RESEARCH ARTICLE

Quantitative Multigene FISH on Breast Carcinomas Identifies der(1;16)(q10;p10) as an Early Event in Luminal A Tumors

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In situ detection of genomic alterations in cancer provides information at the single cell level, making it possible to investigate genomic changes in cells in a tissue context. Such topological information is important when studying intratumor heterogeneity as well as alterations related to different steps in tumor progression. We developed a quantitative multigene fluorescence *in situ* hybridization (QM FISH) method to detect multiple genomic regions in single cells in complex tissues. As a "proof of principle" we applied the method to breast cancer samples to identify partners in whole arm (WA) translocations. WA gain of chromosome arm 1q and loss of chromosome arm 16q are among the most frequent genomic events in breast cancer. By designing five specific FISH probes based on breakpoint information from comparative genomic hybridization array (aCGH) profiles, we visualized chromosomal translocations in clinical samples at the single cell level. By analyzing aCGH data from 295 patients with breast carcinoma with known molecular subtype, we found concurrent WA gain of 1q and loss of 16q to be more frequent in luminal A tumors compared to other molecular subtypes. QM FISH applied to a subset of samples ($n = 26$) identified a derivative chromosome der(1;16)(q10;p10), a result of a centromere-close translocation between chromosome arms 1q and 16p. In addition, we observed that the distribution of cells with the translocation varied from sample to sample, some had a homogenous cell population while others displayed intratumor heterogeneity with cell-to-cell variation. Finally, for one tumor with both preinvasive and invasive components, the fraction of cells with translocation was lower and more heterogeneous in the preinvasive tumor cells compared to the cells in the invasive component.

INTRODUCTION

Genomic instability is a hallmark of cancer, demonstrated decades ago by cytogenetic analyses (Nowell and Hungerford, 1960; Hanahan and Weinberg, 2000). Since then, the technology has improved enormously, and a wealth of various structural chromosomal alterations has been identified in human cancers.

The majority of chromosomal defects in human carcinomas and hematopoietic cancers are due to chromosomal instability, resulting in different types of genomic structural alterations such as pericentromeric or centromeric breaks, inversions, translocations, deletions, and gains. Translocations can either be reciprocal with swapped portions of chromosomal arms resulting in two different derivative chromosomes or noneciprocal where transfer of chromosomal material between arms is unbalanced (Ferguson and Alt, 2001). If no genomic material is gained or lost, this is named balanced translocation, in contrast to unbalanced translocations where gain and/or loss of genetic material are observed.

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Hematological cancers often harbor only a few balanced translocations (Pihan and Doxsey, 2003; Draviam et al., 2004; Mitelman et al., 2007), while solid tumors frequently have more complex structural rearrangements. The detection of translocations by cytogenetic analysis are technically challenging due to the need of viable cells dividing in culture, and solid tumors are challenging to culture, and some do not seem to grow *in vitro* at all, making cytogenetic analysis impossible. New technology, such as next generation sequencing (NGS) reveals chromosomal abnormalities including translocations, but cannot reveal those occurring within large stretches of unspecific genomic content, such as centromere-close regions. In genome wide copy number (CN) analyses such as comparative genomic hybridization arrays (aCGH), chromosomal arms involved in unbalanced translocations are visualized as whole arm (WA) loss or gain. In breast cancer, the most frequent WA alterations found by aCGH are gain of 1q, 8q, and 16p and loss of 16q and 17p (Ried et al., 1995; Chin et al., 2006; Hicks et al., 2006; Jonsson et al., 2010; Russnes et al., 2010), the same chromosomal arms are found by cytogenetic studies to be involved in translocations in breast cancer cell lines (Dutrillaux et al., 1990; Kokalj-Vokac et al., 1993; Tsuda et al., 1997; Tsarouha et al., 1999; Kytola et al., 2000). WA 1q gain is a common alteration in breast cancer and is found by metaphase FISH and cytogenetic analysis to be caused by isochromosome formation or WA translocation with other chromosomal arms (Tsuda et al., 1997; Tirkkonen et al., 1998; Cummings et al., 2000). Chromosome arm 16p is found to be a translocation partner to 1q resulting in a derivative chromosome der(1;16)(q10;p10) and is assumed to be an early event in breast cancer progression, although pre malignant ductal carcinoma *in situ* (DCIS) has not yet been extensively studied (Dutrillaux et al., 1990; Pandis et al., 1992; Tirkkonen et al., 1998; Tsarouha et al., 1999; Cummings et al., 2000; Nordgard et al., 2008). Invasive grade I breast carcinomas have more frequently lost the WA of 16q, while in grade II and grade III tumors alterations of 16q are more complex (Cleton-Jansen et al., 2004; Roylance et al., 2006; Natrajan et al., 2009).

In a previous work we developed a bioinformatic tool called whole arm aberration index (WAAI) to objectively score and identify WA alterations using aCGH data. In a merged set of 595 patients, we found a nonrandom distribution of WA alterations, where 1q and 16q were overall the most frequently altered arms (Russnes et al., 2010). In contrast to cytogenetic analysis, array CGH data cannot reveal whether such alterations are due to a translocation or not.

In recent years, based on gene expression analyses, distinctive breast cancer subgroups, which differ with regard to biological characteristics and clinical outcome, have been identified (Perou et al., 2000; Sorlie et al., 2001; Chin et al., 2006). CN gains and losses affecting most chromosomal arms are associated with the basal-like subtype, while high-level DNA amplification is more frequent in the luminal B and HER2 enriched subtypes, while no or few CN alterations are traits of the normal-like subtype, and WA gains and losses are traits of the luminal A subtype (Perou et al., 2000; Sorlie et al., 2001; Bergamaschi et al., 2006; Chin et al., 2006). To our knowledge, the association between the presence of der(1;16)(q10;p10) and molecular subtype have not yet been investigated, and due to inaccessible cytogenetic analyses on breast tumors and limited read-length by NGS analysis, FISH is still the best way to recognize WA translocations and identify the partners involved.

In this study, we established an *in situ* method, quantitative multigene FISH (QM FISH) to detect structural rearrangements with multiple FISH probes at the single cell level, both for intact nuclei (imprints) as well as for archival tissue with intact morphology. By QM FISH, we investigated the relationship between molecular subtype, clinicopathological features, WA alterations of 1q and 16q and the translocation between the two chromosomes in tumors from a cohort of early stage breast cancer patients with molecular data available. Tissue available from one invasive tumor with a DCIS component made it possible to study the time of occurrence of the translocation for this particular case.

