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

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1 **Combinatorial expression of microtubule-associated EB1 and ATIP3 biomarkers**
2 **improves breast cancer prognosis**
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46 **Keywords**

47 **MAPRE1, MTUS1, diagnosis, prognosis, breast cancer, biomarkers combination**
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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.

Introduction

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

1 instability [7]. In addition, EB1 recruits a large number of regulatory proteins at MT plus ends,
2 thereby orchestrating the regulation of MT dynamics and MT targeting to organelles [8-10].
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4 The pivotal role of EB1 on MT dynamics suggests that alterations of EB1 expression or function
5 may have important consequences in cancer. Indeed, up-regulation of EB1 protein in tumor
6 samples has been reported in breast cancer [11], glioblastoma [12], hepatocarcinoma [13], oral
7 [14] and colorectal cancer [15,16]. In breast cancer, high EB1 protein levels have been shown
8 to correlate with tumor malignancy and high tumor grade [11]. However, the status of *MAPRE1*
9 gene encoding EB1, and its paralogs *MAPRE2* and *MAPRE3*, has not yet been evaluated in
10 breast cancer, and it remains to be established whether EB1 represents a prognostic biomarker
11 of breast cancer patient survival.
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24 Recent studies have identified several structural MAPs as endogenous antagonists of EB1
25 functions at MT plus ends [5]. Among them, the ATIP3 protein encoded by candidate tumor
26 suppressor gene *MTUS1* has been described as a prognostic biomarker down-regulated in
27 invasive breast cancer [17, 18]. ATIP3 is a potent MT-stabilizer that markedly reduces breast
28 tumor growth and distant metastasis [18]. ATIP3 directly binds to EB1 in the cytosol and
29 prevents its turnover and accumulation at growing MT ends, thereby reducing MT dynamics
30 and cell polarity [19]. These data suggest that altered expression of either ATIP3 or EB1 in
31 breast tumors may impact the levels of intracellular ATIP3-EB1 molecular complexes that
32 govern EB1 function at MT plus ends. This raises the interesting possibility that deleterious
33 effects of high EB1 levels in breast tumors may be moderated by high levels of ATIP3 whereas
34 tumors with high-EB1 and low-ATIP3 levels may remain more aggressive.
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51 The present study investigates the value of *MAPRE* genes, alone or in combination with
52 *MTUS1*, for molecular classification, diagnosis and prognosis of breast cancer patients. We
53 show that high *MAPRE1* – but not *MAPRE2* and *MAPRE3* - levels correlate with tumor
54 malignancy and poor clinical outcome for breast cancer patients, and that combinatorial
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analysis of *MAPRE1* and *MTUS1* expression refines breast cancer diagnosis and prognosis
compared to *MAPRE1* alone.

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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

1 characteristics and clinical data are presented in Supplemental Table S5. TMA consisted of
2 replicate 1-mm diameter tumor cores selected from whole-tumor tissue section in two most
3 representative tumor areas (high tumor cell density) of each tumor sample. Alcohol formalin
4 acetic acid-fixed paraffin-embedded samples were analyzed by immunohistochemistry (IHC)
5 staining using EnVision FLEX kit (Dako) according to the manufacturer's instructions. For
6 EB1 staining, heat-mediated antigen retrieval was performed in Ethylene-Diamine-Tetra-
7 Acetic acid (EDTA) buffer pH 6 in water bath for 30 min and monoclonal mouse anti-EB1
8 antibodies (BD Biosciences) diluted 1:300 were incubated overnight at 4°C. ATIP3 staining
9 was performed as previously described [17] using monoclonal anti-MTUS1 antibodies
10 (Abnova) diluted 1 :100. Slides were counterstained with hematoxylin. HeLa cells transfected
11 with control siRNA were used as positive control for EB1 staining, and cells transfected for
12 48h with EB1-specific siRNA (Dharmacon) [19] were considered as negative control. Silencing
13 efficiency was validated by immunoblotting using anti-EB1 antibodies (BD Biosciences)
14 diluted 1 :1000 and anti-alpha-tubulin antibodies (Sigma) diluted 1 :1000 as internal control.
15 EB1 immunoreactivity in tissue sections was classified semi-quantitatively into 5 classes (0,
16 <1, 1, 2, 3) according to the intensity of cytoplasmic staining of tumor cells. Scores for EB1
17 immunostaining were defined as 0 (undetectable staining on high-power field (x40), <1 (only
18 visible on high-power field (x40), 1 (detected on medium-power field (x10-x20) but well
19 visualized on high-power field (x40), 2 (detected on low-power field (x4-x5) but well visualized
20 on medium-power field (x20), and 3 (well visualized on low-power field (x4-x5). Samples were
21 independently evaluated by two pathologists of different institutes. EB1 expression was
22 classified on a semi-quantitative basis, immunostaining was considered weak for a score from
23 0 to 1 and strong for a score of 2 to 3. Scoring for ATIP3 staining was evaluated as described
24 [17].

