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GENOME-WIDE ASSOCIATION STUDY OF LOSS OF HETEROZYGOSITY AND METASTASIS-FREE SURVIVAL IN BREAST CANCER PATIENTS

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One of the factors providing the diversity and heterogeneity of malignant tumors, particularly breast cancer, are genetic variations, due to gene polymorphism, and, especially, the phenomenon of loss of heterozygosity (LOH). It has been shown that LOH in some genes could be a good prognostic marker. *Aim*: To perform genome-wide study on LOH in association with metastasis-free survival in breast cancer. *Materials and Methods*: The study involved 68 patients with breast cancer. LOH status was detected by microarray analysis, using a high density DNA-chip CytoScanTM HD Array (Affymetrix, USA). The Chromosome Analysis Suite 3.1 (Affymetrix, USA) software was used for result processing. *Results*: 13,815 genes were examined, in order to detect LOH. The frequency of LOH varied from 0% to 63%. The association analysis identified four genes: *EDA2R*, *PGK1*, *TAF9B* and *CYSLTR1* that demonstrated the presence of LOH associated with metastasis-free survival (log-rank test, $p < 0.03$). *Conclusions*: The presence of LOH in *EDA2R*, *TAF9B,* and *CYSLTR1* genes is associated with metastasis-free survival in breast cancer patients, indicating their potential value as prognostic markers.

***Key Words*: breast cancer, loss of heterozigosity, microarray, metastasis-free survival, prognosis.**

It is well known that one of the factors determining the individual diversity and heterogeneity of the tumor, including breast cancer, is a normal genetic variability caused by gene polymorphism (single nucleotide polymorphism — SNP). SNP in the tumor tissue can be manifested as the phenomenon of allelic imbalance (AI). Also, the special case — the loss of heterozygosity (LOH) — is often detected. LOH is a loss (structural or functional) of one of the alleles of a heterozygous genotype, resulting in a reduced frequency of heterozygous genotypes compared with genomic DNA. AI is presented by allelic deletions (loss of one copy of the locus), duplication or amplification of one allele [1]. A. Knudson was the first to describe the phenomenon of AI in tumors in 1971. According to his two-hit carcinogenesis model, the inactivation of tumor suppressor genes required two successive mutational events — loss of one allele as a result of LOH and somatic mutation in the other allele. The loss of the allele provides the opportunity of a manifestation of lethal recessive mutation in the remaining allele [2, 3].

In addition, LOH and AI can lead to the activation of oncogenes and inactivation of tumor suppressor genes, which can result in uncontrolled cell growth and metastasis [4, 5]. Currently, allele imbalance and LOH are well explored and shown for many genes in different types of cancer. The biological meaning of LOH in carcinogenesis is suggested to be associated with inactivation of heterozygous loci of pathogenetically significant genes, thus providing tumor progression, including metastasizing [6–12]. From perspective of breast cancer (BC), the most important AI and LOH were shown for cancer-related genes, such as the *ERBB2* (*HER2*) [13], *BRCA1* and *BRCA2* [14–16]. Recently, a large study on LOH in BC was performed for the following genes: *EGFR*, *TERT*, *TP53*, *CASP8*, *PARP2*, *GATA3*, and *BRCA1* [17]. Also, it was demonstrated that the LOH in telomeric sites of chromosomes in BC and ovarian tumor cells appeared to be a good predictor of clinical course of diseases [18]. However, most studies on AI, especially the LOH in BC, were focused on genes, contributing to malignant transformation of normal epithelial breast cells, but not on cancer progression [19].

Thus, the genome-wide LOH study in relation to cancer progression is necessary to understand its contribution to disease outcome and also to find new effective prognostic markers. In this work we performed the genome-wide study on the LOH in association with metastasis-free survival in BC patients.

