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Array comparative genomic hybridisation (aCGH) analysis of premenopausal breast cancers from a nuclear fallout area and matched cases from Western New York

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High-resolution array comparative genomic hybridisation (aCGH) analysis of DNA copy number aberrations (CNAs) was performed on breast carcinomas in premenopausal women from Western New York (WNY) and from Gomel, Belarus, an area exposed to fallout from the 1986 Chernobyl nuclear accident. Genomic DNA was isolated from 47 frozen tumour specimens from 42 patients and hybridised to arrays spotted with more than 3000 BAC clones. In all, 20 samples were from WNY and 27 were from Belarus. In total, 34 samples were primary tumours and 13 were lymph node metastases, including five matched pairs from Gomel. The average number of total CNAs per sample was 76 (range 35–134). We identified 152 CNAs (92 gains and 60 losses) occurring in more than 10% of the samples. The most common amplifications included gains at 8q13.2 (49%), at 1p21.1 (36%), and at 8q24.21 (36%). The most common deletions were at 1p36.22 (26%), at 17p13.2 (26%), and at 8p23.3 (23%). Belarussian tumours had more amplifications and fewer deletions than WNY breast cancers. HER2/*neu* negativity and younger age were also associated with a higher number of gains and fewer losses. In the five paired samples, we observed more discordant than concordant DNA changes. Unsupervised hierarchical cluster analysis revealed two distinct groups of tumours: one comprised predominantly of Belarussian carcinomas and the other largely consisting of WNY cases. In total, 50 CNAs occurred significantly more commonly in one cohort *vs* the other, and these included some candidate signature amplifications in the breast cancers in women exposed to significant radiation. In conclusion, our high-density aCGH study has revealed a large number of genetic aberrations in individual premenopausal breast cancer specimens, some of which had not been reported before. We identified a distinct CNA profile for carcinomas from a nuclear fallout area, suggesting a possible molecular fingerprint of radiation-associated breast cancer.
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The incidence of breast cancer in young women is lower than in the postmenopausal age group. However, carcinomas in these patients are generally more aggressive and associated with poorer prognosis. The aetiology of premenopausal breast cancer is not clear. In a minority of patients, tumours develop on the basis of germline mutations in the BRCA1 and BRCA2 genes. Environmental exposure variables have been extensively studied as a causative factor of human mammary neoplasia. There is significant evidence, derived from diverse populations, that ionising radiation can cause breast cancer. Some examples are patients who received therapeutic radiation to the chest early in life for Hodgkin's disease (HD), patients who were treated with radiation for mastitis and other benign breast diseases, patients who received thymic irradiation, patients who underwent frequent fluoroscopies for pulmonary disease, and atomic bomb survivors (Behrens *et al*, 2000; Clemons *et al*, 2000; Gaffney *et al*, 2001; Land *et al*, 2003; Travis *et al*, 2003). Among these, the best-studied group are women who developed breast cancer after treatment for HD. It was reported that the risk of developing breast cancer was greatest if patients were treated under the age of 30, and hormonal stimulation of the irradiated breast tissue appeared to play an important role (Clemons *et al*, 2000; Travis *et al*, 2003). The risk clearly was dose dependent (Travis *et al*, 2003), and the median latency period was in the range of 15 years (Clemons *et al*, 2000). Similar observations held true for the Japanese atomic bomb survivors. Breast cancer risk was inversely related to age at exposure, there was a linear dose response, and the minimum latency period was 12 years (Land *et al*, 2003). Tumours arising in irradiated women

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may be associated with reduced overall survival (Gaffney *et al*, 2001).

Researchers at Roswell Park Cancer Institute (RPCI), including some of the authors of this paper, have been actively involved in several epidemiologic studies on a more recent group of probands, namely individuals exposed to radiation released by the 1986 Chernobyl nuclear accident (Mahoney *et al*, 2004). Although the reactor was located in Ukraine, neighbouring Belarus received about 70% of the radioactive fallout (Ermak *et al*, 2003). It is well documented that children exposed to that fallout have an increased cancer rate (Peterson *et al*, 1997). One of the most common radiation-induced malignancies is papillary carcinoma of the thyroid, and this tumour type was shown to harbour molecular abnormalities that differed from those in sporadic thyroid cancers (Tuttle and Becker, 2000; Ermak *et al*, 2003). There also was an increase in breast cancer incidence in parts of Belarus after the Chernobyl accident, especially in rural areas and in premenopausal women (Sasnouskaya and Okeanov, 2000).

In theory, breast carcinomas associated with ionising radiation should facilitate insight into the molecular pathogenesis of early-onset breast cancer, yet few such studies have been published. In one paper, post-HD breast carcinomas were found to have more microsatellite alterations compared to sporadic tumours (Behrens *et al*, 2000). In another group of post-HD breast cancer patients, there were no significant differences compared with sporadic cases with regard to BRCA1, BRCA2, oestrogen receptor (ER), PR, HER2 and p53 status (Gaffney *et al*, 2001). Several *in vitro* studies are of relevance. It was shown that human mammary epithelial cells (HMEC) in culture can be transformed by γ-irradiation (Wazer *et al*, 1994). Subsequent studies showed that radiation induced nonrandom chromosomal changes in HMEC (Durante *et al*, 1996; Yang *et al*, 1997). Finally, irradiation of nontransformed MCF-10F cells led to altered expression of 49 of 190 genes assayed (Roy *et al*, 2001).

Comparative genomic hybridisation (CGH) is one tool to investigate the molecular pathology of human tumours. Conventional CGH is based on competitive *in situ* hybridisation of normal metaphase spreads by two differentially labelled whole genomic DNAs, one derived from tumour tissue and the other from a normal reference. Regions of altered DNA copy number (losses and gains) in the tumour are quantitated as ratio changes along metaphase chromosomes. The resolution of this technique is in the range of 10–20 Mb. In this study, we have used array CGH to obtain better resolution to facilitate identification of novel candidate breast cancer genes. High-density bacterial artificial chromosome (BAC)-based arrays developed at RPCI were employed to screen for DNA copy number gains and losses in premenopausal breast cancers from two geographically distinct areas in an attempt to identify genetic changes that may be specific to early-onset tumours and/or radiation exposure.

