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Whole Genome Microarray Analysis in Invasive Ductal Breast Cancer Revealed the most Significant Changes Affect Chromosomes 1, 8, 17 and 20

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Abstract: Despite of the large number of molecular studies in breast cancer, the data are still insufficient for understanding its molecular pathogenesis. The dramatic development of genetics in recent years has made it possible to gain insight into the molecular mechanisms of tumorigenesis. The aims of the study was to determine the type, frequency and fine mapping of unbalanced genomic alterations in ductal carcinoma of the breast. For this study we have used tumor samples of invasive ductal breast cancer to be analysed by comparative genomic hybridization on DNA microarrays. Two approaches were applied in the analysis of significant unbalanced genomic changes: a) identification of clones which presented unbalanced changes ($\log_2 T: H > +0.25$ for gain and < -0.25 for losses) in more than 70% of the tumors; b) establishing clones which harbor amplifications ($\log_2 T: H > +0.5$) or homozygous deletions ($\log_2 T: H < -0.5$) in more than 50% of tumors. Our results showed that the most commonly affected chromosome arms by gains were 20q and 1q. Loss at high frequency was found in 8p. Totally 9 regions with high rate of genetic alterations (7 in short and 2 in long arm) were detected in chromosome 1. Genetic losses in chromosome 8 predominated - 8p11.21 have lost in 100% of the cases. There were regions of chromosome 17 with a frequency of genetic gains over 70% - in 17q12, 17q21.31, 17q22 and a region with a frequency of more than 80% loss in 17q21.32. The long arm of chromosome 20 was affected by significant amplifications in 10 regions, but with the highest frequency of more than 80% in 20q11.21-q11.23, 20q12 and 20q13.12-q13.2. Our study contributed to the fine mapping of genomic imbalances in ductal breast cancer, suggesting the significant regions of genetic gains and losses. The genes in the found regions could represent potential oncogenes and/or tumor-suppressor genes with cancer-emerging role.

Keywords: ductal breast cancer, array CGH, oncogenes and tumor-suppressor genes

INTRODUCTION

Despite of the large number of molecular studies in breast cancer, the data are still insufficient for understanding its molecular pathogenesis. The dramatic development of genetics in recent years has made it possible to gain insight into the molecular mechanisms of tumorigenesis. In breast cancer several different combinations of genes are involved that are likely to determine the aggressiveness of the cancer [1].

Genetic mapping in tumors of the breast tissue established multiclonality, which implies the presence of intratumoral heterogeneity at a high level [2]. Frequent changes occur in the chromosome arms 1p, 3p, 6q and 8p; i(1q) and der t(1, 16) as well as single aberrations such as +7, +8 and +20; DNA amplifications such as homogeneously stained regions (HSR), which are mainly related to 8p [3-5]. Aberrations leading to addition or loss of part of the genome can be determined by molecular cytogenetic

techniques: comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH). Such aberrations are interstitial deletions, duplications, non-reciprocal translocations and gene amplifications.

Comparative genomic hybridization (CGH) is used to examine the entire genome for identification of quantitative changes in DNA sequences – genetic gain/amplifications and losses (deletions). CGH assay is based on competitive hybridization between DNA from the sample (ie. test DNA) and DNA from normal cells (reference DNA) to identify chromosomal regions with additional or absent genetic material in the tumor genome.

Many researchers are trying to identify genes involved in chromosomal regions that show the imbalance in CGH. Most of these studies focus on the amplification where it is expected that the genes are activated (overexpressed). Several candidate - target



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gens are already described in the hot areas - AIB1, BTAK, NABC1 and ZNF217 [6]. They are involved in the amplifications and related overexpression of 20q13 and induce phenotypic effects in the cells which carry these modifications. For more than 20 "hot" amplification regions exist throughout the genome, however, the target genes remain unknown.

Tumors can be classified based on their genomic profile, determined by CGH-microarrays [7-10]. Based on these genomic profiles it is possible to perform:

- selection of certain genes from the affected areas for the study of gene expression;
- identifying different types of genomic instability.

Thus, tumor-specific genomic profiles created "genomic phenotypes" of tumors.

