

Gene expression abnormalities in histologically normal breast epithelium from patients with luminal type of breast cancer

Pavol Zubor · Jozef Hatok · Petra Moricova ·
Karol Kajo · Ivana Kapustova · Andrea Mendelova ·
Peter Racay · Jan Danko

Received: 8 May 2013 / Accepted: 10 November 2014 / Published online: 19 November 2014
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Abstract The gene expression profile of breast cancer has been described as a great breakthrough on the way to comprehend differences in cancer origin, behavior and therapy. However, gene expression profile in histologically normal epithelium (HNEpi) which could harbor genetic abnormalities predisposing breast tissue to develop malignancy was minor scope for scientists in the past. Thus, we aimed to analyze gene expressions in HNEpi and breast cancer tissue (BCTis) in order to establish its value as potential diagnostic marker for cancer development. We evaluated a panel of disease-specific genes in luminal type (A/B) of breast cancer and tumor surrounding HNEpi by qRT-PCR Array in 32 microdissected samples. There was 20.2 and 2.4 % deregulation rate in genes with at least 2-fold or 5-fold over-expression between luminal (A/B) type breast carcinomas and tumor surrounding HNEpi,

respectively. The high-grade luminal carcinomas showed higher number of deregulated genes compared to low-grade cases (50.6 vs. 23.8 % with at least 2-fold deregulation rate). The main overexpressed genes in HNEpi were KLK5, SCGB1D2, GSN, EGFR and NGFR. The significant differences in gene expression between BCTis and HNEpi samples were revealed for BAG1, C3, CCNA2, CD44, FGF1, FOSL1, ID2, IL6R, NGFB, NGFR, PAPP, PLAU, SERPINB5, THBS1 and TP53 gene ($p < 0.05$) and BCL2L2, CTSB, ITGB4, JUN, KIT, KLF5, SCGB1D2, SCGB2A1, SERPINE1 ($p < 0.01$), and EGFR, GABRP, GSN, MAP2K7 and THBS2 ($p < 0.001$), and GSN, KLK5 ($p < 0.0001$). The ontological gene analyses revealed high deregulations in gene group directly associated with breast cancer prognosis and origin.

Keywords Breast cancer · Luminal type · Gene expression · Disease risk

P. Zubor (✉) · P. Moricova · I. Kapustova · A. Mendelova ·
J. Danko

Department of Obstetrics and Gynecology, Jessenius Faculty of
Medicine, Comenius University in Bratislava, Kollarova 2
Martin, 036 01 Bratislava, Slovak Republic, Slovakia
e-mail: zubor@jfmed.uniba.sk

J. Hatok · P. Racay
Department of Biochemistry, Jessenius Faculty of Medicine,
Comenius University in Bratislava, Mala Hora 4 Martin,
036 01 Bratislava, Slovak Republic, Slovakia

K. Kajo
Department of Pathology, Jessenius Faculty of Medicine,
Comenius University in Bratislava, Kollarova 2 Martin,
036 01 Bratislava, Slovak Republic, Slovakia

A. Mendelova
Department of Molecular Biology, Jessenius Faculty of
Medicine, Comenius University in Bratislava, Kollarova 2
Martin, 036 01 Bratislava, Slovak Republic, Slovakia

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23 % of the total cancer cases and 14 % of the cancer deaths [1]. It is not a single disease, but is instead a collection of diseases displaying distinct histopathological features, genetic and genomic variability, and diverse prognostic outcomes.

Although we have knowledge about biological profile of carcinomas, despite decades of research we lack detailed insight into the process of malign transformation of breast cells. One of the best forms is the luminal type of the disease, as for the prognosis [2, 3]. Based on hierarchical clustering analyses performed on microarray-based gene

expression profiles, it is characterized with the high hormone receptor and low HER2 positivity, and in majority of low-grade cell dedifferentiation, as well. This gene expression profile-based taxonomy of breast cancer has been described as a great breakthrough on the way to comprehend the differences in cancer origin, behavior, and individually tailored therapeutic approaches.

The most serious threat for breast as an organ is growth of malign tumor. Until now, contrary to breast cancer tissue, the minimum attention was focused on the molecular analyses of histologically normal breast tissue in aim to predict disease origin or phenotype. Today we can identify many genetic abnormalities and impaired signaling pathways in breast carcinomas and premalign breast lesions (e.g. hyperplasias, in situ lesions), proving their common evolution network. However, recent studies surprisingly revealed that similar genetic, epigenetic and functional alterations in gene expression and protein transcription are present also in histologically normal breast tissue [4–10]. One of the first were detected abnormalities related to allelic instability, loss of heterozygosity, aberrant CpG methylations in tumor suppressor genes, telomere shortening, loss of RAR β expression, aberrant p38 phosphorylation and deregulation of EZH2. Later occurred information related to different gene deregulation and signaling, based on the advancement of microarray technology with ontological clustering [5, 8]. It assumes that by their understanding we could noticeably progress in the detection and prevention of functional deviations of breast epithelium and in processes of early malign conversion. This should be presumable situation for an increased disease risk based on genomic level. Moreover there could be a possibility to predict phenotype (lobular, ductal or basal-like type) or biologic behavior of tumor based on its interaction with breast microenvironment [11–13].

Such knowledge has a great potential in preventive strategies, first of all for high risk women with history of increased breast tissue density, family history of malign diseases or serious systematic disease [14, 15]. The existence of specific „malignancy-risk“ gene signature of deregulated genes in histologically normal breast tissue was described previously [9, 16] bringing the great advantage in detection of early breast carcinogenesis at the time when histological changes of malignancy are not present [6]. Such breast tissue is marked as „molecularly-abnormal, but histologically-normal“ tissue and from the view of gene ontology, the signature consist mainly from the deregulated genes participating in cell processes as cell growth, cell cycle regulation, differentiation, adhesion, DNA synthesis, apoptosis and epithelial–mesenchymal transition. Specifically, there is no association between such malignancy risk signature and hormonal expression,

what makes the signature to be a good model for cancer prediction [9].

Following this we hypothesized that gene expression in histologically normal epithelium (HNEpi) would exhibit a different gene abnormalities compared to cancer tissue (BCTis) and that this gene expressions in HNEpi would predict the disease origin and phenotype. The present study compares global gene expression between one of the prognostic good tumors, luminal type breast cancer and tumor surrounding HNEpi.

Materials and methods

Patient population

This was a prospective study using women ($n = 80$) with histologically confirmed breast cancer referred to the Department of Obstetrics and Gynecology, Jessenius Faculty of Medicine for surgical treatment. All participants were of Caucasian race and residents of the geographic area of Slovakia. After initial consultation all subjects signed an informed consent and subsequently underwent biological sample collection during breast surgery. The retrieved tissue samples were as follows: ($3\text{--}5 \times 5 \times 3\text{--}5$ mm) of cancer tissue and the same extent of histological normal tissue surrounding tumor ≤ 2 cm from tumor margins. The collected material was stored and processed for multigene analysis in breast cancer tissue (BCTis) and histologically normal tissue (HNEpi) surrounding tumor, as well. Of all subjects who fulfilled selection criteria (primary unilateral unifocal invasive ductal cancer ($n = 71/80$) the cases with biologic features determining luminal A and luminal B carcinoma ($n = 34$) were selected for further molecular testing. Of them 16 paired samples retrieved sufficient mRNA for multi-gene expression and were included in the final analyses. The Regional Ethical Committee at the Jessenius Faculty of Medicine registered under IRB00005636 at the Office for Human Research Protection, U.S. Department of Health and Human Services approved the study protocol (codes EK 98/2004, EK 169/2005, EK 423/2008, EK 884/2011). The study was carried out in accordance with the Declaration of Helsinki for experiments involving humans.

