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Intratumoural mRNA expression of genes from the oestradiol metabolic pathway and clinical and histopathological parameters of breast cancer

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Abstract

Introduction The expression of the oestrogen receptor (ER) is one of the more important clinical parameters of breast cancer. However, the relationship between the ER and its ligand, oestradiol, and the enzymes that synthesise it are not well understood. The expression of mRNA transcripts of members of the oestradiol metabolic and signalling pathways including the ER was studied in detail.

Method mRNA transcripts for aromatase (*CYP19*), 17-β-hydroxysteroid dehydrogenase I, 17-β-hydroxysteroid dehydrogenase II, *ERα*, *ERβ*, steroid sulfatase (*STS*), oestradiol sulfotransferase (*EST*), cyclin D_1 (*CYCLD1*) and *ERBB2* were fluorometrically quantified by competitive RT-PCR using an internal standard in 155 breast carcinomas. In addition, the transcripts of *CYP19* were analysed for alternative splicing/usage of exon 1 and an alternative poly A tail.

Results A great variability of expression was observed, ranging from 0 to 2376 amol/mg RNA. The highest levels were observed for *STS* and *EST*, and the lowest levels (close to zero) were observed for the 17-β-hydroxysteroid dehydrogenase isoenzymes. The levels of mRNA expression were analysed with respect to clinical and histopathological parameters as well as for disease-free survival. High correlation of the mRNA expression of *STS*, *EST* and 17-β-hydroxysteroid dehydrogenase in the tumours suggested a common regulation, possibly by their common metabolite (oestradiol). Hierarchical clustering analysis in the 155 patients resulted in two main clusters, representing the ERα-negative and ERα-positive breast cancer cases. The mRNA expression of the oestradiol metabolising enzymes did not follow the expression of the ERα in all cases, leading to the formation of several subclasses of tumours. Patients with no expression of *CYP19* and patients with high levels of expression of *STS* had significantly shorter disease-free survival time ($P > 0.0005$ and $P < 0.03$, respectively). Expression of *ERβ* mRNA was a better prognostic factor than that of *ERα* in this material.

Conclusion Our results indicate the importance of CYP19 and the enzymes regulating the oestrone sulfate metabolism as factors of disease-free survival in breast cancer, in addition to the well-known factors *ER* and *ERBB2*.

Keywords: breast cancer, clustering analysis, disease-free survival, oestradiol metabolism, signalling

Introduction

Large-scale expression analysis of mRNA has proven a powerful tool for morphological classification of tumours of the breast [1] as well as for prediction of disease outcome [2,3]. Expression studies of tens of thousands of transcripts give exciting possibilities to draw molecular portraits of tumours [1] within a given range of expression levels, but are less informative for the absolute amounts of

bp = base pairs; E1S = oestrone sulfate; ER = oestrogen receptor; EST = oestradiol sulfotransferase; HER = human epidermal receptor; HSD = 17-β-hydroxysteroid dehydrogenase; PCR = polymerase chain reaction; RT = reverse transcriptase; STS = steroid sulfatase; TKAT = tumor category.

single transcripts. At the same time, intratumoural mRNA expression of enzymes involved in the oestradiol metabolism has been studied in separate reports on different materials for single genes such as aromatase (*CYP19*) [4], steroid sulfatase (*STS*) [5] and 17-β-hydroxysteroid dehydrogenase I (*HSD1*) [6]. It is difficult, however, to see how these genes are expressed in concert. In the present article, we attempt to quantify the mRNA expression of a number of genes in the oestradiol pathway (Fig. 1) simultaneously by fluorimetric quantitation of RT-PCR using gene-specific internal RNA standards.

