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SHORT COMMUNICATION

Frequent *MYC* coamplification and DNA hypomethylation of multiple genes on 8q in 8p11-p12-amplified breast carcinomas

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Genetic and epigenetic (DNA methylation, histone modifications, microRNA expression) crosstalk promotes inactivation of tumor suppressor genes or activation of oncogenes by gene loss/hypermethylation or duplications/hypomethylation, respectively. The 8p11-p12 chromosomal region is a hotspot for genomic aberrations (chromosomal rearrangements, amplifications and deletions) in several cancer forms, including breast carcinoma where amplification has been associated with increased proliferation rates and reduced patient survival. Here, an integrative genomics screen (DNA copy number, transcriptional and DNA methylation profiling) performed in 229 primary invasive breast carcinomas identified substantial coamplification of the 8p11-p12 genomic region and the *MYC* oncogene (8q24.21), as well as aberrant methylation and transcriptional patterns for several genes spanning the 8q12.1-q24.22 genomic region (*ENPP2*, *FABP5*, *IMPAD1*, *NDRG1*, *PLEKHF2*, *RRM2B*, *SQLE*, *TAF2*, *TATDN1*, *TRPS1*, *VPS13B*). Taken together, our findings suggest that *MYC* activity and aberrant DNA methylation may also have a pivotal role in the aggressive tumor phenotype frequently observed in breast carcinomas harboring 8p11-p12 regional amplification.

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INTRODUCTION

Genomic instability and epigenetic modulations, that is, DNA methylation, histone modifications, microRNA expression, contribute to the neoplastic phenotype by deregulating key gene functions that permit cells to bypass regulatory mechanisms controlling and maintaining normal cellular physiology.[1] Recently, genetic and epigenetic crosstalk has shown to be one of several major driving forces behind tumor initiation and progression.[2–6] However, DNA methylation is considered by some to be a secondary event which locks genes in their inactive/active states only after gene silencing/activation has been achieved by other means.[7–10]

Several well-characterized DNA regions have been investigated extensively in breast cancer for their role in genetic modulations, interactions in molecular pathways and association with unfavorable clinical outcome. These include the 8p11-p12, 8q24 (*MYC*), 11q13 (*CCND1*), 17q12 (*ERBB2*, *GRB7*, *STARD3*) and 20q13 (*ZNF217*, *MYBL2*, *STK6*) amplicons, some of which have become major molecular targets for breast cancer treatment. Regional amplification of the 8p11-p12 genomic region is a common genetic event in solid tumors, for example, breast carcinoma,[11–13] pleuropulmonary blastoma,[14] lung cancer and esophageal squamous cell carcinomas,[15–18] urinary bladder cancer,[19,20] osteosarcoma[21] and pancreatic adenocarcinoma.[17] In breast cancer cell lines, the initiation site and structure of the 8p11-p12 DNA rearrangement involved different mechanisms of gene activation, thereby resulting in the activation of different combinations of candidate genes.[22]

To further define the role, 8p11-p12 regional amplification may have on breast cancer pathophysiology, we examined genome-wide copy number alterations, DNA methylation patterns and transcriptional changes in 229 primary invasive breast tumors. Here, we demonstrate that ~50% of 8p11-p12-amplified tumors also harbor *MYC* amplification, as well as, hypomethylation of genes located in close proximity to the *MYC* gene.

RESULTS AND DISCUSSION

Amplification of the 8p11-p12 genomic region is a common genetic event in breast carcinoma with clinical implications. To assess aberrant transcriptional and DNA methylation patterns in invasive breast carcinomas harboring the 8p11-p12 amplicon, an integrative analysis was performed using DNA copy number, DNA methylation and transcriptome data from 229 primary invasive breast cancer samples previously presented in our work,[23,24] including our own unpublished data. The DNA copy number analysis using array-comparative genomic hybridization data showed recurrent copy number alterations on chromosome bands 8p11-p12 in 83 tumors (36%), including 47/83 high-level gains/amplifications, 20/83 low-level gains and 16/83 heterozygous losses. Copy number alterations were confirmed using a set of overlapping BAC clones building a contig over the 8p11-p12 genomic region. On average, there was a five-fold increase in the number of amplifications observed in lesions containing the 8p11-p12 amplicon compared with those lacking the amplicon ($P = 1.8E-13$). In general, amplification of the 8p11-p12 genomic

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region was predominantly coamplified with 1q, 8q, 11q, 12p, 16p, 17q or 20q, but also occurred as the sole region of amplification in two cases. Notably, 53% ($n = 24$) of 8p11-p12-amplified tumors were coamplified with the *MYC* gene, whereas only 20% ($n = 9$) and 18% ($n = 8$) were coamplified with the *CCND1* and *ERBB2* genes, respectively (Figure 1). Extensive research has been carried out on the coamplification of 8p11-p12 and *CCND1*, but few studies have investigated 8p11-p12 and *MYC* interactions.[22,25]

