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PRECLINICAL STUDY

Combinatorial expression of microtubule-associated EB1 and ATIP3 biomarkers improves breast cancer prognosis

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Abstract

Purpose The identification of molecular biomarkers for classification of breast cancer is needed to better stratify the patients and guide therapeutic decisions. The aim of this study was to investigate the value of *MAPRE1* gene encoding microtubule-end binding proteins EB1 as a biomarker in breast cancer and evaluate whether combinatorial expression of *MAPRE1* and *MTUS1* gene encoding EB1-negative regulator ATIP3 may improve breast cancer diagnosis and prognosis.

Methods Probeset intensities for *MAPRE1* and *MTUS1* genes were retrieved from Exonhit splice array analyses of 45 benign and 120 malignant breast tumors for diagnostic purposes. Transcriptomic analyses (U133 Affymetrix array) of one exploratory cohort of 150 invasive breast cancer patients and two independent series of 130 and 155 samples were compared with clinical data of the patients for prognostic studies. A tissue microarray from an independent cohort of 212 invasive breast tumors was immunostained with anti-EB1 and anti-ATIP3 antibodies.

Results We show that *MAPRE1* gene is a diagnostic and prognostic biomarker in breast cancer. High *MAPRE1* levels correlate with tumor malignancy, high histological grade and poor clinical outcome. Combination of high-*MAPRE1* and low-*MTUS1* levels in tumors is significantly associated with tumor aggressiveness and reduced patient survival. IHC studies of combined EB1/ATIP3 protein expression confirmed these results.

Conclusions These studies emphasize the importance of studying combinatorial expression of EB1 and ATIP3 genes and proteins rather than each biomarker alone. A population of highly aggressive breast tumors expressing high-EB1/low-ATIP3 may be considered for the development of new molecular therapies.

Keywords MAPRE1 · MTUS1 · Diagnosis · Prognosis · Breast cancer · Biomarkers combination

Introduction

Breast cancer is a complex disease whose clinical management relies on well-established clinico-pathological characteristics and molecular biomarkers. The emergence of

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high-throughput techniques for molecular profiling of breast tumors, such as DNA arrays and RNA-seq, has allowed extensive progress in the diagnosis, classification, and prognosis of breast tumors. The availability of large molecular databases now provides the opportunity to rapidly analyze individual and combinatorial expression of genes in cohorts of breast cancer patients, which may accelerate the identification of novel molecular biomarkers that are urgently needed for better stratifying breast cancer patients and deciding the type of therapy to be administered.

The microtubule (MT) cytoskeleton plays a key role in various biological processes such as intracellular transport, cell migration and mitosis, all of which are altered in cancer. MTs are polarized protofilaments that constantly alternate between phases of polymerization (growth) and depolymerization (shrinkage) at their plus ends [1, 2]. This dynamic behavior is essential for MTs to explore the cytosol and ensure cell homeostasis, and is tightly regulated by a wide number of MT-associated proteins (MAPs) [3]. Any defect in these regulatory proteins may alter the organisation and/or function of MTs, with major consequences on cancer initiation or progression.

End-binding protein 1 (EB1) is the leader member of a subfamily of MAPs including EB2 and EB3, encoded by homologous genes designated *MAPRE1, MAPRE2*, and *MAPRE3*, respectively [4]. EB1 and EB3 have been extensively studied, EB1 being ubiquitous and EB3 being predominant in the brain, whereas EB2 remains less characterized [5]. EB1 preferentially binds and accumulates at growing MT ends and is considered a surrogate marker of MT dynamics [6]. Upon binding to MT ends, EB1 accelerates the rate of catastrophe (rapid depolymerization) and thus also functionally contributes to MT-end maturation and dynamic instability [7]. In addition, EB1 recruits a large number of regulatory proteins at MT plus ends, thereby orchestrating the regulation of MT dynamics and MT targeting to organelles [8–10].

The pivotal role of EB1 on MT dynamics suggests that alterations of EB1 expression or function may have important consequences in cancer. Indeed, up-regulation of EB1 protein in tumor samples has been reported in breast cancer [11], glioblastoma [12], hepatocarcinoma [13], oral [14], and colorectal cancer [15, 16]. In breast cancer, high EB1 protein levels have been shown to correlate with tumor malignancy and high tumor grade [11]. However, the status of *MAPRE1* gene encoding EB1, and its paralogs *MAPRE2* and *MAPRE3*, has not yet been evaluated in breast cancer, and it remains to be established whether EB1 represents a prognostic biomarker of breast cancer patient survival.

Recent studies have identified several structural MAPs as endogenous antagonists of EB1 functions at MT plus ends [5]. Among them, the ATIP3 protein encoded by candidate tumor suppressor gene *MTUS1* has been described as a prognostic biomarker down-regulated in invasive breast cancer [17, 18]. ATIP3 is a potent MT-stabilizer that markedly reduces breast tumor growth and distant metastasis [18]. ATIP3 directly binds to EB1 in the cytosol and prevents its turnover and accumulation at growing MT ends, thereby reducing MT dynamics and cell polarity [19]. These data suggest that altered expression of either ATIP3 or EB1 in breast tumors may impact the levels of intracellular ATIP3-EB1 molecular complexes that govern EB1 function at MT plus ends. This raises the interesting possibility that deleterious effects of high EB1 levels in breast tumors may be moderated by high levels of ATIP3 whereas tumors with high-EB1 and low-ATIP3 levels may remain more aggressive.

The present study investigates the value of *MAPRE* genes, alone or in combination with *MTUS1*, for molecular classification, diagnosis and prognosis of breast cancer patients. We show that high *MAPRE1*—but not *MAPRE2* and *MAPRE3*—levels correlate with tumor malignancy and poor clinical outcome for breast cancer patients, and that combinatorial analysis of *MAPRE1* and *MTUS1* expression refines breast cancer diagnosis and prognosis compared to *MAPRE1* alone.

Methods

Breast tumor samples and gene arrays

In a first cohort of patients (Cohort#1) designed for tumor classification, samples were obtained by fine-needle aspiration of breast lesions from patients referred to the breast diagnosis center of the Gustave Roussy Center (Villejuif, France) between 2006 and 2007 [20]. 165 samples, among which 45 benign and 120 malignant tumors, were profiled on a Splice Array (Exonhit, France) [21]. Patient characteristics and probeset intensities are presented in Supplemental Table S1.

The exploratory cohort of breast cancer patients (Cohort#2), designed for prognostic purposes, comprises 150 infiltrating breast carcinomas obtained from patients who were included between 1988 and 1999 in the prospective database initiated in 1981 by the Institut Curie Breast cancer group (Curie, Paris, France) [17]. Samples were analyzed by Affymetrix HG-U133 DNA array hybridization as described [17, 18, 22]. Patients included in the study were aged 33 to 88 and were treated by radiotherapy combined or not with hormono- or chemotherapy after chirurgical resection of the tumor. Immunohistological levels of estrogen receptor (ER) and progesterone receptor (PR) were recorded according to standardized guidelines using 10% as the cut-off for ER- and PR-positive cells [23]. For human epidermal growth factor receptor-2 (HER2), only membrane staining was considered with a 30% cut-off as recommended [24].

