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Intratumor genetic heterogeneity of breast carcinomas as determined by fine needle aspiration and TaqMan low density array

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Abstract. *Background:* Gene expression profiling is thought to be an important tool in determining treatment strategies for breast cancer patients. Tissues for such analysis may at a preoperative stage be obtained, by fine needle aspiration (FNA) allowing initiation of neoadjuvant treatment. To evaluate the extent of the genetic heterogeneity within primary breast carcinomas, we examined whether a gene expression profile obtained by FNA was representative of the tumor. *Methods:* Tumors from 12 consecutive cases of early predominantly estrogen receptor positive (ER+) breast cancer patients undergoing primary surgery were split in halves and FNAs were obtained from each half. A tissue biopsy of the tumors was also snap-frozen for comparison. Non-amplified RNA was investigated by the novel qRT-PCR-based technique, Low Density Array (LDA) using 4 reference genes and 44 target genes. *Results:* Comparison of gene expression at the single gene level in the two FNA samples from each tumor demonstrated various degrees of heterogeneity. However, compared as gene expression profiles, intratumor correlations for 9/12 patients were high and these pairs could in a theoretical blinding of all the FNAs be correctly matched by statistical analysis. High correlations between the gene profiles of tumor FNAs and tissue biopsies from the same patient were observed for all patients. A cluster analysis identified clustering of both the two FNAs and the tissue biopsy of the same 9 patients. *Conclusion:* The overall genetic heterogeneity of breast carcinomas, as sampled by FNA, does not prohibit generation of useful gene profiles for treatment decision making. However, sampling and analysis strategies should take heterogeneity within a tumor, and varying heterogeneity amongst the single genes, into account.

Keywords: Breast cancer, gene expression, genetic heterogeneity, quantitative real-time PCR, fine needle aspiration

1. Introduction

Clinical breast cancer management has been dramatically changed by the initiation of systematic screening programs and the use of improved therapies, such as adjuvant hormonal and antibody-based therapies as well as chemotherapies. These advances are having a major positive impact on patient outcome, and despite the increase in incidence, breast cancer mortality is decreasing in most of the Western world [4,10].

Today, the most important guidelines for prognosis and treatment are age, tumor size, malignancy grade, lymph node involvement, HER2 and hormone receptor status. The advent of novel targeted therapies as well as predictive and prognostic molecular tests have the potential to result in individually tailored treatment regimens that spare patients from serious side effects. Moreover, improved predictive and prognostic tests may avoid the economic burden of these increasingly expensive therapies. Predictive molecular tests are also increasingly required in the neoadjuvant setting, where the preoperative treatment is used in order to reduce the size of surgically-inoperable tumors as well as large operable primary breast cancers. This treatment strategy has the advantage of assessing clinical responses *in vivo* [14,17].

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Biopathologic profiles obtained by techniques such as immunohistochemistry, *in situ* hybridisation etc., are important for cancer management, but gene expression profiles will likely become an essential part of the analytical array allowing further advances in individualized treatments [16,21]. Several new molecular biology techniques show promise in routine diagnostic laboratories since they are objective, quantitative, robust and often allow high-throughput. Such a technique is quantitative real-time reverse trancscriptase PCR (qRT-PCR), proven to be both sensitive and specific. The procedural steps of qRT-PCR technology have been minimized to the point that samples can be analyzed within 24 hours. More recently, a novel qRT-PCR-based technique, Low Density Array (LDA), has been developed that allows simultaneous testing of 384 samples in a micro-titer plate format with primers and probes lyophilized in each reaction well [2,9]. The application of qRT-PCR, especially in the LDA setting, allows minimal handling of the samples, thereby decreasing contamination and operator-induced errors, and allows standardization of method and data interpretation across laboratories [5].

To correctly ascertain the gene expression profile of individual tumors, optimal sampling techniques are critical to ensure that a representative tumor cell population is obtained. In breast cancer, as other solid cancers, a tumor sample to be used for gene profiling and other diagnostic tests may be obtained using fine needle aspiration (FNA), core needle biopsy (CNB) or surgical excision. FNA and CNB have the clinical advantage of being minimally invasive and thus will be important in the neoadjuvant treatment setting [7]. An analytical advantage of FNA is the reported higher percentage of tumor cells (approx. 80%) compared to CNB (50%, the remainder being lymphocytes and stromal cells) [18]. In addition, FNA allows tumor sampling from various tumor sites due to the technical agility of aspiration.

In this study, we investigated whether gene expression data obtained from FNAs of breast carcinomas represent the tumor as a whole rather than reflecting only the individual test site. Although this general representation is often assumed, no study has, to our knowledge, systematically evaluated this. In addition we evaluated whether sufficient material is obtained by FNAs to permit reliable gene expression profiling of non-amplified material using the novel qRT-PCR-based Low Density Array (LDA) technique. Since prediction of responsiveness to endocrine treatment remains a major clinical problem, as emphasized by the fact that 80% of breast cancer patients are ER+, and 30% of these do not benefit from adjuvant Tamoxifen [1], this study focused on genes thought to be involved in this pathway. Forty-four (44) genes on the profile were selected based on their reported involvement in endocrine responsiveness, their status as markers of ER-positive cells, or cell type-specific markers. Our findings should have strong implication for the use of gene expression profiles as a tool for treatment decision-making.

