Article

Hormonal Receptor Status Determines Prognostic Significance of FGFR2 in Invasive Breast Carcinoma

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Table S1. Expression of FGFR2 protein and mRNA in relation to ER/PR status. Nominal variables are presented as raw values followed by percentages of the respective groups, continuous variables are presented as medians and interquartile ranges in brackets;.

Variable	ER-PR-	ER+PR-	ER+PR+	*p*-value
FGFR2 protein [H-score] [1]	0.0 (0.0–26.5)	93.0 (5.0–205.0)	105 (25.0–202.0)	<0.001 *
FGFR2 by H-score quartiles [2]				
0–75	27 (84.4)	31 (42.5)	90 (25.9)	
76–150	2 (6.3)	13 (17.8)	57 (23.5)	<0.001 *
151–225	3 (9.4)	13 (17.8)	45 (18.6)	
226–300	0 (0.0)	16 (21.9)	50 (20.7)	
FGFR2 mRNA [log2] [1]	7.8 (6.6–9.2)	8.5 (7.2–9.4)	8.6 (8.0–9.3)	0.049 *

[1] ANOVA Kruskal-Wallis or ANOVA test, [2] Pearson's chi-squared test, * significant differences.

Table S2. Cox univariate and multivariate overall and disease-free survival analyses according to prognostic clinicopathological features, including FGFR2 status (low vs. high divided by 1st tercile of protein H-score). Hazard ratios are present for nominal variables, while β-parameters for continuous variables. Variables significant only in univariate analyses were incorporated in multivariate analyses. CI—confidence interval, NA—not applicable.

Variable	Hazard ratio (95% CI)/ β-parameter (SE) univariate analysis	*p*-value	Hazard ratio/ β-parameter multivariate analysis	*p*-value
Overall survival				
Age [years]	0.08 (0.01)	<0.001 *	0.05 (0.02)	0.002 *
Grade [ref. G1]	NA	0.987	NA	NA
Ki67 [%]	0.01 (0.02)	0.583	NA	NA
HER2 [ref. no amplification]	1.29 (0.54–3.07)	0.566	NA	NA
Hormonal receptor status [ref. ER+PR+]	4.18 (1.98–8.84)	<0.001 *	2.17 (0.89–5.29)	0.230
FGFR2 status [ref. high]	2.34 (1.26–4.34)	0.007 *	1.26 (0.63–2.52)	0.518
Tumor size [mm]	0.04 (0.01)	<0.001 *	0.02 (0.02)	0.120
Lymph node metastases [ref. absent]	1.90 (0.99–3.65)	0.052	NA	NA
Stage [ref. very early]	30.80 (2.06–460.49)	0.017 *	1.87 (0.05–67.51)	0.937
Disease-free survival				
Age [years]	0.05 (0.01)	<0.001 *	0.03 (0.02)	0.007 *
Grade [ref. G1]	4.31 (1.00–18.52)	0.012 *	3.55 (0.45–27.77)	0.188

Ki67 [%]	0.01 (0.01)	0.949	NA	NA
HER2 [ref. no amplification]	0.92 (0.41–2.05)	0.834	NA	NA
Hormonal receptor status [ref. ER+PR+]	3.88 (1.94–7.73)	<0.001 *	1.63 (0.68–3.90)	0.432
FGFR2 status [ref. high]	2.22 (1.25–3.93)	0.007 *	1.31 (0.65–2.62)	0.448
Tumor size [mm]	0.04 (0.01)	<0.001 *	0.01 (0.01)	0.293
Lymph node metastases [ref. absent]	1.90 (1.04–3.47)	0.036 *	1.49 (0.76–2.93)	0.243
Stage [ref. very early]	2.73 (0.88–8.43)	0.205	NA	NA

*significant differences.

Table S3. List of PR-dependent genes (PR(mol) – "molecular signature") signifying receptor activation and rapid degradation with respective reasons for inclusion.

