Supplementary material

Journal name: Biomarkers in Medicine

Article title: High expression of *DNAJA1 (HDJ2)* predicts unfavorable survival outcomes in breast cancer

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Sample information sheet of the commercial tissue microarray (TMA) used in the study

TMA (BC081116b): Breast carcinoma tissue microarray with adjacent breast tissue, containing 100 cases of breast invasive ductal carcinoma and 10 cases of adjacent breast tissue, single core per case (Biomax Inc. Rockville, MD, USA)

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F9 59 71 F Breast Invasive ductal carcinoma T4N2M0 2 IIIB malignant +++ 0											+	+	
F10 60 47 F Breast Invasive ductal carcinoma T2N1M0 - IIB malignant + - 3+		59		F				2		-	+++	+++	0
	F10	60	47	F	Breast	Invasive ductal carcinoma	T2N1M0	-	IIB	malignant	+	-	3+

									-			
G1	61	66	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
G2	62	35	F	Breast	Invasive ductal carcinoma	T1N0M0	2	1	malignant	++	++	3+
G3	63	47	F	Breast	Invasive ductal carcinoma	T4bN0M0	2	IIIB	malignant	++	+++	3+
G4	64	44	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	++	++	0
G5	65	38	F	Breast	Invasive ductal carcinoma	T2N0M0	1	IIA	malignant	-	-	3+
G6	66	55	F	Breast	Invasive ductal carcinoma	T2N0M0	-	IIA	malignant	*	*	3+
G7	67	46	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+	0
G8	68	56	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	++	+	3+
G9	69	47	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	3+
G10	70	48	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	0
H1	71	66	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	-	3+
H2	72	46	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	++	0
H3	73	59	F	Breast	Invasive ductal carcinoma	T4bN0M0	2	IIIB	malignant	+++	+	0
H4	74	38	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
H5	75	62	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
H6	76	52	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	0
H7	77	54	F	Breast	Invasive ductal carcinoma	T1N0M0	2	I	malignant	+++	++	3+
H8	78	52	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	++	++	3+
H9	79	49	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	3+
H10	80	43	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
11	81	33	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
12	82	47	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+	+++	1+
13	83	32	F	Breast	Invasive ductal carcinoma	T2N0M0	-	IIA	malignant	*	*	*
14	84	38	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+	+++	0
15	85	70	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
16	86	48	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	++	3+
17	87	48	F	Breast	Invasive ductal carcinoma	T3N1M0	2	IIIA	malignant	+++	-	1+
18	88	44	F	Breast	Invasive ductal carcinoma	T4aN0M0	2	IIIB	malignant	-	-	2+
19	89	69	F	Breast	Invasive ductal carcinoma	T3N0M0	2	IIB	malignant	+++	-	0
110	90	49	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+	-	0
J1	91	48	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+	0
J2	92	42	F	Breast	Invasive ductal carcinoma	T2N1M0	2	IIB	malignant	++	-	0
J3	93	42	F	Breast	Invasive ductal carcinoma	T2N2M0	2	IIIA	malignant	+++	+++	0
J4	94	49	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	0
J5	95	68	F	Breast	Invasive ductal carcinoma	T4bN0M0	2	IIIB	malignant	-	-	3+
J6	96	53	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	+++	+++	0
J7	97	49	F	Breast	Invasive ductal carcinoma	T2N0M0	3	IIA	malignant	-	-	3+
J8	98	69	F	Breast	Invasive ductal carcinoma	T2N0M0	3	IIA	malignant	+	-	3+
J9	99	36	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	0
J10	100	29	F	Breast	Invasive ductal carcinoma	T2N0M0	2	IIA	malignant	-	-	3+
K1	101	51	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	++	++	0
K2	102	37	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	++	++	0
К3	103	35	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	++	+	0
K4	104	45	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	-	-	0
K5	105	35	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	+	++	0
K6	106	46	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	+	+	0
K7	107	49	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	+	+	0
K8	108	44	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	+	+	0
К9	109	38	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	+	+	0
K10	110	37	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT	++	+	0
	0	42	М	Adrenal gland	Pheochromocytoma (tissue marker)				Malignant			

• One sample (Position: D6) is overlap with the cases of TMA (BR486; Positions: A8, B8, C8)

