Breast Cancer Res Treat (2013) 140:219–232
DOI 10.1007/s10549-013-2622-y

REVIEW

A meta-analysis of gene expression-based biomarkers predicting outcome after tamoxifen treatment in breast cancer

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Received: 8 May 2013 / Accepted: 21 June 2013 / Published online: 9 July 2013

Abstract To date, three molecular markers (ER, PR, and CYP2D6) have been used in clinical setting to predict the benefit of the anti-estrogen tamoxifen therapy. Our aim was to validate new biomarker candidates predicting response to tamoxifen treatment in breast cancer by evaluating these in a meta-analysis of available transcriptomic datasets with known treatment and follow-up. Biomarker candidates were identified in Pubmed and in the 2007–2012 ASCO and 2011–2012 SABCS abstracts. Breast cancer microarray datasets of endocrine therapy-treated patients were downloaded from GEO and EGA and RNAseq datasets from TCGA. Of the biomarker candidates, only those identified or already validated in a clinical cohort were included. Relapse-free survival (RFS) up to 5 years was used as endpoint in a ROC analysis in the GEO and RNAseq datasets. In the EGA dataset, Kaplan–Meier analysis was performed for overall survival. Statistical significance was set at $p < 0.005$. The transcriptomic datasets included 665 GEO-based and 1,208 EGA-based patient samples. All together 68 biomarker candidates were identified. Of these, the best performing genes were PGR (AUC = 0.64, $p = 2.3\text{E}{-}07$), MAPT (AUC = 0.62, $p = 7.8\text{E}{-}05$), and SLC7A5 (AUC = 0.62, $p = 9.2\text{E}{-}05$). Further genes significantly correlated to RFS include FOS, TP53, BTG2, HOXB7, DRG1, CXCL10, and TPM4. In the RNAseq dataset, only ERBB2, EDF1, and MAPK1 reached statistical significance. We evaluated tamoxifen-resistance genes in three independent platforms and identified PGR, MAPT, and SLC7A5 as the most promising prognostic biomarkers in tamoxifen treated patients.

Keywords Breast cancer · Tamoxifen · Resistance · Biomarker

Electronic supplementary material The online version of this article (doi:10.1007/s10549-013-2622-y) contains supplementary material, which is available to authorized users.

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List of Abbreviations

ASCO	American Society of Clinical Oncology
EGA	European genome–phenome archive
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FFPE	Formalin-fixed, paraffin-embedded
GEO	Gene expression omnibus
NCCN	National Comprehensive Cancer Network
NICE	National Institute for Health and Clinical Excellence
PR	Progesterone receptor
PRISMA	Preferred reporting items for systematic reviews and meta-analyses

PROSPERO	International prospective register of systematic reviews
RFS	Relapse-free survival
ROC	Receiver operating characteristic
SABCS	San Antonio breast cancer symposium
TCGA	The cancer genome atlas

Introduction

The anti-estrogen tamoxifen was the first targeted therapy agent approved for the treatment of breast cancer in 1977. It competes with estrogen receptor (ER) for binding, and subsequently stops the cell cycle in the G0 and G1 phases thus preventing the cell division. Adjuvant tamoxifen can reduce the risks of both breast cancer recurrence and death. According to the current NCCN guidelines, tamoxifen is approved for the endocrine treatment of early and advanced breast cancer in both pre- and post-menopausal women. In addition, tamoxifen could also be used in patients as a risk-reducing tool to prevent breast cancer [1].

Tamoxifen therapy can be designated as targeted therapy because the expected response can be estimated by measuring the expression of the ER. Only ER positive tumors respond to endocrine therapy where the treatment results in a reduction of the annual event rate to 0.62 ($p < 1E-05$) while ER negative tumors will fail to respond at all [2]. The lack of response of ER negative patients was confirmed by a review of four clinical trials [3]. However, only 50 % of patients with ER positive tumors respond to hormonal therapy [4]. In addition, although the lack of expression of ER is highly predictive, its IHC-based determination displays a high inter-laboratory heterogeneity [5]. ER-status determination could be improved by array-based tests which are more objective and display higher reliability [6].

Similar efficacy can be achieved by measuring expression of the progesterone receptor (PR), an estrogen-regulated gene. About 65 % of ER positive tumors is also PR positive while the PR positive ER negative tumors account for only 1–2 % of all patients [7]. Although PR status is predictive for response, this is not significant when the ER status is also included in the analysis [2]. By a retrospective analysis of 155,175 women between 1990 and 2000, the proportion of ER negative PR positive patients decreased what could suggest an improvement in diagnostic procedures [8]. Due to these discrepancies, in contrast to the NCCN, PR is not included in the NICE guidelines (National Institute for Health and Clinical Excellence (UK): http://www.nice.org.uk).

Tamoxifen is converted in vivo into several more active forms including 4-hydroxy-tamoxifen and endoxifen. The CYP2D6 and CYP3A4 isoforms of the cytochrome P450 participate in this conversion, and a large retrospective study identified shorter relapse-free survival (RFS) time in patients who had two variant polymorphisms of CYP2D6 [9]. This was however not confirmed in following studies [10] and therefore current NCCN and ASCO guidelines do not recommend CYP2D6 testing as a tool for determining optimal endocrine treatment [11].

Besides the three markers discussed above (ER, PR, and CYP2D6) there are numerous new candidates many of which have not yet been evaluated in an independent cohort. In present meta-analysis our focus will be on the expression-based markers as by utilizing transcriptomic cohorts published in the last decade we can provide the foundation for an independent validation of these candidates. We have screened the GEO and EGA repositories for breast cancer patients with known follow-up. In addition, we also included the RNAseq datasets published by the TCGA project. We have filtered to include only patients receiving endocrine (tamoxifen) therapy and in these we evaluated 59 tamoxifen response biomarker candidates published in the last 5 years (2007–2012).

