Stable ‘portrait’ of breast tumors during progression: data from biology, pathology and genetics

M Lacroix, R-A Toillon and G Leclercq

Laboratoire Jean-Claude Heuson de Cancérologie Mammaire, Institut Jules Bordet, Université Libre de Bruxelles, 127 boulevard de Waterloo, B-1000 Bruxelles, Brussels, Belgium

(Requests for offprints should be addressed to M Lacroix; Email: Marc.Lacroix@ulb.ac.be)

Abstract

It is widely believed that ductal breast cancer dissemination involves a succession of clinical and pathological stages starting with carcinoma *in situ*, progressing into invasive lesion and culminating in metastatic disease. Such changes have frequently been attributed to the sequential acquisition of various alterations in a single cell followed by clonal selection and expansion, thus leading to intra-tumor diversity. According to this multi-step view, extensive genotype and phenotype (marker expression, grade) shift may occur in the same tumor during progression; this may lead to the co-existence of molecularly and/or pathologically different areas within the same lesion. An increasing amount of data of various natures now appear to challenge this concept: only a few distinct ‘portraits’, in relation to estrogen receptor (ER) status and grade, may be found among tumors. Moreover, although undergoing increasing genetic alteration, most individual lesions largely maintain their phenotype when they evolve from *in situ* to the metastatic state. While many of the data presented here are related to ductal tumors, lobular cancer is also discussed.

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Introduction

In Western countries, about 1 in 11 women will develop a breast carcinoma, generally of ductal origin. Despite considerable progress in the knowledge of tumor biology and treatment, more than two-thirds of patients still succumb to the disease. In most cases, death results from metastasis of breast cancer cells (BCCs). Elucidating the mechanisms that ultimately confer metastatic properties to BCCs remains, therefore, a major research challenge.

It has been widely believed that breast cancer dissemination involves a sequential progression through clinical and pathological stages starting with carcinoma *in situ*, progressing into invasive carcinoma and culminating in metastasis. To explain the transition from one stage to another, a multi-step hypothesis has been proposed. Based on the fact that BCCs are genetically unstable, the hypothesis postulates that tumor-stage changes are

associated with the sequential acquisition of various genetic and, consequently, phenotypic alterations in a single cell followed by clonal selection and expansion. The successive clonal populations are thought to acquire an increasing aggressiveness, with numerous alterations in properties such as proliferation, adhesion, proteolysis, motility, angiogenic ability etc. and loss of estrogen receptor (ER). It is also believed that considerable intra-tumor diversity may result from the co-existence of these clonal populations. In particular, the characteristics of metastatic cells are expected to often be significantly different from those observed in BCCs *in situ*.

In fact, a number of recent data—of pathological, molecular and genetic nature—have revealed that despite increasing genetic alteration, the ‘portrait’ of breast tumors remains amazingly stable during progression, and that no major change appears to explain why a tumor may progress to the metastatic stage. A series of these data are reviewed here.

Phenotype studies in breast tumors—molecular markers and grade

Before examining the occurrence of phenotype changes during tumor progression, it is necessary to specify which phenotype categories will be considered here. The categories are based on: (1) a series of molecular markers related to ER status; (2) grade. The rationale behind this choice is that both ER status and grade have long been used to classify tumors. As a consequence, there is abundant literature examining correlations between them and other parameters such as marker expression or proliferation. A classification based on an ER-positive/ER-negative or a low-grade/high-grade dichotomy hardly suffices to describe the complex spectrum of breast tumors; however, its simplicity will facilitate our main purpose: evaluating the stability of the breast tumor 'portrait'.

Molecular markers

ER appears as a major discriminator in the molecular classification of breast tumors. As a mediator of (anti-)estrogen action, its key role in the biology and treatment of breast cancer is well established. ER level has been evaluated in tumors for more than 30 years (Leclercq *et al.* 2002). The receptor, encoded by the *ESR1* gene, was long believed to be unique, until an isoform named ER-beta and encoded by a specific gene, *ESR2*, was identified. The 'older' isoform, subsequently named ER-alpha, seems to be functionally the most important in breast tumors (Speirs 2002). We have shown that the ER protein content, evaluated in breast tumors by a ligand-binding assay which measures both ER-alpha and -beta isoforms, was linearly correlated with the level of mRNA specific for *ESR1*, while the *ESR2* mRNA was undetectable in samples (Lacroix *et al.* 2001). Here, ER-beta will be considered as being of secondary importance in breast tumors, and the term ER will refer to the alpha isoform, unless otherwise indicated. ER is expressed by about 60–80% of breast tumors ('ER-positive'), while 20–40% are considered ER-negative.

During the last decade, many genes and/or proteins with an expression level positively or negatively correlated to that of ER have been identified in tumors. A series of these genes are listed in Table 1a (positive correlation to ER) and b (negative correlation to ER). From these studies, it appeared that two highly different phenotypes could be found in breast tumors according to their ER status. In addition, the co-existence in the same tumor of markers related to both ER-positive and ER-negative phenotypes was rarely observed. For instance, it is well known that ER and epidermal growth factor receptor (EGFR) levels are inversely correlated in most ductal

tumors. Both receptors are, however, occasionally co-expressed in lesions, but are then, in the vast majority of cases, localized in distinct tumor cells, or in interspersed groups of cells ('mosaic expression', as described for instance in van Agthoven *et al.* 1994). Whether ER-poor/EGFR-rich BCCs were derived from ER-rich/EGFR-poor cells in these tumors is unknown. If this was the case, the observations suggest that such an event is infrequent and does not seem to be related to any significant advantage for progression. Rare co-expressions have also been observed with other pairs of markers related to distinct BCC phenotypes (not discussed here).

A series of proliferation/apoptosis-related markers has also been measured in tumors. In general, their expression levels reflected the fact that mitotic/apoptotic activity is higher in ER-negative than in ER-positive lesions (Keshgegian & Cnaan 1995, Gandhi *et al.* 1998, Lipponen 1999). For instance, the expression of the apoptosis inhibitor BCL2 was correlated to that of ER (Gee *et al.* 1994, Binder *et al.* 1995, Yang *et al.* 1999). P53 levels were repeatedly found to be higher in ER-negative lesions (see notably Rudolph *et al.* 1999a). Proliferation markers KI-67 and topoisomerase II alpha levels were also positively correlated to ER-negative status (Molino *et al.* 1997, Rudolph *et al.* 1999b). Regarding cyclins, while cyclin E was associated with the absence of ER, the inverse was observed for cyclin D1 (Nielsen *et al.* 1997, Barnes & Gillett 1998, Reed *et al.* 1999, Spyrtatos *et al.* 2000, Park *et al.* 2001, Loden *et al.* 2002). The level of the cyclin-dependent kinases inhibitors 1A (P21^{WAF1/CIP1}) and 1B (P27^{KIP1}) was found to be higher in ER-positive tumors (Barbareschi 1999, Reed *et al.* 1999, Barbareschi *et al.* 2000, Oh *et al.* 2001).

The evaluation of multiple markers in multiple tumor samples has recently been facilitated by the use of tissue micro-arrays (TMAs) (Kononen *et al.* 1998, Camp *et al.* 2000, Simon *et al.* 2004). In general, TMA-based data are in good agreement with previous observations. For instance, TMA analysis was applied to 107 breast carcinomas samples, to assess the pattern of expression of ER, PR, P53, ERBB2, MYC, P27^{KIP1}, cyclin D, cyclin E, BCL2, MIB1, MCM2 (minichromosome maintenance protein 2, see Gonzales *et al.* 2003), basal cytokeratins CK5/6, epithelial cytokeratins CK8/18. Cluster analysis of the data classified samples into two main groups: the first ('ER-related') included about two-thirds of the tumors and was characterized by a high expression level of ER, PR, BCL2, cyclin D, P27^{KIP1}, CK8/18, MYC; the second group expressed relatively high levels of P53, ERBB2, CK5/6, and of the proliferation markers MCM2, MIB, and cyclin E (Callagy *et al.* 2003). In another study, 15 markers (ER, PR, P53, ERBB2, EGFR, cyclin A, cyclin D1, cyclin E, BCL2, P21^{WAF1/CIP1}, P27^{KIP1}, CK5/6, CK8/

Table 1a Series of genes differentially expressed in breast tumors – genes expressed at higher levels in ER-positive and/or in low-grade tumors.

