Featured Article

Molecular Profiling of Inflammatory Breast Cancer: Identification of a Poor-Prognosis Gene Expression Signature

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ABSTRACT

Purpose: **Inflammatory breast cancer (IBC) is a rare but particularly aggressive form of primary breast cancer. The molecular mechanisms responsible for IBC are largely unknown.**

Experimental Design: **To obtain further insight into the molecular pathogenesis of IBC, we used real-time quantitative reverse transcription (RT)-PCR to quantify the mRNA expression of 538 selected genes in IBC relative to non-IBC.**

Results: **Twenty-seven (5.0%) of the 538 genes were significantly up-regulated in IBC compared with non-IBC. None were down-regulated. The 27 up-regulated genes mainly encoded transcription factors (*JUN, EGR1, JUNB, FOS, FOSB, MYCN,* and *SNAIL1*), growth factors (*VEGF, DTR/HB-EGF, IGFBP7, IL6, ANGPT2, EREG, CCL3/MIP1A,* and *CCL5/RANTES*) and growth factor receptors (*TBXA2R, TNFRSF10A/TRAILR1,* and *ROBO2*). We also identified a gene expression profile, based on *MYCN, EREG,* and *SHH*, which discriminated subgroups of IBC patients with good, intermediate, and poor outcome.**

Conclusion: **Our study has identified a limited number of signaling pathways that require inappropriate activation for IBC development. Some of the up-regulated genes identified here could offer useful diagnostic or prognostic markers and could form the basis of novel therapeutic strategies.**

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INTRODUCTION

Inflammatory breast cancer (IBC) is the most lethal form of primary breast cancer (1). It is characterized clinically by erythema, dimpling of the skin ("peau d'orange"), and rapid onset (2). The 3-year survival rate is about 40% among patients with IBC, compared with 85% among patients with non-IBC (1). Little is known of the molecular mechanisms underlying the genesis and progression of IBC (3). *TP53* and *ERBB2*, genes involved in breast carcinogenesis, are more frequently altered in IBC than in non-IBC (4). A few genetic alterations, such as *WISP3/LIBC, ARHC/RhoC,* and *CDH1/E-cadherin*, have been specifically linked to the inflammatory phenotype in this setting (5, 6). Several authors have reported that IBC tends to be highly vascularized (7, 8).

The recent advent of efficient tools for large-scale assessment of gene expression has already provided new insights into the involvement of gene networks and regulatory pathways in various tumoral processes (9). cDNA microarrays can be used to test the expression of thousands of genes at a time, whereas real-time reverse transcription (RT)-PCR is a more accurate and quantitative assay method applicable to smaller numbers of selected candidate genes (10, 11).

To obtain further insight into the molecular pathogenesis of IBC, we used real-time quantitative RT-PCR to quantify the mRNA expression of many selected genes in pooled IBC samples, in comparison with pooled non-IBC samples. We assessed the expression level of 538 genes known to be involved in various cellular and molecular mechanisms associated with tumorigenesis, focusing on genes related to angiogenesis and inflammation. Genes of interest were further investigated in 36 individual IBC samples in comparison with 22 non-IBC samples.

MATERIALS AND METHODS

Patients and Samples

IBC samples were surgical biopsy specimens obtained from 36 women with clinical IBC treated at Saint-Louis Hospital (Paris, France). All tumors were diagnosed on the basis of rapidly progressive signs such as localized or generalized induration, redness, and edema of the breast, and thus classified T4d (International Union Against Cancer classification, 1977). All biopsies were done before treatment, and all confirmed the diagnosis of infiltrating carcinoma. All patients underwent a first-line anthracycline-based high-dose chemotherapy followed by the local treatment. At the time of this analysis, 27 patients had relapsed and nine remained disease-free.

As "non-IBC" controls, we used 22 specimens of non-inflammatory locally advanced breast cancers including 6 stage IIb and 16 stage III. These 22 non-IBC controls were all high-grade invasive ductal carcinomas, *i.e.,* Scarff-Bloom-Richardson histopathological grade III. The expression levels of

the 538 genes in IBCs were expressed relative to the expression levels in non-IBCs.