Methods

We have structured our review and meta-analysis according to the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" guidelines published in 2009 (PRISMA) [12]. The original PRISMA flow diagram [12] includes "identification" of data sources, "screening" methods, "eligibility" criteria, and "included" patients. Here, we used an approach in which both the markers to be validated and the data to be used for validation were identified by a search of available publications. This generates a new issue, the combination of these. Therefore, we have extended the PRISMA pipeline by adding a fifth category for "analysis" in Fig. 1.

Identification of tamoxifen resistance biomarkers

We have performed search in Pubmed and in the ASCO and SABCS abstracts to identify published biomarker candidates. In Pubmed, the words "tamoxifen," "resistance," "biomarker," and "breast" were used. The search was narrowed to include only genes published between 1977 and 2012. Only publications in English were considered. The search in the ASCO (*Journal of Clinical Oncology*) and SABCS (*Cancer Research*) abstracts was reduced to include abstracts published between 2007–2012 and 2011–2012, respectively; the reason for the search in the conference proceedings was to identify biomarkers currently under investigation but without any relevant

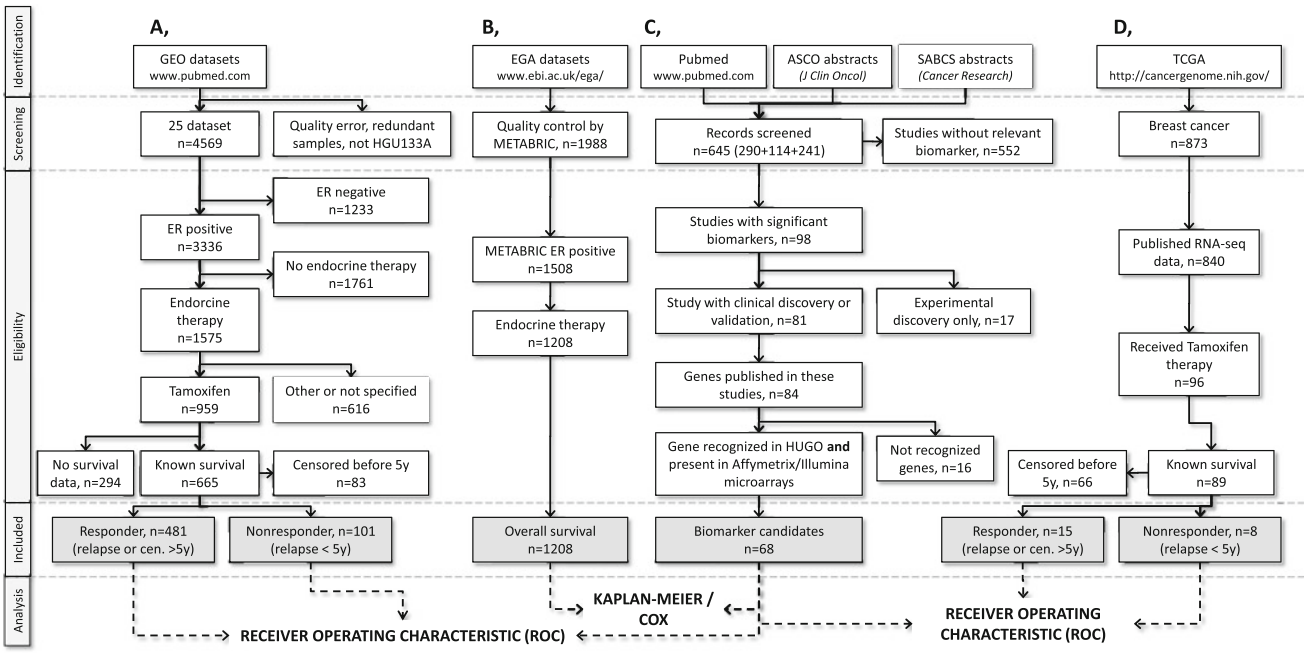

Fig. 1 A flow diagram depicting the processing of GEO (*A*) and METABRIC (*B*) microarrays, the search for biomarker candidates in the published scientific literature as well as recent conference proceedings (*C*), and the selection of patients with RNA-seq data in the NCI-TCGA project (*D*)

peer-reviewed publication. In addition, Pubmed was searched for earlier publications investigating genes described in the ASCO and SABCS abstracts. The unique gene symbol and name was identified for each gene by querying the online repository of the HUGO Gene Nomenclature Committee (http://www.genenames.org).

Construction of GEO-based microarray database

Breast cancer datasets were identified in GEO (http://www.ncbi.nlm.nih.gov/gds) using the GEO platform IDs "GPL96" (for HG-U133A), "GPL570" (for HG-U133 Plus 2.0) and the keywords "breast," "cancer," and "survival". Only datasets including at least 30 patients were considered (*some of the final datasets include less patient as not all patients within one dataset were actually treated by endocrine therapy*), all together 6,197 breast cancer patients were processed. The database quality control and removal of duplicate samples were performed as described previously [13]. The raw CEL files were MAS5 normalized in the R statistical environment (http://www.r-project.org) using the Affy Bioconductor library. MAS5 was used because it performed among the best normalization methods compared to RT-PCR measurements in our previous study [14].

The ER status was determined for each patient using the probe set 205225_at as implemented in http://www.recurrenceonline.com [6]. JetSet was used to identify the most representative Affymetrix probe sets for each gene. JetSet is based on a method calculating principled, unbiased quality scores for probe sets, and we used these scores to define a simple, unambiguous mapping from gene to probe set [15].