Gene name	Gene product name(s)	Higher expression in		References
		ER-positive tumors	Low-grade tumors	
CA12	Carbonic anhydrase XII	*	*	Watson <i>et al.</i> 2003
CDH1	Cadherin type 1, epithelial cadherin (E-cadherin)	*	*	Sommers <i>et al.</i> 1994, Parker <i>et al.</i> 2001
CLDN7	Claudin 7		*	Kominski <i>et al.</i> 2003
DSP	Desmoplakin (DPI, DPII)		*	Sommers <i>et al.</i> 1994, Davies <i>et al.</i> 1999
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	*	*	Bieche <i>et al.</i> 2003
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	*	*	Bieche <i>et al.</i> 2003
ESR1	Estrogen receptor alpha	*	*	Tong <i>et al.</i> 1999, Zafrani <i>et al.</i> 2000, Ringberg <i>et al.</i> 2001
GATA3	GATA sequence binding protein 3	*		Hoch <i>et al.</i> 1999
GREB1	Greb1 protein	*		Ghosh <i>et al.</i> 2000
MDM2	Mdm2, p53 binding protein	*	*	Gudas <i>et al.</i> 1995, Hori <i>et al.</i> 2002
NME1	Protein expressed in non-metastatic cells (nm23A)		*	Hartsough & Steeg 2000, 2001
PDZK1	PDZ domain containing 1	*		Ghosh <i>et al.</i> 2000
PGR	Progesterone receptor	*	*	Hall <i>et al.</i> 1990, Tong <i>et al.</i> 1999, Zafrani <i>et al.</i> 2000
PRDM2	PR domain containing 2 (RIZ1), transcript 1			Du <i>et al.</i> 2001
PRLR	Prolactin receptor	*		Peirce <i>et al.</i> 2001, Gill <i>et al.</i> 2001
PTPRA	Protein tyrosine phosphatase, receptor type, A	*	*	Ardini <i>et al.</i> 2000
RERG	Ras-like, estrogen-regulated, growth-inhibitor	*		Finlin <i>et al.</i> 2001
SLC9A3R1	Solute carrier family 9, isoform 3 regulatory factor 1	*		Stemmer-Rachamimov <i>et al.</i> 2001
STC2	Stanniocalcin 2	*		Bouras <i>et al.</i> 2002
TFAP2C	Transcription factor activator protein 2 gamma	*		Kuang <i>et al.</i> 1998
TFF1	Trefoil factor 1 (pS2, BCEI)	*	*	Tong <i>et al.</i> 1999
TFF3	Trefoil factor 3	*		Gillesby & Zacharewski 1999
TJP1	Tight junction protein 1 (ZO-1)	*	*	Sommers <i>et al.</i> 1994, Hoover <i>et al.</i> 1998

Table 1b Series of genes differentially expressed in breast tumors – genes expressed at higher levels in ER-negative and/or in high-grade tumors.

Gene name	Gene product name(s)	Higher expression in		References
		ER-negative tumors	High-grade tumors	
AKT3	V-akt murine thymoma viral oncogene homolog 3	*		Nakatani <i>et al.</i> 1999
CCNE1	Cyclin E1	*	*	Donnellan <i>et al.</i> 2001
CDH3	Cadherin 3, placental cadherin (P-cadherin)	*	*	Paredes <i>et al.</i> 2002
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	*	*	Hui <i>et al.</i> 2000
CTSB	Cathepsin B	*	*	Lah <i>et al.</i> 2000
CTSL	Cathepsin L	*	*	Lah <i>et al.</i> 2000
DFNA5	Deafness, autosomal dominant 5 (ICERE-1)	*		Thompson & Wiegel 1998
EGFR	Epidermal growth factor receptor	*	*	Hall <i>et al.</i> 1990, Klijn <i>et al.</i> 1992, Walker & Dearing 1999
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (avian)	*	*	Tsuda <i>et al.</i> 1990, Rilke <i>et al.</i> 1991, Tagliabue <i>et al.</i> 1999, Spizzo <i>et al.</i> 2002, Hoque <i>et al.</i> 2002, Hoff <i>et al.</i> 2002
GPX1	Glutathione peroxidase 1	*		Townsend <i>et al.</i> 1991, Esworthy <i>et al.</i> 1995
GSTP1	Glutathione S-transferase pi	*		Moscow <i>et al.</i> 1988
HGF	Hepatocyte growth factor (hepatoietin A; scatter factor)		*	Beviglia <i>et al.</i> 1997
HMG1Y	High-mobility group protein isoforms I and Y		*	Liu <i>et al.</i> 1999
HXB	Hexabrachion (tenascin-C)	*	*	Dandachi <i>et al.</i> 2001
IL8	Interleukin-8	*	*	De Larco <i>et al.</i> 2001
MET	Met proto-oncogene (HGF receptor)	*	*	Beviglia <i>et al.</i> 1997
MSN	Moesin	*		Carmeci <i>et al.</i> 1998
MT1E	Metallothionein 1E	*		Friedline <i>et al.</i> 1998, Jin <i>et al.</i> 2000
PLAU	Plasminogen activator, urokinase	*	*	Grøndahl-Hansen <i>et al.</i> 1993, Tetu <i>et al.</i> 2001, Look <i>et al.</i> 2002
PTGS2	Prostaglandin-endoperoxide synthase 2	*	*	Ristimaki <i>et al.</i> 2002
RARB	Retinoic acid receptor, beta	*	*	Widschwendter <i>et al.</i> 2000
S100A4	S100 calcium binding protein A4 (metastasin)	*	*	Pedrocchi <i>et al.</i> 1994, Sherbet & Lakshmi 1998
SDC1	Syndecan 1	*	*	Barbareschi <i>et al.</i> 2003
SERPINEB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	*	*	Umekita <i>et al.</i> 2002
SERPINE1	Plasminogen activator inhibitor type 1 (nexin)	*	*	Grøndahl-Hansen <i>et al.</i> 1993, Look <i>et al.</i> 2002
SNAI1	Snail homolog 1	*	*	Blanco <i>et al.</i> 2002
STMN1	Stathmin 1 (oncoprotein 18)	*	*	Curmi <i>et al.</i> 2000
STS	Steroid sulfatase	*	*	Miyoshi <i>et al.</i> 2003
TIMP1	Tissue inhibitor of metalloproteinase 1	*	*	Yoshiji <i>et al.</i> 1998, McCarthy <i>et al.</i> 1999, Remacle <i>et al.</i> 2000
VIM	Vimentin	*	*	Sommers <i>et al.</i> 1994, Dandachi <i>et al.</i> 2001

18 and SMA (smooth muscle actin)) were evaluated in 166 breast cancer samples. Tumors expressing the basal CK5/6 showed higher levels of cyclin A, KI-67, P53 and EGFR expression; tumors expressing CK8/18 but not CK5/6 were associated with a higher level of ER, PR, P21^{WAF1/CIP1}, P27^{KIP1}, ERBB2 and BCL2. When cluster analysis was applied to the data, three classes were found. Besides those defined by the presence or absence of CK5/6, a third class appeared which was characterized by ERBB2 over-expression and *ERBB2* amplification (Korsching *et al.* 2002). Thus, a specific 'portrait' may occasionally be associated with ERBB2-overexpressing tumors. This is also found in DNA-micro-array-based gene expression studies (see Micro-array studies in section on Genetic studies on breast tumors).

Grade

One of the most widely accepted classification systems for breast carcinomas is grading. The majority of grading systems, such as those based on the Scarff, Bloom and Richardson (SBR) method, combine histological assessment of nuclear pleomorphism, mitotic activity and tubule formation (Elston & Ellis 2002). Tumors classified as 'grade I' or 'low grade' have well-differentiated attributes, while 'grade III' or 'high-grade' tumors have poorly differentiated attributes. Grade II tumors fall into an intermediate category. High-grade ductal carcinoma have been associated with the highest rate of local recurrence (25–30%), low-grade tumors have very low recurrence (0–5%), while intermediate-grade tumors have a recurrence rate somewhere in between (10–15%) in 12 years of median follow-up (Polyak 2001). Moreover, high-grade tumors recur within a shorter time than the low-grade ones (for instance, in Cserni (2002), the median recurrence time is 88, 42 and 23 months for grades I, II and III respectively).

Grading is not directly based on molecular expression profiles. It may thus be asked whether or not grades are associated with the expression of specific sets of tumor markers, and more precisely if they are correlated to the distinct ER-status phenotypes described above for tumors. It has been repeatedly reported that most ER-positive tumors are of low grade. Conversely, high-grade tumors are mainly ER-negative (see for instance Tong *et al.* 1999, Zafrani *et al.* 2000, Ringberg 2001). Unsurprisingly, several markers whose expression is positively correlated to that of ER in tumors have also been associated with low-grade tumors; these are listed in Table 1a. Conversely, high-grade tumors are characterized by the expression of markers more related to the ER profiles in tumors; these are listed in Table 1b. None of the genes positively correlated to ER in tumors were found associated with high-grade/poorly differentiated carcino-

mas. None of the genes negatively correlated to ER in BCC in tumors was found associated with low-grade/well-differentiated lesions.

Proliferation/apoptosis-related markers appear to reflect the fact that mitotic/apoptotic activity is higher in high-grade/poorly differentiated tumors. For instance, a study examining mitotic index (MI, number of mitoses/1000 nuclei) and apoptotic index (AI, number of apoptosis/1000 nuclei) found MI and AI values to be up to 20-fold and 2.5-fold higher, respectively, in poorly differentiated ductal carcinoma *in situ* (DCIS), as compared with well-differentiated DCIS (Buerger *et al.* 2000a). This is in line with results from other groups (Keshgegian & Cnaan 1995, Gandhi *et al.* 1998). For instance, in an immunohistochemical (IHC) study of 46 DCIS, it was shown that BCL2 was expressed in 12/12, 14/20 and 1/14 well-, moderately and poorly differentiated tumors respectively. For P53, the respective values were 0/12, 6/20 and 12/14, while for KI-67 (Viacava *et al.* 1999) they were 0/12, 10/20 and 14/14. In another report, KI-67 expression was found in only 5% of low-grade but in 70% of high-grade DCIS. For P53, the respective percentages were 5% and 50% (Krishnamurthy & Sneige 2002). Regarding cyclins, while cyclin E has been associated with high-grade tumors, the inverse was observed for cyclin D1 (Loden *et al.* 2002). The level of P21^{WAF1/CIP1} and P27^{KIP1} was found to be higher in low-grade or well-differentiated tumors (Barbareschi 1999, Reed *et al.* 1999, Wu *et al.* 1999, Oh *et al.* 2001, Barbareschi *et al.* 2000).

