Presence of bone marrow micrometastasis is associated with different recurrence risk within molecular subtypes of breast cancer

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ABSTRACT

Expression profiles of primary breast tumors were investigated in relation to disseminated tumor cells (DTCs) in bone marrow (BM) in order to increase our understanding of the dissemination process. Tumors were classified into five pre-defined molecular subtypes, and presence of DTC identified (at median 85 months follow-up) a subgroup of luminal A patients with particular poor outcome ($p = 0.008$). This was not apparent for other tumor subtypes. Gene expression profiles associated with DTC and with systemic relapse for luminal A patients were identified. This study suggests that DTC in BM differentially distinguishes clinical outcome in patients with luminal A type tumors and that DTC-associated gene expression analysis may identify genes of potential importance in tumor dissemination.

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1. Introduction

Genome-wide expression profiling has demonstrated great power in deciphering molecular portraits of human breast tumors and has identified gene signatures that can be correlated to many different aspects of breast cancer such as tumor progression, prediction of outcome and sensitivity to therapy (Ayers et al., 2004; Chang et al., 2005, 2003; Dai et al., 2005; Ma et al., 2003; Sorlie et al., 2001; van't Veer et al., 2002; Wang et al., 2004). Distinct subtypes that are associated with significant differences in overall and disease-free survival have been identified and validated in different patient cohorts (Bertucci et al., 2005; Sorlie et al., 2003; Sotiriou et al., 2003; Yu et al., 2004). Molecular profiling by microarray technologies will improve our understanding of primary tumor biology and the mechanisms behind tumorigenesis, and also provide

improved tools for patient stratification to establish more tailored therapeutic strategies.

Presence of disseminated tumor cells (DTCs) in bone marrow (BM) as an independent prognostic factor for breast cancer patients receiving adjuvant systemic treatment as well as for untreated patients, is now well documented (Braun et al., 2000; Wiedswang et al., 2003; Diel et al., 1996), and supported by a large pooled analysis of more than 4700 patients (Braun et al., 2005). The occurrence of DTC in distant organs, such as BM, has traditionally been considered as rare and relatively late events during primary tumor progression. This has been challenged by recent expression profiling studies that recognized early primary tumors with a "metastatic phenotype" (Bernards and Weinberg, 2002; Ramaswamy et al., 2003; van't Veer et al., 2002; Weigelt et al., 2003), indicating that tumor dissemination may be an early event in tumorigenesis in some patients. Distinct biological properties of the primary tumors may affect the ability for disseminated tumor cells to settle in certain distant organs and subsequently be responsible for metastasis formation. Thus, combined analyses of primary tumors' gene expression patterns, other molecular parameters such as mutation in the TP53 gene and DTC status will increase our understanding of subsequent metastasis formation.

Previously, we reported the independent prognostic value of DTC in BM in early breast cancer in a large cohort of 817 patients (Wiedswang et al., 2003). From this cohort, gene expression patterns of 123 primary tumors prospectively collected during surgery have been examined by using cDNA microarrays. TP53 mutation status and HER2 amplification were also determined. The prognostic importance of DTC in BM was shown to be different among the various molecular subtypes, separating groups with significantly different outcome only in patients expressing the luminal A type tumors. Differentially expressed genes and functional gene sets that provide potentially useful markers for a more in-depth study of the dissemination process were identified.

2. Results

2.1. Primary tumor characteristics vs DTC and relapse

In this sub-cohort of 123 patients with early stage breast cancer, DTCs were detected in 24% of the cases (27/114; nine with missing data) (Table 1). To identify gene expression profiles associated with DTC in BM, we applied two-class SAM, but were not able to find any genes that were significantly differentially expressed between tumors from patients with DTC in BM compared to those with no detectable DTC.

As breast tumors can be separated into different molecular subtypes with distinct biological characteristics accompanied with differences in survival, the association between DTC and molecular characteristics of the primary tumors within the previously defined gene expression-based subtypes was explored. Tumors were assigned to a subtype using correlation to the expression centroids as previously described (Sorlie et al., 2003). As shown in the hierarchical clustering diagram in Figure 1A, all five subtypes of breast tumors were

identified: 41% of the tumors were classified as luminal A, 13% were of the luminal B type, 17% were ERBB2+, 14% were basal-like and 12% were characterized as normal breast tissue-like (Table 1). Four tumors were labeled as unclassified (correlation <0.1). The most striking difference was between luminal A and basal-like samples that were strongly anti-correlated. However, for other samples, particularly a few normal breast-like and ERBB2+ samples, the correlation of each tumor sample to its final designated subtype was not as convincing (Figure 1B).

Genomic DNA from 117 of these tumors was available for TP53 mutation screening. Mutations were found in 32.5% of the cases (Table 1), which is slightly higher than reported for breast cancer in general (IARC TP53 mutation database; Olivier et al., 2002). Of the 38 mutations detected, 27 were missense, three were nonsense, seven were deletions/insertions and one was a putative splice mutation downstream of exon 9 (Supplemental Table 1).

In the further analyses, the four patients with unclassified tumors and the one patient with ductal carcinoma in situ (DCIS) were excluded (n = 118). DTC in BM was detected in all subgroups as shown in Figure 1C. As expected, there was a high frequency of ER/PgR-expression in the luminal A and luminal B subtypes (98% and 94%, respectively) as compared to the ERBB2+ and basal-like subtypes (Figure 1C). Also, a high frequency of TP53 mutations and histological grade 3 was observed among the luminal B, ERBB2+ and basal-like subtypes (43% and 63%, 65% and 70%, 82% and 88%, respectively) compared to luminal A. Although the number of systemic relapses and breast cancer deaths were somewhat lower for patients with luminal A type tumors than for those with other tumor types, a considerable number of luminal A patients also experienced systemic relapse (27%) during the follow-up time of median 85 months.