Many studies use the advantage of the high resolution of the method for more precise mapping of boundaries and size of the amplified region. Once this is known, the candidate oncogenes in this region or in the amplification maximum can be easily identified by a "genome sequence databases". The study of the expression levels of these candidate genes in tumors and cell lines can further determine which of the candidates at most contribute to the phenotype and are "driving" genes in the amplified region [11-13].

The purpose of this study was to determine the type, frequency and fine mapping of unbalanced genomic alterations in ductal carcinoma of the breast by comparative genomic hybridization on microarrays covering the entire genome.

MATERIALS AND METHODS

For this study we have used 10 tumor samples of invasive ductal breast cancer analysed by comparative genomic hybridization on DNA microarrays covering the whole genome with an average density of one BAC clone / 0.8 Mb. Materials and microarrays are kindly provided by the laboratory "Identité et plasticité des tumeurs", Montpellier, France.

Array CGH

The method is based on competitive hybridization of alternatively labeled tumor and normal DNA on BAC clones applied to microchips. The method allows quantitative detection of abnormalities in the genetic material of tumor cells relative to normal, and the determination of the position of the additional or missing DNA sequences according to the affected branch. In this work against each tumor DNA was hybridized DNA from normal female.

DNA isolation, labeling and hybridization

Isolation of DNA by phenol-chloroform extraction is based on the extraction of proteins and other constituents of the cell lysate in the organic phase, DNA remains in the aqueous phase and precipitated with ethanol. Labeling of genomic DNA was performed using a random priming. The presence of labeled DNA was estimated on a spectrophotometer at the following wavelengths: 260 nm for DNA, 550 nm for Cy3 and 650 nm for Cy5. Incorporation of fluorochrome was calculated by Molecular probes software:

<http://www.probes.com/resources/calc/basedyeratio.html>. For subsequent hybridization was taken only samples with incorporation of less than 50 (i.e. 1 fluorochrome per 50 base pairs) or 525 pmol DNA, calculated by the software program.

Mix 700 pmol Cy-5 and 700 pmol Cy-3 labeled probe is precipitated in ethanol in the presence of 250-300 µg of human Cot-1 DNA (Roche Diagnostics, Meylan, France) and 100 µg DNA from herring sperm (Promega, Charbonnières, France). The microchip was then dried and resuspended in 110 µl hibrizol VII (Appligene Oncor, Qbiogen, Illkirch, France). It continues with hybridization and washing of the samples.

Scanning and software analysis of the images

The slides were scanned by GenePix 4100 A. The images were analyzed with the software program GenePix Pro 6.0. Spots on the chip were outlined and defined automatically by the software and manually where necessary. It calculated fluorescent intensities of all spots after elimination of the local background. Intensities of Cy3 and Cy5 were normalized to the entire set spots on the chip and then the ratio Cy3 / Cy5 was calculated (when tumor DNA was labeled with Cy3), taking the average of the ratio of the replicates for each clone (analyzed only in spots less than 15% deviation from the average of the replicates). All these data are processed in program CapWeb array CGH, which is generated by the profile of each tumor with fluorescent log₂ value ratios of all BAC clones on the ordinate and Mb position of BAC clones on the abscissa. Fluorescent ratio values above 0.25 are reported as gain, and over 0.5 - as amplification and values below -0.25 are reported as a loss and below -1 - as homozygous deletion.

RESULTS

In this work the method of array CGH was applied using DNA microarrays covering the entire genome, which allowed full-genome analysis of unbalanced genetic alterations in 10 primary ductal carcinoma of the breast. Microchips we used were commercially available and contain clones of 12 452 bacterial

artificial chromosome (BAC) with an average density of one BAC / 0.8 Mb. Figure 1 shows a single profile with $\log_2 T: N$ ratios for all clones in all chromosomes in tumor № 4. corresponding karyotype of a tumor is presented in Figure 2.

Array CGH analysis found that the average increased copy number (gain and amplification) was 8.3% of the clones, while the average reduced number of copies was 7.8% of clones.

