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Amplification of 8q21 in breast cancer is independent of *MYC* and associated with poor patient outcome

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Copy number gains involving the long arm of chromosome 8, including high-level amplifications at 8q21 and 8q24, have been frequently reported in breast cancer. Although the role of the *MYC* gene as the driver of the 8q24 amplicon is well established, the significance of the 8q21 amplicon is less clear. The breast cancer cell line SK-BR-3 contains three separate 8q21 amplicons, the distal two of which correspond to putative target genes *TPD52* and *WWP1*. To understand the effect of proximal 8q21 amplification on breast cancer phenotype and patient prognosis, we analyzed 8q21 copy number changes using fluorescence *in situ* hybridization (FISH) in a tissue microarray containing more than 2000 breast cancers. Amplification at 8q21 was found in 3% of tumors, and was associated with medullary type ($P<0.03$), high tumor grade ($P<0.0001$), high Ki67 labeling index ($P<0.05$), amplification of *MYC* ($P<0.0001$), *HER2*, *MDM2*, and *CCND1* ($P<0.05$ each), as well as the total number of gene amplifications ($P<0.0001$). 8q21 copy number gains were significantly related to unfavorable patient outcome in univariate analysis. However, multivariate Cox regression analysis did not reveal an independent prognostic value of 8q21 amplification. The position of our FISH probe and data of a previously performed high-resolution CGH study in the breast cancer cell line SK-BR-3 involve *TCEB1* and *TMEM70* as new possible candidate oncogenes at 8q21 in breast cancer.
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Structural and numerical alterations of chromosome 8 have been reported in up to 60% of breast cancers.[1,2] In the majority of cases, these alterations occur as low-level copy number changes, including partial or complete deletions of 8p and gains of 8q.[3] Recurrent high-level amplifications have been found at 8p12, 8q21, and 8q24.[4,5] Gene amplification is an important mechanism for protein overexpression and oncogene activation in tumor cells.[6] At 8q24, the transcription factor v-myc myelocytomatosis viral oncogene homolog (avian) (*MYC*) is generally accepted as the biologically relevant amplification target.[7] Several studies have shown that *MYC* amplification occurs in approximately 5% of breast cancers and it has been linked with high grade, advanced tumor stage, and poor patient survival.[8–11] At 8p12, the fibroblast growth factor receptor (FGFR1) has been suggested as the candidate amplification target in breast and bladder cancer.[12,13] Kallioniemi *et al*[14] first reported amplification of 8q21–q23 in breast carcinomas that occurred independently of *MYC* amplification. Subsequent studies showed that the amplicon was

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not restricted to breast cancer but also occurred in carcinomas of lung, bladder, and prostate.[15–17] High-resolution array CGH in combination with interphase fluorescence *in situ* hybridization (FISH) showed a high variability of amplicons at 8q21–24 with several discontinuous target regions.[18] Rodriguez *et al*[19] identified three separate amplicons within 8q21 in the SK-BR-3 breast cancer cell line by using high-resolution BAC arrayCGH. Putative target genes of two distal regions include *tumor protein D52* (*TPD52*) and the ubiquitin-protein ligase *WWP1*, the amplification of which has been confirmed in clinical breast cancer specimens.[20,21] In contrast, data on the prevalence and clinical relevance of the first (proximal) amplicon involving a 70–80 Mb stretch at 8q21 are lacking in breast cancer. To explore the potential significance of 8q21 amplification in breast cancer, we analyzed a tissue microarray containing more than 2000 breast cancer specimens using a FISH probe that maps to the center of the proximal 8q21 amplicon.

