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Increased MAPK1/3 Phosphorylation in Luminal Breast Cancer Related with *PIK3CA* Hotspot Mutations and Prognosis

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Abstract

INTRODUCTION: While mutations in *PIK3CA* are most frequently (45%) detected in luminal breast cancer, downstream PI3K/AKT/mTOR pathway activation is predominantly observed in the basal subtype. The aim was to identify proteins activated in *PIK3CA* mutated luminal breast cancer and the clinical relevance of such a protein in breast cancer patients. *MATERIALS AND METHODS:* Expression levels of 171 signaling pathway (phospho-)proteins established by The Cancer Genome Atlas (TCGA) using reverse phase protein arrays (RPPA) were in silico examined in 361 breast cancers for their relation with PIK3CA status. MAPK1/3 phosphorylation was evaluated with immunohistochemistry on tissue microarrays (TMA) containing 721 primary breast cancer core biopsies to explore the relationship with metastasis-free survival. *RESULTS:* In silico analyses revealed increased phosphorylation of MAPK1/3, p38 and YAP, and decreased expression of p70S6K and 4E–BP1 in *PIK3CA* mutated compared to wild-type luminal breast cancer. Augmented MAPK1/3 phosphorylation was most significant, i.e. in luminal A for both *PIK3CA* exon 9 and 20 mutations and in luminal B for exon 9 mutations. In 290 adjuvant systemic therapy naïve lymph node negative (LNN) breast cancer patients with luminal cancer, high MAPK phosphorylation in nuclei (HR = 0.49; 95% CI, 0.25–0.95; $P = .036$) and in tumor cells (HR = 0.37; 95% CI, 0.18–0.79; $P = .010$) was related with favorable metastasis-free survival in multivariate analyses including traditional prognostic factors. *CONCLUSION:* Enhanced MAPK1/3 phosphorylation in luminal breast cancer is related to *PIK3CA* exon-specific mutations and correlated with favorable prognosis especially when located in the nuclei of tumor cells.

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Introduction

The Cancer Genome Atlas (TCGA) data on breast cancer have shown that the *PIK3CA* mutation frequency could rise to 45% in luminal A tumors compared to only 9% in basal tumors [1]. Remarkably, the reverse protein phase array (RPPA) data of this study demonstrated that phosphorylation of AKT, S6 and 4 EB-P1, typical markers of PI3K pathway activation (PI3K-AKT signaling pathway in KEGG: http://www.genome.jp/kegg-bin/show_pathway?hsa04151) were highly expressed in basal-like and HER2 molecular subtypes and correlated strongly with INPP4B and PTEN loss, and with *PIK3CA* amplification. Moreover, protein and mRNA signatures of PI3K pathway activation were enriched in basal-like over luminal A breast cancers [1].

Mutations in *PIK3CA* occur predominantly in two hotspot regions, i.e. in the helical and kinase domains encoded by exon 9 and 20, respectively. COSMIC, the catalogue of somatic mutations in cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) reported so far 2745 missense mutations for *PIK3CA* in breast carcinomas, which were located in exon 9 and more frequently in exon 20.

It has been reported that the *PIK3CA* mutation status between primary and corresponding metastatic disease can be discordant [2]. Interestingly, single cell *PIK3CA* mutational analyses revealed heterogeneity in circulating tumor cells and metastases compared to primary breast

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tumors [3]. Additionally, different treatment responses have been observed for the distinct molecular subtypes in relation to *PIK3CA* mutation status. For example, prolonged survival was observed in ER-positive early disease patients treated with adjuvant tamoxifen and in advanced disease patients treated with first-line aromatase inhibitors for those with *PIK3CA* mutant tumors [4,5]. In contrast, *PIK3CA* mutations in HER2+ patients were linked to trastuzumab therapy resistance [6–8]. This resistance might be due to co-occurrence of both oncogenes or to tumor cell heterogeneity with underrepresented trastuzumab responsive HER2+ clones.

In order to understand the apparent disconnection in breast cancer between genetic activation through *PIK3CA* oncogenic driver mutations and its downstream PI3K/AKT/mTOR signaling cascade, we evaluated in especially luminal tumors pathway proteins for their relation with *PIK3CA* mutations. Therefore, TCGA in silico RPPA-database of 171 cancer-related (phospho) proteins from 361 breast cancer specimens were stratified by type of *PIK3CA* mutation and molecular subtypes. Expression or phosphorylation of seven proteins correlated significantly with *PIK3CA* status in luminal breast cancer. Phosphorylated MAPK1/3, as most significant up-regulated protein, was further explored on TMAs containing 721 primary breast cancer specimens, including a subset of luminal tumors from 290 systemic untreated LNN patients, to address the prognostic value of this phosphorylated kinase (Figure 1).