MATERIALS AND METHODS

Patient Cohorts and Samples

Fresh-frozen tissue and/or formalin-fixated paraffin-embedded (FFPE) tissue sections were available from 26 breast cancer samples collected from patients undergoing radical mastectomy at the Karolinska Institute between 1984 and 1991 (the WZ cohort) and between 1995 and 1997 at the Norwegian Radium Hospital (the Oslo1 cohort; MicMa). The MicMa cohort consists of primary operable breast carcinomas mainly of low stage (stage 1 and 2) with CN analysis (aCGH),

molecular subtype and clinical data available (Supporting Information Table 1) (Hicks et al., 2006; Naume et al., 2007; Russnes et al., 2010). In addition, paired end sequencing data from two previously published MicMa samples (Stephens et al., 2009) were available. Previously published aCGH data and molecular subtypes from 295 samples from three cohorts (MicMa: 110 samples [Hicks et al., 2006; Naume et al., 2007; Russnes et al., 2010), Ull: 73 samples (Russnes et al., 2010) and Chin-UCAM 112 samples (Chin et al., 2007)] were analyzed with regard to the WAAI score. aCGH data from the 140 samples in the WZ cohort (Hicks et al., 2006) was used to determine the chromosomal breakpoints. The raw and preprocessed data can be accessed from the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE8757 (Chin-UCAM), GSE20394 (Ull), and GSE19425 (MicMa and WZ).

The institutional and regional ethical boards in both countries approved use of tissue for this project (WZ; DNR 001–64 and MicMa: S97103).

Histopathological assessment of HE sections was performed for all cases (AZ and HGR), the preinvasive lesions were scored in accordance with the Van Nuys Classification (Silverstein et al., 1995). WA gain of 1q and WA loss of 16q was indicated by the WAAI score. As previously reported, a total of 295 patients had both WAAI score and molecular subtype available (Russnes et al., 2010). In short; the raw data from the aCGH analysis were preprocessed and segmented by the PCF (piecewise constant fit) algorithm and the WAAI was calculated. The WAAI score measures gains and losses of whole chromosomal arms, a cutoff value at ± 0.8 was defined, where chromosome arms with WAAI >0.8 and WAAI<-0.8 were assessed as "WA gain" and "WA loss," respectively (Russnes et al., 2010).

From the MicMa cohort, 23 samples with available imprints, aCGH data and molecular subtype were selected. From the WZ cohort, 1 sample with imprint and 2 samples with FFPE sections and aCGH data available were investigated. In addition, the FFPE WZ122 sample section contained normal, invasive and DCIS components.

Construction of Imprints from Fresh-Frozen Tumor Tissue and Control Slides

Imprints were constructed by lightly pressing a semifrozen piece of the tumor onto glass slides (Superfrost plus), followed by 10 min fixation in 1% formaldehyde (2.5% neutral formalin) with 100 mM phosphate buffer (pH 7.5) and 1 mM $MgCl_2$. The slides were rinsed in phosphate buffered saline (PBS) and fixated for 1 hr at 4°C in 70% ethanol/salt (70 ml absolute ethanol mixed with 30 ml 140 mM potassium acetate, 1mM $MgCl_2$). Sections were stored in 70% ethanol at 4°C over night, followed by long-term storage in 70% ethanol at −20°C. Human diploid fibroblast (HDF) cells (The Coriell Institute for Medical Research) were grown according to culture conditions recommended by the manufacturer. Cells were harvested after trypsination, washed and allowed to attach to Superfrost Plus slides at room temperature in a humidified chamber (12×10^3 cells/slide). After 2 hr, additional medium was added and the slides were incubated under coverslip at 37°C over night. Slides with cultured cells were fixed in the same way as imprints, except that 0.4% formaldehyde was used.

Identification of Breakpoints and Design and Production of Probes

As previously described, DNA from the MicMa and WZ cohorts were hybridized to the ROMA (The Representational Oligonucleotide Microarray Analysis) 85k microarray, developed at Cold Spring Harbor Laboratory (Sebat et al., 2004). Frequency plots of chromosome breakpoints on chromosome 1 and 16 were generated from 140 segmented aCGH profiles from the WZ cohort (Hicks et al., 2006) (three segmented profiles are shown in Supporting Information Fig. 1). As aCGH data cannot precisely identify breakpoint locations within the centromeric region due to lack of unique sequence information, breakpoints within the centromeric region are therefore displayed on either side of the flanking regions. For each tumor sample, chromosomal regions specific for each side of the breakpoints were defined by inspecting aCGH data. Using UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), bacterial artificial chromosomes (BAC) were selected to lie as close as possible to the breakpoints (list of the BAC probes used for each sample are listed in Supporting Information Table 2). To improve the FISH signal, 1–4 BAC probes with partially overlapping DNA sequences (in total

covering target region spanning from 320,000 bp to 735,000 bp) were selected, except for the centromeric probe for chromosome 16 and some tumor cases where one BAC gave strong enough signal. The BACs were purchased from RZPD (Deutsches Resourcenzentrum fur Genomforschung GmbH, Berlin) and ImaGenes (GmbH, Berlin) as inserts in plasmids in *Escherichia Coli*. Bacteria were grown according to the manufacturer instructions and the plasmid DNA was isolated using a plasmid maxikit (Qiagen). The quality of each FISH probe was tested on slides with cultured HDF cells (data not shown). Probes for FISH were made by labeling DNA isolated from BAC clones (individual or pooled BACs) with a modified standard protocol for nick translation (Maniatis et al., 1989) or a nick translation kit (Abbott). When making probes for FFPE sections and imprints, BAC DNA was cut with *EcoR1* prior to labeling according to a standard protocol (Maniatis et al., 1989). Fluorescence labeled nucleotides used for labeling were SpektrumGreen-, SpektrumOrange-, SpektrumRed-dUTP (Vysis); Green-dUTP (Enzo); Texas Red-dUTP (Molecular Probes); PromoFluor-415-aminoallyl-dUTP, Promofluor-555-dUTP, Promofluor-590-aminoallyl-dUTP, and Promofluor-647-aminoallyl-dUTP (PromoKine); HyPer5-dCTP (GE Healthcare). Labeled probes were mixed with human cotDNA, salmon sperm DNA and tRNA, precipitated with ethanol and subsequently dissolved in a hybridization buffer containing 50% formamide, 10% dextran sulphate, 2× SSC, 1% Tween 20 and 50mM phosphate buffer (pH 7) or Vysis CEP hybridization buffer (Abbott) to a final concentration of 4–8 ng/μl of each probe.

QM FISH on Imprints and Control Slides

Slides with imprints or cultured cells were washed in PBS, refixated in 0.4% formaldehyde for 10 min, washed in PBS followed by 0.1× PBS, dehydrated in graded ethanol and air-dried. Probemix was added and a coverslip was sealed with rubber cement. Probes and tissue were codenatured at 85–90°C for 5 min followed by hybridization over night at 47°C. Next day, slides were washed in 4× SSPE at 37–72°C for 10–20 min, dehydrated in ethanol, washed in hexan:isopropanol (60:40, v/v) for 10 min, isopropanol for 5 min and finally in absolute ethanol. Slides were briefly air-dried and mounted with Vectashield mounting media with DAPI (4′,6-diamino-2phenylindole, Vector).

QM FISH on FFPE Tissue Slides

Slides with FFPE sections were placed at 65°C for 1 hr to melt the wax before deparaffinization in xylene, and subsequently pretreated according to the citric acid buffer method described by Chin et al. (2003) with some minor modifications. Briefly, deparaffinized slides were treated in 0.2N HCl for 10 min at room temperature prior to citric acid treatment. After pepsin digestion, slides were refixated in 0.4% formaldehyde for 10 min, washed once in PBS, once in 0.1×PBS, dehydrated and air-dried before hybridization. Probes were added to air-dried slides and probes and tissue were codenaturated at 87°C for 10 min. Hybridization was carried out overnight at 47°C. After hybridization slides were washed in 4×SSPE for 10 min at 55°C, followed by 10 min at 63°C. After dehydration in a graded series of ethanol slides were washed in hexan:isopropanol and treated as described above.