MATERIALS AND METHODS

Tissues

In all, 55 frozen samples of breast cancers were obtained from premenopausal patients from Western New York (WNY) and Belarus. Samples from Belarus were collected and snap frozen between August 2002 and January 2003. The tumours were collected from women who resided in the Gomel area since April 1986. They were transported on dry ice to the United States and processed at RPCI. The WNY samples were obtained through the tissue procurement facility at RPCI. Patients with a strong family history of breast cancer and cases with known BRCA1/2 mutations were excluded. This study was approved by the RPCI Institutional Review Board. All samples were evaluated morphologically and only those with more than 50% tumour cellularity were included.

Table 1 Patient characteristics

	WNY	Belarus	*P*-value
Age (median, years)	41.5	45.5	
⩽43	12	10	0.23
>43	8	12	
Caucasian	18	22	
African American	2	0	0.43
Histology			
Ductal	17	20	
Lobular	2	0	0.29
Other/unknown	1	2	
Grade			
1–2	7	5	
3	13	14	0.81
Unknown	0	3	
Size			
T1	4	8	
T2–T4	16	12	0.30
Unknown	0	2	
LN status			
Negative	6	5	0.85
Positive	14	17	
Stage			
I	2	4	
II	11	12	0.71
III	7	6	

WNY = Western New York; LN = lymph node.

Preliminary experiments had shown that >30% tumour cellularity was sufficient. Duplicate assays and dye swapping experiments were performed for a subset of samples, showing very good reproducibility. Genomic DNA was extracted from all samples using TriZol (Invitrogen, Grand Island, NY, USA). In total, 47 samples met the quality criteria and were included in the final analysis. Patient characteristics are shown in Table 1. All Gomel patients were Caucasian, as were the great majority of RPCI patients (except for two African American women). The age of the patients ranged from 24 to 50 years. There were 34 primary tumours and 13 lymph node metastases. This cohort included five paired cases from Belarus.

Her2 protein expression assays

The immunohistochemical assay for Her2 expression in formalin-fixed, paraffin-embedded tumour sections followed the Herceptest protocol (Dako, Carpinteria, CA, USA), and the stains were scored from 0 and 1+ (negative) to 2+ and 3+ (positive), using recommended guidelines. For Western blotting, 50 μg of protein was used per sample (extraction from tissue utilized the TriZol protocol). Protein was resolved over 8% SDS–PAGE and transferred to a PVDF membrane. The blot was blocked in blocking buffer (5% nonfat dry milk, 10 mM Tris (pH 7.5), 10 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. The membrane was then incubated with the Herceptest antibody (Dako) at a dilution of 1 : 500 at 4°C overnight. This was followed by incubation with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD, USA) at a dilution of 1 : 5000 at room temperature for 1 h. Protein bands were visualised by the SuperSignal West Pico Chemiluminescent Substrate kit obtained from PIERCE (Rockford, IL, USA) and exposed with Kodak X-Ray film.

Array-based CGH (aCGH)

The CGH arrays were prepared in the Microarray Core Facility at RPCI according to established protocols (Snijders *et al*, 2001; Cowell and Nowak, 2003; Cowell *et al*, 2004a). A total of 3084 BAC clones were common to all arrays used in this series of experiments (average resolution $\sim$1 Mb). The WNY and Belarussian samples were coded and then assayed concurrently and blindly. Genomic DNA (0.5 μg) was fluorescently labelled by random priming in a 100 μl reaction containing the DNA, random primers solution, appropriate buffers and Cy3- or Cy5-dCTP-labelled nucleotides. Labelling occurred with the addition of appropriate agents and incubation overnight at 37°C. Arrays were hybridised with appropriate solutions for 16 h. Slides were washed, dried and immediately scanned using an Affymetrix 428 scanner (Cowell *et al*, 2004b). Image analysis was performed using the ImaGene version 4.1 software from BioDiscovery Inc. The reference was pooled normal male DNA. Output of the image analysis was processed by an in-house Perl program to calculate log-transformed and normalised mean ratios of test to reference fluorescence intensities. Any BAC that had less than two replicates flagged as good or a standard error greater than 0.15 was excluded. Map positions were identified by querying the human genome sequence (July 2003 Build) at http://genome.ucsc.edu. A sample output showing intensity ratios across the whole genome for an individual tumour is shown in Figure 1.

Data analysis

Copy number aberrations (CNAs) were determined at the clonal level for each individual coded sample in a blinded manner. The genome-wide mean and standard deviation of the log ratios were calculated for the autosomal chromosomes and the X chromosome separately. Thresholds for amplification and deletion were set at two standard deviations from the mean in both directions. Contiguous regions of amplification or deletion were identified by flagging clones on the array based on adjacent chromosomal locations. Recurrent amplifications and deletions were determined by the frequency of these events among all samples or within specific groups.

Unsupervised hierarchical clustering was performed using the TIGR Multi-experiment Viewer (MeV version 2.2) software (Saeed *et al*, 2003). Only clones that were included in at least 45 of 47 samples were considered. A filtered set of 202 clones with a high variability across all samples (standard deviation/mean >0.3) was used for the hierarchical clustering. Clusters were generated using the average linkage method with Pearson's correlation coefficient as the similarity metric.

Association between groups was tested from contingency tables using the χ^2 Pearson statistic. Resulting *P*-values are shown as appropriate.