Patient characteristics and probeset intensities are presented in Supplemental Table S2. Two independent series of breast cancer patients, designated Cohort#3 [25, 26] and Cohort#4 [18] are described in Supplemental Methods and Supplemental Tables S3 and S4.

Immunohistochemical analysis of breast cancer tissue microarray (TMA)

Samples of invasive ductal carcinomas (IDCs) were surgically removed before any radiation, hormonal or chemotherapy treatment at Institut Curie from 2005 to 2006 [27]. Patient characteristics and clinical data are presented in Supplemental Table S5. TMA consisted of replicate 1-mm-diameter tumor cores selected from whole-tumor tissue section in two most representative tumor areas (high tumor cell density) of each tumor sample. Alcohol formalin acetic acid-fixed paraffin-embedded samples were analyzed by immunohistochemistry (IHC) staining using EnVision FLEX kit (Dako) according to the manufacturer's instructions. For EB1 staining, heat-mediated antigen retrieval was performed in Ethylene-Diamine-Tetra-Acetic acid (EDTA) buffer pH 6 in water bath for 30 min and monoclonal mouse anti-EB1 antibodies (BD Biosciences) diluted 1:300 were incubated overnight at 4 °C. ATIP3 staining was performed as previously described [17] using monoclonal anti-MTUS1 antibodies (Abnova) diluted 1:100. Slides were counterstained with hematoxylin. HeLa cells transfected with control siRNA were used as positive control for EB1 staining, and cells transfected for 48 h with EB1-specific siRNA (Dharmacon) [19] were considered as negative control. Silencing efficiency was validated by immunoblotting using anti-EB1 antibodies (BD Biosciences) diluted 1:1000 and anti-alpha-tubulin antibodies (Sigma) diluted 1:1000 as internal control.

EB1 immunoreactivity in tissue sections was classified semi-quantitatively into 5 classes (0, < 1, 1, 2, 3) according to the intensity of cytoplasmic staining of tumor cells. Scores for EB1 immunostaining were defined as 0 (undetectable staining on high-power field (×40), < 1 (only visible on high-power field (×40), 1 (detected on medium-power field (×10–×20) but well visualized on high-power field (×40), 2 (detected on low-power field (×4–×5) but well-visualized on medium-power field (×20), and 3 (well-visualized on low-power field (×4–×5). Samples were independently evaluated by two pathologists of different institutes. EB1 expression was classified on a semi-quantitative basis, immunostaining was considered weak for a score from 0 to 1 and strong for a score of 2–3. Scoring for ATIP3 staining was evaluated as described [17].

Statistical analysis

Statistical analyses were done using JMP-7 and GraphPad Prism 6.0 softwares. The association between clinicopathological characteristics and the expression level of either *MAPRE1, MTUS1*, or their combination were calculated using the Mann–Whitney test for continuous variables and using the chi-squared and the Fisher exact tests for dichotomized variables. Dot plot analyses were done using Mann–Whitney test. Overall survival (OS) and Relapse-free survival (RFS) curves were plotted according to the method of Kaplan–Meier and compared by the log-rank test. $p < 0.05$ was considered statistically significant.

Results

Combinatorial expression of *MAPRE1* and *MTUS1* genes improves breast cancer diagnosis

Expression levels of the *MAPRE1* gene encoding End-Binding protein EB1 were evaluated in a splice-array profiling of 165 breast samples including 120 malignant cancers and 45 benign lesions (Cohort#1) [20]. Only T probesets—that identify expressed exons—were taken into account to avoid potential bias due to alternative exon splicing. Analysis of probesets intensities revealed that *MAPRE1* transcripts are significantly higher in malignant compared to benign breast tumors (Fig. 1a and Supplemental Fig.S1). Heatmap analysis of *MAPRE1* gene expression was then used to classify tumor samples according to high or low levels of EB1 transcripts (Fig. 1b). As shown in Fig. 1c and Table 1, 65% of low-*MAPRE1* expressing tumors were malignant compared to 79,6% of those expressing high-*MAPRE1* levels, further indicating that elevated expression of *MAPRE1* gene is associated with tumor malignancy. In contrast, expression levels of paralog *MAPRE2* and *MAPRE3* genes encoding EB2 and EB3 proteins, respectively, were either decreased (Supplemental Fig.S2a) or unchanged (Supplemental Fig.S2b) in cancer samples compared to benign lesions.

Expression of the *MTUS1* gene, whose major product ATIP3 antagonizes EB1 functions [5, 19], was analyzed in the same samples (Supplemental Table S1). Analysis of *MTUS1* probesets intensities (Fig. 1d and Supplemental Fig.S3) revealed that *MTUS1* levels are significantly decreased in malignant breast tumors compared to benign lesions. Classification of samples into clusters expressing high- and low-*MTUS1* using heatmap analysis (Fig. 1e) further indicated that 93.6% of low-*MTUS1* tumors were malignant compared to 54% of tumors with high-*MTUS1* levels (Fig. 1f; Table 1).

We next evaluated the diagnostic value of combining *MAPRE1* and *MTUS1* gene expression. Tumors expressing