Construction of EGA-based microarray database

Illumina microarrays published by the Metabric project were downloaded from the European genome–phenome archive (EGA) (https://www.ebi.ac.uk/ega/) [16]. The database contains 1,988 patients, the average overall survival is 8.07 years, 76 % of the patients are ER positive and 47.3 % are lymph node positive.

Instead of using the processed table, we have re-run the complete pre-processing for all arrays. First, the raw data were imported into R and summarized using the beadarray package [17]. For annotation, the illuminaHumanv3 database of Bioconductor was used (http://www.bioconductor.org). During summarization, 319 unmapped probes were removed. Then, quantile normalization was performed using the preprocessCore package [18]. For genes with several probes, the one with the highest dynamic range was retained.

Construction of database using RNA-seq data

RNA-seq data for breast cancer patients [19] were published in The Cancer Genome Atlas (TCGA) of the

National Cancer Institute (http://cancergenome.nih.gov/) and we downloaded the pre-processed level 3 data generated using the Illumina HiSeq 2000 RNA Sequencing Version 2 platform. For these samples, expression levels were determined using a combination of MapSplice and RSEM. We have combined the individual patient files in R using the plyr package [20].

Statistical analyses

ROC analysis was performed in the R statistical environment (http://www.r-project.org) using the ROC Bioconductor library. A Kaplan–Meier analysis platform was established previously [21]. For present study, our online available Kaplan–Meier plotter was upgraded to enable future biomarker validation in the 665 tamoxifen-treated patients (http://www.kmplot.com/breast). For the expression of the genes, each percentile (of expression) between the lower and upper quartiles was computed and the best performing threshold was used as the final cutoff in the Cox regression analysis. Kaplan–Meier survival plot, and the hazard ratio with 95 % confidence intervals and log-rank *P* value were calculated and plotted in *R* using Bioconductor packages. To assess correlation to proliferation, Spearman correlation to MKI67 expression was computed within the tamoxifen-treated patients. Multiple testing correction was performed using a step-up method (http://www.kmplot.com/multipletesting/) as described previously [22]. The supplemental material contains R scripts for ROC analysis (Supplemental R script 1.R) and Kaplan–Meier analysis (Supplemental R script 2.R). Statistical significance was set at $p < 0.005$.

Results

Construction of microarray databases

After selection, the microarray files were re-processed from the original raw files. The normalized table of the 665 microarray files of tamoxifen-treated samples downloaded from GEO including the gene expression values for all genes used in the study is available as Supplemental Table 1. The clinical characteristics of the individual datasets used for assessing RFS is listed in Table 1 and the detailed characteristics for each patient are listed in Supplemental Table 2. The normalized table for the 1,208 microarray files of endocrine therapy-treated patients downloaded from EGA is available in Supplemental Table 3 and the detailed characteristics for each patient in Supplemental Table 4.

Table 1 Clinical characteristics of tamoxifen-treated patients of the GEO datasets included in the construction of the transcriptomic database for the meta-analysis of predictive power for relapse-free survival

Dataset	Platform	Reference	Sample size[#]	Median follow-up (RFS)	No. of progressions	Lymph-node positive (%)	Age*	Grade 1/2/3 (%)	Size*	Responser/non-responder/censored	Subtype: basal/luminal A/luminal B/HER2 + (%)
GSE2990	GPL96	[43]	40	4.7 ± 3.0	11	39.5	66.0 ± 10.9	55.2/0/44.7	2.05 ± 1.5	18/7/15	0/82.5/17.5/0
GSE3494	GPL96	[44]	64	6.95 ± 4.4	28	72.6	65.0 ± 10.3	17.5/65.0/17.5	2.4 ± 0.94	35/22/7	0/62.5/37.5/0
GSE6532	GPL96	[45]	69	5.2 ± 2.8	19	33.8	64.0 ± 9.3	0/100/0	2.3 ± 1.4	38/14/17	0/82.6/17.4/0
GSE12093	GPL96	[46]	135	7.1 ± 3.2	20	0	NA	NA	NA	124/11/1	0/77.04/29.96/0
GSE9195	GPL570	[47]	77	8.2 ± 2.8	13	46.8	65.0 ± 9.2	24.1/34.5/41.4	2.1 ± 0.96	67/9/1	1.3/84.4/13/1.3
GSE16391	GPL570	[48]	20	2.2 ± 1.2	3	70	58.5 ± 7.3	8.3/60/58.3	NA	2/3/15	5.0/85.0/10.0/0
GSE17705	GPL96	[49]	196	9.4 ± 3.6	53	41.9	NA	NA	NA	158/28/10	2.5/49.5/48/0
GSE19615	GPL570	[50]	64	6.1 ± 1.4	3	56.3	53.0 ± 12.6	34.8/34.8/31.2	2.05 ± 1.2	43/3/18	0/67.2/32.8/0
Entire database			665	7.1 ± 3.5	150	38.0	62.0 ± 11.2	23.5/49.7/26.8	2.2 ± 1.2	481/101/83	1.05/68.6/30.2/0.15

RFS relapse-free survival, * median ± SD, *NA* data not available, [#]only tamoxifen-treated patients, *GPL96* HG-U133A, *GPL570* HG-U133 Plus 2.0

Biomarker candidates

After screening the published literature and the presentations at large international conferences of the last three years, 98 publications were identified describing biomarker candidates of tamoxifen resistance. Of these, 17 did not have a clinical validation, these were excluded. In the remaining 81 publications, 84 genes were described as new biomarker candidates. Of these, 16 were either not present on the Affymetrix/Illumina microarrays or the published gene symbol was not unambiguously recognized in the HUGO database. The remaining 68 genes were evaluated in the established transcriptomic databases. None of these genes were identified using the microarray cohorts used for construction of the database.