RESULTS

In all, 47 samples that met all quality control criteria including tumour cellularity and patient age were included in the final analysis. Patient charactertistics are shown in Table 1. The two groups from WNY and Gomel were well matched for ethnic background, age, extent of disease (tumour size, nodal involvement, stage), tumour histology, grade and ER status. The average number of CNAs in the breast carcinomas was 76 (37 gains and 39 losses). Tumours from WNY, older patients, and HER2-positive cancers had more deletions and fewer amplifications than tumours from Belarus, younger patients, and HER2-negative cancers, respectively (Table 2). Primary tumours and lymph node metastases, smaller and larger tumours,

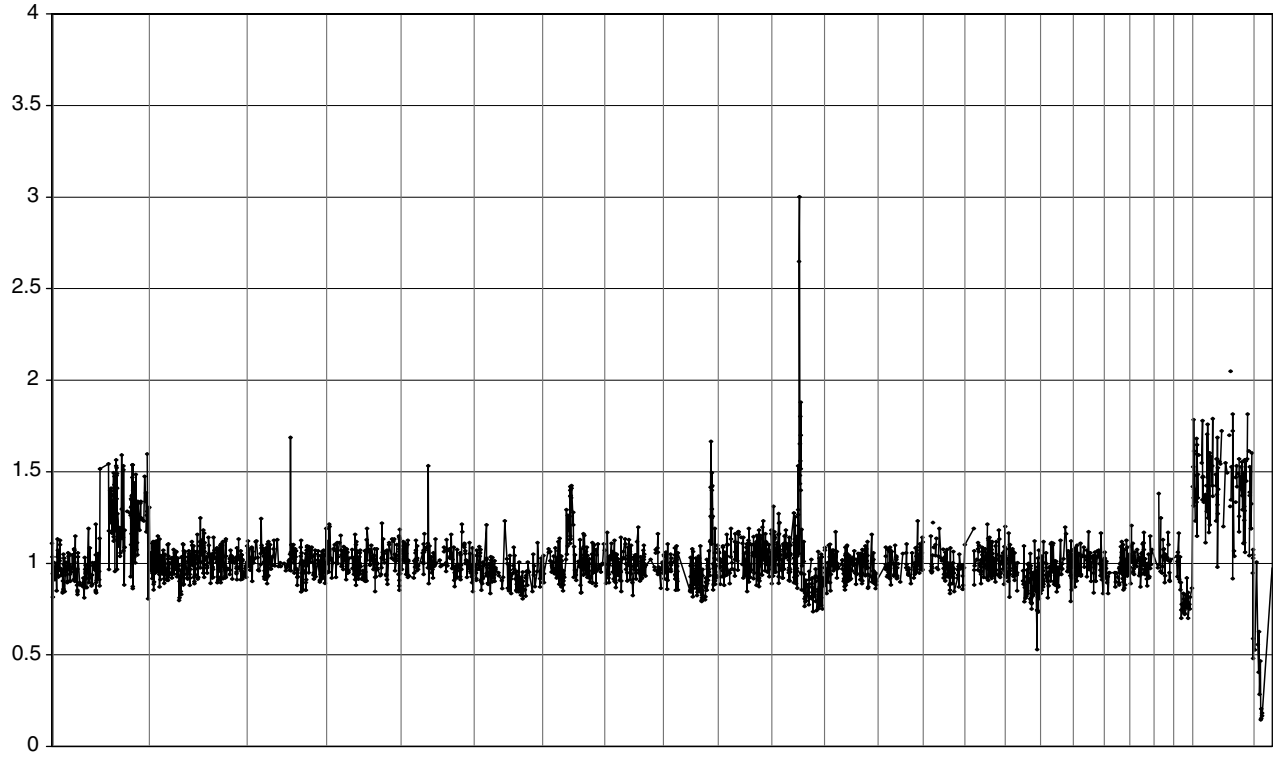

Figure 1 aCGH of a representative tumour. DNA from a primary breast cancer was hybridised with normal male reference DNA. The whole genome is arranged along the *x*-axis from left (1p) to right (X, Y). The chromosomal boundaries are indicated by vertical lines. The *y*-axis is linear. A number of distinct amplifications (e.g. 3q, 9q, 11) and deletions (16q) as well as larger regions of DNA copy gains (e.g. 1q) and losses (11q, 22q) are easily recognised.

ER+ and ER− cases, and carcinomas with and without associated nodal metastases had comparable frequencies of copy number gains and losses.

A representative diagram of genome-wide amplifications and deletions is shown in Figure 1. Most breast cancers displayed both distinct single BAC amplifications and deletions as well as broader areas of DNA gains and losses. A total of 152 CNAs occurred in more than 10% of samples, including 92 copy number gains and 60 losses. The majority of DNA gains (72%) were on the long arms of chromosomes 1 and 8. Most of them were gains of one or two copies, but a subset was higher level amplifications (three copies or more, Table 3). The most common amplifications were at 8q13.2 (49%), at 1p21.1 (36%), and at 8q24.21 (36%). Gains at loci containing the well-known breast oncogenes c-myc (at 8q24.21), HER2 (at 17q21.1), and cyclin D1 (at11q13.3) were also frequent (26, 19, and 13%, respectively) (Table 3). In an initial validation experiment, HER2 amplification detected by array CGH was correlated with protein expression using immunohistochemistry and Western blot analysis (Figure 2). Amplified tumours showed high levels of the Her2 oncoprotein, while nonamplified cases were negative. While the majority of chromosomal gains had previously been described, we identified seven novel recurrent gains (as indicated in Table 3), five of which were in areas on 8q that had not been reported to be common amplification sites in breast cancer.

