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HYPERMETHYLATION OF *TUSC5* GENES IN BREAST CANCER TISSUE

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Aim: **Breast cancer (BC) is one of the most common forms of cancer amongst females. Early diagnosis, prognosis and therapy plays crucial role in the survival of patients with breast cancer. The study was aimed on identification of potential markers for early BC diagnostics by means of genome-wide comparative analysis of gene expression in cancer and normal tissue of breast.** ***Methods*****: The analysis of gene expression in 15 invasive adenocarcinoma specimens and 15 normal breast tissue was conducted using the full-genome microarrays Sentrix HumanWD-6V3 BeadChip (Illumina). Methylation of *TP53INK1* and *TUSC5* promoters was interrogated by the combined bisulfite restriction analysis (COBRA).** ***Results*****: Analysis of gene expression in the samples of breast adenocarcinoma revealed abnormal expression of more than 2,300 genes. While genes *TFF1, S100P, ERBB2, TOP2A, CDF15, HOOK1, DNAJC12, CORO2A* were up-regulated in cancer, decreased expression was found for genes *TUSC5, SFRP1, PPPQR1B, NTRK4, TIMP4, BARD1, AKR1C2, TP53INK1* and others. Analysis of DNA methylation of *TUSC5* by COBRA revealed higher levels of exon 1 methylation (11/12) in samples of breast cancer, whereas the gene was essentially unmethylated in matched normal appearing tissue of breast (2/12). *TP53INK1* gene was methylated neither in cancer nor in normalcy.** ***Conclusion*****: A total of 149 genes exhibited the highest difference in expression in cancer versus normal appearing tissue of breast. Most prominent down-regulated candidates, *TUSC5* and *TP53INK1*, were reported for the first time in breast cancer and may be considered as potential markers of the disease. Aberrant DNA hypermethylation of *TUSC5* suggests epigenetic mechanism of cancer associated down-regulation.**
Key Words**: differentially expressed genes, DNA methylation, breast cancer.**

The majority of breast cancer deaths are associated with metastases. Therefore, early detection of tumors would have substantial preventive effects. The routine diagnostic methods that are currently in use lack sensitivity for the detection of tumors at the early stage. For examples, mammography may lead to false-negative results in up to 31% of cases. Thus, identification of tumor-specific molecular markers is vital for development of new sensitive diagnostic methods that are capable of early detection of tumor cells [1]. Analysis of DNA methylation is one of the promising diagnostic approaches given the facts that: (1) aberrant DNA methylation occurs at any stage of tumorigenesis in various cancers including breast cancer [3]; (2) covalent cytosine modification by methylation is stable and can be readily detected in body fluids; (3) modification of DNA by methylation is functionally involved in regulation of gene expression [2]. Cancer is associated with the global and localized hypermethylation of DNA in GC-rich regions referred to as CpG islands. Taken together, these properties of methylated DNA strongly suggest detection of DNA hypermethylation for cancer diagnostics. For instance, detection of hypermethylated promoters of certain genes has been suggested as potential clinical markers for early diagnostics of prostate cancer (*GSTP1*), prognosis of lung and colorectal cancer (*p16INK4*) and response to temozolomid treatment of glioma patients (*MGMT*) [4]. Therefore, identification of novel markers for early diagnostics, prognosis and therapy of breast cancer is of substantial practical use.

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Abbreviations used: BC – breast cancer.

MATERIALS AND METHODS

Breast tissue samples. A total of 30 samples were analyzed in the study. These included 15 adenocarcinomas of breast along with matched normal-appearing breastspecimens. Fresh-frozen breast tissue samples were obtained, with written informed consent from patients from Odessa State Medical University after approval by the Institutional Review Board (Table 1).

