Novel gene rearrangements in transformed breast cells identified by high-resolution breakpoint analysis of chromosomal aberrations

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Abstract

Chromosomal copy number alterations and chromosomal rearrangements are frequent mutations in human cancer. Unlike copy number alterations, little is known about the role and occurrence of chromosomal rearrangements in breast cancer. This may be due to the fact that chromosome-based breakpoint analysis is widely restricted to cultured cells. In order to identify gene rearrangements in breast cancer, we studied the chromosomal breakpoints in radiation-transformed epithelial breast cell lines using a high-resolution array-based approach using 1 Mb bacterial artificial chromosome (BAC) arrays. The breakpoints were further narrowed down by fluorescence *in situ* hybridisation (FISH) with clones from the 32 k BAC library. The analysis of the cell lines B42-11 and B42-16 revealed rearrangements of chromosomes 7, 8, 10 and 12. We identified the genes *Has2*, *Grid1*, *Ret*, *Cpm*, *Tbx3*, *Tbx5*, *Tuba1a*, *Wnt1* and *Arf3* within the breakpoint regions. Quantitative RT-PCR showed a deregulated expression of all of these candidate genes except for *Tbx5* and *Tbx3*. This is the first study demonstrating gene rearrangements and their deregulated mRNA expression in radiation-transformed breast cells. Since the gene rearrangements occurred in the transformed and tumourigenic cell lines only, it is likely that these were generated in conjunction with malignant transformation of the epithelial breast cells and therefore might reflect early molecular events in breast carcinogenesis. Initial studies indicate that these gene alterations are also found in sporadic breast cancers.

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Introduction

Chromosome rearrangement is a key mechanism for gene alteration and subsequent deregulation of cellular processes such as proliferation, apoptosis, genome stability, angiogenesis and motility, leading to malignant tumour development (Hanahan & Weinberg 2000, Albertson *et al*. 2003). Whereas in breast cancer, numerous studies on copy number alterations exist

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(Climent *et al*. 2007), there is only little knowledge on the role of chromosomal rearrangements in this type of cancer. This might be due to the fact that high-resolution chromosome analysis requires metaphase chromosomes from vital cells, which are not available from archived clinical samples. However, cell culture models are highly informative tools for the investigation of molecular aberrations (Riches *et al*. 1997, Zitzelsberger *et al*. 2001, Climent *et al*. 2007), which moreover can be used for the functional assessment of candidate genes (Neve *et al*. 2006). This study supports the hypothesis that gene rearrangements observed in radiation-transformed breast cell lines partly mirror those occurring in primary breast cancer. In order to identify such gene rearrangements, we made use of an experimental approach, which combines the detection of chromosomal breakpoints by cytogenetic techniques, high-throughput isolation of aberrant chromosomes using fluorescence-activated chromosome sorting (FACS) and high-resolution mapping of chromosomal breakpoints using 1 Mb bacterial artificial chromosome (BAC) arrays. This approach has already been applied in both cancer and standard clinical cytogenetics (Darai-Ramqvist *et al*. 2006, Neve *et al*. 2006, Backx *et al*. 2007).

In this study, we present a comprehensive analysis of chromosome rearrangements in two radiation-transformed human epithelial breast cell lines showing a tumourigenic phenotype. The identification of rearranged candidate genes by chromosome breakpoint analysis in transformed breast cells and their altered gene expression point to an involvement of these gene rearrangements in the development of breast cancer.

Material and methods

Cell lines

Primary cultures of human mammary epithelial cells were established from surgically removed tissue samples from regions remote from the tumour in a patient with breast cancer presenting for mastectomy (Romanov *et al*. 2001). The cells were grown in mammary epithelial cell growth medium (MEGM) with additives (Lonza, Basel, Switzerland) and immortalised by transduction with the human telomerase catalytic subunit (hTERT). The hTERT gene was cloned into the retroviral vector (pBABE-puro), and supernatants from the packaging cell line ψCRIP were used to transduce the mammary epithelial cells (Wyllie *et al*. 2000). A cloned cell line B42-htLG was established following selection with puromycin and subsequently exposed to 20 fractions of 2 Gy gamma irradiation and screened for anchorage-independent growth in soft agar (0.3%). In contrast to the irradiated cell line, the parent line B42 does not produce significant numbers of anchorage-independent colonies. Cloned cell lines were established from individual anchorage-independent colonies derived from the irradiated parent cells, from which two cell lines (B42-11 and B42-16) were used for the current experiments. The cell lines were tested for tumourigenicity by s.c. injection into athymic nude mice, which were subsequently monitored for the induction of tumour growth.

Spectral karyotyping

Metaphase preparations were generated from the radiation-transformed cell lines and from the non-tumourigenic parent cell line B42-htLG according to standard procedures (Zitzelsberger *et al*. 2001). Spectral karyotyping (SKY) analysis was performed as described previously (Zitzelsberger *et al*. 1999).