Statistical analysis

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2 Statistical analyses were done using JMP-7 and GraphPad Prism 6.0 softwares. The association
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4 between clinicopathological characteristics and the expression level of either *MAPRE1*,
5
6 *MTUS1*, or their combination were calculated using the Mann-Whitney test for continuous
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8 variables and using the chi-squared and the Fisher exact tests for dichotomized variables. Dot
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10 plot analyses were done using Mann–Whitney test. Overall survival (OS) and Relapse-free
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12 survival (RFS) curves were plotted according to the method of Kaplan–Meier and compared by
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14 the log-rank test. $P < 0.05$ was considered statistically significant.
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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 high-*MAPRE1* and low-*MTUS1* levels included a majority (97.7%) of

1 malignant samples, compared to 45.2% among those expressing low-*MAPRE1* and high-
2 *MTUS1* (Fig.1g). Of interest, combining *MAPRE1* and *MTUS1* gene expression allowed better
3 tumor classification compared to each biomarker alone (Fig.1g, Table 1). Together these studies
4 point to *MAPRE1* and *MTUS1* as valuable diagnostic biomarkers and suggest that analyzing
5 combinatorial expression of these genes may refine tumor classification and improve breast
6 cancer diagnosis.
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17 **Combinatorial expression of *MAPRE1* and *MTUS1* genes correlates with tumor grade**

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20 Analysis of *MAPRE1* gene levels in invasive breast carcinomas from 150 patients of the Curie
21 Institute (Exploratory Cohort#2) revealed that *MAPRE1* expression is significantly increased
22 in high grade (GIII) tumors compared to those of histological grade I ($p=0.0003$) and grade II
23 ($p=0.0242$) (Fig.2a, Table 2). Tumors were classified into clusters expressing low or high levels
24 of EB1 transcripts according to heat-map analysis of *MAPRE1* probesets intensities (Fig.2b).
25 Results indicate that the percentage of grade-III tumors is 3 times more elevated among high-
26 *MAPRE1* (36,8%) compared to low-*MAPRE1* (10,7%) expressing tumors (Fig.2c, Table 2).
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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, S4d). 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

1 Fig.S4b, S4c, S4e, S4f and Supplemental Table S6, S7), therefore confirming that high-
2 *MAPRE1* correlates with tumor aggressiveness. Of note, *MAPRE1* levels were not significantly
3 different among ER-positive and ER-negative breast tumors of Cohort#2 (Supplemental
4 Fig.S4g) and Cohort#4 (Supplemental Fig.S4h), although in Cohort#3, high *MAPRE1* levels
5 were significantly associated with ER-negative tumors (Suppl Fig.S4i and Supplemental Table
6 S6). Of note, the percentage of ER-negative tumors included in Cohort #2 and #4 is very low
7 (18,7% and 6,6%, respectively) compared to that in Cohort#3 (54,6%), which may explain why
8 results do not reach significance in these two series of patients. Future studies including new
9 cohorts with larger numbers of ER-negative breast cancer samples are warranted to address that
10 question.

11 We then investigated whether *MAPRE2* and *MAPRE3* genes may also be regulated in invasive
12 breast cancer. Probesets intensities for these two genes were examined in each cohort of
13 invasive breast carcinomas when data were available, and results (Supplemental Fig.S5)
14 revealed that *MAPRE2* and *MAPRE3* gene levels do not consistently correlate with tumor grade.
15 Together, these studies indicate that increased levels of *MAPRE1*, but not *MAPRE2* or *MAPRE3*
16 genes, correlate with high tumor grade in breast cancer.

