Subtypes of Breast Cancer Show Preferential Site of Relapse

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Abstract

We explored whether the five previously reported molecular subtypes in breast cancer show a preference for organ-specific relapse and searched for molecular pathways involved. The "intrinsic" gene list describing the subtypes was used to classify 344 primary breast tumors of lymph node–negative patients. Fisher exact tests were used to determine the association between a tumor subtype and a particular site of distant relapse in these patients who only received local treatment. Modulated genes and pathways were identified in the various groups using Significance Analysis of Microarrays and Global Testing. Bone relapse patients were most abundant in the luminal subtypes but were found less than expected in the basal subtype. The reverse was true for lung and brain relapse patients with the remark that absence of lung relapse was luminal A specific. Finally, a pleura relapse, although rare, was found almost exclusively in both luminal subtypes. Many differentially expressed genes were identified, of which several were in common in a subtype and the site to which the subtype preferentially relapsed. WNT signaling was up-regulated in the basal subtype and in brain-specific relapse, and down-modulated in the luminal B subtype and in bone-specific relapse. Focal adhesion was found up-regulated in the luminal A subtype but down-regulated in lung relapse. The five major molecular subtypes in breast cancer are evidently different with regard to their ability to metastasize to distant organ(s), and share biological features and pathways with their preferred distant metastatic site. [Cancer Res 2008;68(9):3108–14]

Introduction

Molecular subtypes in breast cancer were first described by Perou et al. (1) who mapped the phenotypic diversity seen in breast cancer to a specific gene expression pattern. Later studies reported differences in prognosis (2, 3) and chemotherapy response (4) with respect to the subtypes in specific patient cohorts. These observations reinforce the hypothesis that the molecular gene expression patterns specific for a subtype have clinical relevance. Besides the above-mentioned clinical aspects (prognosis and response to therapy), the distant site to which a tumor preferentially metastasizes is of clinical and biological importance. Recently, microarray analyses identified gene expression profiles for bone (5–7) and lung (8) metastasis in breast cancer. One of the reported aspects is that the aggressiveness of a tumor, i.e., the ability to metastasize, is driven by a distinct set of genes, different from those involved in the capacity to home, survive, and proliferate in a particular organ (6, 8). This may tie in with the self-seeding theory (9) and the seed and soil theory of Paget (10). The latter theory proposes that specific organs are in some way predisposed targets for secondary growth. This may reflect the necessity for the primary tumor to express a certain genetic module to invade specific organs. The self-seeding theory offers the view that dislodged cancer cells may either reenter the primary tumor bed or otherwise colonize a distant organ, the latter cell possibly needing additional (genetic) properties. In this respect, we consider multiple affected organs as a first event after removal of the primary tumor as the outcome of the genetic capabilities of the primary tumor.

With the understanding that the reported molecular subtypes in breast cancer reflect phenotypic and genotypic variation of the biology of breast disease, we evaluated in this study expression data of 344 primary breast cancer patients; first to ascertain the presence of the molecular subtypes within our cohort, then to study if samples in a specific subtype showed organ-specific metastasis. If so, analyses were performed to establish if shared biology is present among the subtypes and the site of relapse.

Materials and Methods

Patients. A group of 344 primary breast tumors was used in this study; all from patients with lymph node–negative breast cancer who did not receive any adjuvant systemic treatment. The study was approved by the Medical Ethics Committee of the Erasmus MC Rotterdam, the Netherlands (MEC-02.953) and was performed in accordance to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands.[4] Two hundred eighty-six patients were included from an earlier study (11) that was supplemented with an additional 58 estrogen receptor (ER)-negative patients (12). The mean age at time of surgery was 53 years (SD, 12); 221 patients were ER-positive and 123 patients were ER-negative; 198 patients were premenopausal and 146 were postmenopausal. T_1 tumors (≤2 cm) were present in 168 patients (49%), T_2 tumors (>2–5 cm) in 163 patients (47%), $T_{3/4}$ tumors (>5 cm) in 12 patients (3%), and 1 patient with unknown tumor stage. Of the complete group, 226 patients had no relapse during follow-up (median follow-up time of patients still alive was 101 mo; range, 61–171 mo), 81 patients had a distant relapse to a single organ, and 37 patients had multiple relapse sites (of which 30 patients had two organs affected at first presentation). Including the multiple sites of relapse in a single patient, the 118 relapse patients showed 165 relapses: 71 bone, 30 lung, 18 liver, 14 brain, 12 pleura, 5 skin, and 15 miscellaneous sites. Due to the low numbers, the latter two sites were not evaluated.