Sample information sheet of the commercial tissue microarray (TMA) used in the study

TMA (BR486): Breast invasive ductal carcinoma tissue microarray with matched adjacent normal breast tissue, containing 16 cases of breast invasive ductal carcinoma with matched adjacent normal breast tissue, triplicate cores per case (Biomax Inc. Rockville, MD, USA)

Pos.	No.	Age	Sex	Organ/Anatomic Site	Pathology diagnosis	TNM	Grade	Stage	Туре
A1	1	60	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
A2	2	53	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
A3	3	46	F	Breast	Invasive ductal carcinoma	T1N0M0	1	1	malignant
A4	4	51	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
A5	5	39	F	Breast	Invasive ductal carcinoma	T2N1M0	2	llb	malignant
A 6	6	50	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
A7	7	48	F	Breast	Invasive ductal carcinoma	T2N0M0	1	lla	malignant
A 8	8	34	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
B1	9	60	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
B2	10	53	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
B3	11	46	F	Breast	Invasive ductal carcinoma	T1N0M0	1	1	malignant
B4	12	51	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
B5	13	39	F	Breast	Invasive ductal carcinoma	T2N1M0	2	llb	malignant
B 6	14	50	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
B7	15	48	F	Breast	Invasive ductal carcinoma	T2N0M0	1	lla	malignant
B8	16	34	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
C1	17	60	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
C2	18	53	F	Breast	Cancer adjacent normal breast tissue (breast	-	-	-	NAT
C3	19	46	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
C4	20	51	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
C5	21	39	F	Breast	Cancer adjacent normal breast tissue (adeno sis)	-	-	-	NAT
C6	22	50	F	Breast	Cancer adjacent normal breast tissue (adeno sis)	-	-	-	NAT
C7	23	48	F	Breast	Cancer adjacent normal breast tissue (fibrofat ty tissue and blood vessel)	-	-	-	NAT
C8	24	34	F	Breast	Cancer adjacent normal breast tissue (adeno sis)	-	-	-	NAT
D1	25	31	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
D2	26	53	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
D3	27	52	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
D4	28	50	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
D5	29	46	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
D6	30	37	F	Breast	Invasive ductal carcinoma	T4N2M0	2	IIIb	malignant
D7	31	46	F	Breast	Invasive ductal carcinoma	T2N0M0	3	lla	malignant
D8	32	50	F	Breast	Invasive ductal carcinoma	T2N2M0	3	Illa	malignant
E1	33	31	F	Breast	Invasive ductal carcinoma	T2N0M0	2	lla	malignant
E2	34	53	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
E3	35	52	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
E4	36	50	F	Breast	Invasive ductal carcinoma	T2N1M0	1	llb	malignant
E5	37	46	F	Breast	Invasive ductal carcinoma	T4N0M0	2	IIIb	malignant
E6	38	37	F	Breast	Invasive ductal carcinoma	T4N2M0	2	IIIb	malignant
E7	39	46	F	Breast	Invasive ductal carcinoma	T2N0M0	3	lla	malignant
E8	40	50	F	Breast	Invasive ductal carcinoma	T2N2M0	3	Illa	malignant
F1	41	31	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F2	42	53	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F3	43	52	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F4	44	50	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F5	45	46	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F6	46	37	F	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
F7	47	46	F	Breast	Cancer adjacent normal breast tissue (spars e)	-	-	-	NAT
F8	48	50	F	Breast	Cancer adjacent normal breast tissue (spars e)	-	-	-	NAT
-	0	55	F	liver	Hepatocellular liver cancer (tissue marker)	T3N0M0	3		Malignant

• One sample (Positions: A8, B8, C8) is overlap with the case of TMA (BC081116b; Position: D6)

Primers used for mutation analysis

Primer	Sequence (5'-3')	Product Size (bp)
F	gctccagaagattccacgag	434
R	aggaactgacagcggtccta	434
rimers used for COB	RA	
Bi-F	gttgagtggagaaaagggttttag	373
Bi-R	ccaaccaaaaactaaaaacaac	
Bi-F	gttgagtggagaaaagggttttag	494
Bi-Nested-R	ccaaaacaccaccaacc	
rimers used for Q-R	I-PCR	
TBP-F*	TGCACAGGAGCCAAGAGTGAA	132
TBP-R*	CACATCACAGCTCCCCACCA	152
F	CATGCCAATTTATCGTAGACCA	147
R	TCAGTCTCTTCCACTTCCTTCC	147

DNAJA1 Ensembl Gene ID: ENSG0000086061

(*) Primer sequences were obtained from Gur-Dedeoglu B. et al. (2009).