Materials and methods

Breast Cancer Tissue Microarray

The breast cancer tissue microarray used for this study has been described in detail.[22] In brief, a total of 2197 formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded tumors with a median patient age of 62 (range 26–101) years and a median follow-up time of 68 months (range 1–176) were assembled in a tissue microarray format (Table 1). We punched one tissue cylinder per case with a diameter of 0.6 mm from representative tumor areas of a 'donor' tissue block using a home-made semiautomatic robotic precision instrument. The histological grade was determined according to a modified scoring system by Elston and Ellis (BRE score).[23] Several molecular data used in this study were available from previously published studies. These included amplification data obtained by FISH for *HER2*, *MYC*, *CCND1*, *MDM2,* and *EGFR,* as well as expression data obtained by immunohistochemistry for estrogen receptor (ER), progesteron receptor (PR), and Ki67.[8,22]

The use of these human tissues for protein expression and FISH studies was approved by the local ethics committee of the University of Hamburg.

FISH Analysis

A FISH probe was generated from genomic clone RZPDB737E022003D for 8q21 containing the entire *TMEM70* gene and part of the adjacent *LY96* gene. The probe was labeled with digoxigenin-dUTP by nick translation (Invitrogen). A commercially available pericentromeric probe for chromosome 8 was used as reference (CEP 8Z2 SpectrumOrange, Vysis, Downers Grove, IL, USA). For dual-color FISH analysis, 4-μm sections of the breast cancer tissue microarray blocks were transferred to

Table 1 8q21 amplification and clinicopathological features of invasive breast carcinomas

			8q21 amplification status			
		Analyzable for 8q21 (n)	*Normal (%)*	*Gain (%)*	*Amplification (%)*	P-*value*
All samples		1458	1301 (89)	107 (7)	50 (3)	
Histological type	Ductal carcinoma	1064	944 (89)	84 (8)	36 (3)	
	Lobular carcinoma	174	159 (91)	11 (6)	4 (2)	
	Medullary carcinoma[a]	48	38 (79)	5 (10)	5 (10)	0.03[b]
	Papillary carcinoma	21	20 (95)	0	1 (5)	
	Cribriform carcinoma	43	40 (93)	2 (5)	1 (2)	
	Mucinous carcinoma	32	29 (91)	2 (6)	1 (3)	
	Others	76	71 (93)	3 (4)	2 (3)	
Tumor stage	pT1	493	448 (91)	28 (6)	17 (3)	0.2
	pT2	714	640 (90)	52 (7)	22 (3)	
	pT3	83	68 (90)	10 (12)	5 (6)	
	pT4	161	139 (86)	16 (10)	6 (4)	
Nodal stage	pN0	610	548 (90)	47 (8)	15 (2)	0.21
	pN1	531	471 (89)	36 (7)	24 (5)	
	pN2	78	66 (85)	9 (12)	3 (4)	
Grading	G1	338	307 (91)	25 (7)	6 (2)	<0.0001
	G2	516	472 (91)	30 (6)	14 (3)	
	G3	479	410 (86)	43 (9)	26 (5)	
ER	Negative	332	288 (87)	30 (9)	14 (4)	0.21
	Positive	1053	950 (90)	73 (7)	31 (3)	
PR	Negative	865	764 (88)	68 (8)	33 (4)	0.24
	Positive	484	441 (91)	31 (6)	12 (2)	

[a]Comprises pure medullary carcinomas as well as atypical medullary carcinomas and poorly differentiated ductal carcinomas with strong stromal inflammatory response (medullary-like carcinomas).
[b]Medullary *vs* ductal.

an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA). For proteolytic slide pretreatment, a commercial kit was used (Paraffin pretreatment reagent kit; Vysis). Before hybridization, tissue microarray sections were deparaffinized, air dried, and dehydrated in 70, 85, and 100% ethanol followed by denaturation for 5 min at 74 °C in 70% formamide-2 × SSC solution. After overnight hybridization at 37 °C in a humidified chamber, slides were washed and counterstained with 0.2 μmol/l 4′,6-diamidino-2-phenylindole in an antifade solution. Detection of the digoxigenin-labeled probe was conducted using fluorescent antibody enhancer set (Roche) containing an FITC-conjugated antibody. For each tissue spot, the predominant gene and centromere copy numbers in the tumor cell nuclei were estimated.

A tumor was considered amplified if the ratio of 8q21/centromere 8 was ≥2.0. Ratios of >1.0 and <2.0 were considered as gains and a ratio of ≤1.0 as normal.