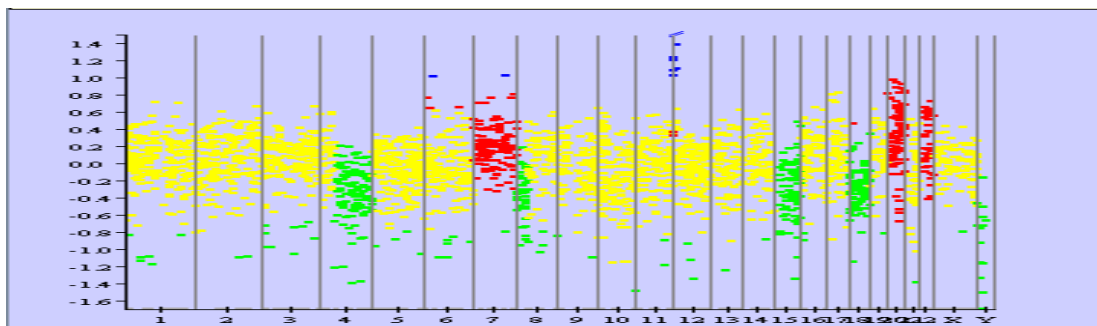


Fig. 1. Single profile with $\log_2 T: N$ ratios for all clones in all chromosomes in tumor № 3. The abscissa represents all chromosomes; genetic gains are colored in red, whereas genetic losses – in green.

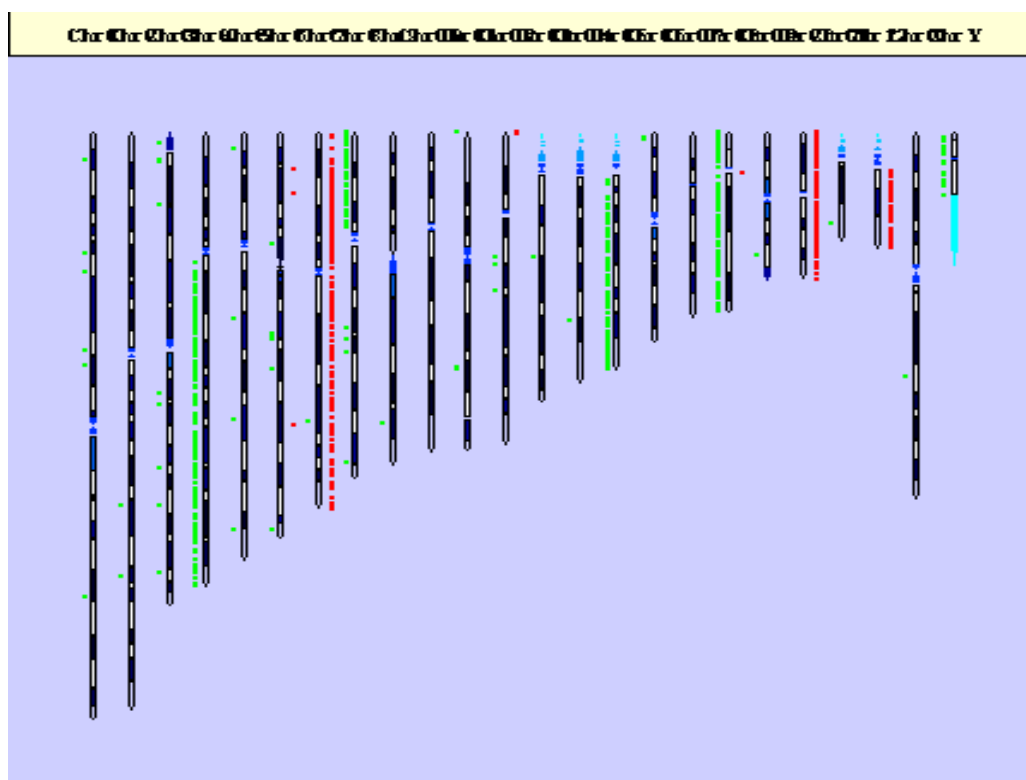


Fig. 2. Corresponding karyotype of tumor № 3 – losses are presented in green and gains in red.

Analysis of genomic instability in the tumors

To assess the degree of genomic instability, tumors were classified into three groups according to the average number of copy number alterations (ACNA), which according to our results was 1319 clones: Group A - instability below ACNA; group B - over ACNA and group C - more than 2 times above ACNA.

