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Contralateral breast cancer can represent a metastatic spread of the first primary tumor: determination of clonal relationship between contralateral breast cancers using next-generation whole genome sequencing

Sara Alkner[1,2*†], Man-Hung Eric Tang[1†], Christian Brueffer[1], Malin Dahlgren[1], Yilun Chen[1], Eleonor Olsson[1], Christof Winter[1], Sara Baker[1], Anna Ehinger[1,3], Lisa Rydén[1,4], Lao H. Saal[1], Mårten Fernö[1†] and Sofia K. Gruvberger-Saal[1*†]

Abstract

Introduction: By convention, a contralateral breast cancer (CBC) is treated as a new primary tumor, independent of the first cancer (BC1). Although there have been indications that the second tumor (BC2) sometimes may represent a metastatic spread of BC1, this has never been conclusively shown. We sought to apply next-generation sequencing to determine a "genetic barcode" for each tumor and reveal the clonal relationship of CBCs.

Methods: Ten CBC patients with detailed clinical information and available fresh frozen tumor tissue were studied. Using low-coverage whole genome DNA-sequencing data for each tumor, chromosomal rearrangements were enumerated and copy number profiles were generated. Comparisons between tumors provided an estimate of clonal relatedness for tumor pairs within individual patients.

Results: Between 15–256 rearrangements were detected in each tumor (median 87). For one patient, 76 % (68 out of 90) of the rearrangements were shared between BC1 and BC2, highly consistent with what has been seen for true primary-metastasis pairs (>50 %) and thus confirming a common clonal origin of the two tumors. For most of the remaining cases, BC1 and BC2 had similarly low overlap as unmatched randomized pairs of tumors from different individuals, suggesting the CBC to represent a new independent primary tumor.

Conclusion: Using rearrangement fingerprinting, we show for the first time with certainty that a contralateral BC2 can represent a metastatic spread of BC1. Given the poor prognosis of a generalized disease compared to a new primary tumor, these women need to be identified at diagnosis of CBC for appropriate determination of treatment. Our approach generates a promising new method to assess clonal relationship between tumors. Additional studies are required to confirm the frequency of CBCs representing metastatic events.

* Correspondence: Sara.Alkner@med.lu.se; Sofia.Gruvberger@med.lu.se
†Equal contributors
[1]Division of Oncology and Pathology, Clinical Sciences Lund, Lund University, MV 404-B2, Lund SE-22381, Sweden
Full list of author information is available at the end of the article

Introduction

Contralateral breast cancer (CBC) is today treated as a new primary tumor, independent of the first breast cancer (BC1). However, whether the second tumor (second breast cancer (BC2)) always is a new independent primary breast cancer, or in some cases could represent a metastatic spread from BC1, remains unclear. Supporting this possibility are recent studies showing the lymph node status of BC1 to influence risk of CBC and the time interval between BC1 and BC2 to affect prognosis after CBC [1–4]. If some CBCs are in fact metastatic events, today's clinical standard of diagnostic work-up and the treatment of patients with CBC as metastasis would need to be revised.

Various characteristics such as X-chromosome inactivation status, *p53* mutations, partial allelotyping, microsatellite instability, and comparative genomic hybridization (CGH) analysis have been used to compare BC1 and BC2 in CBC patients [5–12]. These previous studies suggest that the majority of CBCs appear clonally independent from BC1, although some have features similar enough to the primary tumor that they could potentially represent a metastatic spread [6, 8–10, 13]. However, prior studies utilized methods and techniques that are limited in resolution and were generally not specific enough to demonstrate an unequivocal clonal relationship between BC1 and BC2. For example, while a differing X-chromosome inactivation status indicates two distinct progenitor cells, a similar status must be considered noninformative [13]. Finding the same *p53* mutation in two tumors could indicate a similar origin [6]. However the *p53* gene has mutational hotspots, germline mutations are possible, and certain subtypes, such as basal-like tumors, have a greatly elevated *p53* mutation rate [14], presenting a risk for misclassifications. Consequently, neither X-chromosome inactivation status nor *p53* mutation analysis can with certainty confirm a clonal relationship [5, 6, 12].

Using array CGH, bilateral cancers have been shown to share genomic imbalances [9, 10]. This could indicate a clonal relationship, but another possibility is that their development in a common milieu and genetic background could lead to pathogenetically similar tumors that are in fact independent cancers. Indeed, a high similarity of allelic imbalances has been seen in simultaneously developing breast cancers in monozygotic twins, and allelotypes of CBCs developing synchronously were found to be more alike than metachronous CBCs [7, 15]. Furthermore, the gene expression-based intrinsic subtypes of breast cancer can largely be recapitulated using DNA copy number variation (CNV) profiles [16], presenting a risk that nonclonal tumors or even those from different patients but of the same intrinsic subtype could appear similar by CNV profiles.