The relationship between marker expression and grade has also been investigated by TMA analysis (see below). For instance, in a study of 107 breast cancer samples, the expression of 11 markers showed an association with grade: ER, PR, BCL2, P27^{KIP1} and cyclin D levels were higher in low-grade samples; ERBB2, P53, cyclin E, CK5/6, MCM2 and MIB1 levels were higher in high-grade tumors (Callagy *et al.* 2003). These results are in agreement with previous data.

Concluding remarks on molecular markers and grade

A number of studies have shown that the phenotype categories based on ER status and on grade largely overlap. ER-positive and well-differentiated/low-grade tumors on the one hand, and ER-negative and poorly differentiated lesions on the other hand, share many features. But the two phenotypes defined in each category (ER-positive and -negative, well and poorly differentiated) appear highly different, so that a frequent transition from one to the other during progression seems rather unlikely. However, this does not exclude the possibility that co-expression of the two phenotypes could occasionally occur in the same lesion, as suggested by the

existence of grade II tumors. However, it is largely unknown whether such cases result from phenotype changes during tumor growth, or are derived from two populations that appeared early in tumorigenesis.

Macroscopic homogeneity of breast tumors — stable 'portrait' during progression

According to the multi-step view, progression from primary to metastatic tumor should be accompanied by the sequential acquisition of phenotype changes, allowing BCCs to invade, disseminate and colonize distant sites. Notably, it has been proposed that BCCs *in vivo* might undergo a transition from the ER-positive- to the ER-negative-associated phenotype. Along the same lines, it has been repeatedly proposed that tumor progression is characterized by a shift from the well-differentiated/low-grade to the poorly differentiated/high-grade phenotype. Nevertheless, most investigations have revealed that progression is not accompanied by major changes in marker expression or grade.

Progression to invasiveness and markers/grade

If we hypothesize that progression from *in situ* to invasive carcinoma is often accompanied by extensive phenotype changes, it should then be easy to find in a significant part of invasive tumors both ER-positive- and ER-negative-associated, and both low- and high-grade, compartments. ER-negative-associated markers and high-grade areas should normally be observed more frequently in the invasive than in the *in situ* tumor compartment. In fact, most studies examining this point have revealed a striking similarity between both parts of breast carcinomas (Lampejo *et al.* 1994, Moriya & Silverberg. 1994, Iglehart *et al.* 1995, Douglas-Jones *et al.* 1996, Millis *et al.* 1998, Foster *et al.* 2000, Mommers *et al.* 2001 Warnberg *et al.* 2001.). For instance, grade and tumor marker (P53, ERBB2, KI-67, ER, PR, BCL2 and angiogenesis) expression were compared in 194 pure DCIS, 127 small invasive lesions and 305 lesions with both an invasive and *in situ* component. Grade concordance was high between *in situ* and invasive components of the same tumor. All markers were found to correlate with grade rather than with invasiveness. No marker was clearly associated with the progression from *in situ* to invasiveness. The expression of tumor markers was similar, at 80–90%, in the two components of mixed lesions (Warnberg *et al.* 2001). The DNA content and the expression of ERBB2 were examined simultaneously in both non-invasive and invasive phases of primary breast cancers, by image

analysis. DNA content in the intraductal and invasive components was virtually identical. Expression of ERBB2 was similar in both growth phases, strongly suggesting identity of the ERBB2 genotype (Iglehart *et al.* 1995). An IHC study examined the expression of proteins involved in proliferation and apoptosis (KI-67, cyclin D1, ERBB2, P21^{WAF1/CIP1}, P27^{KIP1}, P53 and BCL2) in 61 DCIS and 53 invasive lesions. More proliferation tended to be observed in invasive cancers. However, well-differentiated DCIS and invasive lesions shared many aberrations in expression of the proliferation-associated proteins, as did poorly differentiated DCIS and invasive lesions. In contrast, many differences were observed between the well- and poorly differentiated lesions (Mommers *et al.* 2001). In a study of 102 patients, a 67% concordance in grade was found between *in situ* and infiltrating components (Millis *et al.* 1998). Another study of 64 cases indicated an 86% grade concordance between both components (Moriya & Silverberg. 1994). These studies and others (Lampejo *et al.* 1994, Douglas-Jones *et al.* 1996) indicated a strong correlation between the grade of DCIS and the grade of infiltrating carcinoma in which both components were present.

It is thus striking that patterns of grade or the other markers did not seem to change significantly during the transition from *in situ* to invasive carcinoma. Invasive cancer seems to occur independently of tumor grade. This is further supported by comparative genetic hybridization and micro-array data (see below).

Recurrence, metastasis

Metastatic and recurrent BCCs are often believed to have accumulated phenotypic alterations, as they are associated with late stages in tumor progression. In addition, metastatic cells may colonize various tissues that are highly different from the breast (bone, lung, brain, etc.) after having completed all steps of a complex process including local invasion, intravasation, resistance to blood pressure, adhesion to blood vessels, and extravasation. This suggests that they have sequentially acquired specific adaptive properties and it has thus been hypothesized that metastatic and recurrent cells could express a phenotype significantly different from that observed in the primary tumor.

Attempts have been made to compare the expression of various markers and/or the histological grade in primary tumors and their corresponding metastases and/or recurrences. It was shown that CK8 and CK19 expression were similar in both primary carcinomas and their lymph node (LN) metastases (Su *et al.* 1996). In an IHC study of 38 LN metastases and their corresponding primaries, very good concordance was found for KI-67 (85%), ER (96%), PR (82%), P53 (76%) and ERBB2

(84%) (Briffod *et al.* 2000). In another comparative IHC study involving 102 LN metastases, an 80% concordance was found for ER (Nedergaard *et al.* 1995). Investigations on a total of 31 LN, 35 lung, 25 skin, 1 liver and 2 contralateral breast metastases revealed good concordance with primaries for ER, PR, P53 and ERBB2 (Barnes *et al.* 1988, Kayser *et al.* 1998, Shimizu *et al.* 2000). This was also the case for ER, PR and EGFR evaluated in 26 LN and 2 distant metastases. In this latter study, expression of ER and EGFR was inverse regarding the individual tumor cells in both primary tumors and metastases (van Agthoven *et al.* 1995). By ligand-binding assay, it has been estimated that no more than 20% of the ER-positive primary tumors will produce ER-negative metastases. It has even been established that the expression of the frequent ER- α variant transcripts is conserved in primary tumors and their matched, concurrent LN metastases (Fuqua 2001). A good concordance was also found for grade; for instance, a study of 102 primaries and LN metastases revealed that both had the same grade (I, II or III) in 79% of cases (Millis *et al.* 1998). In a tissue micro-array analysis, it was found that 77% of ERBB2-positive primary tumors had entirely ERBB2-positive LN metastases, 6.5% had entirely ERBB2-negative metastases and 16.5% had a mixture of ERBB2-positive and ERBB2-negative metastases. For ERBB2-negative primary tumors, a 95% concordance was found for the LN metastases. Moreover, the ERBB2 status within individual tumors was fairly homogenous, as was the status of primary tumors and their metastases (Simon *et al.* 2001).

Along the same lines, several studies have examined grade and marker (ER- α , ER- β , PR, P53, ERBB2 and TFF1 (formerly pS2)) expression in recurrent breast cancers (Millis *et al.* 1998, Horiguchi *et al.* 2000, Shimizu *et al.* 2000, Bijker *et al.* 2001, Jensen *et al.* 2001). Good concordance was found in most cases; for instance, in an analysis of 116 cases of recurrence, only four patients were found to have developed poorly differentiated DCIS or grade III invasive carcinoma after well-differentiated DCIS (Bijker *et al.* 2001). Regarding ER- α and ER- β , their expression was even found to be higher in recurrences than in the corresponding primaries (Jensen *et al.* 2001). In a study of six cases of recurrence, histological type was the same as the initial one. There was concordance in ER, PR, TFF1, ERBB2 and P53 status between the recurrence and the primary cancer (Horiguchi *et al.* 2000). In a study of 49 primaries and recurrences, a 78% grade (I, II or III) concordance was found; in 36 patients who developed both metastasis and recurrence, grade concordance between them was also 78% (Millis *et al.* 1998). In an analysis of 84 patients for which axillary metastases and/or local and/or regional recurrence(s) were found, 78 and 81% concordance was demonstrated

between primaries and their metastases and first recurrences respectively. In the cases where successive (up to six) recurrences were found, there was still a 74% concordance between the last recurrence and the initial tumor sample (Cserni 2002).

Conclusions

In conclusion, breast tumor phenotype does not appear, in most cases, to change extensively during tumor progression from *in situ* carcinoma to secondary site colonization. However, nearly all of the studies described here showed percentages of concordance in the 65–95% range. This indicates the existence of a substantial number of cases in which progression (to invasiveness, to metastasis or recurrence) is accompanied by qualitative changes in marker expression or grade. The fact that BCCs could occasionally undergo profound phenotype alterations has been suggested for years. For instance, it has been proposed that epithelial–mesenchymal transition (EMT) could occur in BCCs. This process would be reminiscent of the transition that is observed during embryonic development at precise times and locations (Boyer *et al.* 2000). EMT in BCCs would consist of the turning-off of genes encoding epithelial markers (ER- α , PR, E-cadherin, tight junction proteins etc.) and the increase of markers such as vimentin. While the reality of EMT has been little substantiated *in vitro*, the possibility that it could happen *in vivo* cannot be definitely excluded.