MATERIALS AND METHODS

The study group. A total of 68 patients aged from 28 to 68 years (median age 53 years) were enrolled in the study (Table 1). The diagnosis of BC was verified morphologically. The tumor stages were IIA–IIIB. In accordance with the Consensus Conference on Neoadjuvant Chemotherapy in Carcinoma of the Breast, April 26–28, 2003, Philadelphia, Pennsylvania [20], all patients underwent 2–4 courses of neoadjuvant chemotherapy (NAC) by FAC scheme (5-fluorouracil, doxorubicin, cyclophosphamide), CAX scheme (cy-

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Abbreviations used: AI – allelic imbalanced; BC – breast cancer; cnLOH – copy-neutral loss of heterozygosity; LOH – loss of heterozygosity; NAC – neoadjuvant chemotherapy; SNP – single nucleotide polymorphism.

clophosphamide, doxorubicin, capecitabine). Surgery was performed 3–5 weeks after the completion of NAC followed by two cycles of adjuvant chemotherapy with FAC regimen. Radiotherapy and/or hormonal treatment were given if required.

The study was carried out in accordance with Helsinki Declaration of 1964 (amended in 1975 and 1983) and was approved by The Ethical Committee of the Institute of Oncology. Signed informed consent was obtained from all participants.

Table 1. The clinicopathological parameters of BC patients (n = 68)

Trait	Value	Number of patients, n (%)
Age, years	⩽ 45	21 (30.9)
	> 45	47 (69.1)
Menstrual status	Premenopausal	36 (52.9)
	Postmenopausal	32 (47.1)
Histological type	Invasive ductal carcinoma	58 (85.3)
	Invasive lobular carcinoma	3 (4.4)
	Medullary carcinoma	2 (2.9)
	Others	5 (7.4)
Tumor size	T1	9 (13.2)
	T2	52 (76.5)
	T3	3 (4.4)
	T4	4 (5.9)
Lymph node status	N0	27 (39.7)
	N1	31 (45.6)
	N2	4 (5.9)
	N3	6 (8.8)
Estrogen receptor	Positive	33 (48.5)
	Negative	31 (42.6)
	No data	4 (5.9)
Progesterone receptor	Positive	35 (51.5)
	Negative	29 (42.3)
	No data	4 (5.9)
HER2	0/+	47 (69.1)
	++	10 (14.7)
	+++	6 (8.8)
	No data	5 (7.4)
Molecular subtype	Luminal B	40 (59.7)
	Triple negative	17 (25.4)
	HER2-positive	10 (14.9)
Histological form	Unicentric	45 (66.2)
	Multicentric	23 (33.8)
NAC regimen	CAX	28 (41.2)
	FAC	40 (58.8)

DNA extraction. Biopsies of tumor tissues were obtained before treatment under ultrasound guidance. The tissues were placed in RNAlater (Ambion, USA), incubated for 24 h at room temperature and stored at –80 °C until DNA and RNA extraction.

DNA was extracted from 68 biopsy specimens of tumor tissues using QIAamp DNA mini Kit (Qiagen, Germany). DNA concentration and purity were assessed using NanoDrop 2000 instrument (Thermo Scientific, USA). The concentration varied between 50 to 150 ng/μl and A260/A280 and A260/A230 ratios were 2.10–2.35 and 2.15–2.40, respectively. The integrity of DNA was assessed using TapeStation instrument (Agilent Technologies, USA); the fragments of the DNA were no longer than 48 kbp, thus suggesting its high integrity.

Microarray analysis. The CytoScan™ HD Array chips (Affymetrix, USA) were used. They contained probes for 2,670,000 markers including 1,900,000 non-polymorphic markers for the analysis of copy number variations and more than 750,000 SNPs. The sample processing, arrays hybridization and scanning were performed according to the manufacturer's protocols for Affymetrix GeneChip® Scanner 3000 7G. The results were analysed using Chromosome Analysis Suite 3.1 software (Affymetrix, USA).

Statistical analysis. Statistical analysis was performed using STATISTICA 8.0 software package (StatSoft Inc., USA). A significance level of $p < 0.05$ was considered for statistically significant differences between treatment groups. p-value < 0.05 was considered statistically significant. The Kaplan — Meier curves were used for the analysis of overall and metastasis-free survival [21]. Comparison of survival curves of the treatment groups was done using the log-rank test. Two-sided Fisher's exact test was used for analyzing comparison of frequencies for qualitative data.

RESULTS AND DISCUSSION

First of all the frequency of LOH in genes from the OMIM (Online Mendelian Inheritance in Man) database was studied (http://omim.org/). A total of 13,815 genes were evaluated for heterozygosity loss in tumors of BC patients. The frequency of LOH varied from 0% to 63% for certain genes, thus 63% of patients showed LOH in some genes. The LOH frequency for 13,815 genes annotated in the OMIM database is presented in Fig. 1. In accordance with the localization of genes on the chromosomes, they are plotted on the abscissa, and the percentage of patients with the LOH in the certain gene is depicted on the ordinate. Fig. 1 clearly shows that the highest incidence of the LOH events is characteristic for genes on chromosomes 17 and X.