Tumors in each group were subdivided depending on the prevalence of genetic losses (1), the genetic gain (2) or equivalent participation of gains and losses (3) (Table 1).

Table 1. Data about number of clones with altered copy numbers in all tumors and grouping them by type of genomic instability.

TUMOR	NUMBER OF GAINED CLONES	NUMBER OF LOSSED CLONES	TOTAL NUMBER OF CHANGED CLONES	GENOMIC INSTABILITY GROUP
Ca01	57	53	110	A3
Ca02	32	35	67	A3
Ca03	98	115	213	A2
Ca04	781	809	1590	B3
Ca05	730	634	1364	B1
Ca06	1287	1131	2418	B1
Ca07	1953	1874	3827	C3
Ca08	826	691	1517	B3
Ca09	913	821	1734	B1
Ca10	762	854	1616	B2

The highest percentage of the analyzed tumors fall into group B of genomic instability, in half of the cases we observed prevalence of genetic gains. Three of the tumors showed a degree of genomic instability lower than average, and only one tumor was assigned to group C of genomic instability. The last had equal participation of genetic gains and losses. These results are a starting point for tracking the effects of treatment and course of the disease in each of the patients to assess the impact of genomic instability on these indicators.

Analysis of aberrations, involving whole chromosomes or chromosome arms

The involvement of chromosomes in large aberrations (gains and losses of whole chromosomes or chromosome arms) is presented in Figure 3 a) for chromosomes 1 to 10 and in Figure 3 b) for chromosome 11 to D.

According to our results, the most frequently affected by large aberrations (aneuploidies of whole chromosomes and chromosomal arms) are chromosomes 8 and 20, and such changes have been found in 90% of tumors studied. They are followed by chromosomes 4 and 15 (60%), 18 and 21 (50%), 5, 7, 13 and X (40%), 11, 12, 16, 19 and 22 (30%), 1 and 17 (20%), 2, 6, 9, 10 and 14 (10%). Chromosome 3 was not affected by large aberrations in any of the tumors.

The most commonly affected whole chromosomes or chromosome arms by gains were chromosome 20 (80%) and chromosome 7 (40% of cases) (see Table 2). In 30% of tumors we discovered aneuploidies in chromosomes 8, 12, 16, 19 and X. These changes were found in 20% of cases for chromosomes 1, 13, 17 and 22. Gains of chromosomes 4, 5, 6, 9 and 11 occur in 10% of the tumors. No large gains were found on chromosomes 2, 3, 10, 14, 15, 18 and 21.