The tumor samples were flash-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Tumor samples containing >70% of tumor cells were considered suitable for the study.

Real-Time RT-PCR

Theoretical Basis. Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a substantial increase in fluorescence is observed. The parameter threshold cycle (C_t) is defined as the fractional cycle number at which the fluorescence generated by cleavage of a TaqMan probe (or by SYBR green dye-amplicon complex formation) passes a fixed threshold above baseline. The increase in fluorescent signal associated with exponential growth of PCR products is detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), using PE Biosystems analysis software according to the manufacturer's manuals.

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (*i.e.,* lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of two endogenous RNA control genes involved in two cellular metabolic pathways, namely *TBP* (GenBank accession NM_003194), which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIID), and *RPLP0* (NM_001002), which encodes human acidic ribosomal phosphoprotein P0. Each sample was normalized on the basis of its *TBP* (or *RPLPO*) content.

We selected *TBP* as an endogenous control because the prevalence of its transcripts is moderate and because there are no known *TBP* retropseudogenes (retropseudogenes lead to co-amplification of contaminating genomic DNA and thus interfere with RT-PCR, despite the use of primers in separate exons). We also selected *RPLP0* because the prevalence of its transcripts is high as compared with *TBP* and because this gene is used widely as an endogenous control for Northern blot analysis (known better as 36B4).

Results, expressed as *N*-fold differences in target gene expression relative to the *TBP* (or *RPLPO*) gene, and termed "N*target*," were determined as $N\textit{target} = 2^{\Delta C_t}\ \textit{sample}$, where the ΔC_t value of the sample was determined by subtracting the average C_t value of the target gene from the average C_t value of the *TBP* (or *RPLP0*) gene (12, 13).

The N*target* values of the samples were subsequently normalized such that the median of the non-IBC N*target* values was 1.

Primers and Controls. Primers for *TBP*, *RPLP0*, and the 538 target genes (list in Supplemental data) were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN).

We conducted searches in database of Expressed Sequence Tags (dbEST), high-throughput genomic sequence (htgs) database, and protein (nr) database to confirm the total gene specificity of the nucleotide sequences chosen as primers and the absence of single nucleotide polymorphisms. In particular, the primer pairs were selected to be unique relative to the sequences of closely related family member genes or of the corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons, if possible. In general, amplicons were between 70 and 120 nucleotides long. Gel electrophoresis was used to verify the specificity of PCR amplicons.

For each primer pair, we performed no-template control and no-reverse-transcriptase control assays, which produced negligible signals (usually >40 in C_t value), suggesting that primer-dimer formation and genomic DNA contamination effects were negligible.

RNA Extraction. Total RNA was extracted from frozen tumor samples with the acid-phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, the 18S and 28S RNA bands being visualized under UV light.

cDNA Synthesis and PCR Reaction Conditions cDNA Synthesis and PCR reaction conditions have been described previously (13).

Statistical Analysis

Because the mRNA levels did not fit a Gaussian distribution, (*a*) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation, and (*b*) relationships between the molecular markers and clinical and histologic parameters were tested using the nonparametric Kruskal-Wallis (14) or Mann-Whitney *U* test (15).

To visualize the capacity of a given molecular marker to discriminate between two populations (in the absence of an arbitrary cutoff value), we summarized the data in a receiver operating characteristic (ROC) curve (16). The area under curve (AUC) was calculated as a single measure for the discriminatory capacity of each molecular marker. When a molecular marker has no discriminatory value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. In contrast, when a molecular marker has strong discriminatory value, the ROC curve moves to the upper left-hand corner, and the AUC is close to 1.

Hierarchical clustering was done with the GenANOVA software (17).

RESULTS

We first determined the mRNA expression level of the 538 genes in an IBC pool and a non-IBC pool. Genes with an expression in the IBC pool differing (>2-fold) from that in the non-IBC pool were then examined for their mRNA expression in 36 individual IBCs relative to 22 non-IBCs. Gene expression levels in the IBCs were expressed relative to the corresponding levels in the non-IBCs.