2.2. Survival analyses according to DTC and primary tumor molecular characteristics

Survival analyses stratified by tumor subtype and DTC status at 85 months median follow-up (range 0.8–127, interquartile range 60) are shown in Figure 2A–J. The survival for the luminal A patients appeared more favorable than those for the other subtypes (Figure 2B), although significantly different only from the ERBB2+ subtype for distant disease-free survival (P = 0.04, log rank test). As shown in Table 2, similar systemic relapse- and breast cancer-death patterns were observed when stratified for systemic treatment, but the small number of patients in each of the subgroups restricts further interpretation. Although clearly evident in the entire patient cohort (Wiedswang et al., 2003), DTC status did not significantly discriminate survival in this smaller sub-cohort (Figure 2C and D). However, the presence of DTC was associated with significantly reduced distant disease-free survival (DDFS) and breast cancer specific survival (BCSS) among the luminal A patients (Figure 2E and F; Table 2). Subdivision by treatment status showed unfavorable outcome for tamoxifen-treated luminal A patients (n = 20) with DTC-positive status (p = 0.002, log rank), whereas among the untreated patients (n = 19), the low frequency of DTC-positive cases (n = 1) makes survival analysis impossible. The association

Table 1 – Molecular and clinicopathological data of the tumor material			
Characteristics		Number of patients	% (Excluding missing)[a]
All		123	100
DTC in BM	Yes	27	23.7
	No	87	76.3
	Missing	9	
Gene profile	Luminal A	50	40.7
	Luminal B	16	13.0
	ERBB2+	21	17.1
	Basal-like	17	13.8
	Normal-like	15	12.2
	Unclassified	4	3.3
TP53	Mutant	38	32.5
	Wild type	79	67.5
	Missing	6	
Her2 – FISH	Positive	20	18.2
	Negative	90	81.8
	Missing	13	
ER/PgR-status	Positive	80	66.1
	Negative	41	33.9
	Missing	2	
Histology	Ductal	97	78.9
	Lobular	23	18.7
	Other infiltrating	2	1.6
	DCIS	1	0.8
Grade	I	16	13.2
	II	58	47.9
	III	47	38.8
	Missing	2	
Tumor status	pT1	55	44.7
	pT2	51	41.5
	pT3-4	13	10.6
	Ptis	1	0.8
	pTX[b]	3	2.4
Nodal status	Negative	50	40.7
	pN1	35	28.5
	pN2	21	17.1
	pN3	10	8.1
	pNX[c]	7	5.7
Menopause	Pre	40	32.5
	Post	77	62.6
	Unknown	6	4.9
Adjuvant treatment	Yes	74	61.7
	No	46	38.3
	Missing	3	
	Tamoxifen	57	46.7
	No Tamoxifen	63	51.6
	Missing	3	
Systemic relapse	Yes	39	32.5
	No	81	67.5
	Missing	3	

Table 1 (continued)			
Characteristics		Number of patients	% (Excluding missing)[a]
Death	From breast cancer	34	27.6
	Other cause	21	17.1
	No	68	55.3

a Missing includes both non-evaluable cases, not performed and information not available.
b pTX defines those cases where no determination of tumor size was possible.
c pNX defines those cases where no axillary dissection was performed but no clinical sign of nodal metastasis existed.

between DTC and clinical outcome within the other subtypes is not clear because of limited sample size (Table 2). However, for patients with ERBB2+ and basal-like tumors the systemic relapse- and breast cancer-death frequencies appeared higher for the DTC-*negative* than for DTC-*positive* patients. Furthermore and in line with this, we found that among the patients with *TP53* mutated tumors (71% of which were ERBB2+ or basal-like), there was a trend towards association between the *presence* of DTC and *improved* DDFS, as compared to the *absence* of DTC ($P = 0.096$, log rank). In contrast, DTC-positive patients with *TP53* wild type primary tumors experienced, at borderline significance, reduced DDFS as compared to the DTC-negative patients with wild type tumors ($P = 0.046$, log rank) (see Supplementary Figure 2).

The observed difference in clinical significance of DTC between patients with tumors with different molecular characteristics was further explored by an updated analysis of the entire study population with known DTC status (the 817-patient cohort). Survival analyses were performed separately for the ER- and/or PgR-positive and the ER/PgR-negative patient groups (assessed by IHC), as surrogate markers for luminal and the basal-like/ERBB2+ subtypes, respectively. As illustrated in Figure 2G–J, a marked difference in both DDFS and BCSS was detected for the ER- and/or PgR-positive patients stratified for DTC ($P < 0.001$), whereas DTC status did not discriminate outcome in ER- and PgR-negative patients. Subdivision according to ER/PgR status within the 123 patient cohort, revealed the same survival pattern as for the entire study population. No difference in outcome between DTC-positive and DTC-negative was detected in the ER- and PgR-negative patients, whereas a marked survival difference was observed for the ER- and/or PgR-positive patients according to DTC status ($P = 0.007$, log rank).