Genetic studies on breast tumors

Karyotype and cytogenetic studies

Breast cancer is characterized by multiple genetic alterations. They may include whole chromosome copy gain or loss (aneuploidy), gain and loss of parts of chromosomes (detected by comparative genetic hybridization and loss of heterozygosity analysis), amplifications or deletions of single genes, insertions and translocations, and mutations of a single or a few nucleotide(s).

Aneuploidy

Aneuploidy is frequent in breast carcinomas. In a study of 127 000 breast tumors, about one-half were found to be diploid or near diploid, the others exhibiting various types of aneuploidy (Wenger *et al.* 1993). In an analysis of 256 patients, 384 modal chromosome numbers were detected, ranging between 29 and 211; 74% of these modal numbers were between 41 and 50, 19% between 51 and 80. Only 3% were lower than 41 and 4% higher than 80 (Teixeira *et al.* 2002). Our cumulative study of breast tumor series (Ried *et al.* 1995, Schwendel *et al.* 1998, Adeyinka *et al.* 1999,

Roylance *et al.* 1999, Davidson *et al.* 2000, Dellas *et al.* 2002, Zudaire *et al.* 2002) emphasized that monosomy was observed mainly for chromosomes 7, 19, 20 and X, while trisomy most frequently concerned chromosomes 4, 18, 19 and X. By analyzing a set of relatively small and partly overlapping series of BCC lines (Forozan *et al.* 1999, 2000, Kytola *et al.* 2000, Davidson *et al.* 2000, Larramendy *et al.* 2000), the same variations were observed, except that loss of chromosome X was relatively less frequent in cell lines. As most available cultured BCC lines are of metastatic origin, this suggests that the qualitative pattern of whole chromosome losses and gains remains constant during tumor progression. But what about the quantitative pattern?

Fluorescence *in situ* hybridization (FISH) analysis of chromosomes 7, 8, 16 and 17 was applied to foci of residual DCIS and a representative area of co-existing invasive neoplasm. Most hybridization pairs (7/12, 58%) showed a gain in chromosomal copy number between the *in situ* and corresponding invasive area, whereas 29% showed no apparent change and 13% showed loss in copy number. Hybridizations from areas of invasive carcinoma, thus, were more frequently characterized by tumor cells with trisomy/polysomy (78%) than neoplastic cells from residual DCIS (50%) and less frequently characterized by cells with monosomy (10% versus 16%). Even when DCIS cells exhibited chromosome trisomy, 65% of hybridizations demonstrated a significantly greater proportion of trisomic cells in the corresponding invasive population (Mendelin *et al.* 1999). In another FISH study, a high proportion (54%) of 214 invasive breast carcinomas displayed aneusomy of chromosome 17. Aneusomy was not associated with survival, suggesting that it is not significantly related to the development of metastases. In contrast, an association was found with grade III carcinoma and ER negativity (Watters *et al.* 2003).

Indeed, although several FISH studies have attempted to identify chromosome gain or loss responsible for breast tumorigenesis and progression, no specific alterations can yet be repeatedly attached to certain histopathological stage. On the other hand, the patterns of aneuploidy may differ according to tumor grade. For instance, in a FISH study of numerical alterations of chromosomes 7, 8, 16 and 17 in 28 DCIS, grade I lesions were characterized by a complete lack of significant chromosome gain, but 29% showed partial (focal) monosomy. Grade III lesions, in contrast, showed partial or complete trisomy/polysomy in 88% hybridizations versus monosomy in only 4%. Grade II DCIS exhibited a mixed pattern of chromosome aneuploidy: 38% hybridizations were disomic, 36% trisomic/polysomic and 26% monosomic (8 out of 10 hybridizations showing complete monosomy occurred in grade II lesions). In morphologically heterogeneous

lesions, higher-grade foci were characterized by chromosome copy gain relative to corresponding lower-grade areas in 17 of 22 (77%) hybridizations (Visscher *et al.* 2000).

In conclusion, despite the fact that most tumors tend to gain chromosomes during progression, a higher proportion of high-grade carcinomas are believed to progress to near-triploidy (Pandis *et al.* 1996). However, no specific chromosome number change has been as yet clearly associated with progression.

An important criticism that is often addressed to karyotype analysis is that it may be biased, at least when it is applied to long-term cell cultures. The resulting karyotypes may represent minor malignant cell clones of the tumors expressing a growth advantage in culture (see for instance Truong *et al.* 1999). It is also possible that some of the simple abnormal karyotypes might be due to mitoses of non-malignant breast lesions (Lundin & Mertens 1998, Persson *et al.* 1999). This underlines the need for additional techniques to detect genetic alterations.

Comparative genomic hybridization (CGH) studies

In addition to changes in chromosome number, DNA losses or gains larger than 10 mb have been detected in tumors by CGH. Table 2 reports the frequency of DNA losses or gains affecting the ten most involved chromosome arms, as determined from a cumulative set of breast tumors (Ried *et al.* 1995, Schwendel *et al.* 1998, Adeyinka *et al.* 1999, Roylance *et al.* 1999, Davidson *et al.* 2000, Loveday *et al.* 2000, Guenther *et al.* 2001, Zudaire *et al.* 2002, Cingoz *et al.* 2003). These data have been confirmed by a recent study of 305 unselected primary invasive breast tumors, according to which the six most commonly observed gains were on 1q (55%), 8q (41%), 16p (40%), 17q (28%), 20q (19%) and 11q (16%) and the three most commonly observed losses were on 13q (27%), 16q (22%) and 8p (18%) (Rennstam *et al.* 2003).

Several studies using CGH analysis on DCIS have demonstrated a large number of chromosomal alterations

Table 2a Chromosome arms most frequently altered in a cumulative series of 542 breast tumors (CGH analysis), in order of decreasing frequency – DNA losses

16q	1p	8p	13q	11q	17p	22q	6q	Xp	Xq
25%	21%	20%	19%	19%	18%	17%	14%	11%	11%

Table 2b DNA gains

1q	8q	17q	20q	16p	11q	12q	7q	6q	3q
51%	46%	24%	24%	22%	21%	17%	15%	15%	15%

including gains on 1q, 6q, 8q, 17q, 19q, 20q and Xq, and losses on 13q, 16q, 17p and 22q. Most of these alterations resemble those identified in invasive ductal carcinoma (IDC), adding weight to the idea that DCIS is a direct precursor lesion of IDC (Aubele *et al.* 2002).

It has been shown that DNA loss at 16q is less frequent in high-grade ductal carcinoma (Roylance *et al.* 1999, 2002, Richard *et al.* 2000, Boecker *et al.* 2001, Cingoz *et al.* 2003) and in ER-negative tumors (Zudaire *et al.* 2002). This constitutes a strong argument against the theory supporting a frequent tumor progression from low to high grade and from ER-positive to ER-negative status. Indeed, it appears unlikely that grade III tumors could arise from grade I tumors through a process involving regain of 16q. Besides similar observations on 16q, Richard *et al.* (2000) also noted a higher frequency of 7q gains in ER-negative carcinomas and of 3q gains in ER-negative and high-grade carcinomas, as well as a lower frequency of 16p gains in ER-negative tumors and of 22q losses in ER-negative and high-grade carcinomas. In an analysis of 22 tumors (DCIS and IDC), ER positivity was significantly higher in cases displaying 16q losses and 20q gains (Cingoz *et al.* 2003). Other investigators pointed out that, contrasting with the higher global frequency of chromosome changes in high-grade carcinomas, 16q losses were more frequent in low-grade carcinomas, while the frequency of 20q gains was the same in both low- and high-grade lesions (Boecker *et al.* 2001). Thus, while several qualitative differences have been highlighted by CGH in ER-positive/low-grade tumors, as compared with ER-negative/high-grade lesions, only one change, loss at 16q, has been repeatedly observed. Whether it is crucially involved in phenotype definition is unlikely, as not all ER-positive/low-grade tumors are characterized by this loss, while it is occasionally observed in ER-negative/high-grade tumors.

Total DNA changes were found to be 1.7-fold more frequent in ER-negative than in ER-positive tumors (Richard *et al.* 2000), while between 1.5- and 3.2-fold more genetic alterations were observed in grade III/high-grade/poorly differentiated than in grade I/low-grade/well-differentiated samples (Schwendel *et al.* 1998, Roylance *et al.* 1999, 2002, Buerger *et al.* 2000a, Richard *et al.* 2000). For instance, amplification of *ERBB2* (17q12), *TOP2A* (17q24), *MYC* (8q23), and *CCND1* (11q13) was more frequently found in high- than in low-grade tumors. However, major amplifications in pure *in situ* carcinoma and in intraductal carcinoma with an invasive component did not differ (Glockner *et al.* 2001). That no specific gross DNA alteration was associated with invasion was confirmed by analysis of a series of ductal (but also lobular) tumors submitted to CGH following microdissection (Buerger *et al.* 2000b).

Loss of heterozygosity (LOH) studies

LOH analysis detects allelic loss at specific loci by a PCR-based screening with polymorphic microsatellite markers spaced across the region of interest. This technique has been widely used to detect the loss of putative suppressor genes.