Expression of the 538 Genes in the IBC and Non-IBC Pools. The IBC and non-IBC pools were each prepared by mixing identical amounts of tumor RNA from eight patients. The mean *TBP* gene C_t values for the eight tumor samples were 25.81 ± 0.33 (IBC pool) and 25.64 ± 0.37 (non-IBC pool).

Very low levels of target gene mRNA that were only detectable but not reliably quantifiable by real-time quantitative

RT-PCR assays, mainly based on fluorescence SYBR green methodology ($C_t > 32$), were observed for 47 (8.7%) of the 538 genes in both the IBC and non-IBC pools.

Forty-eight (9.6%) of the 491 remaining genes were expressed at a different level (>2-fold) in the IBC pool compared with the non-IBC pool; 40 (83.3%) were up-regulated, and 8 (16.7%) were down-regulated.

mRNA Expression of *ESR1/ERα, MKI67,* and 48 Candidate Genes in 36 IBCs and 22 Non-IBCs. The expression level of the 48 dysregulated genes identified by pooled sample analysis was then determined individually in 36 IBCs and 22 non-IBCs. Twenty-seven (67.5%) of the 40 up-regulated genes identified by the pooled sample analysis were significantly up-regulated in the 36 individual IBCs relative to the 22 non-IBCs ($P < 0.05$; Table 1). None of the eight down-regulated genes identified by the pooled sample analysis was significantly down-regulated in the individual IBCs.

The 27 up-regulated genes mainly encoded transcription factors (*JUN, EGR1, JUNB, FOS, FOSB, MYCN,* and *SNAIL1*), growth factors (*VEGF, DTR/HB-EGF, IGFBP7, IL6, ANGPT2, EREG, CCL3/MIP1A,* and *CCL5/RANTES*), and growth factor receptors (*TBXA2R, TNFRSF10A/TRAILR1,* and *ROBO2*).

ROC curve analysis was then used to test the capacity of each of these 27 genes to discriminate between IBC and non-IBC. The overall diagnostic values of the 27 molecular markers was assessed in terms of their AUC values (Table 1). Fig. 1 shows the mRNA levels of the three most discriminatory genes, namely *JUN* (AUC-ROC, 0.908), *EGR1* (AUC-ROC, 0.896), and *DUSP1* (AUC-ROC, 0.896), in each non-IBC and IBC sample.

In the same set of 58 tumors, we also examined the expression of the *ESR1/ERα* and *ERBB2* genes and that of the proliferation-associated gene *MKI67* gene that encodes the proliferation-related antigen Ki-67. *MKI67* shows a trend toward

Table 1 List of the significantly dysregulated genes in IBCs relative to non-IBCs