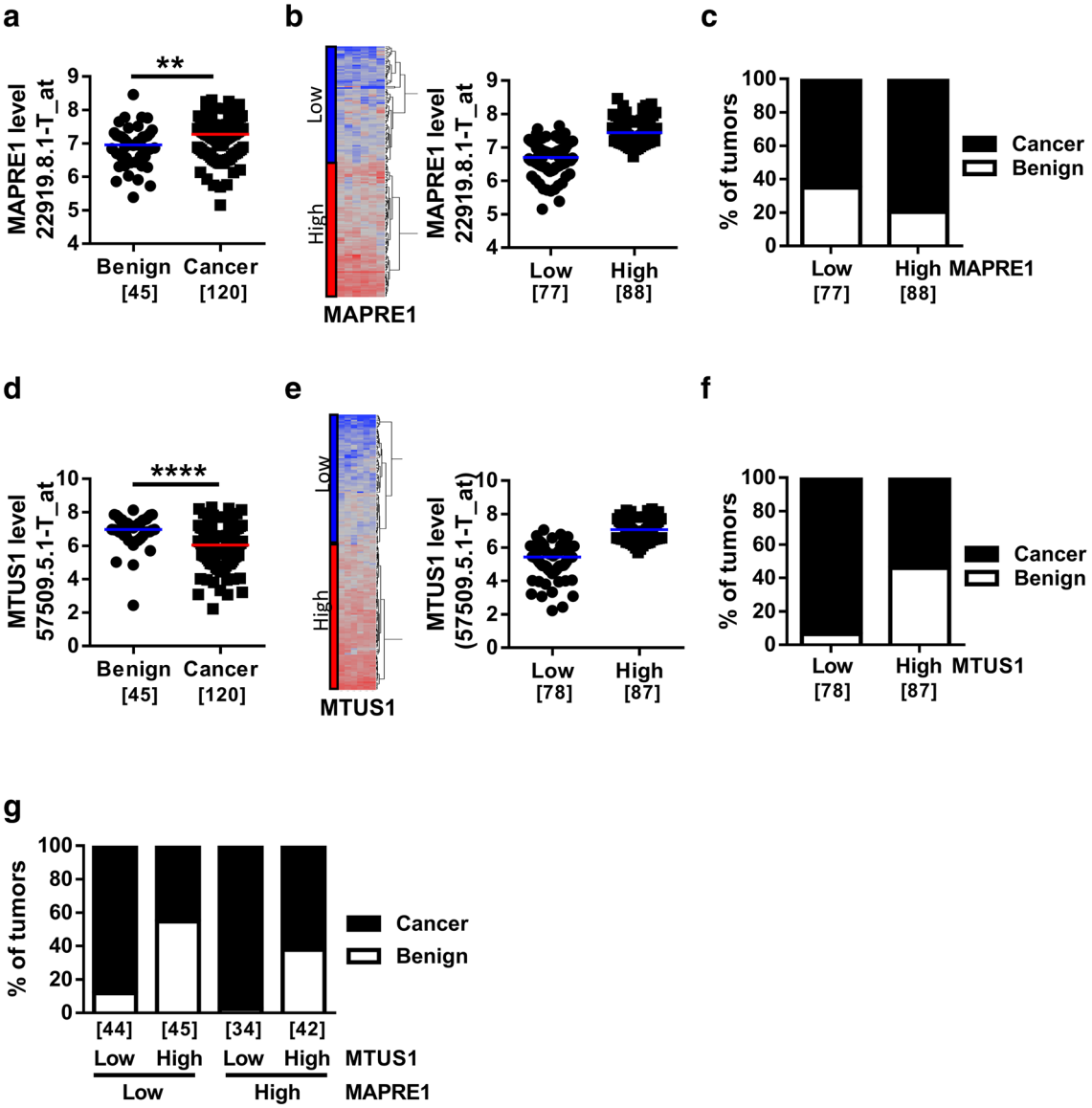


Fig. 1 Combinatorial expression of *MAPRE1* and *MTUS1* genes improves breast cancer diagnosis. **a** Scattered dot plot of MAPRE1 probeset (22919.8.1-T_at) intensity in tumors from patients of cohort #1 (Exonhit) according to tumor malignancy. $^{**}p = 0.0016$. **b** Heat-map and hierarchical clustering of 165 breast tumor samples based on the intensities of 6 MAPRE1 probesets (22919.1.1-T_at, 22919.1.2-T_at, 22919.2.1-T_at, 22919.6.1-T_at, 22919.7.1-T_at, 22919.8.1-T_at). Heat-map illustrates relative expression profiles of *MAPRE1* (column) for each tumor sample (line) in continuous color scale from low (blue) to high (red) expression. Dendogram of the 2 selected tumor groups and the corresponding scattered dot plot of *MAPRE1* expression are shown on the right. **c** Proportion of patients with benign or malignant tumors according to *MAPRE1* level. **d** Scattered dot plot of MTUS1 probeset (57509.5.1-T_at) intensity in tumors from patients of cohort #1 (Exonhit) according to tumor malignancy. $^{****}p < 0.0001$. **e** Heat-map and hierarchical clustering of 165 breast tumor samples based on the intensities of 6 MTUS1 probesets (57509.5.1-T_at, 57509.6.1-T_at, 57509.10.1-T_at, 57509.13.1-T_at, 57509.13.2-T_at, 57509.26.1-T_at). Dendogram of the 2 selected tumor groups and the corresponding scattered dot plot of *MTUS1* expression are shown on the right. **f** Proportion of patients with benign or malignant tumors according to *MTUS1* level. **g** Proportion of patients with benign or malignant tumors according to combinatorial expression of *MAPRE1* and *MTUS1* genes. **a**–**g**- Number of samples in each group is indicated under brackets

high-*MAPRE1* and low-*MTUS1* levels included a majority (97.7%) of malignant samples, compared to 45.2% among those expressing low-*MAPRE1* and high-*MTUS1* (Fig. 1g). Of interest, combining *MAPRE1* and *MTUS1* gene expression allowed better tumor classification compared to each biomarker alone (Fig. 1g; Table 1). Together these studies point to *MAPRE1* and *MTUS1* as valuable diagnostic biomarkers and suggest that analyzing combinatorial expression of these genes may refine tumor classification and improve breast cancer diagnosis.

Table 1 Characteristics of patients from Cohort#1, with high or low *MAPRE1*, *MTUS1* or combinatorial genes expression

Variables	All $N=165$	MAPRE1			MTUS1			Combined MAPRE1/MTUS1		
		Low $N=77$ (46.7%)	High $N=88$ (53.3%)	*p* value	Low $N=78$ (47.3%)	High $N=87$ (52.7%)	*p* value	Cluster 3 $N=43$ (26.1%)	Other clusters $N=122$ (73.9%)	*p* value
Benign	45 (27.3%)	27 (35.1%)	18 (20.4%)	0.0355	5 (6.4%)	40 (46%)	< 0.0001	1 (2.3%)	44 (36.1%)	< 0.0001
Cancer	120 (72.7%)	50 (64.9%)	70 (79.6%)		73 (93.6%)	47 (54%)		42 (97.7%)	78 (63.9%)	

Low and high *MAPRE1* and *MTUS1* levels were determined according to heatmap analysis of Exonhit probesets intensities. Tumors of Cluster 3 expressing high-*MAPRE1* and low-*MTUS1* were compared to tumors of all other clusters

Combinatorial expression of *MAPRE1* and *MTUS1* genes correlates with tumor grade

Analysis of *MAPRE1* gene levels in invasive breast carcinomas from 150 patients of the Curie Institute (Exploratory Cohort#2) revealed that *MAPRE1* expression is significantly increased in high-grade (GIII) tumors compared to those of histological grade I ($p=0.0003$) and grade II ($p=0.0242$) (Fig. 2a; Table 2). Tumors were classified into clusters expressing low or high levels of EB1 transcripts according to heat-map analysis of *MAPRE1* probesets intensities (Fig. 2b). Results indicate that the percentage of grade-III tumors is 3 times more elevated among high-*MAPRE1* (36.8%) compared to low-*MAPRE1* (10.7%) expressing tumors (Fig. 2c; Table 2). Conversely, grade-I tumors were significantly less abundant among high-*MAPRE1* (18.4%) compared to low-*MAPRE1* (49.1%) groups (Table 2), indicating that high transcript levels correlate with high histological grade.

