. The antigen–antibody complex was visualized with 3.3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6] and 0.006% H_2O_2), and counterstained with haematoxylin. As a negative control, normal mouse, rabbit or goat IgG was used instead of the primary antibodies, and no immunoreactivity was detected in these sections (data not shown).

Statistical analysis. Immunoreactivity of Runx2 was detected in the nuclei and the labeling index (LI) was subsequently obtained. Briefly, Runx2 immunoreactivity was evaluated in the nuclei of more than 1000 carcinoma cells for each case, and the percentage of immunoreactivity (i.e. the LI) was subsequently determined. In breast carcinoma cells, Runx2, ER, PR and Ki-67 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated as a LI in the same was as described above. Cases with ER, PR or Ki-67 of more than 10% were considered positive in this study, according to a report on ER.[41] HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and moderately or strongly circumscribed membrane staining of HER2 in more than 10% of carcinoma cells was considered positive.[42] An association between Runx2 immunoreactivity and clinicopathological factors of breast carcinoma patients was statistically evaluated using a correlation coefficient (r) and regression equation, Student's t-test, or a one-way ANOVA and Bonferroni test. Overall and disease-free survival curves were generated according to the Kaplan–Meier method, and the statistical significance was calculated using the log-rank test evaluating 64 cases with the Runx2 LI ≧37% (median value) as Runx2 positive and 56 cases with the Runx2 LI <37% as Runx2 negative in a group of non-distal metastatic breast cancer. Both univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in SAS software (SAS Institute Inc., Cary, NC, USA).

Results

Immunohistochemistry of Runx2. Runx2 immunoreactivity was detected in the nuclei of breast carcinoma cells (Fig. 1a,b), and the mean value of the Runx2 LI in 137 breast carcinoma tissues examined was 43.1% (range, 0–99%) in total. Runx2 immunoreactivity was also detected in non-pathological myoepithelial and ductal cells (Fig. 1c). Ninety-five cases are ER positive (LI ≧ 10%) and 42 cases are ER negative (LI < 10%). Seventy-eight cases are PR positive (LI ≧ 10%) and 59 are PR negative (LI < 10%). The mean value ± SD of the Ki-67 LI in 137 breast carcinoma tissues examined was 21.3 ± 17.9% (range, 0–82%) in total.

Correlation of the Nucleus Runx2 LI with the clinicopathological factors of the cases examined. Table 1 summarizes the correlation of the Runx2 LI in breast carcinoma cells with the clinicopathological parameters in the breast carcinoma cases. Significant association between the Runx2 LI and stage ($P = 0.0004$), histological grade ($P = 0.046$) and HER2 status ($P = 0.002$) of the patients was demonstrated, but there were no significant correlation between the Runx2 LI and age ($P = 0.78$), menopausal status ($P = 0.69$) and lymph node status ($P = 0.66$) of the cases examined. The Runx2 LI tended to be correlated with ER ($P = 0.13$) and PR status ($P = 0.06$), but the correlation did not reach statistical significance. The Runx2 LI also correlated with both clinical stages and histological grades of the patients.

Correlation between the Runx2 LI and clinical outcome in 120 non-distal metastatic breast carcinoma patients (stage I–III). A significant association was detected between the Runx2 LI and recurrence ($P = 0.01$) or overall survival ($P = 0.003$) of the