Statistics

Pearson's chi-squared test and Student's *t*-test were used to study the relationship between 8q21 copy number and clinicopathological or molecular parameters. Survival effect of 8q21 and *MYC* amplification was assessed using Kaplan–Meier curves and log-rank tests. A Cox proportional-hazards model was used to identify independent factors associated with overall survival. Analysis was performed using R statistical software package for Windows (version 2.7.2, R Foundation for statistical computing).

Results

8q21 Amplification Frequency

A total of 1458 (66%) arrayed cancer samples were assessable using FISH (Table 1 and Figure 1). Copy number alterations of the 8q21 locus were found in 157 interpretable breast cancers, including amplification in 50 (3%) tumors and gains in 107 (7%) tumors according to our predefined criteria (Figure 2). Almost all amplified tumors showed clusters of <10 gene copies, but two cases with large clusters of >20 FISH signals were also found.

Association with Clinicopathological and Molecular Features

Amplifications of the 8q21 locus showed significant correlations with various histopathological and molecular features of breast carcinomas. 8q21 copy number changes were related to medullary phenotype ($P=0.03$) and high-grade tumors ($P<0.0001$, Table 1). In addition, 8q21 alterations were related to high Ki67 labeling index ($P<0.05$, Figure 3). Tumors with 8q21 gains or amplifications were characterized by an increased overall frequency of amplifications of other known oncogenes ($P<0.0001$, Figure 4), including *HER2*, *CCND1*, *MDM2* ($P<0.05$ each), and *MYC* ($P<0.0001$, Table 2). The same trend was also found for *EGFR* amplification; however, the low prevalence of *EGFR* amplifications (1%) in our patient set did not allow for a statistically sound analysis. A comparison between the expected and the observed frequency of co-amplifications with at