17 Previous studies have shown that *MTUS1* gene is down-regulated in breast cancer samples
18 compared with normal tissue, and that low-*MTUS1* levels correlate with high tumor grade [17,
19 18]. We thus investigated the impact of combinatorial *MAPRE1* and *MTUS1* expression on
20 histological grade. Based on heatmap classification of 150 tumors of Cohort#2 according to
21 *MAPRE1* and *MTUS1* probesets intensities, four clusters of tumors expressing either low or
22 high levels of each transcript were established (Fig.2d). Comparison with histological grade
23 revealed that cluster 3 - expressing high-*MAPRE1* and low-*MTUS1* levels - is significantly
24 associated with higher percentage of high-grade tumors (44.4% grade-III tumors in cluster 3
25 compared to 18.3%, 2% and 18.2% in clusters 1, 2 and 4, respectively) (Fig.2e, Table 2).

1 Consistently, 46.1% of grade-III tumors were from cluster 3, compared to 18% and 6.4% of
2 grade-II and grade-I tumors, respectively (Fig.2f). Interestingly, among high-*MAPRE1*
3 expressing tumors that are the most aggressive, those with low-*MTUS1* (cluster 3) were
4 predominantly of high grade compared to those with high-*MTUS1* (cluster 4) (Fig.2e),
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6 indicating that combined expression of *MAPRE1* and *MTUS1* genes better distinguishes tumor
7 grade than *MAPRE1* alone. Similar results were obtained using two independent sets of tumors
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9 (Supplemental Tables S6 and S7, Supplemental Fig.S6). Of note, aggressive tumors from
10 cluster 3 represent a substantial proportion of all breast tumors (18% in Cohort#2, 31.5% in
11 Cohort#3 and 23.9% in Cohort#4) (Table 2 and Supplemental Tables S6 and S7).
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24 **Combinatorial expression of *MAPRE1* and *MTUS1* improves breast cancer prognosis**

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26 The prognostic value of *MAPRE1* in breast cancer was assessed by comparing probesets
27 intensities with clinical data of patient survival. The probability of overall survival was
28 significantly reduced in patients with breast tumors expressing high *MAPRE1* compared with
29 low *MAPRE1* levels (HR 2.22; 95% CI 1.19-4.12; p=0.0058) (Fig.3a and Table 3). The Kaplan-
30 Meier plotter tool available online was also used to interrogate public databases [28]. High
31 *MAPRE1* expression correlated with poorer overall survival in 1402 breast cancer patients
32 (Supplemental Fig.S7a) and poorer relapse-free survival in 3115 patients (Supplemental
33 Fig.S7b), further comforting our results that high-*MAPRE1* levels are associated with poor
34 prognosis in breast cancer.
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50 To further investigate whether combinatorial expression of *MAPRE1* and *MTUS1* may have
51 improved prognostic value compared to each single gene, we evaluated the probability of
52 survival among tumor clusters classified according to *MAPRE1* and *MTUS1* probeset intensities
53 as defined previously (Fig.2d). Results indicate that overall survival and relapse-free survival
54 are significantly reduced (p=0.0001) for patients of cluster 3 - expressing high-*MAPRE1* and
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2 low-MTUS1 levels - compared to all other clusters (Fig.3b, 3c, Table 2 and Supplemental
3 Fig.S7c-d, Table S6). Accordingly, the percentage of patients surviving after 5 years was lower
4 for tumors of cluster 3 (42%) compared to those of cluster 1 (61%), cluster 2 (85%) and cluster
5 4 (60%) (Fig. 3d). Thus, combining MAPRE1 and MTUS1 gene levels improves breast cancer
6 prognosis compared to MAPRE1 or MTUS1 alone. Multivariate analyses including ER, PR,
7 HER2, tumor grade, MAPRE1 and MTUS1 gene levels further revealed that MAPRE1/MTUS1
8 gene combination (HR 3.50; CI 1.12-10.85; p 0.015) is an independent indicator of overall
9 survival whereas MAPRE1 alone (HR 1.40; CI 0.40-4.89; p 0.299) is not (Table 3). Notably,
10 MTUS1 (HR 2.7; CI 1.34-5.42; p 0.002) is also identified as an independent prognostic factor,
11 in line with previous studies [18].