Microscopy and Scoring Criteria

Evaluation of signals was performed in an epifluorescence microscope. Selected cells were photographed in a Zeiss Axioplan 2 microscope equipped with an Axio Cam MRM CCD camera and Axio Vision software. Twenty cells from different areas of the individual imprints were selected for thorough examination. As probes were placed on either side of the centromere, not fused signals but a gap between probes on normal or derivative chromosomes are to be expected. To score a case "positive" for translocation, the two probes had to be in the closest proximity of another probe, and the number of "positive" cells was counted (Supporting Information Table 3). Tumors with less than 10% of "positive" cells were regarded as negative. In cases with an obvious heterogeneity with regard to combinations or number of chromosome arms, this was commented on.

DATA Processing and Statistics

Data from aCGH of both the WZ and the MicMa cohort were imported into S-PLUS and normalized and segmented as described by Hicks et al. (2006). The processed data were then further analyzed for breakpoints using Matlab

TABLE 1. Whole Arm Alteration of Chromosome 1q and 16q Determined by WAAI Score with Regard to Molecular Subtypes

WAAI score	Luminal A ($n = 137$)	Luminal B ($n = 33$)	HER2-related ($n = 45$)	Basal-like ($n = 46$)	Normal-like ($n = 34$)
Whole arm gain of 1q only	42 (31%)	8 (24%)	8 (18%)	9 (20%)	4 (12%)
Whole arm loss of 16q only	18 (13%)	4(12%)	0 (0%)	5 (11%)	4 (12%)
Concurrent gain of whole arm 1q and loss of 16q	37 (27%)	3 (9%)	0 (0%)	1 (2%)	6 (18 %)
No whole arm gain of 1q or loss of 16q	40 (29%)	18 (55%)	37(82%)	31 (67%)	20 (59%)
Total	137 (100%)	33 (100%)	45 (100%)	46 (100%)	34 (100%)

$P < 0.001$, $n = 295$.

(Mathworks). For statistical analysis (Fisher's Exact test), SPSS 15.0 was used.

RESULTS

WA Gain of 1q and Loss of 16q Estimated by WAAI Using aCGH Data

From 295 tumor samples with WAAI score and molecular subtype available, 47 tumors had concurrent WA 1q gain and 16q loss, 71 samples had only WA 1q gain and 31 had only WA 16q loss, while 146 tumors did not have WA 1q gain or 16q loss. In combination with molecular subtype information we found 71% of the luminal A tumors to possess either one of the alterations or both compared to only 33% of the basal-like and only 18% of HER2-related tumors (Table 1). From the 47 samples with concurrent alterations, 37 (79%) were luminal A tumors. Importantly, concurrent gain of 1q and loss of 16q were seen in only one basal-like, 6 normal-like and 3 luminal B tumors. Interestingly, none of the HER2-related tumors had concurrent 1q gain and 16q loss.

QM FISH Analysis; Identification of der(1;16)(q10;p10)

The relative position of the centromeric breakpoints of chromosome 1 and 16 differs, by comparing the breakpoints of 140 WZ samples run on aCGH we found the most frequent breakpoints of chromosome 1 to lie close to or within the centromeric region (1p12-1q12), while the most frequent breakpoints of chromosome 16 were located close to the centromere on the p arm (16p11.2–16p11.1) (Supporting Information Fig. 2). To make sure all the probes lie as close to and not within the breakpoints, the probes were manually selected for each individual tumor investigated (Supporting Information Table 2).

QM FISH was performed on imprints from a total of 23 tumors from the MicMa cohort and 2 FFPE and 1 imprint sample from the WZ cohort. Based on the criteria outlined in the material and methods section, tumors were scored positive or negative for der(1;16)(q10;p10). Two MicMa samples, MicMa 300 and 373 had only 12 tumor cells analyzed due to few tumor cells present on the imprints, these two samples were thus excluded from further statistical analysis.

The molecular subtype and WAAI score is together with the WAAI results of with 1q and 16q and the average CN counted for each probe given in Supporting Information Table 3. An example of QM FISH analysis of a tumor displaying the translocation is given in Figure 1. By WAAI score the tumor had WA gain of 1q simultaneously to WA loss of 16q. Two separate experiments were performed with FISH probes selected on each side of the known breakpoints on chromosome 1 and 16 labeled in different color combinations (probe-mix 1 and probe-mix 2). The reference HDF cells displayed two normal copies of both chromosome 1 and 16 for both probe-mix 1 and probe-mix 2, this in contrast to the tumor cells where two normal copies of chromosome 1 but only one normal copy of chromosome 16 was observed. In addition, a colocalization of signals originating from the pericentromeric region of 1q and the pericentromeric region 16p was found identifying der(1;16)(q10;p10). To investigate the relationship between suspected translocation by WAAI score (i.e., concurrent 1q gain and 16q loss), imprints from both diploid and aneuploid invasive breast carcinomas from the MicMa cohort were used. As shown in Figure 2, a translocation was observed in both a diploid and a hyper-diploid tumor, the