Recent studies have shown that breast cancer genomes are highly disorganized and can harbor tens to hundreds of chromosomal rearrangements [17, 18]. Interestingly, chromosomal rearrangements in breast cancer appear to be highly specific, and thus the set of rearrangements in any given tumor can serve as unique "fingerprint" or "barcode" of that tumor. This is in contrast to somatic point mutations, for which there are recurrent mutations across several genes that may be shared between tumors in unrelated persons. Importantly, it has been shown that extensive clonal diversity exists for point mutations within the same breast tumor mass, in contrast to structural chromosomal changes [19]. Therefore, chromosomal rearrangements are ideal for studies of clonal origin.

Until recently, however, a lack of feasible and comprehensive methods to study chromosomal rearrangements has prevented this chromosomal approach from being fully utilized. With the recent advancement of next-generation sequencing, global characterization of chromosomal rearrangements is now possible. Herein we have characterized the genomes of contralateral breast tumors using whole genome sequencing (WGS) to enumerate chromosomal breakpoints, serving as unique tumor barcodes, to determine clonal relationships between CBCs. For the first time we show with certainty that CBC can represent a metastatic spread of the first tumor. Given the poor prognosis of a generalized disease compared with a new primary tumor, these women need to be identified at diagnosis of CBC for appropriate determination of treatment and diagnostic work-up. We hereby present a promising method for determining the clonal relationship between tumors that may be clinically applicable in the near future.

Methods

Patients

Fresh frozen tumor tissue from 10 CBC patients with detailed patient and tumor information [1] and normal blood DNA from three of these patients and seven unrelated persons were obtained from the South Sweden Breast Cancer Group's tumor bank. Nine patients had two invasive tumors, and in Patient 1 BC2 was an in-situ lesion. This patient was included to exemplify a case presumed to be two independent primary tumors. The samples' quality and percentage of tumor cells were controlled with tissue arrays, evaluating an adjacent tissue piece. Paraffin material was available for 13 of 20 tumors, allowing histopathological reevaluation by a pathologist (AE). For the remaining cases these data were abstracted from the patient's chart. The project includes all necessary patients' consent regarding participation and right to publish, and was approved by the Regional Ethical Review Board of Lund (LU240-01). All data were

handled confidentially according to Sweden's Personal Data Act.

Whole genome sequencing and data analysis

Using DNA extracted from frozen tumor samples and normal blood samples, whole-genome paired-end Illumina (San Diego, CA, USA) sequencing libraries were generated, sequenced, and aligned to the human reference genome (see Additional file 1).

Sequencing data were analyzed bioinformatically to identify chromosomal rearrangements in each tumor and normal DNA sample (see Additional file 1). Initial filtering removed centromeric, segmentally duplicated regions, and intrachromosomal rearrangements <7 kb. Germline rearrangements (both ends matching within ±1 kb in any normal sample) were removed. For every comparison between two tumors (whether from the same patient or between patients), each rearrangement was classified as either shared or specific to one tumor as follows: rearrangements were considered shared between two tumors if the genomic coordinates for both ends of the rearrangement matched within ±500 base pairs; or to compensate for potential tumor subclonality (in case a rearrangement was present only in a subclone and therefore represented by fewer reads), each remaining rearrangement in a tumor was computationally "looked-up" in the aligned sequence BAM file of the other tumor, and if ≥1 read pairs matched within ±1 kb on both ends then the rearrangement was classified as shared. In addition, all rearrangements looked up and supported by at least one read pair in the normal samples were removed. The combined shared rearrangement fraction was calculated as the number of shared rearrangements divided by the number in the union of all rearrangements found in the compared tumors.

CNV was evaluated from the WGS data. CNV profiles were compared between samples based on windows delineated by the union of their segmentation breaks taking into account both copy number "state" (gain, normal, or loss) and "slope", corresponding to the difference between the current and previous window's state (see Additional file 1). Windows with the same state and slope were considered shared. The fraction of shared abnormal CNV events between two tumors was calculated after excluding windows with a normal diploid state in both tumors.

The rearrangements of each tumor were plotted using Circos [20], and rearrangement and CNV profiles were drawn using standard R graphical libraries [21]. Detailed methods are available in Additional file 1.

PCR validation of rearrangements

Rearrangements were validated with conventional PCR. Using our in-house SplitSeq bioinformatics pipeline, the local sequence around each breakpoint was retrieved [22]. Rearrangements for validation were randomly selected from all enumerated rearrangements of four patients (Patients 2, 6, 8, and 9). Primers were designed and touchdown PCR was performed on DNA extracted from the BC1 and BC2 (further details in Additional file 1). As germline control, matched normal DNA was used where available (Patient 9) or a normal DNA pool was created from normal lymphocyte DNA extracted from 44 healthy individuals.

Results

Clinical features

Tumor and treatment characteristics are presented in Table 1. Patient 10 was a known *BRCA1* mutation carrier. Patient 3 had a local recurrence from BC2 7 months after diagnosis of CBC, treated with surgery and endocrine therapy. Patient 1 had a local recurrence after BC1 12 months before diagnosis of BC2, treated with surgery, radiotherapy, and endocrine therapy. None of the other patients had any local/regional recurrences. Patient 4 developed mucosa-associated lymphoid tissue lymphoma 61 months after BC2. To avoid the risk of misdiagnosing metastases past this date, the diagnosis date of the lymphoma was considered the last follow-up date. For Patients 1 and 7, BC2 was diagnosed during endocrine therapy.