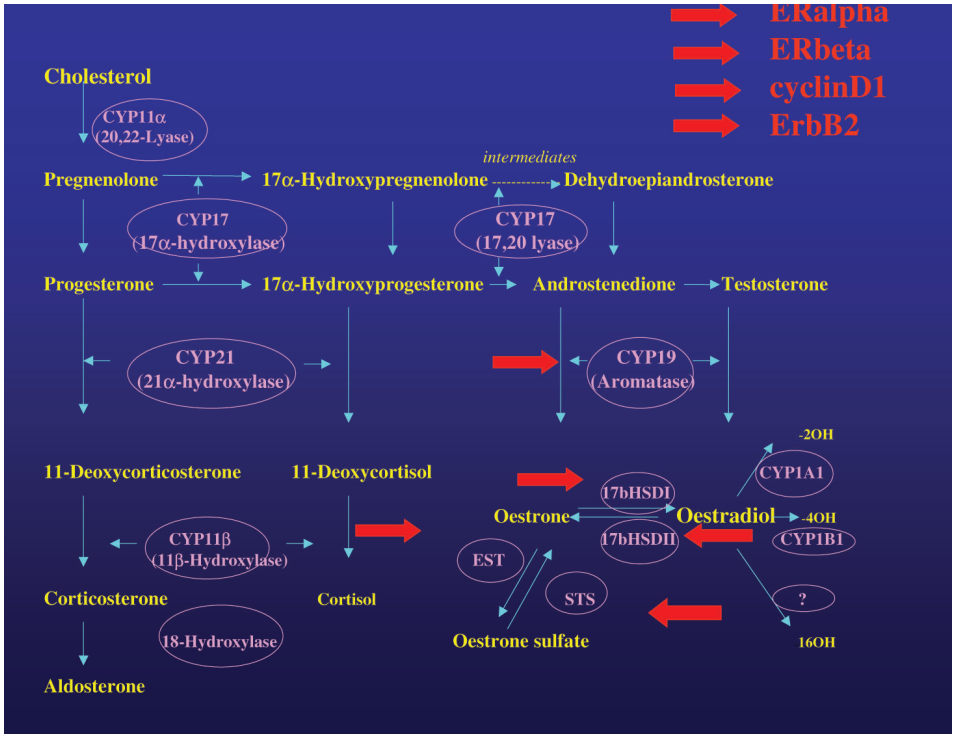
Aromatase (*CYP19*, 15q21) is a key enzyme of the pathway (Fig. 1) and its activity determines the local oestrogen level. Aromatase expression has been suggested to play a role in neoplastic proliferation in both human breast and endometrial carcinomas [7]. Tissue-specific regulation of expression has been studied by several groups, and a switch from an adipose-specific exon 1 (exon 1b or exon I.4) promoter used in nontumour breast tissues to the ovary-specific exon 1 (exon 1c or exon I.2) has been observed in breast cancer tissue [8,9]. Our previous data show that the alternative switch from the usual adipose tissue promoter to an apparently stronger 'ovary' promoter correlates significantly to the *CYP19* mRNA expression level ($P < 0.001$) [4]. Toda and colleagues described alternative RNA processing using different poly A signals of aromatase mRNA in human placenta [10]. In the current investigation, we looked for such poly A variants in breast carcinomas.

HSD1 (17q) catalyses the final conversion of oestrone to oestradiol (Fig. 1). The reverse inactivation of the oestrogenic 17-β-oestradiol to oestrone is catalysed by 17β-hydroxysteroid-dehydrogenase II (HSD2) (6q24) in the human breast as well as in the endometrium [11,12]. Since oestradiol is the most potent oestrogenic end product and since this reaction finalises several pathways by which oestrone can be created, a possible difference in the activity of the enzyme could be of importance for oestrogen levels.

Sulfatation, catalysed by oestradiol sulfotransferase (EST), is an important pathway in the biotransformation of steroid hormones. Oestrone sulfate is the predominant form of oestrogen found in the circulation in women and could thus serve as a precursor for active oestrogens in target tissues by removal of the sulfate group through the action of endogenous *STS* [13,14].

Two isoforms of the human oestrogen receptor (ER) occur, ERα and ERβ, each with distinct tissue and cell patterns of expression (reviewed in [15]). Oestrogen

Figure 1



Oestradiol synthesis pathway from cholesterol. The present study was focused on the right branch of this panel – the final metabolism of oestradiol from androsenedione. Thick arrows indicate the enzymes and activities coded by the mRNA transcripts studied in the present report.

binds to the ER, which modulates the transcription of a series of genes, including genes coding for a number of growth factors such as insulin-like growth factor 1 and transforming growth factor alpha [16,17]. These genes are part of the main gene cluster that defines the group to the luminal subtype A and subtype B breast carcinomas [1]. Oestrogen has been shown to cause upregulation of oncogenes such as c-*myc* through binding to its receptor, and through the Src/p21ras/mitogen-activated protein kinase pathway of c-*fos* and c-*jun*, leading to stimulation of breast cancer cell proliferation [18]. Oestradiol has recently been shown to modulate breast cancer cell apoptosis [19], while androgens have been shown to downregulate *bcl-2* protooncogene expression, providing an alternative mechanism for their inhibitory effect on breast cancer cell growth [20]. *ERβ* has been measurable in normal breast tissue but was very low in breast carcinomas, suggesting that *ERβ* might control the *ERα*-mediated mitogenic activity of oestrogens [21]. Numerous splice variants of *ERβ* have also been described.

Additional proteins under growth factor regulation, including the cell cycle protein cyclin D_1 and ligands for the human epidermal receptor (HER) family, have been shown to interact with the oestrogen receptor either by direct binding or by recruiting co-activators of the SRC-1 family to the ER in the absence of oestrogen itself [22,23].

In the present article, we report a high-precision mRNA expression profile of the genes coding for the enzymes metabolising oestradiol, or coding for its most important receptors ERα and ERβ, as well as modifiers of hormonal response such as cyclin D_1 and HER-2, coded by ERBB2 (Fig. 1, thick arrows) from the same tumours. Internal standards were constructed by insertion of 20–30 bp fragments into *in vitro* synthesised transcripts for every gene: aromatase (*CYP19*), *HSD1*, *HSD2, ERα*, *ERβ*, *STS* and *EST.* To examine additional factors of ER regulation, independent of the presence of oestrogen, we included into our analysis cyclin D_1 (*CYCLD1*) and *ERBB2*. These well-characterised prognostic factors of breast cancer are known to activate the ER or be activated by the ER [24,25].