The highest LOH frequency (from 30 to 63%) was observed in loci of *ZNF267*, *PAGE3*, *MAGEH1*, *RRAGB*, *KLF8*, *UBQLN2*, *SPIN2B*, *SPIN2A*, *FAAH2*, *ZXDB*, *ZXDA*, *ARHGEF9*, *FAM123B*, *MSN*, *MIR223*, *VSIG4*, *HEPH*, *EDA2R*, *AR*, *SLC16A2*, *RLIM*, *KIAA2022*, *ABCB7*, *UPRT*, *ZDHHC15*, *MAGEE2*, and *MAGEE1* genes. It is important to test the functional significance of these genes as tumor suppressors. In concordance with the information of the OMIM database, chromosomes 6, 7, 11 and 14 possess large regions, where the LOH was not observed in any of the 68 BC samples examined. In total, no LOH was detected in 873 out of 13,815 genes, annotated in the OMIM database. Well-known tumor suppressor genes, such as *TP53*, *RB1*, *BRCA1* etc., also showed the low LOH frequency (Table 2).

Table 2. Frequency of LOH in known tumor suppressor genes

Tumor suppressor genes	Frequency of LOH, n (%)
MEN1, TNFAIP3	0 (0.0)
CEBPA, JAK2, WRN	1 (0.7)
CDK6, CDKN2A, DCC, FBXW7, FOXP1, IL2, MSH2, PALB2, SOCS1, SYK	2 (1.4)
BCL11B, BMPR1A, CREB1, CREBBP, CYLD, IDH1, MDM4, NR4A3, PTEN, SDHB, STK11, SUFU, TCF3, TSC1, TSC2, VHL, WT1	3 (2.0)
APC, ATM, CDH11, EXT1, EXT2, NF2, RUNX1, SDHD, SMARCA4	4 (2.7)
CARS, CHEK2, FH, MLH1, NOTCH1, NPM1, NUP98, SMARCB1	5 (3.4)
BLM, BRCA2, CBFA2T3, PML, RB1	6 (4.1)
FLT3, MAP2K4	7 (4.8)
CDH1, SUZ12	8 (5.4)
CDKN2C, NF1, TP53	9 (6.1)
BRCA1	10 (6.8)
GPC3	11 (7.5)
DDX5	12 (8.2)

Fig. 1. A pattern of the LOH presence in the genes, annotated in the OMIM database, in relation to their chromosome localization