2. Materials and methods

2.1. Tissue sampling

Breast carcinomas from 12 consecutive patients undergoing primary surgery at Odense University Hospital were included in the study. Within 30 min of surgery, the tumor was cut in half and FNAs from randomly selected areas within each tumor half were pooled (termed FNA-A and FNA-B). The samples were stored in Hanks buffer (OUH Pharmacy, Odense, Denmark) containing 3% Newborn Calf Serum (Sigma, St. Louis, MO, USA) for a maximum of 12 h at 4°C, after which mononuclear cell counts were determined on a Sysmex-KX-21N machine (Sysmex, Kobe, Japan), and the cells were pelleted by centrifugation at $2.3 \times g$ for 3 min. The cells were stored in MagNa Pure LC mRNA isolation kit I lysisbuffer (Roche, Mannheim, Germany) at −80°C at a concentration of 5×10^6 cells/ml for a maximum of 2 months. A 0.5×0.5 cm tissue sample of the same tumors were snap frozen in isopentane after covering with Tissue Tek (Sakura Finetek, Zoeterwoude, Netherlands) and stored at −80°C until use. A representative formalin-fixed, paraffin-embedded (FFPE) tissue section of each tumor was haematoxylin and eosin (H&E) stained and evaluated by a senior pathologist according to the 2003 WHO-histological criteria of tumors of the breast. In addition, a cryosection of each tumor block was cut and H&E stained to determine the percentage of tumor cells; in all cases the percentage was >50% tumor cells. Tumor tissue (25 mg) was then homogenized in 1 ml lysisbuffer (Roche) using MagNa Lyser Green beads/MagNa Lyser instrument (Roche) for two 15 s pulses at 6,000 rpm, and stored at −80°C for a maximum of 2 months. The study was approved by the ethical committee of Funen and Vejle County (VF20040064).

2.2. Immunohistochemistry (IHC)

IHC for ER, PgR and HER2 was performed on FFPE tissue for all patients. Blocks from both tumor halves that were sampled by FNA were analyzed, except in 3 patients, where only 1 tissue block was available due to lack of sufficient material. HER2 staining was performed using the Hercep Test™ for DakoCytomation Autostainer (Dako, Glostrup, Denmark) according to the manufacturer's recommendations. The antibody NCL-ER-6F11 (Novocastra Ltd., Newcastle, UK), 1:100, and PgR-636 (Dako), 1:200, was used for ER and PgR detection, respectively. For these two antibodies the protocol was as follows: antigen retrieval was performed using Tris-EGTA, pH 9, incubated overnight at 60°C, followed by blockage of endogenous peroxidase by H_2O_2 for 10 min. The primary antibody was incubated for 60 min, and detected by PowerVision (Immunovision Technologies, Fullerton, CA, USA), followed by a nuclear counter staining with Mayers Haematoxylin for 2 min. All immunostaining were performed using the Autostainer (Dako) and known positive and negative controls were included. The steroid receptors were scored positive if $\geqslant$10% of the tumor cells stained. The score for HER2 (0, 1+, 2+ and 3+) was as approved by the FDA, and FISH (*HER2* FISH pharmDx™ Kit, Dako) was performed for patients scored as 2+ to verify amplification.

2.3. RNA purification and cDNA synthesis

RNA was purified from 200 µl of the isolated aspirated cells (FNA) or the homogenized tissue samples by Roche RNA isolation kits for cells or tissue (MagNa Pure LC RNA isolation kit III tissue and MagNa Pure LC RNA isolation kit – high performance) using the MagNa Pure Robot (Roche). This system uses magnetic beads to isolate total RNA and elutes the RNA sample in 100 µl elution buffer (Roche). Total RNA was reverse-transcribed to cDNA using random 9 oligonucleotide primers at 25 µM per reaction. RNA and primers were incubated for 5 min at 70°C and placed on ice. The reaction mix added consisted of 1 mM dNTPs, 1 Unit/µl RNase Inhibitor (Roche), 10 Unit/µl Reverse Transcriptase (Invitrogen Life Technologies, Paisley, UK) and First Strand Buffer × 5 (Invitrogen). The material was subsequently incubated for 10 min at 25°C, followed by 45 min at 37°C, and finally 5 min at 95°C.

2.4. qRT-PCR/LDA

TaqMan® Gene Expression Assays consisting of predesigned primers and probe (Applied Biosystems, Foster City, CA, USA) were used for all qRT-PCR experiments and run on the ABI 7900HT system (Applied Biosystems). The cDNA quality and quantity of each sample was tested by standard qRT-PCR using the genes GUS-B, ABL1 and B2M. The samples were run for 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The samples were then run on the LDAs having a 47+1 (GAPDH) configuration; the 47 genes were ABL1, NM_005157; B2M, NM_004048; BAG1, NM_004323; BCAR1, NM_014567; BCAR3, NM_003567; BCL2, NM_000633; BIRC5, NM_001168; CCNB1, NM_031966; CCND1, NM_053056; CD68, NM_001251; CTSD, NM_001909; CTSL2, NM_001333; CYP19A1, NM_031226/NM_000103; EGFR, NM_005228; ERBB2, NM_004448; ESR1, NM_000125; ESR2, NM_001437; GRB7, NM_005310; GUSB, NM_000181; HOXB13, NM_006361; IGF1R, NM_000875/X04434; IGFBP4, NM_001552; IL17RB, NM_172234/NM_018725; IRS1, NM_005544; KDR, NM_002253; LCN2, NM_005564; MKI67, NM_002417; MYBL2, NM_002466; NCOA3, NM_181659/NM_006534; NFKB1, NM_003998; NFKB2, NM_002502; PGR, NM_000926; PLAU, NM_002658; PLAUR, NM_002659; PTPRC, NM_002838/NM_080921/NM_080922/NM_080923; SCGB2A1, NM_002407; SCGB2A2, NM_002411; SCUBE2, NM_020974; SERPINE/PA-I, NM_000602; SOD3, NM_003102; STC2, NM_003714; STS, NM_000351; SULT1E1, NM_005420; TFF1, NM_003225; TOP2A, NM_001067; VIM, NM_003380; XBP1, NM_005080; GAPDH, NM_002046. All samples were run in triplicate or quadruplicate for 2 min at 50°C, 10 min at 94.5°C, followed by 50 cycles of 30 s at 97°C and 1 min at 59.7°C.