Markers predicting relapse-free survival

The power of the genes to predict RFS was assessed by ROC analysis in two pre-defined cohorts of patients either relapsing before 5 years or not relapsing until 5 years. The ROC analysis has the advantage over Cox regression that it evaluates all available cutoff values and thus its output is representative for the overall performance of the biomarker candidate. The predictive power of each biomarker is listed in Table 2. Higher expression of SLC7A5, HOXB7, TPM4, and CXCL10 was associated with shorter RFS—for all other genes higher expression was correlated to better survival. For the best performing genes, we have completed a Kaplan–Meier analysis using the best performing cutoff to demonstrate their potential to discriminate those with good and bad prognosis. Of the top genes, PGR, FOS, and BTG2 showed negative correlation to MKI67 (coefficients −0.17, −0.22, and −0.20, respectively), MAPT, SLC7A5, TP53, CXCL10, and TPM4 showed a positive correlation (0.14, 0.29, 0.25, and 0.18, respectively). HOXB7 and DRG1 were independent of MKI67 expression. The Kaplan–Meier plots for the strongest genes including PGR, SLC7A5, CXCL10, MAPT, TP53, and HOXB7 are depicted in Fig. 2.

Genes predicting overall survival

We have assessed the power of the genes to predict overall survival in the endocrine-treated patients of the METABRIC dataset. We have not evaluated progression-free survival in these patients as PFS data were not available. Moreover, for the METABRIC patients only the usage of endocrine therapy was published and not the actual protocol. For these reasons, the principal ranking of the genes was made by using the AUC values achieved in the ROC analysis for relapse-free survival. A similar analysis for overall survival was not possible in the GEO-based datasets using Affymetrix microarrays, as only a limited number of tamoxifen-treated patients had overall survival data. Of the best performing genes predicting RFS, only five (PGR, MAPT, SLC7A5 FOS, and CXCL10) were capable to predict overall survival. Five additional genes, EZH2, KRAS, NCOA3, RAF1, and SERPINE1 were only significant when predicting overall survival—for all these genes higher expression resulted in shorter overall survival. The complete results for each gene are presented in Table 2.

Evaluation of RNA-seq data

In TCGA, all together 840 breast cancer patients were available with complete RNAseq results and survival information. The normalized RNA-seq expression values for the genes are listed in Supplemental Table 5 and the clinical data for all RNA-seq patients are available in Supplemental Table 6. Of the 840 breast cancer patients, 89 received tamoxifen treatment. However, due to limited follow-up time many of the patients were censored before 5 years. Therefore, in the ROC analysis 15 responder patients (those not relapsing before 5 years) were compared to 8 non-responder (relapsing before 5 years) patients. Three genes achieved statistical significance in the ROC analysis: ERBB2 (AUC = 0.83, $p = 1.95E{-}04$), EDF1 (AUC = 0.81, $p = 1.14E{-}3$) and MAPK1 (AUC = 0.79, $p = 1.9E{-}3$). None of the remaining genes were significant.

Discussion

ER expression per se is not a positive biomarker as missing expression predicts lack of response, but only half of those expressing it will actually respond to therapy—this has prompted numerous investigators to seek alternative biomarker candidates.

Resistance against endocrine therapy is an important issue, as the majority of breast cancer patients are ER positive and therefore eligible for such treatment. There are several mechanisms of resistance including the decreased expression of ER, the expression of truncated ER receptors, the increased activity of AP1 and of the ER activator molecules, the activation of the MAPK and PIP3 K pathways, and the disturbed regulation of apoptotic machinery [23]. Of the numerous list of candidate genes investigated in our meta-analysis, only ten genes reached statistical significance. Of these, besides the previously discussed and clinically used PGR the most promising candidates were SLC7A5 and MAPT.

Solute carrier family 7, member 5 (SLC7A5) is a membrane-localized amino acid transporter included in the Mammostrat 5-gene IHC-based biomarker assay [24]. The

Table 2 Detailed results for each biomarker candidate including characteristics of one of the initial studies describing the marker as well as performance of the gene to discriminate patients with high or low relapse-free survival time after tamoxifen therapy ($n = 665$) and the power of the gene to predict overall survival after endocrine therapy ($n = 1{,}208$)