LOH in breast cancer has been observed in multiple chromosomal regions, notably 1p, 1q, 3p, 6q, 7q, 8p, 9p, 11q, 13q, 16q, 17p, 17q, 18q and 22q. The highest rate of LOH in DCIS approaches 50–80% and involves loci on chromosomes 16q, 17p and 17q, suggesting that altered genes in these regions may play a role in the development of DCIS. In general, LOH frequency has been found to be higher in IDC than in DCIS (see notably Ando *et al.* 2000). Eighty per cent of the DCIS shared their LOH patterns with invasive carcinomas from the same breast, strongly supporting a precursor relationship between these lesions and the cancers they accompany (Deng *et al.* 1996).

Differences in LOH frequencies according to grade have been repeatedly observed in breast tumors. Thus, Ando *et al.* (2000) observed higher frequency of LOH at 16q in low- and intermediate-grade DCIS, while LOH at 11p and 17p were less frequent for these grades.

The fact that regions showing the highest levels of LOH were different in tumors of different grades has notably been illustrated by Shen *et al.* (2000). In their genome-wide search using laser capture micro-dissected tissue of breast carcinoma, these authors found LOH frequencies as follows:

Well-differentiated lesions: 16q22.1 (47.6%), 16q22.3 (42.1%), 17p12 (37.5%), 11q22.1 (35.0%), 9q22.33 (35.0%), 1q24.2 (35.0%), 12p12.3 (33.3%), 3p22.1 (33.3%), 1q24.2 (32.0%), 1q44 (31.6%).

Moderately differentiated lesions: 11q22.2 (42.9%), 17p12 (42.1%), 14q31.1 (41.7%), 14q32.11 (41.2%), Xq13.3 (40.0%), 16q22.1 (40.0%), 14q24.1 (39.1%), 8p12 (39.1%), 17q21.31 (37.0%), 16q24.3 (37.0%).

Poorly differentiated lesions: 17p13.3 (90.9%), 17p12 (86.7%), 17p13.3 (77.7%), 17p13.2 (73.3%), 4p15.1 (73.3%), 1q32.1 (71.4%), 4q28.2 (70.0%), 17q21.31 (63.2%), 3q23 (62.5%), 1p36.12 (61.1%), 22q12.3 (60.0%), 8q24.3 (60.0%), 3p14.3 (60.0%).

Such results are in agreement with the observations of Ando *et al.* (2000) on 16q and 17p.

Instability at the nucleotide level

Genomic instability may also exist at the nucleotide level, resulting in base substitutions (nucleotide instability, or NIN) or in deletions or insertions of a few nucleotides (micro-satellite instability, or MIN). In breast tumors however, MIN has been observed only in a small subset (<10%) of tumors (Ingvarsson 1999), and there is little

evidence of mutation hotspots to support a significant etiological role of NIN in this type of cancer.

Concluding remarks on karyotype and cytogenetic studies

Breast cancer progression is accompanied by an increase in the number of genetic alterations. However, no specific gross alteration has been clearly and repeatedly associated with a specific tumor stage. In contrast, qualitative and quantitative differences exist between ER-positive and ER-negative tumors, as well as between low- and high-grade lesions. The lower occurrence of 16q loss in grade III cancers, repeatedly observed, appears to challenge the conception of tumor progression from ER-positive to ER-negative status and from low to high grade.

The data presented in this section concern major (high-frequency) DNA changes. Additional studies have demonstrated that a high genetic divergence characterizes BCC *in vivo*. A number of gains or losses of chromosomal material occur in tumors at a low frequency and at many different sites. This is in agreement with the concept of micro-heterogeneity in breast cancer. Multiple karyotypically related as well as unrelated clones (i.e. no single chromosomal abnormality is shared by them) have been found in a high proportion of carcinomas, suggesting that genetic mechanisms are crucially involved in the generation of small cell-to-cell and clone-to-clone variation in tumors (Aubele et al. 1999, Teixeira et al. 2002). Thus, invasive breast cancer may be viewed as a disease with multiple cytogenetic sub-clones and since no specific DNA alteration has been associated with invasion (see for instance Buerger et al. 2000b), it is concluded that complex patterns of non-specific changes are acquired during tumor progression. Accumulation of these minor (low-frequency) alterations distributed along the genome could ultimately overcome the mechanisms preventing cell aggressiveness. It has been found that the number of genomic aberrations is higher in tumors that give rise to recurrences (Dellas et al. 2002). Moreover, analysis of distant metastases (brain) showed that they were characterized by an accumulation of various genetic alterations and increased LOH frequency at all loci examined (Hampl et al. 1998-1999). In another study, the total number of aberrations detected by CGH exclusively in the lymph nodes or distant metastases was higher than in the primary breast tumors (2.5 vs 0.7) (Nishizaki et al. 1997). Analysis of single disseminated tumor cells has also revealed a high genetic heterogeneity, irrespective of whether they resided within the same compartment or within different homing sites, or whether they were isolated on repeated bone-marrow aspirations (Klein et al. 2002).

Micro-array studies

During recent years, gene expression studies have gained considerably from the introduction of massive and simultaneous analysis tools. Micro-array-mediated studies have allowed measuring the level of up to thousands of mRNAs in tumors (Sgroi et al. 1999, Bertucci et al. 2000, 2002, Perou et al. 2000, Ross et al. 2000, Finlin et al. 2001, Gruvberger et al. 2001, Ross & Perou 2001, Sørli et al. 2001, 2003, West et al. 2001, Zajchowski et al. 2001, Lacroix et al. 2002, van de Rijn et al. 2002, van't Veer et al. 2002, Ma et al. 2003, Sotiriou et al. 2003, Lacroix & Leclercq 2004a). The existence of a few major phenotypes in breast cancer has been confirmed by such investigations.

Cluster analyses of micro-array data from series of breast tumors have repeatedly led to the identification of a major 'luminal epithelial-like/ER-positive' subtype, comprising 60–65% of tumors. It was characterized by the high expression of a gene set including ESR1 (the ER-alpha itself) and genes either regulated by estrogens (*LIV-1*, *TFF1*, *TFF3*), or previously identified as co-expressed with ER (*GATA3*, for instance). Other genes correlated to ESR1 expression were *BCL2*, *COX6C*, *CRABP2*, *ERBB3*, *FBP1*, *HNF3A*, *HPN*, *IGFBP2*, *IGFBP5*, *MYB*, *NAT1*, *SELENBP1*, *VAV3* and *XBPI* (Perou et al. 2000, Gruvberger et al. 2001, Ross & Perou 2001, Sørli et al. 2001, West et al. 2001, van't Veer et al. 2002, Lacroix & Leclercq, 2004b; see also Table 3a). Besides the 'luminal epithelial-like/ER-positive' subtype, three subtypes characterized by low or no ESR1 expression were found: a 'normal breast-like', grouping some tumors with samples of normal breast tissue; a 'basal/myoepithelial-like', comprising about 15–20% of tumors, and notably expressing high levels of keratins 5 (*KRT5*) and 17 (*KRT17*); an 'ERBB2+' group, characterized by the high level of expression of several genes in the *ERBB2* amplicon at 17q22.24 including *ERBB2*, *GRB7*, *MLN64* and others. Most tumors expressing a strong 'luminal epithelial-like/ER-positive' signature were of low grade, while the majority of tumors expressing mainly the other signatures were of high grade. Tumors expressing high levels of *KRT5* and *KRT17* (van de Rijn et al. 2002), or *ERBB2* were associated with poor clinical outcome.

Tumors expressing a basal/myoepithelial gene signature, based on micro-array studies, are expected to include the fraction of ductal carcinomas that are not pure myoepithelial cell carcinomas but that are of high grade, and for which a basaloid/myoepithelial cell differentiation and steroid receptor negativity has been demonstrated by IHC (see for instance Jones et al. 2001). Myoepithelial differentiation, high grade and ER negativity are also found in certain meta-plastic carcinomas (spindle-cell carcinomas and matrix-producing carcinomas), and in

Table 3a A list of genes directly correlated to *ESR1* expression in tumors, as determined by micro-array studies

Gene name	Gene product name(s)
<i>AR</i>	Androgen receptor
<i>BCL2</i>	B-cell CLL/lymphoma 2
<i>CCND1</i>	Cyclin D1
<i>CLDN7</i>	Claudin 7
<i>COX6C</i>	Cytochrome c oxidase subunit VIc
<i>CRABP2</i>	Cellular retinoic acid-binding protein 2
<i>CUTL1</i>	Cut-like 1, CCAAT displacement protein
<i>ERBB3</i>	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
<i>ESR1</i>	Estrogen receptor-alpha
<i>FBP1</i>	Fructose-1,6-bisphosphatase 1
<i>GATA3</i>	GATA sequence-binding protein 3
<i>HNF3A/FOXA1</i>	Hepatocyte nuclear factor 3A/Forkhead box A1
<i>HPN</i>	Hepsin (transmembrane protease, serine 1)
<i>IGF2</i>	Insulin-like growth factor 2 (somatomedin A)
<i>IGFBP2</i>	Insulin-like growth factor-binding protein 2
<i>IGFBP5</i>	Insulin-like growth factor-binding protein 5
<i>LIV-1/SLC39A6</i>	Protein LIV-1/Solute carrier family 39 (metal ion transporter), member 6
<i>MYB</i>	V-myb myeloblastosis viral oncogene homolog (avian)
<i>NAT1</i>	<i>N</i> -acetyltransferase 1 (arylamine <i>N</i> -acetyltransferase)
<i>NPY1R</i>	Neuropeptide Y receptor Y1
<i>SELENBP1</i>	Selenium-binding protein 1
<i>SLC9A3R1</i>	Solute carrier family 9, isoform 3 regulatory factor 1
<i>STC2</i>	Stanniocalcin 2
<i>TFF1</i>	Trefoil factor 1 (pS2, BCEI)
<i>TFF3</i>	Trefoil factor 3
<i>TIMP3</i>	Tissue inhibitor of metalloproteinase 3
<i>VAV3</i>	Vav 3 oncogene
<i>XBP1</i>	X-box-binding protein 1

invasive ductal carcinomas with large central acellular zones (Tsuda *et al.* 1999).