Table 2 Distribution of CNAs

	Average gains	Average losses	Average total CNAs
WNY	32[a]	44	76
Belarus	42[a]	35	77
Age under median (⩽43)	43[b]	33[c]	76
Age over median (>43)	33[b]	45[c]	78
Small tumours (T1)	39	36	75
Large tumours (T2–T4)	37	40	77
Lymph node negative	39	37	76
Lymph node positive	35	41	76
Primary tumours	37	41	78
Lymph node metastases	40	33	73
ER positive	31	49	80
ER negative	37	37	74
HER2 negative	41[d]	35	76
HER2 positive	28[d]	46	74
Total	37	39	76

CNA = copy number aberrations; WNY = Western New York. [a]$P = 0.034$. [b]$P = 0.039$. [c]$P = 0.054$. [d]$P = 0.037$.

The largest number of DNA losses was on chromosome 17. In most instances, one allele was lost, but possible homozygous deletions were also observed (Table 4). The most common deletions were at 1p36.22 (26%), at 17p13.2 (26%), and at 8p23.3 (23%). We identified nine novel recurrent losses, seven of which were on chromosome 22 (Table 4).

In the five paired samples, some genetic changes were common to the primary tumour and the lymph node metastasis. The number of shared changes varied markedly from case to case. However, all cases were characterised by a large number of discordant CNAs. Metastatic tumours consistently developed more gains than losses (Table 5).

Unsupervised hierarchical clustering using 202 discriminating BAC clones produced a dendrogram with two distinct arms: one predominantly composed of WNY carcinomas and the other mostly comprised of Belarussian samples ($P < 0.001$) (Figure 3). These two arms were not significantly different with regard to patient age, primary tumours *vs* metastases, tumour size, nodal status, stage, grade, ER, or HER2 status. A total of 50 BAC clones were differentially amplified or deleted in premenopausal breast cancers from WNY and Belarus, and 25 of these contained named genes (Table 6). Of particular interest were 10 BAC clones that were amplified selectively in Belarussian tumours. Moreover, three of these BACs were specifically deleted in WNY

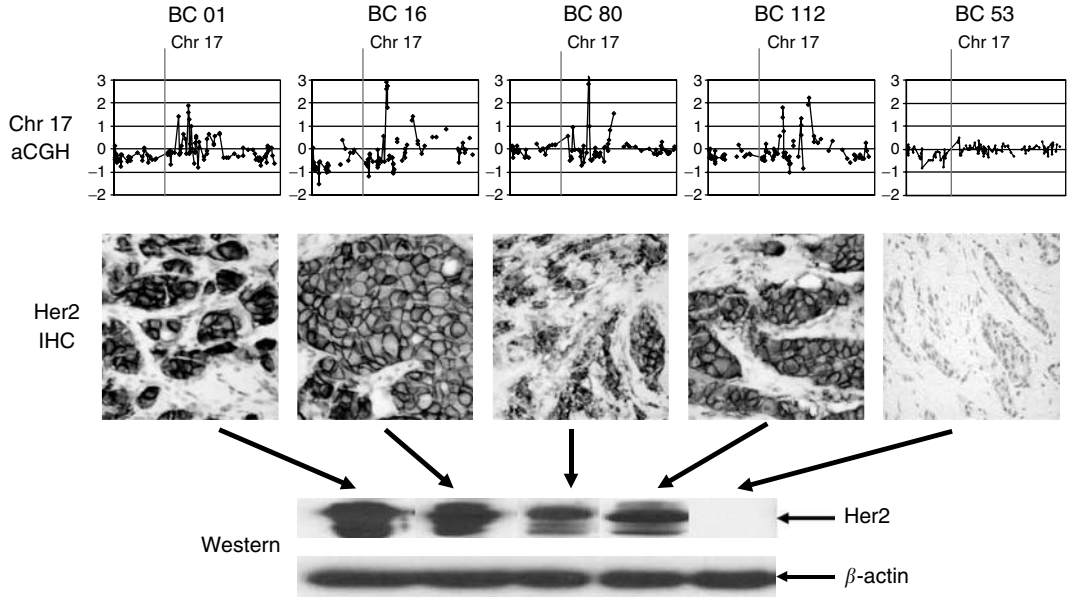

Figure 2 HER2 amplification and overexpression. Nine tumours from eight patients showed amplification at 17q21. The chromosome 17 aCGH profiles of four of them are shown at the top along with one nonamplified sample (the *y*-axis is on a $\log_2$ scale). This was associated with Her2 protein overexpression by immunohistochemistry (middle) and Western blot analysis (bottom).

Table 3 Recurrent gains arranged by chromosomal location ($n = 92$)

