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Analysis of copy number changes on chromosome 16q in male breast cancer by multiplex ligation-dependent probe amplification

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Gene copy number changes have an important role in carcinogenesis and could serve as potential biomarkers for prognosis and targets for therapy. Copy number changes mapping to chromosome 16 have been reported to be the most frequent alteration observed in female breast cancer and a loss on 16q has been shown to be associated with low grade and better prognosis. In the present study, we aimed to characterize copy number changes on 16q in a group of 135 male breast cancers using a novel multiplex ligation-dependent probe amplification kit. One hundred and twelve out of 135 (83%) male breast cancer showed copy number changes of at least one gene on chromosome 16, with frequent loss of 16q (71/135; 53%), either partial (66/135; 49%) or whole arm loss (5/135; 4%). Losses on 16q were thereby less often seen in male breast cancer than previously described in female breast cancer. Loss on 16q was significantly correlated with favorable clinicopathological features such as negative lymph node status, small tumor size, and low grade. Copy number gain of almost all genes on the short arm was also significantly correlated with lymph node negative status. A combination of 16q loss and 16p gain correlated even stronger with negative lymph node status ($n = 112$; $P = 0.012$), which was also underlined by unsupervised clustering. In conclusion, copy number loss on 16q is less frequent in male breast cancer than in female breast cancer, providing further evidence that male breast cancer and female breast cancer are genetically different. Gain on 16p and loss of 16q identify a group of male breast cancer with low propensity to develop lymph node metastases.
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Gene copy number changes have an important role in carcinogenesis and could serve as potential biomarkers for prognosis. In addition, they could provide potential targets for molecular therapy.

Previous studies in male breast cancer showed clear differences in gene copy number changes when compared with female breast cancer, pointing toward differences in carcinogenesis between male and female breast cancer.[1] This emphasizes the importance of identifying biomarkers and therapeutic targets that could aid in clinical management of male breast cancer.

Copy number changes mapping to chromosome 16 have been reported to be the most frequent alteration in female breast cancer. In female breast cancer, aberrations on chromosome 16 have extensively been studied showing association between loss on the long arm of chromosome 16, low-grade ductal and lobular cancer, and favorable prognosis. High-grade ductal cancer often has complex changes, typically small regions of gain together with larger regions of loss.[2–6] Genetic alterations on chromosome 16 in male breast cancer are poorly

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characterized compared with female breast cancer, and only a few studies have been performed[1,7,8] of which the first analyzes only one gene on 16q and the latter studies analyze small series of male breast cancer. These studies report frequent chromosomal imbalances on both the short and long arm of chromosome 16.

In the present study, we aimed to further characterize copy number changes on the long arm of chromosome 16 in relation to the 16p copy number changes in a large group of male breast cancer using a novel multiplex ligation-dependent probe amplification (MLPA) kit with multiple genes on chromosome 16, and to correlate these genomic anomalies with clinicopathological features and patients' outcome.

Materials and methods

Patient Material

All consecutive cases of surgical breast specimens of invasive male breast cancer from 1986 to 2011 were collected from four different pathology labs in the Netherlands (St. Antonius Hospital, Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, and Laboratory for Pathology East Netherlands), as described in more detail previously[1,9] and from three pathology labs in Germany (Paderborn, Cologne, Kassel). Hematoxylin and eosin (HE) slides were reviewed by four experienced observers (PJvD, RK, AM, ML) to confirm the diagnosis and to type and grade according to current standards. Pathological reports were used to retrieve information on age, tumor size, and lymph node status. A total of 145 cases from which the paraffin blocks contained enough tumor for DNA isolation were included. The age of these patients ranged from 32 to 89 years (average 66 years). Tumor size ranged from 0.2 to 7.2 cm (average 2.3 cm). In 120 cases (82%), the lymph node status was known by axillary lymph node dissection or sentinel node procedure and 55% of these patients had lymph node metastases. The majority of cases were diagnosed (according to the WHO) as invasive ductal carcinoma (130/145; 90%). The remaining cases were lobular ($n = 3$), mixed type (ductal/lobular; $n = 3$), invasive cribriform ($n = 3$), papillary ($n = 2$), mucinous ($n = 2$), invasive micropapillary ($n = 1$), or adenoid cystic carcinomas ($n = 1$). According to the modified Bloom and Richardson score,[10] most tumors were grade 2 (44%) or grade 3 (32%). Mitotic activity was assessed as before[11] with a mean mitotic index of 12 per 2 mm^2 (range 0–56). For all cases, hormone receptor and HER2 status were reassessed as described previously.[9] Tissue microarray (TMA) slides were used for immunohistochemical staining of ER, PR, and chromogenic *in situ* hybridization for HER2 assessment, the latter showing HER2 amplification in only five cases (3%). Most tumors were ER positive (131/145; 90%), and PR positivity was also common (97/145; 67%). TMA slides were also stained and scored for E-cadherin, considering cases with no membranous staining as E-cadherin negative. Six cases were scored as E-cadherin negative, three lobular and three ductal carcinomas.