Materials and Methods

Databases and Software Packages

We have used the quantified expression of 171 cancer-related proteins and phosphoproteins by RPPA of 403 primary breast cancer

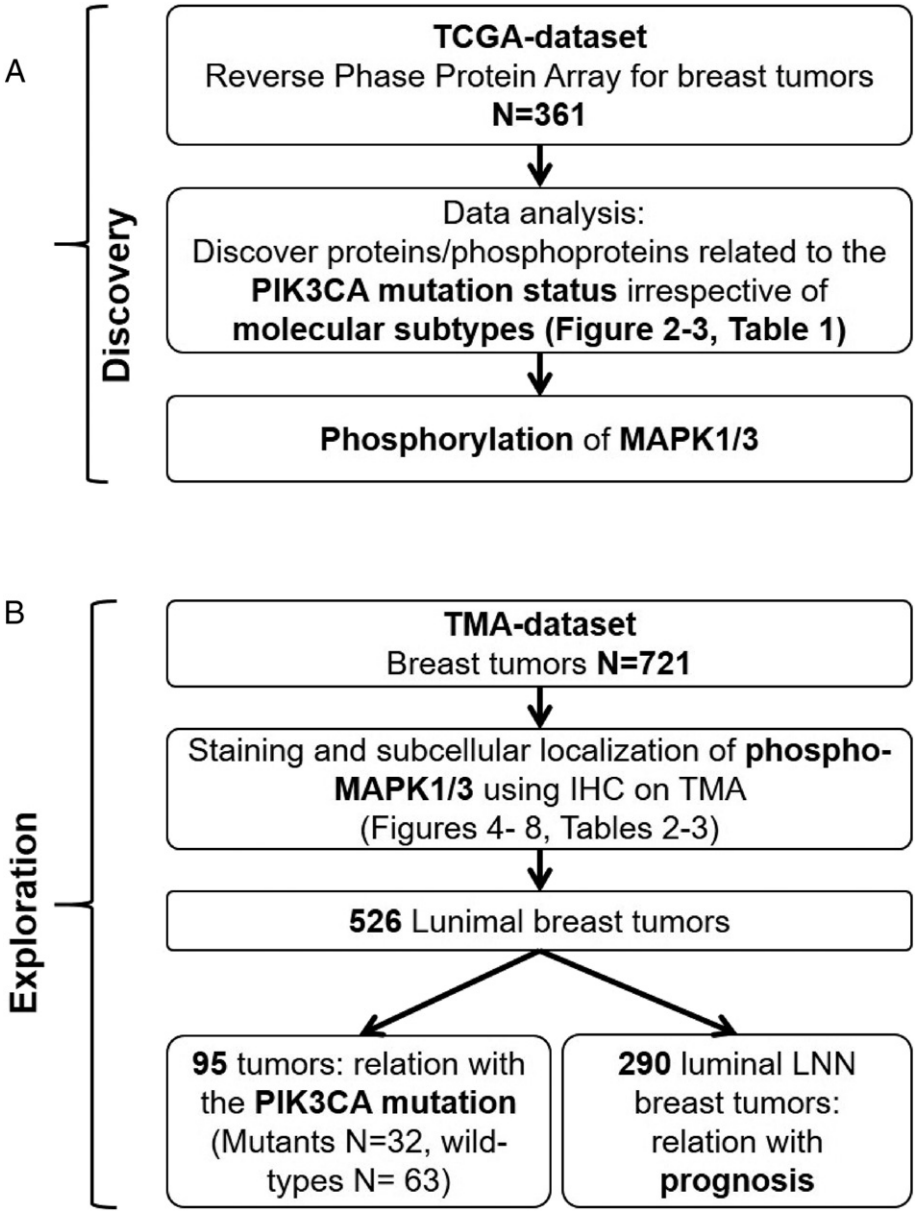

Figure 1. Overview of the study design. Figure 1*A*. In-silico data used from the TCGA consortium to identify potential biomarkers (protein expression and/or phosphorylation patterns related to the *PIK3CA* mutation status adjusted for subtype. Figure 1*B*. Tissue microarrays data used to explore the subcellular localization of the discovered potential biomarker phospho-MAPK1/3 and its correlation with subtypes, *PIK3CA* mutation status and prognosis. Abbreviations: IHC: Immunohistochemistry; TMA: Tissue microarrays; LNN: Lymph Node Negative.

specimens available from TCGA (see Supplemental Table S1). The data used by us were deposited [1] and can be retrieved at TCGA portal at https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. The tumors were selected based on their known *PIK3CA* mutation status and molecular subtype [9]. Of the 403 tumors, 366 specimens with a single *PIK3CA* mutation within the hotspot in exons 9 or 20 or wild-type were selected for further analyses, excluding tumors with *PIK3CA* double mutations. Five additional specimens were excluded, because 4 samples belonged only to the normal-like subtype and one specimen lacked subtype classification. In the remaining 361 tumors, either mutant (N = 94) or wild-type (N = 267) for *PIK3CA*, the protein expression and phosphorylation levels were analyzed irrespective of molecular subtype as well as stratified by subtype (using the PAM50 classification) (Figure 2).