In accordance with published studies, genetic aberrations of the 8p11-p12 region (gain, loss and amplification; $P = 5.0E-6$), including amplification ($P = 4.0E-5$) or loss ($P = 0.005$), were associated with reduced overall survival rates.[26] Conversely, genomic gain was not indicative of unfavorable patient outcome ($P = 0.08$). The amplicon was most prevalent in tumors of large pathologic size ($P = 0.0002$), high genomic grade index status ($P = 0.0004$) and high S-phase fraction ($P = 0.002$; Table 1). There was no significant difference in histologic type, axillary lymph node status, estrogen/progesterone receptor status, human epidermal growth factor receptor 2 (HER2)/*neu* receptor status, triple negative status or molecular breast cancer subtype. These findings are consistent with previous reports showing high cell proliferation (high Ki-67) and high tumor grade in breast carcinomas harboring the 8p11-p12 amplicon. However, Gelsi–Boyer *et al.* [26] did not find a connection with amplification and tumor size. Recently, several studies have found an association between the luminal B molecular subtype and DNA amplification of two genes (*ZNF703* and *FGFR1*) within the 8p11-p12 amplicon. Interestingly, tumors harboring these genetic alterations were also resistant to endocrine therapy.[27–30] However, we show that ~80% of the breast tumors analyzed here were luminal B subtype/estrogen receptor-positive regardless of 8p11-p12 amplicon status. Furthermore, *ZNF703* was generally upregulated in breast carcinomas, particularly in estrogen receptor-positive tumors, compared with normal breast tissue.[24] Functional studies have provided additional evidence for biological effects *in vitro* and *in vivo* using small-interfering RNA-mediated knockdown of candidate genes within the 8p11-p12 genomic region.[27–33] Eight genes *(BAG4*, *C8orf4*, *DDHD2*, *ERLIN2, LSM1*, *PPAPDC1B*, *WHSC1L1* and *ZNF703)* have thereby emerged as targets with oncogenic potential.

To delineate whether aberrant methylation patterns may also has a role in the evolution of breast tumors harboring the 8p11-p12 amplicon, we performed genome-wide DNA methylation analysis on 22/229 tumors (11 tumors harboring the amplicon and 11 tumors lacking the amplicon) using the 450k Infinium Methylation Beadchip (Illumina Inc., San Diego, CA, USA). Of the 382 815 cytosine sites remaining after filtering, ⩽1% ($n = 2066$) were differentially methylated in tumors harboring the 8p11-p12 amplicon compared with samples lacking the amplicon. Eighty-nine percent of aberrantly-methylated cytosine sites were hypermethylated ($n = 1847$) and 11% ($n = 219$) of sites were hypomethylated. The promoter regions (200 and 1500 bp upstream transcriptional start sites, 5′ untranslated region and the first exon) were tightly linked with hypermethylation ($n = 352$ sites, 92%), whereas fewer methylation events occurred further downstream in the body of genes and at the 3′ untranslated region region. The highest number of aberrantly-methylated cytosine sites surrounded CpG islands ($n = 408$) with fewer sites found in the CpG shores (up to 2 kb from CpG islands, $n = 172$) and shelves (2–4 kb from CpG islands, $n = 48$). The majority of aberrant methylation patterns occurred within genes and intergenic regions, whereas few microRNA transcripts were found (Figure 2). We found that differential methylation occurred on all chromosomes including the X chromosome in 8p11-p12-amplified

