Apoptosis-, proliferation, immune function-, and drug resistance- related genes in ER positive, HER2 positive and triple negative breast cancer

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The aim of our study was to examine an association between gene expression assessed using a 23-gene microarray and receptor status of breast cancer samples categorized as ER positive, HER2 positive and triple negative subtypes. The ER positive cohort was subsequently divided into Luminal A, Luminal B HER2 negative and Luminal B HER2 positive subtypes. Core- needle biopsies were collected from 78 female patients with inoperable locally advanced breast cancer or resectable tumors suitable for downstaging, before any treatment. Expressions of 23 genes were determined by means of TagMan Low Density Arrays. Analysis of variance was used to select genes with discriminatory potential between receptor subtypes. We introduced a correction for false discovery rates (presented as q values) due to testing multiple hypothesis. Pairwise post-hoc comparisons of receptor subtypes were performed using Tukey 's HSD test. Five genes out of a 23-gene microarray differed significantly in relation to breast cancer receptor-based subtypes. Among these five genes, we identified: *BCL2* (p=0.0002, q=0.0009), *MKI67* (p=0.0037, q=0.0064), *IGF1R* (p=0.0040, q=0.0064), *FOXC1* (p=0.0113, q=0.0135) and *IRF1* (p=0.0435, q=0.0416) as ones showing ER positive, HER2 positive and triple negative -subtype specific expression profiles. When incorporating Luminal A, Luminal B HER2 negative, Luminal B HER2 positive subtypes into analysis, four genes: *BCL2* (p=0.0006, q=0.0034), *MKI67* (p=0.0078, q=0.0198), *FOXC1* (p=0.0102, q=0.0198) and *IGF1R* (p=0.0174, q=0.0254) were selected. Elevated levels of *IGF1R* and *BCL2* were significantly linked with Luminal A subtype. Triple negative breast cancer subtype was associated with higher expression of *IRF1*, *FOXC1* and *MKI67*. In HER2 positive cohort lower expression of all five analyzed genes was noted.

Key words: breast cancer, gene microarray, receptor subtypes

Heterogeneity of breast cancer is a well established phenomenon [1-3] but is not adequately reflected by traditional clinical and pathological criteria such as tumor size, node involvement, grade, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status [4-6]. In recent years, genetic developments and other more sophisticated methods of characterization of breast cancer have evolved [4, 7-9]. In addition to the three receptor-based subtypes – ER positive, HER2 positive and triple negative, intrinsic subtypes of breast cancer have been identified in recent years – Luminal A, Luminal B, HER2-enriched, Basal-like and their clinico-pathologic definition classified as Luminal A, Luminal B HER2 negative, Luminal B HER2 positive, HER2 positive (non luminal) and triple negative (ductal) [4, 8, 10, 11]. Furthermore, a wide variety of multigene signatures have been validated to individualize decision making and therapeutic algorithms. Extensive research has been conducted on pathways that involve proliferation and apoptosis cascades, immune response genes, chemo- and endocrine response and resistance (the 70-gene signature Mammaprint™, the 21-gene signature Oncotype DX™, Femtelle™, 76-gene signature, Core epithelial-to-mesenchymal transition interactome gene-expression signature, the 7 gene immune response, wound response gene signature,

MapQuant™ Genomic Grade, an anthracycline- based score A-score, etc) [12-22]. The majority of these signatures show comparable performance despite the limited overlap of genes, but are probably less informative for ER negative patients [23], leaving scope for further research.

The aim of our study was to examine an association between 23-gene microarray including *ABCB1, ABCC1, BAX, BBC3, BCL2, CASP3, CYP2D6, ERCC1, FOXC1, GAPDH, IGF1R, IRF1, MAP2, MAPK 8, MAPK9, MKI67, MMP9, NCOA3, PARP1, PIK3CA, TGFB3, TOP2A, YWHAZ* (Table 1) with receptor status of breast cancer samples categorized as ER positive, HER2 positive and triple negative subtypes. The ER positive cohort was subsequently divided into Luminal A, Luminal B HER2 negative and Luminal B HER2 positive subtypes. Lists of genes that represent various biological pathways were assembled from gene datasets [24, 25].