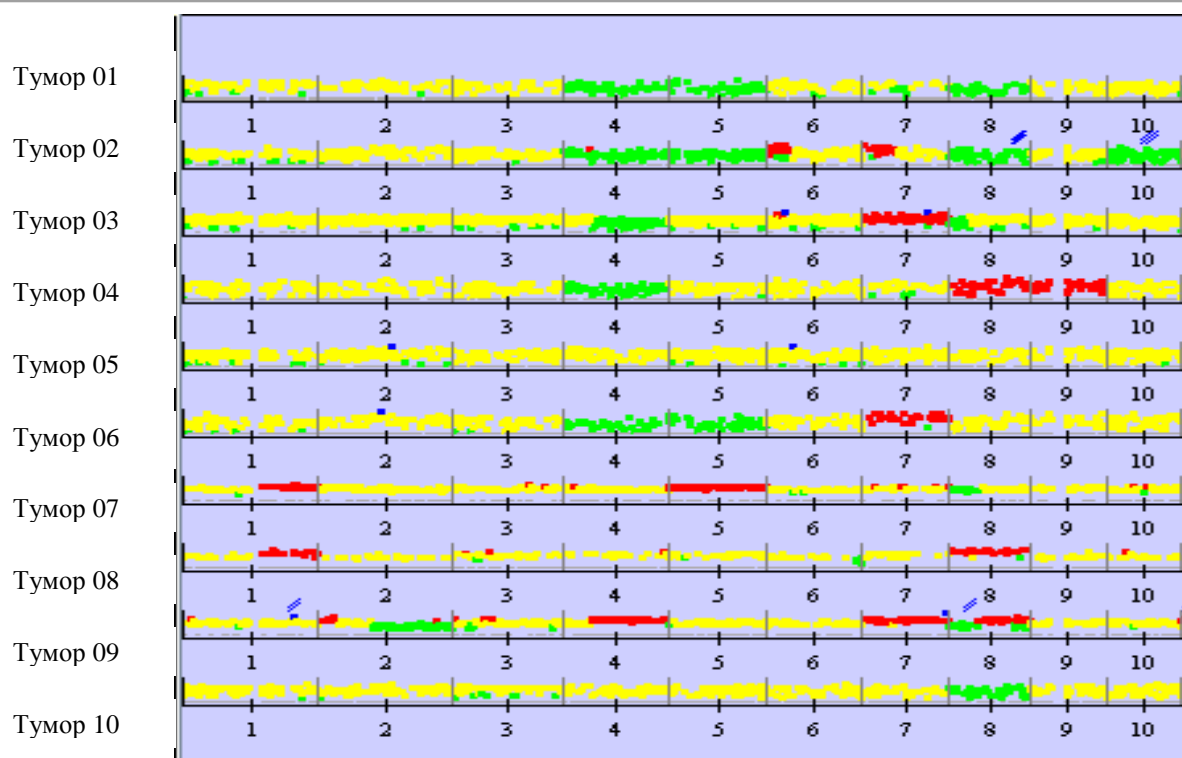


Figure 3 a). Large aberrations in chromosomes 1 to 10. Genetic gains are presented in red and losses – in green.

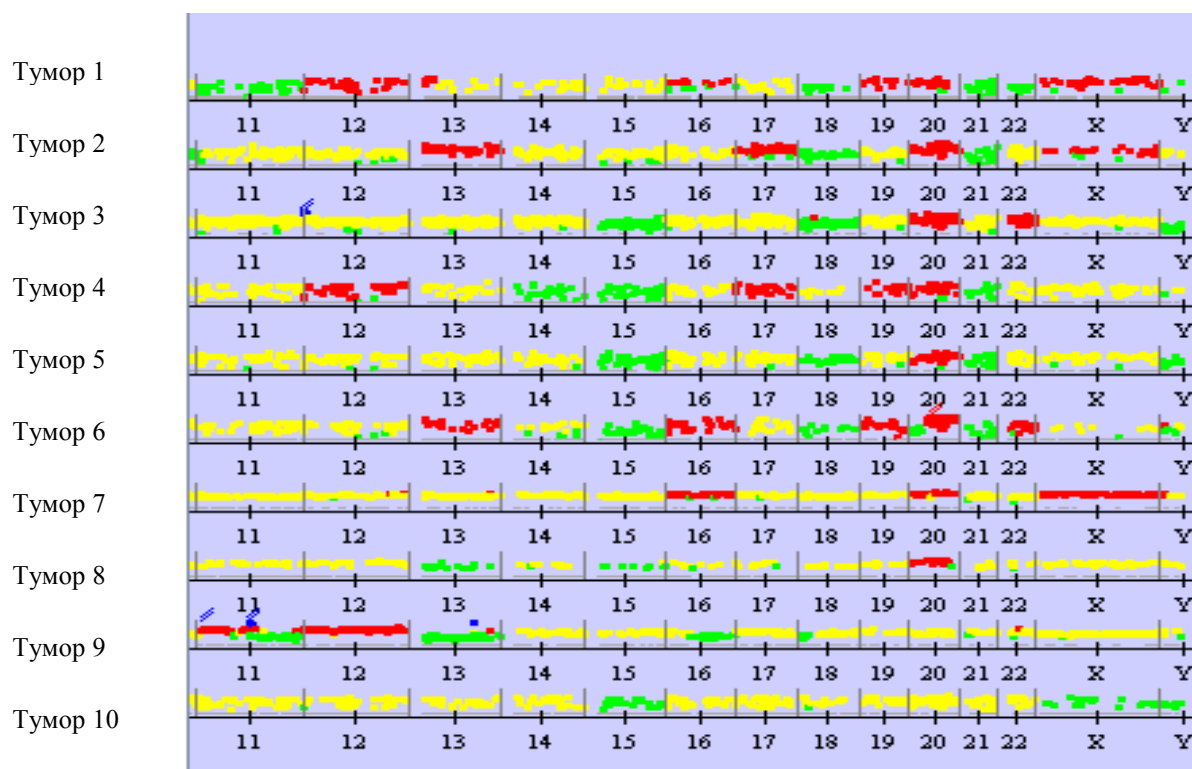


Figure 3 b). Large aberrations in chromosomes 11 to Y. Genetic gains are presented in red and losses – in green.