2.5. Data analysis and statistics

The qRT-PCR raw data were analyzed using the SDS vers. 2.1 software (Applied Biosystems). Ct values for the tumor samples (FNA-A, FNA-B or tissue biopsy) were determined in replicates (triplicate or quadruplicate) to ensure methodological reproducibility. Criteria for objective removal of outliers were set as follows: Ct $<$ 30: replicates must be within 0.5 of each other, 30 $\leqslant$ Ct $\leqslant$ 33: replicates must

be within 1.0 of each other and $33 \leqslant Ct < 37$: all replicates were included. All measurements above Ct 37 and samples showing amplification of only a single replicate were regarded as immeasurable, leaving only those with 2–4 replicates for further analysis. These limits were initially empirically determined, but subsequently experimentally verified (Petersen et al., unpublished data). Replicates of each target gene ($n = 44$) were normalized to the averaged reference genes (GAPDH, ABL1, B2M and GUSB); $\Delta Ct_{target} = Ct_{ref,avg} - Ct_{target}$.

To investigate the genetic heterogeneity of the single genes, we compared the FNA-A and -B samples for each gene and each patient by an unpaired t-test (Microsoft Excel®). The level of significance was set at 5%. The test was not conducted, and was termed 'undetermined', if there were not at least 2 replicates for each of the two halves. Individual gene profiles were determined by averaging the selected, normalized replicates within each sample, resulting in a ΔCt_{target} value for each sample and each target gene, which was further standardized by subtracting the population mean. To study the intra-patient genetic heterogeneity of these profiles, Spearman Correlation coefficient was calculated (STATA, ver. 9, TX, USA) between the FNA-A and FNA-B tumor sample, for all patients. To investigate the clinical impact of the correlation we investigated how many of the tumor halves had the highest correlation with their counterpart among the remaining 23 tumor halves (theoretical blinding). To study the similarity of the profiles obtained by the two sampling techniques, Spearman Correlation coefficients were calculated (STATA, ver. 9) for the averaged profiles of the FNA-A and FNA-B samples per patient (termed FNA_{avg}) and the profiles from the tumor biopsy, for all patients. In addition, all 36 samples (FNA-A, -B and tissue biopsy) were subject to a cluster analysis, using the Spearman correlation as similarity measure and the average linkage clustering method (STATA, ver. 9).

The ΔCt_{target} values were plotted for all 44 genes for visual verification of similarity of expression for FNA-A, FNA-B and the tissue biopsy for each patient. Additionally, the average difference between the average from the 2 FNA samples and the tissue biopsy was computed for each gene giving the $\Delta\Delta Ct$ value ($\Delta\Delta Ct_{target} = \Delta Ct_{FNA,avg(target)} - \Delta Ct_{tissuebiopsy(target)}$).

3. Results

3.1. Characteristics of patients and breast tumor samples

Tumors from 12 consecutive patients undergoing primary surgery for breast cancer were sampled immediately following resection. The tumors were divided in halves and FNAs were obtained from each half. A tissue biopsy was also removed from the intersecting area and snap-frozen. The clinical characteristics of the 12 patients are listed in Table 1.

Comparison of the morphology and histology of the two tumor halves from each patient by H&E staining demonstrated similar patterns and showed that each half contained at least 50% invasive carcinoma. Further, IHC analysis for ER, PgR and HER2 was performed on the majority of tumor halves. As shown in Table 2, similar expression patterns were observed in the two tumor halves in the majority of patients for all three markers. Differences were only observed in 3 of 12 patients stained for PgR in the order of 10–25%,

Table 1
Clinical characteristics of the 12 breast cancer patients

Age	
Average	59
Range	33–90
Tumor size (mm)	
Average	22
Range	8–40
Diagnosis	
Invasive ductal carcinomas (IDC)	9
Invasive lobular carcinomas (ILC)	2
Invasive mucineous carcinomas (IMC)	1
Malignancy grade	
I	4
II	4
III	4
Receptor status*	
ER+/PgR+	11
ER−/PgR−	1
HER2 status†	
Normal	10
Overexpression	2
Lymph node status**	
Positive	7
Negative	5

*>10%, determined by IHC.
**tumor cells detected.
†determined by IHC/FISH.

Table 2
Immunohistochemical analysis of the protein expression of ER, PgR and HER2 for the two tumor halves (A and B) of each patient

	Tumor half	ER (%)	PgR (%)	HER2 score
1		100*	70	2+*
2	A	100	100	2+*
	B	100	100	2+*
3		100*	90*	2+*
4	A	100*	100	0
	B	100*	100	0
5		100	100	1+*
6	A	100	100	0
	B	100	100	1+
7	A	100*	100*	1+*
	B	100*	100*	1+*
8	A	100	5	1+*
	B	100	30	1+*
9	A	100*	20*	2+*
	B	100*	30*	2+*
10	A	100*	100*	1+
	B	100*	100*	1+
11	A	100*	70*	1+*
	B	100*	50*	1+*
12	A	0	0	2+
	B	0	0	2+

For the patients where A/B is not indicated, it was not possible to investigate the heterogeneity with IHC per FNA sampled tumor half, as routine clinical considerations had priority and did not allow division of the tumor due to the small size of the tumor.
* indicate the patients demonstrating genetic heterogeneity for the given gene.

and for 1 of 12 patients stained for HER2 (score of 0 vs. 1+).

RNA purification and cDNA synthesis, with no amplification step, was successful for all 24 FNA samples and 12 tissue biopsies, as determined by standard qRT-PCR using the internal reference genes. cDNA of the 24 FNA samples and 12 tissue biopsies were assayed for gene expression levels of 44 selected genes using the novel qRT-PCR-based LDA technique [2,9]. The LDA analysis of the tissue biopsies demonstrated that over 95% of the genes were measurable in all 12 samples. For the 12 FNA sample pairs, the number of measurable genes was lower, but more than 64% of the genes were measurable in 11 of 12 patients (range: 27–93%).

When examining the individual genes, it became clear that it was primarily the same genes in all patients that were not detected when the amount of material was low (data not shown). This illustrates the differential level of mRNA transcripts in cells; some genes are highly abundant and can be detected in samples with low amounts of total RNA, whereas others cannot.