RNA extraction and microarrray study. After surgical removal, samples were immediately placed in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany), transported to the laboratory and frozen at -80 °C. For RNA isolation, they were homogenized on dry ice using mortar and pestle. Total RNA was extracted using the RNEasy Mini Kit (Qiagen) according to manufacturer's instructions. The quality of isolated patient RNA was controlled using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Full-genome microarrays Sentrix HumanWD-6V3 BeadChip (Illumina inc., USA) were employed for analysis of gene expression. cDNA was synthesized using 1 µg of total RNA obtained from each tissue sample. Hybridisation to the BeadChip was performed according to the manufacturer's instructions. A maximum of 10 µl cDNA was mixed with a 20 µL GEX-HYB hybridization solution. The preheated 30 µl assay sample was dispensed onto the large sample port of each array and incubated for 18 hours at 58°C. Following hybridization, samples were washed according to the protocol and scanned with a BeadArray Reader (Illumina, San Diego, USA). Raw data were exported from the Beadstudio software to CHIPSTER software, whereby data quality assessment, normalization and correspondence cluster analysis were performed. Most prominent candidate genes that were differentially expressed in cancer vs. normal-appearing tissue were identified by filtering

99.95% of all transcripts ($p < 0.005$). Pathway analysis of the resulting genes was performed using IPA Ingenuity software.

DNA isolation, bisulfite conversion and combined bisulfite restriction analysis (COBRA). Twelve samples of breast adenocarcinoma (10 ductal and 2 lobular forms) along with matched normal appearing tissue were used to analyze CpG methylation within exon 1 of *TUSC5* and promoter of *TP53INP1*.

DNA was extracted by using QIAmp DNA Mini Kit and modified by sodium bisulfite with EpiTect Bisulfite kit of Qiagen. Interrogated DNA regions were amplified by PCR by using following primers: TUSC5-forward ATTAGTAAAGTTGTTT, TUSC5-reverse CAAAAAACTCTAAAAAAA; TP53INP1-forward1 ATTTTGGAGAGGGAATA, TP53INP1-reverse1 AAAAACTACTATCTTCCCCACCTC; TP53INP1-forward2 TTTAATTGTTTTTTTTGGTTAGTTTT, TP53INP1-reverse2 ATCCAACCATCACCT-ATA-AATCC. Primers were designed by using Methyl Primer Express v. 1.0 (Applied Biosystems, USA). EpiTect control DNA set (Qiagen) was employed as a reference for totally methylated and unmethylated DNA samples. Amplicons were next incubated with BstUI restriction enzyme (New England Biolabs, USA) as suggested by the manufacturer and analyzed by electrophoresis in 2%-agarose gel. Semiquantitative analysis of TUSC5 and TP53INP1 DNA methylation was performed using the Quantity One software (Bio-Rad, USA) as earlier reported [5]. Methylation degree was estimated according to the equation

$$M = \frac{C}{B + C} \cdot 100,$$

where B and C are amounts of unmethylated and methylated alleles, respectively.

RESULTS AND DISCUSSION

In this study, we aimed at identification of differentially expressed genes in tumors of breast versus normal appearing breast tissue. To this end, genome-wide gene expression profiling was performed (Fig.1). A total of 2300 genes were shown to be deregulated including 759 candidates with log fold change > 2.0. These included DNAJC12, *TOP2A, STAT1, KIAA0101, GGT6, HOOK1, FLJ2315, TTF1, FSIP1, S100P, CRB14, GDF15, RASEF, TP531NP1, SEC14L2, TMEM26, MUC1, SUSD3, SPP1* etc. Significant down-regulation was shown for 73 genes: *TUSC5, TP53INP1, CIDEC, CIDEA, SFRP1, GSTT1, DNAJC12, SOCS2, AQ7, HBB, IGSF1, GPD1, LEP, LPL, AKR1C2, CASP4, NTRK2, PPP1R1B, HOXA5, RBP4* etc. Significant up-regulated candidates included 76 genes: *S100P, TFF1, CORO2A, FLG2351, C10orf106, C15orf48, VANGL2, UGT2B7, SOCS2, SUSD* and others. Deregulation of the following genes in breast adenocarcinoma was reported for the first time: *TUSC5, AQ7, CORO2A, FLJ2351, CIDEA, CIDEC, FAM89A, DDIT4L, C10orf106, C15orf48, MRAP, UGT2B7, CCDC3* and *VANGL2.*