Target Identifier	Gene name	Reason for inclusion to the signature
NM_000926	*PGR* (progesterone receptor)	Progesterone receptor gene transcript - levels of *PGR* mRNA are strongly correlated with PR protein and its downstream pathway activity [1–4]
NM_022970	*FGFR2* (fibroblast growth factor receptor 2)	Main gene of interest in this study – good marker of PR expression in the TCGA analysis - significantly different levels of FGFR2 in ER+PR+ and ER+PR-
NM_005067	*SIAH2* (Seven in Absentia Homolog 2)	PR degradation marker - an ubiquitin E3 ligase involved in degradation of PR, especially in tumours with high activity of PR pathway [5]
NM_198400	*NEDD4* (Neural Precursor Cell Expressed, Developmentally Down-Regulated 4, E3 Ubiquitin Protein Ligase)	PR degradation marker - a ubiquitin E3 ligase reported to be involved in degradation of PR downstream mediators [6]
NM_001993	F3 (Coagulation Factor III, Tissue Factor)	Marker of PR activity – secreted molecule highly up-regulated (18-fold change) by PR [7–9]; displayed significantly different levels between ER+PR+ and ER+PR- in the TCGA analysis
NM_005195	CEBPD (CCAAT/Enhancer Binding Protein Delta)	PR downstream activation marker – transcription factor up-regulated (6-fold change) by PR [7–9]; displayed significantly different levels between ER+PR+ and ER+PR- in the TCGA analysis
NM_000820	GAS6 (Growth Arrest Specific 6)	PR downstream activation marker – secreted molecule highly up-regulated (23-fold change) by PR [7–9]
NM_005980	S100p (S100 Calcium Binding Protein P)	PR downstream activation marker – calcium-binding protein, up-regulated (2–4 fold change) by PR [7–9]
NM_009061	RGS2 (Regulator of G-protein signalling 2)	PR downstream activation marker – GTPase activating protein, up-regulated in the report by Knutson et al, verified *in vitro* [8], significantly different levels between ER+PR+ and ER+PR- in the TCGA analysis
NM_0010371 62	ACOT6 (Acyl-CoA Thioesterase 6)	PR downstream activation marker – involved in metabolism, up-regulated by PR [8]
NM_004235	KLF4 (Kruppel Like Factor, Epithelial Zinc Finger Protein EZF)	PR downstream activation marker – transcription factor up-regulated (6–8 fold change) by PR [7–9]

NM_000196	HSD11B2 (Hydroxysteroid 11-Beta Dehydrogenase 2)	PR downstream activation marker – involved in cholesterol or steroid metabolism and trafficking, highly up-regulated (23-8 fold change) by PR [7–9]
NM_003152	STAT5A (Signal Transducer And Activator Of Transcription 5A)	PR downstream activation marker – transcription factor up-regulated (6 fold change) by PR [7–9]
NM_014737	RASSF2 (Ras Association Domain Family Member 2)	PR downstream activation marker – involved in signal transduction from membrane, up-regulated (10 fold change) by PR, verified *in vitro* [7–9]
NM_001165	BIRC3 (Baculoviral IAP Repeat Containing 3)	PR downstream activation marker – involved in cell cycle and apoptosis, up-regulated (7 fold change) by PR [7–9]
NM_170604	RASGRP4 (RAS Guanyl Releasing Protein 4)	PR downstream activation marker – selected from the TCGA analysis as one of the most significantly different between PR+ and PR- patients, member of RAS guanyl nucleotide-releasing, up-regulated by PR [8]
NM_015409	EP400 (E1A Binding Protein P400)	PR downstream activation marker – the upregulation effect [7–9]; significantly different levels between ER+PR+ and ER+PR- in the TCGA analysis
NM_0011457 77	FKBP5 (FK506 Binding Protein 5)	PR downstream activation marker – involved in chaperones/protein folding up-regulated (3–9 fold change) by PR [7–9]
NM_0010471 60	NET1 (Neuroepithelial Cell Transforming 1, ARHGEF8)	PR downstream activation marker – selected from the TCGA analysis as one of the significantly different between PR+ and PR- patients, involved in signal transduction from membrane [7–9]
NM_0011284 31	SLC39A14 (Solute Carrier Family 39 Member 14)	PR downstream activation marker – selected from the TCGA analysis as one of the significantly different between PR+ and PR- patients
NM_0013635 68	UCK2 (Uridine-Cytidine Kinase 2)	PR downstream activation marker – selected from the TCGA analysis as one of the significantly different between PR+ and PR- patients [7–9]
NM_0010206 58	PUM1 (pumilio RNA binding family member 1)	House-keeping gene recommended by Nanostring
NM_004168	SDHA (succinate dehydrogenase complex flavoprotein subunit A)	House-keeping gene recommended by Nanostring
NM_003194	TBP (TATA-box binding protein)	House-keeping gene recommended by Nanostring