2.3. Gene profiles associated with DTC and systemic relapse

The strong association between DTC and clinical outcome for the luminal A molecular subtype encouraged further analysis of genes associated with DTC within this group of patients. Two-class SAM identified 20 genes (including two replicate probes) highly expressed in tumors associated with positive DTC status (20% FDR) (Table 3). Of these, several are involved in regulation of transcription, transporter activity and ATP
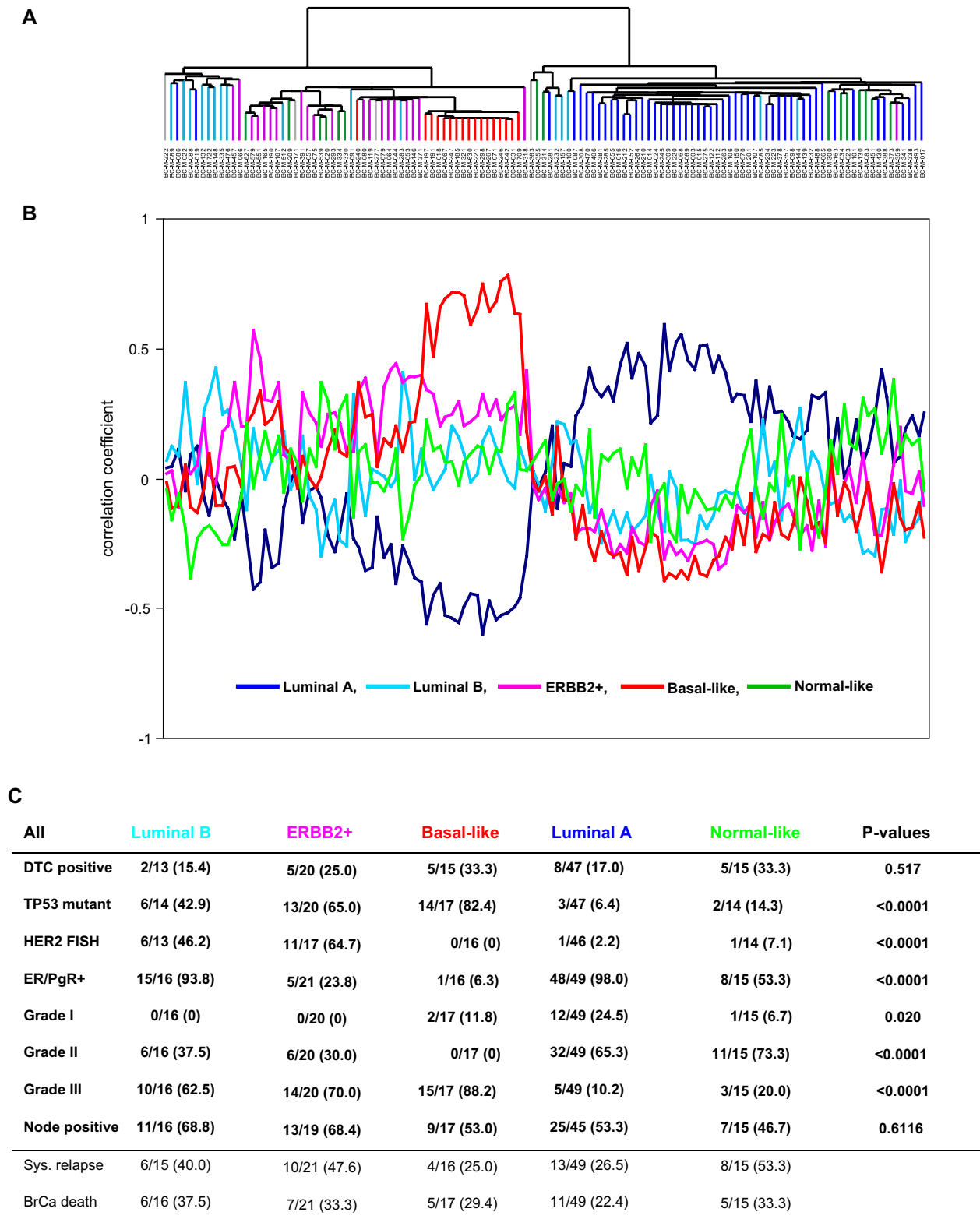
Figure 1 − Subclassification of breast tumor samples using the "intrinsic" gene list. A. Hierarchical clustering dendrogram indicating the relationship between the tumor samples based on gene expression patterns from 494 intrinsic genes. Branches are color-coded according to tumor subtype as indicated in 1B. Grey are unclassified B. Correlation of each sample to each of the five expression centroids as previously identified (Sorlie et al., 2003). C. Clinicopathological data by molecular tumor subtype. Cases with missing parameters within each analysis are excluded. The four unclassified tumors are also excluded.
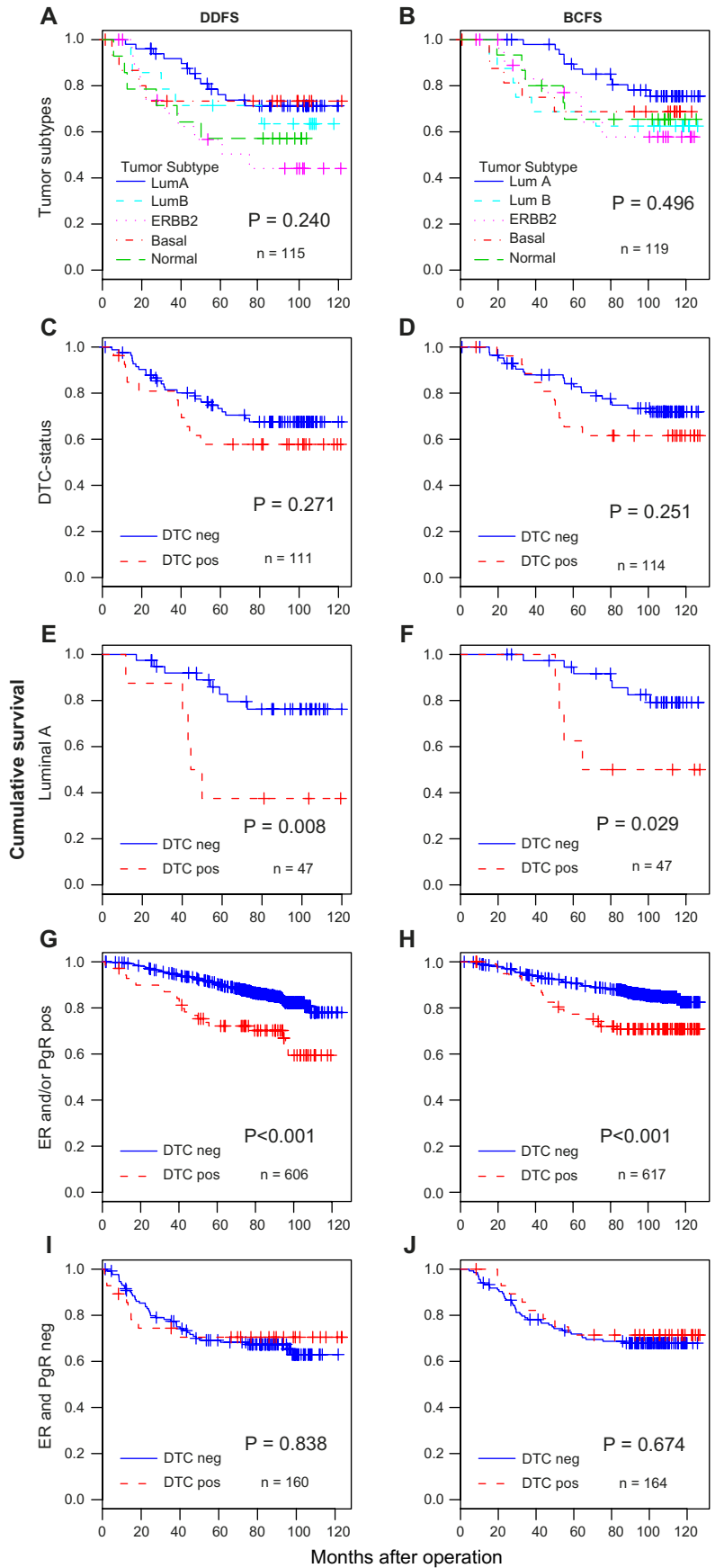
Figure 2 – Overall and disease-free survival analyses by DTC and tumor subtype. Analyses of distant disease free survival (DDFS) (A, C, E, G, I) and breast cancer specific survival (BCSS) (B, D, F, H, J) in patients according to (A, B) molecular tumor subtype; (C, D) DTC-status for all patients subgroups; (E, F) DTC-status for luminal A patients only; (G, H) DTC status in the entire 811-cohort based on ER and/or PgR-positive status; (I, J) DTC-status in the 811-cohort based on ER and PgR-negative status.