Patients and methods

The study was conducted under Institutional Review Board protocol # RNN/159/10/KE/07/09/2010, Medical University of Lodz and all patients gave written informed consent.

Before any treatment, ultrasound guided 14-gauge core-needle biopsies using an ultra automatic biopsy instrument (Pro-Mag™, Angiotech) were collected from 78 female patients with inoperable locally advanced breast cancer or resectable tumors suitable for downstaging, and from two healthy controls at the Cancer Center of Lodz, Copernicus Memorial Hospital between September 2010 and April 2011. Four to – five specimens per lesion were obtained, half of which were frozen immediately at -80 °C, for subsequent RNA extraction, cDNA generation and custom-designed TaqManR gene expression assay. The other samples were paraffin-embedded and reviewed by dedicated breast pathologists in the Department of Pathology, Medical University of Lodz. ER and PR status were determined by immunohistochemistry (IHC) using the Allred score. HER2 status was evaluated by immunohistochemistry or by fluorescence *in – situ* hybridization. HER2-positive tumors were defined as 3+ receptor overexpression on IHC staining and/or gene amplification found on fluorescent *in – situ* hybridization. TNM clinical staging was assessed by mammography, ultrasound of the breast, axilla and abdomen, and chest x-ray. In selected cases, MRI of the breast was performed.

Total RNA extraction and cDNA generation. Total RNA was extracted from samples according to the manufacturer 's RNeasy mini kits protocol (Qiagen, Hilden, Germany). In the initial step, RLT buffer (containing β-mercaptoethanol) was added to Eppendorf tubes containing the frozen samples which were homogenized using a Qiagen homogenizer (TissueRuptor) and centrifuged for 3 min at 14000 rpm. Following the manufacturer 's protocol, a DNase digestion was performed and RNA was quantified using PicoDrop spectrophotometer (Picodrop, Saffon Walden, Cambridgeshire, UK). The quality of RNA samples was analyzed by measuring the ratio of ab-

sorptions at 260/280 nm (an optical density ratio was between 1.9-2.2). The purified total RNA was immediately used for cDNA synthesis or stored at −80 °C.

Generation of cDNA was performed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems Inc., Foster City, CA, USA) following the reverse transcription protocols of the manufacturer. 500 ng of DNAse-treated total RNA was used as starting material, to which was added 2x RT master mix containing 2 μl of 10x RT Buffer, 0.8 μl of 25x dNTP Mix (100 mM), 2 μl of 10x RT Random Primers, 1 μl MultiScribe™Reverse Transcriptase and 1 μl RNase Inhibitor per each 20 μL reaction. Reverse transcription was performed in conditions optimized for use with this kit (25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min). The samples were kept frozen at −20 °C.

Custom-designed TaqMan® Gene Expression Assays. Gene expression was measured using custom-made TaqMan Low Density Arrays (Applied Biosystems Inc., Foster City, CA). The assay comprised of probes selected to measure expression of 23 *a priori* selected genes: *ABCB1, ABCC1, BAX, BBC3, BCL2, CASP3, CYP2D6, ERCC1, FOXC1, GAPDH, IGF1R, IRF1, MAP2, MAPK8, MAPK9, MKI67, MMP9, NCOA3, PARP1, PIK3CA, TGFB3, TOP2* and *YWHAZ.*

The microfluidic cards consisted of 8 ports with 23 different TaqMan primer pair/probe sets arrayed in duplicate in a 384-well microplate. Each well contained a gene-specific forward and reverse primer, as well as a gene-specific probe, which is labeled at the 5' position with 6FAM (reporter dye) and at the 3' position with minor groove binder/non-fluorescent quencher.

RT reactions were performed after adding 500 ng cDNA mixed with 2x TaqMan Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA), loaded on the TLDA card, and analyzed by PCR on the 7900HT instrument using Applied Biosystems Sequence Detection System 2.0 software according to the manufacturer's instructions. Target gene expression data from samples was normalized using 18S RNA to compensate for variability in the amount of RNA and for exclusion of general transcriptional effects.

Molecular analyses were performed in the Department of Molecular Biology, Central Laboratory, Medical University of Lodz.