Microarray analysis. The expression data have been deposited in the National Center for Biotechnology Information/Gene Expression Omnibus database entries GSE2034 and GSE5327. In short, targets were hybridized to Affymetrix HG-U133A chips. Gene expression signals were calculated using Affymetrix GeneChip analysis software MAS 5.0. Global scaling was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[4] http://www.fmwv.nl

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performed to bring the average signal intensity of a chip to a target of 600 before data analysis. For each probe set, intensities were thresholded at 30 and were then expressed relative to the geometric mean of that probe set and were 2-log transformed.

Bioinformatic analysis. The Genbank accession numbers of the original 496 subtype genes were matched to the Affymetrix probe-sets using Unigene cluster numbers. Some of the genes have multiple probe sets present. To ensure analysis of only the informative probe sets, the probe sets that did not vary across all samples were removed, leaving 66% of the probe sets, i.e., the most variable ones. To identify the molecular subtypes in our cohort, these top 66% variable probe sets were used to cluster 344 samples, using average linkage hierarchical clustering with correlation as a distance metric (13). The robustness of the clusters was ascertained as described (14) using the 10th percentile of the variance of log signals of all samples for the Gaussian noise, as recommended (14).

To identify differentially expressed genes and pathways, the samples were grouped according to the site of relapse, e.g., all bone relapse patients versus patients relapsing elsewhere. In a separate analysis, genes and pathways were identified in the subtype groups, e.g., basal-type samples versus the rest. Differentially expressed genes were obtained via a Significance Analysis of Microarrays (SAM) analysis (15). The top 33% variable genes (n = 7,353) were used for input, and 300 permutations of the data were performed to calculate the false discovery rate (FDR). Genes were selected with a 1.7-fold difference or more between groups that showed a FDR of 5% or less (for two analyses, the FDR was set at 25%; see Results). The overlap between the gene lists was statistically evaluated by calculating z-scores based on the hypergeometric distribution.

The Global Test program (16, 17) was used (version 4.2.0) to associate KEGG pathways (18) to the site of relapse and to the molecular subtypes. All P values were corrected for multiple testing and checked by resampling if an equally sized, randomly chosen group of genes is also significant (1,000 samplings). Pathways were considered of interest if the P value of the Global Test, after correcting for multiple testing, and the resampling P value were both below 0.05. Pathway P values mentioned in the text are two-sided P values corrected for multiple testing, except where stated otherwise. The contribution of individual genes in a pathway was evaluated using z-score calculated by the Global Test program. Genes with z-scores that are >1.96 were considered significant contributors to the pathway. R version 2.3.1[5] was used to run the Global Test package.

Results

Subtyping

The intrinsic gene list (1) of the molecular subtypes was linked to the Unigene cluster number, which left 417 unique genes. These matched to 684 probe sets from the U133A chip (Supplementary Table S1). Based on identical Unigene number, these 684 probe sets represent 360 unique genes. To ascertain that these 360 genes still contain the information to distinguish the subtypes, the original data (1) were reclustered using this shorter gene list, consisting of only those intrinsic genes that are present on the Affymetrix U133A chip. Only one sample switched from not labeled in the original data to normal-like in the new clustering; all other samples remained in the same clusters (see Fig. 1). The top 66% variable probe sets were used to cluster 344 primary breast tumors (Fig. 2). In line with the initial reports (1, 2), clustering using the intrinsic genes revealed five main molecular subtypes. Evaluation of the expression patterns and in particular, the expression of *ERBB2, ESR1, ADH1B,* and *KRT5* (see *arrows*; Fig. 2, *left to right*) were used to identify each clustering branch. The two luminal subtypes can be distinguished by the higher expression of the ER and its target genes (e.g., *GATA3, TFF1*, and *NAT1*). Among others, the high expression of *ADH1B* sets the luminal A subtype apart from the luminal B subtype. The erbb2 subtype was identified by the higher expression of the *ERBB2* gene and its chromosomal neighboring genes. The basal subtype can be characterized by the low expression of ER and its target genes, plus the higher expression of the *KRT5* and *KRT17* genes. With four of the cluster groups identified, the last one is the normal-like subtype, which in addition, as described by Perou et al. (1), expresses *ADH1B* abundantly. Thus, samples were labeled (top to bottom) as luminal B (n = 68), luminal A (n = 93), which comprise the first major category, and erbb2 (n = 70), normal-like (n = 27), and basal subtype (n = 86) forming the second major category. The reproducibility of the clusters was studied using previously published methods (14), in which the R measure, which is the proportion of the time a sample pair stays in the same cluster after perturbation and reclustering, is calculated. Using 100 perturbations of the data, we obtained R values of 0.808 and 0.957 for the luminal A and B subtypes, respectively, and 0.982, 0.725, and 0.654, respectively for the basal, erbb2, and normal-like tumors.