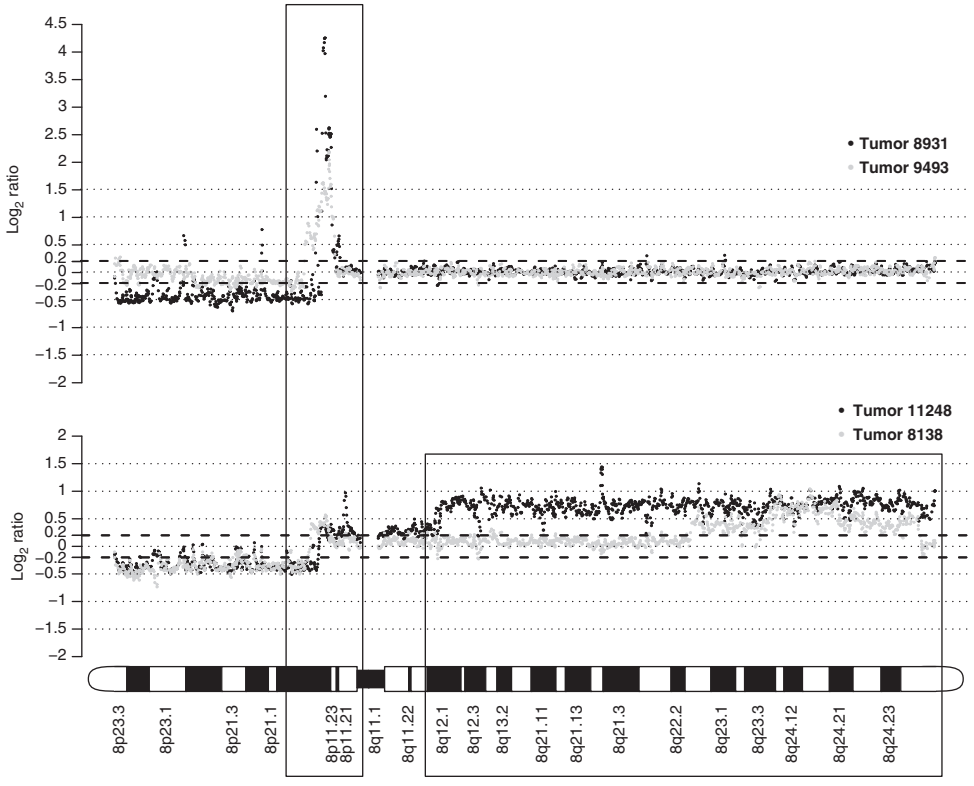

Figure 1. Array-CGH genomic profiles showing recurrent DNA amplification of the 8p11-p12 genomic region in breast carcinoma. The top panel shows focal amplification (log2 ratio > 0.5) of the 8p11-p12 region in two breast tumors. Black dots depict BAC clones spanning chromosome 8 for tumor 8931 and gray dots for tumor 9493. The bottom panel shows amplification of the 8p11-p12 and 8q regions. Black dots depict BAC clones spanning chromosome 8 for tumor 11248 and gray dots for tumor 8138. The *x*-axis shows chromosome 8 from the 8p telomere to the 8q telomere. The *y*-axis shows the log2 ratio value for each BAC clone (tumor gDNA versus normal control gDNA).

Table 1. Correlation between 8p11-p12 DNA amplification and clinicopathological features in breast carcinoma

Characteristics	*Number of tumors (%)*			
	Total tumors (n = 229)	*Neutral DNA dosage*[a] *(n = 71)*	*DNA amplification*[a] *(n = 45)*	P-*value*
Age				
Mean	59	60	60	
Range	30–88	37–79	30–88	
Histologic type				0.7
Ductal	136 (59)	52 (73)	21 (47)	
Lobular	22 (10)	7 (10)	4 (9)	
Other	26 (11)	12 (17)	3 (7)	
Not available	45 (20)	0 (0)	17 (38)	
Axillary lymph node status				0.2
pN0	82 (36)	38 (54)	12 (27)	
pN1	84 (37)	33 (46)	19 (42)	
Not available	63 (28)	0 (0)	14 (31)	
Pathologic tumor size				0.0002
pT1	51 (22)	22 (31)	4 (9)	
pT2	89 (39)	35 (49)	12 (27)	
pT3	51 (22)	11 (15)	20 (44)	
pT4	6 (3)	3 (4)	0 (0)	
Not available	32 (14)	0 (0)	9 (20)	
S-phase fraction				0.002
$\leqslant 6.1$	112 (49)	59 (83)	18 (40)	
>6.1	69 (30)	12 (17)	16 (36)	
Not available	48 (21)	0 (0)	11 (24)	
GGI status				0.0004
Low	45 (20)	31 (44)	6 (13)	
High	73 (32)	26 (37)	29 (64)	
Not available	111 (48)	14 (20)	10 (22)	
Estrogen receptor				0.8
Negative	60 (26)	14 (20)	10 (22)	
Positive	166 (72)	57 (80)	34 (76)	
Not available	3 (1)	0 (0)	1 (2)	
Progesterone receptor				0.7
Negative	108 (47)	31 (44)	21 (47)	
Positive	118 (52)	40 (56)	23 (51)	
Not available	3 (1)	0 (0)	1 (2)	
HER2/neu status				0.6
Negative	199 (87)	61 (86)	37 (82)	
Positive	30 (13)	10 (14)	8 (18)	
Not available	0 (0)	0 (0)	0 (0)	
Triple negative status				1.0
Yes	41 (18)	9 (13)	5 (11)	
No	186 (81)	62 (87)	39 (87)	
Not available	2 (1)	0 (0)	1 (2)	
Subtype				0.9
Luminal subtype A	2 (1)	1 (1)	0 (0)	
Luminal subtype B/ HER2-	101 (44)	47 (66)	31 (69)	
Luminal subtype B/ HER2 +	13 (6)	8 (11)	4 (9)	
HER2/ER-	18 (8)	10 (14)	5 (11)	
Basal-like	16 (7)	5 (7)	5 (11)	
Normal-like	0 (0)	0 (0)	0 (0)	
Not available	79 (34)	0 (0)	0 (0)	

Abbreviations: GGI status, genomic grade index; HER2, human epidermal growth factor receptor 2. *P*-values were calculated using the Fisher's exact test (neutral DNA dosage versus DNA amplification). [a]Tumor specimens included in the analysis with both array-CGH and gene expression microarray data are available.

tumors, where hypermethylation ranged from 72–98% and the highest hypomethylation rates were found on chromosomes 8 and 9 with 28% and 24%, respectively.

Few of the methylation events resulted in aberrant gene expression patterns in 8p11-p12-amplified tumors ($n = 61$, 4.5% of aberrantly-methylated coding RNAs), although disparate methylation-transcriptional patterns were observed for 23/61 genes (38%); 20/23 genes were hypermethylated and overexpressed and 3/23 genes were hypomethylated and underexpressed (Table 2). Univariate Cox regression analysis showed that aberrant transcriptional patterns for 47/61 genes influenced overall survival rates. In addition, only one gene located at 8p11-p12 showed differential methylation and gene expression patterns, that is, *BRF2* was hypermethylated but overexpressed owing to *BRF2* gene amplification in 7/11 cases. Gene Ontology enrichment analysis of the genes with aberrant DNA methylation and gene expression patterns revealed several cancer-related processes, for example, cell differentiation, DNA replication, cell migration and cell adhesion (Table 3).