Two independent series of 130 and 155 invasive carcinoma from breast cancer patients of the Curie Institute (Cohort#3) (Supplemental Table S3) and Gustave Roussy Hospital (Cohort #4) (Supplemental Table S4) were then analyzed. As shown in Supplemental Fig.S4a, *MAPRE1* gene levels were higher in cancer samples compared to 11 normal tissues. In both sets of tumors, *MAPRE1* levels were increased in grade-III versus grade-I/II invasive tumors (Supplemental Fig.S4a, d). Accordingly, the percentage of high-grade tumors was also elevated in high-*MAPRE1* compared to low-*MAPRE1* breast tumors from Cohorts #3 and #4 (Supplemental Fig.S4b, c, e, f and Supplemental Tables S6, S7), therefore confirming that high-*MAPRE1* correlates with tumor aggressiveness. Of note, *MAPRE1* levels were not significantly different among ER-positive and ER-negative breast tumors of Cohort#2 (Supplemental Fig.S4g) and Cohort#4 (Supplemental Fig. S4h), although in Cohort#3, high *MAPRE1* levels were significantly associated with ER-negative tumors (Suppl Fig.S4i and Supplemental Table S6). Of note, the percentage of ER-negative tumors included in Cohort #2 and #4 is very low (18,7% and 6,6%, respectively) compared to that in Cohort#3 (54,6%), which may explain why results do not reach significance in these two series of patients. Future studies including new cohorts with larger numbers of ER-negative breast cancer samples are warranted to address that question.

We then investigated whether *MAPRE2* and *MAPRE3* genes may also be regulated in invasive breast cancer. Probesets intensities for these two genes were examined in each cohort of invasive breast carcinomas when data were available, and results (Supplemental Fig.S5) revealed that *MAPRE2* and *MAPRE3* gene levels do not consistently correlate with tumor grade. Together, these studies indicate that

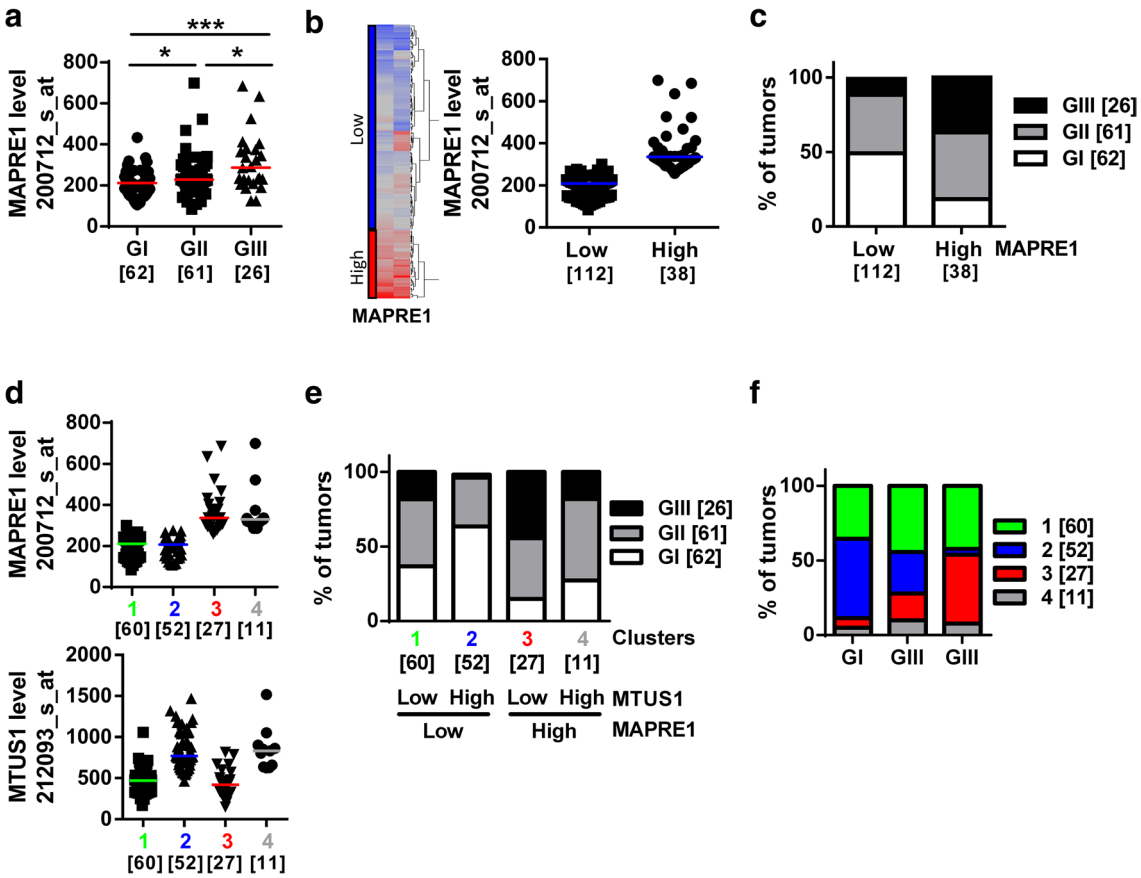


Fig. 2 Combinatorial expression of *MAPRE1* and *MTUS1* genes correlates with tumor grade. **a** Scattered dot plot of *MAPRE1* probeset (200712_s_at) intensity in tumors from patients of exploratory Cohort #2 according to tumor grade (GI, GII, GIII). Median value is indicated by the red line. $*p<0.05$, $***p<0.001$. **b** Heat-map and hierarchical clustering of 150 breast tumor samples based on the intensities of 2 *MAPRE1* probesets (200712_s_at, 200713_s_at). Dendogram of the 2 selected tumor groups and the corresponding scattered dot plot of *MAPRE1* expression are shown on the right. **c** Proportion of patients with grade-I (GI), grade-II (GII) or grade-III (GIII) tumors according to *MAPRE1* level. **d** Scattered dot plot of *MAPRE1* probeset (200712_s_at, upper panel) and *MTUS1* probeset (212093_s_at, lower panel) intensities in tumors classified in 4 clusters of either low or high level of *MAPRE1* and *MTUS1* transcripts. **e** Proportion of patients with grade-I (GI), grade-II (GII) or grade-III (GIII) tumors according to combinatorial expression of *MAPRE1* and *MTUS1* in the four clusters. **f** Proportion of patients in clusters 1–4 according to tumor grade (GI, GII, GIII). **a**–**f**- Number of samples is indicated under brackets

increased levels of *MAPRE1*, but not *MAPRE2* or *MAPRE3* genes, correlate with high tumor grade in breast cancer.