Association with Clinical Data

The association of the site of relapse of the patients with the molecular subtype of the samples, considering bone, lung, liver, brain, and pleura as sites of relapse is shown in Table 1 and Fig. 2. Direct pairwise comparison between subtypes and site of relapse using the Fisher's exact test showed that patients who relapse to bone, which is the most abundant site of relapse, are more frequently found in the luminal subtypes together (P = 0.0031). When the luminal subtypes are analyzed separately, only the luminal A tends to have more bone relapse patients (P = 0.056). Furthermore, bone relapse patients were found less than expected in the basal subtype (P = 0.0001). Compared with other subtypes, lung metastases are also found more than expected in the basal subtype (P = 0.01) but are found less than expected in the luminal A subtype (P = 0.019). The highest number of liver relapse patients was observed in the erbb2 group (6 of 18), but this failed to reach statistical significance (P = 0.17). There is a tendency (P = 0.08) for fewer liver relapse patients in the luminal B subtype. Of the 14 brain metastases, 8 were from basal-type tumors (P = 0.0035), whereas only 2 were found in the luminal subtypes (P = 0.0031). Almost all (10 of 12) metastases to the pleura were found in the luminal subtypes (P = 0.066). All significant P values are retained after correcting for multiple testing using the Holm-Bonferroni method (19).

Molecular Similarities between the Site of Relapse and the Subtype

Differentially expressed genes. SAM (15) analysis was performed to identify differentially expressed genes (Supplementary Table S2). We separately analyzed each distant relapse site versus the remaining sites (e.g., relapse to bone versus other site of relapse). This was also done for each subtype versus the other subtypes, considering only patients with a distant relapse (e.g., basal samples who relapsed versus the other subtype samples with a distant relapse). The number of overlapping genes between the subtypes and the different site of relapses was determined (see Table 2). The observed frequencies of differentially expressed genes in Table 2 follow the pattern in Table 1 to a high degree. For example, the basal subtype shows frequent lung metastases (Table 1), and of the total 67 differentially expressed genes in lung relapse patients, 59 are also found in the basal subtype (Table 2).

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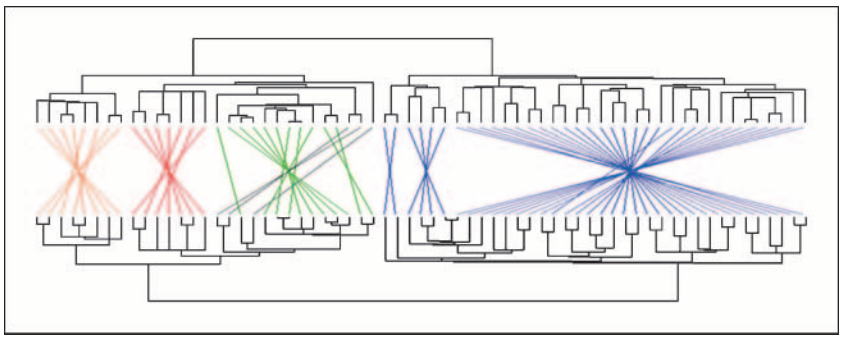

Figure 1. Clustering of original data set of Perou et al. (1) using the Affymetrix-matched, intrinsic gene list. The 360 unique genes of the intrinsic gene list that could be mapped to the Affymetrix platform were used to recluster the original data. The original data (*top dendogram*) was compared with the hierarchical clustering using this shorter gene list (*bottom dendogram*). Lines were drawn between the positions of the samples and colored according to the label used by Perou et al. (1); *orange,* basal subtype; *red,* erbb2 subtype; *green,* normal-like subtype; *black,* unlabeled; *blue,* luminal.