Chromosome sorting

Metaphase chromosomes from cell lines B42-11 and B42-16 were prepared according to standard flow sorting protocols (Ferguson-Smith 1997) with optimised buffers (Ng & Carter 2006).

Chromosome sorting was performed on a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with two water-cooled lasers (Innova 300 and Innova 70, Coherent, Santa Clara, CA, USA) tuned at multi-line u.v. (330–360 nm) to excite Hoechst 33258 and chromomycin A3 (457 nm). Sorting speed was ∼10 chromosomes/s using a 70-μm nozzle tip. For flow karyogram analysis and gating the programme, CellQuest (Becton Dickinson) was used. For each rearrangement, about 500 chromosomes were sorted into PCR tubes.

Amplification of genomic chromosomal DNA

DNA from chromosomes was amplified using the single cell WGA kit (Sigma) according to the manufacturer's protocol. DNA purity and quantity were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Hybridisation of amplified DNA to metaphase spreads

One microgram of WGA amplification product was labelled with digoxigenin by nick translation, hybridised to normal human metaphase spreads and detected using Cy3-conjugated anti-digoxigenin. FISH signals

were analysed using an epifluorescence microscope (AxioVision, Zeiss) and captured using the FISHView software (Applied Spectral Imaging, Neckarhausen, Germany).

Array painting

DNA from aberrant chromosomes (M11-1, M16-1 to -5; Table 1) was hybridised onto 1 Mb BAC arrays according to Fiegler *et al*. (2003) in an automated hybridisation and washing station (HS400, Tecan, Männedorf, Switzerland). Slides were scanned using a GenePix Personal 4100A array scanner (Molecular Devices, Sunnyvale, CA, USA), and feature intensities were extracted using the GenePixPro 6.0 software (Molecular Devices). The datasets were normalised using the MANOR package (Neuvial *et al*. 2006) and segmented using the DNAcopy package (Venkatraman & Olshen 2007).

Confirmatory fluorescence *in situ* hybridisation (FISH) analysis of array-painting results

DNA from BAC clones spanning or flanking the chromosomal breakpoints was labelled either with biotin or with digoxigenin by nick translation according to standard protocols. Hybridisation to metaphase spreads of the tumourigenic cell lines was performed as previously described (Zitzelsberger *et al*. 2001).

Array CGH

DNA was extracted from fresh cultured cells (B42-11, B42-16 and B42-htLG) using the DNA mini kit (Qiagen). Totally, 450 ng male genomic reference DNA (Promega) and 450 ng cell line DNA were labelled and co-precipitated with 135 μg Cot-1 DNA (Roche) prior to hybridisation. Hybridisation and washing were performed as described above.

Data were normalised and segmented using the R-package MANOR (Neuvial *et al*. 2006) and DNAcopy (Venkatraman & Olshen 2007).

Cloning of fusion products using rapid amplification of cDNA ends

In order to analyse the rearranged sequences of *Has2* and *Grid1*, rapid amplification of cDNA ends (RACE)-PCR (Frohman *et al*. 1988) was performed. The reactions were carried out according to the manufacturer's protocols using the 3′ and 5′ RACE systems for RACEs (Invitrogen). Gene-specific 'nested' primers binding within exons 1 and 2 of the gene *Has2* and exon 2 of gene *Grid1* were used for 3′ RACE analysis, and primers binding within exons 3 and 4 of the gene *Grid1* and exon 3 of the gene *Has2* were used for 5′ RACE analysis.

The amplification products were cloned into a TOPO cloning vector (Invitrogen) according to the manufacturer's protocol. Transfected clones were checked for inserted DNA using PCR with M13 forward and reverse primers. Clones showing PCR amplicons of the expected size were grown for 14 h at 37 °C in LB medium (ampicillin) followed by a DNA extraction using the Nucleo Plasmid kit (Macherey-Nagel, Düren, Germany). The plasmid DNA was amplified using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Fosters City, CA, USA) and analysed using an ABI3730 sequencer (Applied Biosystems). DNA sequences were aligned to genomic and cDNA sequences gathered from the Ensembl database using the NTI Vector software (Invitrogen).

RNA isolation and reverse transcription

RNA was isolated from $\sim 5 \times 10^4$ cells by phase separation using Trizol (Invitrogen) and residual DNA was digested using the Turbo DNA-free kit (Ambion, Austin, TX, USA). Five micrograms of total RNA was

Table 1 Summary of marker chromosomes in cell lines B42-11 and B42-16 detected by spectral karyotyping (SKY) and array painting