Histopathological analysis

Histological assessments were performed on 4–5 μm thick hematoxylin and eosin stained sections of formalin-fixed, paraffin-embedded tumors. Typing was evaluated according to the WHO classification of tumours [17], and histological grading as presented by Elston and Ellis [18]. Moreover, tissue sections (4–5 μm thick) from paraffin

blocks were used for immunohistochemical analyses. For detection of estrogen receptors (ER) we used anti-ER (clone ER1D5, Immunotech) and for detection of progesterone receptors (PR) we used anti-PR (clone 1A6, Immunotech). The ER and PR status was interpreted semiquantitatively as positive when >1 % of tumor cells showed positive nuclear staining. HER2 immunohistochemical status was initially analyzed by HercepTest (DakoCytomation, Glostrup). The results were interpreted as follows: 0 = no membrane staining (MS); 1+ = faint, partial MS; 2+ = weak complete MS in more than 10 % of invasive cancer cells; 3+ = intense complete MS in more than 10 % of invasive cancer cells. Patients with 2+ results were re-examined by FISH using the ONCOR HER-2/neu Gene Detection System (Ventana Medical System). The disease stage was classified according to FIGO staging system.

RNA extraction

The tissue samples sized 5 × 5 × 3 mm were retrieved by surgeon and pathologist under sterile conditions during surgery and frozen section for every patient. Obtained tissue samples were immediately placed in plastic tubes with mRNA stabilizing solution (RNAlater[®]; Applied Biosystems/Ambion, USA) and stored frozen at −80 °C for mRNA isolation. The RNA was isolated by using kit (RNeasy Lipid Tissue; Qiagen Inc., USA). The RNA concentration was ascertained by spectrometry using an Ultraspec III instrument (Pharmacia Biotechnology Inc., USA) by 260 nm and the its purity was assessed as the absorbance index $AI = A_{260\text{ nm}}/A_{280\text{ nm}}$. Cases with $AI > 1.8$ were selected for further analyses. The quality of each RNA sample was later determined by microchip electrophoresis (MCE[®]-202 MultiNA; (Shimadzu Biotech, Germany).

The synthesis of cDNA and RT-PCR

The synthesis of complementary (cDNA) needed for PCR amplification was achieved using iScript[™] cDNA Synthesis Kit (Bio-Rad, UK). The reagent mix consisted of following components: 5 × iScript mix (4 μl); iScript Reverse transcriptase (1 μl); RNA template (5 μg) and sterile water (to a total of 20 μl volume). The mixture was gently stirred and incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. The newly synthesized cDNA was diluted and prepared for multigene analysis (84 genes, Table 1; Figs. 1, 2) using the RT² Profiler PCR Array (Qiagen). Five endogenous control genes; beta-2-microglobulin (B2 M), hypoxanthine phosphoribosyltransferase (HPRT1), ribosomal protein L13a (RPL13A),

Table 1 The ontological classification of analyzed genes

Functional group (1)	Genes directly associated with breast cancer development
Analyzed genes	<i>CDKN1A</i> (<i>p21Wap1/Cip1</i>), <i>CLDN7</i> (<i>claudin-7</i>), <i>CLU</i> (<i>clusterin</i>), <i>ERBB2</i> (<i>Her-2</i>), <i>FGF1</i> , <i>FLRT1</i> (<i>fibronectin</i>), <i>GABRP</i> (<i>GABAa</i>), <i>GNAS</i> , <i>ID2</i> , <i>ITGA6</i> , <i>ITGB4</i> (<i>β4 integrin</i>), <i>KLF5</i> (<i>GC Box BP</i>), <i>KRT19</i> (<i>Keratin 19</i>), <i>MT3</i> (<i>metallothionein-III</i>), <i>MUC1</i> (<i>mucin</i>), <i>PTGS2</i> (<i>COX-2</i>), <i>RAC2</i> (<i>p21Rac2</i>), <i>SCGB1D2</i> (<i>lipophilin B</i>), <i>SCGB2A1</i> (<i>mammaglobin 2</i>), <i>SPRR1B</i> (<i>Spr1</i>), <i>THBS1</i> , <i>THBS2</i> , <i>TNFAIP2</i> (<i>B94</i>)
Functional group (2)	Genes associated with hormonal signaling pathways
Analyzed genes	<i>AR</i> , <i>C3</i> (<i>Complement component 3</i>), <i>CCND1</i> , <i>CTSD</i> (<i>cathepsin D</i>), <i>ESR1</i> , <i>ESR2</i> , <i>GATA3</i> , <i>HSPB1</i> (<i>HSP28</i>), <i>KRT18</i> , <i>KRT19</i> (<i>Keratin 19</i>), <i>PGR</i> , <i>SERPINA3</i> (<i>a1-antichymotrypsin</i>), <i>SLC7A5</i> , <i>STC2</i> , <i>TFF1</i> (<i>pS2</i>)
Functional group (3)	Genes associated with breast cancer prognosis
Analyzed genes	<i>BAD</i> , <i>BAG1</i> , <i>BCL2</i> , <i>CCNA1</i> , <i>CCNA2</i> , <i>CCND1</i> , <i>CCNE1</i> , <i>CDH1</i> (<i>E-cadherin</i>), <i>CDKN1B</i> (<i>p27Kip1</i>), <i>CDKN2A</i> (<i>p16INK4a</i>), <i>COL6A1</i> , <i>CTNNB1</i> (<i>b-catenin</i>), <i>CTSB</i> (<i>cathepsin B</i>), <i>EGFR</i> , <i>ERBB2</i> (<i>Her-2</i>), <i>ESR1</i> , <i>ESR2</i> , <i>FAS</i> (<i>TNFRSF6</i>), <i>FASLG</i> (<i>TNFSF6</i>), <i>FOSL1</i> (<i>FRA-1</i>), <i>GATA3</i> , <i>GSN</i> , <i>IGFBP2</i> , <i>IL2RA</i> , <i>IL6</i> , <i>IL6R</i> , <i>IL6ST</i> (<i>glycoprot.130</i>), <i>ITGA6</i> (<i>a6 integrin</i>), <i>JUN</i> , <i>KLK5</i> , <i>KRT19</i> , <i>MAP2K7</i> , <i>MKI67</i> (<i>Ki-67</i>), <i>NGFB</i> (<i>NGF</i>), <i>NGFR</i> , <i>NME1</i> (<i>NM23A</i>), <i>PGR</i> , <i>uPA</i> , <i>PTEN</i> , <i>SERPINB5</i> (<i>maspin</i>), <i>SERPINE1</i> (<i>PAI-1</i>), <i>TGFA</i> , <i>THBS1</i> , <i>TIE1</i> , <i>HIF-1</i> , <i>TOP2A</i> , <i>TP53</i> , <i>VEGFA</i>
Functional group (4)	Genes associated with response to chemotherapy
Analyzed genes	<i>BCL2</i> , <i>BCL2L2</i> , <i>CD44</i> , <i>CTSD</i> (<i>cathepsin D</i>), <i>CYP19A1</i> , <i>DLCI</i> , <i>ESR1</i> , <i>ESR2</i> , <i>HMGB1</i> , <i>KIT</i> , <i>NFYB</i> , <i>PAPPA</i> , <i>PGR</i> , <i>RPL27</i> , <i>VEGFA</i>

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) present on the PCR array were used for normalization. Each replicate cycle threshold (C_T) was normalized to the average C_T of five endogenous controls on a per plate basis. The comparative C_T method was used to calculate the relative quantification of gene expression. The following formula was used to calculate the relative amount of the transcripts in the cancer samples and the control group, both of which were normalized to the endogenous controls:

$$\Delta\Delta C_T = \Delta C_T(\text{BCTis}) - \Delta C_T(\text{HNEpi}) \text{ for RNA samples}$$

ΔC_T is the log² difference in C_T between the target gene and endogenous controls by subtracting the average C_T of controls from each replicate. The fold-change for each treated sample relative to the control sample = 2^{−ΔΔCT}.