Abbreviations: F, forward primer; R, reverse primer.

GEO and Array Express datasets used for survival (OS, RFS and DMFS) analyses by KM-plotter

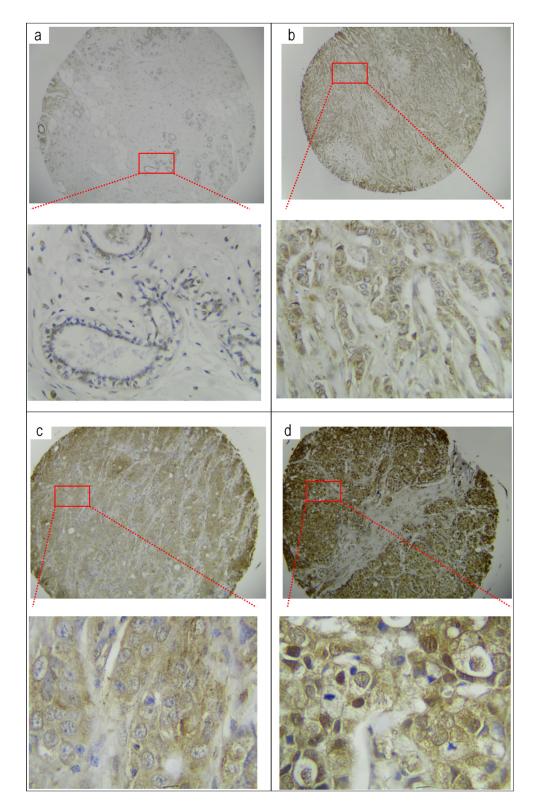
Dataset	Patients used for OS analysis (n)
GSE1456 (n=159)	159
GSE16446 (n=120)	107
GSE16716 (n=47)	7
GSE20271 (n=96)	2
GSE20685 (n=327)	327
GSE20711 (n=90)	88
GSE3494 (n=251)	236
GSE37946 (n=41)	40
GSE42568 (n=121)	104
GSE45255 (n=139)	134
GSE7390 (n=198)	198
Total	1402

Dataset	Patients used for DMFS analysis (n)
GSE11121 (n=200)	200
GSE16446 (n=120)	107
GSE16716 (n=47)	1
GSE17907 (n=54)	38
GSE19615 (n=115)	115
GSE20685 (n=327)	327
GSE26971 (n=276)	256
GSE2990 (n=102)	95
GSE3494 (n=251)	130
GSE45255 (n=139)	136
GSE5327(n=58)	57
GSE6532 (n=82)	66
GSE7390 (n=198)	198
GSE9195 (n=77)	77
Total	1803