Data Analyses and Statistics

Differences between groups were examined with a two-tailed student's t-test or when not normally distributed with the Mann–Whitney

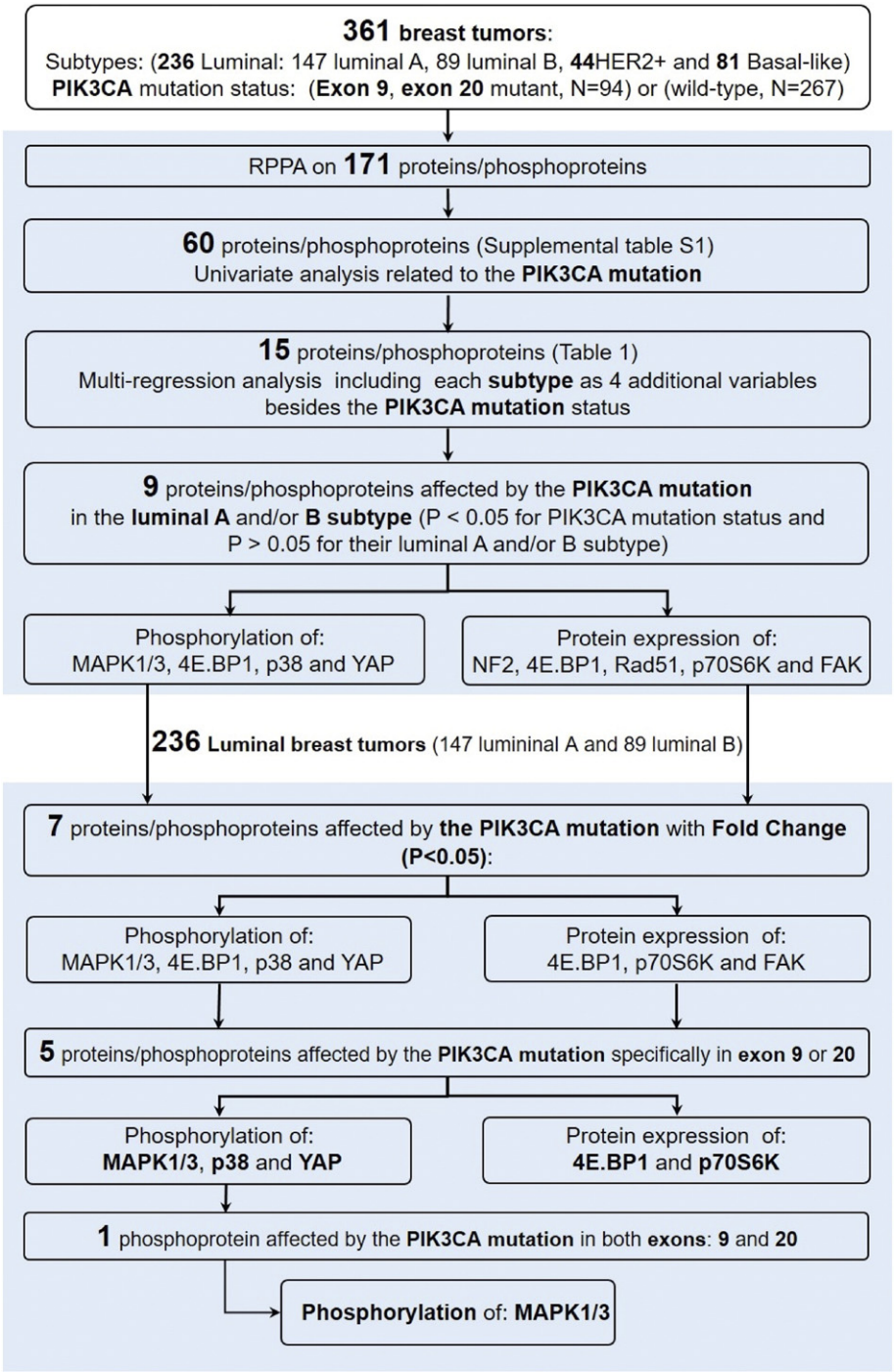

Figure 2. Data analysis flowchart to discover potential protein biomarkers for breast cancer. The figure shows the detailed criteria used to identify the association of the *PIK3CA* mutation with the protein expression and phosphorylation patterns using Reverse Phase Protein Array (RPPA) in silico data from the TCGA consortium on 361 breast tumors and 171 proteins or phosphoproteins.

test. Multiple linear regression analyses were performed on the protein expression and phosphorylation levels to establish their relationships with the *PIK3CA* genotype and molecular subtypes. The residuals of the regression analyses were afterwards checked for their constant variance by the Breusch-Pagan/Cook-Weisberg test for heteroscedasticity and for their normality. In multiple linear regression analyses, the *PIK3CA* wild-type basal subtype was used as reference to which the other subtypes and their *PIK3CA* status were compared. Proteins significant after these regression analyses were subsequently investigated for their relationship with *PIK3CA* genotype in luminal A and B specimens and also for their associations with exon 9 and exon 20 mutations, separately. The protein expression and phosphorylation levels were visualized with boxplots to illustrate the levels in luminal A and B *PIK3CA* wild-type or mutant with the levels obtained in basal wild-types (Figure 3).