Cell line	Marker	**Cytogenetic description** (SKY)	**BAC-based description** (array painting with 1 Mb BAC clone set)
B42-11	M11-1	der(7)(7pter→q11.1::10q11.2→qter)	der(7)(7pter→RP5-905H7::RP11-38B21→qter)
	M16-1	der(4)(4pter→4q31.3::12q24.2→qter)	der(4)(4pter→RP11-28E24::RP11-8A1→qter)
B42-16	M16-2	der(8)(8pter→q24.1::10q23.2→qter)	der(8)(8pter→RP11-115M9::RP11-396M20→qter)
	M16-3	der(10)(12q13.1→q15::10p11.2→q23.2:8q24.1→qter)	der(10)(RP11-89H19→RP11-444B24::RP13-445N5→RP11-113E21::RP11-96B2→qter)
	M16-4	der(12)(12pter→12q13.1::12q15→q24.2::10p12.33→p11.2::8q11.21→q24.1ter)	der(12)(12pter→RP3-417E16::Cancer_3A4→ →RP1-46F2::RP11-16O1→CTB-164I22::RP11-115M9RP11-567J20→qter)

Table 2 mRNA expression levels measured with real-time RT-PCR

Gene	Relative mRNA expression		Localisation	Genomic position	Description[a]	Tumourigenesis-related function	Reference
	B42-11	B42-16					
Has2	2.1	60.8	8q24.13	8:122694719-122722811	Hyaluronan synthase 2	Promotes tumour progression	Udabage *et al.* (2005*a*)
Grid1	31.6	1.1	10q23.2	10:87351731-88116230	Glutamate receptor delta-1 subunit precursor (GluR delta-1)	Promotes tumour progression	Haas *et al.* (2005)
Wnt1	No expression	Expression	12q13.12	12:47658503-47662746	Proto-oncogene protein Wnt-1 precursor	Proto-oncogene	Ozaki *et al.* (2005)
Arf3	0.8	1.1	12q13.12	12:47616259-47637577	ADP-ribosylation factor 3	Unknown	Hirai *et al.* (1996)
Cpm	0.9	1.4	12q15	12:67531227-67612891	Carboxypeptidase M precursor	Unknown	Pessoa *et al.* (2002)
Tbx3	0.9	0.8	12q24.21	12:67531227-67612891	T-box transcription factor TBX3	Promotes tumour growth	Platonova *et al.* (2007)
Tbx5	Expression	No expression	12q24.21	12:113333477-113333712	T-box transcription factor TBX5	Inhibits cell growth	Hatcher *et al.* (2001)
Ret	Expression	Expression	10q11.21	10: 42892528-42945805	Ret proto-oncogene	Receptor tyrosine kinase	Takahashi *et al.* (1988)

[a]http://www.ensembl.org.

reverse transcribed using the Superscript II kit (Invitrogen), and the resulting cDNA was directly used for RT-PCR.

Real-time RT-PCR

We used commercial exon-spanning Taqman probe sets (Applied Biosystems) for the genes *Has2*, *Grid1*, *Wnt1*, *Arf3*, *Cpm*, *Tbx3*, *Tbx5* and *β-actin* (reference gene). Reactions (25 µl) were carried out in triplicates and under standard cycling conditions (40 cycles) in an ABI7300 real-time PCR thermocycler (Applied Biosystems) in combination with the analysis software SDS (Applied Biosystems). Probe sets for exons 10–11 and 12–13 of the *Ret* gene were used as previously described (Rhoden *et al.* 2004).

Relative quantitation was performed using the $\Delta\Delta C$(t) method (Livak & Schmittgen 2001). $\Delta - C_t$ values were calculated relative to the C_t of β-actin and C_t values in the non-transformed B42-htLG (calibrator cell line). If genes were not expressed in the calibrator cell line, expressions are given qualitatively (Table 2).

Validation of gene alterations of Has2, Ret and Grid 1 by FISH analysis of a human breast cancer tissue array

A human breast cancer tissue microarray (TMA, BRC1501, Pantomics Inc., San Francisco, CA, USA) was hybridised with the following YAC and BAC FISH probes: RP11-115N9/RP11-711N15 (*Has2* gene), RP11-369M20/RP11-711N15 (*Grid1* gene) and CEPH-YAC clones 313F4/214H10/344H4 (*Ret* gene). The TMA comprised 5-µm FFPE sections (in duplicate) of 70 mostly high-grade breast cancers. Hybridisation and analysis of the TMA were performed as described earlier (Unger *et al.* 2004). For evaluation, fused and separated FISH signals were scored in each cell to detect gene rearrangements (split signals), the wild-type gene (fused signals) or altered copy number (increased or decreased number of FISH signals). FISH signals were counted as split when the distance between the red and the green signal was >10 µm.

In silico validation of candidate genes in a public available gene expression set

In order to validate gene expressions of the found candidate genes, a publicly available gene expression set GSE3494 (Miller *et al.* 2005) was downloaded from the Gene Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). The cel files that resulted from Affymetrix U133A and U133B Genechip hybridisations were normalised separately using the

R-package gcrma (http://www.bioconductor.org/packages/2.0/bioc/html/gcrma.html), combined and clinical data attached. After subsetting data according to clinically meaningful groups (grade, nodal status and estrogen receptor (ER) status) expression values (of candidate genes *Has2*, *Grid1*, *Ret*) were tested group by group for significantly different levels of expression either using Mann–Whitney test in case of testing two groups or using Kruskal–Wallis test in case of testing three groups. The test result was significant when the *P* value was within the 95% confidence interval. Plots of expressions along with test results are given in the Supplementary data file, see section on supplementary data.