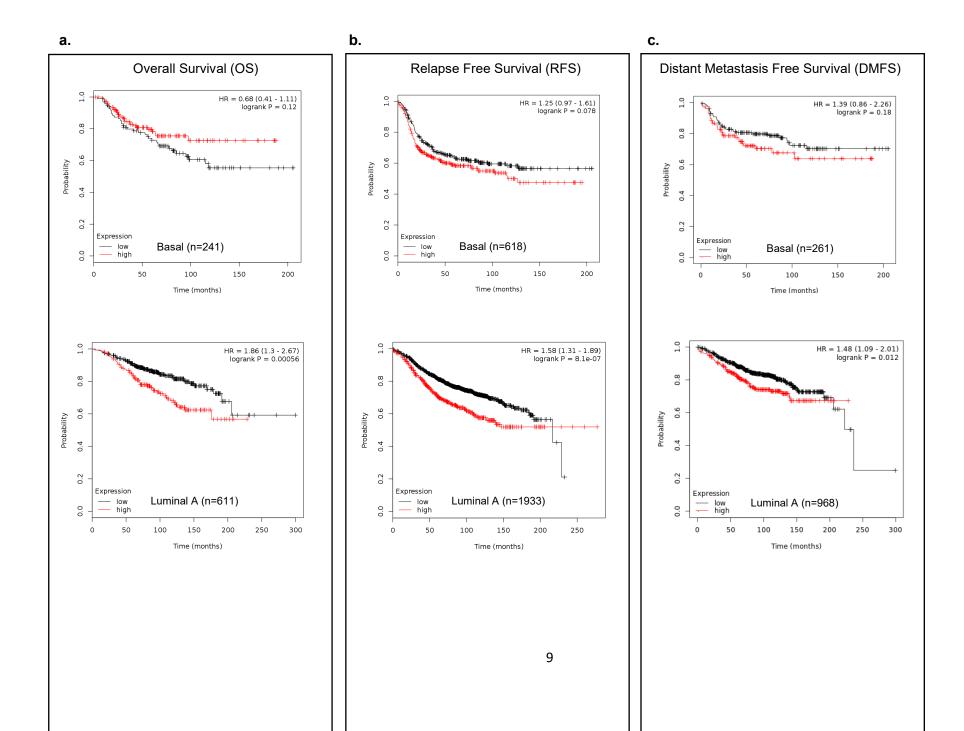
Dataset	Patients used for RFS analysis (n)
E-MTAB-365 (n=537)	426
GSE11121 (n=200)	200
GSE12093 (n=136)	135
GSE12276 (n=204)	204
GSE1456 (n=159)	159
GSE16391 (n=55)	48
GSE16446 (n=120)	107
GSE16716 (n=47)	8
GSE17705 (n=196)	196
GSE17907 (n=54)	38
GSE19615 (n=115)	115
GSE20271 (n=96)	2
GSE2034 (n=286)	286
GSE20685 (n=327)	327
GSE20711 (n=90)	88
GSE21653 (n=240)	230
GSE2603 (n=99)	82
GSE26971 (n=276)	97
GSE2990 (n=102)	102
GSE31519 (n=67)	63
GSE3494 (n=251)	247
GSE37946 (n=41)	40
GSE42568 (n=121)	104
GSE45255 (n=139)	94
GSE4611 (n=153)	151
GSE5327 (n=58)	57
GSE6532 (n=82)	69
GSE7390 (n=198)	198
GSE9195 (n=77)	77
Total	3951

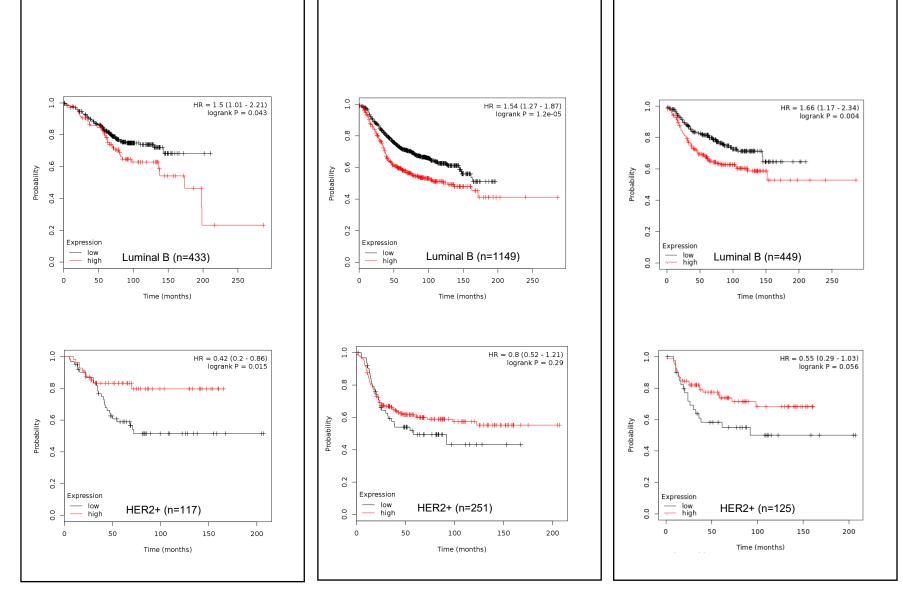
Associations between DNAJA1 protein expression and clinical characteristics of invasive breast carcinoma samples. Immunostaining intensity was graded relatively as: *negative* (score: 0), *weakly positive* (score: 1), *moderately positive* (score: 2), or *strongly positive* (score: 3) stainings. Staining scores of the samples were grouped as "0" vs. "1+2+3".