Materials and methods

Immunohistochemistry

Frozen sections were prepared from fresh tumour tissue and immunostaining for ER protein was performed, applying the avidin–biotin peroxidase complex method. The antibodies were from DAKO (batch M7047; DakoCytomation, Inc. Carpinteria, CA, USA). All series included positive and negative controls. Only cells with nuclear staining were scored as positive. The number of immunopositive cells was semiquantitatively estimated: grade +, 5–10% positive cells; grade ++, 11–50% positive cells; and grade +++, > 50% positive cells. For each sample at least 100, usually more than 1000, tumour cells were analysed.

RNA was prepared from fresh frozen tumours from a consecutive series of 155 breast cancer patients admitted to the City Hospital of Oslo with a mean age at diagnosis of 65 years (range 28–87 years). The samples were collected in the period 1989–1993 and the ethical procedures followed the present day standard. A letter to the patients asking them to donate blood and a tumour specimen was sent, and those patients who consented participated in the study.

Tumour status and lymph node status were characterised based on the pathology reports according to the 1988 tumour node metastasis classification. Fresh frozen tumour tissue (20–50 mg) was dissected and powdered in liquid nitrogen in steel mortars. The powder was then immediately (while still frozen) transferred to 2 ml screw-capped tubes with 0.5 ml Trisol TM (Gibco-BRL, Grand Island, NY, USA). Total RNA was prepared by standard procedures using isopropanol precipitation. Fluorometric quantitation was performed to determine the absolute mRNA contents using fluorescent dye-labelled primers in the presence of an internal standard for each gene: *CYP19*, *HSD1*, *HSD2*, *ERα*, *ERβ*, *STS*, *EST* (*SULT1A3*), *cyclin* D_1, *ErbB2* and *β-actin*. To prepare the internal standard RNA, the modified cDNAs for each gene were constructed by inserting 20–30 bp DNA fragments between the two PCR primer sites. The internal standard RNAs were synthesised *in vitro* with T7 RNA polymerase using the modified cDNA as templates. Total RNAs mixed with a known amount of internal standard RNAs were subjected to reverse transcription with RAV-2 transcriptase (Takara Shuzo Co., Kyoto, Japan) and a gene-specific amplification at 42°C for 40 min. The resulting cDNAs were amplified by PCR using fluorescence-labelled (FAM; Perkin Elmer Co., Foster City, CA, USA), gene-specific, sense primers. Fluorescent PCR products were electrophoresed in a 2% agarose gel and were analysed with a Gene Skanner 362 Fluorescence Fragment Analyzer (ABI; Perkin Elmer Co.). The amount of aromatase mRNA was calculated from the peak areas of the fluorescent products by the internal standard method and was corrected on the basis of β-actin mRNA.

Alternative exon 1 of aromatase

The utilisation of alternative exons 1 of the aromatase gene was investigated by RT-PCR using sense primers specific for exons 1a, 1b, 1c, 1d and a fluorescent dye (FAM)-labelled antisense primer specific for exon 3, according to the previously described protocol [26].

Alternative poly A tail of aromatase

One common fluorescent-labelled primer (5′-FAM AGC AAC ATT CAT AGT CTT TG) and the poly A-specific primers 5′-CCA CAC TAA TTG AGC TAA GC-3′ (long transcript) and an equimolar mix of 5′-$(T)_{15}$GAACA-3′ and 5′-$(T)_{15}$GGGAA-3′ (short transcript) were used to simulta-

Table 1

Descriptive statistics of mRNA expression of oestrogen metabolising enzymes (amol/mg RNA) in the tumours of a set of 155 breast cancer patients

Transcript	Mean	Median	Minimum	Maximum	Standard deviation
Aromatase (CYP19)	59.24	12.2	0.00	750.00	13.92
17β-Hydroxysteroid dehydrogenase I	0	0.0	0.00	0.95	0.10
17β-Hydroxysteroid dehydrogenase II	0	0.0	0.00	0.27	0.00
Oestrogen receptor α	4.31	1.2	0.00	29.50	6.20
Oestrogen receptor β	0.16	0.0	0.00	2.33	0.28
Steroid sulfatase	335.09	204.5	0.00	2376.34	391.18
Oestradiol sulfotransferase	124.49	104.6	0.00	436.00	94.34
Cyclin D_1	0.46	0.2	0.00	4.73	0.68
ERBB2	0.38	0.0	0.00	7.23	1.10

neously amplify two different RT PCR products with length 352 bp and 422 bp, respectively. cDNA derived from the variant short transcript [27] inserted at an *Eco*RI site in a pUC118 vector was used as the positive control for amplifying the alternative poly A site.

Statistical analysis

Clinical parameters as well as mRNA levels and data on alternative splicing were collected in a SPSS file (SPSS Inc., Chicago, IL, USA), where the initial descriptive statistics, cross tab (chi-square test) and Pearson correlation analysis were performed. Survival analysis was performed using Kaplan–Meier plots in SPSS with log-rank statistics. The follow-up interval was 12 years (around 140 months; the disease-free survival was in months to first recurrence, the longest being 111 months). Patients were under observation every 6 months for the first 5 years, then once a year for the next 2–3 years. After that the information about the patients' status was collected in the Cancer Registry of Norway and updated every year. Hierarchical clustering and visualisation were performed using EPCLUST (http://ep.ebi.ac.uk/ep/epclust/). mRNA levels were calculated relative to the median, and clustering analysis of levels above/below the median was performed.