Table S4. Clinical and pathological characteristics of PR(mol-) versus PR(mol+) subgroups within ER+ patients. Nominal variables are presented as raw values followed by percentages of the respective groups, continuous variables are presented as medians and interquartile ranges in brackets.

Variable	PR(mol-) $n = 110$ (31.8)	PR(mol+) $n = 204$ (59.0)	*p*-value
Age [years] [1]	65.0 (58.0–70.0)	63.0 (52.0–71.2)	0.125
Menopausal status [2]			
Pre	8 (8.2)	25 (13.3)	0.197
Post	90 (91.8)	163 (86.7)	
Grade [2]			
1	10 (9.1)	30 (14.7)	0.039 *
2	70 (63.6)	141 (69.1)	
3	30 (27.3)	33 (16.2)	
Ki67 [%] [1]	22.0 (10.0–40.0)	12.0 (5.0–22.0)	0.003 *
HER2-positivity [2]	19 (17.3)	13 (6.4)	0.002 *
Tumour size [mm] [1]	22.5 (17.0–30.0)	20.0 (15.0–25.0)	0.012 *
T feature [2]			0.007 *

pT1	29 (42.6)	99 (60.0)	
pT2	33 (48.5)	63 (38.2)	
pT3/4	6 (8.8)	3 (1.8)	
Metastases present	37 (34.6)	68 (34.0)	0.919
N feature [2]			
pN0	70 (65.4)	135 (67.5)	0.682
pN1	24 (22.4)	47 (23.5)	
pN2-3	13 (12.2)	18 (9.0)	
Staging [2]			
Very early (Stage IA)	36 (33.3)	86 (43.0)	0.220
Early (IB-IIIA)	64 (59.3)	104 (52.0)	
Advanced (IIIB-C, IV)	8 (7.4)	10 (5.0)	
Multifocality [2]	8 (11.8)	27 (16.4)	0.372
DCIS present [2]	35 (31.8)	56 (27.5)	0.416
DFS events [3]	14 (14.1)	21 (12.5)	0.354
Disease-free survival [years]	3.8 (2.3–4.9)	4.3 (2.8–6.7)	
Deaths [3]	11 (10.0)	20 (9.8)	0.551
Overall survival [years]	4.0 (2.8–5.5)	4.7 (3.0–6.7)	

[1] Mann-Whitney U test, [2] Pearson's chi-squared test, [3] log-rank test, * significant differences.

Table S5. Expression of FGFR2 protein and mRNA in PR(mol-) versus PR(mol+) patients within ER+ subgroup. Nominal variables are presented as raw values followed by percentages of the respective groups, continuous variables are presented as medians and interquartile ranges in brackets.

Variable	PR(mol-) *n* = 110 (35.0)	PR(mol+) *n* = 204 (65.0)	*p*-value
FGFR2 protein [H-score] [1]	103.5 (21.0–210.0)	105 (22.0–201.5)	0.739
FGFR2 by H-score quartiles [2]			
0–75	44 (40.0)	77 (37.8)	0.929
76–150	22 (20.0)	47 (23.0)	
151–225	20 (18.2)	38 (18.6)	
226–300	24 (21.8)	42 (20.6)	
FGFR2 mRNA [log2] [1]	8.3 (7.2–9.3)	8.7 (8.1–9.4)	0.008 *

[1] Mann-Whitney U test, [2] Pearson's chi-squared test, * significant differences.