Interestingly, 11 genes spanning the 8q12.1-q24.22 genomic region were differentially methylated and expressed, of which nine genes (*IMPAD1*, *NDRG1*, *PLEKHF2*, *RRM2B*, *SQLE*, *TAF2*, *TATDN1*, *TRPS1*, *VPS13B*) were hypomethylated and overexpressed, the *ENPP2* gene was hypermethylated and underexpressed and the *FABP5* gene was hypermethylated but overexpressed. As the 11 genes were also coamplified with the 8p11-p12 region in at least one tumor specimen, we examined whether DNA copy number, DNA methylation or both had an impact on gene expression (Figure 3). We found that hypomethylation alone frequently enhanced gene expression patterns. However, hypomethylation and DNA amplification of the same transcript further enhanced expression levels. These findings suggest that genes in the 8q region are frequently targeted by more than one mechanism for activation in breast tumors harboring 8p11-p12 amplification. Consequently, *ENPP2* was the only example showing lower expression levels when hypermethylated (at four different cytosine sites in the promoter region) despite amplification of the gene in 2/11 samples harboring the 8p11-p12 amplicon. These results indicate that aberrant methylation patterns may be a secondary event to further lock genes in their inactive or active states only after they have already been silenced or activated by other means.[7–10] The *ENPP2* gene was an exception to this phenomenon because hypermethylation occurred at four different cytosine sites in the promoter region of the gene, resulting in lower transcriptional levels despite amplification of the gene in 2/11 samples harboring the 8p11-p12 amplicon. However, 8/11 genes (*FABP5, NDRG1, PLEKHF2*, *RRM2B*, *SQLE*, *TAF2*, *TATDN1, TRPS1)* may not be distinctive of 8p11-p12 amplification as they were also differentially regulated in *MYC* coamplified tumors.

Several of the aberrantly-methylated genes spanning the 8q arm have been previously associated with cancer-related processes. In addition to gene amplification shown in the present study, *VPS13B* frameshift mutations have also been identified in gastric and colorectal cancers, as well as *TRPS1-LASP1*, *PLEC1-ENPP2* and *TATDN1-GSDMB* fusion genes in breast carcinoma.[34–36] Recently, gain of *TRPS1*, *TATDN1* and *SQLE* DNA copy numbers in estrogen receptor-positive, *ERBB2*-amplified breast tumors have been reported, and elevated SQLE levels were associated with distant metastasis-free survival.[37–39] TRPS1, a transcription factor that belongs to the GATA gene family, in which, protein expression is inhibited by androgens via the androgen receptor in prostate cancer and demonstrates high expression levels in both normal breast and tumor tissue. In breast tumors, TRPS1 expression is associated with ER, PgR, GATA3, HER2/*neu* expression and favorable clinical outcome.[40,41] Elevated NDRG1 protein levels have been associated with shorter disease-free and overall survival, cell differentiation and breast cancer progression.[42–44] In contrast, Han *et al.*[45] demonstrated that *NDRG1* methylation in breast cancer is associated with a more aggressive phenotype. Interestingly, substantial NDRG1 phosphorylation is found in Akt inhibitor-resistant breast cancer cell lines,

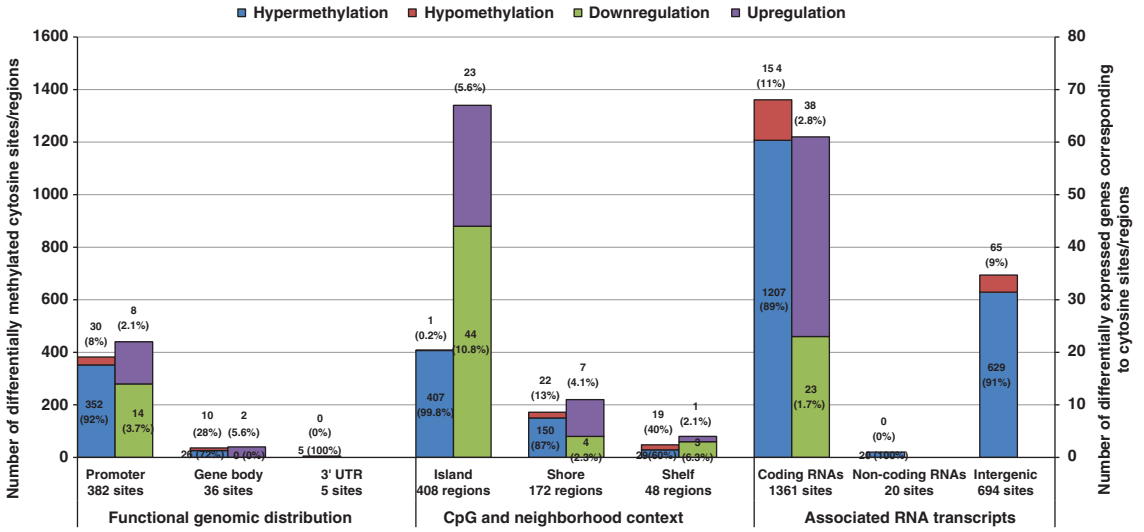

Figure 2. DNA methylation patterns in 8p11-p12-amplified tumors. The distribution of aberrant methylation (hyper- and hypomethylation, $Q<0.05$) and gene expression patterns (downregulation and upregulation, $Q<0.01$) among the 2066 differentially-methylated cytosine sites in 8p11-p12-amplified tumors. Transcripts were categorized into functional genomic regions (promoter region (between 200 and 1500 bp upstream of transcriptional start sites, 5′ untranslated region, first exon), gene body and 3′ untranslated region region) and regions surrounding CpG islands (CpG islands, 2 kb from CpG islands (CpG shores) and 2–4 kb from CpG islands (CpG shelves)).