Results

mRNA expression

Competitive RT-PCR was used to quantify the amounts of mRNA of nine different transcripts as well as β-actin in breast carcinomas. In the series of 155 tumour samples two independent quantitations from the same mRNA preparations were performed for *CYP19* (aromatase), giving a correlation of $r=0.811$ ($P<0.0001$). Quantitation of mRNA of the *ERα* by competitive RT-PCR was in concordance with the immunohistochemistry data for total ERα protein ($P<0.0001$). The absolute amount of each of the studied transcripts (amol/mg RNA) gave a high correlation to that adjusted by β-actin, suggesting a uniformity of the samples and the mRNA preparation procedures. A great variability of expression among the patients was observed, ranging from 0 to 2376 amol/mg RNA, with the highest levels being observed for *STS* and *EST*, and the lowest levels (close to zero) observed for both *HSD* isoenzymes (Table 1).

Correlations of expression levels of the various transcripts were studied using Pearson correlation (Table 2). A high correlation was observed between the expression of EST and STS ($r=0.252$, $P<002$). While the levels of EST correlated with those of *ERα* ($r=0{,}550$, $P<0.0001$), the levels of STS correlated with *ERβ* ($r=274$, $P<0.001$). Expression of *HSD1* mRNA levels also correlated with both EST and STS mRNA expression ($P<0.003$ and $P<0.0001$, respectively). Furthermore, mRNA levels were calculated as relative to the median and clustering analysis of levels above/below the median was performed (Fig. 2). Two distinct clusters of the ER-positive and ER-negative samples were observed. The mRNA expression of the oestradiol metabolising enzymes, however, did not follow the expression of the ER in all cases, leading to the formation of several subclasses of tumours. Cyclin D_1 fell into the same cluster together with *EST* and *ERα* ($P<0.0001$).

mRNA expression and clinical and histopathological parameters

Expression of mRNA was then studied in relation to different clinical and histopathological parameters: tumour size (tumor category [TKAT]), lymph node status, distant metastases, stage of disease, site, and histological type (Table 3). Data were analysed in two ways using the SPSS data editor: by comparing the levels of the different

Table 2

Pearson correlation of the expression levels of mRNA coding for oestradiol metabolising enzymes

	CYP19	STS	HSD1	HSD2	EST	ERα	ERβ	ERBB2	Cyclin D_1
CYP19	1	0.081	−0.051	−0.054	0.021	−0.057	0.065	−0.071	−0.047
STS		1	0.333**	0.027	0.252**	0.045	0.274**	0.215**	0.195*
HSD1			1	−0.051	0.242**	0.107	0.047	0.003	0.164*
HSD2				1	−0.065	−0.144	0.050	−0.022	0.000
EST					1	0.550**	−0.082	0.089	0.422**
ERα						1	−0.089	−0.104	0.502**
ERβ							1	−0.031	−0.029
ERBB2								1	0.010
Cyclin D_1									1

Data presented as significance (two-tailed). CYP19, aromatase; STS, steroid sulfatase; HSD1, 17β-hydroxysteroid dehydrogenase I; HSD2, 17β-hydroxysteroid dehydrogenase II; EST, oestradiol sulfotransferase; ERα, oestrogen receptor α; ERβ, oestrogen receptor β. *Correlation significant at the 0.05 level (two-tailed). **Correlation significant at the 0.01 level (two-tailed).