Paraffin material was available for both tumors in Patients 3, 4, 5, 6, 7, and 8 and for BC1 in Patient 2. Here histopathological markers have been reevaluated with immunohistochemistry by a pathologist. The cutoff value for estrogen receptor (ER)/progesterone receptor (PR)-positivity was >10 % positive cells. For the remaining tumors these data are from the patient's chart. For most of these patients (Patients 1, 9, and 10) ER and PR were measured with immunohistochemistry. For Patient 2 BC1 ER and PR were measured in cytosol (ER 140 fmol/mg protein, PR 170 fmol/mg protein; cutoff value for ER/PR-positivity = 25 fmol/mg protein).

Chromosomal rearrangements

WGS was performed on the 10 CBC pairs employing a strategy that combined low sequence coverage paired-end sequencing with larger fragment sizes for improved physical genome coverage. The median sequence coverage was 6.5 (range 1.8–11.2) and the median physical coverage was 14.7 (range 6.9–28.4) (Additional files 1 and 2). The total number of rearrangements detected in each tumor varied widely, from 15 to 256 with a median of 87 per tumor (Fig. 1a, Table 2; all plots in Additional file 3).

We were first interested in ascertaining how often two unrelated tumors from different individuals display identical chromosomal rearrangements because this would

Table 1 Clinical characteristics

Patient	Tumor	Laterality	Age at BC1	Histology	Multicentric	Size (mm)	Lymph node metastasis[a]	ER	PR	HER2[b]	Ki67	NHG	Treatment	Time interval BC1 – BC2 (months)	Metastasis after BC2[c] (months, location)
1	BC1	Left	61	Lobular	No	32	No (0/4)	Pos	Pos	UK	UK	2	Tam	61	Yes (15, lung, liver, skeleton)
	BC2	Right		DCIS	Yes	14	No (0/3)	NA	NA	NA	NA	3	None		
2	BC1	Left	41	Ductal	No	15	No (0/10)	Pos	Pos	UK	UK	2	None	5	No (249)
	BC2	Right		Ductal	No	14	No (0/5)	Pos	Pos	2+	9 %	3	None		
3	BC1	Left	83	Ductal	No	17	No (0/11)	Neg	Neg	Neg	30 %	3	None	23	No (8)
	BC2	Right		Ductal	No	25	No (0/12)	Neg	Neg	Neg	75 %	3	None		
4	BC1	Left	51	Ductal	No	25	Yes (9/21, PG)	Neg	Pos	3+	14 %	3	RT, CT (7 CMF)	24	No (61)
	BC2	Right		Ductal	No	25	Yes (7/28, PG)	Pos	Pos	Neg	16 %	3	RT, Tam		
5	BC1	Left	43	Ductal	No	10	No (0/23)	Neg	Neg	Neg	80 %	3	RT, CT (9 CMF)	22	Yes (13, liver)
	BC2	Right		Ductal	No	20	No (0/20)	Neg	Neg	Neg	27 %	3	RT		
6	BC1	Right	51	Lobular	Yes	70	Yes (3/8, PG)	Pos	Pos	Neg	28 %	2	RT, CT (9 CMF)	17	Yes (5, brain)
	BC2	Left		Lobular	Yes	12	Yes (23/26, PG)	Pos	Neg	Neg	22 %	2	CT (3 Doxo–2 T)		
7	BC1	Right	52	Ductal	No	16	No (0/11)	Pos	Pos	Neg	21 %	3	RT, Tam	30	No (66)
	BC2	Left		Ductal	No	16	No (0/1)	Neg	Neg	Neg	40 %	3	CT (6 FEC)		
8	BC1	Left	46	Lobular	No	80	Yes (33/34, PG)	Pos	Pos	Neg	4 %	3	RT, CT (9 FEC)	11	Yes (16, lung)
	BC2	Right		Lobular	Yes	55	Yes (11/25, PG)	Pos	Pos	Neg	5 %	2	RT, Tam + oophorectomy		
9[d]	BC1	Right	63	Ductal	No	28	Yes (2/13)	Pos	Pos	Neg	UK	2	Tam–AI	0	Yes (62, skeleton)
	BC2	Left		Ductal	No	55	Yes (1/12)	Pos	Pos	Neg	UK	3	RT, Tam–AI		
10	BC1	Right	32	Ductal	No	23	No (0/1)	Neg	Neg	UK	UK	3	RT, CT (6 FEC)	35	Yes (13, skin)
	BC2	Left		Ductal	No	45	Yes (12/12)	Neg	Neg	Neg	UK	2	RT		

AI aromatase inhibitor, *BC1* first breast cancer, *BC2* second breast cancer, *CMF* cyclophosphamide, methotrexate and fluorouracil, *CT* chemotherapy (cycles and regime used in parentheses), *DCIS* ductal carcinoma in situ, *Doxo* doxorubicin, *ER* estrogen receptor, *FEC* fluorouracil, epirubicin, and cyclophosphamide, *HER2* human epidermal growth factor receptor 2, *NA* not applicable, *Neg* negative, *NHG* Nottingham histological grade, *PG* periglandular growth, *Pos* positive, *PR* progesterone receptor, *RT* radiotherapy, *T* docetaxel, *Tam* tamoxifen, *UK* unknown