Genes	Gene definition	Gene characterisation	Non-IBC ($n = 22$)	IBC ($n = 36$)	*P**	*ROC-AUC*
JUN	Jun oncogene	Transcription factor	1.0 (0.2–10.4)†	8.3 (0.8–230.7)	$<10^{-6}$	0.908
EGR1	Early growth response 1 (Krox-24)	Transcription factor	1.0 (0.1–5.2)	13.8 (0.3–625.1)	$<10^{-6}$	0.896
DUSP1	Dual specificity phosphatase 1 (CL100, MKP-1)	Signaling transduction	1.0 (0.2–4.8)	13.7 (0.6–303.0)	$<10^{-6}$	0.896
JUNB	Jun B oncogene	Transcription factor	1.0 (0.1–4.2)	6.7 (0.6–47.9)	$<10^{-6}$	0.893
FOS	Fos oncogene	Transcription factor	1.0 (0.3–15.9)	30.1 (0.4–1051.5)	$<10^{-6}$	0.886
FOSB	Fos B oncogene	Transcription factor	1.0 (0.1–19.7)	40.5 (0.2–1580.4)	0.000002	0.876
VEGF	Vascular endothelial growth factor	Angiogenesis	1.0 (0.1–2.4)	2.9 (0.5–13.8)	0.000008	0.852
DTR/HB-EGF	Diphtheria toxin receptor (heparin-binding EGF-like growth factor)	Growth factor	1.0 (0.3–4.9)	4.7 (0.4–56.4)	0.00004	0.824
TBXA2R	Thromboxane A2 receptor	Angiogenesis	1.0 (0.4–4.0)	4.4 (1.2–22.2)	0.00004	0.824
PTGS2/COX2	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)	Angiogenesis	1.0 (0.2–22.9)	10.7 (0.2–256.3)	0.00004	0.824
IGFBP7	Insulin-like growth factor binding protein 7 (IGFBP-rP1/MAC25)	Growth factor	1.0 (0.1–2.8)	3.1 (0.3–23.1)	0.00012	0.803
IL6	Interleukin 6	Growth factor	1.0 (0.2–20.6)	9.3 (0.4–93.11)	0.00020	0.793
NOS3	Nitric oxide synthase 3, endothelial (ENOS)	Oxidative stress	1.0 (0.2–3.6)	3.5 (0.6–9.6)	0.00029	0.785
MAP3K8/COT	Mitogen-activated protein kinase kinase kinase 8	Signaling transduction	1.0 (0.3–3.0)	2.7 (0.1–11.6)	0.00068	0.768
RASGRF1	Ras protein-specific guanine nucleotide-releasing factor 1	Signaling transduction	1.0 (0.1–108.0)	6.6 (0.7–183.5)	0.0012	0.755
KAI1	Kangai 1 (CD82 antigen)	Cell motility	1.0 (0.2–4.1)	3.4 (1.0–10.5)	0.0014	0.752
THBD	Thrombomodulin	Angiogenesis	1.0 (0.2–4.6)	3.6 (0.5–23.6)	0.0014	0.751
PPARGC1	Peroxisome proliferative activated receptor, gamma, coactivator 1 (PGC1)	Transcription factor coactivator	1.0 (0.0–14.0)	3.0 (0.4–156.6)	0.0024	0.739
ANGPT2	Angiopoietin 2	Angiogenesis	1.0 (0.2–4.7)	3.0 (0.3–10.7)	0.0024	0.739
EREG	Epiregulin	Growth factor	1.0 (0.0–173.2)	16.1 (0.2–10744.5)	0.0037	0.729
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a (TRAILR1, DR4)	Apoptosis	1.0 (0.4–2.3)	2.7 (0.2–17.9)	0.0043	0.725
ROBO2	Roundabout homolog 2	Growth factor receptor	1.0 (0.0–771.6)	14.8 (0.0–1521.1)	0.0073	0.711
CCL3/MIP1A	Chemokine (C-C motif) ligand 3	Growth factor	1.0 (0.1–5.6)	2.0 (0.2–31.2)	0.019	0.684
MYCN	N-myc oncogene	Transcription factor	1.0 (0.1–41.8)	3.1 (0.2–144.4)	0.021	0.682
CCL5/RANTES	Chemokine (C-C motif) ligand 5	Growth factor	1.0 (0.1–5.0)	3.2 (0.1–33.1)	0.029	0.672
SNAIL1	Snail homolog 1	Transcription factor	1.0 (0.3–5.3)	2.2 (0.5–7.3)	0.043	0.660
H19	H19, imprinted maternally expressed untranslated mRNA	Development	1.0 (0.1–13.4)	6.4 (0.1–58.6)	0.046	0.657
MKI67	Proliferation-related Ki-67 antigen	Cell cycle regulation	1.0 (0.1–3.7)	1.6 (0.2–21.2)	NS (0.07)	0.644
ESR1/ERα	Estrogen receptor 1 (α)	Nuclear receptor	1.0 (0.0–21.1)	0.4 (0.0–21.3)	NS (0.18)	0.606

* Kruskal-Wallis test.

‡ Median (range) of gene mRNA levels.