Table 3

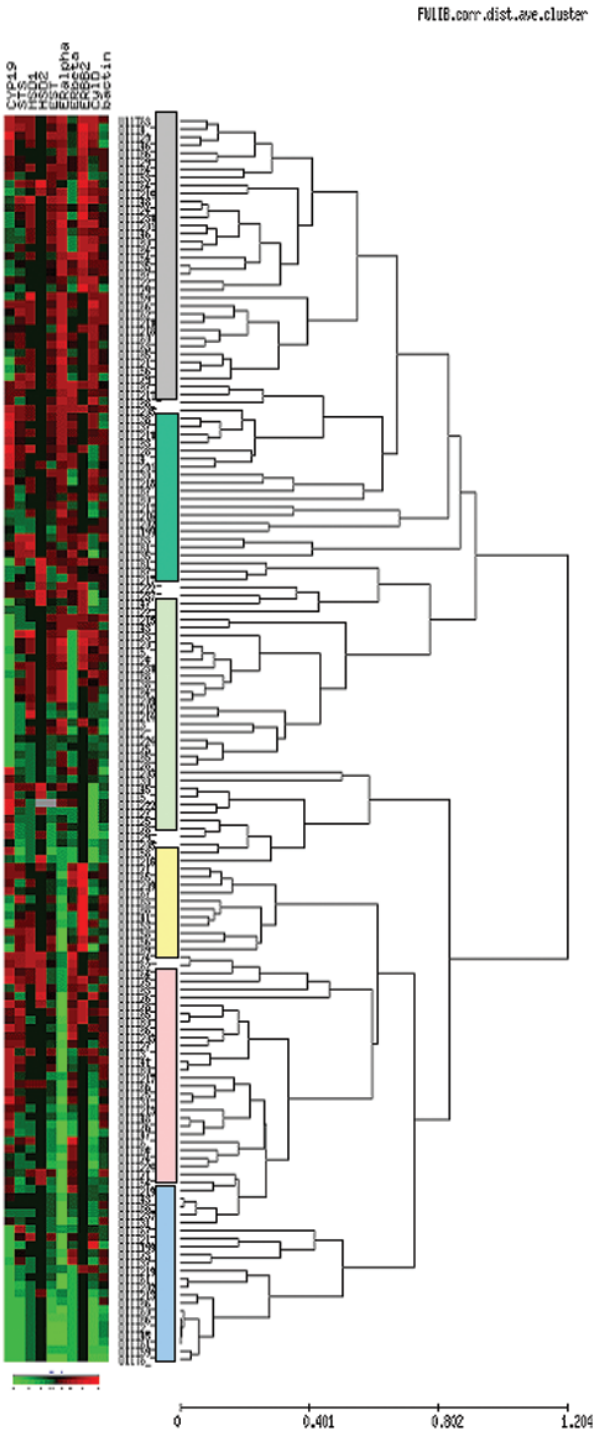
Mean values of mRNA expression of oestrogen metabolising enzymes (amol/mg RNA) in relation to the clinical and histopathological parameters of a set of 155 breast cancer patients

Clinical parameter	*n*	CYP19	STS	HSD1	HSD2	EST	ERα	ERβ
Age								
< 50 years	48	37.60	316.3	0.0027	0.0250	140.49	2.20	0.22
> 50 years	107	84.30	347.3	0.0198	0.0080	100.28	4.96	0.14
Stage								
I	81	77.50	265.7	0.0136	0.0970	93.81	4.60	0.20
II	57	45.90	304.5	0.2580	0.1170	135.22	4.70	0.16
III	17	13.60	382.1*	0.0970	–	122.98	2.04	0.13
Tumour category								
1	61	73.30	285.3	0.0140	0.0120	98.30	4.20	1.19
2	55	67.80	223.7	0.0273	0.1090	113.30	4.70	0.16
3	29	14.30	409.1	0.0090	–	139.20	3.60	0.15
4	10	6.3***	446.3**	0.0140	–	194.8***	2.70	0.04
Lymph node status								
1	90	30.80	303.7	0.0260	0.0980	128.60	5.40	0.18
2	65	47.00	409.2	0.0150	0.1260	119.72	2.80	0.17
Histology								
Ductal	87	63.40	384.1	0.0210	0.0110	119.32	4.64	0.17
Lobular	16	45.90	262.5	0.0160	0.0094	121.64	4.34	0.16

CYP19, aromatase; STS, steroid sulfatase; HSD1, 17β-hydroxysteroid dehydrogenase I; HSD2, 17β-hydroxysteroid dehydrogenase II; EST, oestradiol sulfotransferase; ERα, oestrogen receptor α; ERβ, oestrogen receptor β. *$P < 0.01$ (stage III versus stage I). **$P < 0.01$ (tumour category 3 and 4 versus tumour category 1 and 2). ***$P < 0.05$ (tumour category 3 and 4 versus tumour category 1 and 2).

transcripts between the different clinical categories using box plots, and by categorising these levels into two categories (below and above the median) or into quartiles and comparing their distribution among the clinical categories using cross tabs. mRNA levels of STS and EST were significantly higher in patients with TKAT 3 and TKAT 4 than

Figure 2



Clustering analysis of levels of mRNA relative to the median. Two distinct clusters of the oestrogen receptor (ER)-positive and ER-negative samples were observed. The mRNA expression of the oestradiol metabolising enzymes, however, did not follow the expression of the ER in all cases, leading to the formation of several subclasses of tumours: grey, ERα$^+$/CYP19$^-$; dark green, ERα$^+$/CYP19$^+$; light green, ERα$^+$/CYP19$^-$/ERβ$^-$; yellow, ERα$^-$/CYP19$^+$/ERβ$^-$; pink, ERα$^-$/CYP19$^+$/ERβ$^+$; blue, ERα$^-$/CYP19$^-$/ERβ$^-$.

in those patients with TKAT 1 and TKAT 2 ($P<0.01$) (Table 3) and with higher stage of the disease ($P<0.01$).

mRNA expression of CYP19 aromatase

A considerable difference in the expression of *CYP19* was observed. Twenty-five per cent of the tumours had no expression of aromatase at all, and 23% had overexpression of the transcript (50–800 CYP19 mRNA amol/mg RNA). A surprisingly high fraction of postmenopausal women (25%, in contrast to only 5% among premenopausal women in our study) did not express *CYP19* in their tumours. These results are of importance for the strategies for treatment with aromatase inhibitors in postmenopausal patients.