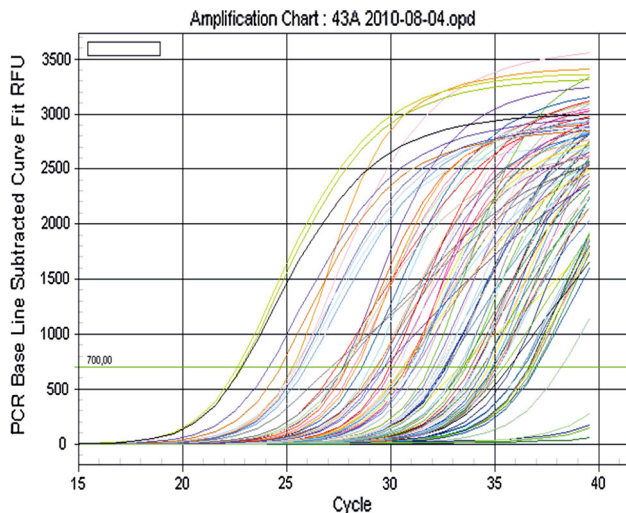


Fig. 1 Amplification plot of quantitative genes expression ($n = 84$) based on threshold cycles for representative cancer tissue sample. Baseline for threshold cycles is set to 700 (green horizontal line). Every gene that crosses baseline responds to specific color lines and individual Ct value. (Color figure online)

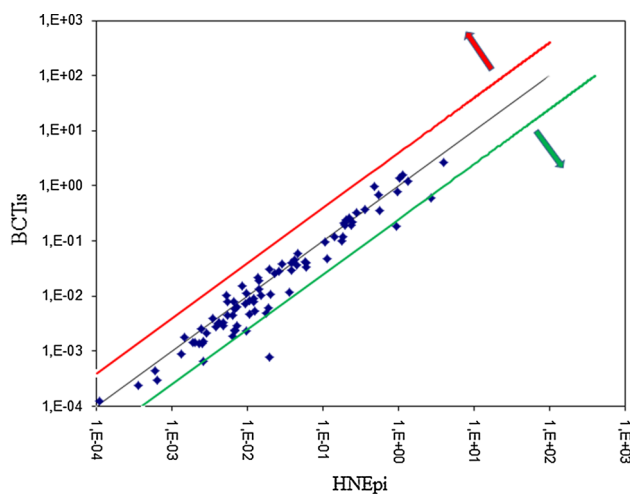


Fig. 2 The scatter plot of gene expression analysis in BCTis and HNEpi tissue samples in women with luminal type of breast cancer. Red line boundary line of gene overexpression in BCTis; green line boundary line of gene overexpression in HNEpi. (Color figure online)

Statistical analysis

The difference in gene expressions in analyzed tissue samples was determined by students-*t* test and parametric statistic tests using iQ5 optical system software 2.1 (Bio-Rad Inc., UK). The statistical level of significance was set to $p \leq 0.05$.

Results

We have performed 2,688 gene expressions in BCTis and HNEpi samples of all 16 luminal type breast cancer

subjects. There were present 17 deregulated genes (20.2 %) with minimal 2-fold deregulation rate and 2 (2.4 %) with 5-fold deregulation rate in samples of HNEpi. Except one gene (*THBS2*) all genetic abnormalities were present only in HNEpi samples, no significant overexpression was detected in BCTis samples. The main overexpressed genes in HNEpi samples were *KLK5* (24.22 fold), *SCGB1D2* (5.14 fold), *GSN* (4.28 fold), *EGFR* (4.10 fold) and *NGFR* (4.03 fold). The significant differences in gene expression between BCTis and HNEpi samples were revealed for *BAG1*, *C3*, *CCNA2*, *CD44*, *FGF1*, *FOSL1*, *ID2*, *IL6R*, *NGFB*, *NGFR*, *PAPPA*, *PLAU*, *SERPINB5*, *THBS1* and *TP53* gene ($p < 0.05$) and *BCL2L2*, *CTSB*, *ITGB4*, *JUN*, *KIT*, *KLF5*, *SCGB1D2*, *SCGB2A1*, *SERPINE1* ($p < 0.01$), and *EGFR*, *GABRP*, *GSN*, *MAP2K7* and *THBS2* ($p < 0.001$), and *GSN*, *KLK5* ($p < 0.0001$). The borderline significance ($p < 0.07$) was observed for *CDKN2A* and *RPL27* gene (Table 2; Figs. 3, 4).

Focusing on biologic aggressiveness of the disease we have divided the luminal type of breast carcinomas into low-(G1-2) and high-grade cases (G3). In low-grade group we have observed a 2-fold overexpression rate in 23.8 % genes and in 2.4 % genes with at least 5-fold overexpression rate. The highest deregulation rate was revealed for *KLK5*, *SCGB1D2*, *NGFR*, *ITGB*, *GABRP*, *GSN* and *EGFR* gene. The significant differences ($p < 0.05$) were detected for *CCNA2*, *CDH1*, *CDKN2A*, *ITGB4*, *JUN*, *MAP2K7*, *MKI67*, *PLAU*, *PTGS2*, *SCGB2A1*, *SERPINA*, *STC2*, *TGFA*, *THBS1* and *VEGFA*, ($p < 0.01$) for *BAG1*, *CTSB*, *IL6*, *IL6R*, *NGFB*, *NGFR* and *PGR* gene, ($p < 0.001$) for *EGFR*, *GABRP*, *GSN*, *KIT*, *KLK5*, *RPL27*, *SCGB1D2* and *THBS2* gene, and ($p < 0.0001$) for *KLF5* gene (Table 3). In high-grade group there was 50.6 % genes with at least 2-fold overexpression and 16.9 % with 5-fold overexpression rate. Except *SERPINEA3*, all abnormalities were present only for HNEpi samples. The most expressed genes in HNEpi samples was *GNAS*, *KLK5*, *MT3*, *SPRR1B*, *CLU*, *GABRP*, *EGFR*, *KLF5*, *NGFR*, *PAPPA* and *CCNA1* gene. Significant difference ($p < 0.05$) was observed for *BCL2L2*, *CDKN1A*, *CLDN7*, *EGFR*, *GSN*, *MAP2K7*, *SCGB2A1*, *TP53* gene and *KLK5* gene ($p < 0.01$), (Table 3).