Previous studies have shown that *MTUS1* gene is down-regulated in breast cancer samples compared with normal tissue, and that low-*MTUS1* levels correlate with high tumor grade [17, 18]. We thus investigated the impact of combinatorial *MAPRE1* and *MTUS1* expression on histological grade. Based on heatmap classification of 150 tumors of Cohort#2 according to *MAPRE1* and *MTUS1* probesets intensities, four clusters of tumors expressing either low or high levels of each transcript were established (Fig. 2d). Comparison with histological grade revealed that cluster 3—expressing high-*MAPRE1* and low-*MTUS1* levels—is significantly associated with higher percentage of high-grade tumors (44.4% grade-III tumors in cluster 3 compared to 18.3%, 2%, and 18.2% in clusters 1, 2, and 4, respectively) (Fig. 2e; Table 2). Consistently, 46.1% of grade-III tumors were from cluster 3, compared to 18% and 6.4% of grade-II and grade-I tumors, respectively (Fig. 2f). Interestingly, among high-*MAPRE1* expressing tumors that are the most aggressive, those with low-*MTUS1* (cluster 3) were predominantly of high grade compared to those with high-*MTUS1* (cluster 4) (Fig. 2e), indicating that combined expression of *MAPRE1* and *MTUS1* genes better distinguishes tumor grade than *MAPRE1* alone. Similar results were obtained using two independent sets of tumors (Supplemental Tables S6 and S7, Supplemental Fig.S6). Of note, aggressive tumors from cluster 3 represent a substantial proportion of all breast tumors (18% in Cohort#2, 31.5% in Cohort#3 and 23.9% in Cohort#4) (Table 2 and Supplemental Tables S6 and S7).

Table 2 Characteristics of patients from Cohort #2, with high or low *MAPRE1* or *MAPRE1/MTUS1* combinatorial genes expression

Variables	MAPRE1				Combined MAPRE1/MTUS1		
	All *N*=150	Low *N*=112 (74.7%)	High *N*=38 (25.3%)	*p* value	Cluster 3 *N* =27 (18%)	Other clusters *N*=123 (82%)	*p* value
ER							
Pos	119 (79.3%)	93 (83%)	26 (68.4%)	0.153	19 (70.4%)	100 (81.3%)	0.419
Neg	28 (18.7%)	17 (15.2%)	11 (28.9%)		7 (25.9%)	21 (17.1%)	
Missing	3 (2%)	2 (1.8%)	1 (2.6%)		1 (3.7%)	2 (1.6%)	
PR							
Pos	82 (54.7%)	67 (59.8%)	15 (39.5%)	0.0712	10 (37%)	72 (58.5%)	0.121
Neg	63 (42%)	41 (36.6%)	22 (57.9%)		16 (59.3%)	47 (38.2%)	
Missing	5 (3.3%)	4 (3.6%)	1 (2.6%)		1 (3.7%)	4 (3.3%)	
HER2							
Pos	21 (14.1%)	14 (12.5%)	7 (18.9%)	0.12	5 (18.5%)	16 (13.1%)	0.161
Neg	105 (70.5%)	77 (68.7%)	28 (75.7%)		21 (77.8%)	84 (68.9%)	
Missing	23 (15.4%)	21 (18.8%)	2 (5.4%)		1 (3.7%)	22 (18%)	
Grade							
I	62 (41.3%)	55 (49.1%)	7 (18.4%)	0.0004	4 (14.8%)	58 (47.2%)	0.0002
II	61 (40.7%)	44 (39.3%)	17 (44.7%)		11 (40.7%)	50 (40.6%	
III	26 (17.3%)	12 (10.7%)	14 (36.8%)		12 (44.4%)	14 (11.4%)	
Missing	1 (0.67%)	1 (0.67%)				1 (0.8%)	
Recurrence							
No	91 (60.7%)	74 (66.1%)	17 (44.7%)	0.02	10 (37%)	81 (65.9%)	0.0055
Yes	59 (39.3%)	38 (33.9%)	21 (55.3%)		17 (63%)	42 (34.1%)	
Death							
No	77 (51.3%)	65 (58%)	12 (31.6%)	0.015	8 (29.6%)	69 (56.1%)	0.0097
Yes	56 (37.3%)	35 (31.3%)	21 (55.3%)		17 (62.9%)	39 (31.7%)	
Missing	17 (11.3%)	12 (10.7%)	5 (13.2%)		2 (7.4%)	15 (12.2%)	
5-years survival							
No	41 (27.3%)	25 (22.3%)	16 (42.1%	0.0228	13 (48.15%)	28 (22.8%)	0.0248
Yes	91 (60.7%)	75 (67%)	16 (42.1%)		11 (40.7%)	80 (65%)	
Missing	18 (12%)	12 (10.7%)	6 (15.8%)		3 (11.1%)	15 (12.2%)	

Low and high *MAPRE1* levels were determined according to heatmap analysis of Affymetrix probesets intensities. Tumors of Cluster 3 expressing high-*MAPRE1* and low-*MTUS1* were compared to tumors of all other clusters

Combinatorial expression of *MAPRE1* and *MTUS1* improves breast cancer prognosis

The prognostic value of *MAPRE1* in breast cancer was assessed by comparing probesets intensities with clinical data of patient survival. The probability of overall survival was significantly reduced in patients with breast tumors expressing high *MAPRE1* compared with low *MAPRE1* levels (HR 2.22; 95% CI 1.19–4.12; $p=0.0058$) (Fig. 3a; Table 3). The Kaplan–Meier plotter tool available online was also used to interrogate public databases [28]. High *MAPRE1* expression correlated with poorer overall survival in 1402 breast cancer patients (Supplemental Fig.S7a) and poorer relapse-free survival in 3115 patients (Supplemental Fig.S7b), further comforting our results that high-*MAPRE1* levels are associated with poor prognosis in breast cancer.

To further investigate whether combinatorial expression of *MAPRE1* and *MTUS1* may have improved prognostic value compared to each single gene, we evaluated the probability of survival among tumor clusters classified according to *MAPRE1* and *MTUS1* probeset intensities as defined previously (Fig. 2d). Results indicate that overall survival and relapse-free survival are significantly reduced ($p=0.0001$) for patients of cluster 3 - expressing high-*MAPRE1* and low-*MTUS1* levels—compared to all other clusters (Fig. 3b, c; Table 2 and Supplemental Fig.S7c, d, Table S6). Accordingly, the percentage of patients surviving after 5 years was lower for tumors of cluster 3 (42%) compared to those of cluster 1 (61%), cluster 2 (85%), and cluster 4 (60%) (Fig. 3d). Thus, combining *MAPRE1* and *MTUS1* gene levels improves breast cancer prognosis compared to *MAPRE1* or *MTUS1* alone. Multivariate analyses

Table 3 Univariate and multivariate analyses of the correlation between clinical parameters, *MAPRE1* and *MTUS1* levels, and survival time of patients from Cohort#2