Similarly, large numbers of overlapping genes are found between the bone relapse group and luminal A, B, and erbb2 subtypes. Noteworthy, the 12 genes found up-regulated in the overlap of bone relapse patients and the erbb2 subtype are almost entirely different from those found in the overlap between bone and the two luminal subtypes. The basal subtype has also a large number of genes in overlap with the bone relapse group; however, their expression points in the opposite direction in line with the strong negative association of this subtype with relapse to bone.

Apart from the number of overlapping genes, we also indexed shared functions of the differentially expressed genes for the overlap of several groups. Reviewing the genes found in the overlap of bone relapse patients with a luminal B subtype, we noted ER-related genes (such as *TFF1* and *GATA3*) as the most dominant attribute. Remarkably, the 12 genes found up-regulated in the overlap of bone relapse patients and erbb2 subtype are different from those found in the luminal B subtype, and has only one gene in common with the luminal A/bone overlap. Interesting genes in this list are *PERLD1* (located in the ERBB2 amplicon), and chromosomal neighbors on *11q13, SCGB2A2* (mammaglobin 1), and *SCGB1D2* (lipophilin B). Genes related to the extracellular remodeling system were prominent in breast cancer patients relapsing to lung and breast cancers of the basal subtype. Also, notable in this list are again the abovementioned genes on *11q13*, which are up-regulated in bone relapse and erbb2 subtype tumors but down-regulated in lung relapse and basal subtyped tumors. Analyzing basal tumors as well as tumors relapsing to brain, we noticed 6 of 11 up-regulated genes are involved in cell cycle. None of these genes were down-regulated in luminal A/B, suggesting that we are not merely looking at marker of basal cancers. Common characteristics of the genes down-regulated in brain relapse patients and basal subtyped tumors are nuclear and growth factor receptors (*AR, ERBB3, ERBB4,* and *ITPR1*) and several members from the solute carrier family.

Pathway mapping. To evaluate whether the processes that govern the metastasis to a certain organ are also present in the subtypes, we mapped KEGG (18) pathways to all site of relapse and subtype groups using the Global Test package (16, 17). Although KEGG contains fewer entries than Gene Ontology (20, 21), in our view, it is more suited for our purpose because this curated