		IHC Score (0) n (%)		IHC Score (1+2+3) n (%)
Tumor stage		<u>x_r</u>		
T	TI VIT-II	2 (22.22) 7 (6.60)	P= 0.147	7 (77.78) 99 (93.40)
Т	TI-TII III-TIV	5 (5.43) 4 (17.39)	P=0.0773	87 (94.57) 19 (82.61)
Lymph node			1 -0.0775	
stage	N0 NI-NII	7 (7.53) 2 (9.09)	P=0.6814	86 (92.47) 20 (90.91)
Grade	GI GII-GIII	0 (0) 8 (8.70)	P=0.6018	16 (100) 84 (91.30)
Stage	SI SII-SIII	2 (25) 7 (6.54)	P=0.1193	6 (75) 100 (93.46)
	gative ositive	4 (7.40) 4 (9.52)		50 (92.60) 38 (90.48)
	gative ositive	5 (10.20) 2 (4.26)	P=0.7262 P=0.4361	44 (89.80) 45 (95.74)
	gative ositive	3 (9.37) 4 (6.25)	P=0.4361 P=0.6827	29 (90.63) 60 (93.75)

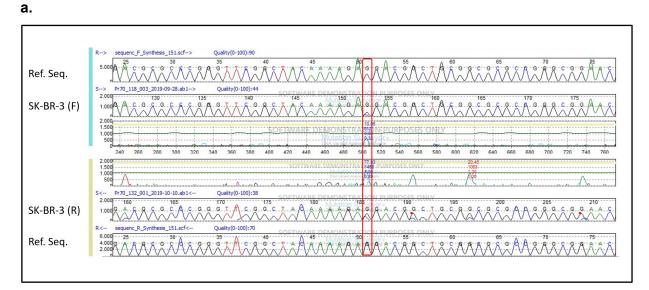


Representative photographs from DNAJA1 immunohistochemistry study performed with commercial tissue microarrays. 400X magnification of the selected regions (red rectangulars) were shown for each tissue spot. (a) Expression was lost in normal breast tissue. (b, c, d) Invasive Ductal Carcinoma tissues shows weak to strong cytoplasmic staining (brown).

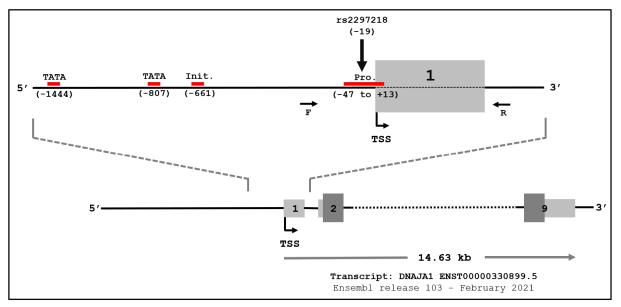




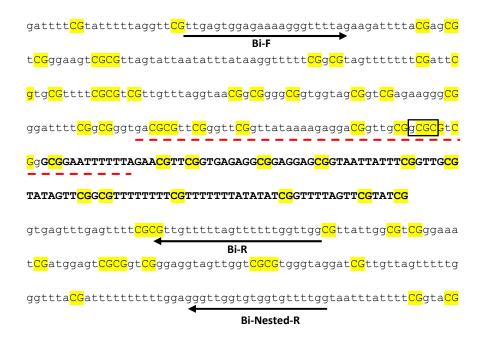
Kaplan-Meier plots of OS (a), RFS (b) and DMFS (c) in breast cancer subtypes according to *DNAJA1* mRNA expression. The probeset ID selected for *DNAJA1* expression was 200881_at. Reliability of the analysis: Highly reliable analysis (>500 samples), neutral analysis (500-200 samples), preliminary analysis (200-50 samples) (Györffy et al. 2010).