The Cox proportional hazard model was used in univariate analysis to compute the hazard ratio (HR) for metastasis-free survival (MFS). The HR was presented with its 95% confidence intervals (95% CI). Survival curves were generated using the Kaplan–Meier method and a log rank test was applied to test for differences. MFS was defined as the time elapsed between the date of diagnosis and the date of distant metastatic relapse. In multivariate analysis, the biomarker was added to the base model of traditional prognostic factors for early breast cancer disease, i.e., age at diagnosis, menopausal status, tumor size, and tumor grade. Analyses were executed in STATA statistical package, release 14 (STATA Corp., College Station, TX). All *P*-values were two-sided, $P < .05$ was considered statistically significant.

Immunohistochemistry and Evaluation of MAPK1/3 Phosphorylation

Tissue microarrays (TMAs) of formalin-fixed, paraffin-embedded primary breast cancer specimens were described previously [11], and were immunocytochemically stained according to the procedures previously applied by us [10,11]. Briefly, tissue samples were used from patients with primary operable breast cancer between 1985 and 2000. This TMA contained a cohort of 817 patients. Tumors were included for analyses if histologic subtype, Bloom-Richardson score for tumor differentiation grade, estrogen and progesterone receptor status (ER, PgR), HER2/neu, epidermal growth factor receptor (EGFR) and cytokeratin 5 (CK5) status were available. Above selection criteria resulted finally in the evaluation of triplicate tissue core biopsies from 721 patients with primary breast cancer. These included 526 luminal breast tumors amongst other subtypes, of which 290 were from lymph node negative patients who received no (neo)adjuvant systemic therapies as described previously (van der Willik et al., AJCR 2016). This retrospective study with coded tumor tissues was approved by the Erasmus MC medical ethics committee at Rotterdam, The Netherlands (MEC 02.953). TMAs were stained with the primary rabbit polyclonal antibody for MAPK1/3 phosphorylation at 1:200 dilution (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101 from Cell Signaling Technology). The antibody was incubated overnight at 4 °C after 20 minutes antigen retrieval at pH 6.0. Subsequently, the TMA-slides were incubated with a secondary antibody (Rabbit Envision + System, HRP (DAKO)) and staining was visualized using diaminobenzidine (DAB).

Both nuclei and cytoplasm were scored for MAPK1/3 phosphorylation by two independent observers with regard to staining intensity and to the estimated proportion of tumor cells with positive nuclei and/or cytoplasm within a core biopsy. These scores were categorized to explore staining pattern differences. Negative versus positive staining was evaluated for both nuclei and cytoplasm separately, and combined since a subset of specimens showed heterogeneity in tumor cell staining (see Figure 4). Positive staining was specified for its intensity (weak, moderate, strong) and/or proportion of staining. To have enough cases for proper evaluation, groups were taken together to obtain more robust categories. For that reason, negative and weak staining were combined into one category (weak), and moderate and strong staining into another category (strong). In a similar

Table 1. Protein Alterations in Breast Cancer Related with *PIK3CA* Genotype Irrespective of Molecular Subtype

P-Values in Statistical Analysis for Protein Alterations in Relation to PIK3CA Mutation Status Alone or Combined with Molecular Subtypes in 361 Tumor Specimens

		Student t-test or Mann–Whitney *U* Test	Multiple Linear Regression (Basal *PIK3CA* Wild-Types as Reference)			
Protein	Alteration	*PIK3CA*	*PIK3CA*	HER2	Luminal A	Luminal B
MAPK1/3	Phosphorylation of T202/Y204	<0.001	<0.001 ‡	0.883	0.054	0.933
4E.BP1	Expression	<0.001	0.001	0.155	0.002	0.679
p70S6K	Expression	0.010	0.009†,‡	0.014	0.137	<0.001
p38	Phosphorylation of T180/Y182	<0.001	0.001	0.053	0.58	0.001
YAP	Phosphorylation of S127	0.003	0.001	0.003	0.12	<0.001
4E.BP1	Phosphorylation of T70	<0.001	0.049 ‡	0.734	<0.001	0.591
FAK	Expression	0.021	0.048	0.018	0.394	0.007
NF2	Expression	0.014	0.025	0.391	0.684	0.751
Rad51	Expression	<0.001	0.016†,‡	0.195	<0.001	0.268
PR	Expression	<0.001	<0.001 †	0.974	<0.001	<0.001
Akt	Phosphorylation of S473	0.012	0.002	0.138	0.013	<0.001
Bcl.xL	Expression	0.018	0.002†,‡	0.836	0.011	0.003
MIG.6	Expression	0.002	0.017 ‡	0.002	0.043	<0.001
Cyclin_E1	Expression	<0.001	0.008 ‡	<0.001	<0.001	<0.001
S6	Expression	<0.001	0.039	0.004	<0.001	<0.001