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

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24 To further evaluate the impact of EB1-ATIP3 combination on patient survival, serial sections
25 of the TMA were stained with anti-ATIP antibodies and samples were classified according to
26 strong or weak EB1 and ATIP3 immunostaining (Fig.3g and Supplemental Table S5). Results
27 indicate that the probability of survival is significantly reduced in patients with tumors
28 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.

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Discussion

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2 This study examined the diagnostic and prognostic value of *MAPRE* genes expression in breast
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4 cancer, either alone or in combination with candidate tumor suppressor *MTUS1* gene. We found
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6 that *MAPRE1*, but not *MAPRE2* nor *MAPRE3*, is up-regulated in malignant tumors compared
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8 to benign lesions, and in high-grade compared to low-grade invasive carcinoma. These results
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10 are consistent with previous studies [10] reporting EB1 as an oncogenic protein up-regulated in
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12 malignant and high-grade breast tumors. In contrast to this latter study however, our data did
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14 not clearly correlate EB1 levels with ER status in breast tumors and more studies are required
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16 to investigate this question. Results presented here further indicate that high expression of
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18 *MAPRE1* gene and encoded protein EB1 correlates with reduced survival of the patients,
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20 pointing out this molecule as a novel prognostic biomarker in breast cancer.
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26 We further demonstrate here that combinatorial expression of *MAPRE1* and *MTUS1* genes
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28 significantly improves breast cancer diagnosis and prognosis compared to *MAPRE1* and
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30 *MTUS1* alone. Combining data on *MAPRE1* and *MTUS1* expression both at the gene and
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32 protein levels allowed to identify a group of aggressive high-grade tumors with high-EB1 and
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34 low-ATIP3 levels, that represent 20% to 30% of all breast tumors and are associated with
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36 reduced patient survival rates. While a number of studies have identified gene signatures
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38 associated with poor prognosis of breast cancer patients [29, 30], this is to our knowledge the
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40 first demonstration that combinatorial expression of two defined biomarkers with known
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42 associated molecular mechanisms may be used as a tool to select populations of cancer patients
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44 for personalized therapy. Further studies are warranted to evaluate the feasibility of using
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46 *MAPRE1* and *MTUS1* biomarkers as diagnostic and prognostic tools in the clinic.
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53 On a molecular basis, these results are supported by our previous findings that ATIP3 acts as a
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55 brake on EB1 functions through binding and reducing EB1 turnover at MT growing ends [19].
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58 In the absence of ATIP3, EB1 accumulates at MT ends and forms large comet-like structures
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1 that specify increased MT dynamics [18]. Results on breast cancer patients favor a model in
2 which in breast tumors with low ATIP3 levels, the brake is turned off and the oncogenic activity
3 of EB1 is increased. This opens the way to novel molecular therapeutic strategies involving
4 delivery of active domains of ATIP3 to target the population of high-EB1/low-ATIP3 breast
5 tumors that remain of poor prognosis. Alternative approaches may rely on the design or
6 discovery of small molecule modulators of EB1 binding at MT ends to compensate for ATIP3
7 loss in the target population of breast tumors identified here. An integrative approach based on
8 the discovery of small molecule scaffolds that target EB1 interactions with other partners at MT
9 ends has indeed been recently described [31].
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21 In conclusion, we show here for the first time that combinatorial expression of MT-associated
22 EB1 and ATIP3 biomarkers improves breast cancer prognosis compared to each biomarker
23 alone, and may be useful for better stratifying the patients for targeted molecular therapy.
24 Further studies are warranted to analyze combinations of *MAPRE1* and *MTUS1* genes
25 expression in other types of cancers such as glioblastoma [11], hepatocarcinoma [12], and oral
26 [13] and colorectal [15] cancers, in which high-EB1 protein levels have been shown to correlate
27 with poor clinical outcome.
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1 **Compliance with ethical standards**