Table 2. Frequency for gains of whole chromosomes or chromosome arms.

CHROMOSOME	TRISOMY	GAIN OF SHORT ARM	GAIN OF LONG ARM	TOTAL GAINS
chromosome 1	0	0	20%	20%
chromosome 2	0	0	0	0
chromosome 3	0	0	0	0
chromosome 4	0	0	10%	10%
chromosome 5	10%	0	0	10%
chromosome 6	0	10%	0	10%
chromosome 7	30%	10%	0	40%
chromosome 8	20%	0	10%	30%
chromosome 9	10%	0	0	10%
chromosome 10	0	0	0	0
chromosome 11	0	10%	0	10%
chromosome 12	30%	0	0	30%
chromosome 13	20%	0	0	20%
chromosome 14	0	0	0	0
chromosome 15	0	0	0	0
chromosome 16	30%	0	0	30%
chromosome 17	20%	0	0	20%
chromosome 18	0	0	0	0
chromosome 19	30%	0	0	30%
chromosome 20	40%	0	40%	80%
chromosome 21	0	0	0	0
chromosome 22	20%	0	0	20%
chromosome X	30%	0	0	30%

The highest incidence of large losses (Table 3) had chromosomes 8 and 15 (60%), followed by chromosomes 4, 18, and 21 (50%), chromosome 5 (30%) and chromosomes 11 and 13 (20%). Low

frequency loss was observed at 2, 10, 14, 20, 22 and X chromosome (10%). Absence of deletions had chromosomes 1, 3, 6, 7, 9, 12, 16, 17 and 19.

Table 3. Frequency for genetic losses of whole chromosomes and chromosome arms.

CHROMOSOME	MONOSOMY	LOSS OF SHORT ARM	LOSS OF LONG ARM	TOTAL LOSSES
chromosome 1	0	0	0	0
chromosome 2	0	0	10%	10%
chromosome 3	0	0	0	0
chromosome 4	40%	0	10%	50%
chromosome 5	30%	0	0	30%
chromosome 6	0	0	0	0
chromosome 7	0	0	0	0
chromosome 8	30%	30%	0	60%
chromosome 9	0	0	0	0
chromosome 10	10%	0	0	10%
chromosome 11	10%	0	10%	20%
chromosome 12	0	0	0	0
chromosome 13	20%	0	0	20%
chromosome 14	10%	0	0	10%
chromosome 15	60%	0	0	60%
chromosome 16	0	0	0	0
chromosome 17	0	0	0	0
chromosome 18	50%	0	0	50%
chromosome 19	0	0	0	0
chromosome 20	0	10%	0	10%
chromosome 21	50%	0	0	50%
chromosome 22	10%	0	0	10%
chromosome X	10%	0	0	10%

Analysis of significant gains and losses and their fine mapping in ductal breast cancers

In this study, two approaches were applied in the analysis of significant unbalanced genomic changes:

a) identification of clones which presented unbalanced changes ($\log_2 T: H > +0.25$ for gain and < -0.25 for losses) in more than 70% of the tumors;

b) establishing clones which harbor amplifications ($\log_2 T: H > +0.5$) or homozygous deletions ($\log_2 T: H < -0.5$) in more than 50% of tumors.

Genetic loss in 1p36.33, 1p32.3 and 1p32.1 occurred in 100% of the cases and in the 1p22.1-p13.3 and 1q23.3-q41 – in 80% (Table 4). In the short arm of

chromosome 8 we detected high frequency of loss for 8p11.21 and 8p23.1-p12. In the long arm, loci in 8q21.13, 8q21.3 and 8q22.1-q24.23 are characterized with the same frequency of losses (Table 4).