Table 2 – The systemic relapse- and breast cancer-death rates according to tumor subtypes, DTC status and adjuvant systemic treatment

	Subtype		Systemic relapse	Breast cancer death
Adjuvant−	Luminal A		4/19 (21%)	3/19 (16%)
	Luminal B		0/3 (0%)	0/3 (0%)
	ERBB2+		2/6 (33%)	2/6 (33%)
	Basal-like		2/8 (25%)	3/9 (33%)
	Normal-like		3/8 (38%)	2/8 (25%)
Adjuvant+	Luminal A		9/30 (30%)	8/30 (27%)
	Luminal B		6/12 (50%)	6/13 (46%)
	ERBB2+		8/15 (53%)	5/15 (33%)
	Basal-like		2/8 (25%)	2/8 (25%)
	Normal-like		3/5 (60%)	3/5 (60%)
All patients	Luminal A	DTC+	5/8 (63%)	4/8 (50%)
		DTC−	8/39 (21%)	7/39 (18%)
	Luminal B	DTC+	1/2 (50%)	1/2 (50%)
		DTC−	4/11 (36%)	4/11 (36%)
	ERBB2+	DTC+	1/5 (20%)	1/5 (20%)
		DTC−	8/15 (53%)	6/15 (40%)
	Basal-like	DTC+	1/5 (20%)	1/5 (20%)
		DTC−	3/9 (33%)	3/10 (30%)
	Normal-like	DTC+	3/5 (60%)	2/5 (40%)
		DTC−	3/9 (33%)	2/10 (20%)

binding. When expression data from these genes were used in a hierarchical clustering analysis, the luminal A samples formed two main branches and all eight tumors associated with DTC clustered together in one branch (Figure 3). Of these eight patients, five experienced systemic relapse of the disease. Similarly, SAM was also applied to identify genes correlated with distant relapse in the luminal A patients irrespective of DTC status. Here, we identified 64 genes (76 UniGene clusters) with an FDR of 5% that showed significantly different expression patterns between tumors from patients who experienced systemic relapse and those who did not (see Supplemental Figure 1 for the complete cluster diagram and Supplemental Table 2 for the list of genes). Only two of the 20 genes (LONP2 and MCM6) associated with DTC status for the luminal A patients were also among the genes associated with systemic relapse.

To further explore the primary tumor characteristics of the luminal A type in association with DTC, we used Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) to identify potentially interesting regulatory modules or co-expressed gene sets that may be important for cancer dissemination. A gene set representing the CREB pathway was identified (FDR = 0.138) whose genes were enriched in tumors associated with positive DTC status. A similar analysis for distant relapse in luminal A tumors failed to identify any significant gene sets with an FDR < 0.25.