Phosphorylation and expression levels of 171 proteins measured with reverse phase protein arrays (RPPA) obtained from the TCGA database, were evaluated for their relationship with *PIK3CA* genotype and molecular subtype. Student t-test and Mann–Whitney test analyses defined protein alterations that are significantly related to *PIK3CA* status in all 361 tumor specimens. Multiple linear regression evaluated protein levels in relation to *PIK3CA* status combined with molecular subtype and used the wild-type basal-like subtype as reference. Only 15 protein alterations were significant for *PIK3CA* mutation status after linear regression. Of these, 9 proteins were of interest because their levels were altered due to *PIK3CA* ($P < .05$) but not by luminal subtype ($P > .05$).

P-value based on Mann–Whitney *U* test;

† Residuals failed test for heteroskedasticity.

‡ Residuals failed test for normality.

way, the categories for proportions were dichotomized into less than 20% positivity (low) versus more than 20% positive nuclei or cytoplasm (high).

PIK3CA Mutation Analysis

PIK3CA status were established by SnaPshot® multiplex assays (Life technologies) as described previously [5]. For only 105, of which 95 luminals, out of the 721 cases, (sufficient) DNA was available from the TMA core biopsies to identify *PIK3CA* exon 9 and exon 20 mutations.

Results

Protein Expression and Phosphorylation Related to PIK3CA Genotype and Molecular Subtypes

Student t-test or Mann–Whitney test analyses of proteins in 361 tumors, showed that 60 proteins had significant differences in expression or phosphorylation levels between tumors mutant (N = 94) or wild-type (N = 267) for *PIK3CA* (Supplemental Table S1). Of these 60 proteins, 15 protein changes were significant ($P < .05$) in multiple linear regression analyses for *PIK3CA* status when molecular subtypes were taken into account (Figure 2, Table 1).

To identify protein alterations correlated with mutation and not with subtype, only proteins were studied further for which expression level or phosphorylation status was affected by the *PIK3CA* mutation status and not significantly by subtype. Of the 15 proteins, the residuals of 11 proteins showed that the assumptions for constant variance (4 failed tests for heteroscedasticity) were valid allowing extrapolation of findings (Table 1). Subsequently, 9 proteins had P values <.05 for *PIK3CA* mutation status and $P > .05$ for luminal A and/or B subtypes (Table 1). Of these, MAPK1/3 phosphorylation and NF2 expression had significant different levels between *PIK3CA* wild-types and mutants irrespective of molecular subtype.

The other proteins were differentially expressed or phosphorylated due to *PIK3CA* status and subtype when compared to the wild-type basal subtype. Only significant for *PIK3CA* mutation status and