BAC clone (RP11-)	Chr. loc.	#Samples (%)	#Samples with ⩾3 copy gains	Genes
727M5	1p21.1	17 (36)	0	
35B4	1q21.1	10 (21)	1	AB033071, AF379633, AF379636, LOC64182, AK075065
439A17	1q21.1	9 (19)	1	BC034024
196G18	1q21.2	5 (11)	1	
307C12	1q22	5 (11)	1	
120D12	1q23.1	8 (17)	0	FCRH1, CD5L, AF329488
452O22	1q23.1	8 (17)	1	SPTA1, AF060556, M11049
520H16	1q23.1	7 (15)	0	
223F11	1q23.3	8 (17)	1	DDR2
403P14	1q23.3	8 (17)	0	
506O24	1q23.3	8 (17)	0	
572K18	1q23.3	8 (17)	0	
404F10	1q23.3	7 (15)	0	SLAMF1, CD48, SLAMF7
528G1	1q23.3	6 (13)	0	
80D6	1q24.1	6 (13)	0	LMX1A
54B9	1q24.1	5 (11)	0	
162H11	1q25.2	6 (13)	1	RASAL2
317P15	1q25.3	5 (11)	1	RGS16, RGS8
335O13	1q32.1	8 (17)	1	PPFIA4, MYOG, FMOD, MYBPH, CHIT1
148K15	1q32.1	6 (13)	1	*MDM4, LRRN5*
294K24	1q32.1	6 (13)	1	PTPN7, ARL10B
150L7	1q32.1	5 (11)	0	PKP1, DKFZp434B1231
243M13	1q32.1	5 (11)	0	ABO18299, CNTN2
564A8	1q32.2	9 (19)	1	IL20, IL24, TOSO, MGC4309, MGCPIGR, FKSG87, AY063126
167J2	1q32.2	8 (17)	0	AY320401
328D5	1q32.2	7 (15)	3	CD34
357P18	1q32.2	5 (11)	0	
35C1	1q32.2	5 (11)	1	CR1, CR2
211K12	1q41	5 (11)	0	
35D17	1q41	5 (11)	0	PTPN14
115K6	1q43	7 (15)	1	
182B22	1q43	6 (13)	0	
150L22	1q44	9 (19)	2	AKT3, SDCCAG8, BC019085
194A15	3q25.31	5 (11)	1	TIPARP
88L18	5p15.1	5 (11)	0	
36H5	5p15.31	5 (11)	0	AK090679
43B19	6q26	8 (17)	1	LPA
90P13	7p22.3	7 (15)	1	
331D5	**7q36.3**	**11 (23)**	**4**	**DNAJB6, PTPRN2**
83O14	**8q11.23**	**9 (19)**	**2**	**ST18**
182B21	**8q12.3**	**8 (17)**	**1**	
252M13	**8q12.3**	**6 (13)**	**2**	
566L6	**8q13.2**	**23 (49)**	**0**	**AB095942**
120N14	**8q13.3**	**5 (11)**	**1**	**SULF1**
65J24	8q21.11	9 (19)	1	
203C23	8q21.11	6 (13)	0	LY96
531A24	8q21.11	5 (11)	1	
594N15	8q21.12	9 (19)	3	PKIA
79H23	8q21.12	8 (17)	3	IL7, CGI-62
93J13	8q21.12	8 (17)	1	
523D2	8q21.12	7 (15)	3	
80C11	8q21.13	14 (30)	3	FABP5, BT007449
214E11	8q21.13	10 (21)	2	TPD52
93E11	8q21.13	8 (17)	3	
219B4	8q21.2	5 (11)	1	E2F5, BC030701, AK056185
480D6	8q21.3	12 (26)	3	MMP16, DKFZp761D112
435I11	8q21.3	12 (26)	3	NBS1, DECR1, C8orf1
122C21	8q21.3	8 (17)	2	CBFA2T1
118O8	8q21.3	5 (11)	1	CBFA2T1
413N8	8q22.1	10 (21)	2	SDC2, AF119386, AF107833
30J11	8q22.1	7 (15)	2	CDH17, RAD54B
700M17	8q22.1	7 (15)	2	CDH17, GEM
27I15	8q22.1	6 (13)	2	
347C18	8q22.1	6 (13)	2	FLJ20530, TP53INP1
498C11	8q22.1	5 (11)	2	
125O21	8q22.2	13 (28)	3	STK3, KCNS2, BC034778
131D12	8q22.2	7 (15)	1	
208E21	8q22.2	6 (13)	1	COH1
680F3	8q22.3	6 (13)	1	
12K18	8q22.3	5 (11)	1	ODF1, TIEG

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Table 3 (*Continued*)

BAC clone (RP11-)	Chr. loc.	#Samples (%)	#Samples with ⩾3 copy gains	Genes
762A3	8q23.3	8 (17)	3	TRPS1
150N13	8q24.13	7 (15)	1	
229L23	8q24.13	7 (15)	1	
145G10	8q24.21	17 (36)	5	
237F24	8q24.21	12 (26)	3	MYC
128G18	8q24.21	9 (19)	2	
184M21	8q24.22	13 (28)	0	TG, SLA
316E19	8q24.22	12 (26)	0	TG, SLA, S40807
28A4	8q24.23	5 (11)	0	PFKP, PITRM1, BC021698
298E9	10p15.2	8 (17)	1	
120P20	11q13.3	13 (28)	4	
300I6	11q13.3	6 (13)	3	CCND1, FGF19
91P18	11q13.4	9 (19)	1	
461N23	13q32.2	6 (13)	1	GPR18, EB12
455F5	16p11.2	5 (11)	0	ALDOA, TBX6, CORO1A
521P1	17q11.2	5 (11)	1	CCL8, CCL13, CCL1
94L15	17q21.1	9 (19)	7	ERBB2, STARD3, TCAP, PNMT, CAB2, GRB7, ZNFN1A3, MGC14832, BT006964
209H21	20p11.23	5 (11)	1	CSRP2BP, ZNF133
17F3	20q12	5 (11)	1	PTPRT
465L10	20q13.12	5 (11)	0	PLTP, ZNF335, SLC12AA5
560A15	20q13.31	5 (11)	1	
181G18	**21q21.1**	**6 (13)**	**0**	

Chr. loc. = chromosomal location. Bold abnormalities indicate previously undescribed recurrent gains. Shaded rows indicate the same chromosomal band.

cases. Two BAC clones with known genes were significantly more often amplified, and 13 were more frequently deleted in WNY tumours.

DISCUSSION

There is a paucity of data on genomic changes in early-onset breast cancer. The study reported here benefitted from a unique population, namely premenopausal women from Belarus who were exposed to significant fallout radiation from the 1986 Chernobyl nuclear accident. It has been suggested that Chernobyl-related cancers may be the best model to study the carcinogenic effect of low-dose radiation (Ermak *et al*, 2003). We were able to compare the genomic abnormalities in the Belarussian cancers to those in similar tumours from a nonexposed area that were well matched for ethnic background, age, tumour characteristics, and extent of disease.