That ER status reflects major differences in tumor gene expression patterns and phenotypes was notably illustrated by Gruvberger *et al.* (2001) and West *et al.* (2001) who showed that the number of genes that discriminated tumors according to their ER status was high. Moreover, only a small proportion of these discriminator genes were known to be regulated by estrogens, suggesting that mechanisms underlying *ESR1* gene expression are, indeed, common to many genes. Detailed analysis of the transcription of such genes could be valuable in understanding the molecular mechanisms underlying *ESR1* expression, which remain largely unclear. Among the genes positively correlated to ER were those correlated to *ESR1* expression in the 'luminal epithelial-like/ER-positive' phenotype (see above), but also *AR*, *CCND1*, *CUTL1*, *IGF2*, *NPY1R*, *SLC9A3R1*, *STC2* and *TIMP3* (see Table 3a). Among those negatively correlated to ER were *CX3CL1*, *CDH3*,

Table 3b A list of genes inversely correlated to *ESR1* expression in tumors, as determined by micro-array studies

Gene name	Gene product name(s)
<i>CDH3</i>	Cadherin 3, placental cadherin (P-cadherin)
<i>CX3C1</i>	Chemokine (C-X3-C motif) receptor 1
<i>EGFR</i>	Epidermal growth factor receptor
<i>FABP7</i>	Fatty acid-binding protein 7, brain
<i>GALNT3</i>	UDP- <i>N</i> -acetyl-alpha-D-galactosamine:polypeptide <i>N</i> -acetylgalactosaminyltransferase 3 (GalNAc-T3)
<i>GSTP1</i>	Glutathione <i>S</i> -transferase pi
<i>HMGIIY</i>	High-mobility group protein isoforms I and Y
<i>KRT7</i>	Keratin 7
<i>LAD1</i>	Ladinin 1
<i>LCN2</i>	Lipocalin 2 (oncogene 24p3)
<i>S100A8</i>	S100 calcium-binding protein A8 (calgranulin A)
<i>S100A9</i>	S100 calcium-binding protein A9 (calgranulin B)
<i>SERPINB5</i>	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
<i>SLPI</i>	Secretory leukocyte protease inhibitor (antileukoproteinase)
<i>SOD3</i>	Superoxide dismutase 3, extracellular

EGFR, *FABP7*, *GALNT3*, *GSTP1*, *HMGIIY*, *KRT7*, *LAD1*, *LCN2*, *S100A8*, *S100A9*, *SERPINB5*, *SLPI* and *SOD3* (see also Table 3b).

According to micro-array studies, only a few distinct breast tumor classes seem to exist. This suggests that phenotype transition from one class (for instance the 'luminal epithelial-like/ER-positive') to another is unlikely to occur in the same tumor during progression. Tumor phenotypes seem to be defined very early in the development of the lesions. This is further supported by micro-array-mediated analysis of invasion.

Micro-array investigations have also aimed to define the genes, if any, contributing to the invasive phenotype of breast tumors. However, in a study of 36 ductal tumors, extensive similarities at the transcriptome level were found among the distinct stages of progression (atypical hyperplasia, carcinoma *in situ*, invasive carcinoma), supporting the hypothesis that alterations that confer on tumors their potential for invasive growth are already present in the pre-invasive stages. Contrasting with stage, different tumor grades were associated with distinct gene expression signatures, suggesting that tumor grade is unlikely to change significantly during progression. A few genes were found to have an increased expression both in high tumor grade and in the carcinoma *in situ*/invasive carcinoma transition. They included genes involved in the cell cycle, centrosomal function and DNA repair (Ma *et al.* 2003).

In another study, comparison of gene expression changes between cancer cells at the periphery and in the center of breast cancers was performed using a combination of micro-dissection and micro-array analysis

(Zhu *et al.* 2003). Of 1176 genes analyzed, only 22 changed their expression levels in the periphery relative to the central region: 15 were up-regulated (including *VIM* and *AHRC*, encoding the small GTPase RhoC) and 7 were down-regulated (including *TSG101*) (arbitrary threshold of 1.5-fold or greater). RhoC has already been found to have increased expression in more motile, invasive and metastatic tumors, and in the most lethal form of the locally advanced breast cancer, inflammatory breast cancer (Kleer *et al.* 2002). *VIM* up-regulation might indicate the initiation of epithelial-mesenchymal conversion at the periphery. *TSG101* has been previously proposed as a tumor suppressor gene, but it seems that its expression could rather be needed for activities associated with aspects of tumor progression (Wagner *et al.* 2003, Zhu *et al.* 2004). Whether the expression of these genes was altered under the influence of normal surrounding tissue is presently unknown.

While a few changes in gene expression and, possibly, phenotype at the invasive front of tumors are suggested by the previous example, various micro-array studies indicate that the ability of BCC to metastasize to distant sites could indeed be an early and inherent genetic property. For instance, a 70-gene expression signature was found to be a strong independent factor in predicting a short interval to distant metastases. Those breast cancer patients presenting with a good prognostic fingerprint had a 95% chance of surviving the next decade, whereas those with a bad fingerprint had only a 55% chance of surviving (van de Vijver *et al.* 2002, van't Veer *et al.* 2002).

Other investigators identified aggregate patterns of gene expression (called 'meta-genes') allowing the classification of breast tumors by their likelihood of having associated LN metastases at diagnosis and by 3-year recurrence risk (Huang *et al.* 2003).

Bone marrow (BM) is a common homing organ for metastatic BCCs. Micro-array analysis of 83 breast tumor samples showed distinct profiles between BM-positive ($n = 23$) and BM-negative ($n = 60$) lesions. Nine genes were up-regulated while 77 were down-regulated in BM-positive tumors. In the same study, the expression profile associated with lymphatic metastasis was also studied. Forty-four genes were found to distinguish between LN-positive and LN-negative lesions. Again, the number of up-regulated genes in LN-positive tumors was smaller ($n = 9$) than the number of down-regulated genes ($n = 35$), suggesting that transcriptional repression of genes is important for metastasis. Of interest, the gene signature associated with LN metastasis was distinct from the signature associated with BM micro-metastasis, with only nine genes in common, suggesting that the two routes of dissemination could be governed by different molecular determinants (Woelfle *et al.* 2003).

There are studies suggesting that metastatic signatures could be common to cancers of various origins. Thus, a 17-gene expression signature that distinguished primary from metastatic adenocarcinomas was found from lung, breast, prostate, colorectal, uterus and ovary cancers. It was applied to 279 primary solid tumors (lung, breast, prostate, lymphoma and medulloblastoma). Those tumors carrying the gene expression signature were most likely to be associated with metastasis and poor clinical outcome ($P < 0.03$) (Ramaswamy *et al.* 2003).

All this suggests that the clinical outcome of individuals with cancer can be predicted using the gene expression profiles of primary tumors at diagnosis. It is proposed that some tumors could be pre-ordained to spread, while some would have a favorable combination of initiating events making them less likely to disseminate. The existence of early-expressed metastatic signatures is a further argument against the widely accepted idea that metastatic potential is acquired relatively late during multi-step tumorigenesis. It supposes that not just a few rare cells in the tumor acquire metastatic ability, but that all cells within such tumors have this ability to metastasize. It must be mentioned, however, that the metastatic signatures found by different groups have only a few, if any, genes in common, raising some questions about their potential use as clinical tools.

Concluding remarks on micro-array studies

Based on their pattern of gene expression, it appears that breast tumors may be grouped in a limited number of distinct classes largely correlated to ER status and grade. A transition of tumors from one to another of these classes seems unlikely, considering the number of differences in gene expression that discriminate them. Moreover, no 'mixed class' has been observed. Micro-array data also suggest that a (very) few genes could be susceptible to allowing distinction between *in situ* and invasive breast tumors. Other genes, such as ERBB2, KRT5 and KRT17, seem to be associated with higher aggressiveness.