From the view of gene ontology we have revealed that in HNEpi samples in women with luminal type of breast cancer were the most frequently deregulated the genes from the third group (genes associated with breast cancer prognosis), and genes from the first group (genes associated with breast cancer origin); $n = 9$ and $n = 5$ deregulated genes with at least 2-fold overexpression, respectively. Particular differences (qualitative and quantitative) in gene expression from HNEpi samples between low- and high-grade cases. If considered only cases with at least 2-fold deregulation rate we have found that cases in

Table 2 The result of multigene analysis in BCTis and HNEpi tissue samples of all luminal type A and B cases

LUMINAL type A, B	AVG AC _i (Ct(GOD) - Ave Ct (HKG))		2 ^{-ΔC_t}		Fold Difference	T-TEST	Fold Up- or Down- Regulation
	BCTis	HNEpi	BCTis	HNEpi	BCTis / HNEpi	p value	BCTis / HNEpi
AR	6.47	6.71	1.13E-02	9.58E-03	1.18	0.8888	1.18
BAD	7.10	6.75	7.26E-03	9.30E-03	0.78	0.3669	-1.28
BAG1	4.44	4.60	4.61E-02	4.13E-02	1.12	0.0216	1.12
BCL2	6.90	6.58	8.40E-03	1.05E-02	0.80	0.6796	-1.25
BCL2L2	4.63	4.08	4.04E-02	5.92E-02	0.68	0.0028	-1.47
C3	7.66	5.81	4.94E-03	1.78E-02	0.28	0.0384	-3.60
CCNA1	7.44	7.23	5.75E-03	6.67E-03	0.86	0.2402	-1.16
CCNA2	6.02	6.89	1.54E-02	8.44E-03	1.82	0.0193	1.82
CCND1	7.01	7.25	7.76E-03	6.58E-03	1.18	0.7713	1.18
CCNE1	9.43	9.07	1.45E-03	1.86E-03	0.78	0.2210	-1.28
CD44	4.88	4.09	3.41E-02	5.86E-02	0.58	0.0147	-1.72
CDH1	1.41	1.48	3.78E-01	3.58E-01	1.06	0.9023	1.06
CDKN1A	2.27	2.40	2.07E-01	1.90E-01	1.09	0.5523	1.09
CDKN1B	6.59	6.07	1.04E-02	1.49E-02	0.70	0.2472	-1.43
CDKN2A	4.68	5.12	3.90E-02	2.87E-02	1.36	0.0685	1.36
CLDN7	8.22	7.74	3.35E-03	4.67E-03	0.72	0.4629	-1.39
CLU	7.27	7.11	6.46E-03	7.23E-03	0.89	0.4282	-1.12
COL6A1	-0.28	-0.40	1.21E+00	1.32E+00	0.92	0.7127	-1.09
CTNNB1	3.05	2.83	1.21E-01	1.40E-01	0.86	0.8757	-1.16
CTSB	-0.47	-0.03	1.38E+00	1.02E+00	1.35	0.0046	1.35
CTSD	2.38	2.11	1.92E-01	2.32E-01	0.83	0.8300	-1.21
CYP19A1	8.33	7.94	3.11E-03	4.07E-03	0.76	0.7138	-1.31
DIC1	7.82	7.32	4.42E-03	6.26E-03	0.71	0.1685	-1.42
EGFR	8.75	6.71	2.32E-03	9.53E-03	0.24	0.0007	-4.10
ERBB2	8.63	8.70	2.53E-03	2.40E-03	1.06	0.5960	1.06
ESR1	5.16	5.29	2.80E-02	2.55E-02	1.10	0.2520	1.10
ESR2	9.39	8.59	1.49E-03	2.59E-03	0.58	0.1317	-1.73
FAS	4.65	4.74	3.98E-02	3.75E-02	1.06	0.1256	1.06
FASLG	8.01	8.20	3.87E-03	3.39E-03	1.14	0.5748	1.14
FGF1	6.77	6.38	9.19E-03	1.20E-02	0.76	0.0435	-1.31
FLRT1	10.13	9.55	8.93E-04	1.33E-03	0.67	0.2790	-1.49
FOSL1	9.48	8.77	1.40E-03	2.29E-03	0.61	0.0496	-1.64
GABRP	7.33	5.72	6.20E-03	1.90E-02	0.33	0.0010	-3.06
GATA3	9.52	8.64	1.36E-03	2.50E-03	0.55	0.8026	-1.83
GNAS	12.04	11.47	2.37E-04	3.52E-04	0.67	0.1323	-1.48
GSN	0.70	-1.39	6.13E-01	2.63E+00	0.23	<0.0001	-4.28
HMG1	6.21	6.15	1.35E-02	1.41E-02	0.95	0.8051	-1.05
HSPB1	0.37	0.05	7.74E-01	9.67E-01	0.80	0.9399	-1.25
ID2	3.30	2.50	1.01E-01	1.77E-01	0.57	0.0426	-1.74
IGFBP2	4.61	4.14	4.11E-02	5.68E-02	0.72	0.9045	-1.38
IL2RA	8.25	7.91	3.30E-03	4.16E-03	0.79	0.2668	-1.26
IL6	6.99	6.40	7.87E-03	1.18E-02	0.67	0.0768	-1.50
IL6R	7.77	6.56	4.59E-03	1.06E-02	0.43	0.0154	-2.31
IL6ST	5.05	5.69	3.01E-02	1.93E-02	1.56	0.3379	1.56
ITGA6	2.40	2.36	1.89E-01	1.95E-01	0.97	0.7178	-1.03
ITGB4	9.09	7.31	1.84E-03	6.29E-03	0.29	0.0022	-3.41
JUN	8.46	7.13	2.84E-03	7.13E-03	0.40	0.0057	-2.51
KIT	8.74	7.24	2.34E-03	6.62E-03	0.35	0.0021	-2.83
KLF5	7.54	6.36	5.38E-03	1.22E-02	0.44	0.0025	-2.27
KLK5	10.31	5.71	7.90E-04	1.91E-02	0.04	<0.0001	-24.22
KRT18	2.17	2.05	2.23E-01	2.42E-01	0.92	0.5197	-1.09
KRT19	-0.66	-0.17	1.58E+00	1.12E+00	1.41	0.1841	1.41
MAP2K7	8.69	7.23	2.42E-03	6.66E-03	0.36	0.0009	-2.75
MKI67	6.99	7.54	7.89E-03	5.39E-03	1.46	0.1241	1.46
MT3	11.12	10.74	4.49E-04	5.84E-04	0.77	0.1832	-1.30
MUC1	3.04	2.48	1.22E-01	1.79E-01	0.68	0.8263	-1.47
NFYB	5.27	5.47	2.60E-02	2.26E-02	1.15	0.2148	1.15
NGFB	11.68	10.62	3.04E-04	6.35E-04	0.48	0.0187	-2.09
NGFR	10.60	8.59	6.45E-04	2.60E-03	0.25	0.0106	-4.03
NME1	3.41	3.23	9.39E-02	1.07E-01	0.88	0.9944	-1.14
PAPPA	8.90	8.48	2.09E-03	2.80E-03	0.75	0.0405	-1.34
PGR	5.74	6.14	1.87E-02	1.42E-02	1.32	0.1616	1.32
PLAU	4.08	4.43	5.93E-02	4.65E-02	1.28	0.0119	1.28
PTEN	2.05	2.34	2.41E-01	1.97E-01	1.22	0.3650	1.22
PTGS2	5.08	4.71	2.96E-02	3.81E-02	0.78	0.3760	-1.29
RAC2	8.43	7.70	2.89E-03	4.81E-03	0.60	0.2329	-1.66
RPL27	-1.40	-1.96	2.63E+00	3.88E+00	0.68	0.0614	-1.48
SCGB1D2	2.46	0.10	1.82E-01	9.36E-01	0.19	0.0014	-5.14
SCGB2A1	4.40	3.15	4.73E-02	1.13E-01	0.42	0.0034	-2.38
SERPINA3	1.62	1.85	3.24E-01	2.77E-01	1.17	0.3775	1.17
SERPINB5	6.44	4.81	1.15E-02	3.57E-02	0.32	0.0476	-3.10
SERPINE1	5.53	6.21	2.16E-02	1.35E-02	1.59	0.0084	1.59
SLC7A5	9.15	9.44	1.76E-03	1.44E-03	1.23	0.9451	1.23
SPRR1B	9.42	8.97	1.46E-03	1.99E-03	0.73	0.3501	-1.37
STC2	1.91	2.20	2.67E-01	2.18E-01	1.22	0.4649	1.22
TFF1	1.47	0.87	3.60E-01	5.48E-01	0.66	0.6150	-1.52
TGFA	8.47	8.06	2.81E-03	3.74E-03	0.75	0.1000	-1.33
THBS1	0.51	0.91	7.00E-01	5.33E-01	1.31	0.0114	1.31
THBS2	0.06	1.06	9.60E-01	4.79E-01	2.01	0.0072	2.01
TIE1	12.95	13.16	1.27E-04	1.09E-04	1.16	0.1198	1.16
TNFAIP2	7.77	7.56	4.57E-03	5.30E-03	0.86	0.4307	-1.16
TOP2A	6.60	7.56	1.03E-02	5.28E-03	1.95	0.1505	1.95
TP53	6.56	5.66	1.06E-02	1.98E-02	0.54	0.0188	-1.86
VEGFA	4.78	4.48	3.65E-02	4.49E-02	0.81	0.3270	-1.23