Intrinsic Subtypes

Immunohistochemical stainings were used to classify the tumors into five different subtypes: luminal type A (ER + and/or PR + , HER2- and Ki-67 low), luminal type B (ER + and/or PR + , and HER2 + and/or Ki67 high), HER2 driven (HER2 + and ER − /PR −), basal like (ER − /PR − /HER2 − , and CK5/6 + and/or CK14 + and/or EGFR +), and unclassifiable triple negative (negative for all six markers) as described before.[9]

DNA Extraction and MLPA Analysis

Representative tumor areas were identified in HE-stained slides and corresponding tumor areas (at least 1 cm^2) were dissected with a scalpel from 8 μm paraffin slides.[12] DNA was extracted by overnight incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56 °C, boiled for 10 min and centrifuged. Five microlitres of this DNA solution was used for MLPA analysis. MLPA was performed according the manufacturers' instructions (MRC-Holland, Amsterdam, The Netherlands), using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The novel X043-1A kit (MRC-Holland), containing 6 probes for 6 16p genes and 28 probes for 21 16q genes, was used. All tests were performed in duplicate. Negative reference samples (normal breast and blood) were included in each MLPA run as before.[1] The PCR products were separated by electrophoresis on an ABI 3730 capillary sequencer (Applied Biosystems). Mean probe peaks were used for final gene copy number analysis with Genescan v4.1 (Applied Biosystems) and Coffalyser v9.4 (MRC-Holland) software. Cut-off values were set as before with 1.3–2.0 for gene copy number gain, > 2.0 for amplification and < 0.7 for loss. Values between 0.7 and 1.3 were regarded normal.[13,14] Whole arm loss was defined as copy number loss of $> 75\%$ of all the probes, as defined before using array-comparative genomic hybridization.[15] Partial loss on the long arm of the chromosome was defined as any probe showing copy number loss.

To define smallest regions of overlap (SRO) between areas of copy number loss we analyzed the cases according to the previous definition, with an additional threshold defining retention 0.8–1.2,[14,16] values between 0.7–0.8 and 1.2–1.3 were regarded as gray areas.

Statistics

Statistical calculations were performed using IBM SPSS for Windows version 20.0. Associations between gene copy number and clinicopathological characteristics were calculated with Pearson's χ^2 (or Fisher's exact test when appropriate) for categorical variables. Grade, tumor size, and mitotic count were dichotomized. Unsupervised hierarchical clustering using the statistical program R (http://www.r-project.org) was performed to identify relevant clusters. We used the maximum distance and Ward's clustering method, and calculated the stability of the clusters with pvclust as before.[1]

Information regarding prognosis and therapy was requested from the Integral Cancer registration, the Netherlands (IKNL). Survival data were available for 100 cases with a mean follow-up of 5.6 years. Therefore, survival analysis was based on 5-year survival rates. For univariate survival analysis, Kaplan–Meier curves were plotted and analyzed with the log rank test. Multivariate survival analysis was done with Cox regression including the variables that were significant in univariate analysis.

Results

Copy Number Analysis by MLPA

In 10 cases the amount of DNA was insufficient, leaving 135 cases of male breast cancer for further analysis. Gene copy number status of the analyzed genes is presented in Table 1 and Figure 1. In 23/135 (17%) of cases, there were no copy number alterations in any of the studied genes on 16q; 2 of these had partial gain of 16p.

The most common 16q alteration was copy number loss (71/135; 53%), either partial arm loss (66/135; 49%), or whole arm loss (5/135; 4%). Twenty-seven cases with partial 16q arm loss (27/66, 41%) also had partial gain of 16q. A total of 49% of the cases ($n = 66$) had partial gain of 16q and in 38 cases this combined with copy number gain of 16p.