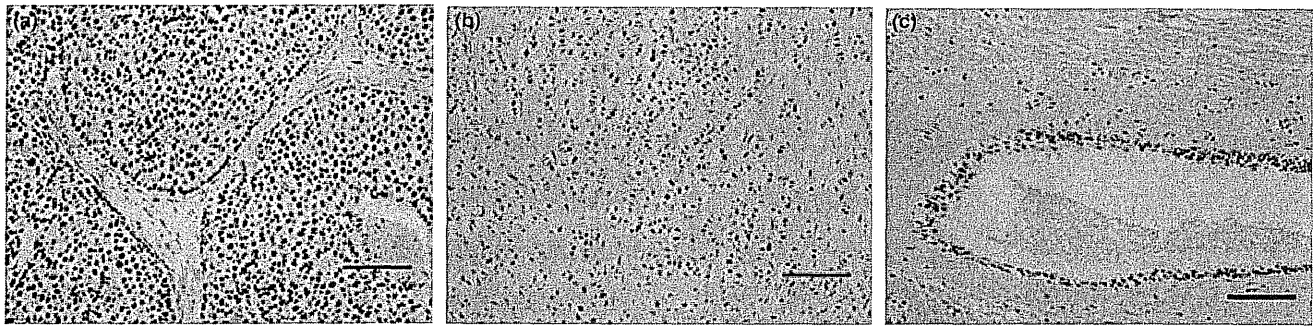

Fig. 1. Immunohistochemistry for Runx2. Runx2 immunoreactivity was detected in the nuclei of both carcinoma and stroma cells. (a) Case I with Stage III showed a labeling index (LI) = 97.0. (b) Case II with Stage II showed a LI = 37.0. (c) Normal mammary epithelial cells also include nucleus Runx2 positive. Bar, 100 μm.

Table 1. Summary of an association between the nuclear Runx2 LI of carcinoma cells and the clinicopathological parameters in 137 breast cancers

	n	Runx2 LI	*P* value
Age (22–81 years)	137		0.78 ($r = -0.024$)
Menopausal status			
Premenopausal	50	43.1 ± 4.1	0.69
Postmenopausal	87	45.1 ± 2.9	
Stage			
I	34	35.3 ± 4.6	**0.0004**
II	67	42.0 ± 3.4	
III	19	47.2 ± 5.1	
IV	17	68.8 ± 5.7	
Tumor size			
<2.0 cm	18	49.8 ± 6.3	0.68
≥2.0 cm	94	46.5 ± 3.0	
Lymph node status			
Positive	17	45.5 ± 3.6	0.66
Negative	120	43.4 ± 3.2	
Distant metastasis			
Positive	60	68.8 ± 5.7	**<0.0001**
Negative	77	40.9 ± 2.5	
Histological grade			
1 (well)	27	34.7 ± 5.6	**0.046**
2 (moderate)	63	43.4 ± 3.6	
3 (poor)	47	51.2 ± 3.6	
ER status			
Positive	95	41.6 ± 2.9	0.13
Negative	42	49.3 ± 3.8	
PR status			
Positive	78	40.3 ± 3.2	0.06
Negative	59	49.2 ± 3.4	
HER2 status			
Positive	30	58.3 ± 5.4	**0.002**
Negative	107	40.5 ± 2.5	
Ki-67 LI (0–82%)			**0.004** ($r = 0.25$)

Data considered significant ($P < 0.05$) in the univariate analysis are shown in bold. Significant values were examined in the multivariate analysis in the present study. ER, estrogen receptor; LI, labeling index; PR, progesterone receptor.

patients in 120 breast carcinoma patients at stage I, II and III (Fig. 2a,b). In particular, among these 120 patients, ER negative cases (23 Runx2 positive and 20 Runx2 negative), the Runx2 LI was markedly associated with an increased risk of clinical recurrence ($P = 0.03$) (Fig. 2c) and overall survival of the patients (P not calculated because no patients died in the ER negative/Runx2 positive group) (Fig. 2d). However, among the ER-positive cases (41 Runx2 positive and 36 Runx2 negative), no significant association was detected between the Runx2 LI and an increased risk of recurrence ($P = 0.55$) (Fig. 2e) and overall survival ($P = 0.39$) (Fig. 2f).

In a Univariate analysis, the Runx2 LI evaluated as a continuous variable also turned out to be a significant prognostic factor ($P = 0.049$ in disease-free survival and $P = 0.004$ in overall survival), and an independent prognostic factor when it was included in a multivariate analysis instead of the dichotomized variable ($P = 0.01$ and $P = 0.04$, respectively) (Table 2). Because no cases had received administration of trastuzumab agent in this study, HER2 positive was a remarkably poor prognostic factor.[43]

Discussion

In the present study, the Runx2 LI in breast carcinoma cells was significantly associated with stage ($P = 0.0004$) and histological grade ($P = 0.046$) of the patients examined. These findings indicate the possible roles of Runx2 in the biological behavior of breast carcinoma patients, including those without metastasis. We also demonstrated that the prognosis or clinical outcome of cases associated with a high Runx2 LI is generally poor. In particular, in 120 cases not associated with distant metastasis, a significant positive association was detected between the Runx2 LI and both the risk of recurrence and overall survival of patients. Furthermore, this association was more pronounced in the group of 43 ER-negative cases (36%). This group of ER-negative carcinoma included HER2 positive and basal-like subgroups of breast carcinoma. These findings also suggested that Runx2 could serve as a marker of aggressive biological behavior and its inhibition might open a new strategy of therapy for these cases.