3.2. Analysis of individual genes of the FNA pairs

To determine the intratumoral heterogeneity of the individual genes across the 12 patients, we plotted the number of patients having either (1) a significant difference in gene expression, (2) no significant difference in gene expression or (3) remained undetermined (Fig. 1).

For all genes we found more patients with a significant difference in gene expression than the 5% to be expected under chance conditions. However, the number of patients with a significant difference varied substantially from gene to gene. As shown in Fig. 1, some genes, such as NCOA3, BCL2, IL17RB and BCAR1, showed no significant difference in 66% of the patients. In contrast, the genes CTSD, ERBB2, SCUBE2, VIM and XBP1 showed significant differences between the two tumor halves in 66% or more of the patients. Two genes, SULT1E1 and CYP19A1, could not be statistically compared in any of the patients, as they were not expressed in most of the samples.

3.3. Analysis of gene profiles of the FNA pairs

To investigate the impact of the heterogeneity at the gene level on the validity of the overall profile, we compared the gene profile of the two tumor halves per patient using Spearman Correlation (Fig. 2). All but three pairs (patients 2, 10 and 11) had correlations of at least 0.48, and in $7/12$ the correlation exceeded 0.72. The three pairs with a low correlation had all in common, that at least one of the profiles showed a rather low variation (i.e. all standardized ΔCt values were close to 0), especially compared to the pairs with a correlation above 0.9. Thus, these patients exhibit a profile close to the mean profiles, which diminishes the possibility of high correlations. The 9 patients (8 invasive ductal carcinoma (IDC) and 1 invasive mucinous carcinoma (IMC)) with a correlation above 0.48 always exhibit the highest correlation with its rightful counterpart, when their halves were compared to all other tumor halves ($n = 24$–1). For the 6 remaining halves (patients no. 2 invasive lobular carcinoma (ILC), no. 10 (ILC) and no. 11 (IDC)), we found 1, 1, 2, 4, 4 and 4, respectively, with a higher correlation than the correct match.

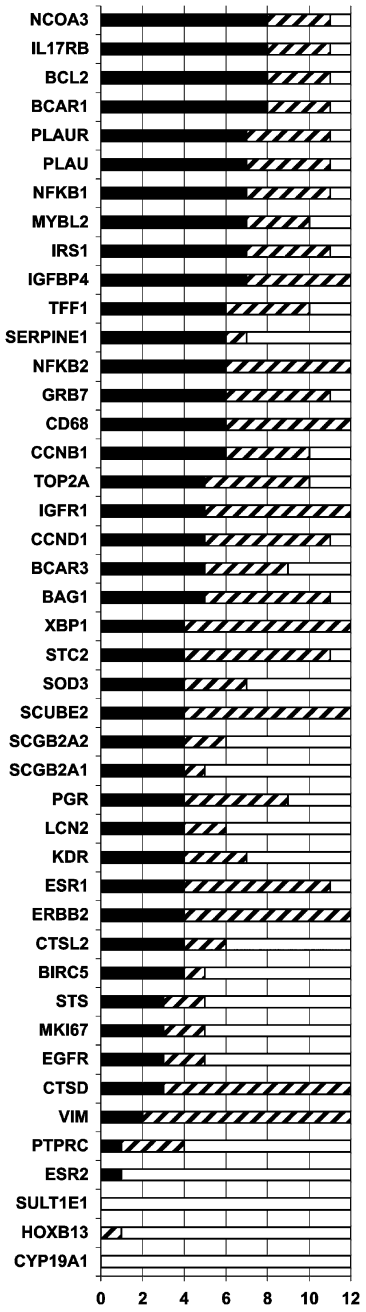

Fig. 1. Analysis of intratumoral heterogeneity by statistical comparison of individual gene expression levels in matched pairs of FNA samples from 12 breast cancers. For each gene (y-axis) and the number of patients (x-axis), the distribution of genes showing either no significant difference in gene expression ($p > 0.05$, independent t-test, black column), or a significant difference in expression ($p < 0.05$, hatched column) between the tumor halves are shown. Some gene pairs within an individual could not be statistically evaluated as the gene expression in at least one sample was under the detectable limit (white column).

3.4. Comparison of gene profiles obtained from FNA as compared to tissue biopsies

To investigate the compatibility of tumor gene expression profiles obtained from FNA with that from a tissue biopsy of the same patient, the correlation of the profiles between the average of the 2 FNAs and the tissue biopsy was investigated (Fig. 3). Only genes that were measurable by both sampling techniques were included in each profile (the FNA results being the limiting factor in all). A correlation of above 0.40 was observed for $11/12$ patients, and 5 of these exhibited correlations above 0.69. The remaining patient exhibited a correlation of 0.43 when only one of the FNAs (FNA-A *vs.* tissue biopsy) was analyzed, whereas the correlation for the other FNA (FNA-B *vs.* tissue biopsy) was negative. Further, a cluster analysis of the three samples (FNA-A, -B and tissue biopsy) of each of the 12 patients demonstrated that all three samples of 9 patients clustered (Fig. 4). The 3 patients, in which no clustering was observed, were the same as those that did not find their counterpart in the FNA-pair analysis mentioned above (patient no. 2, 10 and 11). In two of these patients (no. 2 and 11), one of the FNAs clustered with the corresponding tissue biopsy.

To estimate the difference in gene expression observed between the sampling techniques, we compared directly the ΔCt values between the three samples within each patient. Figure 5 shows three representative patients (2 ER+ and 1 ER$-$), illustrating that the ΔCt values generally tended to be close together. The difference between the average of the two FNA samples and the biopsy ($\Delta\Delta$Ct value) was estimated. The 40 genes that were measurable across the patients, and therefore could be compared, were grouped as follows: 25 genes had $\Delta\Delta$Cts between 0 and 1.0, 8 genes had $\Delta\Delta$Cts between 1.1 and 2.0, and 7 genes had $\Delta\Delta$Cts between 0 and -1.29, which equivalents to maximally a 2.5-fold difference (using the $2^{-\Delta\Delta Ct}$ method [13]), demonstrating the limited variability among sampling techniques for these genes.