By utilising high-density aCGH technology with the capacity to screen for DNA copy changes across the entire genome at high resolution, we identified a larger number of chromosomal abnormalities than previous breast cancer studies. Links to genomic databases helped to identify candidate genes for further investigation. A small number of published studies applied aCGH technology to breast cancer cells, but only to a very limited extent (Pinkel *et al*, 1998; Albertson *et al*, 2000; Kauraniemi *et al*, 2001; Hyman *et al*, 2002; Pollack *et al*, 2002; Lage *et al*, 2003). Almost all of these utilised a significantly smaller number of specimens. Some of them focused only on cell lines (Hyman *et al*, 2002; Lage *et al*, 2003) or on individual chromosomes (Pinkel *et al*, 1998; Albertson *et al*, 2000; Kauraniemi *et al*, 2001). We are not aware of any published reports on array or conventional CGH analysis of DNA abnormalities in irradiated cells. Likewise, our study may be the first to screen for somatic genetic changes specifically in premenopausal tumours, providing new insight into the molecular pathology of early-onset breast cancer. The average number of total CNAs in our cohort of tumours was 76 (range 35–134), which is approximately eight times the average number of CNAs in breast cancers detected by conventional CGH (Isola *et al*, 1995; Kuukasjärvi *et al*, 1997; Aubele *et al*, 2000a, b; Zudaire *et al*, 2002), demonstrating the superior sensitivity of our assay. This is in keeping with the density of the BAC arrays used (some 3000 elements, average resolution $\sim$1 Mb).

Several groups have used conventional CGH to probe for genetic aberrations in invasive breast carcinomas. Tirkkonen *et al* (1998) found that the pattern of chromosomal gains and losses depended on tumour size, grade, and receptor status. They described gain on 1q as an early event and gain on 8q as a marker for advanced breast cancers. We have identified similar recurrent gains on 1q and 8q, which were the most commonly affected chromosomal arms in our series (Table 3). In a study of T2 ($>$2 cm) node-negative breast cancers, a high overall number of genetic aberrations was correlated with poor prognosis, and an increased copy number at 8q and 20q13 indicated an aggressive phenotype (Isola *et al*, 1995). Another similar study of invasive ductal carcinomas found that gains on 1q, 11q, 17q, and 20q were associated with poor prognosis (Zudaire *et al*, 2002). These DNA changes were common in our series as well, and this observation is in agreement with the more aggressive clinical course of premenopausal breast cancer. Nine of 47 tumours showed amplification of a BAC clone including HER2. This copy gain rate (19%) is in keeping with numerous previous studies on HER2 amplification in breast cancer and proved to be among the most common abnormalities in our study. Additional commonly amplified BACs included the well-known breast cancer oncogenes c-myc (at 8q24.21) and cyclin D1 (at 11q13.3), further demonstrating adequate sensitivity of our method. Novel recurrent CNAs with potential target genes included gains at 3q25.31, 6q26, 7q36.3, 13q32.2, and 16p11.2 (Table 3). Seven recurrent amplification loci, most of which were found on 8q, had not been reported to be frequent events in breast carcinomas.

Recurrent deletions were less common, and no single event was found in more than 26% of the samples. Losses were most frequently observed on chromosome 17, which is in keeping with published cytogenetic data. However, nine of the remaining recurrent deletions, including seven on chromosome 22, had not been reported before (Table 4). Potential target genes included

Table 4 Recurrent losses arranged by chromosomal location ($n = 60$)

BAC clone (RP11-)	Chr. loc.	#Samples (%)	#Samples with possible homozygous deletions	Genes
6B16	1p31.1	8 (17)	0	
238O13	1p34.3	5 (11)	0	THRAP3
104J13	1p35.2	5 (11)	1	
4O6	1p35.3	5 (11)	1	FGR, G1P3, MGC34648
692J20	1p35.3	5 (11)	0	PTAFR, DNAJC8, ATP1F1, SESN2
285H13	1p36.11	6 (13)	2	
426M1	1p36.22	12 (26)	2	TNFRSF8, TNFRSF1B, BC042167
21N6	2q24.3	10 (21)	0	
425J9	3p21.31	7 (15)	0	MYL3, PTHR1, HYPB
745J15	**7p11.2**	**7 (15)**	**2**	**PSPH, CCT6A, SUMF2, PHKG1**
148K1	7q36.1	9 (19)	0	ACCN3, FASTK, ABCB8, ASB10, CDK5
89M8	8p21.2	7 (15)	1	RHOBTB2, TNFRSF10B
232J22	8p21.2	5 (11)	0	BNIP3L
288N10	8p21.2	5 (11)	0	
110I16	8p21.3	9 (19)	0	
235I5	8p23.1	5 (11)	0	GATA4, FDFT1, NEIL2
240A17	8p23.3	11 (23)	0	DLGAP2
181G12	9p21.1	8 (17)	0	
228B15	9q34.11	9 (19)	0	SH2D3C, CDK9, FPGS, ENG
104F15	10q22.1	5 (11)	1	LRRC20, EIF4EBP2, NODAL
37L21	10q24.31	7 (15)	0	PEO1, MRPL43, SEMA4G, BC053373
892K21	11q23.3	10 (21)	0	
238I24	**14q32.33**	**5 (11)**	**2**	
16O9	15q15.1	6 (13)	2	OIP5, ANKT, CIA30, ITPKA, LTK, KIAA0252
442O1	16q24.1	10 (21)	0	ZDHHC7, KIAA0513, BC030280
9A21	17p13.1	8 (17)	1	SENP3, EIF4A1, MPDU1, CD68, SSAT2, SHBG, FXR2, AK027742, BC006380
104H15	17p13.1	6 (13)	0	CENTB1, TNK1, NLGN2, FGF11, POLR2A, CHRNB1, KAISO-L1, PLSCR3, MGC40107, BC040900
1D5	17p13.1	5 (11)	0	ALOX12B
89A15	17p13.1	5 (11)	0	RPL26, MYH10
231G16	17p13.2	11 (23)	2	
208J12	17p13.2	12 (26)	4	TRPV3, CARKL, CTNS
61B16	17p13.3	11 (23)	1	CT120, GEMIN4
216P6	17p13.3	9 (19)	0	ABR, NXN, TIMM22
26N16	17p13.3	7 (15)	0	CGI-150, TIMM22
433M14	17p13.3	6 (13)	0	
4F24	17p13.3	6 (13)	0	RPA1
356I8	17p13.3	5 (11)	0	
818O24	17p13.3	5 (11)	0	
73F15	17q11.2	6 (13)	1	CRLF3
58O9	17q21.2	5 (11)	1	GJC1, CTEN
266I24	17q21.31	8 (17)	4	PSME3, AOC2, AOC3, G6PC, RPL27, IFI35, AK055784
506G7	17q21.31	7 (15)	1	
36J16	17q21.32	10 (21)	0	HOXB13, NDP52, FLJ35808
388C12	17q25.3	7 (15)	0	
12I11	19p13.12	7 (15)	2	TPM4, HSH2, AP1M1, KIP3, MEL, FLJ25328, DKFZP586O0120, BT007184
56K21	19p13.12	5 (11)	2	CD97, DDX39, PRKCL1
298C17	19p13.2	8 (17)	0	EIF3S4, DNMT1, P2RY11
31N2	19p13.2	6 (13)	1	KIAA1198
283B8	19q13.2	9 (19)	1	
2J15	19q13.32	7 (15)	1	CALM3, GNG8, PTGIR
236B14	19q13.33	7 (15)	0	
17I20	19q13.33	5 (11)	2	SULT2B1, RPL18, SPHK2, DBP, CA11, FUT2
87L13	19q13.42	6 (13)	1	
36N5	**22q11.21**	**5 (11)**	**1**	**SDF2L1**
213L15	**22q12.1**	**7 (15)**	**1**	**KREMEN1**
5O6	**22q12.1**	**5 (11)**	**1**	
206B19	**22q13.2**	**5 (11)**	**1**	**SREBF2**
49M22	**22q13.31**	**7 (15)**	**1**	**ARHGAP8**
61L22	**22q13.31**	**6 (13)**	**0**	**SMC1L2, FLJ20635**
111312	**22q13.33**	**5 (11)**	**0**	**MLC1, MOV10L1, PANX2**