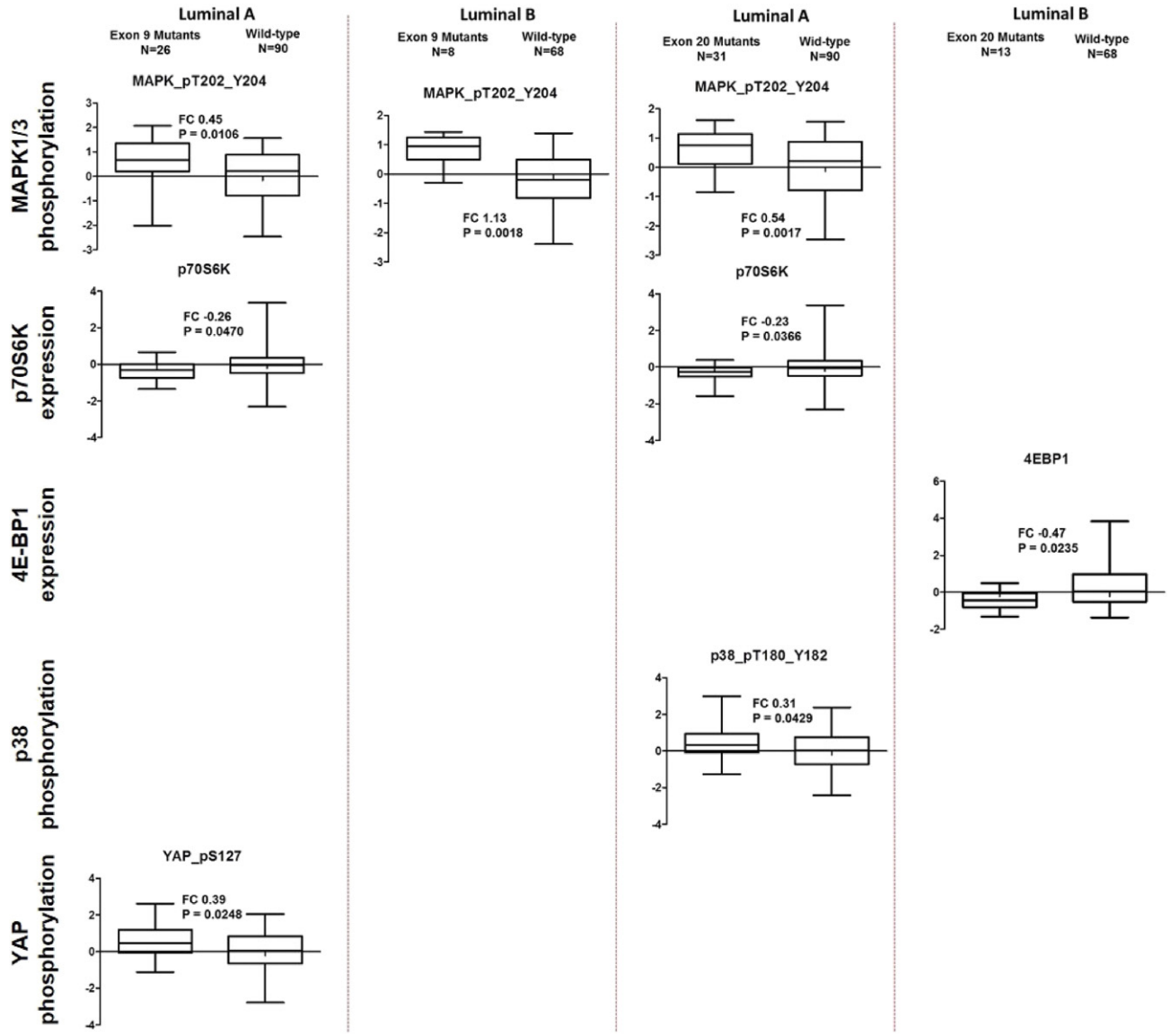

Figure 3. Protein alterations in *PIK3CA* exon-specific mutated luminal breast cancer. Differential phosphorylation and expression of 5 proteins due to *PIK3CA* mutation status but not molecular subtype as established in the multi-regression analysis (Table 1). The figure presents fold changes (FC) and *P*-values of non-parametrical Mann–Whitney *U* tests for MAPK1/3, p38 and YAP phosphorylation and expression of p70S6K and 4E–BP1 in luminal A and B breast cancer with exon 9 or exon 20 *PIK3CA* mutations.