Gene		Study				Relapse-free survival			Overall survival		
Symbol	Full name	Exp.	No. of patients	Method	Ref.	Affymetrix probe ID	AUC	*p* Value	Illumina probe ID	HR	*p* Value
Genes with significant power to predict relapse-free survival after tamoxifen treatment											
PGR	Progesterone receptor	No	587	IHC, microarray	[51]	208305_at	**0.64**	**2.3E−07**	1811014	**0.67**	**1.7E−04**
		No	458	Microarray	[52]						
		No	402	Microarray, IHC	[53]						
MAPT	Microtubule-associated protein tau	No	32	Microarray, IHC	[54]	203929_s_at	**0.62**	**7.8E−05**	2310814	**0.7**	**7.2E−04**
SLC7A5	Solute carrier family 7, member 5	No	1,044	Microarray, IHC	[55]	201195_s_at	**0.62**	**9.2E−05**	1720373	**1.6**	**1.6E−05**
FOS	FBJ murine osteosarcoma viral oncogene homolog	No	71	IHC	[33]	209189_at	**0.61**	**1.6E−5**	1669523	**0.65**	**8.7E−05**
TP53	Tumor protein p53	No	1,044	Microarray, IHC	[55]	201746_at	**0.60**	**1.2E−03**	1779356	0.94	0.57
BTG2	BTG family member 2	Yes	60	IHC	[56]	201236_s_at	**0.60**	**8.0E−04**	1770085	0.86	0.15
HOXB7	Homeobox B7	Yes	127	IHC	[31]	204779_s_at	**0.59**	**1.1E−03**	1702125	1.19	0.09
DRG1	N-myc downstream regulated 1	No	1,044	Microarray, IHC	[55]	202810_at	**0.59**	**1.4E−03**	1658259	0.95	0.63
CXCL10	Chemokine (C-X-C motif) ligand 10	No	912	Microarray, IHC	[57]	204533_at	**0.58**	**3.7E−03**	1791759	**1.4**	**5.6E−04**
TPM4	Tropomyosin 4	Yes	432	shRNA screening, microarray	[34]	209344_at	**0.59**	**2.7E−03**	1653180	1.17	0.13
Non-significant genes											
AR	Androgen receptor	No	n.a.	Microarray	[58]	211110_s_at	0.57	0.01	1767351	0.99	0.9
ATF2	Activating transcription factor 2	No	1,351	Microarray	[59]	205446_s_at	0.51	0.36	1748271	1.17	0.14
BAD	BCL2-associated agonist of cell death	No	402	IHC	[60]	209364_at	0.55	0.07	1738652	1.01	0.94
BAG1	BCL2-associated athanogene	Yes	292	IHC	[61]	202387_at	0.57	0.02	1733970	0.78	0.02
BAP1	BRCA1 associated protein-1	Yes	432	shRNA screening, microarray	[34]	201419_at	0.52	0.23	1768363	1.00	1
BCL2	B-cell CLL/lymphoma 2	No	108	IHC, PCR	[62]	203685_at	0.58	0.0086	1801119	**0.60**	**8.6E−07**
		No	n.a.	Microarray	[58]						
		No	1,082	Microarray, IHC	[63]						
CALM1	Calmodulin 1	No	n.a.	Microarray	[58]	211984_at	0.56	0.03	1778242	1.18	0.12
CALM2	Calmodulin 2	No	n.a.	Microarray	[49]	207243_s_at	0.55	0.07	1687858	0.90	0.32
CALM3	Calmodulin 3	No	n.a.	Microarray	[49]	200623_s_at	0.53	0.18	1666385	0.99	0.95
CCND1	Cyclin D1	No	70	IHC	[64]	208712_at	0.53	0.16	1688480	1.1	0.3
CD24	CD24	No	60	IHC	[65]	266_s_at	0.53	0.17	2060413	1.14	0.22
CDH1	E-cadherin	No	794	IHC	[66]	201131_s_at	0.51	0.34	1770940	1.08	0.44
CDK10	Cyclin-dependent kinase 10	Yes	87	Meta-analysis	[67]	203468_at	0.51	0.38	1741459	1.3	0.03
		Yes	432	shRNA screening, microarray	[34]						

Table 2 continued

Gene		Study				Relapse-free survival			Overall survival		
Symbol	Full name	Exp.	No. of patients	Method	Ref.	Affymetrix probe ID	AUC	*p* Value	Illumina probe ID	HR	*p* Value
CDKN1A	Cyclin-dependent kinase inhibitor 1A	No	108	IHC, PCR	[62]	202284_s_at	0.54	0.10	1784602	0.90	0.32
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	No	1,044	Microarray, IHC	[55]	201884_at	0.55	0.05	1670959	1.3	0.01
CXCL12	Chemokine (C-X-C motif) ligand 12	No	33	IHC	[68]	203090_at	0.52	0.23	1791447	0.79	0.33
CXCR3	Chemokine (C-X-C motif) receptor 3	No	912	Microarray, IHC	[57]	207681_at	0.50	0.44	1797975	1.2	0.08
EBAG9	Estrogen receptor binding site associated antigen 9	Yes	n.a.	IHC	[69]	204274_at	0.57	0.01	1791896	0.85	0.13
EDF1	Endothelial differentiation-related factor 1	Yes	432	shRNA screening, microarray	[34]	209059_s_at	0.55	0.04	1726169	1.00	0.97
ESR1	Estrogen receptor 1	No	1,129	IHC/FISH/RT-PCR	[70]	205225_at	0.57	0.01	1678535	0.85	0.13
		No	97	FISH	[71]						
		No	394	IHC, FISH	[72]						
		No	n.a.	Microarray	[49]						
ESR2	Estrogen receptor 2 (ER beta)	No	1,129	FISH	[70]	211120_x_at	0.51	0.38	1740045	1.01	0.96
		No	n.a.	Microarray	[49]						
ESRRG	Estrogen-related receptor gamma	No	50	Microarray	[73]	207981_s_at	0.50	0.48	1661994	0.95	0.59
EZH2	Enhancer of zeste homolog 2	Yes	688	IHC	[74]	203358_s_at	0.57	0.02	**1708105**	**1.5**	**4E−04**
		Yes	344,109	Microarray, ChIP	[75]						
FKBP4	FK506 binding protein 4, 59 kDa	No	24,511	Proteomic screening, IHC	[76]	200895_s_at	0.52	0.25	1782045	1.19	0.09
FOXA1	Forkhead box A1	Yes	344,109	Microarray, ChIP	[65]	204667_at	0.51	0.39	1766650	0.89	0.26
		Yes	108	IHC	[77]						
		Yes	n.a.	IHC	[78]						
FAS	Fas cell surface death receptor	No	215	PCR	[79]	204781_s_at	0.57	0.02	1808132	1.02	0.85
FASLG	Fas ligand (TNF superfamily, member 6)	No	215	PCR	[79]	210865_at	0.50	0.49	1781824	1.13	0.25
GPER	G protein-coupled estrogen receptor 1	No	208	IHC	[80]	210640_s_at	0.53	0.19	**2384056**	**0.76**	**0.0092**
GRN	Granulin	Yes	n.a.	IHC	[81]	211284_s_at	0.50	0.49	1724250	1.06	0.58
IGF1R	Insulin-like growth factor 1 receptor	No	32	Microarray, IHC	[54]	203628_at	0.58	0.0051	1675048	0.82	0.06
HER2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	No	2,379	Meta-analysis	[82]	216836_s_at	0.51	0.33	2352131	1.02	0.87
		No	402	Microarray, IHC	[53]						
HER3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	No	402	Microarray, IHC	[53]	202454_s_at	0.52	0.29	1751346	1.08	0.44
HOXB13	Homeobox B13	No	60	Microarray	[83]	209844_at	0.53	0.15	1742677	1.19	0.09
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Yes	432	shRNA screening, microarray	[34]	204009_s_at	0.55	0.03	**1728071**	**1.8**	**1.2E−07**