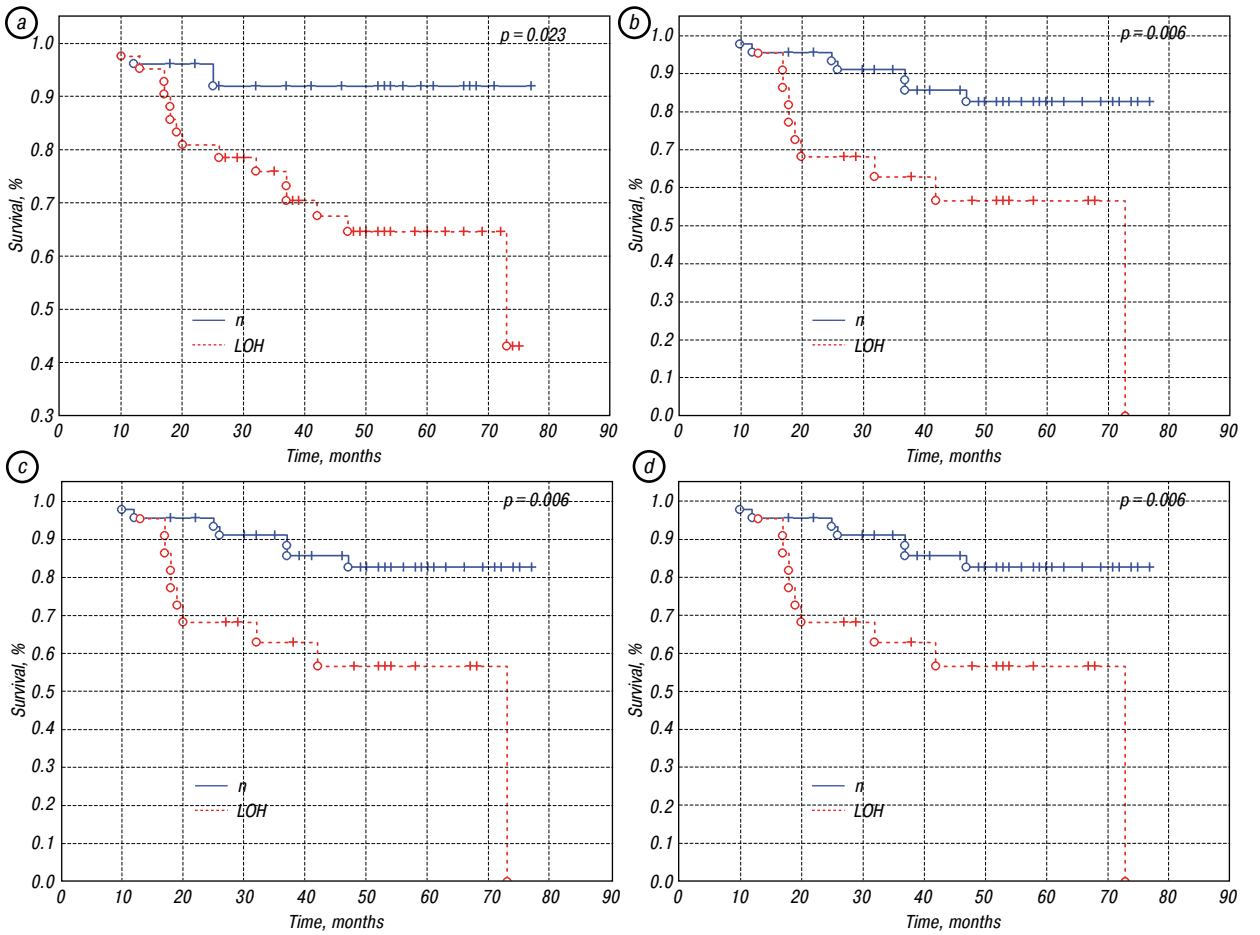

Fig. 2. Metastasis-free survival of BC patients with the detected LOH in a set of genes: (*a*) Ectodysplasin A2 Receptor (*EDA2R*) gene; (*b*) phosphoglycerate kinase 1 (*PGK1*) gene; (*c*) RNA polymerase II, TATA Box-Binding Protein-Associated Factor (*TAF9B*) gene; (*d*) cysteinyl leukotriene receptor 1 (*CYSLTR1*) gene.
Note: a red line — patients with the LOH in genes examined; a blue line — patients with the normal status of genes examined. *p* — a significance value, calculated by a log-rank test

The next step of investigation was to analyze the relationship between distant metastasis of breast tumors and the LOH frequency in OMIM genes. Distant metastases occurred in 17 (25%) of the 68 patients within 10 to 77 months from the time of diagnosis. The two-, three- and five-year metastasis-free survival rates were 86.8; 82.4, and 76.5%, respectively.

The patients were divided into two groups: the first group consisted of 17 patients with metastases and the second group consisted of 51 patients without metastases.

Based on a difference between the frequency of the normal status of the gene and the LOH in patients with metastases and without metastases, we have selected genes in which this difference reached the maximum value. In result of our study seven genes were selected, namely, *FGD1*, *GNL3L*, *TRO*, *EDA2R*, *PGK1*, *TAF9B,* and *CYSLTR1* for those the difference ranged from 25.5 to 35.3%.

The next step was to evaluate the association between the LOH and metastasizing, using a Fisher's exact test. The association between the LOH and metastasizing was demonstrated only for 4 genes: *EDA2R*, *PGK1*, *TAF9B,* and *CYSLTR1*. Using the Kaplan — Meier survival analysis, we showed that the metastasis-free survival rate in patients with the LOH in these genes was significantly lower, compared with those observed in the group of patients, who had a normal status of these genes (Fig. 2).