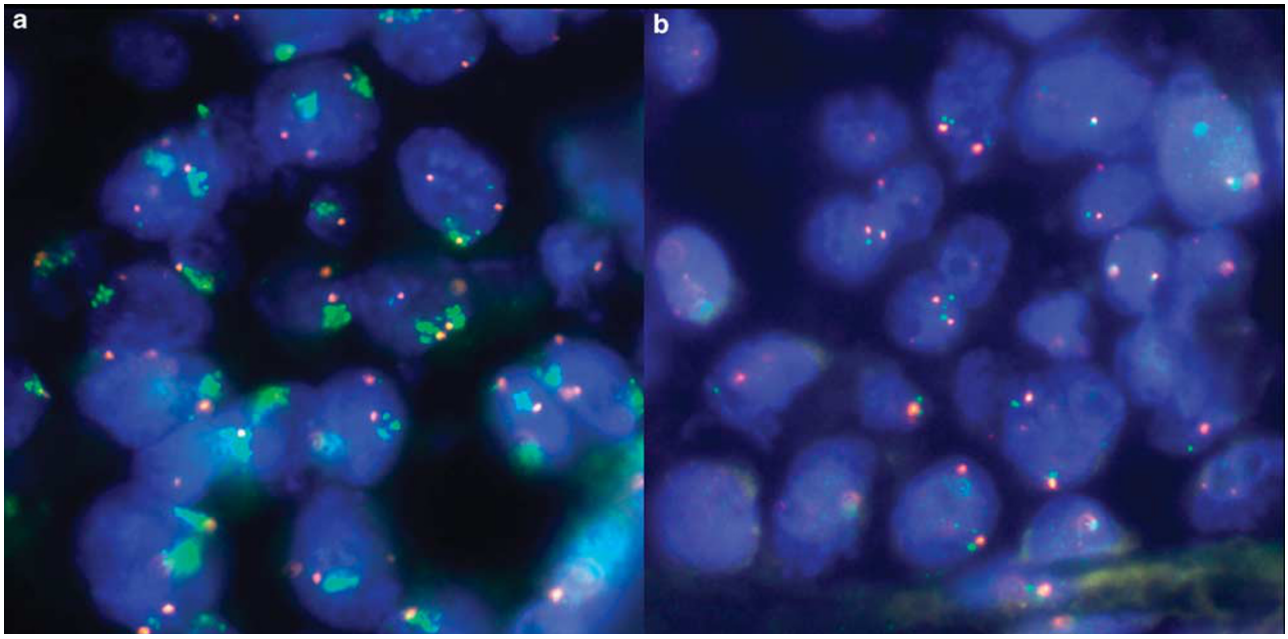

Figure 1 Examples of breast cancers with (**a**) and without (**b**) 8q21 amplification: red signals indicate copy number of chromosome 8 and green signals indicate 8q21 copy number. FISH analysis, × 630 magnification.

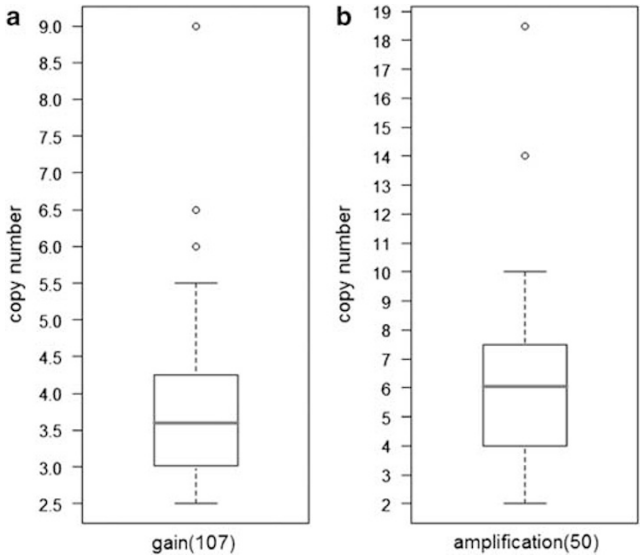

Figure 2 8q21 copy number range in 157 breast carcinomas with gains (**a**) and amplifications (**b**) according to our predefined criteria.

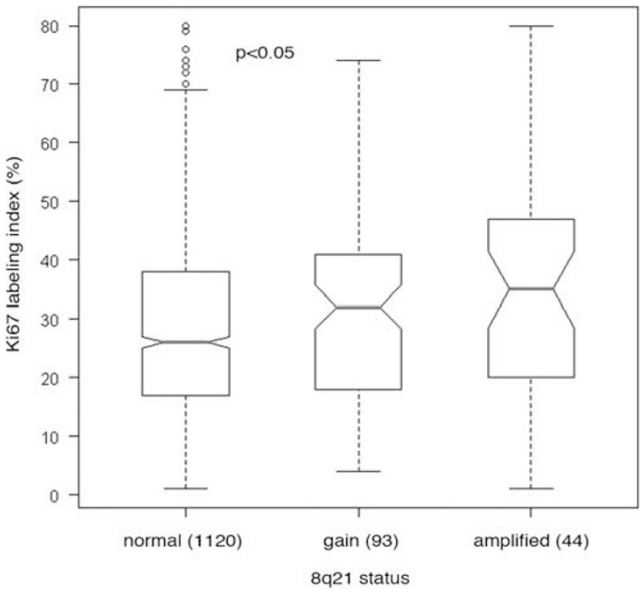

Figure 3 Relationship between Ki67 labeling index and 8q21 amplification status in breast carcinomas.

least one of the other genes revealed that tumors with 8q21 amplification had a twofold increased likelihood to develop other amplifications (expected probability 1.5%, observed probability 2.7%; $P=0.0053$). There was no association between aberrations of the 8q21 locus and tumor stage, presence of lymph node metastases, or hormone receptor status ($P>0.05$ each).

Association with MYC Amplification

Data on *MYC* amplification were available for the breast cancer tissue microarray from a previous study with a total number of 121 (5%) *MYC*-amplified tumors.[8] A subset of 1132 tumors with data available for both 8q21 and 8q24 (*MYC*) were included in the current study. A combined analysis of *MYC* and 8q21 identified 90 tumors with amplifications of *MYC* and/or 8q21. Of these, 28 tumors were amplified for 8q21 only and 54 for *MYC* only. Co-amplification of both genes was found in the remaining eight tumors.