which can be reversed by the mTORC1/2 inhibitor, MLN0128, in breast cancer xenograft models.[46,47] p53-mediated induction of DNA damage-associated genes, such as RRM2B, can promote resistance of cancer cells to genotoxic therapy, which can be prevented by inhibiting histone deacetylases that can in turn inhibit ataxia telangiectasia-mutated kinase and p53 activation and their downstream targets.[48–50] The *TAF2* gene is involved in general transcription processes and is the DNA binding component of the transcription factor II D transcription factor complex.[51]

In summary, we have identified the enrichment of *MYC* amplification and hypomethylation of genes on cytoband 8q in 8p11-p12-amplified tumors. These findings indicate that the aggressive phenotype observed in invasive breast tumors harboring the 8p11-p12 amplicon may not only be a consequence of altered activity of amplified genes in the genomic region, but also a result of *MYC* coamplification and aberrant DNA methylation patterns on chromosome 8q.

MATERIALS AND METHODS

Tumor specimens

Primary invasive breast carcinoma specimens ($n=229$) corresponding to 185 patients diagnosed from 1988–1999 were obtained from the fresh-frozen tumor bank at the Sahlgrenska University Hospital Oncology Lab in accordance with the Declaration of Helsinki and approved by the Medical Faculty Research Ethics Committee (Gothenburg, Sweden). The 229 cases were compiled from three independent array-comparative genomic hybridization microarray datasets, including two published (138/229 tumors) and one unpublished (91/229 tumors) studies.[23,24] The clinicopathological features of the 229 cases are shown in Table 1. Each tumor specimen was assessed for the presence of malignant cells using May–Grünwald Giemsa staining (Chemicon International, Temecula, CA, USA) on touch preparations. Highly representative specimens containing $>70\%$ neoplastic cell content were included in the microarray and fluorescence *in situ* hybridization analyses.

Genomic and transcriptome profiling

Genomic profiling of the tumor specimens was performed using whole-genome tiling 38K array-comparative genomic hybridization microarrays, as previously described.[23,24] Data preprocessing, normalization and data analysis were performed as previously described using $\log_2$ ratio thresholds set at $+0.2$, $\geqslant +0.5$, -0.2 and $\leqslant -1.0$ for low-level gain, high-level gain/amplification, heterozygous loss and homozygous deletion (henceforth referred to as gain, amplification, loss and deletion), respectively.[24] Total RNA samples from 150/229 tumor specimens were isolated and profiled using Illumina HumanHT-12 Beadchips (Illumina Inc.) as previously described.[24] Enriched gene ontology terms associated with differentially regulated genes were set to $P<0.05$, analyzed further using the gene ontology database (http://www.geneontology.org). The dataset was stratified into the molecular breast cancer subtypes using the five centroids (normal-like, basal-like, luminal subtype A, luminal subtype B and human epidermal growth factor receptor 2/estrogen receptor-negative (HER2/ER−)) and genomic grade index (high, low), as previously described.[52–54] Luminal subtype B was further stratified according HER2 status as determined by array-comparative genomic hybridization; HER2+ was set to $\log_2$ ratio $\geqslant +0.5$ and HER2− was set to $\log_2$ ratio $< +0.5$.[55] Univariate Cox proportional hazard models were calculated for statistically significant genes using overall survival.

Fluorescence *in situ* hybridization

Probe labeling and hybridization were performed as described elsewhere[56] using locus-specific bacterial artificial chromosome (BAC; BACPAC Resources, Oakland, CA, USA) probes to verify gene amplification. Dual-color fluorescence *in situ* hybridization was performed on touchprint and metaphase preparations using cohybridized biotin-16-deoxyuridine triphosphate (dUTP) and dioxigenin-11-dUTP-labeled probes. Analysis was performed using a Leica DMRA2 fluorescent microscope (Leica, Wetzler, Germany) equipped with an ORCA Hamamatsu CCD (charged-couple devices) camera (Hamamatsu City, Japan) and filter cubes specific for fluorescein isothiocyanate, Rhodamine and ultraviolet for DAPI visualization. Digitalized black and white images were acquired using the Leica CW4000 software package (Leica).