A switch from the usual adipose tissue promoter to an apparently stronger 'ovary' promoter has been previously observed in these samples and was associated with the levels of mRNA expression ($P<0.0001$) [4]. We also detected an alternative poly A tail in 15 of the tumour samples. The type of poly A tail was not associated to either alternative use of promoter, or mRNA levels.

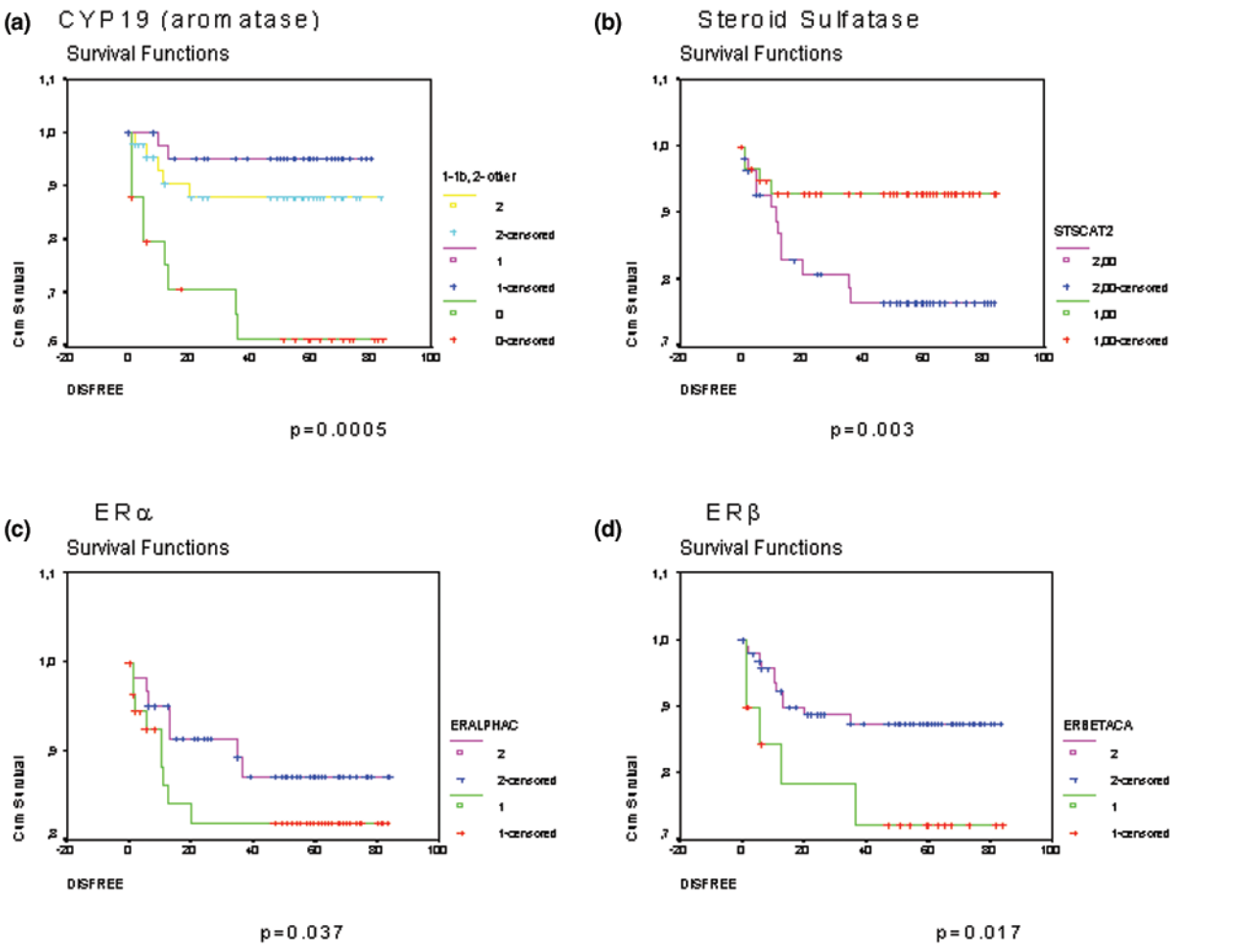
mRNA expression and disease-free survival

The levels of mRNA expression were also analysed as factors for disease-free survival. Patients with expression of tumour aromatase (*CYP19*) had a better prognosis than patients with no expression of this transcript ($P<0.0005$) (Fig. 3a). Furthermore, patients whose aromatase gene was using the normal adipose tissue promoter had longer survival than those patients with a switch of promoters. Lack of expression of aromatase was by far the strongest prognostic factor in this material of the predominantly early stages of breast cancer. High levels of expression of STS were also strongly correlated with short disease-free survival ($P<0.03$) (Fig. 3b). Expression of *ERβ* mRNA was a better prognostic factor than that of *ERα* in these tumours (Fig. 3c,d). *ErbB2*, whose amplification and overexpression is a well-documented modifier of survival, was also a strong prognostic factor in our material ($P<0.01$) (Fig. 4).