[a]Number of positive lymph nodes/number of investigated nodes in parentheses

[b]HER2 determined with immunohistochemistry (Herceptest) where score 0–1 has been classified as negative. For score 2+ and 3+ the individual score is given in the table

[c]If the patient developed metastases, the time interval between BC2 and diagnosis of metastasis (months) and the site of the first metastasis is given within parentheses. In patients who do not develop metastases, the follow-up period (months) is given within parentheses

[d]Patient 9 was diagnosed with her left and right breast cancer simultaneously (synchronous contralateral breast cancer), hence it cannot be said which tumor was first

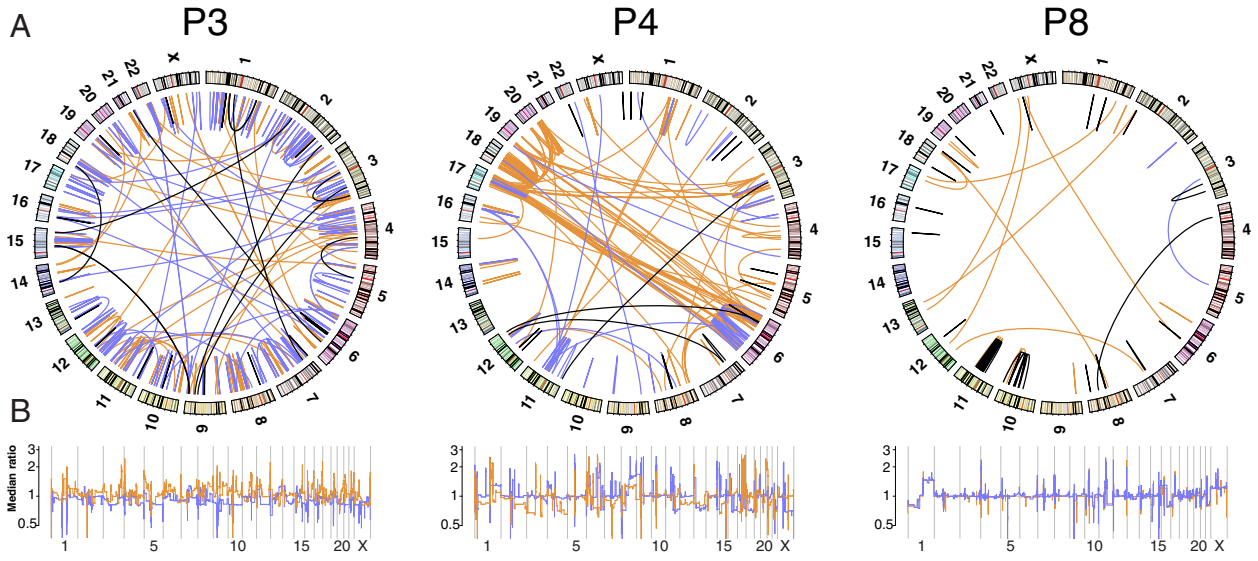

Fig. 1 a Chromosomal rearrangements for three patients visualized in Circos plots. **b** Copy number profiles for three patients. *Orange* denotes events (rearrangements or copy number traces) specific to BC1, *blue* denotes events specific to BC2, and *black* denotes events shared between BC1 and BC2. *P3* Patient 3, *P4* Patient 4, *P8* Patient 8

be an indication of overlap occurring by chance or due to incomplete filtering of germline events. For this, all possible pairings of tumors between patients (excluding the true within-patient pairing) were exhaustively compared. This comparison showed that 0–25 rearrangements (median 4) matched between any two random tumors with a combined shared fraction (i.e., the fraction of rearrangements shared between BC1 and BC2 divided by the union of all rearrangements detected in both tumors) ranging from 0 to 18 % (median 2 %; Table 2). For the comparisons of matched patient CBCs, on the other hand, the number of shared rearrangements between BC1 and BC2 ranged between 6 and 68 (median 28) and the combined shared fraction varied between 6 and 76 % (median 13 %).

Patient 8 was a clear outlier and had the most similar CBCs, with 76 % shared rearrangements between the two tumors (68 shared rearrangements), 19 rearrangements unique to BC1, and three rearrangements unique to BC2 (Fig. 1a, Table 2). Patient 8's extreme degree of similarity of chromosomal rearrangements unequivocally supports BC2 being the result of a metastatic spread from BC1. Furthermore, in a separate study of matched primary and distant metastases using the same experimental approach, we have found that the average primary breast cancer shares well over one-half of its chromosomal rearrangements with the distant metastasis it seeds (results not shown; Tang and Gruvberger-Saal, manuscript under review). Therefore, the 76 % shared rearrangements between CBCs in Patient 8 is highly consistent with known primary-metastasis pairs.