Although the presence of DTC is a strong risk factor for future metastases, a significant portion of the patients do not experience relapse. Different biological properties of the disease might explain this difference. Hence, we searched for differentially expressed genes in tumors from patients with DTC who later experiences systemic relapse ($n = 5$) compared to those who were free of the disease within the follow-up

time ($n = 3$). Despite the small sample size, SAM identified one gene, CXXC4 which is a negative regulator of the Wnt receptor pathway, with q-value 0% and that were considerably higher expressed in the tumors from the DTC-positive patients with systemic relapse.

3. Discussion

One of the challenges when studying breast tumor dissemination is to understand the molecular and functional characteristics of a cancer disease that results in cells that reside in bone marrow and later develop into overt metastases. Associations between DTC and certain primary tumor characteristics such as size, differentiation and vascular invasion have previously been noted (Naume et al., 2001; Gerber et al., 2001; Mansi et al., 1999). This study focus on the association between gene expression patterns in primary breast tumors and presence of DTC in BM.

We observed that the presence of DTC in BM was associated with poor outcome in the luminal A subgroup while this was not found for the ER-negative/TP53-mutated group of tumors, which for a large part represents the basal-like and ERBB2+ subtypes. One possible explanation for this difference is that non-luminal A patients with ER-negative/TP53-mutated tumors *and presence of* DTC may respond better to systemic adjuvant treatment compared to luminal A/TP53 wild type DTC-positive patients. It is known that ER-negative patients achieve complete remissions more frequently than the ER-positive patients in the neoadjuvant setting (Colleoni et al., 2004). On the other hand, ER-negative patients have in general an increased risk of early relapse as compared to the ER-positive patients (Colleoni et al., 2004; Thorpe et al., 1987), and TP53 mutation is associated with reduced effect of some chemotherapeutic agents (Aas et al., 1996; Andersson et al., 2005; Geisler et al., 2001). Furthermore, studies have indicated that DTC may be refractory to chemotherapy, although this was shown in very high-risk patients (Braun et al., 2000). Another explanation might be that the ER-negative/TP53-mutated patients more frequently have clinically relevant tumor cell dissemination to other distant organs irrespective of the BM findings. Indeed, a higher frequency of visceral metastasis was observed among the ER-negative/DTC-negative patients than the ER-negative/DTC-positive patients (21% vs 14%) in the whole patient cohort. This is in contrast to the frequency of visceral metastasis among ER-positive patients according to DTC status (6% in DTC-negative vs 14% in DTC-positive) (Naume et al., unpublished observations). Although we used pan-cytokeratin monoclonal antibodies (AE1/AE3) to detect DTC, it cannot be excluded that tumorigenic cells in BM are differentially stained by the immunocytochemical technique across tumor subtypes due to different marker expression.

Another interesting hypothesis for the different significance of DTC among the subgroups could be a different frequency – or different relevance – of cancer stem cells or progenitor cells (Al Hajj et al., 2003). If cancer stem cells are present in the circulation or in BM in the ER-negative/TP53-mutated patients at extremely low frequencies, whereas differentiated, non-tumorigenic cells shedded from the primary tumor are much more frequently present, the clinical

Table 3 – Gene information on the 20 genes correlating with DTC status from SAM analysis

Symbol	Name	UG cluster	Clone ID	GeneID (LocusLink)	Cytoband	GO: molecular function/biological process
DIP2B	DIP2 disco-interacting protein 2 homolog B (Drosophila)	Hs.505516	IMAGE:489031	57609	12q13.12	Ligase activity/metabolism
MCM6	Minichromosome maintenance deficient 6 homolog (S. cerevisiae)	Hs.444118	IMAGE:1587847	4175	2q21	ATP binding/DNA replication
PLEK2	Pleckstrin 2	Hs.170473	IMAGE:453710	26499	14q23.3	
FOXI1	Forkhead box I1	Hs.87236	IMAGE:1901720	2299	5q34	Transcription factor activity/development
SYT13	Synaptotagmin XIII	Hs.436643	IMAGE:23443	57586	11p12-p11	Transporter activity/transport
PRDM6	PR domain containing 6	Hs.135118	IMAGE:259607	93166	5q23.2	DNA binding/transcription regulation
LONP2[a]	Lon peptidase 2, peroxisomal	Hs.651202	IMAGE:181787	83752	16q12.1	ATP binding/proteolysis
ZNF185	Zinc finger protein 185 (LIM domain)	Hs.16622	IMAGE:855079	7739	Xq28	Zinc ion binding
ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	Hs.444389	IMAGE:2272922	9583	8p21.3	Hydrolase activity/nucleic acid metabolism
PPP2R2C[a]	Protein phosphatase 2, regulatory subunit B (PR 52), gamma isoform	Hs.479069	IMAGE:399593	5522	4p16.1	Hydrolase activity/signal transduction
LANCL2	LanC lantibiotic synthetase component C-like 2 (bacterial)	Hs.595384	IMAGE:429647	55915	7q31.1-q31.33	ATP binding/regulation of transcription
SFTPD	Surfactant, pulmonary-associated protein D	Hs.253495	IMAGE:1601355	6441	10q22.2-q23.1	Bacterial binding/alveolus development
TSGA10	Testis specific, 10	Hs.120267	IMAGE:745499	80705	2q11.2	Porin activity/spermatogenesis
B3GALT2	UDP-Gal:betaGlcNAc beta-1,3-galactosyltransferase, polypeptide 2	Hs.518834	IMAGE:1556976	8707	1q31	Beta-1,3-GalTase activity/protein amino acid glycosylation
SEZ6L	Seizure related 6 homolog (mouse)-like	Hs.194766	IMAGE:2244016	23544	22q12.1	
KCND2	Potassium voltage-gated channel, Shal-related subfamily, member 2	Hs.21703	IMAGE:770427	3751	7q31	Voltage-gated potassium channel activity/cation transport
VSTM2	V-set and transmembrane domain containing 2	Hs.335933	IMAGE:1914847	222008	7p11.2	
FANCL	Fanconi anemia, complementation group L	Hs.631890	IMAGE:713058	55120	2p16.1	Ligase activity/DNA repair
FDPS	Farnesyl diphosphate synthase	Hs.335918	IMAGE:1655450	2224	1q22	transferase activity/cholesterol biosynthesis
VRK2	Vaccinia related kinase 2	Hs.651156	IMAGE:824117	7444	2p16-p15	Protein serine-threonine kinase activity/amino acid phosphorylation

a The 20 genes were represented by 22 clones; LONP2 and PPP2R2C were represented twice each; and GO, gene ontology.