Breast cancer development consists of many sequential steps, including primary tumor growth, neovascularization around the tumor, invasion, extravasation and subsequently formation of bone metastasis.[24] Many *in vitro* studies demonstrated that Runx2 might participate in these steps in multiple fashions. Regulation or modification of VEGF secretion by Runx2 was reported in neovascularization.[44] Regulation of several MMP secretion by Runx2 was also postulated to be linked with subsequent invasion of carcinoma cells.[45,46] Runx2 was proposed to subsequently mediate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone and might be involved in the formation of a vicious cycle.[2] All of the above might be related to an adverse clinical outcome for patients but little has actually been demonstrated in clinical cases of human breast carcinoma. In the present study, we demonstrated a significant correlation of the status of Runx2 expression in carcinoma cells with the histological grade and stage of patients. In addition, the possibility of potential involvement of Runx2 in earlier phases of breast cancer development was also raised in the present study.

In the present study, the groups of patients with elevated Runx2 expression were significantly associated with a poor prognosis in the ER-negative group of patients, while this association was not detected in the ER-positive carcinoma patients. In our cohort of ER-positive breast cancer patients, 34 of 95 cases (35.8%) received administration of tamoxifen, while in that of the ER-negative breast cancer patients, six of 42 cases (14.3%) did. Estrogenic depletion affects not only breast carcinoma cells but also the entire body of patients. It is true that SERM can prevent the systemic effects of estrogen depletion to some extent, but ovarian suppression and administration of AI result in marked side-effects, especially in the skeletal system causing development of clinically significant osteoporosis in some patients.[36–39]

Results of various *in vitro* studies have shown that suppression of estrogenic activation caused maturation of osteoclasts in a direct manner.[47] Estrogen, acting via ER, causes upregulation of Fas ligand (FasL) in osteoclast progenitors (pOC) and/or OC themselves.[47] The increased FasL levels cause apoptosis because these OC lineage cells also express Fas. Fas ligand expression is also reported to be diminished or even suppressed without estrogens in these systems, and the life span of OC was actually reported to be elongated in the absence of estrogen in a culture medium. These OC might stimulate osteoblasts to form bone via poorly defined factors but the resorptive effects of these OC are usually considered dominant.[47]

Results of several reported studies suggest that anti-estrogen therapy did amplify the maturation of OC, resulting in the development of osteoporosis. Osteoporosis is a disease of increased bone turnover, in which the bone-resorbing activity of OC outpaces the bone-forming activity of osteoblasts, resulting in the loss of predominantly trabecular bone.[47] Both of these cell types are reported to respond to estrogen, but results of previous studies suggest that the response of human bone to estrogen