mRNA expression and menopausal status

Given that the biosynthesis of oestradiol and its metabolism are very different in premenopausal and postmenopausal women, we analysed the differences in the expression of the enzymes studied according to menopausal status (see Additional files). While all the main findings presented in Table 2 were also found in the postmenopausal group, which was also larger in size, some of the correlations were lost in the premenopausal group. Most notably lost was the correlation of mRNA expression of cyclin D_1 with several of the oestradiol synthesising enzymes and *EST* with *STS*. Multiple correlations were observed in the postmenopausal group, suggesting a tighter coregulation of the oestrogen synthesis enzymes at the mRNA level in the tumours of post-

Figure 3



Kaplan–Meier analysis of disease-free survival of breast cancer patients dependent on the expression of mRNA coding for oestradiol metabolising enzymes. **(a)** CYP19 (aromatase) using the conventional, adipose tissue-specific first exon (curve 1), abnormally using the ovarian-specific first exon (curve 2), and with the 0-null expression (curve 0). **(b)** Steroid sulfatase with expression below (curve 1) and above (curve 2) the median. **(c)** Oestrogen receptor (ER)α below (curve 1) and above (curve 2) the median. **(d)** ERβ below (curve 2) and above (curve 1) the median.

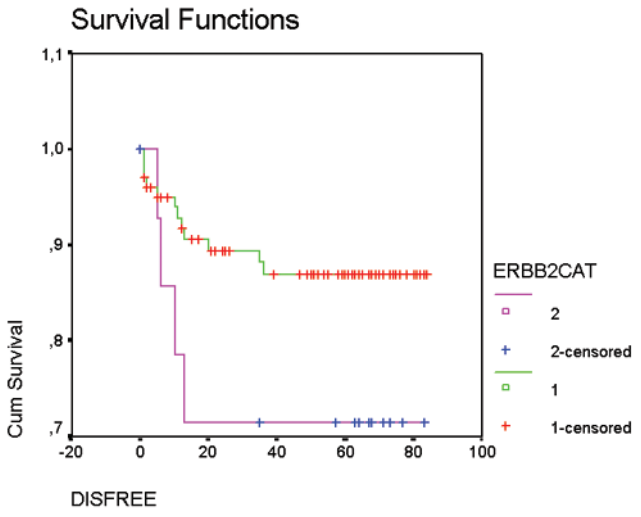
menopausal breast cancer patients than in those of premenopausal breast cancer patients. Additional experiments will be conducted to address this interesting topic.

Discussion

Endogenous oestradiol is synthesised in the ovarian granulosa cells of premenopausal breast cancer patients or in the stromal adipose cells of the breast of postmenopausal breast cancer patients, and is synthesised in minor quantities in peripheral tissue. These cells, as well as breast cancer tissue, express all the necessary enzymes for the synthesis of oestradiol (CYP17, CYP11a, CYP19 and HSD), as well as enzymes of its further metabolism such as EST and oestrogen sulfotransferase. We have conducted a study to absolutely quantify the mRNA levels of the transcripts of several important proteins in the synthesis of oestradiol: *CYP19* (aromatase), *HSD1*, *HSD2*, *ERα*, *ERβ*, *STS* and *EST*. Two other breast cancer-related regulators, cyclin D_1 (*CYCLD1*) and *ERBB2*, have also been included in the study.

The hierarchical clustering analysis revealed two main clusters (the ER-positive and ER-negative tumours of the breast), in concordance with results from large-scale microarray analysis. The mRNA expression of oestrogen metabolising genes did not directly follow the expression of the ER: we observed a cluster of ER-positive tumours with low (below median) expression of these enzymes, as well as ER-negative tumours with high expression of oestradiol synthesising enzymes. This information may help to further nuance the prediction of response to hormonal treatment in addition to ER status.

Figure 4



Disease-free survival of breast cancer patients as a function of mRNA expression of ERBB2 below (curve 1) and above (curve 2) the median. $P = 0.011$.

Our results of increased levels of EST mRNA were in concordance with metabolic data *in vivo* showing high concentrations of the product of sulfotransferase activity (oestrone sulfate [E1S]) in tumours of the breast [13,28]. Similar data from fibroadenomas and breast cysts [29] suggest that oestrone and oestradiol can be converted to E1S within the breast cysts *in vivo* at remarkably high speed. Furthermore, the formation of E1S (EST activity) was shown to be upregulated by a number of growth factors, such as the epidermal growth factor, insulin-like growth factor type 1, and acidic and basic fibroblast growth factors in the ER-positive MCF7 cells but not in the ER-negative MDA-MB-231 cells. The cytokines tumour necrosis factor alpha and interleukin-1b also increase the sulfate formation in ER-positive cell lines but not in ER-negative cell lines [30]. We can indirectly confirm this ER dependence in humans *in vivo* because the expression of mRNA of EST in the breast cancer tissue correlated with the ER expression in our sample material. The amounts of *STS* and *ERα* mRNA seemed to be coregulated in the present study and in another independent cohort of breast cancer patients analysed in our laboratory (data not shown). They also correlated with mRNA levels of *HSD1*. This observation possibly reflects that all three enzymes have a common substrate (oestradiol) and could be under common regulation depending on the oestradiol levels.