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5
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7
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17
18 LERMIT, the association GEFLUC and Prolific.
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25 **Compliance with current laws**

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27 The authors declare that all experiments presented here comply with the current laws of
28
29 France
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35 **Conflict of interests :**

36
37 The authors declare that they have no conflict of interests.
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42 **Ethical approval :**

43
44 This article does not contain any studies with animals performed by any of the authors.
45
46 All procedures used in studies involving human participants have been previously published
47
48 elsewhere and were in accordance with ethical standards of the Institut Curie and Gustave
49
50 Roussy committees, and with 1964 Helsinki declaration.
51
52 Informed consent was obtained from all individual participants included in this study.
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Legends to Figures

Figure 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. Dendrogram 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). Dendrogram 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.

Figure 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). Dendrogram 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 to 4 according to tumor grade (GI, GII, GIII).
- a-f- Number of samples is indicated under brackets.

Figure 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 dendrogram in Fig2b.
 - b- Overall survival curves for patients from exploratory Cohort #2, with tumors expressing low or high *MAPRE1* and *MTUS1* levels, according to clusters 1 to 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.

Legends to Tables

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

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.

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

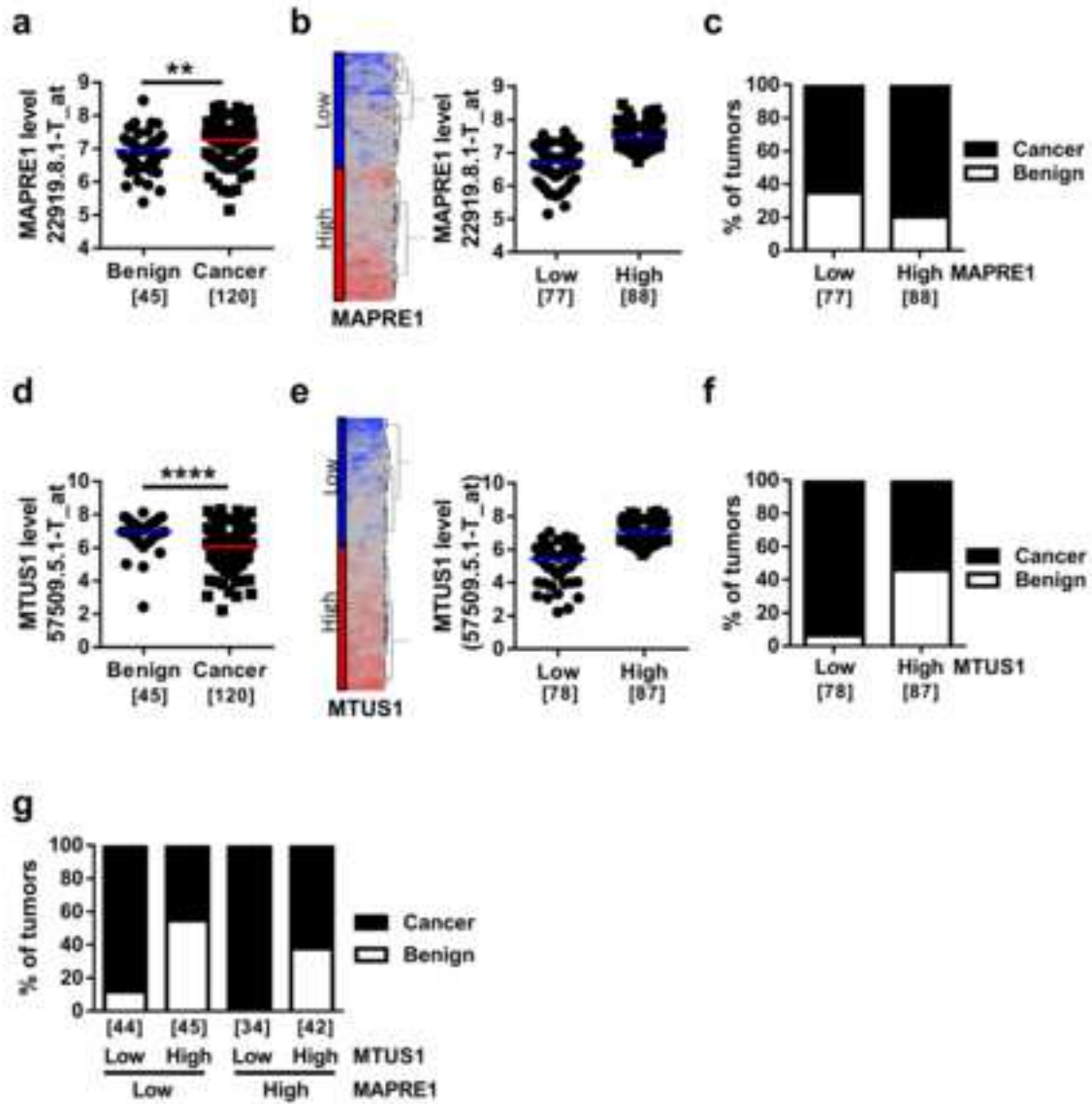
Low and high *MAPRE1* levels were determined according to heatmap analysis of Affymetrix probesets intensities. Tumors of Cluster 3 expressing high-*MAPRE1* and low-*MTUS1* were compared to tumors of all other clusters.