Results

We have identified chromosomal rearrangements and copy number alterations in the radiation-transformed breast cell lines B42-11 and B42-16 producing small tumours in nude mice (tumour induction in each of the three mice tested per cell line). No tumours developed from cells of the parent cell line B42-htLG in any of the eight nude mice tested indicating a tumourigenic phenotype for the radiation-transformed cell lines. Copy number changes were detected using 1 Mb BAC arrays, whilst the chromosomal breakpoints were fine-mapped and confirmed by FISH and mRNA expression levels of the resulting candidate genes were measured by quantitative RT-PCR. Further, we cloned the rearrangement products of the genes Has2 and Grid1 using RACE.

Isolation of marker chromosomes

All markers of the cell lines B42-11 and B42-16 identified by SKY (Fig. 1, cytogentic description of cell lines is given in Supplementary data, see section on supplementary data, section 2) were successfully isolated by FACS. A cytogenetic description and a schematic illustration of the marker chromosomes are

Figure 1 Structural aberrations in the radiation-transformed cell lines B42-11 and B42-16. (A) Marker chromosomes (indicated by red arrows) identified by spectral karyotyping (SKY) in the cell line B42-11 (upper panel) and B42-16 (lower panel). B42-11 shows one marker chromosome M11-1 (mar(7)) and B42-16 marker chromosomes M16-1 (mar(4)), M16-2 (i(8)(q)), M16-3 (mar(10)) and M16-4 (mar(12)). Schematic illustration of chromosomal rearrangements in B42-11 demonstrating a translocation (B) and in B42-16 exhibiting complex exchange aberrations (C). Thin black lines with cytogenetic band designation indicate positions of chromosomal breakpoints. Chromosomal fragments indicated by a red cross are deleted in the cell lines. Blue lines indicate exchange processes between chromosomes at the marked breakpoints. Grey small boxes indicate candidate genes, which may be affected by the chromosomal rearrangement, whilst red arrows indicate elevated mRNA expression. (D) Marker chromosomes of cell lines B42-11 and B42-16 drawn in 500 band resolution according to their ISCN cytogenetic description using the CyDAS online analysis site (http://www.cydas.org/OnlineAnalysis, Hiller B, Bradtke J, Balz H & Rieder H 2005).

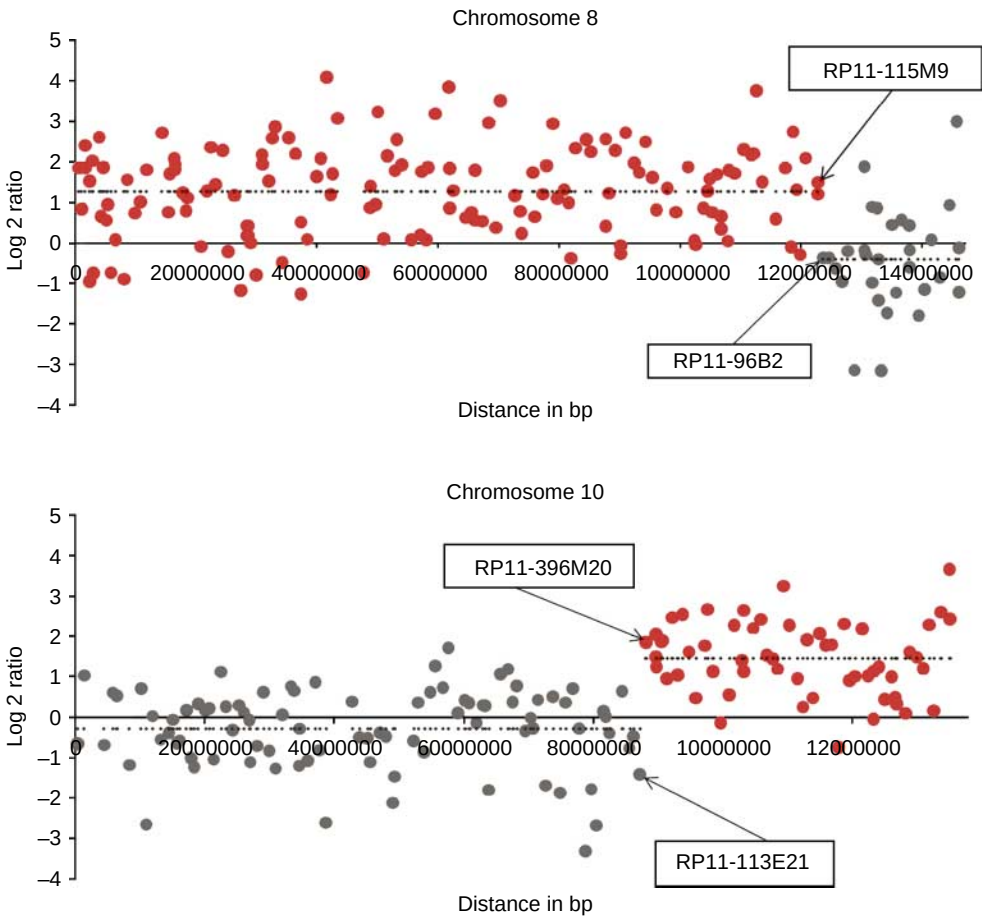

Figure 2 Array-painting analysis of marker M16-2 (t(8;10)) from cell line B42-16. Array profiles indicate hybridisation signals of the marker chromosome (green dots). BAC clones flanking the breakpoint region are indicated by arrows.