4. Discussion

Diagnosis, prognosis and therapeutic decision-making in the future will likely depend on profiles of a limited number of tumor-expressed genes, which requires reliable gene expression profiles reflecting the entire tumor. In breast cancer, an attractive method of obtaining representative tissue samples at a preopera-

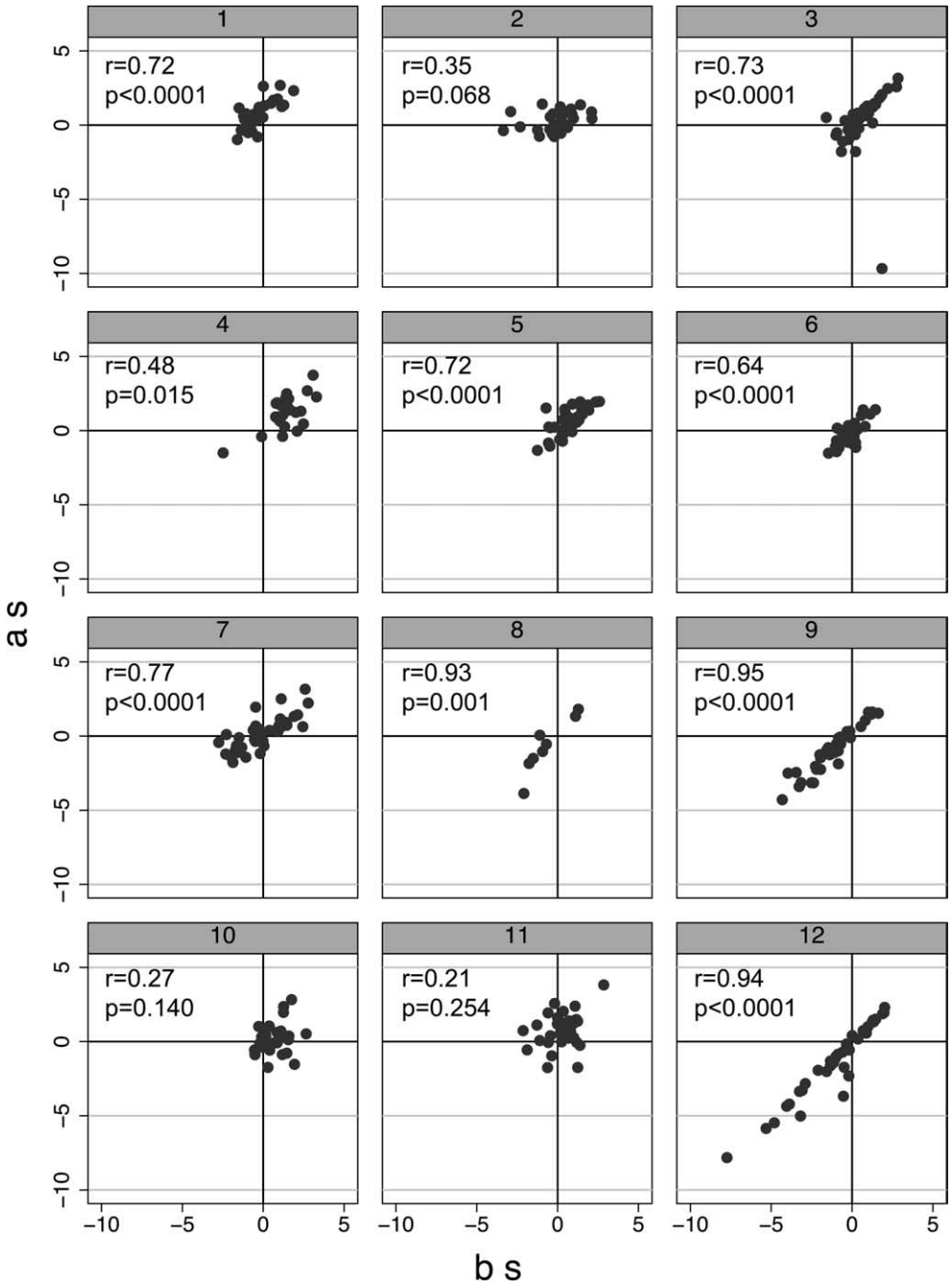

Fig. 2. Scatterplot and Spearman Correlations of the intra-patient FNA pairs. The tumors were split in half and sampled by FNA (termed FNA-A (a s) and FNA-B (b s)). High intra-patient correlations were observed in 9 patients, while tumor halves from patients no. 2, 10 and 11 could not be statistically matched, indicating genetic heterogeneity. Only patient no. 12 was ER−; the remaining were 100% ER+.

tive stage is FNA, which allows initiation of neoadjuvant treatment based on in-depth tumor characterization. In this study, we systematically evaluated the genetic heterogeneity of breast carcinomas sampled by FNA using gene expression profiles obtained by the qRT-PCR-based LDA technique, which exhibits high sensitivity and specificity and enables standardization across laboratories [5]. Our study showed that gene expression examined at the single gene level yielded considerable heterogeneity within the tumors. For exam-