Epigenetic alterations

Epigenetic alterations are heritable modifications of gene expression that do not involve mutation. They include hypermethylation of CpG island-rich promoters, which contributes to the transcriptional inactivation of a number of tumor-related genes in many types of cancer (Malik & Brown 2000, Widschwendter & Jones 2002). A series of genes frequently hypermethylated in breast cancers are listed in Table 4. The heritability of methylation states and the secondary nature of the decision to attract or exclude methylation suggest that DNA methylation is adapted for the cellular memory

Table 4 A list of genes for which CpG island promoter hypermethylation has been demonstrated in tumors

Gene name	Gene product name(s)
<i>APC</i>	Adenomatosis polyposis coli
<i>ARHI</i>	Ras homolog gene family, member I
<i>ASC</i>	Apoptosis-associated speck-like protein containing a CARD
<i>BRCA1</i>	Breast cancer 1, early onset
<i>CCND2</i>	Cyclin D2
<i>CDH1</i>	Cadherin 1, epithelial cadherin (E-cadherin)
<i>CDH13</i>	Cadherin 13, H-cadherin (heart)
<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A (p16)
<i>DAPK1</i>	Death-associated protein kinase 1
<i>ESR1</i>	Estrogen receptor-alpha
<i>FABP3</i>	Fatty acid-binding protein 3 (MDGI)
<i>FHIT</i>	Fragile histidine triad gene
<i>GJB2</i>	Gap junction protein, beta 2, 26kD (connexin 26)
<i>GPC3</i>	Glypican 3
<i>GSN</i>	Gelsolin (amyloidosis, Finnish type)
<i>GSTP1</i>	Glutathione S-transferase pi
<i>HIC1</i>	Hypermethylated in cancer 1
<i>HOXA5</i>	Homeo box A5
<i>HSH1N1</i>	High in normal-1
<i>IL6</i>	Interleukin-6
<i>KLK10</i>	Kallikrein 10
<i>MGMT</i>	Methylguanine-DNA methyltransferase
<i>NME1</i>	Protein expressed in non-metastatic cells 1 (nm23A)
<i>PGR</i>	Progesterone receptor
<i>PLAU</i>	Plasminogen activator, urokinase
<i>PRDM2(1)</i>	PR domain-containing protein 2 (RIZ1), transcript 1
<i>PRKDCBP</i>	Protein kinase C, delta-binding protein (SRBC)
<i>PRSS8</i>	Protease, serine, 8 (prostasin)
<i>RARB(2)</i>	Retinoic acid receptor, beta (transcript 2)
<i>RASSF1(A)</i>	Ras association (RalGDS/AF-6) domain family 1 (transcript A)
<i>SERPINB5</i>	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
<i>SFN</i>	Stratifin
<i>SLC19A1</i>	Solute carrier family 19 (folate transporter), member 1
<i>SNCG</i>	Synuclein, gamma (breast cancer-specific protein 1)
<i>SYK</i>	Spleen tyrosine kinase
<i>TFF1</i>	Trefoil factor 1 (pS2, BCEI)
<i>TIMP3</i>	Tissue inhibitor of metalloproteinase 3
<i>WT1</i>	Wilms tumor 1

For references, see Widschwendter *et al.* 2002, Paz *et al.* 2003.

(Bird 2002). Hypermethylation could participate in the development and the preservation of specific cell phenotypes, by definitely ‘bolting’ specific sets of genes.

Few studies have examined potential correlations between promoter hypermethylation and tumor stage or

grade. However, in a laser capture micro-dissection-assisted analysis of 16 specimens with intraductal and invasively growing breast cancer, promoter hypermethylation of *CDKN2A* (p16), *SFN* (stratifin), *RASSF1A* and *CCND2* (cyclin D2) was found to be largely conserved between both compartments. This suggests that in most cases the epigenetic inactivation takes place before invasive growth develops (Lehmann *et al.* 2002). A number of hypermethylated genes are associated with the distinctive phenotypes observed in tumors. For instance, *ESR1*, *PGR*, *CDH1*, *TFF1* etc. are associated with the ER-positive/low-grade phenotype. In contrast, *CDKN2A*, *GSTP1*, *PLAU* etc. are preferentially found in ER-negative/high-grade tumors. The fact that the expression of many genes for which promoter methylation has been shown is correlated to the ER status was demonstrated by Yan *et al.* (1999). These authors performed a methylation profile analysis of 7776 CpG islands, which led to the identification of CpG island clusters that can significantly distinguish ER–/PR– from ER+/PR+ breast tumors. Thus, epigenetic events might significantly contribute to stabilize the phenotype of tumors. As we have concluded that the BCC phenotype is unlikely to change significantly during progression, we conclude that hypermethylation is not expected to play a key role in this progression.

Conclusion on genetic/epigenetic studies

(Epi)genetic studies have not revealed major changes in the gross DNA alterations or in the gene expression patterns of breast tumors during progression. Tumor progression to invasiveness and metastasis probably results from the accumulation by *in situ* carcinoma of various minor and localized genetic or epigenetic events. This would eventually alter the molecular balances controlling cell adhesion, migratory ability, proteolysis and/or angiogenesis. Such evolution is suggested by the known micro-heterogeneity of tumor tissues.

Tumor size and progression

Pre-invasive cells express almost all of the features associated with a full-blown cancer phenotype: sustained cell proliferation, disregard of growth and differentiation controlling signals, evasion of apoptosis, immortalization and induction of angiogenesis. However, they (apparently) lack the ability to invade surrounding tissue. How is this property acquired?

Tumor size may play a role in the acquisition of invasiveness. In fact, and with the possible exception of *BRCA1*-associated tumors (Foulkes 2004), a relation has been repeatedly found between tumor size, on the one hand, and LN status and reduced survival, on the other hand (see for instance Carter *et al.* 1989, Hayes *et al.* 2002).

When growing, a tumor accumulates genetic alterations (see for instance Sato *et al.* 1991). This may allow the emergence of different cell sub-populations sharing essentially the same 'portrait', but exhibiting minor phenotype differences. One may speculate that a local complex co-operation between these sub-populations might favor invasion. A growing *in situ* tumor is also believed to exert a mechanical stress on its neighboring basement membrane. Moreover, BCC accumulation in a confined space might lead to local concentrations of various secreted molecules (for instance metalloproteinases (MMPs)) high enough to overcome the mechanical and molecular resistance expressed (for instance through secretion of MMP inhibitors) by the surrounding normal cells.

Interactions between tumor cells and their cellular environment

It is now widely accepted that tumor evolution is highly dependent on interactions (by direct contact or through paracrine signaling) between BCCs and other cell types present in their vicinity. BCCs modulate stromal cell activity. In turn, the stromal micro-environment profoundly influences many steps of tumor progression. In various experimental tumor models, the micro-environment affects the efficiency of tumor formation, the rate of tumor growth, the onset of angiogenesis, the extent of invasiveness and the ability of tumor cells to metastasize (Elenbaas & Weinberg 2001). Among the cell types with which BCCs may interact are normal breast epithelial cells, blood cells, vascular endothelial cells and, at metastatic sites, specialized cells from brain, lung, liver, bone, bone marrow etc. (Lacroix *et al.* 1996, 2000, Sierra *et al.* 1997, Siwek *et al.* 1997, Dano *et al.* 1999, Yoneda 2000, Moore 2001, Deugnier *et al.* 2002, Toillon *et al.* 2002a,b, Ben-Hur *et al.* 2002, Blot *et al.* 2003). Among others, myoepithelial cells and stromal fibroblasts are thought to be implicated in the first steps of invasion.

Myoepithelial cells have been seen as 'natural tumor suppressors' (Deugnier *et al.* 2002, Barsky 2003). Surrounding the mammary ducts, they deposit extra-cellular matrix components, express high amounts of several proteinase inhibitors and appear responsive for limiting invasive behavior. The loss of this cell type, observed only in invasive tumors, should permit subsequent invasion and tumor progression (Sternlicht *et al.* 1997, Xiao *et al.* 1999, Barsky 2003). However, the mechanisms by which BCCs may reduce the amount of myoepithelial cells in their neighborhood remain largely unknown.

BCCs also have paracrine interactions with their surrounding stromal fibroblasts. In tumors, these latter are often phenotypically different from normal fibroblasts. For example, they may express smooth muscle

differentiation with increased motility into collagen gel 'myofibroblasts' (Wang & Tetu 2002). Myofibroblasts, which comprise a predominant stromal cell type in breast tumors, are often seen in close association with the myoepithelium surrounding carcinoma *in situ*. Under the influence of BCCs, stromal (myo)fibroblasts can increase their production of various components of the urokinase (uPA) system (Schnack Nielsen *et al.* 2002) and of MMPs (Heppner *et al.* 1996). Since BCCs themselves are able to produce proteolysis-related molecules (uPA, uPAR, PAI-1, MMPs, matriptase/ST14) (Oberst *et al.* 2001), this is could lead to a considerable local matrix degradation and cancer progression (Dano *et al.* 1999).

Lobular breast cancer

Lobular tumors represent a minority (5–10%) of all breast carcinomas, but their occurrence appears to have increased steadily and disproportionately in recent years, possibly in association with increased use of combined hormone replacement therapy (Li *et al.* 2003a, Verkooijen *et al.* 2003). Compared with the ductal type, the characteristics of lobular tumors and the mechanisms of their progression have been less investigated. While additional studies will be necessary to draw firm conclusions, current data on lobular cancer will be summarized here.

Lobular tumors are usually composed of small monomorphic round cells, without significant nuclear atypia or abundant cytoplasm. Cells are most often arranged in single files. When present, invasion typically occurs in a manner that does not destroy anatomic structures or excite a substantial connective tissue response. Cells infiltrate alone or in files. Targetoid arrangements around non-neoplastic ducts may be observed. Besides the usual type, several variants have been described (signet-ring, alveolar, solid and others). The most studied of these, the pleomorphic variant, is characterized by a marked nuclear enlargement and pleomorphism, and small nuclei. It has moderate to high nuclear grade, contrasting with the low grade found in the usual type (Weidner & Semple 1992, Frykberg 1999, Soslow *et al.* 2000). In addition, lobular cancer may occasionally be observed in association with low-grade ductal cancer.