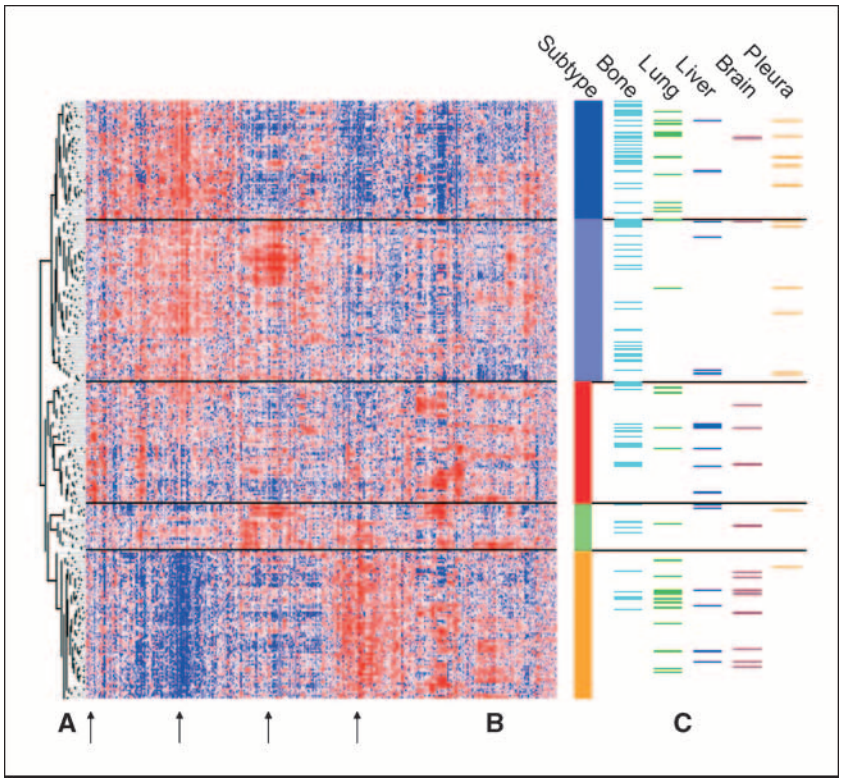

Figure 2. Association of subtypes and site of relapse. Subtypes in 344 primary breast cancers. *A,* dendogram after hierarchical clustering. *B,* clustered expression data of the Affymetrix intrinsic genes; *rows,* tumor samples; *columns,* genes; *red,* relative high expression; *blue,* relative low expression. Arrows indicate from left to right the position of *ERBB2, ESR1, ADH1B,* and *KRT5*. *C,* subtypes (*first column*). *Dark blue,* luminal B; *light blue,* luminal A; *red,* erbb2; *green,* normal-like; *orange,* basal subtype. The remaining columns indicate the site of relapse of a patient; *light blue,* bone relapse; *green,* lung relapse; *blue,* liver relapse; *maroon,* brain relapse; *orange,* pleura relapse.

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Table 1. Frequencies of site of relapse in the molecular subtypes

Subtype	Site of relapse					
	Bone	Lung	Liver	Brain	Pleura	Total
Luminal B	26 (36.6)	11 (36.7)	2 (11.1)	1 (7.1)	5 (41.7)	45
Luminal A	22 (31.0)	2 (6.7)	4 (22.2)	1 (7.1)	5 (41.7)	34
Erbb2	14 (19.7)	4 (13.3)	6 (33.3)	3 (21.4)	0 (0.0)	27
Normal	4 (5.6)	1 (3.3)	2 (11.1)	1 (7.1)	1 (8.3)	9
Basal	5 (7.0)	12 (40.0)	4 (22.2)	8 (57.1)	1 (8.3)	30
Total	71	30	18	14	12	145

NOTE: Numbers between parentheses are column percentages, e.g., 36.6% of bone relapses are in the luminal B subtype.

database contains well-established pathways, whereas Gene Ontology groups genes with a similar function.

We tested the same groups of samples as were used in the SAM analysis. All pathways were corrected for multiple testing and verified by resampling (see Materials and Methods for details). We focused on pathways with a high percentage of influential genes, and found two pathways standing out (see Supplementary Table S3). "Focal adhesion" is found significant in the luminal A and normal-like subtype (both $P < 0.0001$) and in tumors from patients with a lung relapse ($P = 0.001$). We subsequently concentrated on analyzing focal adhesion genes in lung relapse patients and luminal A subtype (Fig. 3), as the normal-like subtype only harbors one lung relapse patient. The 10 significant genes (z-score, >1.96) of focal adhesion that are higher expressed in the lung relapse patients are associated with signaling and particularly with signaling toward cell survival. Of the 16 down-regulated genes in the lung relapse patients, 14 are found significantly higher expressed in the luminal A subtype group (Fig. 3, *arrows*). Six of these 14 genes are extracellular matrix genes.

The other pathway of interest was "WNT signaling." This pathway was found highly relevant ($P < 0.0001$) in both luminal B and basal subtypes, as well as in the brain relapse ($P = 0.007$) and bone relapse ($P < 0.0001$) patients, with the remark that the comparative P value for the bone relapse group was of borderline significance ($P = 0.058$). The 26 genes of the WNT signaling pathway that are significant (z score, >1.96) in at least three of the four above-mentioned groups show a remarkable consistent pattern; genes up- or down-regulated in tumors of brain relapse patients and of the basal subtype are, with one exception, expressed in opposite direction in luminal B and bone relapse subgroups. The core part of the significant genes of the WNT signaling is depicted in Fig. 4, with inhibitors, receptors, the β-catenin/APC signaling complex, and downstream genes all identified as significant (the WNT ligands themselves were not found significant in more than three groups). All these genes, except *APC*, were found higher expressed in tumors of the basal subtype and in tumors of patients with a brain relapse.