The very low mRNA levels of *HSD1* and *HSD2* strongly suggest a post-translational mechanism of protein stabilisation because high protein expression and metabolic activity of HSD1 have been previously reported in breast cancer tissue [31]. It has been shown that the HSD1 activity in the endometrium is elevated during the secretory phase, as compared with the level during the proliferative phase, and that the elevation is in response to progesterone via the progesterone receptors. GATA-3 responsive sequences have been identified in the 5′ flanking regulatory area of *HSD1* [32]. Comparing patterns of gene expression in cells with or without expression of the ER by the human cDNA array revealed clustering of a transcription factor GATA-3 together with ER. This was hypothesised to regulate, in association with the ER, genes critical to the hormone-responsive breast cancer phenotype [33]. Specific HSD oxidation–reduction activity has been discussed to play a role in the transition from hormone dependence to hormone independence of breast cancer [34].

Expression of *STS* has previously been shown to be a strong prognostic factor for disease-free survival in breast cancer [5,13] and in ovarian cell adenocarcinoma [35]. The steroid sulfatase is an important target for the development of new drugs for the treatment of endocrine-dependent breast cancers. One such compound, 2 methoxyoestrone-3-*O*-sulfamate, was shown to inhibit the growth of MCF-7 cells by causing arrest in the G(2)/M phase and apoptosis [36]. Previous analysis has shown a strong inverse relationship between the length of the disease-free interval and the plasma levels of E1S and the ratios of E2/E1 and E1S/E1 in postmenopausal patients with recurrent disease [37].

The previously reported high correlation of the expression of CYP19 (aromatase) mRNA and the switch from the adipose tissue-specific promoter to the ovary-specific promoter was confirmed in this new independent measurement [4]. Furthermore, in the present study the alternative usage of exon 1 was also associated with shorter disease-free survival. However, the patients with worse prognosis had no expression of CYP19 mRNA at all. This is a somewhat paradoxical result since the present data and our previous data [4] suggest that, in the course of the disease, a switch of promoter occurs and results in higher expression levels. The process, however, may occur in a given time frame of the disease on the way to hormone-independent breast cancer.

The poor prognosis of patients with null expression of aromatase may also reflect prior treatment exposures of the patients. This is a consecutive series of patients, predominantly stage I and stage II, who had received a standard treatment of tamoxifen. Those patients who responded poorly were offered treatment with an aromatase inhibitor. We have no exhaustive data on treatment response in order to verify whether patients with null aromatase expression in their tumour respond poorly to such standard therapeutic procedure. Further studies designed to particularly address treatment response may be necessary. A recent study showed that failure of the antitumour

activity of tamoxifen in patients with breast cancer is actually determined by both the levels of and the interaction between the ER coactivator amplified in breast cancer-1 (AIB1) and the epidermal growth factor-related protein human epidermal receptor (HER, coded by ERBB2) [38]. Members of the HER family are attractive therapeutic targets because they are overexpressed and/or deregulated in many solid tumours. Activation of HER1/epidermal growth factor receptor mediated through ligand binding triggers a network of signalling processes that promote tumour cell proliferation, migration, adhesion and angiogenesis, and that decrease apoptosis. Various approaches are being investigated to target members of the HER family, particularly HER1/epidermal growth factor receptor and HER2 (ERRB2).

Conclusion

Expression studies of tens of thousands of transcripts give exciting possibilities to draw molecular portraits of tumours within a given range of expression levels, but they are less informative for the absolute amounts of single transcripts. Our results complement these data by providing absolute quantitative analysis of physiologically important genes with very low expression levels, such as ERβ and the hydroxysteroid dehydrogenases, which remain 'invisible' by the high-density hybridisation arrays. These results also point to the importance of CYP19 (aromatase) and the enzymes regulating the oestrone sulfate metabolism as factors of disease-free survival in breast cancer, in addition to well-known factors such as the ER and ERBB2.

Additional files

The following Additional files are available online:

Additional file 1
A word document that contains two tables for correlation between mRNA expression of oestradiol metabolising enzymes in A. premenopausal breast cancer patients and B. postmenopausal breast cancer patients. A tighter co-regulation of the oestrogen synthesis enzymes at mRNA level was observed in the tumors of post-menopausal than pre-menopausal breast cancer patients. These results are however preliminary and additional experiments will be conducted to address this interesting topic.
See http://breast-cancer-research.com/content/supplementary/bcr746-S1.doc

Additional file 2
Clustering analysis of levels of mRNA in premenopausal women. Hierarchical clustering and visualization was performed using EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/). mRNA levels were calculated as relative to median and clustering analysis of levels above/below median was performed.
See http://breast-cancer-research.com/content/supplementary/bcr746-S2.bmp

Additional file 3
Clustering analysis of levels of mRNA in postmenopausal women. Hierarchical clustering and visualization was performed using EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/). mRNA levels were calculated as relative to median and clustering analysis of levels above/below median was performed.
See http://breast-cancer-research.com/content/supplementary/bcr746-S3.bmp

Competing interests

None declared.

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