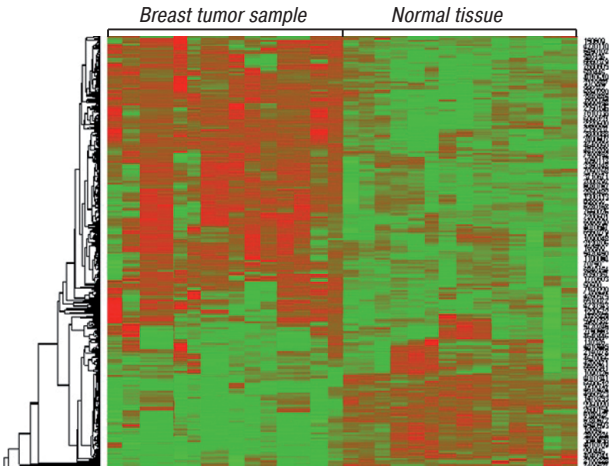

Fig. 1. Unsupervised clustering of gene expression data (rows) for breast cancer samples (columns at the left) and matched normal appearing tissue (columns at the right). In the heatmap, up-regulation is shown in red, down-regulation is designated in green.

TP53INP1 and *TUSC5* were among most prominent down-regulated candidates in breast adenocarcinoma. TP53INP1 is stress-inducible protein that is responsible for phosphorylation of p53 in response to double-strand DNA breaks. *TUSC5* is a presumable tumor suppressor. However, it function is poorly understood. Given the fact that aberrant DNA hypermethylation is a frequent epigenetic event in cancer that is associated with loss of gene function, we hypothesized that *TP53INP1* and *TUSC5* may be epigenetically silenced. We addressed methylation of CGCG sites in exon 1 of *TUSC5* and promoter of *TP53INP1* by combined bisulfite restriction analysis. Hypermethylation of CGCG site in *TUSC5* was detected in 11 out of 12 samples of breast cancer with mean 51.4±3.1% methylation. Significantly lower methylation load was observed in normal appearing breast tissue of same patients (30.5±1.9%, $p<0.002$, Fig.2). *TUSC5* methylation in two normal samples was higher (43% and 41%) that in the rest of normal specimens, what may be explained by contamination of these samples with tumor cells (Table 2). These findings suggest epigenetic mechanism of *TUSC5* deregulation. No DNA methylation was detected in *TP53INP1* promoter both in cancer and normalcy suggesting an alternative mechanism of its deregulation

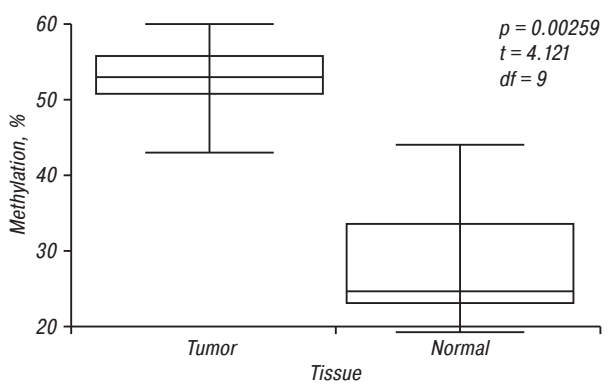

Fig. 2. Comparison of *TUSC5* gene methylation status in breast cancer and normal tissue in the same patients.

Table. Methylation level of *TUSC5* in brest cancer and matched normal appearing tissue

Sample ID	Percent DNA methylation	
	Breast cancer	Normal appearing breast tissue
1	67	28
2	45	29
3	65	31
4	48	24
5	51	32
6	56	45
7	51	23
8	49	24
9	24	41
10	53	34
11	51	27
12	53	28
	51.4 ± 3.1	30.5 ± 1.9 p = 0.0029

Our results are concordant with a report by Weng et al. [6], who have shown loss of *TP53INP1* expression in esophageal carcinoma. However, in contrast to other malignancies, where *TP53INP1* was shown (e.g., esophagus cancer and melanoma [7]), methylation could not be detected in our samples.

In conclusion. a total of 149 genes exhibited the highest difference in expression in cancer versus normal appearing tissue of breast. Most prominent down-regulated candidates, TUSC5 and TP53INK1, were reported for the first time in breast cancer and may be considered as potential markers of the disease. Aberrant DNA hypermethylation of TUSC5 suggests epigenetic mechanism of cancer associated down-regulation.

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