Negative value overexpression in HNEpi, positive value overexpression in BCTis (tumor), blue color 2-fold overexpression in HNEpi, pink color 2-fold overexpression in BCTis, red color significant difference, green color borderline significance

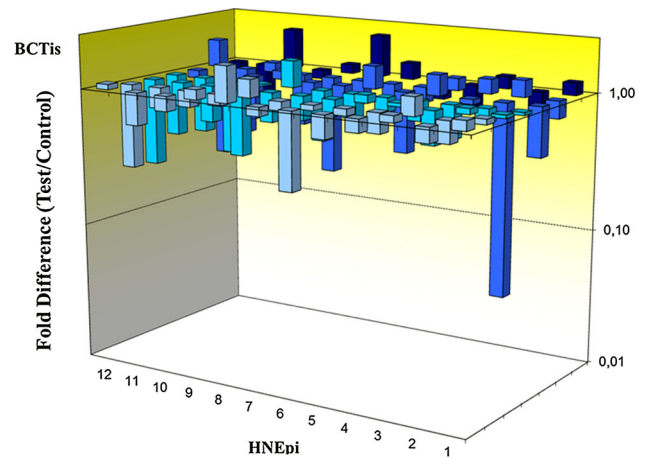


Fig. 3 The 3D plot of gene expression analysis in BCTis and HNEpi tissue samples in women with luminal type of breast cancer

high-grade group expressed much more genes in particular studied groups, (10 vs. 5 in 1st group; 7 vs. 2 in 2nd group; 20 vs. 8 in third group; and 8 vs. 1 in 5th group). Patients with low-grade carcinomas apart of same expressed genes to high-grade cases showed moreover the overexpression of C3 (complement 3), ITGB4 (β4-integrin) and TFF1 (pS2) gene in HNEpi and PGR, PLAU, CCNA2 and THBS2 in BCTis samples. On the other side, in high-grade cases we have detected high expressions for genes involved in apoptosis, cell cycle control and immune pathways in HNEpi samples, e.g. FOSL1 (FRA-1), FASLG, MKI67, IL2RA, TGFα, CCNA1 (cyclin A1), CCNE1 (cyclin E1) and CDKN1B (p27Kip1) gene, (Table 4).

Furthermore, apart from gene ontology we have analyzed the gene expressions in linkage to main cellular pathways, as well. The following processes were reviewed: hormonal signaling activity, epithelial-mesenchymal transition, angiogenesis, cell adhesion, proteolysis, apoptosis, cell cycle regulation, DNA impairment and transcription activity. We have found that the hormonal signaling, angiogenesis, cell adhesion and transcription pathways were more expressed in HNEpi in high-grade cases whereas other functional pathways showed more-less similar gene abnormalities, Table 5. Nevertheless, this difference should represent a phenotype-specific cancer gene signature in assessment the risky samples for the development of luminal low- or high-grade carcinomas.

Discussion

Breast cancer evolution is a complex process of continual changes. The pathologists for long time described morphological changes of this event and established many evolutionary theories in line with breast carcinogenesis.

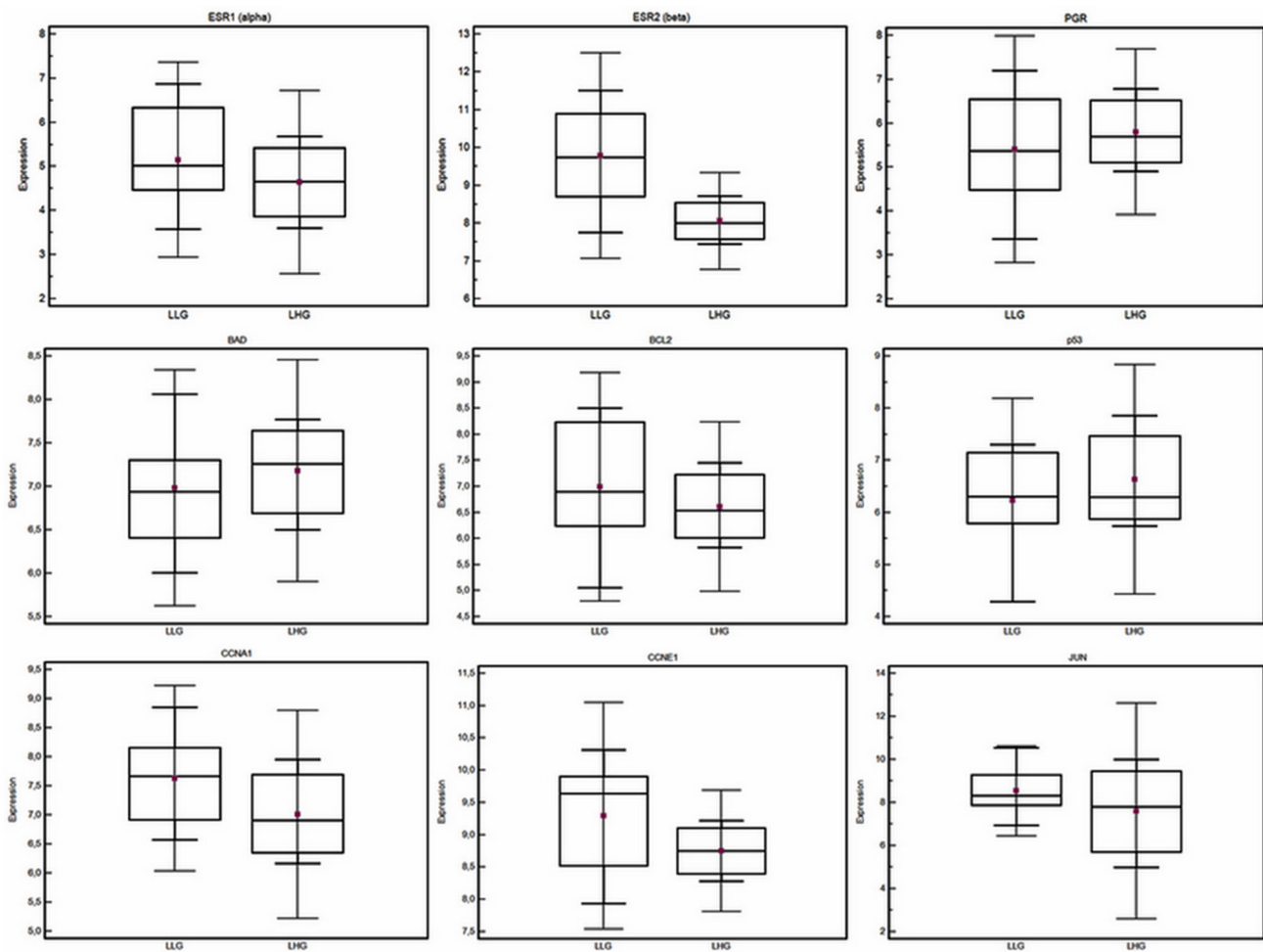


Fig. 4 Expression (ΔC_t) of selected genes in cell specific signaling pathways in HNEpi samples from patients with luminal breast cancer. *Upper row* hormonal signaling pathway; *middle row* apoptotic pathway; *lower row* cell cycle regulation pathway. Box-and-whisker plot, the *central box* represents the values from the lower to upper

quartile (25 to 75 %). The *middle line* represents the median. The *central marker* represents the mean, a line extends from the minimum to the maximum value, inner fences represents *error bars* for mean ($\pm 2SD$). *LLG* luminal low-grade type, *LHG* luminal high-grade type