Statistical analysis. Expression ratios were computed for each gene by dividing the values obtained in each patient by those from healthy tissue samples. Gene expression data underwent logarithmic transformation and standardization by dividing their difference from group mean by respective standard deviations. This provided a homogenous group of variables with similar scales and ranges of values. Univariate comparisons of gene profile between receptor subtypes were performed using analysis of variance (ANOVA) with p values verified by false discovery rates (FDR) to correct for multiple hypotheses testing. Genes that showed different expression depending on receptor subtype in ANOVA entered post-hoc analyses with Tukey's HSD test to determine which subgroup

Table 1. Summary of the studied genes. * 3 subtype analysis included ER positive, HER2 positive and triple negative subtypes. ** 5 subtype analysis included Luminal A, Luminal B HER2 negative, Luminal B HER2 positive, HER2 positive and triple negative subtypes.

Gene symbol	Full name	Function	p (3 subtype analysis*)	q (3 subtype analysis*)	p (5 subtype analysis**)	q (5 subtype analysis**)
Apoptosis- related genes						
BAX	Bcl-2 associated X protein	Apoptotic activator	0.1394	0.0707	0.3131	0.1409
BBC3	PUMA- p53 upregulated modulator of apoptosis; Bcl-2 binding component 3	Essential mediator of p53-dependent and p53-independent apoptosis	0.6605	0.1581	0.7469	0.2080
BCL2	B-cell CLL/Lymphoma 2	Suppression of apoptosis	**0.0002**	0.0009	**0.0006**	0.0034
CASP3	Caspase 3; apoptosis-related cysteine peptidase	Execution-phase of cell apoptosis	0.4828	0.1358	0.6955	0.2034
Proliferation- related genes						
FOXC1	Forkhead box C1	Embryonic and ocular development; regulation of cellular functions in breast cancer	**0.0113**	0.0135	**0.0102**	0.0198
IGF1R	Insulin-like growth factor 1 receptor	Tyrosine kinase activity; anti-apoptotic agent enhancing cell survival; mediates pre- and postnatal growth	**0.0040**	0.0064	**0.0174**	0.0254
MAPK8	Mitogen activated protein kinase 8; C-Jun kinase 1; JNK1; Jun N-terminal kinase	Response to activation by environmental stress and pro-inflammatory cytokines, T-cell proliferation, apoptosis and differentiation	0.1388	0.0707	0.2972	0.1409
MAPK9	Mitogen activated protein kinase 9; C-Jun kinase 2; JNK2	Stress- activated serine-threonine kinase, involved in cancer and inflammation, increases the stability of p53 in non-stressed cells	0.8494	0.1909	0.5685	0.1750
MKI67	Ki-67	Proliferation- related antigen	**0.0037**	0.0064	**0.0078**	0.0198
MMP9	Matrix metalloproteinase 9; type IV collagenase, GELB gelatinase B	Breakdown of extracellular matrix, tissue remodeling, proliferation, migration, angiogenesis, differentiation, metastasis	0.1626	0.0707	0.1882	0.1376
TGFB3	Transforming growth factor beta 3	Suppression and promotion of tumorigenesis	0.3169	0.1010	0.4742	0.1569
Immune function genes						
IRF1	Interferon regulatory factor 1	Immune function gne; apoptosis, tumor suppression;	**0.0435**	0.0416	0.1432	0.1376
Drug resistance/ metabolism – related genes						
ABCB1	ATP-binding cassette sub-family B member 1	Decreased drug accumulation in multidrug-resistant cells; development of resistance to anticancer drugs	0.1895	0.0734	0.2152	0.1399
ABCC1	MRP1- multidrug resistance protein; ATP-binding cassette sub-family C member 1	Multidrug resistance	0.1165	0.0707	0.0623	0.0729
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	Drug metabolism	0.8781	0.1909	0.9590	0.2550
ERCC1	Excision repair cross complementing 1	DNA repair	0.3960	0.1184	0.3385	0.1414
MAP2	Microtubule associated protein 2	Stabilization of microtubules	0.1550	0.0707	0.1820	0.1376
NCOA3	Nuclear receptor coactivator 3; AIB-1	Coactivation of nuclear receptors such as steroids (ER), histone acetyltransferase activity	0.6157	0.1581	0.4830	0.1569
PARP1	Poly-(ADP ribose) polymerase 1	Base excision repair pathway, DNA metabolism	0.6612	0.1581	0.2805	0.1409
PIK3CA	Phosphoinositide- 3 kinase, catalytic, alpha polypeptide	Lipid kinase, involved in proliferation, cell survival and migration, cooperation with the mTOR (mammalian target of rapamycin) pathway	0.2608	0.0891	0.4698	0.1569
TOP2A	Topoisomerase II alpha	Control of topology of DNA strands; development of drug resistance	0.1599	0.0707	0.2586	0.1409
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zet	Anti-apoptotic gene; chemoresistance to anthracyclines	0.1996	0.0734	0.3698	0.1442
Endogenous control						
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Endogenous control; carbohydrate metabolism				