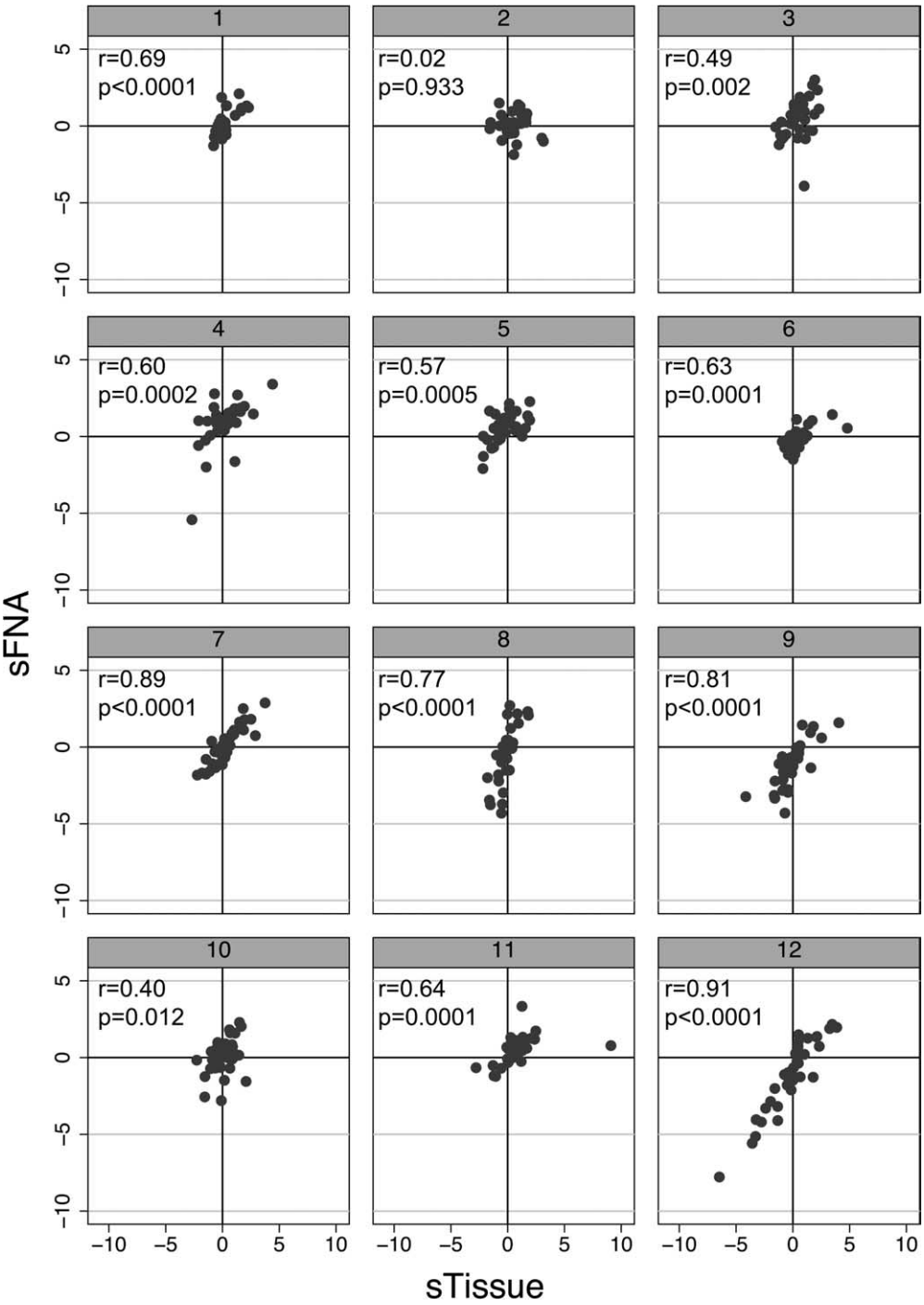

Fig. 3. Scatterplot and Spearman Correlations between the gene expression profiles obtained by FNA (averaged of FNA-A and FNA-B (sFNA)) and gene expression profiles obtained by tissue biopsy (sTissue) per patient. Only patient no. 12 was ER−; the remaining were 100% ER+.

ple, ER, PgR and ERBB2/HER2 showed significant heterogeneity in 42–66% of patients ($p < 0.05$, t-test), which parallels earlier observations at the protein level using immunohistochemistry [6,15,19]. This underscores one of the difficulties in using single genes as markers, i.e., they are prone to false positive/negative