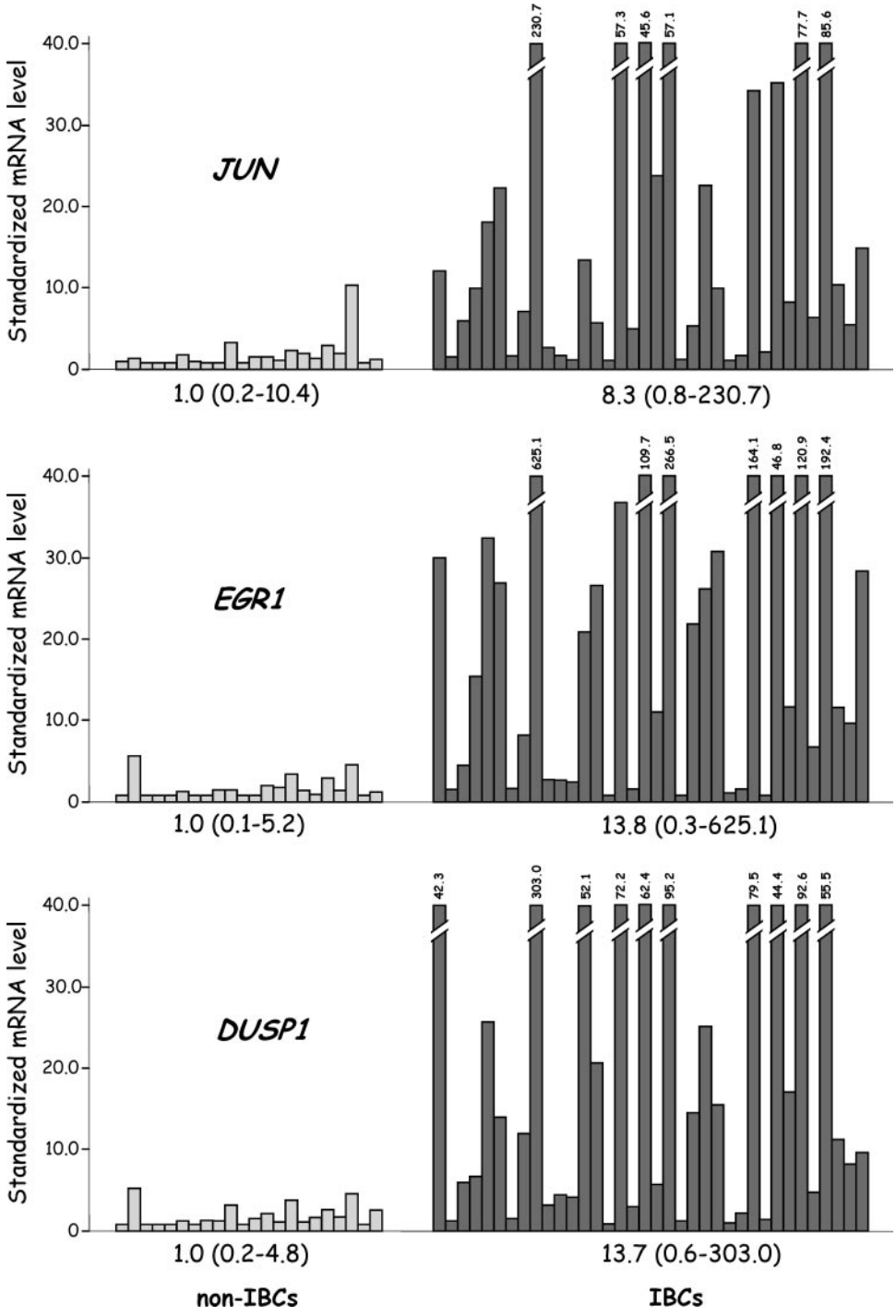

Fig. 1 mRNA levels of *JUN*, *EGR1*, and *DUSP1* in 22 individual non-IBCs (*gray bars*) and 36 IBCs (*black bars*). Median values (and ranges) are indicated for each tumor subgroup.

overexpression in IBC compared with non-IBC ($P = 0.07$), whereas *ESR1/ER*α and *ERBB2* expressions were similar in the two tumor types.