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

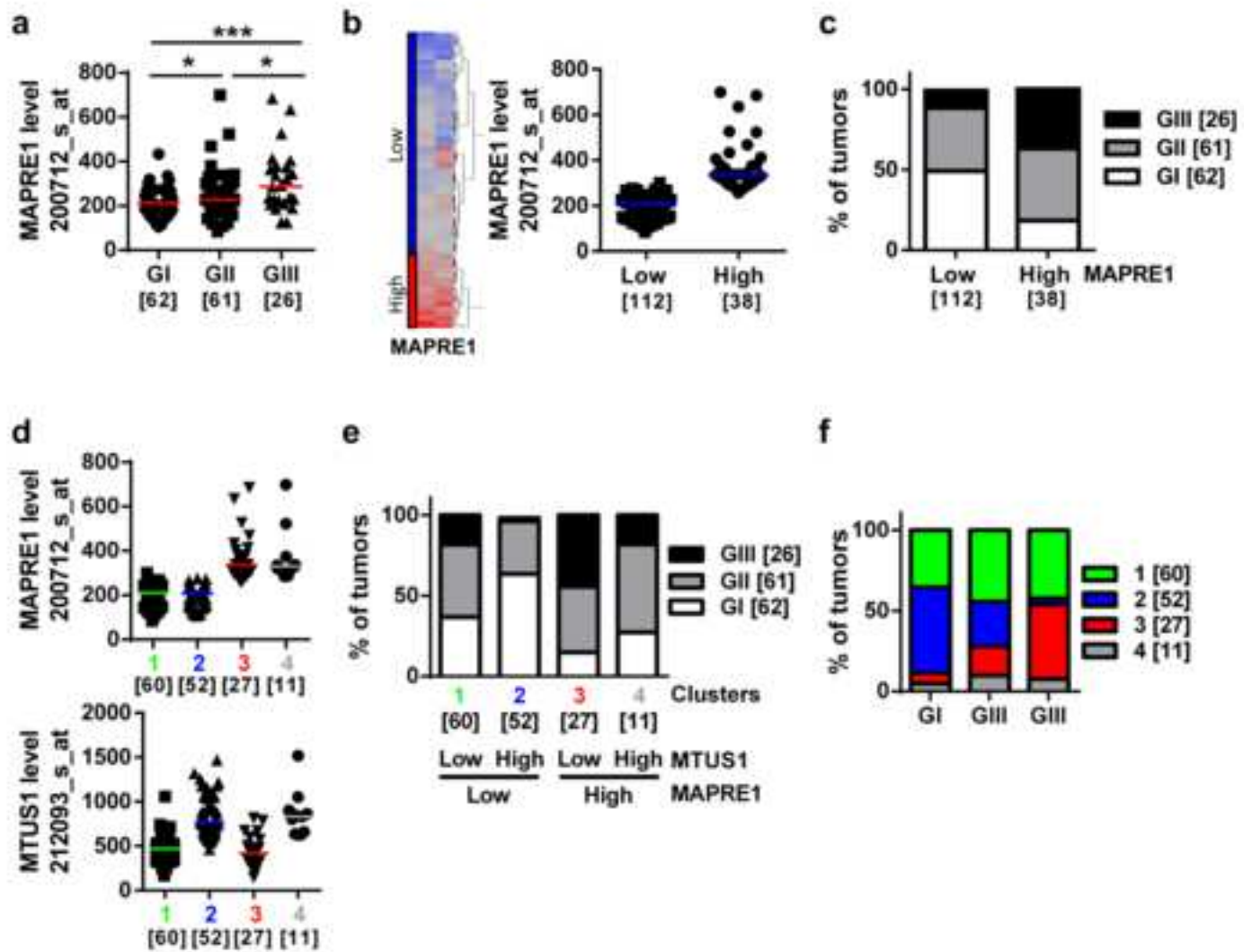
A star indicates multivariate analysis including *MAPRE1/MTUS1* combination. HR: Hazard Ratio; CI: Confidence Interval; p: p-value; p-values that reach significance are in bold.