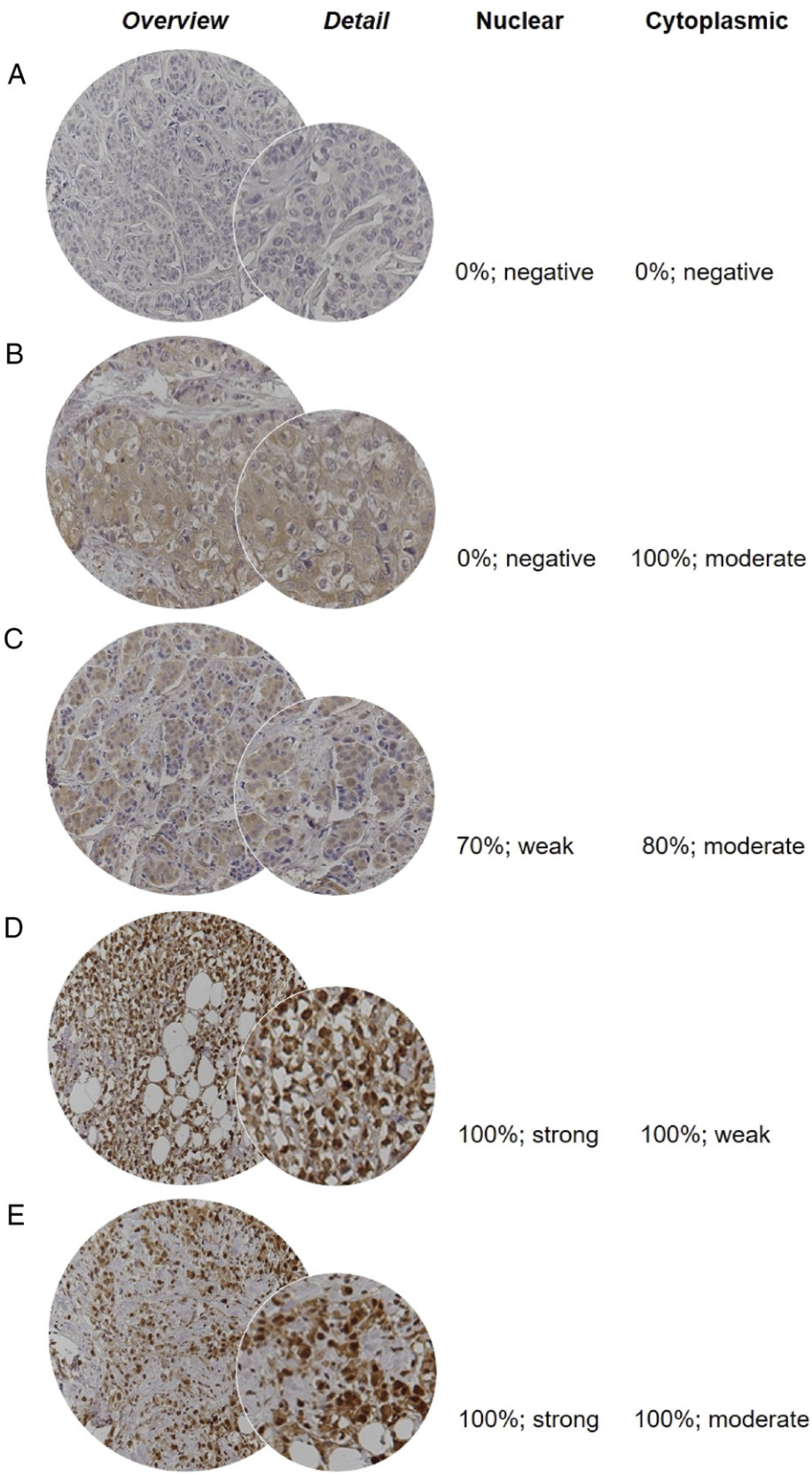

Figure 4. Phosphorylated MAPK1/3 staining patterns examples defined by intensity and proportion of MAPK1/3 phosphorylated tumor cells. Figure 4*A*. Negative for both nuclear and cytoplasmic. Figure 4*B*. Negative for nuclear and 100% moderate cytoplasmic intensity. Figure 4*C*. 70% weak nuclear and 80% moderate cytoplasmic intensity. Figure 4*D*. 100% strong for both nuclear and cytoplasmic intensity. Figure 4*E*. 100% strong nuclear and 100% moderate cytoplasmic intensity.

independent of subtype in luminal B were the altered levels for Rad51 expression and 4E–BP1 expression and phosphorylation, and in luminal A the p38 and YAP phosphorylation and expression of p70S6K and FAK. The applied (non)parametric tests have demonstrated significant proteins alterations in the TCGA dataset. For the findings on p70S6K, however, these may not be extrapolated due to test limitations and/or assumptions.

PIK3CA Genotype Related Protein Alterations in Luminal Breast Cancer

The altered expression and phosphorylation levels of the 9 selected proteins were further investigated in the luminal subsets, i.e. 147 luminal A and 89 luminal B tumors (Supplemental Table S2). Two proteins, i.e. NF2 and RAD51, showed no significant fold changes in this luminal subset when evaluated for all mutants together, and were excluded from further analyses. In *PIK3CA* mutated specimens compared to wild-type specimens significant fold changes were observed for the remaining 7 proteins with increased MAPK1/3 phosphorylation seen in luminal A and B cancers, decreased 4E–BP1 expression and phosphorylation in luminal B tumors, and decreased p70S6K and FAK expression and increased p38 and YAP phosphorylation in luminal A typed specimens.

Protein Expression and Phosphorylation Related to PIK3CA Exon 9 and 20 Mutations

Five of the 7 proteins altered in the luminal subset, all but FAK expression and phosphorylation of 4E–BP1, were significantly related to exon specific mutations when compared to wild-type counterparts (Figure 3, Supplemental Table S2).