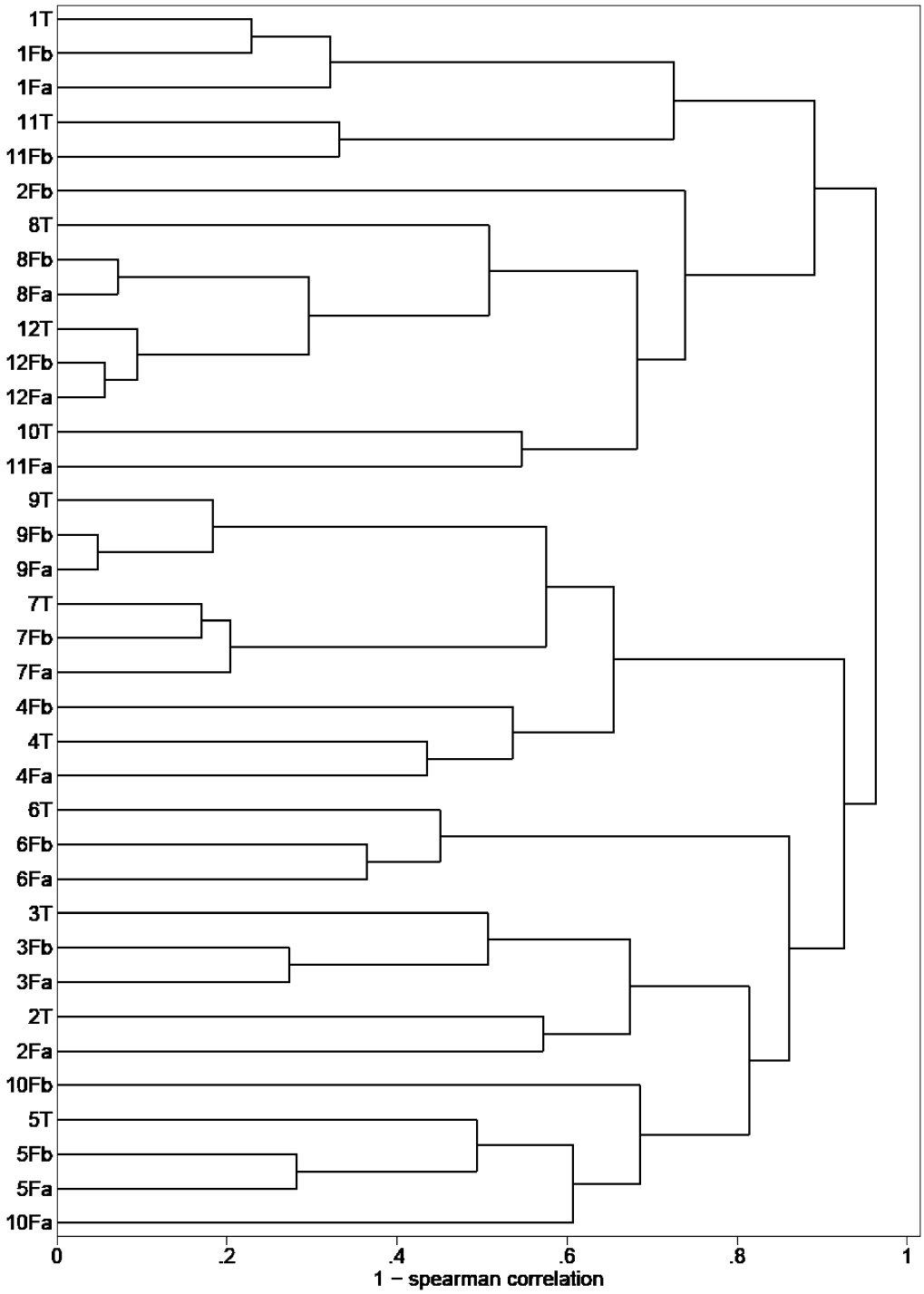

Fig. 4. Cluster analysis of the three samples per patient (FNA-A, -B and tissue biopsy) for each of the 12 patients. Clustering was observed for 9 of 12 patients, whereas only one FNA-sample and the tissue biopsy clustered for patient no. 2 and 11. Fa: FNA-A. Fb: FNA-B. T: tissue biopsy.

results depending on the intra-tumor site of sampling.

An essential question is whether the observed genetic heterogeneicity at the single gene level is due to either methodological errors or inherent differences in the two tumor halves, which also could be observed by morphological and immunohistochemical analysis

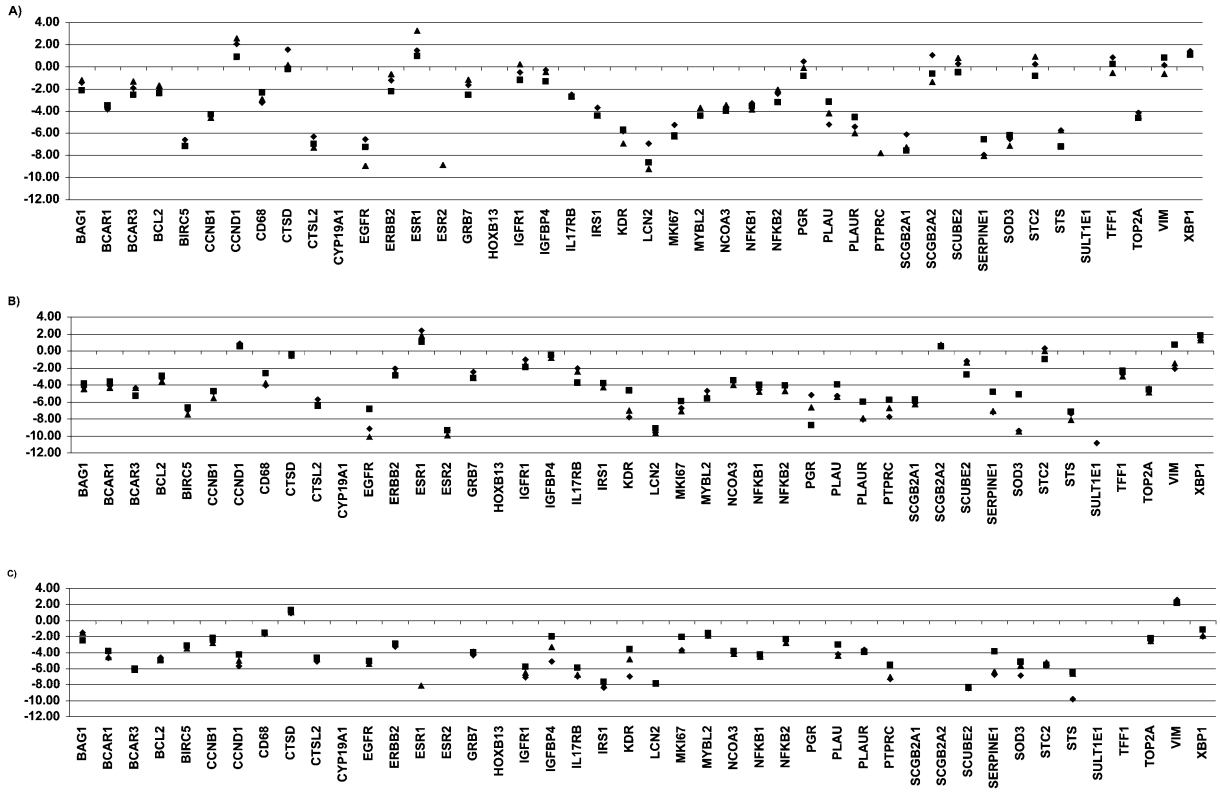

Fig. 5. Normalized gene expression (ΔCt) values obtained by FNAs or tissue biopsy samples for the 44 individual genes. Three representative patients shown: (A) no. 7 (ER+), (B) no. 9 (ER+) and (C) no. 12 (ER−). ◆ FNA-A, ▲ FNA-B and ■ the tissue biopsy.

of the two tumor halves. There seems no plausible cause for methodologically introduced heterogeneity observed between the tumor halves as the halves were treated identically throughout the process, strict precautions were taken to prevent cross-contamination and replicates were run for confirmation of reproducibility of results. Further, FNA contains primarily tumor cells [18] and the selected target genes are primarily expressed by tumor cells, minimizing the likelihood that the genetic heterogeneity is due to admix of non-cancerous cells. In addition, there seemed no inherent differences in the two tumor halves as examined by H&E and IHC.