Seven of the remaining CBC patients had much fewer shared rearrangements between BC1 and BC2 with combined shared fractions between 6 and 15 % (median 10 %), most consistent with these CBCs having developed as independent primary tumors (Fig. 1a, Table 2; Additional file 3). However, Patients 2 and 6 had a rearrangement overlap (39 % and 46 %) between that seen for unmatched randomized tumor pairs and true tumor-metastasis pairs (including Patient 8). The cases of Patients 2 and 6 are highly suspicious for contralateral metastasis, but to classify them with certainty normal DNA samples would be needed to rule out any remaining germline rearrangements. Interestingly, for Patient 1, where BC2 was an in-situ lesion and included in the study as a CBC pair presumably representing two independent lesions, 15 % of rearrangements matched between the tumors. This overlap was considerably lower than the overlap for Patient 8 (76 %) but well within the range between CBC pairs when excluding the three most similar patients (Patients 2, 6, and 8) (6–13 %).

The similarities and differences in chromosomal rearrangements between tumors are well illustrated in a barcode plot, where all nonredundant identified rearrangements are arranged and their presence in each tumor is denoted by a line (Fig. 2). For example, the two tumors from Patient 3 were highly eventful yet displayed distinct rearrangement barcodes spread across the whole genome (Figs 1a and 2). Many tumors exhibited localized areas of high density of rearrangements suggestive of one catastrophic shattering event (chromothripsis). Chromothripsis is thought to contribute to

Table 2 Information on rearrangement and copy number variation

Patient	Tumor	Shared rearrangements	Total rearrangements	Fraction of rearrangements (%)	Combined shared fraction of rearrangements (%)	Fraction of shared aberrant copy number events (%)
1	BC1	7	15	47	15	18
	BC2	7	40	18		
2	BC1	53	64	83	39	15
	BC2	53	124	43		
3	BC1	28	151	19	9	10
	BC2	28	203	14		
4	BC1	17	256	7	6	18
	BC2	17	75	23		
5	BC1	27	67	40	13	20
	BC2	27	170	16		
6	BC1	62	86	72	46	23
	BC2	62	111	56		
7	BC1	33	128	26	12	22
	BC2	33	173	19		
8	BC1	68	87	78	76	28
	BC2	68	71	96		
9	BC1	20	84	24	10	25
	BC2	20	128	16		
10	BC1	6	58	10	7	28
	BC2	6	30	20		
Summary						
Paired tumors	Median	28	87	24	13	21
	Minimum	6	15	7	6	10
	Maximum	68	256	96	76	28
Random pairs	Median	4	75	4	2	13
	Minimum	0	6	0	0	6
	Maximum	25	254	43	18	22

BC1 first breast cancer, *BC2* second breast cancer
Summarized numbers for randomized matching

oncogene activation and tumor suppressor loss, and as a consequence is an important driving force for early cancer development and would probably be shared between clonal tumors. For Patient 8 this appears to be true; the two tumors share identical chromothripsis-like rearrangement patterns in two localized hotspots on chromosomes 10 and 11 (Figs 1a and 2). On chromosome 11 alone the two tumors share 38 identical intrachromosomal rearrangements, while only five are specific to BC1 and none to BC2. Patient 4, on the other hand, has localized areas on 1q, 6p, and chromosomes 17 and 18 of chromothripsis-like patterns that only appear in BC1, whereas BC2 has specific high-density areas on 6q. Patient 9, with a combined percentage of shared rearrangements of 10 %, has very distinct heavily rearranged areas on 11q and chromosome 12 unique to BC1, while BC2 has areas suggesting chromothripsis on chromosomes 1, 3, and 11p that are not being shared with BC1, further indicative of their nonclonal origin.

A number of cancer-associated genes (e.g., *RB1*, *RARA*, *CDKN2A*, and *PTEN*) were found to be involved in the enumerated rearrangements [23], probably contributing to tumorigenesis (Additional file 4).

Indicative of a suitable reliability of our analysis pipeline, 68 rearrangements out of 86 (79 %) were confirmed by PCR across the breakpoint junction as either specific to BC1, specific to BC2, or shared. However, nine of these rearrangements (13 %) were also identified in the matched normal DNA, or if unavailable were identified in a pool of 44 normal DNA samples. Therefore, although our pipeline results in a good accuracy of calls based on the sequencing data, owing to the fact that matched normal DNA were unavailable for most patients and