Table S6. Multivariate analyses of the combined effect of FGFR2 (protein) status and PR(mol) status on the poor prognostic associations characterised for FGFR2(low). The analysis involves only ER+ patients. Hazard ratios with confidence intervals are present for overall and disease-free survival, β-parameters and standard deviation for Ki67 proliferation index and odds ratios (OR) with confidence intervals for tumor grade.

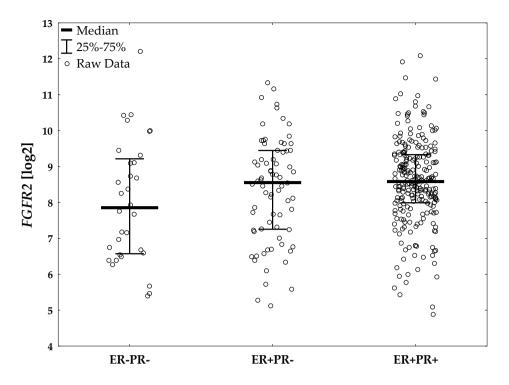| Feature	FGFR2 status (high as reference)	PR(mol) status (PR(mol+) as reference)
Tumor grade (probability for grade 3 regarding grade 1)	OR 4.76 (95% CI 1.69–12.50); $p = 0.003$	OR 2.86 (95% CI 1.15–7.14); $p = 0.023$
Ki67 proliferation index	B = 5.1 (±1.77), p=0.005	β = 5.1 (±1.77), $p = 0.005$
Overall survival	HR 2.25 (95% CI 1.11–4.58), $p = 0.025$	HR 1.32 (95% CI 0.63–2.77), $p = 0.462$
Disease-free survival	HR 2.07 (95% CI 1.06–4.05), $p = 0.034$	HR 1.50 (95% CI 0.76–2.98), $p = 0.246$

Figure S1. *FGFR2* mRNA levels compared between hormonal receptor status subgroups (ER-PR- vs. ER+PR- vs. ER+PR+), *p* = 0.049 from ANOVA test.
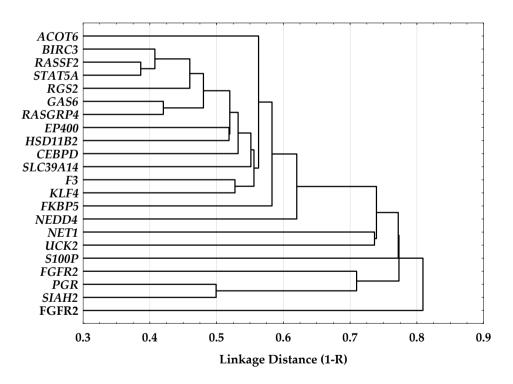