given in Table 1. The DNA of sorted chromosomes was amplified and subsequently FISH hybridised to normal human metaphase spreads in order to verify the origin of the sorted chromosomes. Regions of the metaphase chromosomes showing a hybridisation signal represent the chromosomal composition of the isolated marker chromosomes (data not shown).

Array painting and identification of candidate genes

In total, six marker chromosomes were cytogenetically characterised by flow-sorting and hybridised to 1 Mb BAC arrays (array painting). An exemplary illustration of such an analysis is given in Fig. 2, while Table 1 summarises the results of the array-painting analyses. The region in between breakpoint-flanking BAC clones (given in Table 1) identified the candidate genes *Has2* (chromosome 8), *Grid1*, *Ret* (both chromosome 10), *Cpm*, *Tbx3/5*, *Wnt1* and *Arf3* (chromosome 12). These were further investigated by quantitative RT-PCR in order to detect aberrant mRNA expression levels. All chromosome rearrangements were fine-mapped and verified by FISH with BAC clones (Supplementary data, see section on supplementary data) on metaphase preparations of the cell lines. This analysis also revealed the deletion of a small region comprising the gene *Tbx5*, what explains the absent mRNA expression of *Tbx5* (Table 2) in the cell line B42-16. A schema showing array-painting results and illustrating exchange processes in both radiation-transformed cell lines is given in Fig. 1.

Array CGH analysis

All cell lines have been investigated for copy number changes by array CGH using 1 Mb BAC arrays (Table 3). In total, gains on chromosomes 7 and 8 and a loss on chromosome 10 were identified in

Table 3 Summary of array CGH results

Chromosome	Region start (clone name)	Region end (clone name)	Region start (bp)	Region end (bp)	Region start (cytogenetic band)	Region end (cytogenetic band)	Size (Mb)
B42-11							
Gains							
7	CTB-164D18	RP4-725G10	178219	56275548	7p22	7p11.2	56.1
8	RP11-338B22	CTD-2115H11	477653	43438715	8p23.3	8p11.1	42.9
Losses							
8	RP11-114M5	RP11-24H3	59452746	99366374	8q12.1	8q22.2	39.9
10	CTC-306F7	RP11-124O11	249607	42829196	10p15	10q11.2	42.6
B42-16							
Gains							
8	RP11-45M12	RP11-263C6	3889636	29104558	8p23	8p12	25.2
8	RP11-350F16	CTC-489D14	47816618	146028921	8p11.1	8q24.3	98.2
12	RP11-438N16	CTC-221K18	112653797	132282931	12q24.1	12q24.3	19.6
13	RP11-264F20	RP11-245B11	46538919	113940806	13q14	13q34	67.4
X	RP11-457M7	RP5-966K21	2789811	57960019	Xp22.33	Xp11.21	55.2
Losses							
4	RP11-164P12	CTC-963K6	191030215	191076731	4q31.3	4q35.2	38.3
10	CTC-306F7	RP11-37P5	249607	16206799	10p15	10p13	16.0
X	RP13-34C21	RP11-218L14	62268206	154713754	Xcen	Xqter	92.4

B42-11. In B42-16, gains on chromosomes 8, 12 and 13 and losses on chromosomes 4, 10 and X were detected. An isochromosome 8q, which was already seen in SKY, became apparent in the cell lines B42-11, B42-16 and the parent cell line B42-htLG.

Quantitative RT-PCR

Transcriptional expression of the candidate genes *Has2*, *Grid1*, *Wnt1*, *Arf3*, *Cpm*, *Tbx3*, *Tbx5* and *Ret* was determined by quantitative real-time PCR (Table 2). *Ret*, which usually is not expressed in epithelial breast cells (Esseghir *et al*. 2007), was clearly overexpressed in both cell lines, B42-11 and B42-16. Also *Has2* and *Grid1* were overexpressed in B42-11 and B42-16 relative to B42-htLG. Expression of the gene *Wnt1* was not seen at all in B42-11, whereas in B42-16 it was detected. A slight overexpression of *Cpm* was found in B42-16, whilst in the same cell line *Tbx5* was not expressed at all (Table 2).