deviated from the remainder in expression values. Statistical computations were performed in Statistica 9.0 PL (Statsoft, Tulsa, OK, USA). Hierarchical clustering of gene expression ratios was used to visualize expression patterns in the analyzed groups. The GenePattern online suite (http://genepattern. broadinstitute.org/gp/pages/index.jsf) was used for this purpose. Q values representing FDRs were computed in R using the qvalues package. A p value less than 0.05 was considered as statistically significant. For comparisons of gene expression profiles, a q value less than 0.05 was necessary to deem a particular result as significant rather than an incidental one due to testing multiple hypothesis.

Results

Patients enrolled in the study were aged between 32-82 years old, mean age 60.2 years. Histopathological tumor types were: invasive ductal breast cancer (67 patients) , invasive lobular cancer (eight patients), mucinous cancer (one patient), adenoid cystic cancer (one patient), unknown (one patient). Tumor grades were: Gx – 1, G1 – 1, G2 – 12 and G3 – 64 patients. Tumor stage was: stage I in two patients, IIA in six, IIB in 13, IIIA in 19, IIIB in 25, IIIC in four and IV in 9 individuals. Receptor status was: ER positive in 47 patients, ER negative in 31 patients, PR positive in 38 patients, PR negative in 40 patients, HER2 positive in 13 patients, HER2 negative in 61 patients. In four cases HER2 status was unknown and these patients were not included in statistical analysis. Surrogates of intrinsic subtypes were: Luminal A (34 patients), Luminal B HER2 negative (7 patients), Luminal B HER2 positive (5 patients), Triple Negative (20 patients), HER2 positive (8 patients).

After neoadjuvant chemotherapy or hormonal therapy, pathologic complete response (pCR) was found in 12% of patients, near pCR- in 16.7%, partial pathologic response- in 43% and no response or progression in 28.3% of patients.

Statistical analysis showed that five genes out of a 23-gene microarray differed significantly in relation to ER positive, HER2 positive, and triple negative breast cancer subtypes. Among these genes, we identified: *BCL2* (p=0.0002, q=0.0009), *MKI67* (p=0.0037, q=0.0064), *IGF1R* (p=0.0040, q=0.0064), *FOXC1* (p=0.0113, q=0.0135) and *IRF1* (p=0.0435, q=0.0416). Expressions of these five genes were compared between receptor subtype groups – results of these comparisons are shown in Figure 1. When incorporating Luminal A, Luminal B HER2 negative, Luminal B HER2 positive subtypes into analysis, four genes: *BCL2* (p=0.0006, q=0.0034), *MKI67* (p=0.0078, q=0.0198), *FOXC1* (p=0.0102, q=0.0198) and *IGF1R* (p=0.0174, q=0.0254) were identified (Fig. 2).

P values of the remaining genes in the 23- gene microarray- (*ABCB1, ABCC1, BAX, BBC3, CASP3, CYP2D6, ERCC1, MAP2, MAPK 8, MAPK9, MMP9, NCOA3, PARP1, PIK3CA, TGFB3, TOP2A, YWHAZ)* did not reach a level of statistical significance (Table 1).

Hierarchical clustering of gene expression ratios in the compared patient groups showed considerable heterogeneity of the patients categorized into the 5 subtypes of receptor status (Figure 3).