As most tumor halves showed homogeneity at the protein level using IHC, the use of qRT-PCR is confirmed as being more sensitive to the amounts of mRNA in the cells, as methodological causes are excluded. As seen in Table 1, 7, 2 and 8 patients were found to have heterogeneous expression between the tumor halves at the mRNA level of ER, PgR and HER2 respectively, although only 0, 2 and 1 patients, ER, PgR and HER2 respectively, were found to exhibit heterogeneous expression at the protein level (Table 2). These findings demonstrate the greater sensitivity of investigating gene expression levels for sub-grouping of patients [11].

When gene expression data were analyzed as profiles of multiple genes, this heterogeneity was much less decisive. In 18 out of 24 FNA samples, the profile of one tumor half exhibited the highest correlation to its rightful counterpart. For the remaining 6 tumor halves, blinded statistical analysis was unable to match with their rightful counterpart as they exhibited profiles that were close to the population average. A cluster analysis supported the findings by Spearman Correlation, identifying the same 9 patients, in which the FNA-A, -B and tissue biopsy clustered. Since nearly all tumors (11/12) were ER+, it was expected that these genes would be expressed at similar levels as they were selected based on their reported involvement in ER pathway and/or role in endocrine responsiveness. In this setting, it was not surprising that 3/12 patients were not matched with their correct counterparts in the blinded study. In addition, 2 of the 3 patients in whom the two FNAs did not match were ILC. This may relate to the characteristic diffuse infiltrat-

ing growth pattern of this subtype, where the cancer cells grow in so-called Indian files surrounding benign ductal glandular tissue and as single tumor cells situated in fibrous stroma. FNAs from this subtype may exhibit greater variation in the cancer cell-to-normal stromal/epithelial cell ratio and thus show more heterogeneous gene expression profiles.

Overall, our study support the use of gene profiles instead of single genes to compensate for single-gene variation, and when using FNA it is important to perform tumor sampling from different parts of tumor in order to avoid incorrect decisions due to neglecting heterogeneity within a tumor.

As expected, the FNA sampling technique yielded smaller amounts of material, and thus smaller amounts of RNA, than the tissue biopsies. The breast tumor FNAs yielded a median of 1.4 million cells (0.2–2.06 million cells, $n = 24$), while the 25 mg tumor tissue biopsies (approx. 0.25 cm^3) were estimated to contain approximately 250 million cells (1 cm^3 tumor $=$ 10^9 cells [8]). This resulted in $>95\%$ of the genes being detected in all patients using tissue biopsies, while only $> 64\%$ of the genes were detected in $11/12$ patients when sampled by FNA. However, compared to earlier studies these values are highly acceptable [3, 12]. To our knowledge, no other investigators have examined the efficacy of using non-amplified RNA from breast cancer FNAs for quantitative-PCR analysis. In a study by Assersohn et al. [3], the feasibility of using FNA for microarray analysis in neoadjuvant treated patients was investigated, finding that adequate RNA for successful microarray analysis could only be obtained in 4 of 27 patients (15%). Similar success rates in obtaining RNA of sufficient quality for successful microarray analysis was observed in two other cancer FNA studies (10% [20] and 39% [12]), even when RNA was amplified. The comparatively lower amount of RNA required for LDA analysis is an important issue that overcomes previous limitations when using FNA for microarray analysis.

Importantly, we observed that in different patients the same genes failed to be detected when the amount of sample material was low. This illustrates the differential level of mRNA transcripts in cells; some genes are abundant and thus detectable in samples with low amounts of total RNA, whereas others are not. These observations highlight the importance of gene selection in that some genes might be too sensitive to the RNA concentration to provide information in most clinical samples. In case a low-abundant gene is found to have crucial informative value, amplification can be considered prior to LDA. In these incidences, it is recommended that the qRT-PCR primer-probe location is close to the poly-A tail of the mRNA if amplification is conducted with oligo(dT)-primers, or using gene-specific amplification primers of the qRT-PCR site, to ensure reliable quantitative results.

The above mentioned study by Assersohn et al. indicated a median Pearson correlation of 0.69 between the gene expression of the 4 FNAs and the corresponding tumors, which they considered good [3]. These correlations are comparable to the values observed for the FNA pairs in our study. Since this was a non-selective, consecutive study sampling of newly diagnosed patients, one ER− patient (no. 12) was included who, as illustrated by the scatterplots in Figs 2 and 3, exhibited a distinct gene expression pattern compared to the rest, demonstrating the feasibility of this assay to differentiate between breast cancer subpopulations.

In addition to investigate heterogeneity, this study examined the possibility of assessing FNA samples for endocrine responsiveness using gene expression analysis by LDA in order to determine which genes should be further investigated in such a predictive test. Identification of non-responders could be offered a different treatment than Tamoxifen as aromatase inhibitors, e.g. Letrozol, which inhibits the synthesis of ligand for the ER *in vivo*.

Some genes exhibit pronounced heterogeneous expression, and in these cases the cut off limit may be set higher to avoid false positive or false negative results. This underscores the importance of testing genes and validating their expression pattern before their use as parameters in clinical diagnosis and treatment decisions.

5. Conclusion

Neoadjuvant therapy is being applied more frequently in order to reduce the size of surgically-inoperable tumors as well as large operable primary breast cancers. A preoperative characterization of tumors, conducted within 24 hours that could aid the clinician in determining the optimal neoadjuvant therapy is an attractive concept. Herein, we found that although some genes are very heterogeneously expressed, the composition as a profile enables them to be distinct, providing patients with their own tumor gene expression signatures. To avoid false positive/negative results the heterogeneity of individual genes could advantageously be pre-tested and taken into account in

the design of the gene expression profile. To reduce the impact of the heterogeneity, FNA samples should be obtained from several different areas of the tumor. Our study supports the concept of treatment-based decisions by gene expression profiles.

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