Figure S2. Hierarchical tree-clusterisation of genes included in PR-dependent molecular signature. All patients with good quality RNA were included in this analysis. Linkage distance is showed as 1-R (Spearman).
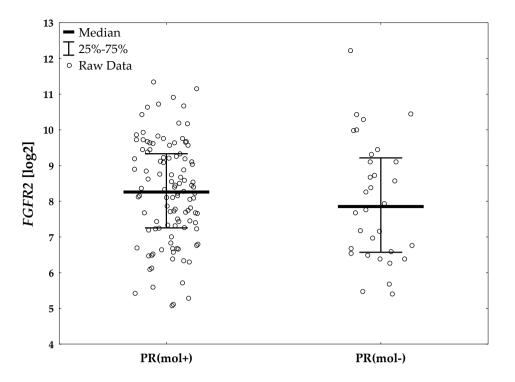
Figure S3. *FGFR2* mRNA levels compared between estrogen receptor status and progesterone receptor molecular activity status (ER-PR(mol-) vs. ER+PR(mol-) vs. ER+PR(mol+)), *p* = 0.002. *p*-value from ANOVA test.
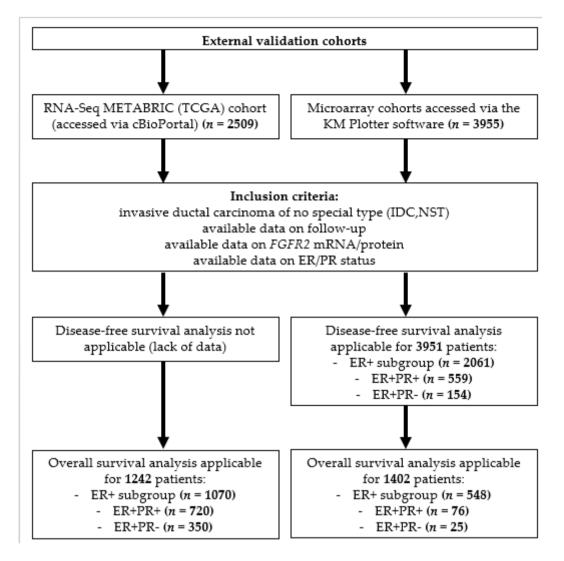
Figure S4. Flowchart of the in silico verification with indication of numbers of patients from the external databases included in every analysis. "ER+" subgroup included all ER+ patients regardless of PR status and it comprised ER+PR+ and ER+PR- subgroups. IDC, NST – invasive ductal carcinoma of no special type, ER – estrogen receptor protein status, PR – progesterone receptor protein status, FGFR2 – fibroblast growth factor receptor 2 protein.
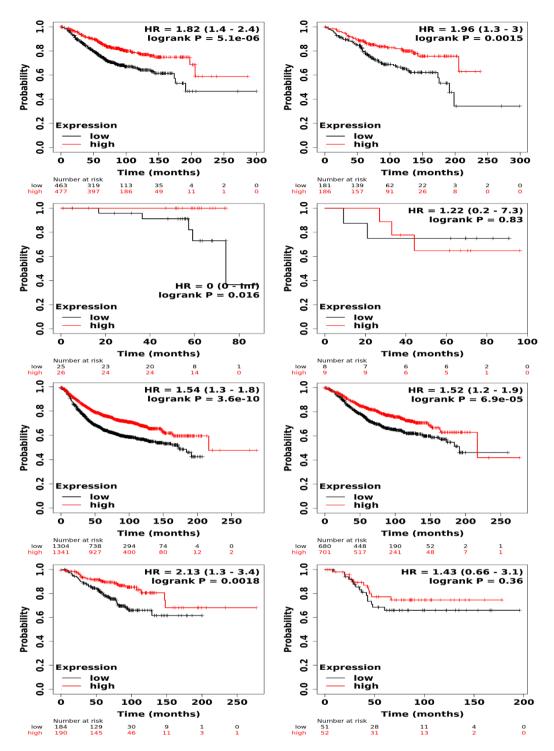
Figure S5. Kaplan-Meier curves for OS (**a–d**) and PFS (**e–h**) regarding FGFR2 microarray mRNA levels. "ER+" subgroup included all ER+ patients regardless of PR status and it comprised ER+PR+ and ER+PR- subgroups. FGFR2low stands for 1st tercile and FGFR2high for 2nd-3rd terciles. Plots were generated using online open access tool KM plotter (encompassing patients different than those included in TCGA database). (**a**) OS probability in all 1402 breast cancer patients, (**b**) OS probability in all 548 ER+ breast cancer patients, (**c**) OS probability in all 76 ER+PR+ patients, (**d**) OS probability in all 25 ER+PR- patients., (**e**) PFS probability in all 3951 breast cancer patients, (**f**) PFS probability in all 2061 ER+ breast cancer patients, (**g**) PFS probability in all 559 ER+PR+ patients, (**h**) PFS probability in all 154 ER+PR- patients.

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