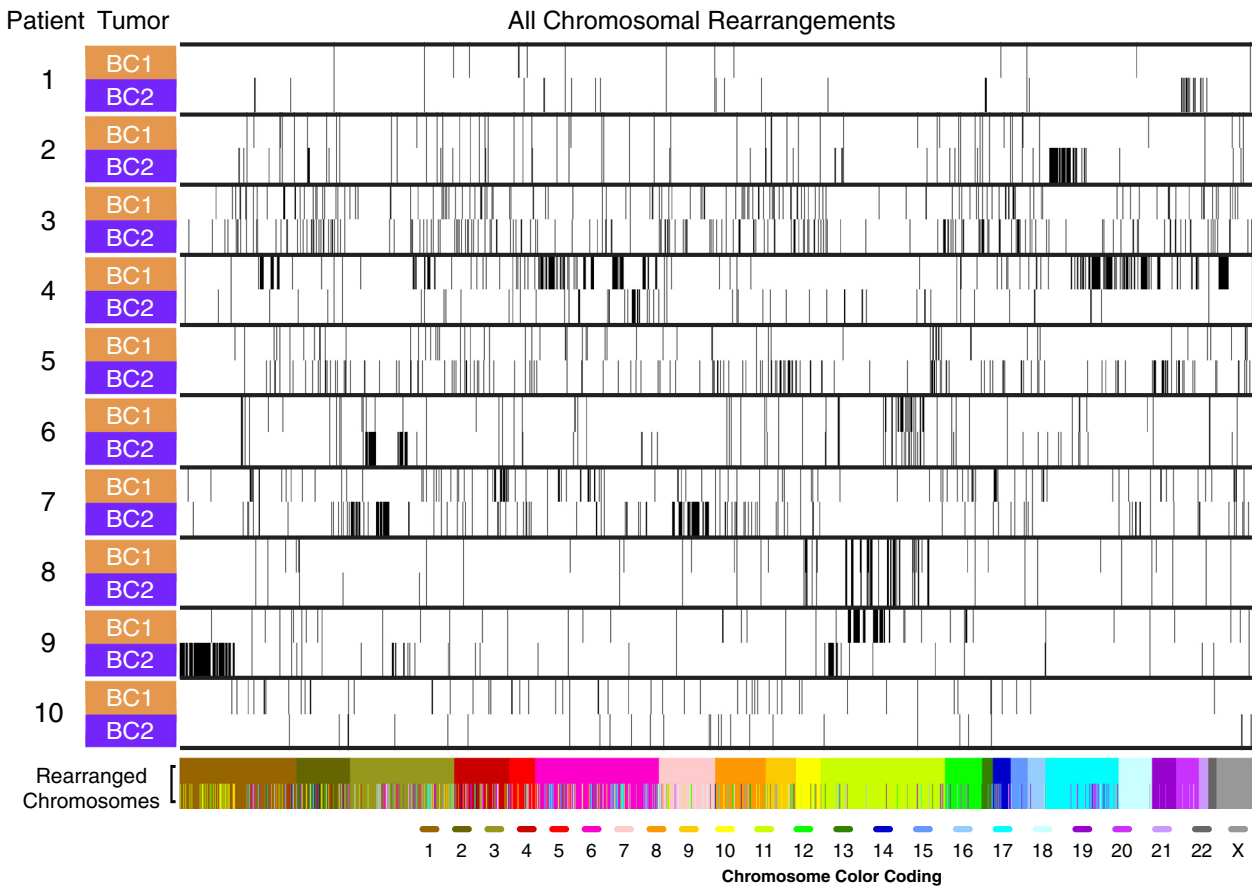

Fig. 2 Tumor genetic barcodes of chromosomal rearrangements for individual tumors. All nonredundant chromosomal rearrangements were included and their presence in each tumor denoted by a line. The rearrangements were plotted in the order of genomic location for the end of the fusion that appears first in the genome. Color coding below indicates which two chromosomes where involved in each rearrangement. *BC1* first breast cancer, *BC2* second breast cancer

thus were not sequenced, our results are underestimating to some extent the contribution of rearrangements that are present in the germline.

Copy number variation

CNV profiles were also evaluated using the WGS data (Fig. 1b; Additional file 3). The fraction of shared abnormal copy number events between CBC pairs ranged between 10 and 28 % (median 21 %; Table 2). When exhaustively comparing all pairings of tumors from different patients, the fraction of shared aberrant copy number events ranged between 6 and 22 % (median 13 %), which was only slightly lower than that seen for the matching tumor pairs. Again, the CBCs from Patient 8 had the highest fraction of shared aberrations (28 %) and the CNV profiles appeared similar (Fig. 1b; Additional file 3). Patient 1, with BC2 representing an in-situ lesion, shared 18 % of the aberrant copy number events between the two tumors.

Clonal relationship between contralateral tumors

The analysis of genomic clonal similarities between tumors can be visualized in two dimensions (Fig. 3). When looking at chromosomal rearrangements, the majority of the CBC pairs (blue) cluster together just slightly off the cloud of randomly-paired tumors (red) (Patients 2 and 6 showed a somewhat higher similarity as discussed above). Patient 8, on the other hand, is clearly distantly separated from the other CBC pairs (including Patient 1 with an in-situ BC2) and from the randomized pairings.

For the CNV events this separation is not evident and no patient CNV profiles are more similar than some of the tumor pairings from different individuals. Based on the analysis of rearrangements, however, we can conclude that BC2 from Patient 8 in fact is highly clonally related to BC1.