Table 2 continued

Gene		Study				Relapse-free survival			Overall survival		
Symbol	Full name	Exp.	No. of patients	Method	Ref.	Affymetrix probe ID	AUC	*p* Value	Illumina probe ID	HR	*p* Value
MAPK1	Mitogen-activated protein kinase 1	No	743	Microarray	[84]	212271_at	0.55	0.05	2235283	1.12	0.29
		Yes	304	Microarray, IHC	[85]						
		No	108	IHC	[86]						
		No	n.a.	Microarray	[49]						
MAPK14	Mitogen-activated protein kinase 14	Yes	39	IHC	[36]	202530_at	0.57	0.01	2388090	0.90	0.32
MKS1	Meckel syndrome, type 1	No	556	IHC	[87]	218630_at	0.50	0.49	1737953	1.15	0.18
MT1-X	Metallothionein 1X	No	60	IHC	[88]	204326_x_at	0.55	0.05	1775170	1.03	0.78
NCOA3	Nuclear receptor coactivator 3	No	349	Microarray, IHC	[89]	209061_at	0.55	0.07	**1708805**	**1.5**	**2.4E−04**
		No	297	Microarray, IHC	[90]						
NCOR2	Nuclear receptor corepressor 2	Yes	77	Microarray	[91]	207760_s_at	0.52	0.27	2340052	0.99	0.9
NF1	Neurofibromin 1	Yes	432	shRNA screening, microarray	[34]	210631_at	0.56	0.02	1726387	1.05	0.67
IDN3 (NIPBL)	Nipped-B homolog	Yes	432	shRNA screening, microarray	[34]	212483_at	0.56	0.05	2264625	1.15	0.17
IL17RB	Interleukin 17 receptor B	No	60	Microarray	[83]	219255_x_at	0.52	0.28	1767523	0.82	0.05
NRIP1	Nuclear receptor interacting protein 1	Yes	n.a.	IHC	[59]	202599_s_at	0.51	0.36	1718629	0.80	0.04
PAK1	p21 protein-activated kinase 1	No	214	PCR	[92]	209615_s_at	0.52	0.30	1767365	1.2	0.05
PAWR	PRKC apoptosis WT1 regulator	No	?	RNAseq	[93]	204005_s_at	0.52	0.30	1806907	1.3	0.09
PLAU	Plasminogen activator, urokinase	No	n.a.	ELISA	[94]	205479_s_at	0.52	0.24	1656057	1.2	0.05
PTEN	Phosphatase and tensin homolog	No	404	ISH	[95]	204054_at	0.50	0.46	1701134	0.94	0.56
		Yes	432	shRNA screening, microarray	[34]						
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	No	318	IHC	[96]	201244_s_at	0.52	0.27	**1813489**	**1.4**	**0.0023**
		Yes	432	shRNA screening, microarray	[34]						
RARG	Retinoic acid receptor, gamma	Yes	432	shRNA screening, microarray	[34]	204189_at	0.52	0.22	1737433	1.05	0.64
RRAS2	Related RAS viral (r-ras) oncogene homolog 2	No	450	Microarray, IHC	[97]	212589_at	0.53	0.20	2077623	0.82	0.07
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	No	n.a.	ELISA	[94]	202628_s_at	0.55	0.07	**1744381**	**1.4**	**1.7E−03**
SMC3	Structural maintenance of chromosomes 3	Yes	432	shRNA screening, microarray	[34]	209259_s_at	0.58	0.0057	1718807	0.95	0.64
SRC	v-src sarcoma viral oncogene homolog	No	392	IHC	[98]	213324_at	0.51	0.40	1729987	1.17	0.13
		No	n.a.	Microarray	[49]						
TMPRSS2	Transmembrane protease, serine 2	Yes	432	shRNA screening, microarray	[34]	205102_at	0.52	0.22	1791123	1.21	0.07

Table 2 continued

Gene		Study				Relapse-free survival			Overall survival		
Symbol	Full name	Exp.	No. of patients	Method	Ref.	Affymetrix probe ID	AUC	*p* Value	Illumina probe ID	HR	*p* Value
UBA3	Ubiquitin-like modifier activating enzyme 3	Yes	432	shRNA screening, microarray	[34]	209115_at	0.52	0.26	2324157	0.90	0.3
WNT5A	Wingless-type MMTV integration site family, member 5A	No	564	IHC	[99]	213425_at	0.53	0.14	1800317	0.88	0.22