DNA methylation profiling

In total, 22/229 tumor samples harboring ($n=11$) or lacking the 8p11-p12 amplicon ($n=11$) were profiled using Illumina Infinium Human Methylation 450 Beadchips (Illumina Inc) according to the manufacturer's instructions. The estimated methylation level for specific cytosine sites (average beta) was calculated as a ratio between the intensities of methylated and unmethylated alleles and ranged from 0 (null methylated) to 1 (completely methylated). Delta beta values were calculated using (average beta values$_{\text{8p11–p12-amplified tumors}}$−average beta values$_{\text{8p11-p12 nonamplified tumors}}$). Cytosine sites located on the Y chromosome or containing single-nucleotide polymorphisms were removed. Differential DNA methylation was determined using the IMA package in R/Bioconductor (Bioconductor, FHCRC, Seattle, WA, USA) with thresholds set at: $\geqslant \pm 0.14$ delta beta value and Bonferroni adjusted at $P<0.05$.[57]

Table 2. Differentially-methylated genes in 8p11-p12-amplified breast tumors

Gene symbol	*Chromosome*	*Delta beta value*[a]	*Gene expression (n = 22)*[b]	*Gene expression (n = 150)*[c]	*Cox coefficient (n = 150)*[d]	*Cox P-value (n = 150)*[d]	*DNA copy number 8p11-p12-amplified tumors (n = 11)*[e] *amplification/ loss/normal*	*DNA copy number 8p11-p12 nonamplified tumors (n = 11)*[f] *amplification/ loss/normal*
BAMBI	10p12.1	Hypomethylated	Overexpressed			NS	0/0/11	0/0/11
CXCL12	10q11.21	Hypermethylated		Underexpressed	−0.498	3.76E−05	0/0/11	0/0/11
HTRA1	10q26.13	Hypermethylated		Underexpressed	−0.400	1.57E−04		
CRYAB;HSPB2	11q23.1	Hypermethylated	Underexpressed	Underexpressed		NS	0/4/7	0/0/11
PTHLH	12p11.22	Hypermethylated	Underexpressed	Underexpressed		NS	1/0/10	0/0/11
HOXC13	**12q13.13**	**Hypermethylated**		**Overexpressed**	**0.397**	**3.96E−04**	**0/1/10**	**0/0/11**
PABPC3	**13q12.13**	**Hypermethylated**		**Overexpressed**	**0.643**	**1.12E−06**	**0/4/7**	**0/0/11**
TRAPPC6B	**14q21.1**	**Hypermethylated**		**Overexpressed**	**0.866**	**1.80E−05**	**0/2/9**	**0/0/11**
BMP4	14q22.2	Hypermethylated		Underexpressed	−0.379	0.001	0/3/8	0/0/11
BATF	**14q24.3**	**Hypomethylated**		**Underexpressed**	**−0.502**	**0.002**	**0/4/7**	**0/0/11**
ELL3	15q15.3	Hypomethylated		Overexpressed	0.432	0.009	0/0/11	0/0/11
SEPHS2	16p11.2	Hypomethylated		Overexpressed	0.495	0.003	0/0/11	0/0/11
SPN	**16p11.2**	**Hypermethylated**		**Overexpressed**	**0.254**	**0.064**	**0/0/11**	**0/0/11**
SPAG9	**17q21.33**	**Hypermethylated**	**Overexpressed**	**Overexpressed**	**0.628**	**0.000**	**1/0/10**	**0/0/11**
DHX40	**17q23.1**	**Hypermethylated**	**Overexpressed**		**0.599**	**0.004**	**2/0/9**	**0/0/11**
CCDC47	17q23.3	Hypomethylated		Overexpressed	0.671	0.001		
ICAM2	17q23.3	Hypermethylated	Underexpressed	Underexpressed	−0.441	0.012	1/0/10	0/0/11
CYGB	17q25.1	Hypermethylated	Underexpressed		−0.433	0.013	0/0/11	0/0/11
NFIX	19p13.2	Hypermethylated	Underexpressed		−0.289	0.023	0/0/11	0/0/11
NFIC	19p13.3	Hypermethylated	Underexpressed			NS	0/1/10	0/0/11
CACNG6	**19q13.42**	**Hypermethylated**	**Overexpressed**			**NS**	**0/0/11**	**0/0/11**
PHGDH	1p12	Hypermethylated	Underexpressed		0.313	0.002	0/0/11	0/0/11
CHI3L2	1p13.3	Hypermethylated		Underexpressed		NS	0/0/11	0/0/11
COL11A1	**1p21.1**	**Hypermethylated**	**Overexpressed**			**NS**	**0/1/10**	**0/0/11**
AGL	1p21.2	Hypomethylated	Overexpressed	Overexpressed	0.337	0.013	0/1/10	0/0/11
PODN	1p32.3	Hypermethylated		Underexpressed	−0.366	0.001	0/2/9	0/0/11
MMP23A;MMP23B	1p36.33	Hypermethylated		Underexpressed	−0.415	0.003	0/1/10	0/0/11