Discussion

By harnessing the power of WGS-detected tumor-specific chromosomal rearrangement barcodes, we have

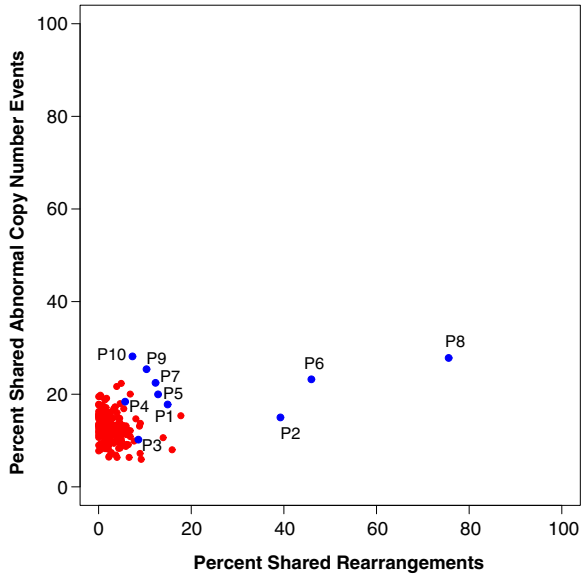

Fig. 3 Fraction of shared abnormal copy number events between BC1 and BC2 plotted against the fraction of shared chromosomal rearrangements between BC1 and BC2. All comparisons between tumors from the same patient are plotted in *blue*, while all possible random pairings of tumors from different individuals are shown in *red*. *P* Patient

for the first time conclusively demonstrated that breast cancer can arise as a metastatic clone from a tumor in the contralateral breast. Among the 10 CBC pairs in this study, the Patient 8 CBC pair exhibited 76 % rearrangement similarity between the left and right tumors. Our analyses showed that chromosomal rearrangements rarely recurred among randomly paired samples (median shared fraction, 2 %; median shared number, four rearrangements; Table 2), and hence chromosomal rearrangements uniquely characterized the tumors. In a separate study of primary-metastasis pairs using the same experimental approach, we have found that the average primary breast cancer appears to share about 50 % or more of its chromosomal rearrangements with the distant metastasis it seeds (Tang and Gruvberger-Saal, manuscript under review). This is highly consistent with the genetic chromosomal rearrangement fingerprint of Patient 8, and thus we can confidently conclude a clonal relationship between BC1 and BC2 for this patient.

However, two patients showed intermediate levels of rearrangement similarity (39 and 46 %). This is higher than that seen in unmatched randomized pairing, but on the lower end of what we have seen for primary-metastasis pairs (including Patient 8). A limitation in our study is the unavailability of matching normal DNA for every CBC patient. WGS analysis of matching normal DNA would have allowed for complete filtering of all germline chromosomal-rearrangement events and CNVs. Although the vast majority of germline events have been removed by our data filtering steps and by comparison with unmatched normal DNA samples from the Swedish population, matched normal DNA would probably have clarified the clonal relationship in these two uncertain cases. Nevertheless, the purpose of this study was to show that contralateral metastasis does exist and can be reliably detected with newer technologies. Owing to the extreme degree of similarity of chromosomal rearrangements in Patient 8, there is no doubt of the clonal relationship between BC1 and BC2 in this patient. Further studies are needed, however, to determine the prevalence of "metastatic CBC", optimal diagnostic work-up and treatment. In addition, further comparisons between known primary-metastasis pairs versus unrelated tumor pairs are required to find the optimal cutoff value for the fraction of shared rearrangements indicating clonality, and to make sure that all CBCs actually representing metastatic events are correctly identified.

Clinically, CBC events are today considered to be two separate tumors, and adjuvant treatment is recommended on the basis of the clinicopathological characteristics of BC1 and BC2 individually. However, since metastatic breast cancer has worse prognosis than and a different treatment regimen from that for localized breast cancer, our finding gives a new important perspective on the clinical management for women diagnosed with CBC. A more careful diagnostic work-up is necessary in order to determine an appropriate treatment strategy, and we have developed a novel approach to ascertain the metastasis status of a CBC that is affordable and can be performed in a clinically relevant time frame. In addition, access to normal DNA would not be a problem in the clinic, increasing sensitivity and specificity of the analysis.

Whether a patient with a contralateral metastasis has an equally poor prognosis as a patient with distant metastases at other sites (lung, liver, brain, etc.) or whether the outcome associated with a contralateral metastasis, if treated correctly, could be more similar to that of a local or regional recurrence are questions that deserve further study. If a contralateral metastasis represents a cancer still prone to thriving mainly in breast tissue, or possibly a local lymphatic spread to the contralateral breast, we may with the right treatment still be able to prevent further spread and a fatal outcome. If, on the other hand, a contralateral metastasis is already a sign of an incurable metastatic disease, treatment may instead be focused on quality of life rather than intense adjuvant therapy.

Among our 10 patients, six developed generalized disease within 5–62 months from BC2. Patient 8, in whom our analysis determined BC2 to be a contralateral metastasis, was diagnosed with lung metastases 16 months after BC2. Since she was treated with radiotherapy, tamoxifen,

and oophorectomy for BC2, the endocrine treatment given could have helped in delaying development of further metastases. Our study is too small to draw any conclusions regarding prognosis after contralateral metastasis, but it is an important question with significant clinical implications, which needs to be further evaluated.