	Univariate analysis			Multivariate analysis			Multivariate analysis*		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
ER− versus +	3.2	1.64–6.2	**0.0003**	1.89	0.81–4.36	0.068	1.9	0.83–4.3	0.062
PR− versus +	2.2	1.23–3.9	**0.0036**	1.5	0.59–3.78	0.195	1.5	0.59–3.78	0.195
HER2 + versus −	2.8	1.31–5.94	**0.0036**	1.73	0.67–4.45	0.128	1.67	0.61–4.55	0.158
Grade III versus I/II	4.1	1.95–8.6	**< 0.0001**	2,00	0.86–4.6	0.051	1.98	0.86–4.57	0.055
MAPRE1 high versus low	2.22	1.19–4.12	**0.0058**	1.4	0.40–4.89	0.299			
MTUS1 low versus high	3.07	1.7–5.5	**0.0001**	2.7	1.34–5.42	**0.0018**			
MAPRE1 MTUS1 combination cluster 3 versus 2	5.46	2.12–13.97	**0.0002**				3.5	1.12–10.85	**0.015**

A star indicates multivariate analysis including *MAPRE1*/*MTUS1* combination

HR hazard ratio, *CI* confidence interval, *p* *p*-value, *p*-values that reach significance are in bold

including ER, PR, HER2, tumor grade, *MAPRE1*, and *MTUS1* gene levels further revealed that *MAPRE1*/*MTUS1* gene combination (HR 3.50; CI 1.12–10.85; $p = 0.015$) is an independent indicator of overall survival whereas *MAPRE1* alone (HR 1.40; CI 0.40–4.89; $p = 0.299$) is not (Table 3). Notably, *MTUS1* (HR 2.7; CI 1.34–5.42; $p = 0.002$) is also identified as an independent prognostic factor, in line with previous studies [18].

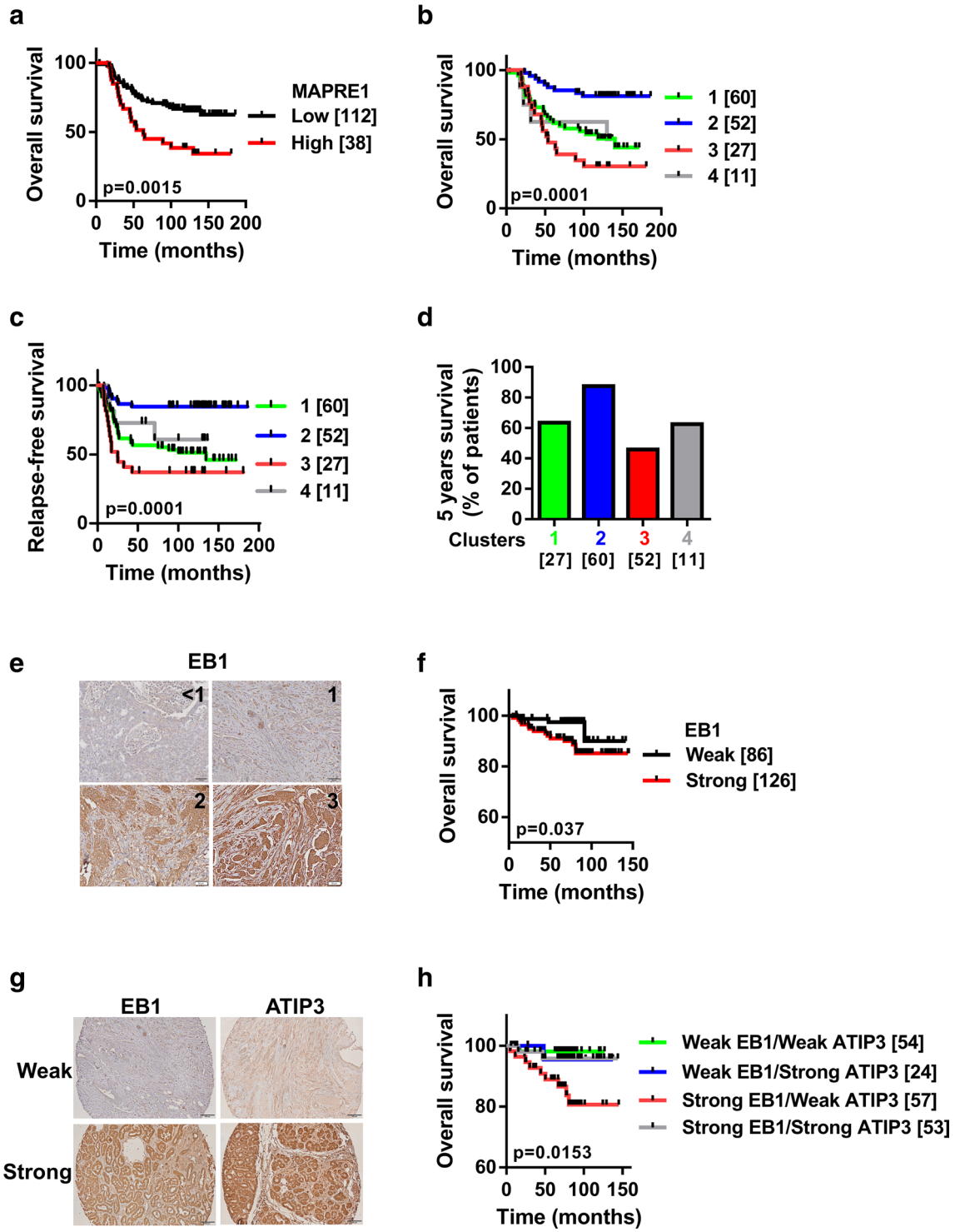
We then turned to IHC experiments to evaluate whether combined expression of EB1 and ATIP3 at the protein level may also improve breast cancer prognosis. Experimental conditions set up for EB1 staining (Supplemental Fig.S7e) were used to detect EB1 expression in patient biopsies. As expected, EB1 immunostaining was weak in normal breast and was markedly increased in the cytosol of tumor samples (Supplemental Fig.S7f). Tissue microarrays from 212 invasive breast tumors were then analyzed (Supplemental Table S5) and EB1 immunostaining (Fig. 3e) was compared with data for patients survival. Results show that the probability of overall survival was reduced in patients with tumors showing strong EB1 staining (HR 3.46; CI 1.065–7.284; $p = 0.037$) (Fig. 3f; Table 4), therefore validating the EB1 protein as a prognostic biomarker in breast cancer.

To further evaluate the impact of EB1-ATIP3 combination on patient survival, serial sections of the TMA were stained with anti-ATIP antibodies and samples were classified according to strong or weak EB1 and ATIP3 immunostaining (Fig. 3g and Supplemental Table S5). Results indicate that the probability of survival is significantly reduced in patients with tumors expressing strong-EB1 and weak-ATIP3 compared to all other groups ($p = 0.0153$) (Fig. 3h), which comforts the data obtained at the mRNA level (Fig. 3b). Consistent with previous results on DNA array analyses, this group of aggressive breast tumors represents 30.4% of all tumor samples (Table 4). Together, these data confirm DNA array results and demonstrate the improved prognostic value of combinatorial EB1/ATIP3 expression in breast cancer.