The mRNA levels indicated in Table 1 (calculated as described in Materials and Methods) show the abundance of the target relative to the endogenous control (*TBP*), to normalize the starting amount and quality of total RNA. Similar results were obtained with a second endogenous control, *RPLP0* (also known as 36B4). Indeed, the 27 up-regulated genes were also significantly up-regulated in the IBCs relative to the non-IBCs.

mRNA Expression of the 48 Candidate Genes in IBC, According to Relapse. Twenty-seven (75%) of the 36 patients with IBC relapsed, a proportion in keeping with published data (18). Comparison of median mRNA levels of the 48 genes between patients who relapsed ($n = 27$) and those who did not relapse ($n = 9$) identified three genes with significantly different

Table 2 List of the significantly dysregulated genes in IBC patients who had relapsed relative to IBC patients who did not relapse

Genes	Gene definition	Gene characterisation	IBC without relapse (n = 9)	IBC with relapse (n = 27)	P *	ROC-AUC
MYCN	*N*-myc oncogene	Transcription factor	12.6 (0.4–144.4) †	2.0 (0.2–85.6)	0.018	0.765
EREG	Epiregulin	Growth factor	34.9 (1.2–10744.5)	14.7 (0.2–3507.7)	0.026	0.753
SHH	Sonic hedgehog homolog	Growth factor	0.0 (0.0–4.7)	2.1 (0.0–1099.1)	0.045	0.726

* Mann-Whitney's *U* test.
† Median (range) of gene mRNA levels.

expression ($P < 0.05$), namely *MYCN, EREG,* and *SHH* (Table 2). *MYCN* was the most discriminatory gene (AUC-ROC 0.765). It is noteworthy that *SHH* mRNA levels were higher in patients who relapsed, whereas *MYCN* and *EREG* mRNA levels were lower in patients who relapsed.

Finally, hierarchical clustering of the samples, based on the expression of these three genes, subdivided the patient population into three groups with significantly different outcomes (P = 0.003; Fig. 2): 16 patients with poor outcome (all but 1 relapsed), 11 with intermediate outcome (9 relapsed) and 9 patients with good outcome (only 3 relapsed).

DISCUSSION

We first used real-time quantitative RT-PCR to quantify the mRNA expression of 538 selected genes in pooled IBC samples relative to pooled non-IBC samples. The 48 genes of interest thus identified were then investigated in 36 individual IBCs and 22 non-IBCs. Using the same approach, we have shown the involvement of several altered molecular pathways in the genesis of prostate and liver cancer (10, 11).

Real-time quantitative RT-PCR is a promising alternative to cDNA microarrays for molecular tumor profiling. In particular, real-time RT-PCR is far more precise, reproducible, and quantitative than cDNA microarrays. Real-time RT-PCR is also more useful for analyzing weakly expressed genes (such as *EREG, ROBO2,* and *SHH* in the present study). Finally, real-time RT-PCR requires smaller amounts of total RNA (about 2 ng/target gene) and is therefore suitable for analyzing small tumor samples or microdissected tumor samples.

We studied a number of genes involved in various cellular and molecular mechanisms associated with tumorigenesis and known to be altered (mainly at the transcriptional level) in various cancers. These genes encode proteins involved in cell cycle control, cell-cell interactions, signal transduction pathways, apoptosis, angiogenesis, etc. (list in Supplemental data). After scrutinizing the literature, we also included many genes involved in angiogenesis and inflammation.

Among the 538 genes analyzed, 27 (5.0%) showed substantial up-regulation in IBC compared with non-IBC, suggest-