implication of DTC in BM would be reduced. In contrast, if the luminal A subtype originates from a more differentiated cell type, it is possible that DTC in BM for this particular subtype more frequently represents more differentiated tumorigenic progenitor cells that have acquired ER expression. This corresponds well with bone as the most frequent metastatic site in ER+ breast cancers.

The disparate information of DTC in luminal A vs the ER-negative/TP53-mutated tumors initiated a further exploration of the gene expression patterns in the luminal A tumors. Among the 20 genes identified by SAM to be correlated with DTC status, there are genes involved in transport (SYT13 and KCND2), ATP-binding (MCM6, LONP2 and LANCL2) and in regulation of transcription (FOXI1 and PRDM6) (source: Diehn et al., 2003). These were all highly expressed in DTC-positive tumors, which is compatible with a more progressive phenotype.

Only two genes, LONP2 (LONPL) and MCM6, were common to both gene profiles associated with DTC and with systemic relapse. LONP2 (Lon peptidase 2) is a peroxisomal ATP-dependent protease, possibly involved in the biogenesis of peroxisomes (Kikuchi et al., 2004). The relevance for tumor dissemination is unclear, but peroxisome proliferation is regulated by PPARs which belong to the steroid hormone receptor superfamily and signaling cross-talk has been observed between PPARs and the estrogen receptor (Keller et al., 1995). Microsome maintainance protein 6 (MCM6) is involved in initiation of DNA replication. In support of our results, this protein has been shown to be overexpressed in several malignant diseases and associated with reduced survival and metastasis formation (Schrader et al., 2005; Winnepenninckx et al., 2006).

GSEA identified only one gene set, the CREB pathway, as enriched in luminal A tumors associated with DTC in BM. CREB binds the cyclic AMP response element (CRE) and activates transcription in response to a variety of extracellular signals. The CREB signaling pathway is involved in the induction of cyclin D1 protein levels (D'Amico et al., 2000), which is associated with estrogen receptor positive disease and reduced survival (Al-Kuraya et al., 2004). Interestingly, it has been
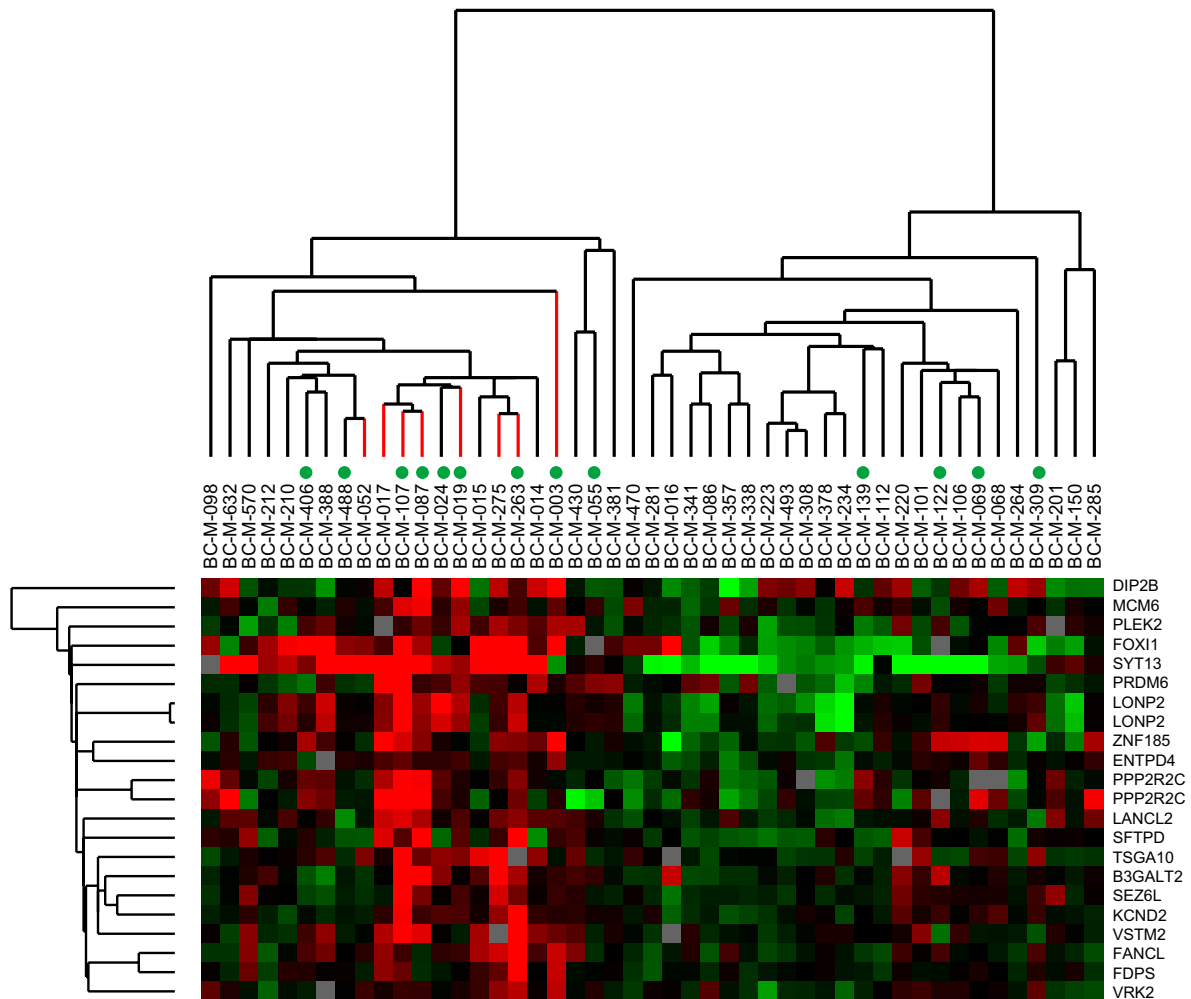
Figure 3 – Hierarchical clustering diagram of genes associated with DTC. A. Cluster diagram showing the relationship of 20 the genes (22 clones) correlating with DTC status in 47 luminal A tumors, as identified by SAM. Samples associated with DTC in BM are color-coded with red branches; green dots indicate tumors from patients who later experienced systemic.