The old model of breast malignancy originated from the neoplastic changes in *terminal duct-lobular unit*—TDLU [19] and for decades was thereafter process of breast carcinogenesis interpreted from the view of basic histomorphological changes favoring progressive linear model of mammary carcinogenesis. In this model normal breast epithelial cells of TDLU have transformed via hyperplasia with or without atypia to in situ lesions and later into invasive carcinoma [20]. The main role in this process took premalign lesions as atypical ductal hyperplasia and low-grade ductal carcinoma in situ, or atypical lobular hyperplasia and lobular carcinoma in situ. However, the presence of certain type of premalign lesion was difficult to address based only histopathologic features due to its subjective interpretation and specific role of usual duct hyperplasia, which was long time considered for precursor of atypical ductal hyperplasia and DCIS. It is obvious that this old model of breast carcinogenesis was built on

histopathological description of premalign lesions. The change in interpretation of breast malign epithelial transformation have come with the progress in molecular and genetic diagnostic methods, which by time enriched the diagnostic algorithm in clinical praxis of pathologists. The outcomes of these methods have point on the insufficiency of linear model of breast carcinogenesis and brought knowledge about wide molecular network complexity of breast carcinogenesis. Genetic abnormalities described in this malign conversion have become inseparable part of such events and therefore can serve as markers for early malignancy [21]. The close cooperation between pathologists and molecular biologists have resulted in description of new and wide accepted *multi-step* model of breast carcinogenesis, which integrate morphological, immunohistochemical, molecular and genetic information on this process. In this model are depicted two particular etiopathological pathways of breast cancer origin; low- and high-

Table 3 The result of multigene analysis in BCTis and HNEpi tissue samples of all Luminal type A and B low- vs high-grade cases. Negative value = overexpression in HNEpi; positive value = overexpression in BCTis (tumor); blue color = 2-fold overexpression in HNEpi; pink color = 2-fold overexpression in BCTis; red color = significant difference; green color = borderline significance

Gen of Interest	Low Grade Luminal type			High Grade Luminal type		
	Fold Difference	Fold Up-or Down-Regulation	T-TEST	Fold Difference	Fold Up-or Down-Regulation	T-TEST
	BCTis / HNEpi	BCTis / HNEpi	p value	BCTis / HNEpi	BCTis / HNEpi	p value
AR	1.12	1.12	0.4767	0.40	-2.51	0.2239
BAD	0.86	-1.16	0.3657	0.64	-1.56	0.2404
BAG1	1.16	1.16	0.0028	0.50	-2.00	0.7637
BCL2	0.80	-1.25	0.9005	0.87	-1.15	0.1560
BCL2L2	0.75	-1.33	0.1287	0.46	-2.18	0.0484
C3	0.33	-2.99	0.1528	0.77	-1.29	0.3819
CCNA1	0.79	-1.27	0.1878	0.22	-4.60	0.3763
CCNA2	2.11	2.11	0.0357	0.47	-2.15	0.8133
CCND1	1.18	1.18	0.1397	0.51	-1.95	0.5225
CCNE1	0.70	-1.44	0.6491	0.20	-5.12	0.1750
CD44	0.66	-1.52	0.1745	0.72	-1.39	0.1221
CDH1	1.21	1.21	0.0422	1.23	1.23	0.3576
CDKN1A	0.97	-1.03	0.9534	1.74	1.74	0.0315
CDKN1B	0.84	-1.09	0.9079	0.39	-2.55	0.2459
CDKN2A	1.36	1.36	0.0272	0.63	-1.58	0.6425
CLDN7	0.74	-1.36	0.8629	0.59	-1.70	0.0285
CLU	0.95	-1.05	0.2273	0.15	-6.81	0.2896
COL4A1	1.24	1.24	0.2289	0.88	-1.13	0.2999
CTNNB1	1.14	1.14	0.1653	0.45	-2.20	0.1833
CTSB	1.40	1.40	0.0011	1.10	1.10	0.7061
CTSD	0.74	-1.36	0.9623	1.36	1.36	0.5003
CYP19A1	0.98	-1.02	0.8666	0.20	-5.02	0.4302
DLC1	0.67	-1.50	0.2789	0.26	-3.83	0.2451
EGFR	0.26	-3.88	0.0007	0.17	-5.88	0.0450
ERBB2	1.33	1.33	0.7002	0.77	-1.30	0.2108
ESR1	1.16	1.16	0.1545	1.10	1.10	0.2588
ESR2	0.64	-1.56	0.1153	0.18	-5.68	0.3618
FAS	0.96	-1.04	0.3454	1.16	1.16	0.8258
FASLG	1.09	1.09	0.7084	0.45	-2.23	0.4644
FGF1	0.73	-1.38	0.0941	0.30	-3.38	0.1174
FLRT1	0.68	-1.47	0.4379	0.40	-2.53	0.2001
FOSL1	0.72	-1.39	0.1259	0.23	-4.28	0.2026
GABRP	0.24	-4.21	0.0002	0.15	-6.48	0.0870
GATA3	0.98	-1.02	0.3094	0.52	-1.94	0.1646
GNAS	0.70	-1.43	0.2534	0.08	-13.24	0.1441
GSN	0.26	-3.89	0.0003	0.26	-3.83	0.0476
HMGBl	1.08	1.08	0.7401	0.37	-2.72	0.6144
HSPB1	0.82	-1.22	0.7982	0.71	-1.41	0.2504
ID2	0.60	-1.66	0.2370	0.65	-1.55	0.8541
IGFBP2	0.83	-1.20	0.8852	0.89	-1.12	0.1957
IL2RA	0.64	-1.55	0.3111	0.25	-3.94	0.1957
IL6	0.57	-1.76	0.0065	0.58	-1.73	0.2406
IL6R	0.43	-2.31	0.0095	0.28	-3.55	0.1416
IL8	1.54	1.54	0.2674	0.65	-1.54	0.6644
ITGA6	1.36	1.36	0.9097	0.59	-1.68	0.2000
ITGB4	0.23	-4.31	0.0212	0.67	-1.50	0.1262
JUN	0.44	-2.29	0.0289	0.33	-3.03	0.1613
KIT	0.28	-3.59	0.0006	0.20	-5.03	0.1689
KLF5	0.53	-1.90	-0.0001	0.19	-5.16	0.0921
KLK3	0.06	-17.76	0.0006	0.08	-12.05	0.0036
KRT18	1.05	1.05	0.9236	1.38	1.38	0.9157
KRT19	1.37	1.37	0.6367	1.40	1.40	0.5105
MAP2K7	0.33	-3.02	0.0307	0.36	-2.78	0.0370
MK167	1.50	1.50	0.0457	0.42	-2.39	0.4470
MT3	0.91	-1.10	0.1358	0.11	-8.98	0.2210
MUC1	1.82	1.82	0.0684	1.02	1.02	0.4376
NFYB	1.21	1.21	0.1862	0.78	-1.28	0.7672
NGF8	0.55	-1.81	0.0029	0.20	-4.91	0.1643
NGFR	0.21	-4.76	0.0059	0.43	-3.32	0.1903
NME1	0.90	-1.11	0.2766	1.42	1.42	0.6870
PAPPA	0.73	-1.37	0.0947	0.19	-5.34	0.2338
PGR	2.32	2.32	0.0083	0.38	-2.61	0.5056
PLAU	2.29	2.29	0.0137	1.05	1.05	0.8129
PTEN	1.17	1.17	0.2229	0.82	-1.22	0.4446
PTGS2	0.68	-1.47	0.0145	0.29	-3.47	0.4019
RAC2	0.70	-1.43	0.8023	1.19	1.19	0.4407
RPL27	0.66	-1.50	0.0002	0.89	-1.12	0.2622
SCGB1D2	0.06	-15.81	0.0007	0.83	-1.20	0.3489
SCGB2A1	0.32	-3.08	0.0137	0.23	-4.34	0.0176
SERPINA3	1.17	1.17	0.0430	2.13	2.13	0.5392
SERPINB5	0.48	-2.08	0.3121	0.22	-4.46	0.2386
SERPINE1	1.79	1.79	0.0653	0.90	-1.11	0.4396
SLC7A5	1.09	1.09	0.8198	0.20	-5.12	0.2640
SPRR1B	0.74	-1.35	0.8999	0.14	-6.90	0.3795
STC2	2.99	2.99	0.0302	0.31	-3.24	0.3692
TFPI	0.34	-2.95	0.1393	0.30	-3.29	0.6943
TGFA	0.77	-1.30	0.0476	0.34	-2.96	0.4090
THBS1	1.23	1.23	0.0491	1.91	1.91	0.2214
THBS2	2.54	2.54	0.0009	1.90	1.90	0.5970
TIE1	1.65	1.65	0.2022	n/a	n/a	n/a
TNFAIP2	0.60	-1.68	0.2953	0.60	-1.68	0.2925
TP2A	1.76	1.76	0.1952	0.53	-1.88	0.6481
TP53	0.64	-1.57	0.5221	0.57	-1.77	0.0454
VEGFA	1.13	1.13	0.0192	0.66	-1.51	0.2400