None of the cases showed a whole arm loss of 16p, whereas six cases (4%) had partial 16p loss. In 56 cases (42%), there was gain of 16p, either partial (39/135; 29%) or whole arm gain (17/135; 13%). Two cases (2%) showed whole arm gain of both 16q and 16p. In two cases (2%) there was no other alteration than copy number gain on 16p.

Of all cases, 26% (35/135) had both loss of 16q (either partial or whole arm loss) and gain of 16p (either partial or whole arm gain); 11 also had partial gain of 16q.

Loss was present in varying frequencies in all genes on 16q but in only one gene on 16p (*CREBBP*). Gene loss was most common for *CDH11*, *MLYCD*, *FOXF1*, *SPG7,* and *FANCA*. Copy number gain was most frequently seen on the short arm but was also present throughout chromosome 16 (Figure 1).

Table 1 Correlation between gene copy number losses by multiplex ligation-dependent probe amplification and clinicopathological features in 135 male breast cancers

Gene	*Location*	*Grade G1*	*Size <2.0*	*Lymph node negative*
TSC2	16p13.3 exon 2			
CREBBP	16p13.3 exon 12			
ABAT	16p13.2 exon 4			
ABCC6	16p13.11exon 13			
PALB2	16p12.1 exon 6			
VKORC1	16p11.2 exon 1			
VPS35	16q11.2 exon 13			
ABCC12	16q12.1 exon 28			
CYLD	16q12.1 exon 19			0.009
SALL1	16q12.1 exon 3			
RBL2	16q12.2 exon 2	0.017		0.001
MMP2	16q12.2 exon 14			0.017
SLC12A3	16q13 exon 12			0.02
GPR56	16q13 exon 10		0.04	0.009
CDH11	16q22.1 exon 3			
CDH11	16q22.1 exon 8			
TK2	16q22.1 exon 6			
CDH1	16q22.1 exon 1			0.047
CDH1	16q22.1 exon 11			0.021
CDH1	16q22.1 exon 14			
ZFHX3	16q22.3 exon 3			
WWOX	16q23.1 exon 4			
WWOX	16q23.1 exon 10			
CDH13	16q23.3 exon 1			
MLYCD	16q23.3 exon 2			0.015
MLYCD	16q23.3 exon 3			0.019
IRF8	16q24.1 exon 9			0.017
FOXF1	16q24.1 exon 2			
FBXO31	16q24.2 exon 4			
SPG7	16q24.3 exon 3			
SPG7	16q24.3 exon 4			0.003
FANCA	16q24.3 exon 20			0.006
FANCA	16q24.3 exon 43			
GAS8	16q24.3 exon 6			

Only significant *P*-values are shown.

Of all the cases with any loss on 16q, 58/71 (82%) showed alternating regions of retention and loss with more than two areas of loss. Two cases showed loss starting at the most centromeric region on the 16q arm alternated by gray areas and no retention. Two showed terminal loss of 16q starting from the *CYLD* gene position with retention on the area centrometric of this probe, and another case showed terminal loss starting from the *MMP2* gene position with gray areas to the *CYLD* gene position and retention on the area centrometric of this probe. Eight cases showed two regions of loss with one or two areas of retention. The latter cases were used to determine SRO.

Copy Number Alterations and Clinicopathological Features

As shown in Table 1, copy number losses of the 16q genes *CYLD*, *RBL2*, *MMP2*, *SLC12A3*, *GPR56*, *CDH1* (exons 1 and 11), *MLYCD*, *IRF8*, *SPG7* (exon 4), and *FANCA* (exon 20) were significantly correlated with