shown that CREB can increase the synthesis and deposition of non-collagenous bone-matrix proteins such as osteocalcin (OC) and bone sialoprotein (BSP) in prostate cancer (Huang et al., 2006), which may help sustain growth and survival of cancer cells residing in the bone marrow (Karadag et al., 2004).

Certain molecular characteristics of the individual disease might explain the ability for disseminated tumor cells to become tumorigenic. The limited number of DTC-positive patients with luminal A type tumors in this data set ($n = 8$), restricts our possibilities for a detailed exploration. However, CXXC4 (IDAX) was found to be highly expressed in the tumors from patients with DTC and a subsequent systemic relapse, as compared to the DTC-positive patients without a subsequent relapse. This gene encodes a negative regulator of the Wnt-signaling pathway by its binding to Dishevelled (DVL), which consequently reduces β-catenin activation (Hino et al., 2001). CXXC4 is therefore an interesting candidate gene for further study of the metastatic process within the luminal A subtype.

A combined analysis of gene expression and DTC in BM in primary tumors using cDNA arrays was reported by Woelfle et al. (2003). A total of 12 DTC-negative cases and seven

DTC-positive were selected for analysis in a retrospective study of ER-positive stage pT2 tumors. The results showed differential expression of HIF-1α, genes in the RAS pathway and certain cytokeratins. In contrast to our results, the signature for DTC was mainly characterized by transcriptional repression. This discrepancy between the genes identified in our study as compared to the results from Woelfle et al. may also be due to different experimental procedures and microarray platform used, differences in patient selection and statistical methods employed and the inherent nature of co-variables in microarray studies (Ein-Dor et al., 2005; Pusztai, 2006; Tan et al., 2003). The prospective design in our study and the use of whole genome cDNA arrays should yield the most optimal information. However, both studies are exploratory and the identified genes need to be evaluated in larger patient cohorts. Nevertheless, our results underline the importance of assessing the molecular heterogeneity of tumors when searching for associations between markers for progression and clinical outcome. Our results form a foundation for a planned further exploration of the identified genes, gene signatures and pathways in the entire cohort of 817 patients using tissue microarrays.

4.   Experimental procedures

4.1.   Patients, sampling of biomaterial and analysis of paraffin embedded primary tumors

Patients treated for localized breast cancer were included in this project (from 1995 to 1998) and have previously been described (Wiedswang et al., 2003). After informed, written consent, bone marrow (BM) aspiration was performed in general anesthesia, just prior to primary surgery for suspected breast cancer. If possible, parts of the tumor specimens were fresh frozen at −80 °C immediately after surgery. The routine selection of patients to adjuvant treatment was based upon the prevailing National Guidelines, where postmenopausal hormone receptor (HR) positive patients received tamoxifen only, postmenopausal HR negative patients received CMF and premenopausal patients received CMF followed by tamoxifen if HR positive. Five patients received high dose chemotherapy and another five, preoperative chemotherapy due to large tumor size. After completed primary therapy, the patients were followed at 6–12 months intervals. A total of 920 patients were included in the study, clinical correlation to DTC status was originally reported from 817 (Wiedswang et al., 2003) and includes now an updated follow-up of 811 patients (median follow-up 85 months). Fresh frozen tissue samples were available from 123 individuals. Of these, 114 had known "disseminated tumor cell" (DTC) status. The remaining did not have a conclusive DTC result, but were included in the microarray analysis. One patient was diagnosed with ductal carcinoma in situ. The study was approved by the Regional Ethical Committee. The primary tumor and axillary lymph nodes collected during surgery were processed on a routine diagnostic basis, as previously described. Automatic immunostaining was performed using mouse monoclonal antibodies against ER and PgR (clones 6F11 and 1A6, respectively, Novocastra, Newcastle upon Tyne, UK). Immunopositivity was recorded if ≥10% of the tumor cell nuclei were immunostained. Amplification of the HER2 gene was assessed by FISH (fluorescence in situ hybridization) on tissue microarray sections using the PathVysion HER-2 DNA Probe kit (Vysis Inc., Downers Grove, IL 60515, USA). Pretreatment was performed according to an in-house protocol modified after Chin et al. (2003), and the remaining of the procedure, including scoring, was performed as described in the Kit package insert, defining a HER2/centromer 17 ratio of ≥2.0 as HER2 positivity. A complete listing of all experimental samples can be found in Supplemental Table 1.