Discussion

Medical literature on Forkhead box C1 (*FOXC1*), initially described in eye development, is scarce [25, 26]. Our study has demonstrated elevated *FOXC1* mRNA levels in core biopsies from triple negative breast cancers. Consistently, Ray and Giuliano et al. from John Wayne Cancer Institute have shown that *FOXC1* may be a pivotal prognostic biomarker of basal- like breast cancer which encompasses 60% to 90% of triple negative breast cancers [26, 27]. On the basis of gene expression analysis of publicly available human breast cancer microarray data sets, overall survival was significantly worse in tumors with elevated *FOXC1* mRNA levels and coincided with the basal- like subgroup clustered by International Genomics Consortium [26, 27]. *FOXC1* is also involved in brain development and tumorigenesis which might explain why triple negative tumors more often metastasize to the brain. Ray and Giuliano et al. examined the function of *FOXC1* in the breast cancer cells- elevated expression of *FOXC1* in MDA-MB-231 basal-like breast cancer cells increased cell proliferation, migration and invasion [26, 27]. Taube et al. from MD Anderson Cancer Center have shown that *FOXC1* induces the epithelial-to-mesenchymal transition, exhibiting stem cell characteristics with metastatic potential [19].

IRF1 interferon regulatory factor 1 mediates interferon and other cytokine effects, promotes apoptosis through p53-dependent and independent pathways and caspase activation. Functionally, overexpression of *IRF1* inhibited carcinogenesis in human breast cancer xenografts [25, 27]. Cavalli et al. have observed that low *IRF1* mRNA expression was associated with poor clinical outcome and correlated with risk of recurrence and death [28]. These authors did not compare *IRF1* levels between receptor- based subtypes. In our study, elevated *IRF1* was found in triple negative breast cancers in comparison with ER positive and HER2 positive cohorts. Currently, we are correlating expressions of immune function genes with response to neoadjuvant chemotherapy in these group of patients and pathologic complete response could be a surrogate for good prognosis. Teschendorff et al. have demonstrated a positive and negative association of immune signatures with good prognosis in ER negative and ER positive disease, respectively [29]. In the studies conducted by Rody, Pusztai et al., ER(-) tumors with high expression of immune function metagenes seemed to respond better to neoadjuvant chemotherapy [30]. Furthermore, Ascierto et al. have hypothesized that a network of genes involved in B, T cell development, interferon signaling, and adaptive and innate immune responses, at the tumor site can predict a risk of distant relapse in breast cancer patients even regardless of the status of ER, PR or HER2 [20].
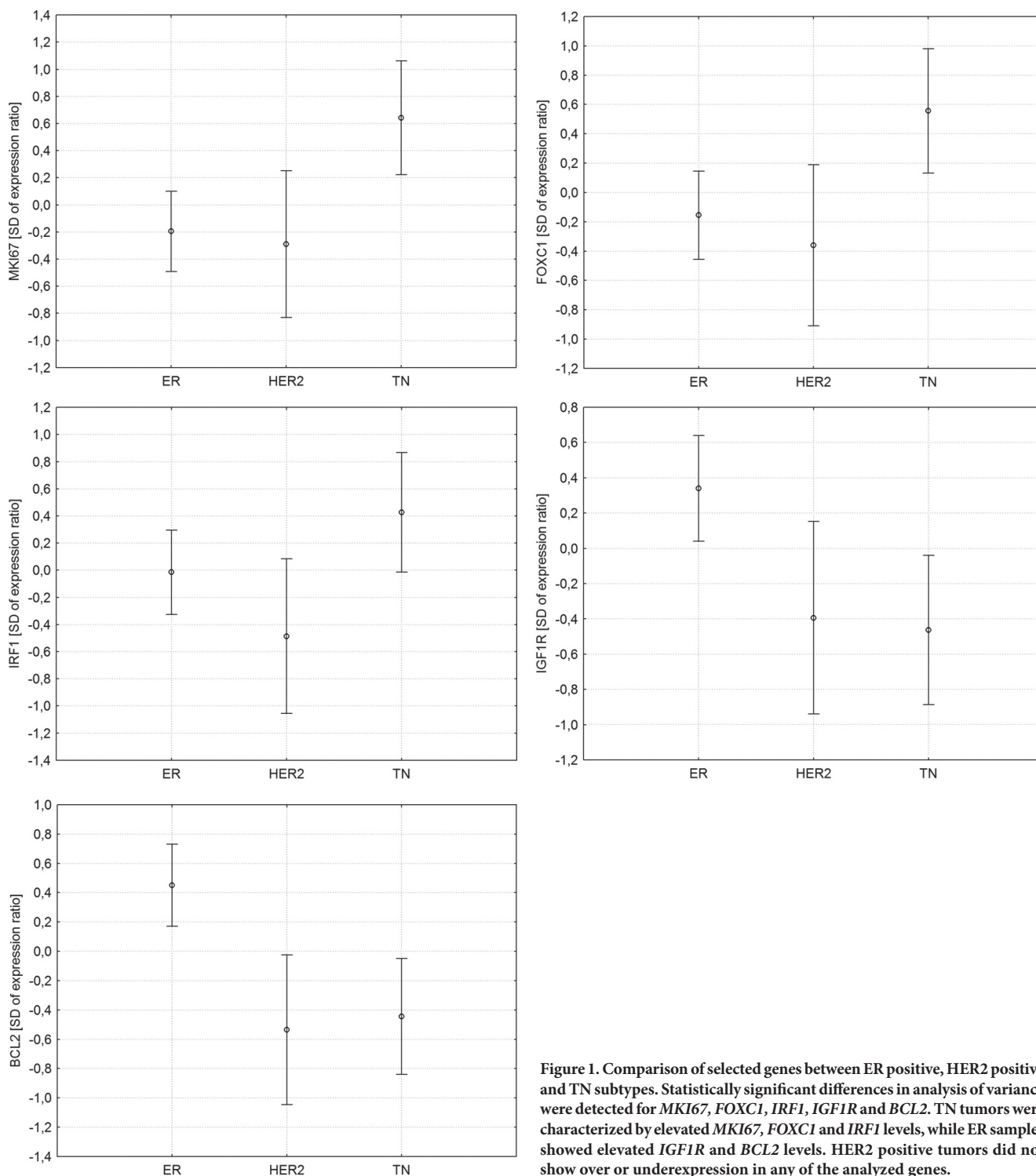
Figure 1. Comparison of selected genes between ER positive, HER2 positive and TN subtypes. Statistically significant differences in analysis of variance were detected for *MKI67, FOXC1, IRF1, IGF1R* and *BCL2*. TN tumors were characterized by elevated *MKI67, FOXC1* and *IRF1* levels, while ER samples showed elevated *IGF1R* and *BCL2* levels. HER2 positive tumors did not show over or underexpression in any of the analyzed genes.