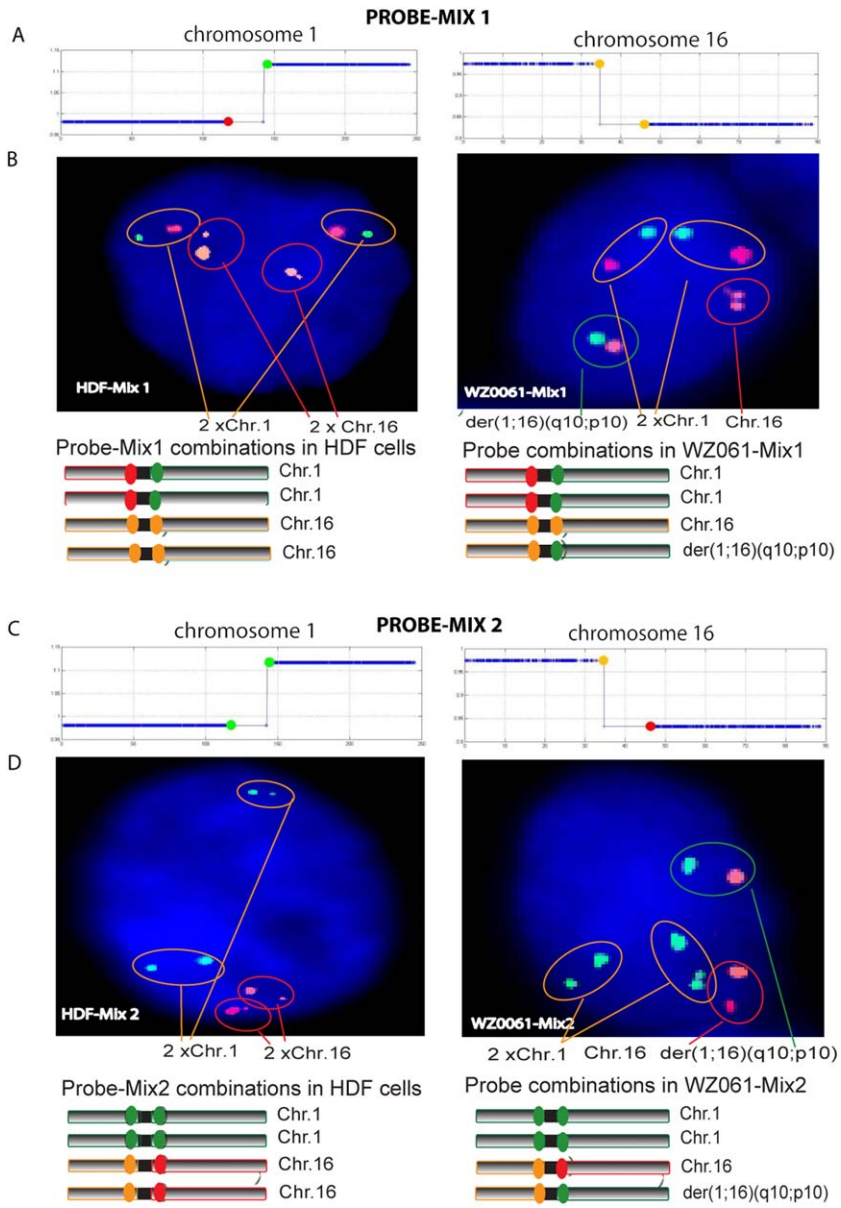

Figure 1. Selection of BAC probes for one tumor to identify der(1;16)(q10;p10). (A) aCGH profile of chromosomes 1 and 16 for sample WZ061 and the probe-mix 1 fluorescence combination. BAC probes were selected on each side of the breakpoints close to the centromeres, and labeled with different fluorescence (1p labeled with red, 1q labeled with green, and 16p and 16q were labeled with orange). (B) QM-FISH performed on HDF cells and sample WZ061 with probe-mix1. The first cell, HDF shows 2 intact copies of chromosome 1 and 16. Sample WZ061 and shows two copies of chromosome 1, one copy of chromosome 16, and one derivative chromosome der(1;16)(q10;p10). The chromosomal rearrangements for both cells are illustrated in the panel below. (C) Shows the aCGH profile of chromosome 1 and 16 for sample WZ061 and the probe-mix 2 fluorescence combinations. (1p and 1q labeled with green, 16p labeled with orange and 16q labeled with red). (D) QM-FISH with probe-mix 2 performed on HDF cells and sample WZ061.The HDF cell shows two copies of chromosome 1 and 16. The WZ061sample with probe-mix 2 shows two copies of chromosome 1, one copy of chromosome 16 and one derivative chromosome der(1;16)(q10;p10). The chromosomal rearrangement is illustrated in the panel below each cell.

latter (MicMa089) showed more complex alterations with one copy of 16p not involved in a translocation with 1q but probably with an unknown partner. Aneuploid tumors with multiple copies of chromosome 1 and 16 are difficult to evaluate, due to extra copies of the selected chromosomes and hence more probe signals, illustrated in Figure 3. This tumor (MicMa122) displayed a cell-to-cell variation in CN of normal chromosome 1 and 16 as well as for the derivative chromosome.

As shown in Table 2, the translocation was observed significantly more frequently in tumors with concurrent WAAI scores, of eight samples observed with translocation seven were of luminal

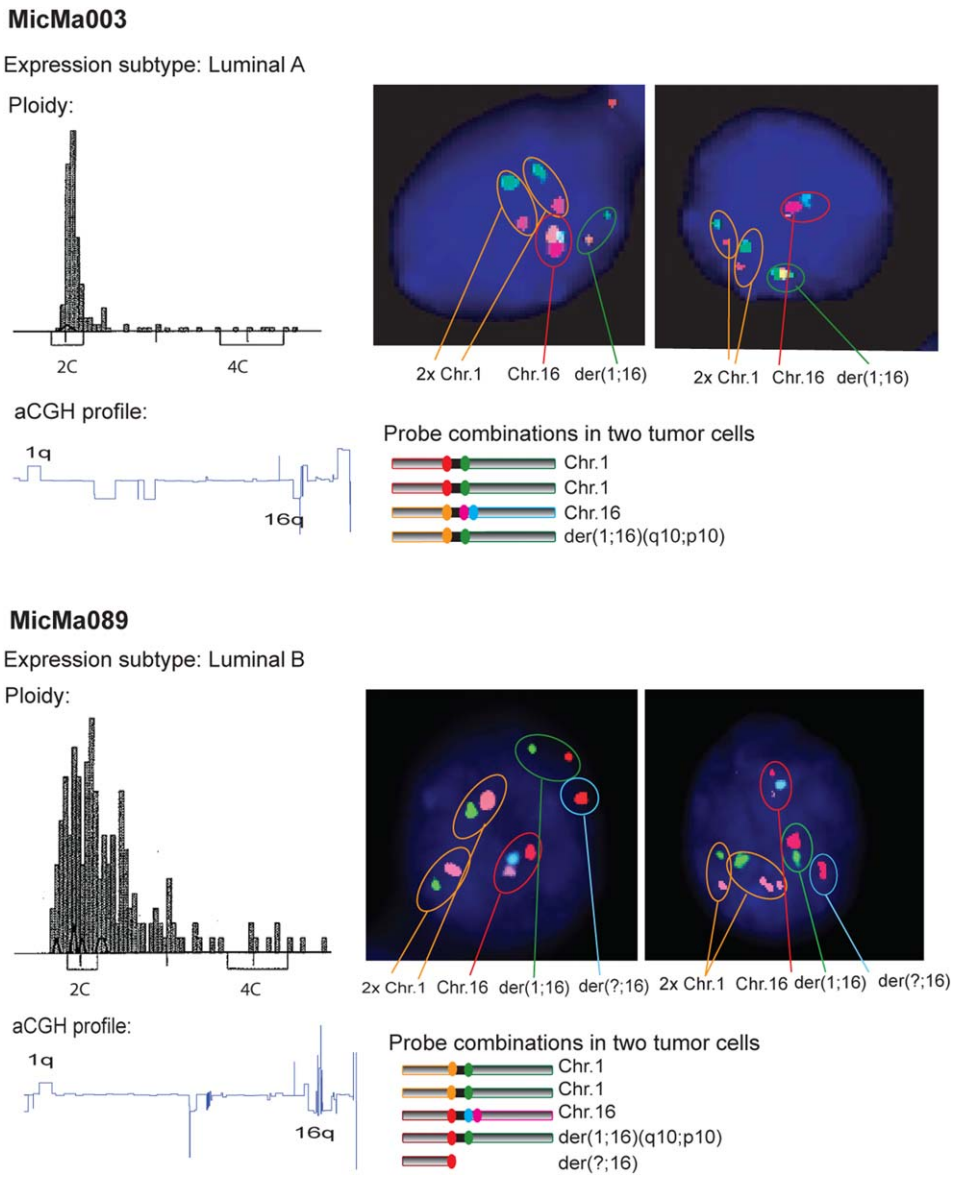

Figure 2. QM FISH with 1p, 1q, 16p, 16cent and 16q probes in a diploid and a hyper diploid tumor. MicMa003 (diploid) and MicMa089 (hyper diploid) with known ploidy, molecular subclass and aCGH profile have WA gain of 1q and loss of 16q seen both by WAAI score and by visual inspection of aCGH profiles. QM FISH performed on imprints from both tumors identified a derivative chromosome der(1;16)(q10;p10) as well as normal copies of chromosomes 1q and 16q from each tumor. Each combination of probes is encircled, (orange: chromosome 1, red: chromosome 16, green: der(1;16) and blue: single chromosomal arm with unknown partner). The schematic drawings under the images show suggested chromosome arm combinations in the two tumors.