An important issue is how one may identify patients with a high risk of BC2 representing a metastasis instead of a new primary tumor. Are there clinical indications as to when contralateral metastasis should be suspected and further investigation with WGS is warranted? Patient 8 had a very large BC1 (80 mm), widespread lymph node metastasis, and both tumors were lobular type and had similar hormone receptor, HER2, and Ki67 expression. Further studies will show whether these characteristics could be indicators of a risk for contralateral metastasis. Of note, the Nottingham histological grade of Patient 8 was classified as grade 3 in BC1 and grade 2 in BC2. This is particularly interesting since, using the traditional criteria summarized by Chaudary et al. [24], bilateral carcinomas are considered independent if: the subsequent tumor has an in-situ component; or the lesions are of distinct histological subtypes; or the subsequent cancer has a better degree of differentiation; or there is no evidence of local, regional, or distant metastases from the ipsilateral lesion. Clearly, these clinical criteria do not accurately detect the contralateral metastasis in our material. Furthermore, for seven of the remaining eight invasive CBC pairs, the CBCs shared the same histological subtype and did not have a better degree of differentiation for BC2 (data on in-situ component unknown) and would consequently be classified as possible metastases. Routine pathological markers do not therefore appear to adequately separate new primaries from contralateral metastases.

Since CBC tumors arise and develop in the same genetic and environmental background, and genetic predispositions such as a germline BRCA1 mutation (also associated with basal-like tumors) are more common [25], it is conceivable that bilateral tumors more often will be of the same intrinsic subtype and may accumulate similar patterns of mutations and copy number aberrations, even when they arise as independent tumors. Indeed, while CNV profiles carried some information on genomic similarities in our data, none of the matched pairs had significantly more similar CNV profiles than some unrelated tumors. However, using the rearrangement barcodes, even Patient 10, a germline *BRCA1* mutation carrier, shared very few rearrangements between BC1 and BC2 and a different clonal origin could be determined (Additional files 3 and 4).

Conclusion

By using next-generation sequencing and an analytical strategy based on chromosomal rearrangements, we can for the first time show strong evidence that some CBCs indeed represent a metastatic spread of BC1. Our approach generated a unique tumor barcode that can assess the clonal relationship between tumors. This is a promising new method not only for management of CBCs, but also in a variety of other cancer types where the question of clonality and tumor heterogeneity raises important clinical issues. Although our study had the disadvantage of a lack of matched normal DNA for most patients, this would not be a problem in the clinical setting, allowing for complete filtration of germline defects and increasing specificity and sensitivity of the method. Further studies are needed in order to identify the optimal cutoff level to with certainty not miss any CBCs actually representing a metastatic event.

CBCs are today treated as two individual tumors, but if BC2 instead represents a metastatic disease state the patient would have a worse prognosis and require a different treatment than a new primary breast cancer. With intensified treatment for these patients there may be a possibility to prevent further spread, and avoid development of a generalized incurable breast cancer.

Additional files

Additional file 1: presents a detailed description of supplemental methods. (DOCX 30 kb)

Additional file 2: is Table S1 presenting sequencing statistics. (DOCX 14 kb)

Additional file 3: is Figure S1 showing chromosomal rearrangements visualized using Circos and copy number profiles for all 10 CBC patients. *Orange* denotes events (rearrangements or copy number traces) specific to BC1, *blue* denotes events specific to BC2, and *black* denotes events shared between BC1 and BC2. *P* Patient. (PDF 1524 kb)

Additional file 4: is Table S2 presenting a list of genes affected by rearrangements and found in the COSMIC Catalogue of somatic mutations in cancer [26]. (XLS 116 kb)

Abbreviations

BC1: First breast cancer; BC2: Second breast cancer; CBC: Contralateral breast cancer; CGH: Comparative genomic hybridization; CNV: Copy number variation; ER: Estrogen receptor; PR: Progesterone receptor; WGS: Whole genome sequencing..

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

SA, M-HET, LR, LHS, MF, and SKG-S contributed to study conception and design. All authors contributed to collection and assembly of data. SA, M-HET, CB, MD, YC, EO, CW, AE, LR, LHS, MF, and SKG-S contributed to data analysis and interpretation. SA, M-HET, MF, and SKG-S had main responsibility for manuscript writing, but all authors were involved in drafting the manuscript. All authors read and approved the final manuscript, as well as revised it critically for intellectual content. All authors agree to be accountable for all aspects of the work.

SB contributed to collection and assembly of data, and contributed to data analysis and interpretation. She was also involved in drafting the manuscript, and has read and approved the final manuscript, as well as revised it critically for intellectual content.

Authors' information
SA and M-HET share first author status, and MF and SKG-S share last author status.

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Author details
[1]Division of Oncology and Pathology, Clinical Sciences Lund, Lund University, MV 404-B2, Lund SE-22381, Sweden. [2]Skåne Clinic of Oncology, Skåne University Hospital Lund, Lund SE-22241, Sweden. [3]Department of Pathology and Cytology, Blekinge County Hospital, Karlskrona SE-37185, Sweden. [4]Clinic of Surgery, Skåne University Hospital Lund, Lund SE-22241, Sweden.

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