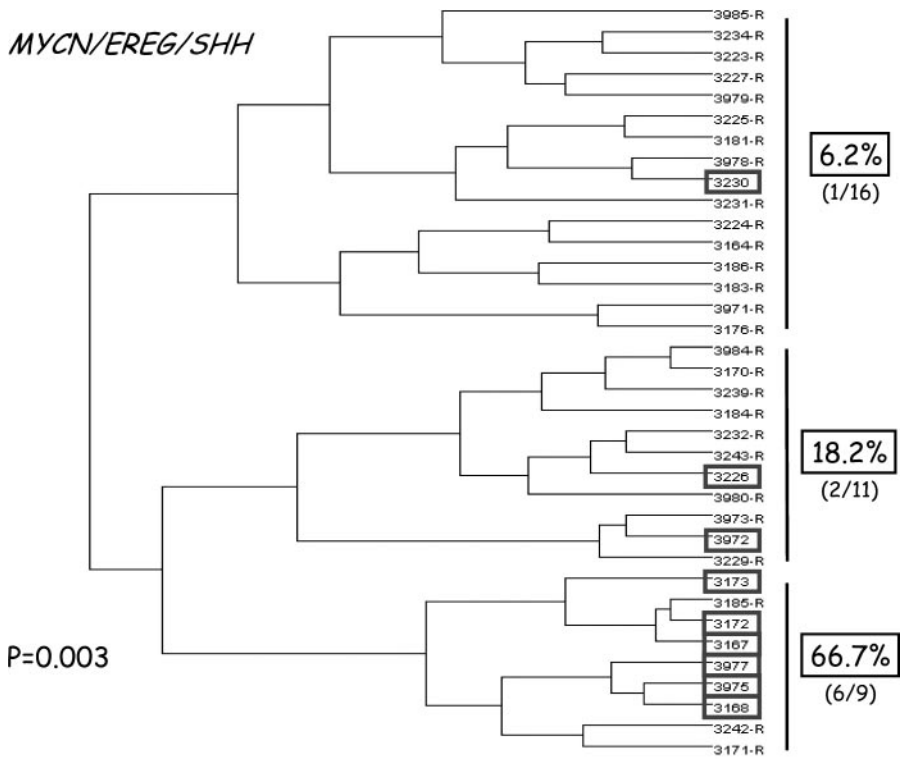

Fig. 2 Dendrogram of 27 IBC patients who relapsed (*R*) and 9 IBC patients who did not relapse (*circled*), constructed by hierarchical clustering, according to *MYCN, EREG,* and *SHH* expression. Percentages of relapse-free patients in each IBC subgroup are indicated on the right.

ing that several signaling pathways are specifically involved in IBC (Table 1).

Some results of this study are in partial agreement with those reported in the IBC literature. First, *MKI67* was overexpressed in IBC (but not significantly), supporting higher cell proliferation rates than in non-IBC. *MKI67* encodes Ki-67 antigen, a large protein of unknown function that is a classical histopathological marker associated with cell proliferation. Paradiso *et al.* (19) have reported higher proliferative activity (mesuared by [^{3}H]thymidine autoradiographic labeling index) in IBC than in non-IBC.

Second, genes involved in angiogenesis (*VEGF, TBXA2R, PTGS2/COX2, THBD/thrombomodulin,* and *ANGPT2/angiopoietin 2*) were also up-regulated. Several authors have reported that IBC has strong angiogenic potential, using different approaches and experimental systems including the following: analysis of microvessel density and endothelial cell proliferation in IBC (6) and analysis of histologic characteristics and molecular basis of human IBC xenograft in nude mice (7, 8). However, we found that other best-known major angiogenic genes (*VEGF2, VEGF3, VEGF4, VEGFR1, VEGFR2,* and *VEGFR3*) had similar expression levels in IBC and non-IBC.

Likewise, IBC and non-IBC showed similar expression levels of the best-known inflammatory cytokines (see list in Supplemental data), including *IFNG, TNF, IL1A, IL1B, IL8*, and *IL10*, tending to confirm the hypothesis that the inflammatory phenotype of IBC is attributable to blockage of the dermal lymphatics by the tumor infiltrate and not to infiltration by inflammatory cells (3). It is noteworthy, however, that *IL6* and the genes encoding the chemokines CCL3/MIP1A and CCL5/RANTES were up-regulated (Table 1). Interestingly, the gene *CCR5* encoding the specific receptor of both CCL3/MIP1A and CCL5/RANTES was also up-regulated in IBC, although not significantly ($P = 0.11$), and had a very similar expression pattern to *CCL3* and *CCL5* ($r = +0.539$, $P = 0.00078$ and $r = +0.891$, $P < 10^{-6}$, respectively; Spearman rank correlation test). Moreover, the two other CCR5 ligands (*CCL4/MIP1B* and *CCL8/MCP-2*), although not significantly up-regulated in IBC, also had a very similar expression pattern to *CCR5* ($r = +0.889$, $P < 10^{-6}$ and $r = +0.738$, $P < 10^{-6}$, respectively; Spearman rank correlation test) in IBC samples.