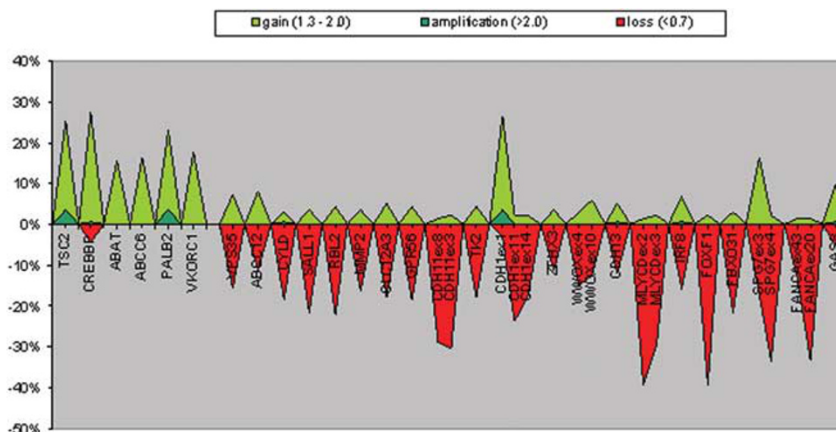

Figure 1 Copy number changes by multiplex ligation-dependent probe amplification (MLPA) in 27 genes on chromosome 16 in 135 male breast cancers.

negative node status. Losses of *GPR56* were associated with small tumor size (T1) and loss of *RBL2* with lower grade (G1). Age and mitotic count were not correlated with copy number changes in any of the studied genes (data not shown).

As shown in Table 2, copy number gains of almost all genes on 16p were also significantly associated with negative lymph node status, as was true for some genes on 16q (*VPS35*, *ABCC12,* and *GPR56*).

Copy number loss (partial loss and whole arm loss) on 16q was significantly correlated with lymph node negative status ($n=112$; $P=0.032$) irrespective of changes on 16p. When the cases had copy number loss on 16q combined with copy number gain on 16p, the correlation was even stronger ($n=112$; $P=0.012$).

Loss on the long arm of chromosome 16 was not significantly different between grades and was present in 56% (18/32) of grade 1 tumors, 36% (21/59) of grade 2 tumors, and 73% (32/44) of grade 3 tumors.

Of the six E-cadherin-negative cases, two had no alterations on the *CDH1* gene, one lobular and one ductal tumor. Two cases, both lobular tumors, had loss of for least one probe of the *CDH1* gene, one case had copy number loss on the *CDH1* exon 11 and *CDH1* exon 14 probes, together with gain on the *CDH1* exon 1 probe and the second one had loss on the *CDH1* exon 11 probe. The two remaining cases, both ductal tumors, had gain on the *CDH1* exon 1 probe. There was no correlation between alterations on the *CDH1* gene and expression of E-cadherin by immunohistochemical staining.

Cluster Analysis

Unsupervised hierarchical clustering revealed two stable clusters ($P<0.001$) (Figure 2). Cluster A consisted of 78 cases and was characterized by partial and whole arm loss on 16q; 85 *vs* 6% in cluster B. Combined loss on 16q and gain on 16p was also found in a higher percentage (40%) in cluster A than that in cluster B. All of the genes analyzed on 16q showed loss in a significantly higher percentage in cluster A than that in cluster B.

The male breast cancer cases in cluster A showed lymph node metastasis in a significantly lower percentage compared with cluster B (45 *vs* 70%; $P=0.008$). Cluster B consisted of 57 cases and was characterized by a higher percentage of copy number gain; 72 *vs* 35% in cluster A. Cases showing no aberrations on chromosome 16 were also more frequently found in cluster B. Distribution of other clinicopathological features was not significantly different between the clusters.

Intrinsic Subtype Analysis

The vast majority of cases were classified as luminal type A (102/135; 76%), whereas 25 cases were of luminal type B (18%). The remaining cases were basal like (3/135; 2%) or unclassifiable triple negative (5/135; 4%). There were no HER2-driven cases. Cases with 16q loss (partial and whole arm loss) showed a similar intrinsic subtype distribution (73% luminal A, 23% luminal B, and 4% unclassified triple negative). Combined loss on 16q (partial and whole arm loss) and gain on 16p occurred only in luminal type, with an overrepresentation of luminal A cases (89%). The luminal type A cases were evenly dispersed over clusters A and B, as were the unclassifiable triple-negative cases. All three basal-like cases clustered in cluster B ($P=0.040$).