Fig. 3 Combinatorial expression of *MAPRE1* and *MTUS1* genes improves breast cancer prognosis. **a** Overall survival curves for patients from exploratory Cohort #2, with tumors expressing low or high *MAPRE1* levels, relative to the dendogram in Fig. 2b. **b** Overall survival curves for patients from exploratory Cohort #2, with tumors expressing low or high *MAPRE1* and *MTUS1* levels, according to clusters 1–4 defined in Fig. 2d. **c** Relapse-free survival curves for patients from exploratory Cohort #2, with tumors expressing low or high *MAPRE1* and *MTUS1* levels as in (**b**). **d** Proportion of patients remaining alive after 5 years with tumors expressing low or high *MAPRE1* and *MTUS1* levels as in (**b**). **e** Representative images of EB1 staining on breast tumor Tissue MicroArray (TMA) using anti-EB1 monoclonal antibody. Numbers on the right upper corner of each image indicate intensity of the EB1 staining from weak (< 1–1) to strong EB1 expression (2–3). A bar represents 50 µm. **f** Overall survival curves for patients from TMA with tumors expressing weak or strong EB1 levels, relative to IHC classification. **g** Representative images of IHC on breast tumor Tissue MicroArray (TMA) using anti-EB1 (left) or anti-ATIP3 (right) antibodies showing weak and strong immunostaining. A bar represents 100 µm. **h** Overall survival curves for patients from TMA, with tumors expressing weak or strong EB1 and ATIP3 levels according to IHC classification. **a**–**h**- Number of tumors in each group is indicated under brackets

Discussion

This study examined the diagnostic and prognostic value of *MAPRE* genes expression in breast cancer, either alone or in combination with candidate tumor suppressor *MTUS1* gene. We found that *MAPRE1*, but not *MAPRE2* nor *MAPRE3*, is up-regulated in malignant tumors compared to benign lesions, and in high-grade compared to low-grade invasive carcinoma. These results are consistent with previous studies [10] reporting EB1 as an oncogenic protein up-regulated in malignant and high-grade breast tumors. In contrast to this latter study however, our data did not clearly correlate EB1



levels with ER status in breast tumors and more studies are required to investigate this question. Results presented here further indicate that high expression of *MAPRE1* gene and encoded protein EB1 correlates with reduced survival of the patients, pointing out this molecule as a novel prognostic biomarker in breast cancer.

We further demonstrate here that combinatorial expression of *MAPRE1* and *MTUS1* genes significantly improves breast cancer diagnosis and prognosis compared to *MAPRE1* and *MTUS1* alone. Combining data on *MAPRE1* and *MTUS1* expression both at the gene and protein levels allowed to identify a group of aggressive

Table 4 Characteristics of patients included in Tissue Microarray analysis, with strong or weak expression of EB1 or EB1/ATIP combinations

Variables	EB1				Combined EB1/ATIP3			
	All *N*=212	Weak *N*=86 (40.6%)	Strong *N*=126 (59.4%)	*p* value	All *N*=194	Strong EB1/ Weak ATIP3 *N* =59 (30.4%)	Other groups *N*=135 (69.6%)	*p* value
ER								
Pos	154 (72.6%)	63 (73.3%)	91 (72.2%)	0.868	141 (72.7%)	46 (78%)	95 (70.4%)	0.247
Neg	58 (27.4%)	23 (26.7%)	35 (27.8%)		53 (27.3%)	13 (22%)	40 (29.6%)	
Grade								
I	38 (17.9%)	19 (22.1%)	19 (15.1%)	0.216	32 (16.5%)	11 (18.6%)	21 (15.6%)	0.847
II	76 (35.9%)	33 (38.4%)	43 (34.1%)		73 (37.6%)	21 (35.6%)	52 (38.5%)	
III	98 (46.2%)	34 (39.5%)	64 (50.8%)		89 (45.9%)	27 (45.8%)	62 (45.9%)	
Death								
No	187 (88.2%)	80 (93%)	107 (84.9%)	**0.043**	175 (90.2%)	48 (81.4%)	127 (94.1%)	**0.0067**
Yes	17 (8%)	3 (3,5%)	14 (11.1%)		13 (6.7%)	9 (15.3%)	4 (3%)	
Missing	8 (3.8%)	3 (3.5%)	5 (4%)		6 (3.1%)	2 (3.4%)	4 (3%)	

Weak and strong EB1 and ATIP3 levels were scored by IHC as indicated in the Methods. Tumors classified as strong-EB1 and weak-ATIP3 were compared to tumors of all other groups

high-grade tumors with high-EB1 and low-ATIP3 levels, that represent 20–30% of all breast tumors and are associated with reduced patient survival rates. While a number of studies have identified gene signatures associated with poor prognosis of breast cancer patients [29, 30], this is to our knowledge the first demonstration that combinatorial expression of two defined biomarkers with known associated molecular mechanisms may be used as a tool to select populations of cancer patients for personalized therapy. Further studies are warranted to evaluate the feasibility of using *MAPRE1* and *MTUS1* biomarkers as diagnostic and prognostic tools in the clinic.

On a molecular basis, these results are supported by our previous findings that ATIP3 acts as a brake on EB1 functions through binding and reducing EB1 turnover at MT growing ends [19]. In the absence of ATIP3, EB1 accumulates at MT ends and forms large comet-like structures that specify increased MT dynamics [18]. Results on breast cancer patients favor a model in which in breast tumors with low ATIP3 levels, the brake is turned off and the oncogenic activity of EB1 is increased. This opens the way to novel molecular therapeutic strategies involving delivery of active domains of ATIP3 to target the population of high-EB1/low-ATIP3 breast tumors that remain of poor prognosis. Alternative approaches may rely on the design or discovery of small molecule modulators of EB1 binding at MT ends to compensate for ATIP3 loss in the target population of breast tumors identified here. An integrative approach based on the discovery of small molecule scaffolds that target EB1 interactions with other partners at MT ends has indeed been recently described [31].

In conclusion, we show here for the first time that combinatorial expression of MT-associated EB1 and ATIP3 biomarkers improves breast cancer prognosis compared to each biomarker alone, and may be useful for better stratifying the patients for targeted molecular therapy. Further studies are warranted to analyze combinations of *MAPRE1* and *MTUS1* genes expression in other types of cancers such as glioblastoma [11], hepatocarcinoma [12], and oral [13] and colorectal [15] cancers, in which high-EB1 protein levels have been shown to correlate with poor clinical outcome.

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Compliance with ethical standards

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

Ethical approval The authors declare that all experiments presented here comply with the current laws of France.