It should be noted that all of the genes identified in the study were located on the X chromosome (http://www.genecards.org/). *EDA2R* gene was localized on the long arm of the X chromosome (Xq12), while *PGK1, TAF9B,* and *CYSLTR1* genes were localized in Xq21.1.

Deletions with LOH can provide a decrease in the expression of genes with LOH at the expense of to gene deficiency. Amplification with LOH results in an increase in gene expression. The copy-neutral LOH (cnLOH) may increase or decrease the expression of the gene with cnLOH. cnLOH might confer a phenotypic advantage for tumor cells due to gain of imprinting through duplication of a methylated allele (decrease the expression of the gene with cnLOH), loss of imprinting through duplication of unmethylated allele (increase the expression of the gene) or homozygosity of an initial heterozygous mutation [22]. The expression of genes with LOH it was necessary to explore in order to understand the impact of LOH on gene expression.

In the study Ching *et al*. [23] was to explicate the underlying chromosomal copy number alterations and LOH implicated in a cohort of Malaysian hospital-based primary breast carcinoma samples (n = 70) using a SNP-array platform. The most prevalent gains (⩾ 30%) were detected at the 8q arm, whilst the most ubiquitous losses (⩾ 20%) were noted at the 8p and 17p regions. The frequency of deletion with LOH of *PGK1, TAF9B,* and *CYSLTR1* genes were 10%. cnLOH was characterized as the most prevailing LOH event, in which the most frequent distributions (⩾ 30%) were revealed at 3p21.31, 5q33.2, 12q24.12, 12q24.12-q24.13, and 14q23.1. The frequency of cnLOH *EDA2R* gene (ectodysplasin A2 receptor) was 24%, which along with *TNFR1* and *Fas* (CD95) is one of the receptors of death. Yan *et al.* [24] showed that *EDA2R* gene appeared to activate expression of tumor necrosis factor gene. Studies suggest that *EDA2R* is a potential downstream effector of p53-induced apoptosis in cancer cells [25, 26] and may therefore be a potential tumor suppressor, it is down-regulated in breast and colorectal cancers [27, 28], and mutations and promoter hypermethylation of *EDA2R* have been identified in colorectal cancer cells [25, 26]. A recent study conducted in 2016 using a microarray CytoScan HD Array showed that the presence of cnLOH in *EDA2R* gene and in several other genes was associated with ameloblastoma cases [29].

The protein encoded by *PGK1* gene is a glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. Additionally, this protein is secreted by tumor cells where it participates in angiogenesis by functioning to reduce disulfide bonds in the serine protease, plasmin, which consequently leads to the release of the tumor blood vessel inhibitor angiostatin (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PGK1&keywords=PGK1). CpG-island methylation was evaluated on a 56-gene cancer-specific biomarker microarray in metastatic *vs* non-metastatic BC in a multi-institutional case series of 123 BC patients. This identified 11 genes (including the *PGK1* signaling kinase gene) as the highest differentially methylated genes between progressing and non-progressing BC [30]. The *PGK1* gene showed differential splicing in TNBC, non-TNBC (luminal A and B) and HER2-positive BC subtypes [31].

The *TAF9B* (TATA-Box Binding Protein Associated Factor 9b) is involved in transcriptional activation as well as repression of distinct but overlapping sets of genes. Among it related pathways is RNA Polymerase II transcription initiation. Microarray screening identified a *TAF9B* gene whose expression was significantly changed by combination treatment with inhibitors of LSD1 and HDAC of human BC cells [32]. Collectively, p53, SNHG1, sno-miR-28, and TAF9B form a regulatory loop which affects p53 stability and downstream p53-regulated pathways [33].

CYSLTR1 (Cysteinyl Leukotriene Receptor 1) is involved in mediating bronchoconstriction via activation of a phosphatidylinositol-calcium second messenger system. Upregulation of this gene is associated with asthma and dysregulation may also be implicated in cancer. Patients with breast tumors characterized by high CysLT1R and low CysLT2R expression levels exhibited increased risk of cancer-induced death in univariate analysis for both the total patient group (hazard ratio [HR] = 2.88, 95% confidence interval [CI] = 1.11–7.41) [34].

Thus, the presence of LOH in *EDA2R, PGK1, TAF9B,* and *CYSLTR1* genes were shown to be associated with low metastasis-free survival in patients with BC, indicating their potential value as prognostic markers.

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