grade directions. Thus detailed understanding of origin, as well as evolutionary mechanisms in mammary carcinogenesis have claimed the change in view of breast pathologist and surgeons on this disease and called for assessment the role of breast stem cells taking active role in early stages of mammary carcinogenesis. In line with this the need for differences between stem cells and normal epithelial cells was noted [22] and the role of tissue microenvironment took on importance. This was achieved thank to implementation of genetic testing in modern senology [23]. The results of this implementation is current knowledge of multigene expression and epigenetic regulation in multi-step model of mammary carcinogenesis.

It is obvious that triggering of carcinogenesis as well as progression into variable phenotypes and biologic forms of disease is initiated by the accumulation of structural and/or functional genetic abnormalities. For this reason we can find some specific genetic changes in breast carcinomas and breast tissue (gene overexpressions), which have impact on selected cellular functions and signaling activity taking role in processes as DNA integrity control, cell growth, detoxication, adhesion, migration, immune response and apoptosis. For years the mutations in cell critical genes were considered for major risk factors in impairment of above mentioned cellular pathways. Later it was revealed that not always the structural changes in genes are required for disease origin and that similar changes can be present with the epigenetic deregulations and multigene expressions. This findings changed the opinions on breast carcinogenesis and stimulated formulation of new theories assimilating the roles of structural DNA changes together with the changed gene signaling due to its epigenetic modulation or aberrant expression [24, 25]. By this we passed long way in understanding breast carcinogenesis from the progressive linear model based on histopathological features through multi-step model of morphological distinct evolutionary pathways based on structural genetic changes to multi-step multi-clonal evolution model with the participation of structural (mutation, deletion, amplification) and functional deregulations (expression, epigenetic modification) of genome at the level of breast epithelial cells, mammary stroma and tissue microenvironment.

After this knowledge a wide progress on the field of gene expression studies in solid carcinomas started, including breast cancer. Here was the importance of such studies augmented by the information that during process of mammary carcinogenesis from epithelial luminal cell to invasive carcinoma up to 90 % of all genetic abnormalities in luminal cells are present in early phase of malignancy (transition of TDLU to DCIS). Only 10 % of stromal and

Table 5 Gene deregulation in HNEpi and BCTis according to functional activity pathways

Functional activity	Overexpression in HNEpi		Overexpression in BCTis	
	LLG	LHG	LLG	LHG
Hormonal signaling			AR	AR
		C3		
		CCNE1		
			CCND1	CCND1
			CTNNB1	CTNNB1
		CTSD		CTSD
			ESR1	ESR1
		ESR2		
				KRT19
				KRT19
				KRT19
	EMT Angiogenesis			CTNNB1
			CTNNB1	CTNNB1
		EGFR		
			ERBB2	ERBB2
		IL6		
		JUN		PLAU
			PTEN	PTEN
			SERPINE1	SERPINE1
			THBS1	THBS1
			VEGFA	VEGFA
		BCL2		
Cell adhesion				CDH1
			P16INK4a	P16INK4a
			CTNNB1	CTNNB1
		EGFR		
			ERBB2	ERBB2
			PTEN	PTEN
			THBS1	THBS1
		CTSD		PLAU
				CDH1
		BAD		BAD
		BCL2		BCL2
	Proteolysis Apoptosis			CDH1
				P21/WAF1
			P16INK4a	P16INK4a
		IL6		
		JUN		
			MUC1	MUC1
		NME1		NME1
		P53		P53

Table 5 continued

Functional activity	Overexpression in HNEpi		Overexpression in BCTis	
	LLG	LHG	LLG	LHG
Cell cycle regulation		BCL2		BCL2
		CCNA1		CCNA1
			CCND1	CCND1
		CCNE1		CCNE1
		P21/WAF1		P21/WAF1
			P16INK4a	CDKN2A
		JUN		JUN
			KI67	KI67
			PTEN	PTEN
		P53		P53
DNA impairment			CCND1	CCND1
		P21/WAF1		P21/WAF1
		P53		P53
			CTNNB1	CTNNB1
Transcription			AR	AR
			ESR1	ESR1
		ESR2		ESR2
		GATA3		GATA3
		JUN		JUN
			PGR	PGR
		P53		P53

LLG luminal low-grade type, LHG luminal high-grade type, EMT epithelial-to-mesenchymal transition

0 % epithelial genetic changes were noted in late phase of breast carcinogenesis, transition of in situ to invasive form. These information point on the fact that majority of genetic alterations occur in advance of histologically detected changes by pathologist. Moreover, it seems that mammary stroma and its gene expression is in regulatory role of mammary carcinogenesis on superior level compared to epithelial gene expressions on which may have direct signaling influence [26]. Thus, the central regulatory role of tissue microenvironment in process of early breast carcinogenesis cannot be omitted. However, the gene expressions in histologically normal breast tissue (HNEpi) in epithelial or stromal cells have been analyzed insufficiently and only few studies directly examined the gene expressions in HNEpi compared to healthy or malignant breast tissue [4–10, 13, 27, 28].

Following this, we have focused our investigation on gene expression analysis in HNEpi among women with luminal breast carcinomas in aim to find differences

between gene expressions and in ambition to find gene signature reflecting malignancy risk and tumor biology profile. Our results brought specific findings on different gene expressions between histologically normal and malign breast tissue and the existence of particular differences related to tumor aggressiveness. The existence of malignancy risk signature may help in selection of patients at risk for luminal breast cancer as well as for prediction of low- or high-grade phenotype of the disease. The implication of this marker is enormous, especially in cases with negative histology after core biopsy for breast tumor. From the view of gene ontology we have revealed that in HNEpi samples in women with luminal type of breast cancer were the most frequently deregulated the genes from the third group (genes associated with breast cancer prognosis), and genes from the first group (genes associated with breast cancer origin); $n = 9$ and $n = 5$ deregulated genes with at least 2-fold overexpression, respectively. Particular differences (qualitative and quantitative) were revealed in gene expression from HNEpi samples between low- and high-grade cases. Furthermore, apart from gene ontology we have analyzed the gene expressions in linkage to main cellular pathways, as well. We have found that the hormonal signaling, angiogenesis, cell adhesion and transcription pathways were more expressed in HNEpi in high-grade cases whereas other functional pathways showed more-less similar gene abnormalities. Moreover, our results represents the unique findings reflecting to molecular subtypes of breast cancer and tumor aggressiveness.