Research involving human and animal rights This article does not contain any studies with animals performed by any of the authors. All procedures used in studies involving human participants have been previously published elsewhere and were in accordance with ethical standards of the Institut Curie and Gustave Roussy committees, and with 1964 Helsinki declaration.

Informed consent Informed consent was obtained from all individual participants included in this study.

References

1. Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312:237–242
2. Howard J, Hyman AA (2003) Dynamics and mechanics of the microtubule plus end. Nature 422:753–758
3. Akhmanova A, Steinmetz MO (2008) Tracking the ends: a dynamic protein network controls the fate of microtubule tips. Nat Rev Mol Cell Biol 9:309–322
4. Su L-K, Qi Y (2001) Characterization of human MAPRE genes and their proteins. Genomics 71:142–149
5. Nehlig A, Molina A, Rodrigues-Ferreira S, Honoré S, Nahmias C (2017) Regulation of end-binding protein EB1 in the control of microtubule dynamics. Cell Mol Life Sci 74(13):2381–2393
6. Vaughan KT (2005) TIP maker and TIP marker; EB1 as a master controller of microtubule plus ends. J Cell Biol 171(2):197–200
7. Maurer SP, Cade NI, Bohner G, Gustafsson N, Boutant E, Surrey T (2014) EB1 accelerates two confirmational transitions important for microtubule maturation and dynamics. Curr Biol 24(4):372–384
8. Galjart N (2010) Plus-end-tracking proteins and their interactions at microtubule ends. Curr Biol 20:R528–R537
9. Jiang K, Toedt G, Montenegro Gouveia S, Davey NE, Hua S, van der Vaart B et al (2012) A proteome- wide screen for Mammalian SxIP Motif-containing microtubule plus-end tracking proteins. Curr Biol 22:1800–1807
10. Kumar P, Wittmann T (2012) + TIPs: SxIPping along microtubule ends. Trends Cell Biol 22:418–428
11. Dong X, Liu F, Sun L, Liu M, Li D, Su D et al (2010) Oncogenic function of microtubule end-binding protein 1 in breast cancer. J Pathol 220(3):361–369
12. Berges R, Baeza-Kallee N, Tabouret E, Chinot O, Petit M, Kruczynski A et al (2014) End-binding 1 protein overexpression correlates with glioblastoma progression and sensitizes to Vinca-alkaloids in vitro and in vivo. Oncotarget 5(24):12769–12787
13. Orimo T, Ojima H, Hiraoka N, Saito S, Kosuge T, Kakisaka T et al (2008) Proteomic profiling reveals the prognostic value of adenomatous polyposis coli-end-binding protein 1 in hepatocellular carcinoma. Hepatology 48(6):1851–1863
14. Kumar M, Mehra S, Thakar A, Shukla NK, Roychoudhary A, Sharma MC et al (2016) End Binding 1 (EB1) overexpression in oral lesions and cancer: a biomarker of tumor progression and poor prognosis. Clin Chim Acta 459:45–52
15. Stypula-Cyrus Y, Mutyal NN, Dela Cruz M, Kunte DP, Radosevich AJ, Wali R et al (2009) End-binding protein 1 (EB1) upregulation is an early event in colorectal carcinogenesis. FEBS Lett 588(5):829–835
16. Sugihara Y, Taniguchi H, Kushima R, Tsuda H, Kubota D, Ichikawa H et al (2012) Proteomic-based identification of the APC-binding protein EB1 as a candidate of novel tissue biomarker and therapeutic target for colorectal cancer. J Proteom 75(17):5342–5355
17. Rodrigues-Ferreira S, Di Tommaso A, Dimitrov A, Cazaubon S, Gruel N, Colasson H et al (2009) 8p22 MTUS1 gene product ATIP3 is a novel anti-mitotic protein underexpressed in invasive breast carcinoma of poor prognosis. PLoS ONE 4(10):e7239
18. Molina A, Velot L, Ghouinem L, Abdelkarim M, Bouchet BP, Luissint AC et al (2013) ATIP3, a novel prognostic marker of breast cancer patient survival, limits cancer cell migration and slows metastatic progression by regulating microtubule dynamics. Cancer Res 73(9):2905–2915
19. Velot L, Molina A, Rodrigues-Ferreira S, Nehlig A, Bouchet BP, Morel M et al (2015) Negative regulation of EB1 turnover at microtubule plus ends by interaction with microtubule-associated protein ATIP3. Oncotarget 6(41):43557–43570
20. André F, Michiels S, Dessen P, Scott V, Suciu V, Uzan C et al (2009) Exonic expression profiling of breast cancer and benign lesions: a retrospective analysis. Lancet Oncol 10(4):381–390
21. Fehlbaum P, Guihal C, Bracco L, Cochet O (2005) A microarray configuration to quantify expression levels and relative abundance of splice variants. Nucleic Acids Res 33:e47
22. Reyal F, Stransky N, Bernard-Pierrot I, Vincent-Salomon A, de Rycke Y, Elvin P et al (2005) Visualizing chromosomes as transcriptome correlation maps: evidence of chromosomal domains containing co-expressed genes–a study of 130 invasive ductal breast carcinomas. Cancer Res 65:1376–1383
23. Hammond ME, Hayes DF, Wolff AC, Mangu PB, Temin S (2010) American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Oncol Pract 6(4):195–197
24. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS et al (2018) human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. J Clin Oncol 36(20):2105–2122
25. Maire V, Némati F, Richardson M, Vincent-Salomon A, Tesson B, Rigaill G et al (2013) Polo-like kinase 1: a potential therapeutic option in combination with conventional chemotherapy for the management of patients with triple-negative breast cancer. Cancer Res 73:816–823
26. Maire V, Baldeyron C, Richardson M, Tesson B, Vincent-Salomon A, Gravier E et al (2013) TTK/hMPS1 is an attractive therapeutic target for triple-negative breast cancer. PloS ONE 8:e63712
27. Lodillinsky C, Infante E, Guichard A, Chaligné R, Fuhrmann L, Cyrta J et al (2016) p63/MT1-MMP axis is required for in situ to invasive transition in basal-like breast cancer. Oncogene 35(3):344–357
28. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q et al (2010) An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients. Breast Cancer Res Treat 123:725–731
29. Alexe G, Alexe S, Axelrod DE, Bonates TO, Lozina II, Reiss M et al (2006) Breast cancer prognosis by combinatorial analysis od gene expression data. Breast Cancer Res 8(4):R41
30. Espinosa E, Vara J, Navarro IS, Gámez-Pozo A, Pinto A, Zamora P et al (2011) Gene profiling in breast cancer: time to move forward. Cancer Treat Rev 37(6):416–421
31. Almeida TB, Carnell AJ, Barsukov IL, Berry NG (2017) Targeting SxIP-EB1 interaction: an integrated approach to the discovery of small molecule modulators of dynamic binding sites. Sci Rep 7(1):15533