MMP23B	1p36.33	Hypermethylated		Underexpressed	−0.415	0.003	0/1/10	0/0/11
EXOC8	1q42.2	Hypomethylated	Overexpressed	Overexpressed	0.734	2.58E−05	1/1/9	0/0/11
SYCP2	20q13.33	Hypomethylated	Overexpressed	Overexpressed	0.447	3.15E−05	2/0/9	0/0/11
GREB1	2p25.1	Hypomethylated	Overexpressed		−0.268	0.050	0/2/9	0/1/10
C2orf40	2q12.2	Hypermethylated	Underexpressed	Underexpressed	−0.268	0.005		
SATB2	**2q33.1**	**Hypermethylated**	**Overexpressed**			**NS**	**0/0/11**	**0/0/11**
KIF1A	**2q37.3**	**Hypermethylated**	**Overexpressed**			**NS**	**0/0/11**	**0/0/11**
TF	3q22.1	Hypermethylated	Underexpressed			NS	0/1/10	0/0/11
TAPT1	4p15.32	Hypomethylated		Overexpressed		NS		
SORBS2	**4q35.1**	**Hypomethylated**	**Underexpressed**	**Underexpressed**	**−0.326**	**0.004**		
PIK3R1	**5q13.1**	**Hypermethylated**		**Overexpressed**	**0.536**	**3.96E−04**	**0/0/11**	**0/0/11**
CARTPT	5q13.2	Hypermethylated	Underexpressed			NS		
PCSK1	5q15	Hypermethylated	Underexpressed		−0.326	0.002	0/1/10	0/0/11
PAM	5q21.1	Hypermethylated	Underexpressed	Underexpressed	−0.340	0.024	0/0/11	0/0/11
DMXL1	**5q23.1**	**Hypermethylated**	**Overexpressed**	**Overexpressed**	**0.620**	**9.99E−06**	**0/0/11**	**0/0/11**
H2AFY	**5q31.1**	**Hypermethylated**	**Overexpressed**	**Overexpressed**	**0.831**	**3.15E−07**	**0/0/11**	**0/0/11**
DOCK2	**5q35.1**	**Hypomethylated**		**Underexpressed**		**NS**	**0/0/11**	**0/0/11**
SCGB3A1	5q35.3	Hypermethylated	Underexpressed		−0.240	0.001	0/0/11	0/0/11
USP49	**6p21.1**	**Hypermethylated**		**Overexpressed**	**0.310**	**0.031**	**1/0/10**	**0/0/11**
SCAND3	**6p22.1**	**Hypermethylated**	**Overexpressed**		**0.492**	**0.015**		
ID4	**6p22.3**	**Hypermethylated**		**Overexpressed**	**0.492**	**1.95E−05**	**0/0/11**	**0/0/11**
RARS2;ORC3L	6q15	Hypomethylated		Overexpressed	0.822	1.85E−05	2/1/8	0/0/11
LRP11	6q25.1	Hypomethylated		Overexpressed	0.638	3.05E−04	0/1/10	0/0/11
C7orf28A	7p22.1	Hypomethylated		Overexpressed	0.775	1.85E−05	0/0/11	0/0/11
LFNG	7p22.3	Hypermethylated		Underexpressed	−0.668	4.55E−06	0/0/11	0/0/11
PON3	7q21.3	Hypermethylated	Underexpressed	Underexpressed	−0.305	3.19E−05	1/0/10	0/0/11
NRCAM	7q31.1	Hypomethylated		Overexpressed	0.336	0.024	0/0/11	0/0/11
NDUFA5	**7q31.32**	**Hypermethylated**		**Overexpressed**	**0.771**	**1.48E−04**	**0/1/10**	**0/0/11**
LEP	**7q32.1**	**Hypermethylated**		**Overexpressed**	**0.357**	**0.003**	**0/0/11**	**0/0/11**
RARRES2	7q36.1	Hypermethylated		Underexpressed	−0.272	0.020	0/0/11	0/0/11
BRF2	**8p11.23**	**Hypermethylated**	**Overexpressed**	**Overexpressed**		**NS**	**7/2/2**	**0/0/11**
IMPAD1	8q12.1	Hypomethylated	Overexpressed	Overexpressed	1.067	2.70E−07		
FABP5	**8q21.13**	**Hypermethylated**		**Overexpressed**		**NS**	**1/0/10**	**0/0/11**
PLEKHF2	8q22.1	Hypomethylated	Overexpressed	Overexpressed	0.393	4.47E−04	3/0/8	0/0/11
VPS13B	8q22.2	Hypomethylated		Overexpressed	0.878	7.06E−06		
RRM2B	8q22.3	Hypomethylated	Overexpressed	Overexpressed	0.452	0.001	3/0/8	0/0/11
TRPS1	**8q23.3**	**Hypermethylated**	**Overexpressed**	**Overexpressed**	**0.320**	**0.008**	**6/0/5**	**0/0/11**
TRPS1	8q23.3	Hypomethylated	Overexpressed	Overexpressed	0.320	0.008	6/0/5	0/0/11
ENPP2	8q24.12	Hypermethylated	Underexpressed	Underexpressed	−0.258	0.017	2/0/9	0/0/11
TAF2	8q24.12	Hypomethylated		Overexpressed	1.164	1.34E−06	2/0/9	0/0/11
SQLE	8q24.13	Hypomethylated	Overexpressed	Overexpressed	0.566	2.95E−07	2/0/9	0/0/11
TATDN1	8q24.13	Hypomethylated	Overexpressed	Overexpressed	0.783	2.89E−06	5/0/6	0/0/11
NDRG1	8q24.22	Hypomethylated		Overexpressed	0.843	5.03E−11	2/1/8	0/0/11
WDR44	Xq24	Hypomethylated		Overexpressed	0.937	4.75E−06	3/0/8	0/0/11