Table 2. Overlap in differentially expressed genes as identified by SAM

		No	Bone		Lung		Brain		Liver*		Pleura*	
			Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
			119	410	23	44	45	104	7	11	6	33
	Up	211	52	0	0	8	0	41	0	5	2	0
Luminal B	Down	747	1	273	11	8	35	0	6	0	0	21
	Up	108	10	0	0	23	0	5	0	2	1	0
Luminal A	Down	18	0	5	2	0	4	0	0	0	0	0
	Up	187	12	28	0	6	2	4	5	0	0	7
Erbb2	Down	5	0	2	0	0	0	0	0	0	0	0
	Up	597	1	61	1	14	3	0	0	0	0	2
Normal	Down	8	0	0	0	0	0	1	0	0	0	0
	Up	814	0	331	21	0	40	0	2	0	0	18
Basal	Down	891	109	0	0	38	0	97	1	8	4	1

NOTE: All genes have a fold difference of ≥1.7 and a FDR of ≤5%.

*For the liver and pleura samples, we did not obtain differentially expressed genes with a FDR of ≤5%. To allow for a comparison, we lowered the stringency by setting the FDR at 25%. Gray boxes are significant according to the hypergeometric distribution ($P < 0.05$) with matching direction of expression (i.e., up- or down-regulated in both site of relapse and subtype). The identity of the genes is listed in Supplementary Table S2.

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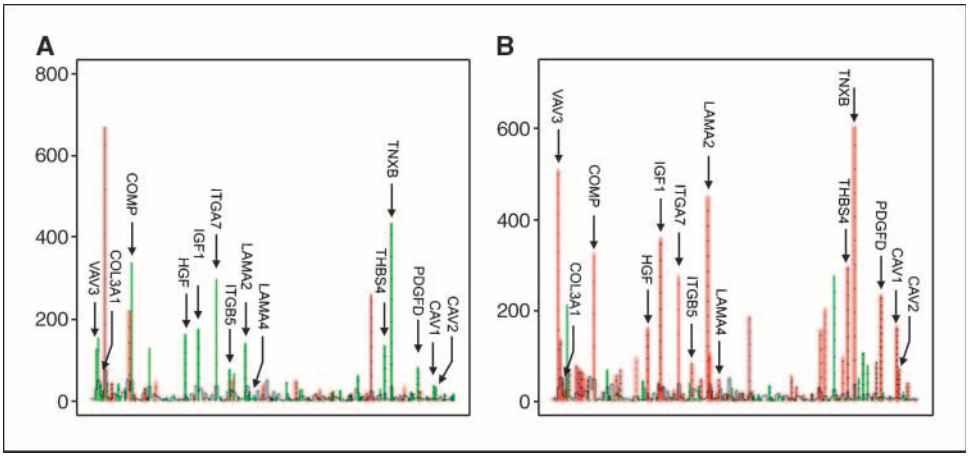

Figure 3. Pathway analysis plot: influential genes in focal adhesion. Genes annotated to the focal adhesion pathway in KEGG and their association with tumors from lung relapse patients and luminal A subtype samples. Each bar at the X-axis represents a gene in the focal adhesion pathway. The height of the bar indicates the contribution (influence) of each individual gene of the pathway with the clinical variable. Horizontal markers in a bar indicate one SD away from the reference point; two or more horizontal markers in a bar indicates a statistically significant association of the corresponding gene with the clinical end point. *A,* lung relapse patients versus patients with relapses at other sites. *Red bars,* genes higher expressed in tumors from lung relapse patients; *green bars,* genes higher expressed in tumors of patients with other relapse sites. *B,* luminal A subtype versus the other subtypes. *Red bars,* genes higher expressed in luminal A; *green bars,* genes higher expressed in the other subtypes. *Arrows,* genes significantly lower expressed in tumors of lung relapse patients but higher expressed in the luminal A subtype.

Discussion

In a landmark paper, Perou et al. (1) reported the presence of molecular subtypes in breast cancer that showed differences in the expression of important molecular markers, in aggressiveness (2, 3), and in response to a specific chemotherapy regimen (4). In the current study, we aimed to determine whether the molecular subtypes showed a preference to relapse to specific distant organs. Some tumors may have abilities to home and proliferate in multiple organs and as such, we included all simultaneously detected metastases (as first event) occurring in different organs in our analysis. Furthermore, the ability to home, adhere, extravasate, survive, and proliferate in a certain distant organ requires a different set of genes as the ability of a tumor to metastasize (5, 6, 8). We verified this by checking that the site of relapse did not show an association with prognosis (data not shown), which means for this study, that pairing of a site of relapse to a subtype is not based on common prognostic outcome. Although outside the scope of this article, it may be that other biological relevant breast cancer signatures, such as the wound-response (22), stromal (23), and hypoxia signatures (24, 25), are characteristic of a phenotype that may facilitate a metastasis in a specific organ. A recent example is the implication of hypoxia and *HIF-1* in osteolytic bone metastases in an animal model (26). However, due to the proven relevance to the breast cancer field, we focused our efforts on the molecular subtypes.