Survival Analysis

Grade 3 ($P=0.026$), high mitotic count (>8; $P=0.028$), large tumor size (>2.0 cm; $P=0.031$), and luminal type B ($P=0.042$) were correlated with

Table 2 Correlation between gene copy number gains by multiplex ligation-dependent probe amplification and clinicopathological features in 135 male breast cancers

Gene	*Location*	*Grade G1*	*Size <2.0*	*Lymph node negative*
TSC2	16p13.3 exon 2			0.032
CREBBP	16p13.3 exon 12			
ABAT	16p13.2 exon 4			0.017
ABCC6	16p13.11exon 13			0.004
PALB2	16p12.1 exon 6			0.021
VKORC1	16p11.2 exon 1			
VPS35	16q11.2 exon 13			0.040
ABCC12	16q12.1 exon 28	0.022		0.010
CYLD	16q12.1 exon 19			
SALL1	16q12.1 exon 3			
RBL2	16q12.2 exon 2			
MMP2	16q12.2 exon 14			
SLC12A3	16q13 exon 12			
GPR56	16q13 exon 10			0.014
CDH11	16q22.1 exon 3			
CDH11	16q22.1 exon 8			
TK2	16q22.1 exon 6			
CDH1	16q22.1 exon 1			
CDH1	16q22.1 exon 11			
CDH1	16q22.1 exon 14			
ZFHX3	16q22.3 exon 3			
WWOX	16q23.1 exon 4			
WWOX	16q23.1 exon 10			
CDH13	16q23.3 exon 1			
MLYCD	16q23.3 exon 2			
MLYCD	16q23.3 exon 3			
IRF8	16q24.1 exon 9			
FOXF1	16q24.1 exon 2			
FBXO31	16q24.2 exon 4			
SPG7	16q24.3 exon 3		0.039	
SPG7	16q24.3 exon 4			
FANCA	16q24.3 exon 20			
FANCA	16q24.3 exon 43			
GAS8	16q24.3 exon 6			

Only significant *P*-values are shown.

a decreased 5-year survival. In univariate survival analysis alterations on chromosome 16 did not predict survival. The cluster groups formed by unsupervised hierarchical clustering were not associated with survival. In multivariate Cox regression, only tumor size and mitotic count emerged as independent prognostic factors.

Discussion

The aim of the present study was to characterize copy number changes on the long arm (and to a lesser extent the short arm) of chromosome 16 in a large group of male breast cancer using a novel MLPA kit. Fifty-three percent of male breast cancer cases lost at least some part of 16q. This is a much lower percentage than previously described for female breast cancer (70% in luminal A tumors, 75% overall in invasive ductal cancer),[3,4,6] especially when considering the high ratio of luminal A tumors in male breast cancer compared with female breast cancer. Most cases of male breast cancer showed at least partial loss of 16q. Of the 135 cases, 27 had regions of both loss and gain on 16q. These complex changes have been described to be more frequent in high-grade ductal cancer,[6] but in the present male breast cancer study these changes were randomly distributed over low- and high-grade tumors.

Cleton-Jansen *et al*[16] previously described loss of heterozygosity (LOH) mapping at 16q in a large cohort of female breast cancer cases, and found a similar percentage of 16q loss in male breast cancer compared with our results in male breast cancer; 53 *vs* 52%. However, ER-positive tumors were more prevalent in their group with LOH on 16q. This suggests a higher percentage of loss when corrected for ER positivity. We found a clearly lower percentage of whole arm losses in male breast cancer; 4 *vs* 28% compared with female breast cancer. These results must however be interpreted with caution as the MLPA technique and the LOH PCR techniques use different markers and probes in different exact locations on 16q for the analysis.

Cleton-Jansen *et al*[16] defined SRO in order to determine the location of a putative tumor suppressor gene targeted by LOH. Two SRO were defined at region 16q24.3 and one at 16q22.1. When analyzing our data set with an additional threshold defining retention, we found a similar SRO at region 16q24.3 and two smaller SRO based on losses at regions 16q12.1 and 16q21. In male breast cancer, there seem to be more complex losses with only three cases showing a single area of loss on 16q. Copy number loss of several genes on 16q was significantly correlated with negative lymph node status (*CYLD*, *RBL2*, *MMP2*, *SLC12A3*, *GPR56*, *CDH1*, *MLYCD*, *IRF8*, *SPG7*, *FANCA*), small tumor size (*GPR56*), and low tumor grade (*RBL2*). *CYLD* and *RBL2* are known tumor suppressor genes.[16–20] The latter is also known to be involved in several malignancies including female breast cancer.[19,20] *CDH1*, also known as E-cadherin, is a cell-to-cell adhesion glycoprotein. Loss of function is thought to contribute to progression of cancer by increasing proliferation, invasion, and/or metastasis.[21] The *SPG7* gene codes for a mitochondrial metalloprotease and *FANCA* encodes a protein involved in the preservation of genomic integrity through the *FA*/*BRCA* pathway.[22–24] Both genes have been mapped to 16q23.3, previously described to be a region of frequent LOH in sporadic breast and prostate cancer.[22] In view of the present results, losses of these genes on 16q seem to have a role in tumorigenesis in the male breast as well.