A subtype and only one was luminal B (Table 3). A significant relationship with the presence of a the derivative chromosome der(1;16)(q10;p10) with ER and PgR status was also seen (Supporting Information Table 4).

QM FISH on FFPE Tissue Sections

The QM FISH method was modified and applied on FFPE tissue slides making it possible to investigate CN alterations and structural rearrangements with regard to morphology. The translocation resulting in der(1;16)(q10;p10) chromosomes were visualized in one FFPE invasive tumor samples from the WZ cohort (WZ055, Fig. 4). QM FISH analysis of WZ055 showed one normal copy of chromosome 1, one normal copy of chromosome 16 and one derivative chromosome der(1;16)(q10;p10). The QM-FISH method was applied to the WZ122 sample, which contains normal, preinvasive tumor (DCIS) and invasive tumor components (Fig. 5). The der(1;16)(q10;p10) was

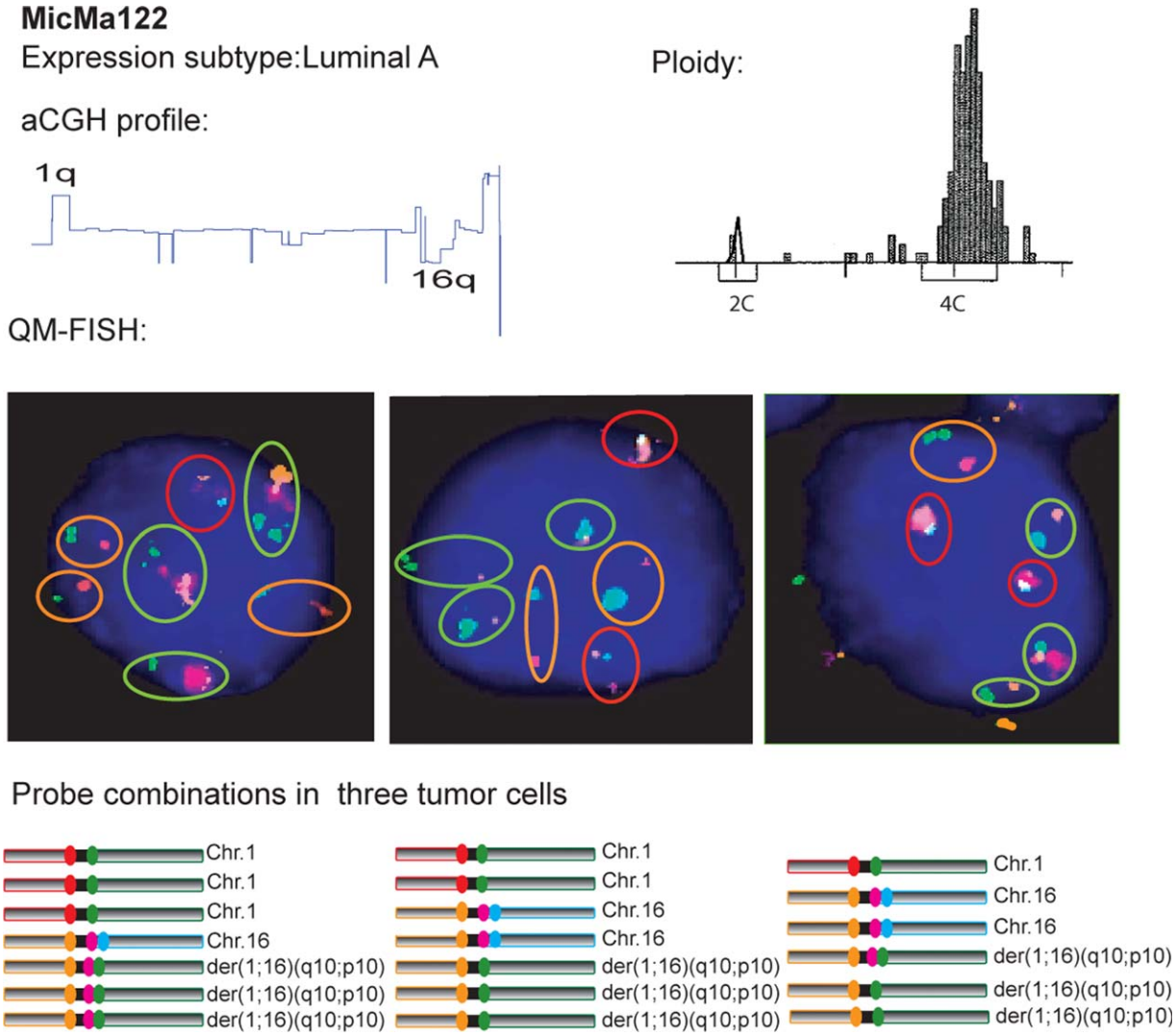

Figure 3. QM FISH with 1p, 1q, 16p, 16cent and 16q probes in an aneuploid tumor. MicMa122 (aneuploid) had WA gain of 1q and loss of 16q seen both by WAAI score and by visual inspection of aCGH profile. QM FISH on imprint from the sample showed that the combination of probes varied from cell to cell, visualized here with three cells where all had three copies of the derivative chromosome der(1;16)(q10;p10), but the number of normal combination of 1p/1q and 16p/16q varies. Each combination of probes is encircled (orange: chromosome 1, red: chromosome 16, green: der(1;16)(q10;p10)), below the QM FISH images schematic drawings of suggested chromosome arm combinations are displayed.

observed in the DCIS and invasive components, but as expected not in the normal tissue. Cells from four different areas from each tissue component were counted with regard to the 16p/16q CN ratio. The normal areas had a 16p/16q ratio ranging from 0.85 to 1, while the invasive areas ranged from 1.7 to 1.9. The four DCIS areas showed a higher variation ranging from 1.2 to 1.9 (Supporting Information Fig. 3A). The average 16p/16q ratio in the DCIS areas was 1.4, indicating the presence of two populations; otherwise the ratio should be closer to 1 or 2, as the DCIS also contains normal cells (Supporting Information Fig. 3B). The QM FISH analysis showed that cells were either genomic "normal" with intact chromosome 16 or had loss of 16q and that these two cell types were topographically interspersed (Supporting Information Fig. 3C).