Bold: significance below $p = 0.005$

IHC immunohistochemistry, *AUC* area under the curve, *HR* hazard ration, *Exp* gene identified in an experimental or in a clinical study

test classifies SLC7A5 as positive when it is expressed by more than 10 % of the invasive tumor cells. The gene was identified in a previous study also employing a sizeable cohort of patients for evaluating 700 computationally identified target genes [25]. Besides SLC7A5 two additional genes of the Mammostrat five-gene panel (TP53 and DRG1) were also significant, while the remaining two genes failed to deliver a decisive correlation. However, no other group has yet identified SLC7A5 as a gene correlated to endocrine sensitivity or progression in breast cancer. In our analysis, SLC7A5 was correlated to RFS and higher expression also resulted in shorter overall survival thereby suggesting a feasible option to be embattled by a targeted therapy to circumvent tamoxifen resistance.

Microtubule-associated protein tau (MAPT) is a protein promoting microtubule assembly having additional unknown cellular functions via its yet unclear involvement in cell cycle [26]. ER influences MAPT expression in human breast cancer cell lines, and the expression of MAPT was increased when the cells were stimulated with tamoxifen. [27]. In this study, the expression of MAPT also correlated to sensitivity to taxanes and silencing of MAPT increased cellular sensitivity to taxanes. Despite being identified as correlated to tamoxifen resistance in a relatively small cohort, MAPT delivered the highest significance of the previously unemployed genes in both correlation to relapse-free survival and to overall survival.

Among the remaining top candidate genes are TP53, BTG2, FOS, HOXB7, DRG1, CXCL10, and TPM4. The tumor suppressor TP53 is one of the most studied gene which is mutated in over 70 % in HER2 and basal subtypes but only 12 and 29 % of ER positive Luminal A and Luminal B subtypes, respectively [28]. Besides TP53, FOS BTG2, PGR, DRG1 and HOXB7 also regulate the transcription, but only TP53 and BTG2 affect directly the cell cycle as well. HOXB7 is overexpressed in breast cancer [29] and also has a role in DNA repair [30]. Moreover, HOXB7 is also involved in cell proliferation and differentiation and HOXB7 antagonism was recently shown to circumvent tamoxifen resistance [31]. BTG2 participates in DNA repair, is a negative regulator of cell proliferation, and is also an ESR2 effector [32]. FOS is a transcription factor participating in both acquired and primary endocrine resistance [33]. TPM4 was identified by a shRNA screen as one of the genes whose silencing caused sensitivity to endocrine therapy [34]. TPM4 and CXCL10 influence cell motility. All together despite some overlapping biological roles, the significant genes seem to be involved in distinct functional pathways.

Another important question is the correlation of the best genes to the Luminal A and Luminal B subtypes. Both of these molecular groups are ER positive, but they fundamentally differ as the Luminal B samples generally show