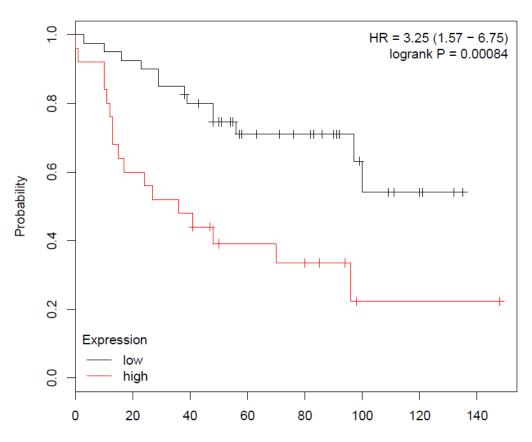


(a) Heterozygous SNP (rs2297218) in the promoter region of *DNAJA1* in SK-BR-3 cell line. "SK-BR-3 (F)": Sequencing with forward primer; "SK-BR-3 (R)": Sequencing with reverse primer; "Ref. Seq": *DNAJA1* genomic sequence (NCBI Gene ID: 3301) (b) The position of the SNP relative to the promotor motifs and location of the primers are depicted. Promotor motifs are estimated by EPD "Search Motif Tool" from -2000 bp to 100 bp relative to TSS (*P* value = 0.001) (https://epd.epfl.ch//index.php). UTRs are presented as light gray boxes, translated exons are presented as dark gray boxes. TSS: Transcription start site; TATA: TATA-box; Init: Initiator; Pro: Core promoter sequences; F: Forward primer; R: Reverse primer.



Bisulfite-modified sequences of the *DNAJA1* gene analysed by COBRA assay. All the CGs are suggested to be methylated and shown in yellow uppercase font. Arrows underlie the position of COBRA primers. Box shows the cutting site (GCG^C) of the *HhaI* restriction enzyme in Bi-F/Bi-R PCR amplicon (373 bp). The expected *HhaI* digestion fragments are 215 bp and 158 bp. Promotor region estimated by EPD (https://epd.epfl.ch//index.php) is shown as red dashed line. 1st UTR exon is depicted as bold uppercase font.

DNAJA1 (P31689)



Supplemental Figure S5

Kaplan-Meier plot of *Overall Survival* in breast cancer according to DNAJA1 protein expression (n=65) (Uniprot ID: P31689) (Györffy et al., 2010; Tang et al., 2018)

Supplemental Method Info.

Immunohistochemistry (IHC) study

Novolink[™] Min Polymer Detection System Kit was used according to the instructions (Cat. No: RE7290-K) (Novolink, Inc., CA, USA). Briefly, TMAs were baked at 60°C overnight to avoid tissue loss during staining procedures. The slides were deparaffinized by incubating them in xylene for 20 min., followed by rehydration in a graded series of ethanol. Sections were cooked in citrate buffer (10mM, pH 6.0) for antigen retrieval. Endogenous peroxidases were blocked with the solutions provided with the kit. TMAs were incubated overnight at 4°C with the primary antibody (rabbit polyclonal Anti-DNAJA1; Cat. No: 11713-1-AP) (Proteintech Group, Inc., IL, USA) (dilution 1:80). Slides were then incubated with Novolink[™] Polymer for 30 minutes and peroxidase activity was developed with DAB working solution for 5 minutes. All slides were counterstained with hematoxylin. Anti-DNAJA1 antibody showed cytoplasmic dark brown staining with different degree of positivity. The tissue microarrays (TMAs) includes tissue spots of "Adrenal gland (Pheochromocytoma)" and they are positively stained with the anti-DNAJA1 antibody.

Immunopositivity of the cells was assessed by qualitative scoring system in which antigen expression are interpreted by two observers (TA, FCI) independently according to an arbitrary scoring range (0 to 3) (Lebok et al. 2019). Briefly, samples with complete absence of staining or staining intensity of 1+ in up to 30% of the cells were scored as negative (score 0); samples with a staining intensity of 1+ in more than 30% or 2+ in up to 30% of the cells were scored as weakly positive (score 1); samples with a staining intensity of 2+ in more than 30% or 3+ in up to 30% of the cells were scored as moderately positive (score 2); samples with a staining intensity was 3+ in more than 30% of the cells were scored as strongly positive (score 3). Statistical analyses were performed by GraphPad software by using *Fisher Exact Test* with a significance level of P < 0.05.