Cloning of aberrant gene products

In order to identify the fusion partners of the rearranged genes *Has2* and *Grid1*, we used RACE PCR followed by sequencing of the resulting amplification products. With respect to the breakpoints of chromosomes 8 and 10, it turned out that a part (exons 3–16) of the gene *Grid1* (10q23.1-23.2) was fused to an intronic sequence of the gene *Zhx2* (8q24.13), which is located ~1 Mb distal to *Has2* (*8q24.13*). RACE amplification of *Has2* revealed a breakpoint between exons 2 and 3 of the gene, but it was not possible to map the fusion sequence to a particular gene. However, neither 5′ RACE using gene-specific primers binding within exons 1 and 2 of *Has2* nor 3′-RACE using primers specific for sequences of exons 1 and 2 of *Grid1* resulted in successful amplification.

TMA analysis

The prevalence of rearrangements of the genes *Grid1*, *Has2* and *Ret* was determined by FISH using breakpoint-specific probes on a TMA comprising 70 mostly high-grade invasive ductal breast cancer cases. Every section (duplicate cores) was scored for separated (rearranged) FISH signals (Fig. 3). Twenty-eight percentage (19 out of 68 cases) of cases showed alteration of *Has2* (rearrangement or copy number amplification), 15% (10 out of 67) rearrangement of *Grid1* and 13% (8 out of 62) rearrangement of *Ret* (Table 4).

In silico validation of candidate genes in a public available gene expression set

The dataset was stratified according to grade (Bloom-Richardson), nodal status and ER status. Group-wise gene expressions of the genes *Ret*, *Has2* and *Grid1* were statistically tested.

Expression of *Has2* was significantly increased in grade 3 cases compared with grade 1 and 2 cases (*P* value: 0.035). *Grid1* showed elevated expression in grade 3 compared with low-grade (grade 1 and grade 2 cases combined, *P* value: 0.03) and in node-positive