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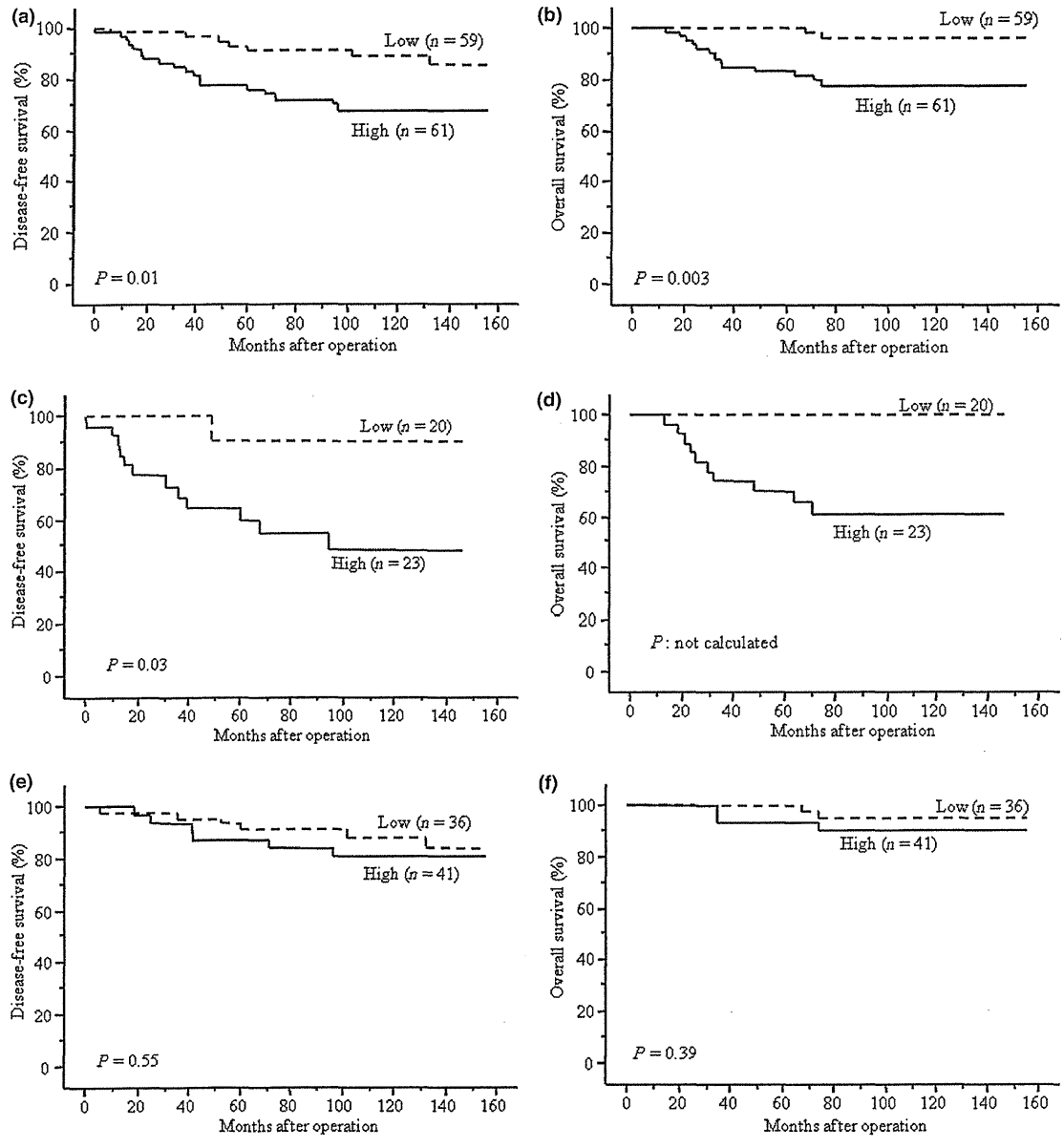

Fig. 2. Disease-free (a) and overall (b) survival of 120 cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity (Kaplan–Meier method), respectively. Disease-free (c) and overall survival (d) of 77 estrogen receptor (ER)-positive breast carcinoma cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity, respectively. Disease-free (e) and overall survival (f) of 43 ER negative breast carcinoma cases associated with non-distal metastatic breast carcinoma according to nucleus Runx2 immunoreactivity, respectively.

withdrawal is at least in part mediated by a network of inflammatory and osteoclastogenic cytokines, including tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), released by stromal/osteoblast lineage cells and T cells.[48,49]

Decreased levels of estrogens usually result in increased production of the cytokine IL-7 by osteoblasts, which stimulates proliferation of T cells and their secretion of both TNFα and receptor activator of NF-κB Ligand (RANKL).[49] Tumor necrosis factor α stimulates osteoblasts to increase their synthesis of RANKL, which results in the differentiation and activation of OC. Tumor necrosis factor α also acts directly on pOC, synergizing with RANKL for OC differentiation.[49] Additional pro-osteoclastogenic cytokines and growth factors are also expressed in T cells and other peripheral blood mononuclear cells.[49,50]

It is practically very difficult to confirm that bone-metastatic breast cancer cells are also associated with elevated Runx2 expression in the cases with high Runx2 expression in primary breast carcinoma cells because clinically the availability of specimens for both primary and bone metastasis are in general rare. However, metastatic breast carcinoma cells associated with high Runx 2 expression may facilitate the process of osteoporosis in the bone microenvironment by RANKL secretion of osteoblasts via PTHrP secretion,[30] but further investigations are required for clarification.

HER2 status of patients turned out to be a strong independent factor because administration of Herceptin had not been used in any of the patients examined in this retrospective study. Runx2 LI in carcinoma cells was also markedly correlated with the