DISCUSSION

Studies of genomic alterations of cancer genomes have until recently mainly been dependent on cytogenetic analysis, Sanger sequencing or array based analyses. For identification of structural alterations such as translocations, cytogenetic analyses have low resolution and are limited by the need of viable cells dividing *in vitro*. Array

TABLE 2. The Observation of der(1;16)(q10;p10) by QM FISH in Breast Carcinomas with Regard to WAAI Scores

	1q gain only (WAAI)	16q loss only (WAAI)	1q and 16q concomitant (WAAI)	No 1q or 16q (WAAI)
der(1;16)(q10;p10) observed ($n = 8$)	2/8 (25%)	0/8 (0%)	6/8 (75%)	0/8 (0%)
no der(1;16)(q10;p10) observed ($n = 13$)	2/13 (15%)	1/13 (8%)	2/13 (15%)	8/13 (62%)

$P = 0.005$ by Fisher's exact test, $n = 21$.

TABLE 3. The Observation of der(1;16)(q10;p10) by QM FISH with Regard to Molecular Subtype

	Luminal A	Luminal B	HER2-related	Basal-like
der(1;16)(q10;p10) observed ($n = 8$)	7/9 (78%)	1/4 (25%)	0/2 (0%)	0/6 (0%)
no der(1;16)(q10;p10) observed ($n = 13$)	2/9 (22%)	3/4 (75%)	2/2 (100%)	6/6 (100%)

$P = 0.006$ by Fisher's exact test, $n = 21$.

based techniques have only been able to detect CN alterations and to indicate breakpoints, but not to identify the partners involved, for instance in WA translocations. In this study, we have established and tested a method for interphase FISH using multiple probes on tumor imprints and paraffin sections to detect centromere close whole chromosome arms translocations. During the last decades, modern techniques such as NGS have revealed structural genomic alterations down to the single base level. As still most NGS technologies sequence shorter segments (hundreds of bases), detection of rearrangements close to centromeres are easily missed due to repetitive sequences that cannot be accurately mapped. This is illustrated by two of the MicMa samples in this study which previously were analyzed by paired-end sequencing (Stephens et al., 2009) and the results illustrated in genome-wide circos plots, displaying somatic rearrangements like insertion, deletions and translocations, but no translocation between chromosomes 1 and 16 was identified (Figure 6). However, QM-FISH performed on imprints from the same samples detected the translocation between chromosome 1 and 16 in multiple cells from both samples (Fig. 6). Cytogenetic as well as molecular analyses such as aCGH or single nucleotide polymorphism array (SNP arrays) have identified gain of WA of 1q and loss of 16q as common alterations both in invasive carcinomas and in pre malignant lesions (Chin et al., 2006; Pandis et al., 1992). Invasive lobular carcinomas (ILC) have for example lost the long arm of chromosome 16 (16q) in more than 60% of the cases, correlating with loss of E-cadherin expression, a protein important for cell adhesion and motility (Vos et al., 1997). The loss of 16q is also found in a substantial proportion of invasive ductal carcinomas, as well as in premalignant lesions such as columnar cell lesions, atypical ductal hyperplasia and DCIS (Simpson et al., 2005). A translocation resulting in a der(1;16)(q10;p10) is considered an early event in mammary carcinogenesis although it has not previously been seen in DCIS by cytogenetic analysis and FISH methods (Dutrillaux et al., 1990; Pandis et al., 1992; Tirkkonen et al., 1998; Tsarouha et al., 1999; Cummings et al., 2000). As DCIS often are small lesions and difficult to cultivate, *in situ* techniques are important for investigations of these lesions. By applying QM FISH on one case with both normal breast epithelium, DCIS and invasive areas in a FFPE tissue slide, we found der(1;16)(q10;p10) in the invasive part as well as in the DCIS region but not in the normal epithelium. Interestingly, we did not find the translocation in all DCIS cells and the cell-to-cell variation in the DCIS was much higher compared to invasive carcinoma (Fig. 5). Although only one case was analyzed, it is tempting to speculate the alteration to be associated with progression to invasive disease.

In aCGH data WA gain and/or loss is identifiable by visual inspection or by objective scores such as WAAI. We wanted to study the relationship between WA gain of 1q and loss of 16q with presence of a centromere close translocation. By WAAI we found WA alterations of 1q and/or 16q across all five molecular subtypes, but most frequently in luminal A and luminal B samples. Concurrent WA gain of 1q and WA 16q loss was most