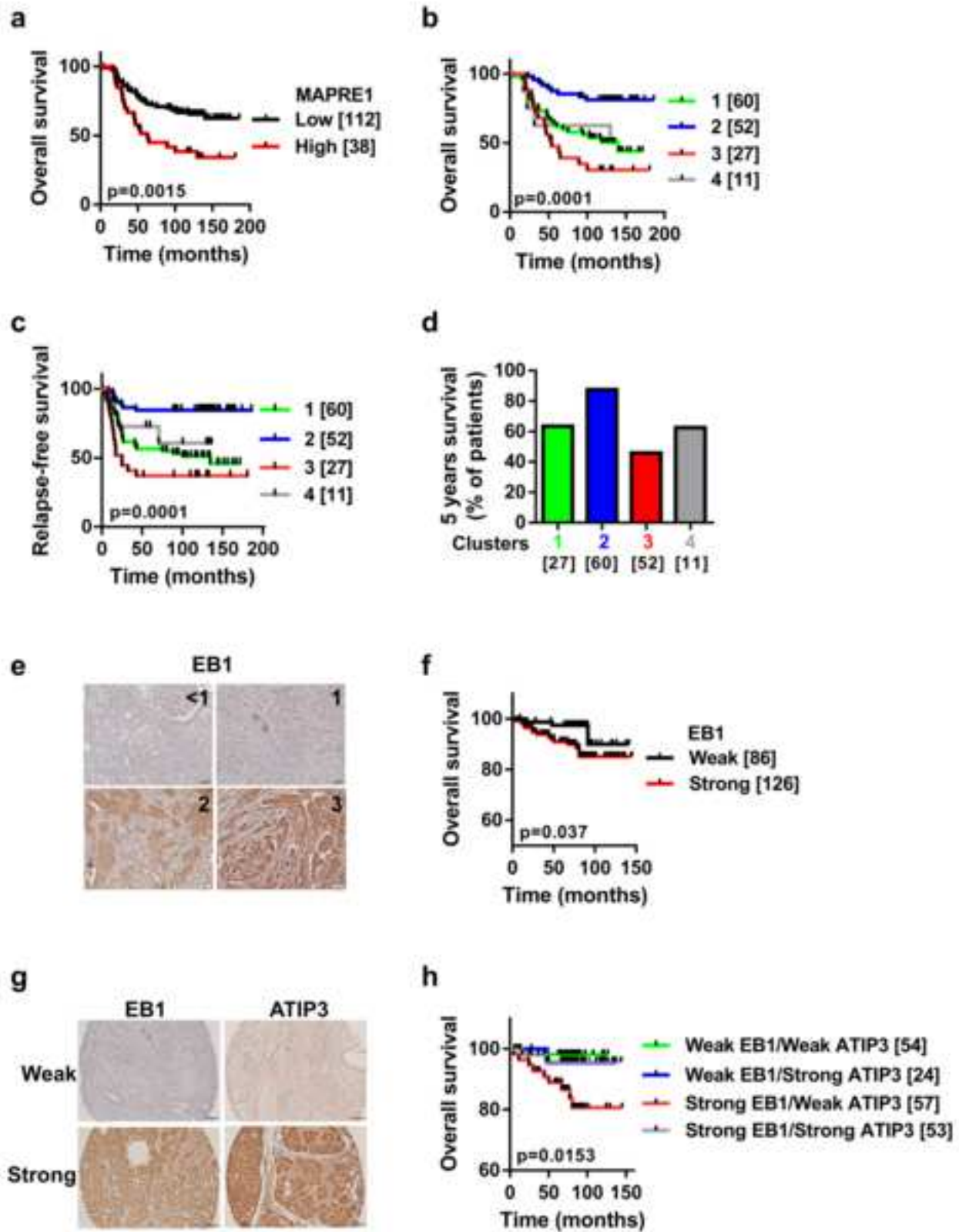
Table 4. Characteristics of patients included in Tissue Microarray analysis, with strong or weak expression of EB1 or EB1/ATIP combinations.