Chr. loc. = chromosomal location. Bold abnormalities indicate previously undescribed recurrent losses. Shaded rows indicate the same chromosomal band.

KREMEN1 (on 22q12.1), which is a component of the Wnt signalling pathway (Mao *et al*, 2002).

It is conceivable that the novel chromosomal gains and losses described here may be the hallmark of premenopausal breast cancer. While our high-density BAC arrays appeared to have adequate sensitivity, an initial validation experiment suggested that the detected abnormalities may also be specific and verifiable by alternate techniques. The tumours with amplification of the

Table 5 Copy number gains and losses in paired primary breast cancers and their nodal metastases

	Genetic changes common to both primary and metastasis		Discordant genetic changes			
			In primary only		In nodal metastasis only	
Paired case #	Gains	Losses	Gains	Losses	Gains	Losses
1	63 (33)	9 (7)	16 (11)	9 (8)	30 (25)	12 (11)
2	19 (13)	10 (7)	21 (15)	26 (25)	31 (30)	15 (13)
3	20 (11)	1 (1)	48 (38)	37 (30)	42 (35)	22 (20)
4	7 (5)	9 (5)	25 (20)	34 (31)	36 (33)	24 (23)
5	13 (7)	0 (0)	13 (12)	91 (63)	42 (29)	4 (3)

Number of amplified or deleted BAC clones (number of affected chromosomal bands in parentheses).

HER2 locus also showed overexpression of the Her2 oncoprotein, while the nonamplified tumours showed no immunoreactivity (Figure 2).

Our series included five matched pairs of primary tumours and nodal metastases from Belarus. As expected, a number of chromosomal abnormalities were common to both the metastasis and the parental tumour, although the number of shared CNAs varied significantly from case to case (Table 5). Importantly, all cases were characterised by a large number of discordant events. Primary tumours developed a smaller, larger, or similar number of CNAs compared to the secondary lesions. They tended to have more deletions than losses. In contrast, nodal metastases consistently were marked by a larger number of amplifications over deletions. These findings suggest that metastases may form at variable points in the evolution of a breast cancer, and that primary tumour and metastasis independently develop additional genetic changes. This adds to the growing body of evidence that metastatic breast cancers may be biologically distinct from their parental tumours.

As detailed in Table 2, the genetic changes were not evenly distributed among the premenopausal breast cancers. While chromosomal gains and losses were not dependent on tumour site or size, nodal involvement, or ER status, younger age and HER2 negativity were associated with a smaller number of deletions and more amplifications. Likewise, tumours from Belarus had more DNA gains and fewer deletions than carcinomas from WNY. One of the most interesting observations was that breast cancers from Gomel had a DNA profile that was distinct from that of an age- and stage-matched control group treated at RPCI. Strikingly, when all 47 cases were subjected to unsupervised hierarchical clustering, two distinct groups emerged: one mostly comprised of Belarussian breast cancers and one mainly comprised of cases from WNY (Figure 3). This segregation was statistically highly significant ($P<0.001$). All of the other variables (age, extent of disease, grade, receptor status) were similarly distributed in the two arms. In all, 50 BAC clones were differentially amplified or deleted in the two groups of tumours, and half of these contained named genes. In total, 10 chromosomal gains were specific to the Belarussian samples, and it is tempting to speculate that these may represent the molecular hallmark of radiation associated breast cancer. Potential target genes included the MDM2-related gene MDM4 (at 1q32.1) and SULT1A3 (at 16p11.2) encoding an enzyme involved in the metabolism of catecholamines and related compounds (Hildebrandt *et al*, 2004). WNY tumours were characterised by a significantly higher number of deleted BAC clones. It is unclear whether the chromosomal