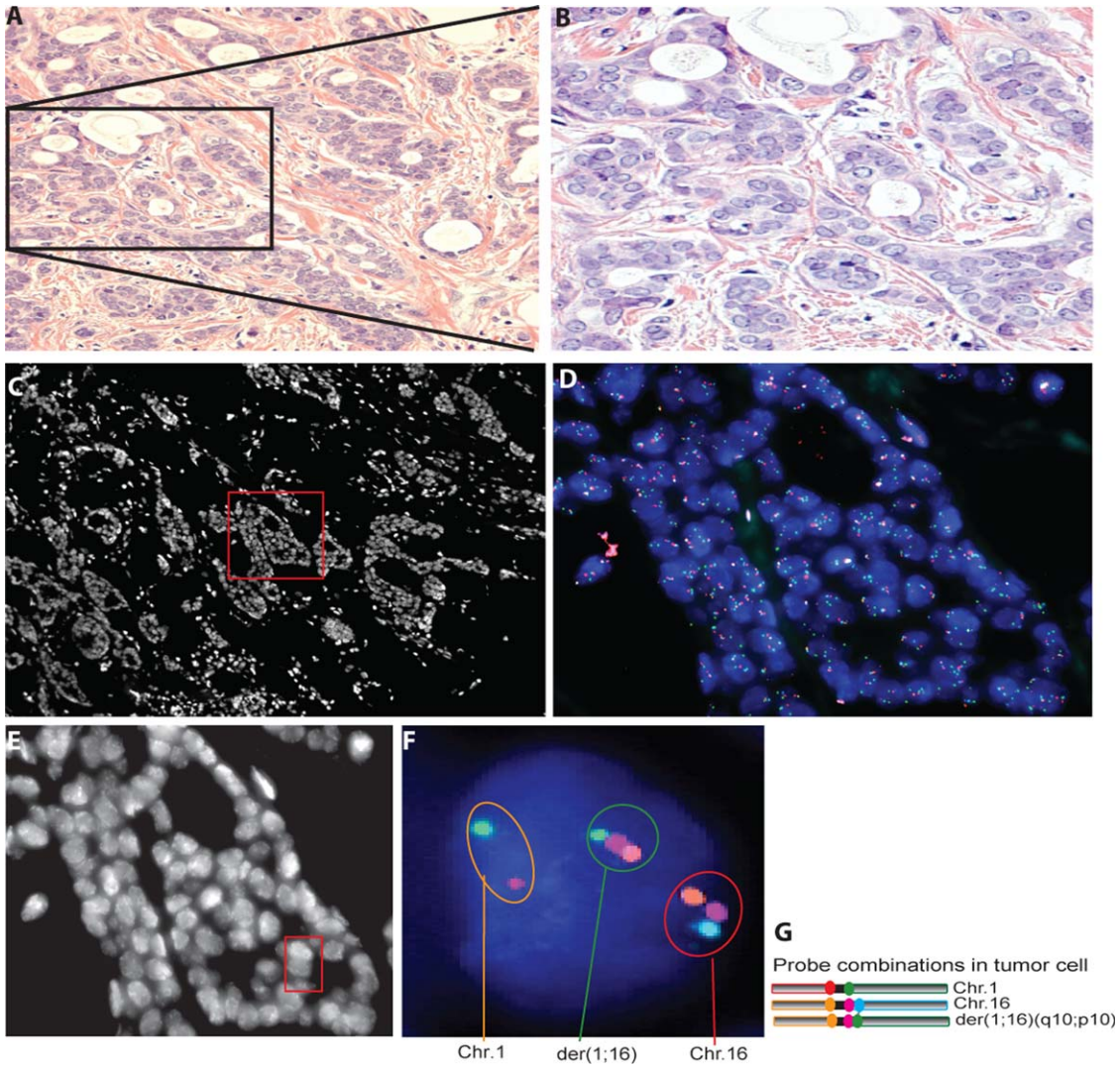

Figure 4. QM FISH on FFPE sections. QM FISH of invasive carcinoma cells in sample WZ055. (A) Shows the 20× magnification of the HE stained section (B) 40× magnification of the outlined area (C) 10× magnification of a DAPI stained area of the tumor (D) the outlined area from in 63× magnification in fluorescence staining. (E) Shows the outlined area from 63× magnification in DAPI. (F) example of a cell which shows one normal copy of chromosome 1, one normal copy of chromosome 16 and a derivative chromosome der(1;16)(q10;p10). A schematic drawing of suggested chromosome arm combinations is illustrated in (G).

frequent in luminal A tumors and very rarely observed in basal-like and HER2-related tumors, in line with the different molecular characteristics of these subtypes (Perou et al., 2000; Sorlie et al., 2001). Next, we used QM FISH to identify translocation between chromosoml arms 1q and 16p, and found a significant correlation between concurrent WAAI result of 1q and 16q from these two chromosomes and presence of a derivative chromosome der(1;16)(q10;p10). Among those with concurrent events, luminal A tumors were overrepresented compared to luminal B, basal-like and the HER2-enriched subtypes, and a centromere close translocation was mainly found in luminal A tumors. We also found a significant correlation between presence of der(1;16)(q10;p10) with ER and PgR status (Supporting Information Table 4), which was to be expected as these parameters are closely linked to molecular subtype (Supporting Information Table 4). These findings indicate that the molecular subclasses have distinct genomic alterations and follow specific paths in their carcinogenesis, probably initiated at the preinvasive stage.

This sample series is too small but the data indicate that a derivate chromosome due to a translocation between 1q and 16p is a common trait for the luminal A subtype.

Detecting centromere close translocation by QM FISH is time consuming mostly due to the

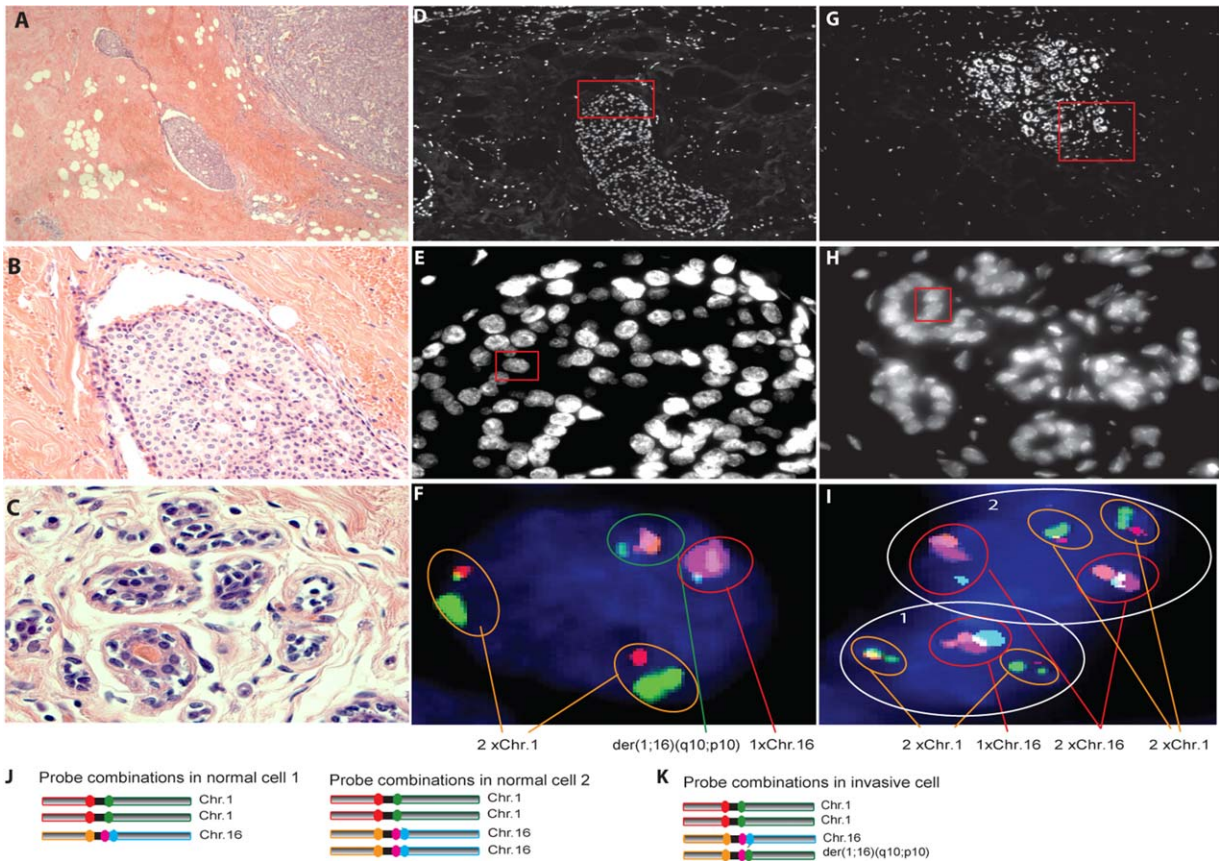

Figure 5. QM FISH of normal breast epithelium and DCIS in FFPE sections from invasive tumor. QM FISH on normal breast epithelium in FFPE tissue section from WZ122. (A) overview 5× image with invasive, DCIS and normal breast epithelium (HE). (B) 20× magnification of the DCIS area (HE). (C) 40× magnification of normal breast epithelium (HE). (D) 10× magnification DCIS area, DAPI. (E) 63× magnification of the outlined area (DAPI). (F) shows the outlined cell, showing two normal copies of chromosome 1, one normal copy of chromosome 16 but only one chromosome with der(1;16)(q10;p10). (G) 10× magnification of normal epithelium (DAPI). (H) outlined area in 63× magnification (I) outlined area in showing two cells, the upper cell (#2) with two intact copies of both chromosomes 1 and 16, and the lower cell (#1) with two copies of chromosome1 but, only one copy of chromosome 16, most likely due to allelic loss during sectioning. Schematic drawings of suggested chromosomal arm combinations are illustrated for the normal epithelium (J) and for cell in the DCIS area (K).

slight variation in breakpoint between different tumors and the need for customized probes. As shown in Supporting Information Figure 1, the position of the breakpoints detected by aCGH in tumors varied slightly, and the need for probes with close spatial proximity to the chromosomal breakpoints were crucial to distinguish translocated chromosomes from normal ones.