Finally, IBC and non-IBC showed similar expression levels of the genes *WISP3/LIBC, ARHC/RhoC,* and *CDH1/E-cadherin*, which has previously been described as specifically altered in IBC (5, 6).

Our results suggest that specific molecular signaling pathways are altered in IBC compared with non-IBC. We found that most components of the AP-1 transcription factor family, including *JUN, JUNB, FOS,* and *FOSB*, were up-regulated (Table 1). AP-1 has been implicated in a variety of tumoral processes, including inflammation, cell transformation, invasive growth, angiogenesis, and metastasis (20). Interestingly, we also observed the up-regulation of major downstream target genes of the altered AP-1 pathway, *i.e., DTR/HB-EGF* (21), *PTSG2/COX2* (22), and *MAP3K8/COT* (23). AP-1 transcription factor activation may be attributable to many different signaling pathways, including the binding of IL6 (the *IL6* gene was up-regulated in our study) to its receptor (24).

Other early growth-response genes that code for transcription factors (*MYCN* and *EGR1*) or nonreceptor-type protein-tyrosine phosphatase (*DUSP1*) were up-regulated in IBC. Like AP-1, *ERG1* (also known as *KROX24*) *and DUSP1* (also known as *CL100* and *MKP-1*) are mainly activated by hypoxia, environmental stress, and proinflammatory cytokines (25, 26). It is noteworthy that *HIF1A* (Hypoxia-inducible factor 1α) gene, encoding a key transcription factor induced by hypoxia (27), was also overexpressed in IBC but not significantly ($P = 0.06$). Taken together, our results suggest that intratumoral hypoxia, possibly attributable to avid embolus formation, may be a prominent feature of IBC. Hypoxia is thought to drive angiogenesis and to be an important contributor to radiation and drug resistance (27).

We attempted to identify markers of IBC aggressiveness by comparing the gene transcription profiles according to relapse status, and we identified a three-gene expression signature predictive of relapse (*MYCN, EREG,* and *SHH*).

EREG (epiregulin) belongs to the EGF growth factor family. This ERBB ligand, like DTR/HB-EGF (also up-regulated in our study), binds both ERBB1 and ERBB4 (28). *SHH* codes for the most important molecule of the Hedgehog-Gli signaling pathway. Inappropriate activation of the Hedgehog-Gli signaling pathway occurs in several malignancies, including pancreas, brain, and skin tumors (29, 30). The third gene of our gene expression signature, *MYCN*, is an unexpected gene involved in breast tumorigenesis. Indeed, *MYCN* alteration is classically described in neuroblastoma and retinoblastoma, in opposite to *MYC* (not up-regulated in our IBC study) that is altered in numerous malignancies including breast cancer. Interestingly, *MYCN* was recently identified as a major direct target of SHH pathways in medulloblastoma, a childhood-onset brain tumor (31). However, we observed a negative correlation between *SHH* and *MYCN* expression in IBC ($r = -0.411$, $P = 0.012$; Spearman rank correlation test). Taken together, these results point to complex regulation of *MYCN* expression by *SHH*, through various Gli transcription factors (GLI, GLI2, GLI3, or GLI4), specific to each cancer type.

In conclusion, this study points to the involvement of several altered molecular pathways in IBC tumorigenesis. Additional studies are necessary to identify the genetic or epigenetic mechanisms responsible for the altered gene expression and to determine the cells types (tumoral epithelial cells or various stromal including fibroblasts, macrophages, lymphocytes, or other cells) responsible for the altered expression of each gene. We identified a gene expression signature of poor-prognosis IBC that warrants validation in larger series. Finally, some of the genes identified here could form the basis for novel IBC treatment strategies. In this regard, it is noteworthy that *EREG* and *SHH* encode secreted proteins that could be studied in human xenograft models of IBC.

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