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.



Rodrigues-Ferreira et al. Figure 1





Variables	All N=165	MAPRE1		p value	MTUS1		p value	Combined MAPRE1/MTUS1		p value
		Low N=77 (46,7%)	High N=88 (53,3%)		Low N=78 (47,3%)	High N=87 (52,7%)		cluster 3 N =43 (26,1%)	other clusters N=122 (73,9%)	
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%)	

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

Low and high *MAPRE1* and *MTUS1* levels were determined according to heatmap analysis of probesets intensities.

Tumors of Cluster 3 expressing high-*MAPRE1* and low-*MTUS1* were compared to tumors of all other clusters

Variables		All N=150	MAPRE1		p value	Combined MAPRE1/MTUS1		p value
			Low N=112 (74,7%)	High N=38 (25,3%)		cluster 3 N =27 (18%)	other clusters N=123 (82%)	
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%)	

Table 2. Characteristics of patients from Cohort#2 with high or low MAPRE1 or combined MAPRE1/MTUS1 genes expression

Low and high MAPRE1 and MTUS1 levels were determined according to heatmap analysis of probesets intensities.

Tumors of Cluster 3 expressing high-MAPRE1 and low-MTUS1 were compared to tumors of all other clusters

	Univariate Analysis			Multivariate Analysis			Multivariate Analysis*		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
ER - vs +	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 - vs +	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 + vs -	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 vs 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 vs low	2.22	1.19-4.12	0.0058	1.4	0.40-4.89	0.299			
MTUS1 low vs high	3.07	1.7-5.5	0.0001	2.7	1.34-5.42	0.0018			
MAPRE1 MTUS1 combination cluster 3 vs 2	5.46	2.12-13.97	0.0002				3.5	1.12-10.85	0.015

Table 3. Univariate and multivariate analysis of the correlation between clinicopathological parameters, MAPRE1 and MTUS1 level, and survival time of patients from Cohort #2

* multivariate analysis including MAPRE1/MTUS1 combination

HR: Hazard Ratio, CI: Confidence Interval, p: p-value

p-values that reach significance are in bold

Variables		EB1			p value	Combined EB1/ATIP3			p value
		All N=212	Weak N=86 (40,6%)	Strong N=126 (59,4%)		All N=194	Strong EB1/Weak ATIP3 N=59 (30,4%)	other groups N=135 (69,6%)	
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%)	

Table 4. Characteristics of patients from TMA, with weak or strong immunostaining of EB1 or combined EB1/ATIP3

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.



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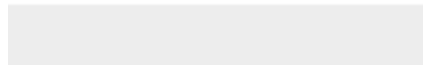
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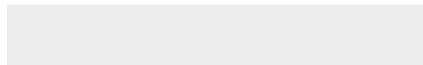
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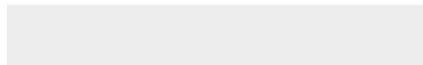
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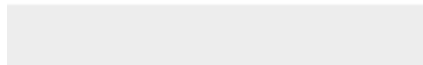
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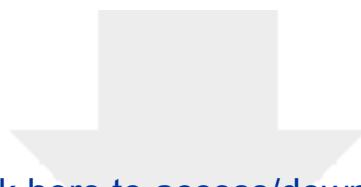




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