In our study expression of *MKI67* – a marker of tumor cell proliferation, was significantly higher in triple negative carcinomas compared with ER positive and HER2 positive tumors, which is consistent with other research results [31,

32]. In commercially available assays such as Oncotype DX, elevated *MKI67* increases the recurrence score [16].

Insulin- like growth factor 1 receptor (*IGF1R*), with tyrosine kinase activity, mediates biologic effects of insulin-
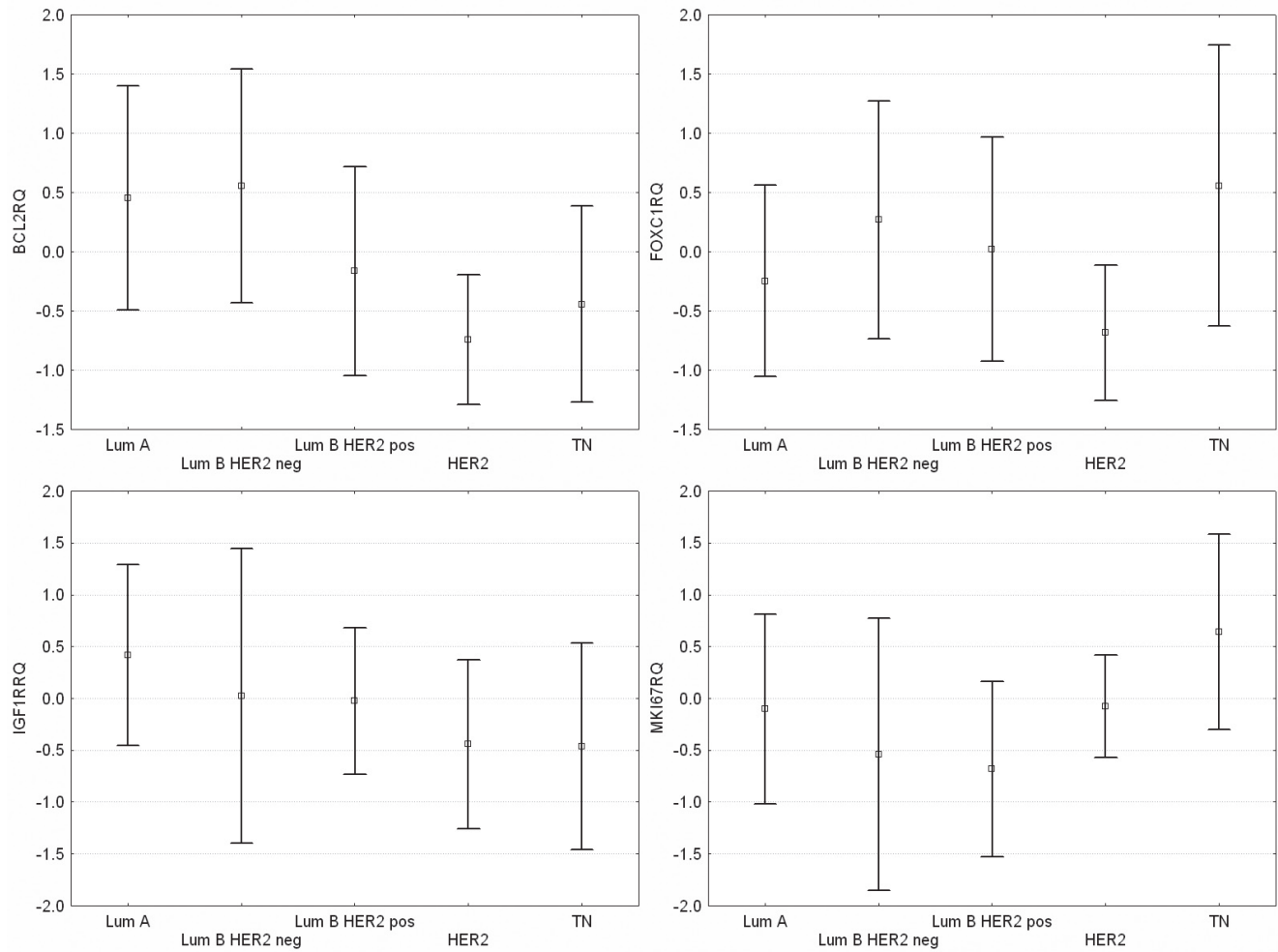
Figure 2. Comparison of selected genes between Luminal A, Luminal B HER2 negative, Luminal B HER2 positive, HER2 positive and TN subtypes. Elevated levels of BCL2 and *IGF1R* were a feature associated with Luminal A subtype, while elevated *FOXC1* and *MKI67* were observed in TN tumors.
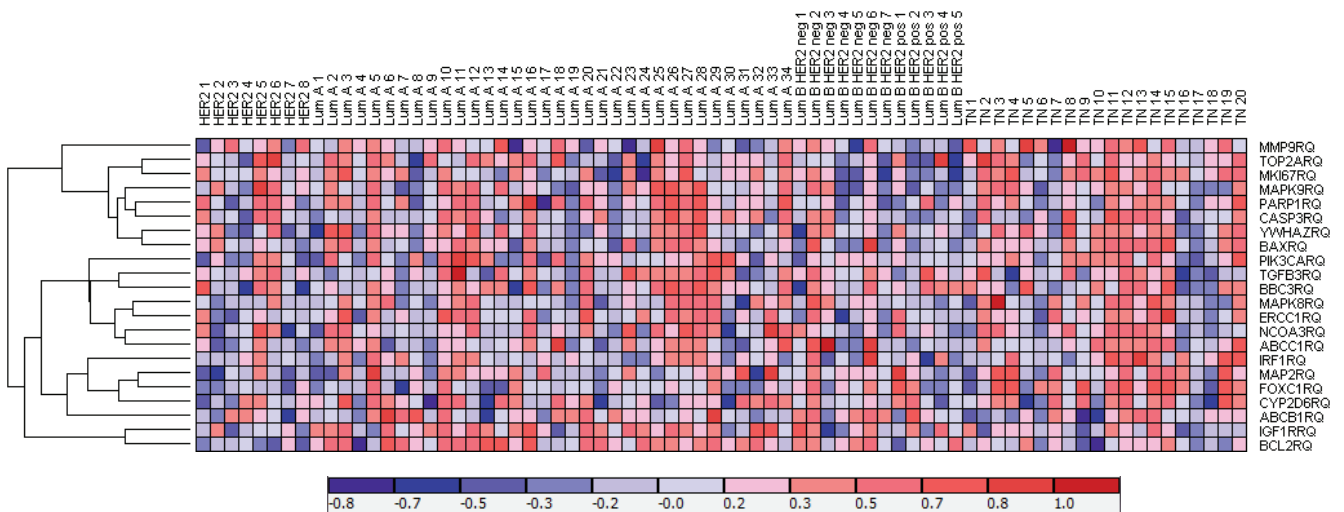