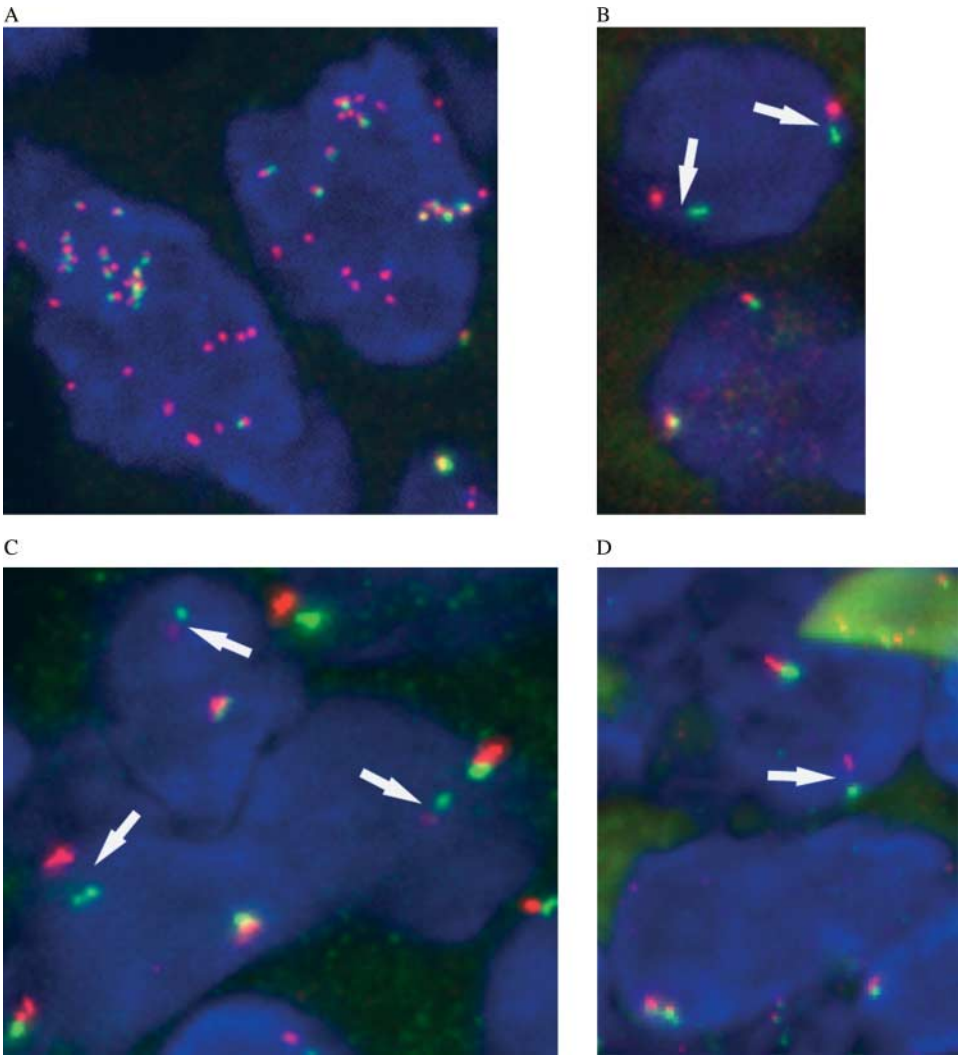

Figure 3 Fluorescence *in situ* hybridisation on a FFPE breast cancer tissue array. FISH probes (BACs RP11-115M9, red and RP11-497O14, green) flanking the chromosomal breakpoint of *Has2* in B42-16 were hybridised onto FFPE breast cancer tissues. (A) Two polyploid cells of a breast cancer specimen with multiple red FISH signals representing a high level amplification. (B) Two breast cancer cells from which the upper exhibits a rearrangement of *Has2* (split red and green signals, indicated by white arrow) and the lower shows a normal hybridisation pattern (fused signals). (C) A rearrangement of grid1 is demonstrated by split FISH signals (indicated by white arrow) in a breast cancer cell. (D) A rearrangement of the ret locus is indicated by split FISH signals (indicated by white arrow) in a breast cancer cell.

grade 1/2 compared with node-negative grade 1/2 cases (*P* values: 0.007 and 0.03). Expression levels of the *Ret* gene were significantly higher (*P* value: 0.04) in ER-positive cases compared with ER-negative cases. Plots of results are given in Supplementary data, see section on supplementary data (section 1).

Discussion

A combined approach of high-resolution breakpoint analysis of marker chromosomes, PCR-based characterisation of chromosomal breakpoints and FISH was used to investigate chromosomal rearrangements and changes in expression of resulting candidate genes in two radiation-transformed cell lines (B42-11 and B42-16). A significant prevalence of the alterations which we identified from an *in vitro* model was demonstrated by FISH on a set of clinical breast cancers. Furthermore, we could show by *in silico* expression analysis that the genes *Has2*, *Grid1* and *Ret* are differentially expressed in clinically relevant groups of breast cancers.

The parent cell line B42-htLG did not induce tumours in nude mice and showed a stable karyotype 47,XX,i(8)(q). The isochromosome 8q has already been reported to be associated with an immortal phenotype

Table 4 Summary of interphase FISH results on a breast cancer tissue microarray (TMA)

Gene	BAC/YAC clones used as DNA probes	Nr. of tumour tissues analysed	Nr. of cases with gene rearrangement (%)	**Cases with gene alteration** (position on array)
Grid1	RP11-369M20 RP11-711N15	67	10 (15)	A6$^{CIS/HER}$, C9$^{ID/AR/HER}$, E4$^{ID/AR/ER/PR}$, E6$^{ID/ER/HER}$, E14$^{ID/HER}$, G9$^{ID/HER}$, G11^{ID}, G15$^{ID/AR/ER}$, I6$^{IL/HER}$, I7$^{ID/M}$
Has2	RP11-115M9 RP11-497O14	68	19* (28)	A6$^{CIS/HER/*}$, A9$^{ID/ER/PR/HER*}$, A11$^{ID/AR/ER/PR}$, A12$^{ID/HER}$, A14^{ID}, C8$^{ID/AR/HER}$, C9$^{ID/M/AR/HER}$, C10$^{ID/AR/ER/PR/HER}$, C12$^{ID/HER}$, E3^{ID}, E5$^{ID/HER}$, E9$^{ID/HER}$, G4$^{ID/AR/ER}$, G5$^{ID/M/HER/*}$, G12$^{ID/ER/HER}$, G15$^{ID/AR/ER}$, I12$^{ID/AR/ER/PR}$, I14$^{ID/M/AR/ER/PR/HER}$, I15$^{ID/HER}$
Ret	313F4 214H10	62	8 (13)	A14$^{ID/M}$, C12$^{ID/HER}$, E9$^{ID/HER}$, E13$^{ID/AR/ER/HER}$, G9$^{ID/HER}$, G12$^{ID/ER/HER}$, I12$^{ID/AR/ER/PR}$, I13$^{ID/AR/ER/PR/HER}$

M, metastasising tumour: either N1-3 or M1; AR, androgene receptor-positive case; ER, estrogene receptor-positive case; PR, progesterone receptor-positive case; HER, HER2/neu amplification; ID, invasive ductal carcinoma; IL, invasive lobular carcinoma; CIS, ductal carcinoma *in situ*.
*Three cases showed an amplified HAS2 signal instead of a translocation.

of epithelial breast cells (Caruso *et al*. 2001). Consequently, the marker chromosomes in the transformed cell lines (Table 1) must have developed during radiation-induced malignant transformation and can be attributed to the tumourigenic phenotype of the cells. The complex nature of the rearrangements of the marker chromosomes M16-3 and M16-4 in the cell line B42-16 could be classified as a translocation between chromosomes 10 and 12 with an additional terminal deletion of 10p and a deletion of the gene *Tbx5* on 12q24.2. The lack of expression of *Tbx5* (Table 2) supports the idea that copy number alterations often lead to altered gene expression of the affected gene.