According to a series of data on proliferation, biology and genetics, lobular tumors in general appear to exhibit many similarities with low-grade tumors, but few with high-grade ductal tumors. Among lobular tumors, the usual type expresses a more well-differentiated and a less-proliferative phenotype than the pleomorphic type.

For instance, proliferation and apoptotic indexes were shown to be higher in ductal (in general) than in lobular cancers. These indexes were higher in pleomorphic than in

the usual lobular tumors (Kruger *et al.* 1999, Frolik *et al.* 2001, Arpino *et al.* 2004).

As also frequently observed in the low-grade ductal type: (1) the great majority of *in situ* and invasive lobular tumors express significant levels of ER, PR, BCL2, TFF1 and TFF3; (2) they are rarely ERBB2- and P53-positive, and their vimentin, VEGF and EGFR levels are low or null (Domagala *et al.* 1990, Poulsom *et al.* 1997, Lee *et al.* 1998, Frolik *et al.* 2001, Rosenthal *et al.* 2002, Arpino *et al.* 2004). The most noticeable feature distinguishing lobular and (low-grade) ductal tumors is the absence of E-cadherin expression in the former (Berx *et al.* 1996, Vos *et al.* 1997, Lehr *et al.* 2000, Goldstein *et al.* 2001, Wahed *et al.* 2002). In two independent gene expression studies comparing ductal and lobular carcinomas, the sole common discriminator identified was *CDH1*, which was significantly down-regulated in lobular samples (Korkola *et al.* 2003, Zhao *et al.* 2004). While marker expression is essentially similar in the usual lobular cancer and its variants (Soslow *et al.* 2000), the pleomorphic type has been found to have less ER and PR, and more P53 positivity than the usual type (Radhi 2000).

Using CGH, a higher number of genetic alterations has been observed in invasive, as compared with *in situ* lobular tumors. On the other hand, a frequent concomitant 1q gain and 16q loss appears to occur in both ductal and (*in situ* and invasive) lobular carcinomas. This has been associated with ER and PR presence and low proliferation (Etzell *et al.* 2001, Rennstam *et al.* 2003, Farabegoli *et al.* 2004). In lobular cancers, the most frequent losses are found at 16q followed by 17p, as also observed by LOH studies in well-differentiated ductal cancers (see above and in Shen *et al.* 2000).

Most studies, including many of those cited above, have indicated that when invasive and *in situ* components are present in lobular tumors, both are of the same type (usual, pleomorphic etc.) (see also Sneige *et al.* 2002) and are very similar, based on their expression of biological markers and their pattern of genetic alterations. For instance, a frequent hypermethylation of the five cancer-related genes *RASSF1A*, *HIN1*, *RARB*, *CCND2* and *TWIST*, has been observed in *in situ* as well as in invasive lobular cancers (Fackler *et al.* 2003).

A major difference between lobular and ductal tumors is their associated pattern of metastasis. Lobular carcinomas frequently metastasize to ovary, gastrointestinal tract, peritoneum and bone marrow; they less frequently colonize the lungs and the central nervous system (Borst & Ingold 1993, Arpino *et al.* 2004, Ferlicot *et al.* 2004). This difference could be essentially due to the lack of *CDH1* expression in lobular cancer, as compared with the ductal type (see above). In the absence of this adhesion molecule, the permeation of cancer cells through tissues

could be facilitated (Goldstein 2002). It has been shown that *CDH1* expression may favor the appearance of intralymphatic tumor emboli, which are rarely observed in lobular cancer (Gupta *et al.* 2003). In addition to the specific metastatic pattern, the absence of *CDH1* could also explain most of the peculiar cytological aspects of lobular cancer cells.

In summary, numerous features of lobular tumors, especially when they are of the usual type, are also observed in low-grade ductal tumors. No major difference seems to be associated with the progression of this type of cancer.

The origin of tumors — breast cancer stem cells

Breast tumors may exhibit different ‘portraits’ that are essentially maintained during progression from *in situ* to metastasis. On the other hand, it is now widely admitted that most tumors are clonal and represent the progeny of a single cell. Therefore, it may be asked whether the distinct tumor phenotypes are already present in the original tumor-initiating cells, or if phenotype divergence occurs at a later stage of tumor evolution from a common precursor.

Various studies on normal mammary tissue have identified a population of supra-basal cells that are able to generate both themselves and differentiated luminal epithelial and myoepithelial cells. These stem cells have a long life and a large replicating potential, making them good candidates for the cells of origin of cancer (Stingl *et al.* 2001, Boecker & Buerger 2003, Clarke *et al.* 2003, Dontu *et al.* 2003, Petersen *et al.* 2003, Smalley & Ashworth 2003).

Observations have long suggested that not all transformed cells composing a breast tumor are able to regenerate the tumor upon transplantation. The existence of ‘breast cancer stem cells’ (BCSCs) has recently gained more credibility through an elegant series of experiments by Al-Hajj *et al.* (2003). These authors identified in several tumors a sub-population of cluster of differentiation (CD)44+ CD24–/low epithelial-specific antigen (ESA)+ cells, of which as few as 200/1000 were consistently able to form tumors in mice. In contrast, 20 000 cells from other sub-populations were unable to do so. CD44 is a marker of basal/myoepithelial cells. CD24 and ESA are found in luminal epithelial cells. Thus, BCSCs express some (but not all) markers representative of both phenotypes, and it is conceivable that the progeny of these cells could evolve, probably rapidly, towards only one of these phenotypes. The final phenotype could result from specific events. For instance, tumors induced in transgenic mice by components of the WNT1 signaling pathway (wnt-1, beta-catenin, c-myc) were found to contain both luminal epithelial and

myoepithelial tumor cells; in contrast, no myoepithelial tumor cells were observed in tumors induced by ERBB2 (Rosner *et al.* 2002, Li *et al.* 2003b). Most BRCA1-associated breast tumors have a basal-like 'portrait'. To explain this, it has been hypothesized that an intact BRCA1 is needed to allow breast cells to acquire a luminal epithelial phenotype (Foulkes *et al.* 2003). Cells in which BRCA1 is truncated would be committed to a basal-like lineage.

The existence of 'monophenotypic' self-renewing BCSCs from which distinct tumor 'portraits' could be obtained may have important therapeutic implications. For instance, ER-positive tumors could indeed be composed of a bulk of ER-expressing cells associated with a very few BCSCs. In such a case, anti-estrogen-based therapy would be efficient only on ER-positive cells, allowing BCSCs to later reconstitute the tumor. New therapeutic targets should be identified that could be exploited to eliminate BCSCs from patients.

On the other hand, the increasing evidence that tumors maintain most of their 'portrait' during progression should also have consequences on their treatment. Different phenotypes mean different expression patterns of various drug targets, or modulators of drug action: components of estrogen metabolic pathways, proteinases, transporter proteins etc. The efficacy of strategies developed against such molecules is expected to be largely predictable from the most precise determination of the tumor phenotype.

General conclusions

Many attempts have been and are still being made to identify critical events responsible for the development and progression of breast cancer. In spite of this, the mechanisms underlying notably tumor invasion and BCC dissemination remain largely unclear. One of the current progression models for ductal tumors proposes that carcinoma *in situ* may evolve into invasive ductal carcinoma and subsequently produce metastases through an accumulation of molecular abnormalities possibly allowing extensive phenotype changes and gain of aggressiveness. To describe this progression, the 'clonal hypothesis' has generally been well received in the breast cancer community.

However, the data presented here indicate that most breast carcinomas cannot be viewed as a collection of a few successive clonal populations being associated with the major stages of progression. Rather unexpectedly, *in situ* and invasive components of carcinomas appear very similar, and this similarity has also been repeatedly observed in metastases, regardless of their localization, and in recurrences. In fact, at any step of their progression, breast tumors may be rather considered as

collections of cell sub-populations exhibiting the same general pattern of gross recurrent genetic alterations and sharing the same major phenotypic features. Regarding phenotype, a few major 'portraits' may be identified. Tumors characterized by expression of ER and a series of correlated markers are generally associated with low proliferation/apoptosis indexes and a low grade (well-differentiated, luminal epithelial phenotype). Tumors characterized by the lack (or very low amounts) of ER are generally associated with high proliferation/apoptosis indexes and a basal-like, poorly differentiated aspect.

Although the tumor phenotype remains essentially stable, genetic alterations accumulate during progression. Micro-heterogeneity exists, due to minor (low-frequency) DNA changes, generally restricted to small sub-populations of BCCs. This could result in minor phenotype differences. Invasion could proceed from a local complex co-operation between different sub-populations. Moreover, dialogue between tumor cells and their surrounding normal cells could also play an important role in the establishment of biological conditions propitious to cell dissemination.

There are also data, obtained notably from micro-array studies, suggesting that breast tumors do not really progress, as they could possess very early the ability to invade and metastasize. According to such a view, the distinction between *in situ* and invasive carcinoma would not reflect a significant difference in the properties of BCCs. To reach definite conclusions on the most pertinent model, future investigations should exploit the most recent analysis techniques (including micro-dissection) to examine genotype and phenotype of individual BCCs close to the invasion front and in invaded tissues.

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