Figure 3. Results of hierarchical clustering of gene expression ratios (RQ). Considerable variability of expression profiles within the analyzed categories is evident, with the TN and HER2 groups being the most homogenous

like growth factors- key factors of cell growth, survival and transformation [24, 25]. We have shown that *IGF1R* mRNA levels were significantly higher in core biopsies from Luminal A tumors versus triple negative and HER2 positive carcinomas. This observation may be clinically relevant since several studies are currently ongoing with monoclonal antibodies or tyrosine kinase inhibitors directed against this target [33]. Fu et al. have demonstrated that high *IGF1R* status correlated with low grade, negative axillary lymph nodes, positive hormone receptor, negative HER2, lower *MKI67* and luminal subtype [34]. Hartog et al. have revealed divergent effects of *IGF1R* expression on the prognosis of ER(+) cancers versus triple negative invasive ductal breast carcinoma. *IGF1R* expression in ER(+) tumors is strongly related to a favorable disease- free survival and breast cancer specific survival, but to a shorter survival in triple negative carcinoma [35]. Increased *IGF1R* mRNA levels according to intrinsic subtypes were found in 53% of Luminal A, 24% of Luminal B, 13% of HER2 and 10% of triple negative tumors, respectively [36]. On the basis of research on insulin/IGF-1R, a new approach to individualized cancer therapy termed metabolo-genomics is proposed which incorporates features of both cell metabolism and gene profiling. These metabolo-genomic signatures are preferentially associated with Luminal A tumors [37, 38].

Studies on prognostic significance of *BCL2* expression in triple negative versus non- triple negative breast carcinomas are limited [39]. Our findings confirmed increased expression of *BCL2*, an anti-apoptotic, tumorigenic protein, in core biopsies from Luminal A tumors in comparison with triple negative and HER2 positive lesions, which is in accordance with data shown by Koronakis et al [40]. Other authors have shown conflicting correlations [39]. These different results in breast cancer phenotypes may be determined by the balance between the dual function of *BCL2* protein: inhibition of programmed cell death and initiation of cell cycle [39]. In future work, we intent to investigate whether *BCL2* is involved in resistance to hormonal therapy and chemotherapy. An association of receptor-based subtypes with gene expression shown in our study may be an essential prerequisite for further assessment of predictive markers of response to neoadjuvant chemo- or hormonal therapy in breast cancer patients.

To summarize, we conclude that elevated levels of *IGF1R* and *BCL2* are significantly linked to Luminal A subtype. Triple negative breast cancer subtype are associated with higher expression of *IRF1, FOXC1* and *MKI67*. In HER2 positive cohort lower levels of all these genes were found. In view of the small sample size, we need to be cautiously optimistic about presenting classification systems distinguishing between respective receptor subtypes. Initial assessment of selected gene utility in the separation of receptor subtype groups showed a 65-75% accuracy before incorporating any clinical factors into the analysis (data not shown). Further studies which focus on validation of our findings are ongoing and will attempt to include multigene signatures within

the receptor- based classification in order to design targeted therapies in the genomic, proteomic, metabolomic and transcriptomic era.

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