The candidate genes identified from breakpoint analysis showed a deregulated expression except for *Tbx3*, *Arf3* and *Cpm* (Table 2) indicating the importance of the identified gene rearrangements for gene expression in these cell lines. *Grid1* represents a subunit of the glutamate receptor GRID1 (Haas *et al*. 2005), *Has2* a Hyaluronan synthase (Haas *et al*. 2005), *Arf3* an ADP-ribosylation factor (Hirai *et al*. 1996), *Cpm* a carboxypeptidase (Pessoa *et al*. 2002) and *Tbx5* a T-box transcription factor (Hatcher *et al*. 2001). *Wnt1* expression and subsequent activation of the Notch signalling pathway have already been reported to play a significant role in breast carcinogenesis (Collu & Brennan 2007), whilst some of the other candidate genes have not been reported in conjunction with breast carcinogenesis so far. Of particular interest are the rearrangements of *Has2*, *Grid1* and *Ret*, because of their significantly elevated expression in the transformed cell lines.

Has2 was translocated one megabase distal to the *Grid1*-containing region on chromosome 10. The breakpoint of *Has2* occurred between exon 2 and 3 affecting the largest (putatively cytosolic) domain of the protein. The DNA sequence fused to *Has2* could not be ascribed to a particular gene, and therefore, the mechanism of activation in the B42-16 cell line remains unclear. *Has2* encodes for a high molecular weight hyaluronan (Itano *et al*. 1999), which at increased expression levels reduces contact inhibition of cell growth and promotes cell migration and thus contributing to features of a transformed phenotype such as anchorage-independent and invasive growth (Itano *et al*. 2002, Zoltan-Jones *et al*. 2003). Consequently, in breast cancer, an elevated *Has2* expression was shown to be associated with increased cellular proliferation and an invasive phenotype (Udabage *et al*. 2005*a*,*b*, Cook *et al*. 2006). In our studied set of primary breast cancers, *Has2* was altered in 28% of cases (TMA), and expression was elevated in the grade 3 tumours of the analysed *in silico* validation set (Miller *et al*. 2005). This is in accordance with another study correlating *Has2* overexpression with an unfavourable outcome (Tammi *et al*. 2008). In this context, gene rearrangement of *Has2* represents a novel mechanism of its activation and offers considerable potential as a therapeutic target in breast cancer.

The location of the chromosomal breakpoint within the *Grid1* gene (between exon 2 and 3) indicates an alteration of the ligand-binding N-terminal domain of the resulting protein. *Grid1* belongs to a group of glutamate receptors that were believed to exclusively

play a role in the central nervous system (CNS). However, it was recently shown that the glutamatergic system is also involved outside the CNS in malignant processes (Rzeski *et al*. 2002, Haas *et al*. 2005, Kalariti *et al*. 2005).

From a clinical perspective rearrangement of *Grid1* in a substantial subset of breast cancers and elevated expression in grade 3 and low-grade lymph-node-positive breast cancers makes the gene, which is reported in this context for the first time attractive as a potential tumour marker.

Another candidate gene from this study is the receptor tyrosine kinase (TK) *Ret*, which was surprisingly overexpressed in both transformed cell lines, although only B42-11 exhibited a rearrangement of the gene. In addition both cell lines expressed the ligand-binding (EC) and TK domains of *Ret* at almost similar levels, which is remarkable in case of B42-11, since in papillary thyroid carcinomas with RET/PTC rearrangement usually only the TK domain is expressed (Ciampi & Nikiforov 2007). Thus, it is likely that in breast cells *Ret* is activated by a different mechanism or also an activation of the *Ret*-EC domain occurs in case of a rearrangement. In a study on mostly Her2/PR-positive breast cancers, it was recently reported that *Ret* is co-expressed with its co-receptor Gfra1 and its ligand GDNF (Esseghir *et al*. 2007), and another study demonstrated overexpression of *Ret* in breast cancer cell lines showing RET-induced anchorage-independent growth (Boulay *et al*. 2008). Both studies suggest interaction of RET with the ER pathway. This is supported by the finding that *Ret* expression is associated with positive ER status in the expression set from Miller *et al*. (2005). With regard to RET as a therapeutic target for TK inhibitors such as sunitinhib, activation by rearrangement might be of particular interest. The above-mentioned studies would not pick up *Ret* expression after rearrangement due to the methodology they have used. Therefore, *Ret* expression in breast cancer is likely to be underestimated, and usage as a therapeutic target or diagnostic/prognostic marker should be taken into consideration.

Our study demonstrated that the molecular analysis of chromosomal breakpoints in radiation-transformed epithelial breast cell lines is able to reveal novel breast cancer candidate genes and that rearrangement of these lead to altered gene expression. Prevalence of rearrangement and subsequent upregulation of *Grid1*, *Has2* and *Ret* could be validated in sporadic breast cancer. This provides evidence that genomic alterations and gene rearrangements in particular can be used for the identification of attractive candidates in diagnosis and treatment of breast cancer.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0065.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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