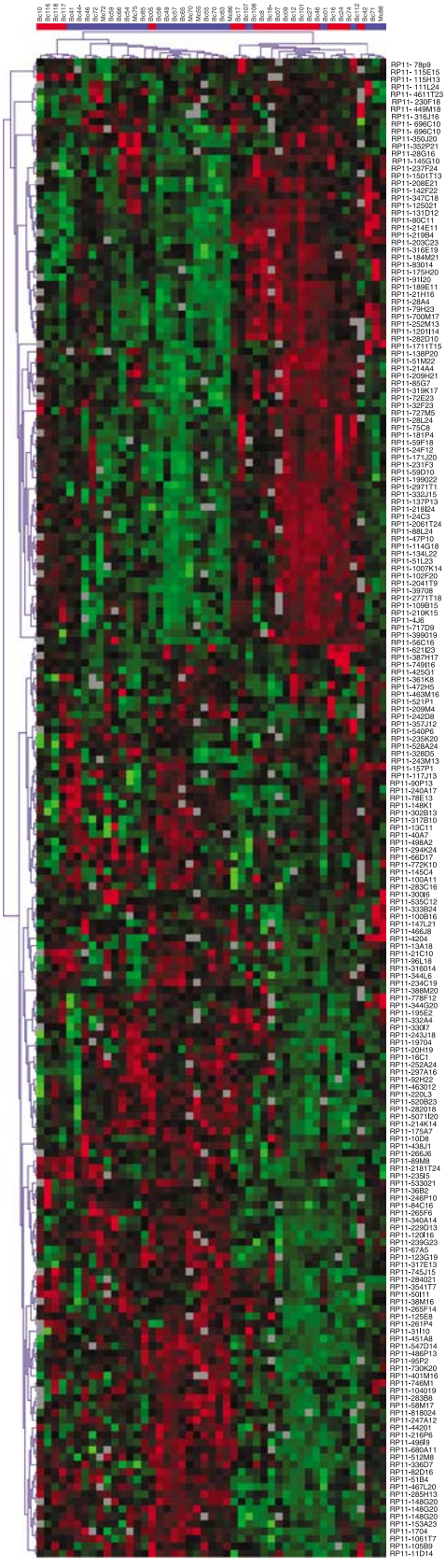

Figure 3 Cluster analysis (dendrogram). Unsupervised hierarchical clustering based on 202 BAC clones (vertical) yielded two main arms (horizontal): 26 tumours predominantly from Belarus (blue) on the left and 21 tumours mainly from WNY (red) on the right.

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Table 6 Genetic changes distinguishing premenopausal breast cancers from WNY and Gomel, Belarus

			WNY		Belarus	
BAC clone (RP11-)	**Chr. loc.**	**Candidate genes**	**Ampl.**	**Del.**	**Ampl.**	**Del.**
425I11	8q21.3	DECR1, C8orf1	9		2	
122C21	8q21.3	CBFA2T1	6		2	
157P1	20q13.33	OSBPL2, ADRM1, LAMAS	0		**8**	
148K15	1q32.1	MDM4	0		**6**	
461N23	13q32.3	EBI2, GPR18	0		**6**	
150L7	1q32.1	PKP1	0		**5**	
243M13	1q32.1	CNTN2, RBBP5, ABO18299	0		**5**	
455F5	16p11.2	SULT1A3, ALDOA, TBX6, CORO1A, MGC5178	0		**5**	
465L10	20q13.12	PLTP, ZNF335, SLC12A5, NCOA5	0		**5**	
252A24	16q22.3	PSMD7, GLG1	0	6	**3**	1
2I16	5q35.3	COL23A1	1	4	**5**	0
58M17	16p13.2	USP7	0	3	**4**	0
73F15	17q11.2	CRLF3		6		0
126L15	7q22.1	ZAN, EPHB4, ACHE		3		0
211E17	11p15.4	TRIM3, ILK, TAF10, CLN2, PCDH16		3		0
35J17	19q13.42	ZNF331		3		0
571M6	12q14.1	CDK4		3		0
746M1	17p11.2	USP22, DKFZp5660084, C17orf35		3		0
36J16	17q21.32	NDP52, HOXB13		8		2
208J12	17p13.2	TRV1, CARKL, CTNS		8		2
61B16	17p13.3	CT120, GEMIN4		8		3
213L15	22q12.1	KREMEN1		6		1
2J15	19q13.32	CALM 3, PTIGR, GNG8		6		1
89M8	8p21.2	RHOBTBN2, TNFRSF10B		6		1
298C17	19p13.2	EIF3S4, DNMT1, P2RY11		6		1

WNY = Western New York; Chr. loc. = chromosomal location; Ampl. = amplification; Del. = deletion. In all, 25 (out of 50) distinguishing BAC clones contained named genes (listed). Ten BAC clones (bold) were selectively amplified in Belarussian breast cancers and may represent signature events related to radiation exposure.

abnormalities in the tumours from Gomel are important in their pathogenesis or merely markers of genomic susceptibility to radiation damage. There are no associated epidemiologic or dosimetry data for the individual samples from Belarus so that, at this juncture, we cannot be sure that the specific changes in the tumour DNA are in fact due to radiation exposure. While the Belarussian population is ethnically homogeneous (Ermak *et al*, 2003), and while the racial background of the breast cancer patients from the Gomel area was similar to that in the WNY cohort, we cannot rule out that other endogenous or exposure variables such as smoking or diet may account for the difference in genomic abnormalities, although there is no evidence that such environmental factors impact on the pattern of chromosomal aberrations in breast carcinomas.

In conclusion, our study significantly adds to the existing body of knowledge by (a) detailing a number of previously undescribed recurrent chromosomal gains and losses in premenopausal breast cancers; (b) focusing on a unique cohort of breast carcinomas associated with prolonged low-dose radiation exposure; and (c) describing a set of CNAs that are specific to tumours from a nuclear fallout area. Our findings may provide the basis for future studies on the molecular pathogenesis of early-onset breast cancer.

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