We found genetic gains along the entire chromosome 17, comprising several clones. In the long arm there was an area with a high percentage of amplification - in 17q11.2-q22 and 17p13.3 (over 70% of tumors) (Table 5), while high frequency loss was found in 17p11.2 and 17q21.31-q24.3 (Table 4).

In our study we observed in the short arm chromosome 20 a region with amplification of 80% frequency - 20p11.21. The long arm of chromosome was affected by significant amplifications in 10 regions, but with the highest frequency of more than 80% in 20q11.21-q11.23, 20q12 and 20q13.12-q13.2 (Table 5).

Among the important gains there were clones containing known oncogenes such as MUC1 gene in 1q21, LASP1 (17q11-q21.3) and HER-2 (17q21.1), ZNF217 and AURKA (STK6) at 20q13.2-q13.3.

Table 4. Genomic regions with more than 70% frequency of genetic loss

Locus	Start	End
1p36.33	579 118	696 613
1p32.3	51 595 439	51 736 659
1p32.1	58 891 581	59 033 455
1p22.1-p13.3	91 775 342	108 930 877
1q23.3-q41	159 470 259	214 931 488
8p23.1-p12	11 571 054	38 425 535
8p11.21	42 287 375	42 448 698
8q11.21-q21.3	51 933 535	93 370 656
8q21.13	82 451 505	82 622 516
8q21.3	90 221 558	90 371 675
8q21.3	93 214 273	93 370 656
8q22.1-q24.23	95 287 683	139 301 569
17p11.2	21 014 529	21 191 516
17q21.31-q24.3	38 944 628	68 436 847
20q12-q13.12	38 611 620	43 171 967

Table 5. Genomic regions with more than 70% frequency of genetic gain

Locus	Start	End
17p13.3	2 027 295	2 250 415
17q11.2-q22	23 921 094	50 904 855
20p11.21	23 173 081	23 348 803
20q11.21-q11.23	30 040 110	36 711 090
20q12	38 611 574	40 190 025
20q13.12-q13.2	42 555 465	51 966 525

DISCUSSION

Genomic aberrations with altered DNA copy number at sub-microscopic level is a hallmark of epithelial tumors, including breast cancer. The introduction of comparative genomic hybridization in 1992 has opened new possibilities for the study of the genome and in particular, the methodology has contributed significantly to the development of research on solid tumors as it removes the need for the cultivation of cells for analysis of chromosomes.

The most common large aberrations in breast cancer

According to our results, the most commonly affected chromosome arm by gains were 20q and 1q. Loss at high frequency was found in 8p. Microstructural aberrations (less than 5Mbp) were found in more than 70% of tumors in the following regions:

- for gains: 17q11.2-q22, 17p13.3, 20p11.21, 20q11.21-q11.23, 20q12 and 20q13.12-q13.2
- for losses: 1p36.33, 1p32.3, 1p32.1, 1p22.1-p13.3, 1q23.3-q41, 8p11.21, 8p23.1-p12, 8q21.13, 8q21.3, 8q22.1-q24.23, 17p11.2, 17q21.31-q24.3 and 20q12-q13.12.

K. Renstam and colleagues studied chromosomal gains and losses through CGH analysis in 305 unselected invasive breast cancer. The most commonly affected chromosome arms were: + 1q (55%), + 8q (41%), + 16p (40%), + 17q (28%), -13q (27%), -16q (22%), + 20q (19%), -8p (18%) and + 11q (16%) [14].

According to other research groups, the most common gains are in 1q, 8q, 11q, 17q, 20q and losses in 6p, 8p, 9p, 13q, 16q [15-17].

M. Tirkonen and colleagues through CGH analysis detected changes in the number of copies of DNA sequences that are common to different cell subpopulations in tumors and thus they suggested pathways of developing breast cancer. In their study with the highest percentage of genetic additions have emerged 1q (67%) and 8q (49%). Other regions with such aberrations were detected in 16p, 19q, 19p, and 20q13 (over 18%). Genetic losses in over 18% of tumors were found in 8p, 16q, 13q, 17p, 9p, Xq, 6q, 11q, and 18q [18].