The Affymetrix-matched, intrinsic gene list, although comprised of fewer genes, correctly classified the samples in the original data set. Therefore, we and others (27), show that the intrinsic gene list

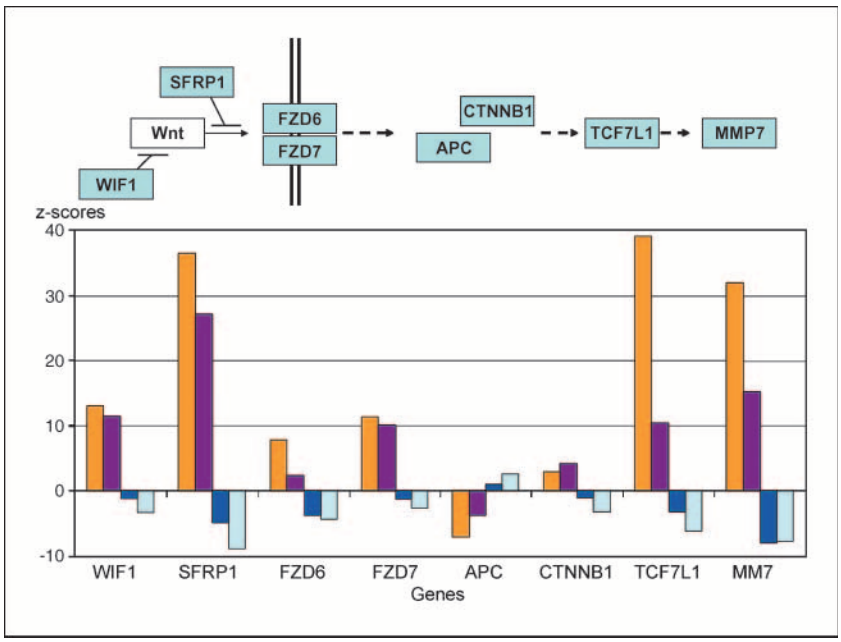

Figure 4. Selected genes of WNT signaling. Important molecules of the WNT signaling pathway that are implicated in the basal and luminal B subtypes and in tumors from bone and brain relapse patients. *Capped lines,* inhibitory effect on the protein-protein interaction. The bottom graph depicts z-score values. These z-scores are a measure for the unlikelihood of the null hypothesis that the gene is not associated with the clinical attribute. Positive and negative z-values indicate genes significantly higher or lower expressed in the corresponding group, respectively. *Orange bar,* basal subtype; *maroon,* brain relapse patient; *blue,* luminal B subtype; *light blue,* bone relapse patient.

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extracted by Perou and colleagues from a relatively small, heterogeneous patient group can be translated across microarray platforms, and as reported here, retains its value even in a large, homogenous (lymph node negative) sample cohort. Thus, the intrinsic gene list proves a robust indicator of the five major molecular subtypes present in breast cancer. Association of the subtypes with clinical data showed that the site of distant relapse was not randomly distributed across the subtypes. The large numbers of genes differentially expressed between the five molecular subtypes confirm that the underlying biology of the subtypes is indeed very different. Also, the many differentially expressed genes in the tumors that relapse to various sites support the view that specific biological processes are involved in organ-specific metastasis.