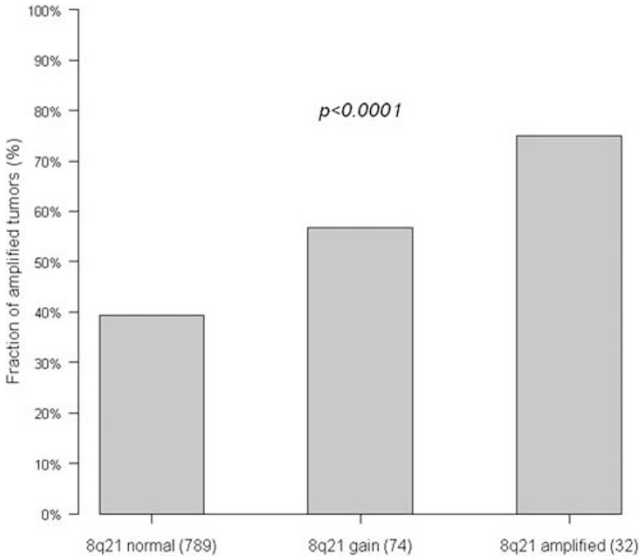

Figure 4 Fraction of breast cancers showing at least one amplification of *CCND1*, *HER2*, *MYC*, *MDM2,* or *EGFR* in relation to the presence of 8q21 gain or amplification.

Prognostic Significance of 8q21Amplification

8q21 amplification was strongly associated with adverse prognosis in univariate survival analysis (Figure 5a). There was no effect of 8q21 gains on patient survival ($P=0.48$). Furthermore, we analyzed the overall patient survival in the subset of 1132 tumors with complete copy number data for 8q21 and *MYC* (Figure 5b). In this subgroup, no statistically relevant survival differences could be found between tumors with *MYC* amplification and tumors with 8q21 amplification, with or without included co-amplifications ($P=0.255$ and $P=0.15$, respectively). However, the adverse effect of 8q21 amplification was retained and a tendency to worse outcome in 8q21-amplified tumors was observed. 8q21/q24 co-amplification ($n=8$) was too rare for further statistically analysis. A multivariate analysis including the established prognostic markers of breast cancer (pT, pN, and BRE grade) and the 8q21 or 8q24 (*MYC*) amplification status did not reveal an independent prognostic value of either locus (Table 3).

Discussion

A recently published study using high-resolution array CGH on SK-BR-3 breast cancer cells reported

Table 2 Relationships between 8q21 amplification and other molecular features in breast carcinomas

			8q21 amplification status			
		Analyzable for 8q21 (n)	*Normal (%)*	*Gain (%)*	*Amplification (%)*	P-*value*
CCND1	Non-amplified[a]	1043	964 (92)	60 (6)	19 (2)	0.021
	Amplified	280	245 (87)	24 (9)	11 (4)	
HER2	Non-amplified[a]	994	898 (90)	66 (7)	30 (3)	0.0034
	Amplified	216	178 (82)	26 (12)	12 (6)	
MYC	Non-amplified[a]	1073	961 (90)	84 (8)	28 (3)	<0.0001
	Amplified	62	49 (79)	5 (8)	8 (13)	
MDM2	Non-amplified[a]	1241	1108 (89)	96 (8)	37 (3)	0.003
	Amplified	79	66 (84)	5 (6)	8 (10)	
EGFR	Non-amplified[a]	1300	1158 (89)	100 (8)	42 (3)	
	Amplified	12	8 (67)	2 (17)	2 (17)	

[a]Includes tumors with normal copy number and gains.