Copy number gain of almost all genes on the short arm was also significantly correlated with negative lymph node status as was copy number loss of 16q ($n = 112$; $P = 0.032$), irrespective of changes on 16p. This is in line with findings in female breast cancer.[25] When loss of 16q and gain of 16p were

Figure 2 Unsupervised hierarchical clustering of copy number changes in genes on chromosome 16 in 135 male breast cancer patients. The identified clusters of patients (horizontal) and genes (vertical) are depicted in different color.

both present, the correlation was even stronger ($n=112$; $P=0.012$). However, other than in female breast cancer, we found no or few correlations between 16q loss and other favorable clinicopathological features such as low grade, low mitotic count, or small tumor size in male breast cancer.

Previous studies in male breast cancer used the comparative genomic hybridization method to analyze genetic alterations.[7,8] Largely in line with these two studies, we found similarities but also differences in cytogenetic aberrations between female breast cancer and male breast cancer. In the study conducted by Rudlowski *et al,*[8] gain on 16p and loss of 16q was reported in a lower percentage of cases compared with our results; 36% and 42% *vs* 31% and 53%, respectively. The difference in percentages could be due to the more sensitive technique used in our study. Tommasi *et al*[7] used a more sensitive CGH technique (array CGH) and found in concordance with our study a lower frequency of loss on chromosome 16 in male breast cancer compared with female breast cancer. The 16p11.2–p11.1 cytoband location was reported by Tommasi *et al*[7] to be frequently gained in male breast cancer. The single gene *VKOR1C*, located on 16p11.2, included in our assays was however found to be gained in a lower percentage (12 *vs* 40%). The *PALB2* gene, also analyzed by Tommasi *et al*,[7] was identified previously as a moderate penetrance breast cancer susceptibility gene, accounting for about 1% of BRCA1/2-negative familial early-onset breast cancer.[26] In our male breast cancer group, we found no copy number loss on the *PALB2* gene. These different results could be explained by the different techniques used, as we looked only at one specific exon on both genes (*VKORC1* exon 1 of 3 and *PALB2* exon 6 of 13).

In nonhierarchical cluster analysis, the two major clusters reflected the correlation between copy number loss on 16q, especially when combined with 16p gain and lower frequency of lymph node metastasis. Similar to all of the genes analyzed on 16q, loss of the *MMP2* gene was found in a higher percentage of tumors in cluster A (28%) compared with tumors in cluster B (0%). Matrix metalloproteinases (MMPs) promote tumor progression and metastasis in invasive cancers by degradation of the extracellular matrix, and loss of MMPs could in part account for the decreased propensity to develop lymph node metastasis.

Reasoning from the genes, we found a high percentage of copy number gain on *CDH1* exon 1 (coding for its signal peptide) and *SPG7* exon 3. In four patients there was even a combined gain on *CDH1* with consecutive losses on *CDH1* exon 13 and exon 14. These genes lie in regions previously reported as regions of copy number polymorphism and segmental duplications, which define hotspots of chromosomal rearrangement. These earlier findings could explain our results.[27] Copy number gain of almost all genes on the short arm also showed a significant correlation with lymph node negative status as was true for several individual genes on 16q.

Loss of 16q was often associated with gain of 16p. According to conventional cytogenetics, in most cases this is due to isochromosome formation and typically requires a loss of the whole arm,[28] which was only seen in three (3/35) of our cases. In eleven of these cases there was also partial gain on 16q. These results suggest that the combination of loss of 16q with gain of 16p may not entirely be explained by this mechanism, and is a reflection of more complex rearrangements.

In conclusion, copy number loss on 16q and gain of 16p identify a group of male breast cancer with low propensity to develop lymph node metastasis. Although most male breast cancer are of luminal intrinsic type, losses on 16q occur much less frequently in male breast cancer than in female breast cancer, providing further evidence that male breast cancer and female breast cancer are genetically different. 16q losses do not seem to have prognostic value in male breast cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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