In four samples with concurrent WA events (MicMa 089, 122, 373, and 388) we could not detect the der(1;16)(q10:p10), this can be explained by translocations involving other chromosomal arms which were not investigated. Another plausible explanation is intratumor heterogeneity. Imprints for FISH are from a neighboring part of the piece used for DNA extraction for aCGH analysis and might represent a different clone. It is also important to note that less than 60 cells were examined per tumor, and an alteration present in only a small subpopulation would by chance be impossible to identify. In two samples (MicMa 220, 232) with der(1;16)(q10;p10), the WAAI score only indicated WA gain of 1q, but not 16q loss. As the WAAI scores are dependent on the log2 ratio of the aCGH data, tumors with a high degree of normal cell contamination are susceptible for false negative results of WAAI, and this can explain the discrepancy between the translocation seen and the WAAI score detected. In addition, samples with high genomic complexity probably due to aneuploidy had multiple signals and were visually challenging to score. Ploidy data were only available for some of the tumors analyzed as shown in Supporting Information Table 1. The pure diploid tumor of luminal A (MicMa003) type had less complex alterations

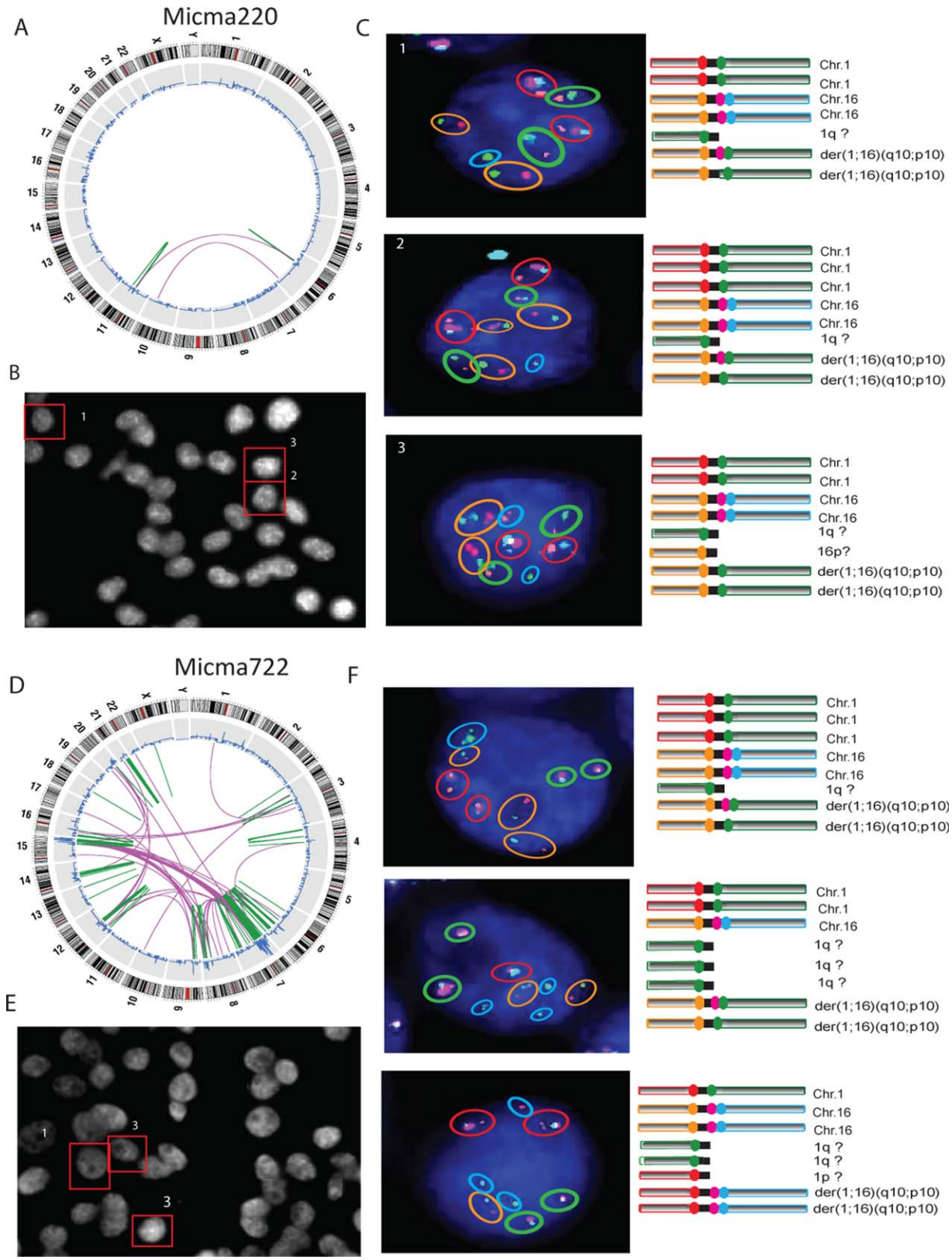

Figure 6. Centromere close translocations in paired-end sequencing data. Two MicMa samples (Micma 220, Micma 722) have been analyzed with paired-end sequencing, and the results are shown as circos plot. QM-FISH were performed with probes tailored to lie as close as possible to the centromeric breakpoint on chromosome 1 and 16 found from aCGH profiles. The circos plots did not indicate any translocation between chromosome 1 and 16, but this translocation was detected with QM-FISH probes labeled with 1p (red), 1q (green), 16p (orange), 16centromer (purple) and 16q (blue). (A) MicMa 220's circos plot (paired-end sequencing) (B) 63X overview image from the imprints with three outlined cells, (C) the outlined cells with schematic illustrations of chromosomal arm combinations. (D) MicMa722's circos plot (paired-end sequencing), (E) 63X overview image from the imprints with three outlined cells, (F) the outlined cells from with schematic illustrations of chromosomal arm combinations. The colored circles index chromosomes, with orange: chromosome 1, red: chromosome 16, green: der(1;16)(q10;p10) and blue is single chromosomal arm without known partner.

than the hyperdiploid, luminal B tumor (MicMa089) seen both by aCGH and by QM FISH analysis (Fig. 2).

In this work, we developed QM FISH, a QM *in situ* hybridization technique recognizing structural genomic alterations in intact cell nuclei in both imprints and FFPE tissue. The technique was used on breast carcinoma samples with detailed aCGH data as well as other molecular data available. We tailored multiple FISH probes close to the centromere on chromosome arms 1q and 16q and hybridized tissue slides with five different probes simultaneously, and found tumors with concurrent WA gain of chromosome arm 1q and loss of chromosome arm 16q by aCGH analysis were frequently of luminal A subtype and had a translocation resulting in a derivative chromosome der(1;16)(q10;p10). In one tumor, this translocation was also seen in DCIS cells close to the invasive part, but in contrast to the invasive tumor cells, DCIS cells with and without the translocation were intermingled. In summary, QM FISH is a method to evaluate CN variation as well as structural genomic rearrangements in a cell specific manner making it possible to investigate both intra tumor heterogeneity as well as tumor progression in both fresh and archival tissue samples.

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