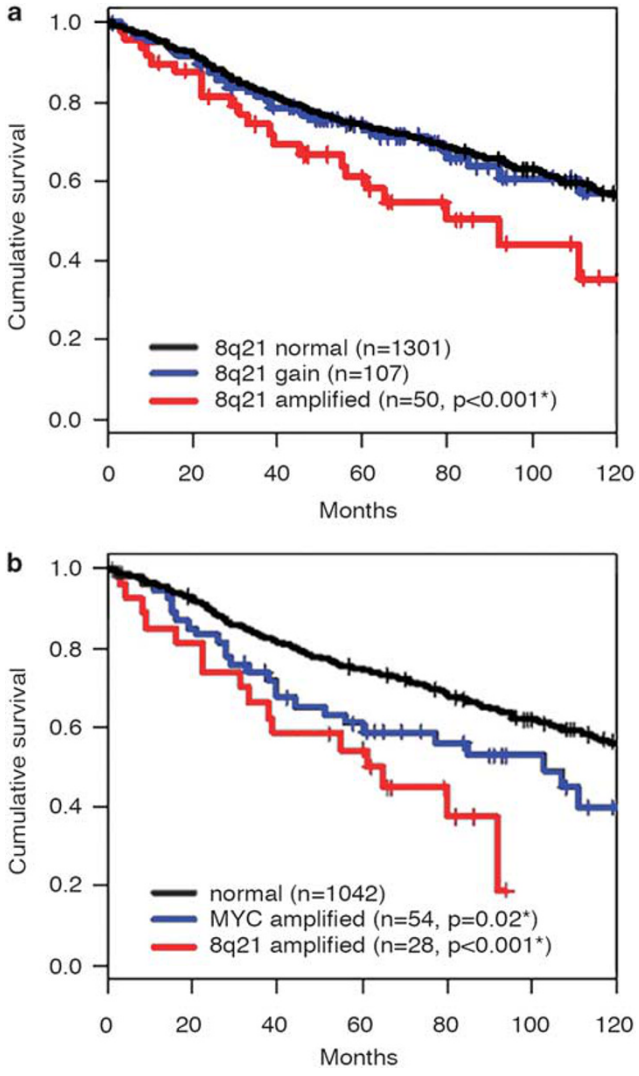

Figure 5 Prognostic significance of 8q21 amplifications in 1458 (**a**) assessable breast cancers at our tissue microarray and a subgroup of 1132 tumors (**b**) with complete copy number information for 8q21 and *MYC* (* *vs* normal).

Table 3 Multivariate analysis of overall survival in breast carcinomas according to T stage, nodal status, grading and amplification status of 8q21 and *MYC* (8q24)

Parameter	*Hazard ratio for overall survival*	*95% Confidence interval*	P-*value*
Tumor stage			
pT1 *vs* pT2	1.37	1.01–1.86	0.045
pT1 *vs* pT3	1.4	0.87–2.23	0.16
pT1 *vs* pT4	1.94	1.29–2.90	0.0013
Nodal stage			
pN0 *vs* pN1	2.13	1.61–2.81	<0.001
pN0 *vs* pN2	4.6	3.06–6.89	<0.001
BRE grade			
G1 *vs* G2	1.5	0.99–2.27	0.053
G1 *vs* G3	3.4	2.31–5.01	<0.001
8q21 amplification *vs* normal	1.66	0.96–2.86	0.07
MYC amplification *vs* normal	0.94	0.61–1.45	0.78

three separate amplicons within 8q21.[19] Studies on clinical breast cancer specimens suggested *TPD52* and *WWP1* as amplification target genes in this chromosomal region.[20,21] In concordance with the SK-BR-3 mapping study, *TPD52* and *WWP1* are located within the two distal 8q21 amplicons. The specific aim of this study was to gain more insight into the potential significance of the third (proximal) amplicon located between 70 and 80 Mb (8q21.11).