Analysis of significantly changed regions

According to B. Orsetti and colleagues, chromosome 1 is associated with copy number changes in 50-60% of breast cancer cases. Their research aimed to identify the specific changes by combining two methods - CGH on DNA microarrays and expression analysis. The results indicate that the short arm of chromosome 1 in the majority of cases is affected by genetic losses, while the long arm - almost entirely by

genetic gains. Using fine mapping they found the shortest regions of overlapping for 19 regions of chromosome 1 (with size of 170 kbp to 3.2 Mbps) for genetic gains (one at 1p and 18 in 1q) and 20 regions - for genetic losses (all in 1p). To identify candidate oncogenes the authors examined RNA expression profiles of 307 genes located in 1q. The research team has identified 30 candidate genes showing significant overexpression associated with increased number of DNA copies [19]. According to our results, a total of 9 regions characterized by a high rate of genetic alterations (7 in short and 2 in long arm) were detected in chromosome 1.

According to Rodriguez and colleagues genomic changes in chromosome 8 are common in breast cancer cell lines and tumors. By CGH-microarrays using BAC clones with a density of 100 kb they were able to identify changes in the copy number of chromosome 8. BAC-clones, covering both established areas of amplification (8q21 and 8q24) in cell lines of breast cancer, were distributed in 9 subregions including 6 amplicons and 3 dropped areas, as all were analyzed by FISH as well. Scientists have found that two regions in 8q24 (125.3-126.5 Mb and 128.1-129.1 Mb) are amplified in 87.5% of the cell lines. Through further research with 30 samples of breast cancer, the team of Rodriguez has found a new gene TRMT12, which was overexpressed twice more in 87% of tumors. TRMT12 represents homologue of the yeast gene, which encodes mRNA methyltransferase involved in post-transcriptional modification of tRNA for the amino acid phenylalanine (Phe). According to the research team, studying the biological effects of this modified expression allows to find new pathways of tumorigenesis [20].

In breast cancer chromosome 17 is strongly affected by the rearrangements. It was found that the short arm of the chromosome is affected by common genetic losses, while in the long arm complex combinations of gains and losses are observed. Orsetti and colleagues aimed to establish quantitative changes in chromosome 17, using CGH on DNA microarrays with density of 1 clone per 0.5 Mbps, in 22 cases of primary tumors and 30 cell lines of breast cancer. They divided chromosome at 13 consensus segments: 4 regions with losses, 6 regions with gains and 3 mixed areas. They compared with the changes in the expression of 358 known genes on the long arm of chromosome 17 and found that at least half of them are significantly affected by changes in the copy numbers. The team identified overexpression of 85 genes as a result of amplification, underexpression of 67 genes as a consequence of deletions and reduced expression of 32 genes, when they are affected by genetic gains [21].

In many types of cancer it has been observed recurring genetic gains or amplifications in the long arm of chromosome 20 (20q). This suggests the existence of a gene or genes that are important for the tumor phenotype when they are overexpressed. Hodgson and colleagues in an effort to discover oncogenes aimed to investigate the whole chromosome 20. Their results have shown a correlation between many areas with changes in the copy number of 20q and poor clinical prognosis. The research team found that this contributes to the development of the cancer phenotype, especially in terms of characteristics such as immortality, genomic instability, apoptosis and enhanced proliferation. Efforts aimed at discovery of new genes on chromosome 20 revealed candidate genes that may contribute to oncogenic development [22].

K. Collins and colleagues confirmed that the gene amplification occurs in most solid tumors and is associated with poor prognosis. Amplification at 20q13.2 is common for many types of tumors, including breast cancer and is the most comprehensive research segment of the human genome. The complex structure of the amplicon was discovered, which suggests the existence of at least two genes resulting in tumorigenesis. One of them, ZNF217, is able to immortalize human mammary epithelial cells when overexpressed. Through further research on the region the research team of Collins has received evidence of additional leading gene - pfoldin 4 (PFDN4), coregulated genes conserved noncoding regions and associated repetitive regions of genomic instability in this locus [23].

Conclusion

Our study contributed to the fine mapping of genomic imbalances in ductal breast cancer, suggesting the significant regions of genetic gains and losses are those affecting chromosomes 1, 8, 17 and 20. We precisely defined their boundaries. The genes in the found regions could represent potential oncogenes and/or tumor-suppressor genes with cancer-emerging role.

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