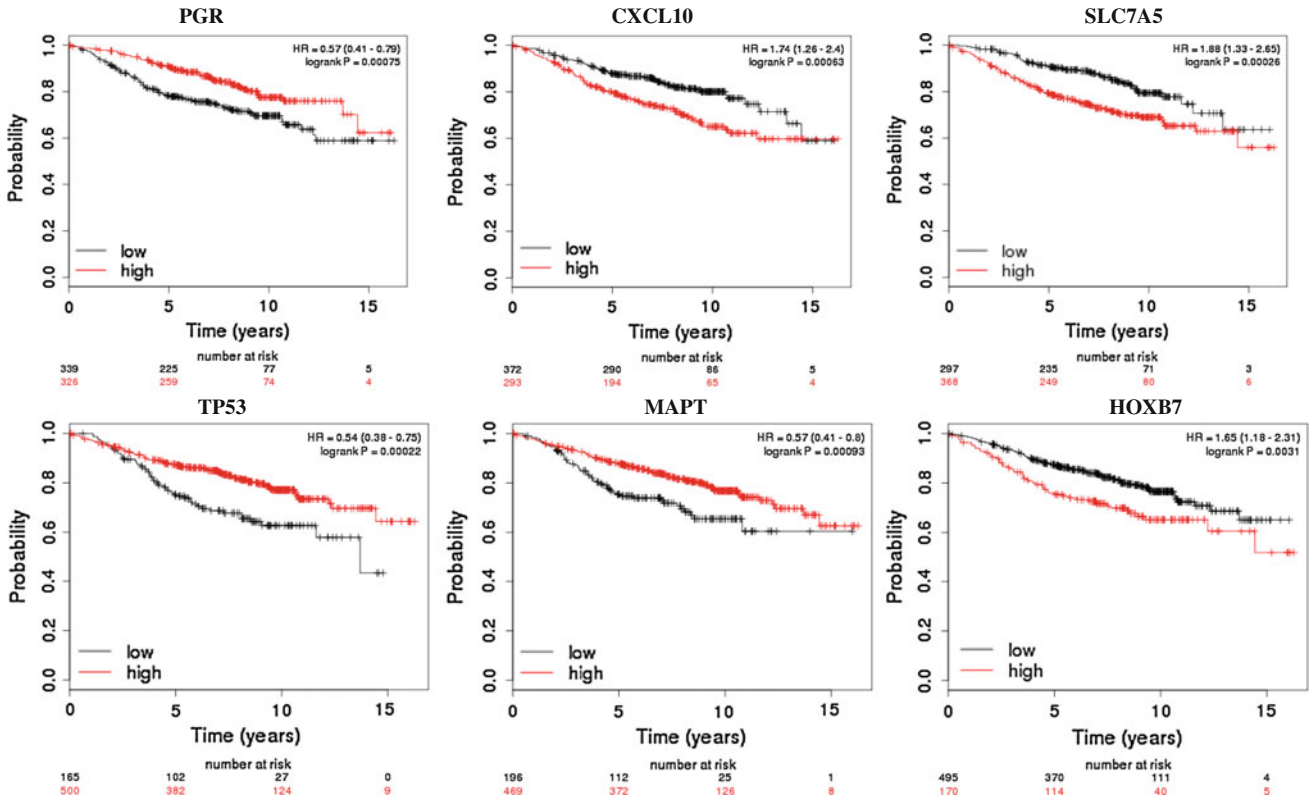

Fig. 2 Kaplan–Meier survival curves for relapse-free survival in patients with tamoxifen endocrine therapy for a selection of the best performing genes (see complete list in Table 2). Genes with higher expression correlated to better prognosis (like PGR) are probably estrogen targets and thus are independent biomarker candidates. Genes with higher expression correlated to worse prognosis (like SLC7A5) represent potential therapeutic targets for combinatorial therapy to circumvent endocrine resistance

high proliferation by displaying higher MKI67 expression. We also assessed the correlation of the top genes to MKI67 and only two genes (HOXB7 and DRG1) were not related to MKI67 at all—these genes could be promising biomarker candidates independently of the tumors' molecular subtype.

The most remarkable negative result is the lack of correlation between ERBB2 expression and survival after endocrine treatment in the microarray datasets. Previously, the clinical endocrine resistance was correlated to HER2 and HER1 overexpression [35]. The reason behind this possible correlation might be a cross-talk between the downstream components of the signal transduction pathways. Furthermore, the higher expression of common downstream genes (p38, MAPK, and ERK) has also been correlated to resistance [36]. These observations provide the background for several ongoing clinical trials in which tamoxifen is combined with HER2- or EGFR-inhibitors (trastuzumab, gefitinib, and lapatinib).

In contrast, by analyzing the RNA-seq data, ERBB2 (and two additional genes, EDF1 and MAPK1) achieved a high discriminative power for predicting RFS in tamoxifen-treated breast cancer patients. RNA-seq can provide a more robust estimate of genes expression with higher dynamic range and sensitivity as compared to other methods [37]. However, due to the limited number of patients actually passing the eligibility threshold for this analysis, these findings must be validated in a larger cohort and we therefore have also omitted to display the detailed result in the tables.

Interestingly, ESR1 itself was not significant when predicting survival after tamoxifen treatment. However, the correlation might be obscured by the fact that only ESR1 positive patients are eligible for endocrine therapy. Thus, the important implication we can draw is the potential of low-ER positive tumors to respond to endocrine therapy. This observation is in line with recent studies reporting benefit of endocrine therapy in patients with minimal ESR1 expression [38]. It can thus be suggested that the majority of high-ER and a substantial group low-ER expressing tumors stimulate the corresponding signaling pathways (also resulting in higher PGR expression) and behave as luminal-type breast cancers being responsive to anti-estrogen treatment.

Besides gene expression-based biomarkers one could also measure gene polymorphisms related to tamoxifen resistance. Besides CYP2D6, ESR1 has also been investigated in a recent SABCS abstract [39]. In this study, the authors paradoxically observed that rare ESR1

homozygous polymorphisms were associated with lower recurrence. As the gene expression dataset do not allow to make extrapolations for gene polymorphisms, we were not able to evaluate these findings.

We have performed a validation of predictive biomarker candidates by using survival data in endocrine therapy-treated patients. By using a cutoff to define responders and non-responders, our analysis is based on assessment of prognosis in cohorts of patients. We have compensated for this limitation by performing a ROC analysis which is independent of a given cutoff value. In this, the results give an overall estimate of the markers potential as a biomarker. However, this was not possible for overall survival, so there we had to rely on the results of a Kaplan–Meier analysis. For identifying the most significant results, an alternative to the used maximally selected logrank procedure is the computation of a twofold a cross-validation [40]. However, we rejected the null hypothesis for the top genes using results of the ROC analysis, and therefore omitted computation and reporting of the two cut-points obtained in a cross-validation.

The application of a cutoff 5 year was selected as the current NCCN guidelines suggest a 5 year initial tamoxifen therapy. Several studies show that ER positive patients show a constant recurrence rate over time after an initial peak after 3 years [41, 42]. However, increasing the threshold in our analysis would also increase the proportion of censored patients. The usage of the 5 year threshold to divide the patients into two cohorts provided a good balance between reliability and feasibility.

Finally, we must also note another important limitation of our meta-analysis: dosage and treatment length data were not available for the patients of the transcriptomic datasets. Moreover, additional systemic therapies were not documented for these patients. Therefore, the potential heterogeneity of included patients might represent a bias for our study.

Conclusions

We performed a validation of tamoxifen treatment outcome predictor candidates. The majority of the genes failed to reach significance and are therefore unlikely to represent robust biomarkers. Those most significant include PGR, MAPT, SLC7A5, FOS, TP53, and five additional genes. Our results suggest the role of alternative pathway activation in the resistance. The potential of these genes to predict survival after tamoxifen by using immunohistochemistry of formalin-fixed, paraffin-embedded (FFPE) samples should be evaluated using the same patient samples in an independent clinical validation study.

Acknowledgments Our work was supported by the OTKA PD 83154 Grant, by the Predict Project (Grant No. 259303 of the EU Health.2010.2.4.1.-8 call), and by the KTIA EU_BONUS_12-1-2013-0003 Grant.

Ethical Standards We declare that the experiments comply with the current laws of Hungary.

Conflict of interests There are no disclaimers. The authors declare that they have no conflict of interest.

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