In international comparisons was the extent of gene deregulation rate in our study (minimal of 2-fold change, luminal type) similar to the rate reported by Dahl et al. [7] (20.2 vs. 30 %). However, they did not differentiate the tumor type, tumor molecular subtype, stage, uni (multi) focal appearance, tumor grade (low–high grade) and lymph nodal status. Apart of this, they revealed high level of gene deregulation in HNEpi in process of breast carcinogenesis, the findings that we can confirm, mainly in relation to high grade lesions.

Similar results to ours presented Grigoriadis et al. [5], who revealed in luminal breast carcinomas high deregulation rate of genes in BCTis and HNEpi in signaling pathways encoding cell cycle regulation, transcription, MAPK regulated mitogene activity (*FGF4*, *IL1A*, *IL1B*, *NGFB*, *TGFB1*, *TGFB3*), JAK-STAT and cytokine signaling pathways (*IL6*, *IL10*, *OSM*, *SPRY2*, *CXC*, *CC*), genes of kallikrein system (*KLK5*, *KLK7*, *KLK8*, *KLK10*) and RAS-regulatory pathway (*RAP1A*, *RALB*, *RAB5B*, *RAB4A*, *RAB2*). It is obvious that we have achieved more-less similar results. The gene expression (*GATA3*, *PGR* a *ER1*, *ER2*) in luminal breast carcinomas was evaluated also by Schummer et al. [6], who concluded that *GATA3* overexpression is associated not only with luminal phenotype of

the disease, but also with worse prognosis, thus confirming our results of higher *GATA3* expression in high-grade carcinomas.

The significant differences in gene expression between HNEpi and BCTis revealed also Pau et al. (2010) [10] for genes *CD24*, *CD36*, *CD9*, *TACSTD1*, *TACSTD2*, *HBB*, *LEP*, *LPL*, *AKR1C1*, *AKR1C2* a *AKR1C3* with minimal 2-fold overexpression rate ontologically belonging to genes playing role in regulation of progesterone, fatty-acid, carboxyl and organic acids. However, these results were achieved from ethnically heterogeneous population (Malay, China, India) and heterogeneous breast carcinomas (invasive ductal/lobular type, ER ±, HER2 ±, Grade 1–3) what may have impact on different findings from our results and results from other studies analyzing Caucasian population.

In our work we have revealed different gene deregulation in HNEpi for low- and high grade luminal cancer. This findings may help to identify an aggressive phenotype of the disease, moreover could serve as adjunct information in classification carcinomas of grade 2 into low- or high grade group. By such approach we are getting closer to understanding of true biologic features of breast malign disease. Similarly to our study, Sortiriou et al. [29], identified overexpressions in genes regulating cell cycle and proliferation responsible for worse prognostic outcome in grade 2 ER + breast carcinomas. These conclusions stimulated others in search for others genetic markers and Dahl et al. [7] revealed deregulations in genes coding synthesis of nuclear proteins (PPAPDC1A and KPNA2) and showed their association with shorter overall survival and disease free interval in patients with breast cancer. The prognostic scope in in situ and invasive breast carcinomas analyzed also Ma et al. [30] who detected alterations in gene expression in HNEpi in epithelial as well as in stromal cells during mammary carcinogenesis. They indicated for the most deregulated genes those related to epithelial-mesenchymal transition, mainly MMP1, MMP2 and MMP14 as a result of basal membrane disruption. This findings represents worse prognosis for patients. Although we did not directly analyzed genes encoding MMP, we have detected a high overexpression of *CTNNB1* gene in HNEpi from patients with luminal high grade disease. Our findings were confirmed by Sgroi [31] on analysis of gene expression in premalign breast lesions.

As luminal type of breast cancer with ER positivity is considered prognostically good type of the disease, there were several studies in past focused on gene expression in HNEpi among women with ER + and ER- breast cancer with the aims to find out possible risk factors for disease origin. Following this Tripathi et al. [8] noted significant differences in gene expression between HNEpi and BCTis in women with ER + breast cancer, mainly for

genes involved in transcription (*FOS*, *JUN*, *ATF3*), translation (*EIF1*, *EIF5*), DNA control (*GADD45B*), cell cycle regulation (*CCN1*, *CEBPD*, *YPEL5*), metabolic and enzyme activity (*ODC1*, *ALDH1A3*), cell adhesion (*CLDN1*, *CYR61*, *FLRT2*), cytokine regulation (*CCL2*, *CXCL1*, *CXCL2*) and RNA binding activity (*CSDE1*, *HNRPA1*), which were overexpressed in HNEpi compared to BCTis samples. Similarly Graham et al. [27] revealed the significant difference in gene expression between BCTis and HNEpi samples among women with breast cancer, women with BRCA mutations after prophylactic mastectomy and in women after reduction mammoplasty. Despite weakness of the study (analysis of luminal and HER2 positive carcinomas together without stratification) was the difference in gene expressions in HNEpi for all groups significant and independent of possible tumor influence. Subsequently they defined these expressions as a possible early marker of disease risk, not the effect of tumor itself.

The advantage of gene expressions in HNEpi is possibility to develop a specific gene signature responsible for the development of different phenotypes of breast cancer. This idea is not timeless, as there exist yet first studies analyzing gene profile in HNEpi in relation to invasive ductal or lobular cancer (aspirin—*ASP* and collagen triple helix repeat containing 1—*CTHRC1* gene overexpression), and invasive medullar breast cancer (insulin growth factor binding protein (*IGFBP*) group genes). From the view of gene ontology are that genes taking role in epithelial-mesenchymal transition, growth-stimulation, TGF-beta and Wnt signaling pathway [12, 32]. Nevertheless, the great advance of gene profiling in HNEpi is the possibility of detection cancer sub-type growing patterns. Graham et al. [4] found that there is 25-53 % genetic abnormalities which allows differentiation between ER positive and ER negative breast carcinomas from gene expressions in HNEpi. The most deregulated genes were *ESR1*, *GATA3*, *CX3CL1*, *CXCL13*, *TNFSF11*, *EREG*, *CYP24A1*, *STC1*, *CLCA2*, *MYBPC1* and *FABP7*. The weakness of the study was the use of samples with variable tumor nuclear grading.

In conclusion we can say that gene expressions in HNEpi are becoming the scope of interest for scientists in search for markers of early breast carcinogenesis. These findings offer speculative ideas about existence of possible malignancy risk signature in “histologically normal, but molecularly abnormal” breast tissue where such signatures would represent a great breakthrough on the way to comprehend the differences in cancer origin, phenotype, behavior and therapeutic or chemopreventive approaches. Our work represents one of few studies focusing on gene expressions in HNEpi among women with breast cancer and is the second that have straight link to luminal breast carcinomas and tumor grading aggressiveness. However,

despite the indicated trends, the limited number of paired samples used in this study lacks sufficient statistical power for definitive identifying an aggressive phenotype of the disease based on these gene expression- deregulation profiling, as some gene expressions may be individual dependent, it means that more samples in further studies are needed for confirmation of our results.

Acknowledgments The authors thank Jana Guranova for her excellent technical support. This work was supported by Grant UK 243/2012 and VEGA 1/0243/12 from Ministry of Education.

Conflict of interest The authors declare no conflict of interests.

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