Abbreviation: NS, not statistically significant. Genes not correlating between DNA methylation and transcriptional patterns are shown in bold text. [a]Delta beta value (8p11-p12-amplified tumors versus nonamplified tumors) >0.14 are indicated by hypermethylation and <-0.14 are indicated by hypomethylation; Bonferroni adjusted *P*-value $P<0.05$. [b]Gene expression microarray log2 ratio for the 22 tumors (8p11–p12-amplified tumors versus nonamplified tumors) >0.58 are indicated by overexpression and <-0.58 are indicated by underexpression. [c]Gene expression microarray log2 ratio for the 150 tumors (8p11-p12-amplified tumors versus nonamplified tumors) >0.58 are indicated by overexpression and <-0.58 are indicated by underexpression. [d]Univariate Cox proportional hazard regression models using the gene expression data for the 150 tumors and overall survival rates. [e]Array-CGH log2 ratio thresholds set at $\geqslant +0.5$, -0.2 and between $+0.5$ and -0.2 for amplification, loss and normal copy number, respectively. [f]Array-CGH log2 ratio thresholds set at $\geqslant +0.5$, –0.2 and between $+0.5$ and -0.2 for amplification, loss and normal copy number, respectively.

Table 3. Significantly enriched Gene Ontology (GO) terms identified by integrated DNA methylation and expression profiling in 8p11-p12-amplified breast tumors

Category	*GO term*	P-*value*	*Gene count*
Biological process			
GO:0032099	Negative regulation of appetite	8.32E − 05	2
GO:0045671	Negative regulation of osteoclast differentiation	2.48E − 04	2
GO:0045060	Negative thymic T-cell selection	2.48E − 04	2
GO:0008343	Adult feeding behavior	4.93E − 04	2
GO:0006935	Chemotaxis	0.00203	4
GO:0007281	Germ cell development	0.002871	2
GO:0006260	DNA replication	0.003236	4
GO:0007420	Brain development	0.003323	3
GO:0030335	Positive regulation of cell migration	0.007046	2
GO:0006366	Transcription from RNA polymerase II promoter	0.014531	3
GO:0016337	Cell-cell adhesion	0.030651	2
GO:0008544	Epidermis development	0.042861	2
Molecular function			
GO:0043169	Cation binding	0.011569	2
GO:0008083	Growth factor activity	0.013173	3
GO:0001104	RNA polymerase II transcription factor activity	0.013842	3
GO:0005179	Hormone activity	0.021768	2
GO:0004252	Serine-type endopeptidase activity	0.030247	3
GO:0006351	Transcription regulator activity	0.038615	2
Cellular component			
GO:0005615	Extracellular space	3.46E − 05	10
GO:0005576	Extracellular region	2.89E − 04	17
GO:0043005	Neuron projection	0.002871	2
GO:0009897	External side of plasma membrane	0.079513	2
GO:0005634	Nucleus	0.081344	22
GO:0005794	Golgi apparatus	0.08346	6
GO:0005768	Endosome	0.096152	2
GO:0005578	Proteinaceous extracellular matrix	0.107112	3
GO:0005737	Cytoplasm	0.33593	19

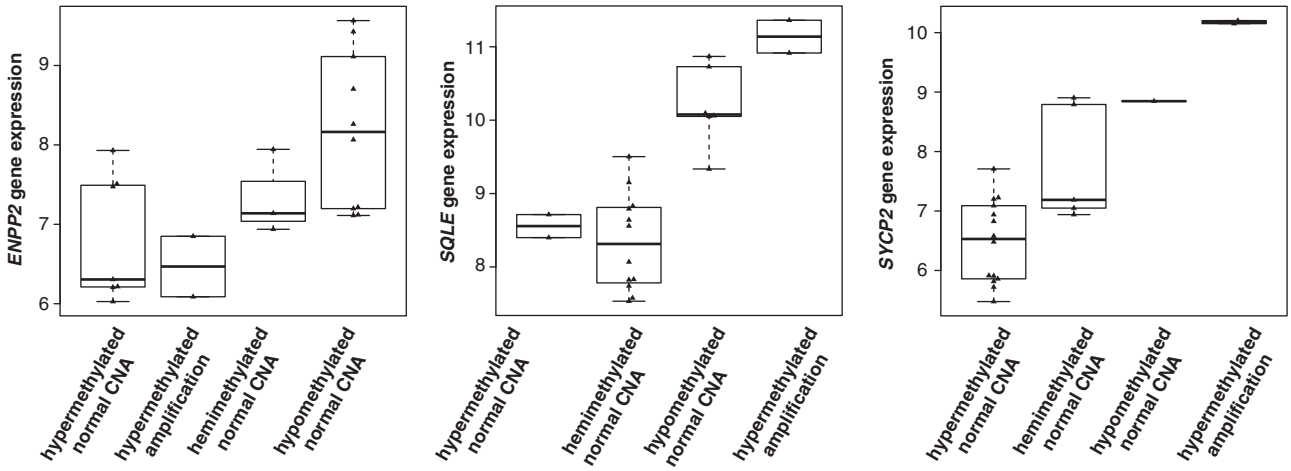

Figure 3. The effect of aberrant DNA copy number and DNA methylation on gene expression. Box plots showing the relationship between DNA copy number (CNA), methylation status and gene expression for three candidate genes (*ENPP2*, *SQLE* and *SYCP2*) in 22 tumor samples. *X*-axis, methylation and CNA status; *Y*-axis, gene expression signal intensity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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