Amplification driver genes often map to central portions of an amplified region.[24] A FISH probe mapping directly to the center of the proximal 8q21 amplicon identified in SK-BR-3 breast cancer cell line was therefore used for this study. The high number of analyzed breast cancer samples in combination with the extensive database collected during previous studies enabled a comprehensive

comparison of the presence or absence of the proximal 8q21 copy number gains with multiple clinicopathological and molecular features, including survival data.[8,22] The associations with high grade, tumor cell proliferation, medullary phenotype, and poor clinical outcome argue for relevant biological role of at least one gene in the proximal 8q21 region. The comparatively weaker link between 8q21 gains and these parameters as well as the lack of prognostic significance underscores the biological effect of 8q21 amplification. Medullary and medullary-like cancers are well known for their high proliferative activity and expression of other relevant tumor proteins, such as EGFR and CD117.[25,26] This tumor entity comprises a heterogeneous subgroup of basal-like carcinomas, consisting of pure medullary carcinomas, atypical medullary carcinomas, and poorly differentiated ductal carcinomas with strong stromal inflammatory response.[27] Accumulation of 8q21 amplifications in medullary-like breast carcinomas constitutes another argument in favor of a biological uniqueness of this rare subtype of breast cancer.

The relatively high number of 8q21 amplifications in our examination (3%) may be viewed as an indirect argument for our FISH probe mapping not so far from the target gene of the proximal 8q21 amplicon. The BAC for the used FISH probe contains entire *transmembrane protein 70* (*TMEM70*), which is one interesting candidate target gene in the region. The gene encodes a small 30 kD protein located at the inner mitochondrial membrane. *TMEM70* wild-type protein is necessary for regular biogenesis and assembly of the ATP synthase, as shown in some mitochondrial disorders with decreased activity of this protein.[28,29] Enhanced activity of ATP synthase results in elevated levels of reactive oxygen species (ROS) in the cell. High intracellular ROS was described in many cancer types, including breast carcinomas.[30–32] It has been suggested that high ROS levels cause elevated expression of the transcription factor HIF-1α, which is also implicated in breast tumor development.[33,34] Stabilization of HIF-1α with increased aerobic glycolysis (*Warburg* effect) has a central role in many common human cancer types.[35] Therefore, it might be that *TMEM70* amplification with elevation of ROS led to a growth advantage of breast tumor cells.

Transcription elongation factor B, polypeptide 1 (*TCEB1*) is another interesting candidate oncogene. *TCEB1* locates in the close proximity of the hybridized region, only 4-kb upstream from *TMEM70*. The gene encodes the protein elongin C, which serves as a cofactor for activation of transcriptional elongation by RNA polymerase II.[36] *TCEB1* was suggested to have oncogenic potential in prostate cancer.[37] One study has described *TCEB1* overexpression using quantitative RT-PCR in *TCEB1*-amplified SK-BR-3 cells.[38]

Although the co-amplification rate of 8q21 and *MYC* (13%) in our study was somewhat higher than the co-amplification rate of 8q21 and other analyzed amplicons (4–10%), amplifications of 8q21 and 8q24 occurred independently in most cases. *MYC* was not amplified in 28 of 36 8q21-amplified cancers, confirming the independent nature of the 8q21 amplicon. Several previous studies, including reports using the same tissue microarray as in this study, have shown a nonrandom accumulation of amplifications of different genomic regions in certain breast cancers that are considered to show an 'amplifier' phenotype.[2,8,39–41] Amplification of the 8q21 locus is probably also part of a spectrum of breast carcinomas with high genomic instability and frequent amplifications.

TPD52 and *WWP1*, the most promising candidate target genes in the two other 8q21 amplicons in breast cancer, map 6 Mb and 12.5 Mb distal from our FISH probe. Amplification and overexpression of these genes were recently also found to be associated with short patient survival in breast cancer.[42,43] Although it has been hypothesized that each of these genes can cause a malignant phenotype in breast cancer by its own, it is also possible that a cumulative effect of multiple 8q21 genes contributes to the adverse prognosis in tumors with high genomic instability and many gene amplifications.

In summary, amplification of 8q21 occurs in a small but significant subgroup of genomic-instable breast carcinomas with poor prognosis. Copy number changes in 8q21 are independent of *MYC* and represent a separate amplicon in this chromosomal segment. Possible candidate oncogenes within this region include *TCEB1* and *TMEM70*.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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