Taken all three types of analysis together, i.e., the number of overlapping genes, the function of differentially expressed genes, and the identified pathways, all show consistent results when analyzing the subtypes and the site of relapse, which suggests that shared biology has been unraveled. The most prominent results that emerged relate to the biology of brain, lung, and bone relapse. The WNT pathway was associated with patients relapsing to brain and bone, as well as with the basal and luminal B subtypes. The findings suggest that active WNT/β-catenin signaling contributes to basal breast tumors metastasizing to brain, whereas the absence of WNT/β-catenin signaling allows for luminal B–type tumors to metastasize to bone. However, the activation of β-catenin may not originate from WNT ligands, as we find inhibitors of WNT ligands and negative regulatory WNT receptors, i.e., *FZD6* (28) overexpressed in the basal tumors relapsing to brain (Fig. 4). Overexpression of these negative regulators, however, could be the results of negative feedback due to activation of the WNT pathway (29). Taken together, these data suggest that a specific route of the WNT pathway may be active in basal tumors relapsing to brain. Interestingly, in brain tissue itself, a large body of evidence points to an important involvement of WNT/β-catenin signaling in the development and maintenance of normal brain tissue as well as in brain tumorigenesis [among others, reviewed by Fogarty et al. (30)]. Thus, the active WNT/β-catenin signaling by breast cancer cells metastasizing to brain could point to mimicry which, if proven, supports the view that the seed grows better in the soil it resembles (10). Along the same line, one could speculate that luminal B tumors, in which WNT/β-catenin signaling is down-regulated, thus, lack the specific genetic module that facilitates a brain relapse and thus relapse elsewhere.

For the lung relapse patients, we found that the focal adhesion signaling cascade is an important modulator of organ-specific relapse. Focal adhesions are specific types of large protein complexes through which the cytoskeleton of a cell connects to and communicates with the extracellular matrix. Of the focal adhesion genes that were annotated by KEGG (18), many are up-regulated in the luminal A subtype and down-regulated in tumors from patients who had a lung relapse. Because very few patients in the luminal A subtype had relapses to the lung, it seems the involved focal adhesion molecules impede a lung relapse. These observations, together with the high frequency of extracellular matrix genes that were found significantly differentially regulated, imply that luminal A–like tumor cells lack the ability to create a specific microenvironment surrounding the metastasizing cells, necessary for invading and proliferating in lung tissue. Similar findings were also reported by Minn et al. (8) who described many genes involved in the tumor cell microenvironment, which were found differentially expressed in lung metastasis in both a mouse model and primary breast tumors.

Finally, despite the fact that the patients with a relapse to the bone were not overrepresented in any of the subtypes, we find in agreement with earlier findings by us (5) and others (6), a clear association with ER status. This is also echoed in the list of differentially expressed genes in bone relapse samples, which holds many ER-target genes. Our findings in this cohort are further in line with the recently (5, 6) revealed involvement of transforming growth factor β and fibroblast growth factor (FGF) signaling, TFF proteins, *IL11*, and *CTGF* in bone relapse. Noteworthy is the fact that the genes up-regulated in the bone relapse patients of the erbb2 subtype are entirely different from those found in the luminal subtypes. This suggests that the erbb2 subtype may metastasize to bone via processes different from luminal tumors. Also, among the list of differentially expressed genes in erbb2 tumors that metastasize to bone are two members of the secretoglobin family, mammaglobin 1 and lipophilin B, which are both on *11q13*, a common amplified region in breast cancer (which includes the genes cylin D1, FGF 3 and 4, and cortactin). Both mammaglobin and lipophilin B are implicated as important breast cancer markers (31–33) and are next to being up-regulated in bone relapse patients of the erbb2 subtype also significantly down-regulated in lung relapse patients. This could suggest that mammaglobin, lipophilin B, or possibly one or more other genes in the 11q13 amplicon modulate the ability of erbb2 tumors to relapse to bone instead of lung.

Although many gene expression studies on breast cancer are available, none provided all essential information to validate our results. Nevertheless, an interesting observation in this respect, is reported by Minn et al. (34). In this collaborative study on lung metastasis in breast cancer, the tumors that expressed the lung metastasis signature (developed by Minn et al.) were very often of the basal subtype, which agrees with our results. However, this observation was partly based on our own data set; thus, we do not regard this as a true independent validation.

In conclusion, the observations reported here indicate that the five major molecular subtypes in breast cancer are not only distinct with regard to primary tumor characteristics, tumor aggressiveness, and response to certain types of chemotherapy, they are also clearly different with regard to their ability to metastasize to distant organ(s). In depth data mining uncovered shared biology and gene expression patterns specific for a subtype and its preferred distant metastatic site. Many identified genes agree with the notion that the tumor microenvironment plays an important role in distant metastasis which, together with specific attractors such as chemokines (35, 36) and specific signaling pathways (e.g., WNT/β-catenin signaling and focal adhesion), are important determinants involved in homing, survival, and proliferation of the tumor cells in their new niche. The genetic modules involved in these three parts are balanced differently in the breast cancer subtypes and, thus, facilitate metastasis to different organs.

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