4.2.   Preparation of the bone marrow and immunocytochemical staining

A total of 40 ml BM was aspirated from anterior and posterior iliac crests bilaterally, 10 ml per site, and processed as described previously (Wiedswang et al., 2003). After separation by density centrifugation, mononuclear cells (MNCs) were collected and cytospins prepared (5×10^5 MNC/slide). The immunocytochemical staining was performed as previously described (Wiedswang et al., 2003). Briefly, four slides (2×10^6 BM MNC) were incubated with the anti-cytokeratin monoclonal antibodies AE1 and AE3 (Sanbio, Uden, The Netherlands), and the same number of slides was incubated with an isotype-specific irrelevant control monoclonal antibody. The visualization-step included the alkaline phosphatase/anti-alkaline phosphatase reaction, and the slides were counterstained with hematoxylin to visualize nuclear morphology.

4.3.   Detection of DTC

The cytospins were manually screened by light microscopy using the 10× lens. All immunostained cells were closely evaluated by one of the pathologists (E.B.). Cells were scored as tumor cell (TC) only when the morphology was compatible with such, i.e. clearly enlarged nucleus as compared to surrounding hematopoietic cells and/or the presence of TC clusters. If lacking hematopoietic characteristics, cells with typically strong and/or irregular cytoplasmatic staining partially covering the nucleus, were also recorded as TC, according to published guidelines (Borgen et al., 1999). In difficult cases, a second pathologist (J.M.N.) was consulted and consensus obtained. The presence of positive cells classified as TC both in the AE1/AE3-stained slides and in the corresponding negative control slides resulted in exclusion of nine patients from diagnostic conclusion.

4.4.   TP53 mutation analysis

Genomic DNA was extracted by standard phenol/chloroform protocols on an automatic DNA isolation system (AB340, Applied Biosystems). The coding region covering exons 2 through 11 of the TP53 gene was screened for mutations by Temporal Temperature Gel Electrophoresis (TTGE) as described in detail elsewhere (Sorlie et al., 2004). Fragments with aberrant migration patterns were sequenced using the AB Prism 373 and 377 Automatic Sequencing systems (Applied Biosystems) to identify the nature of the mutations.

4.5.   DNA microarray analysis

Total RNA was isolated from primary tumor tissues using the TRIzol reagent (Invitrogen). The integrity and quality of RNA samples were evaluated on the 2100 Bioanalyzer (Agilent) and the concentration measured using a NanoDrop spectrophotometer (NanoDrop Technologies). Amplification and labeling of RNA from tumor with Cy5 and from the Universal Human Reference (Stratagene) with Cy3 were performed as previously described (Zhao et al., 2002). Hybridization of labeled cRNA to arrays containing 42,000 features representing 24271 unique cluster IDs (UniGene Build Number 173) produced at the Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/jsp/home.jsp) was performed at 65 °C overnight as previously described. The hybridized arrays were scanned on an Agilent DNA microarray scanner and images analyzed by GenePix Pro v 4.1. All procedures are available at http://genome-www.stanford.edu/breast_cancer/ and all raw data can be obtained from the Stanford Microarray Database (SMD) (http://genome-www5.stanford.edu//). The data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE3985. A subset of

20 tumors has been extensively analyzed using three different DNA microarray platforms (Sorlie et al., 2006).

4.6. Data processing

For the SAM and GSEA analyses, normalized (intensity dependent loess with print tip stratification) log 2-transformed gene expression ratios were retrieved from SMD (http://genome-www5.stanford.edu/), filtered for spot intensity over background at least 1.5 in both channel 1 and channel 2, and finally, filtered for those genes that fulfilled the spot filter criteria in at least 85% of the experiments. For subtype classification, expression data for the intrinsic gene set (Sorlie et al., 2003) were extracted and both genes (559 clones representing 494 unique genes, weighted by UniGene clusters ID) and arrays were median-centered and clustered using average-linkage hierarchical clustering (Eisen et al., 1998). Based on the previously defined gene expression centroids, correlations with the five different breast tumor subtypes were calculated using the Correl function in Excel. These correlations were calculated based on the 549 clone IDs that were common between the two data sets. Each breast tumors sample was assigned to a tumor subclass based on the highest correlation value (<0.1 remained unclassified).

4.7. Statistical analyses

A database was established after permission from the Regional Committee of Ethics and the National Data Inspectorate. The endpoints for the survival analyses were breast cancer specific survival (BCSS) and distant disease-free survival (DDFS) measured from the date of surgery to breast cancer-related death or systemic relapse or otherwise censored at the time of the last follow-up visit or at non-cancer-related death. Metastases to the skeleton, liver, lungs or brain were recorded as systemic relapses. Kaplan–Meier survival curves for time to BCSS and DDFS were constructed and P-values were computed by the log rank test. Cox proportional hazard regression analysis was used for univariate estimation of prognostic impact of the relevant variables. For these analyses, the R software v. 2.4.0 was used. The Pearson chi-square test was used to compare categorical variables. Furthermore, two-class unpaired Statistical Analysis of Microarrays v. 3.0 (SAM) (Tusher et al., 2001) t-statistics was performed with 400 permutations using the K-nearest neighbor imputer ($N = 10$) to identify differentially expressed genes between tumor groups. Finally, Gene Set Enrichment Analysis v. 2 (GSEA) (Subramanian et al., 2005) was applied to identify significantly differential expression of pre-defined gene sets using the C2 collection of gene sets from the MSigDB.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molonc.2007.03.004.

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