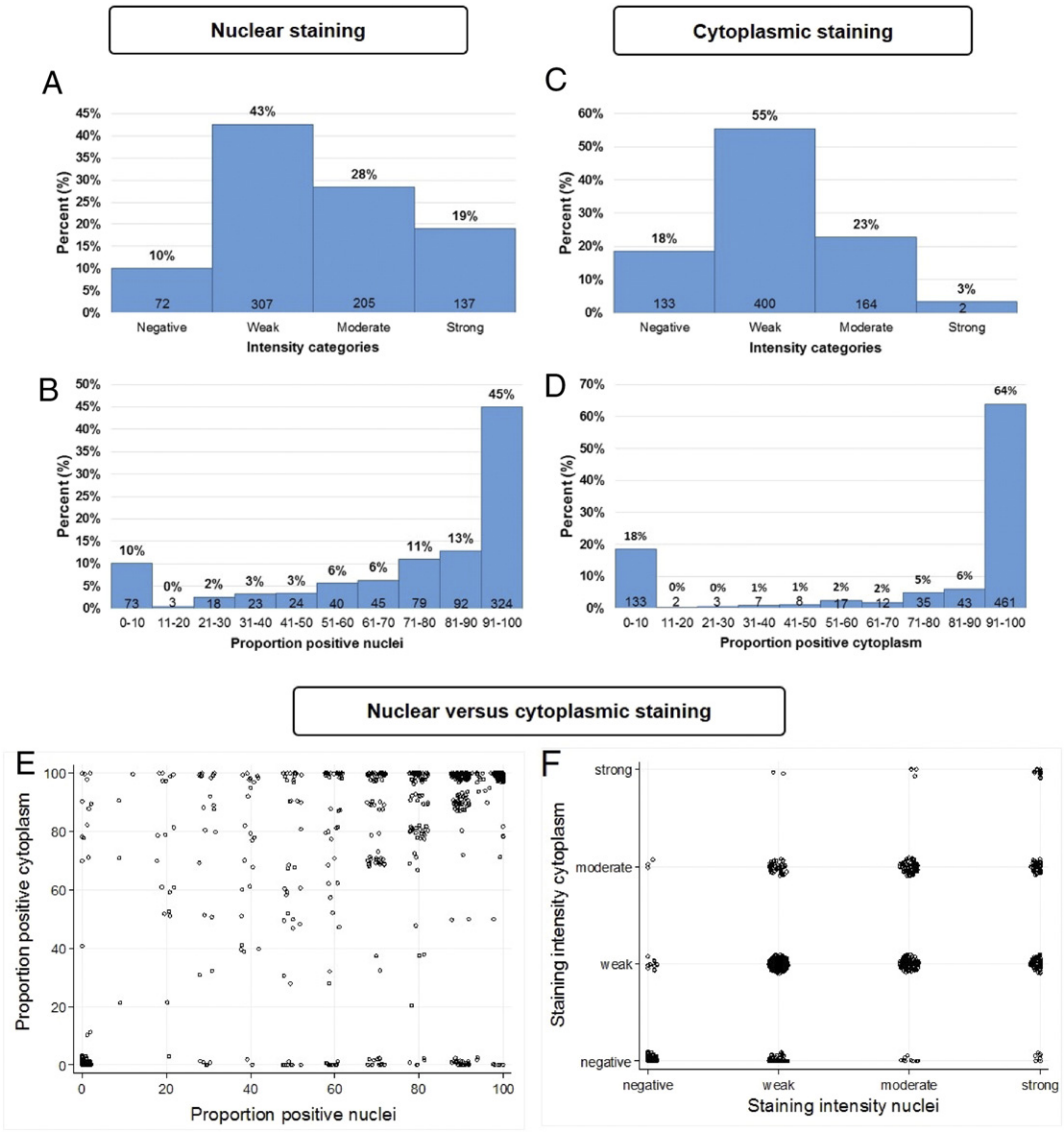

Figure 5. MAPK1/3 phosphorylation immunohistochemical staining evaluation. Tissue microarrays containing primary breast tumor core biopsies from 721 breast cancer patients were evaluated for nuclear, cytoplasmic and tumor cell MAPK1/3 phosphorylation. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 5, *A* and *B*) and cytoplasmic staining (Figure 5, *C* and *D*) defined by intensity (Figure 5, *A* and *C*) and by proportion (Figure 5, *B* and *D*). Additionally, it illustrates the number of cases comparing MAPK1/3 phosphorylation between cytoplasmic and nuclear staining for proportion (Figure 5*E*) and for intensity (Figure 5*F*).

Tumors with *PIK3CA* exon 9 hotspot mutations had increased MAPK1/3 and YAP phosphorylation and decreased p70S6K expression in luminal A, and only increased MAPK1/3 phosphorylation in luminal B (Figure 3). Tumors harboring exon 20 mutations had increased MAPK1/3 and p38 phosphorylation in luminal A and decreased expression of p70S6K in luminal A while only 4E–BP1 was decreased in luminal B. The differences in phosphorylation observed in our in silico analyses for p38 and YAP in relation to exon-specific *PIK3CA* mutations, have not been yet reported by others to our knowledge.

Tumor Cell Heterogeneity and Subcellular Localization of MAPK1/3 Phosphorylation

Protein functioning is not only defined by their expression and phosphorylation status, but may also depend on (sub)cellular localization. The RPPA findings, as described above, however, do not reveal which cell types and cellular compartments express proteins. To address this disadvantage, we examined MAPK1/3 phosphorylation for tumor cell heterogeneity and its subcellular localization in TMAs containing core biopsies of 721 primary tumor tissue specimens (Figures 4 & 5). Immunohistochemical staining for

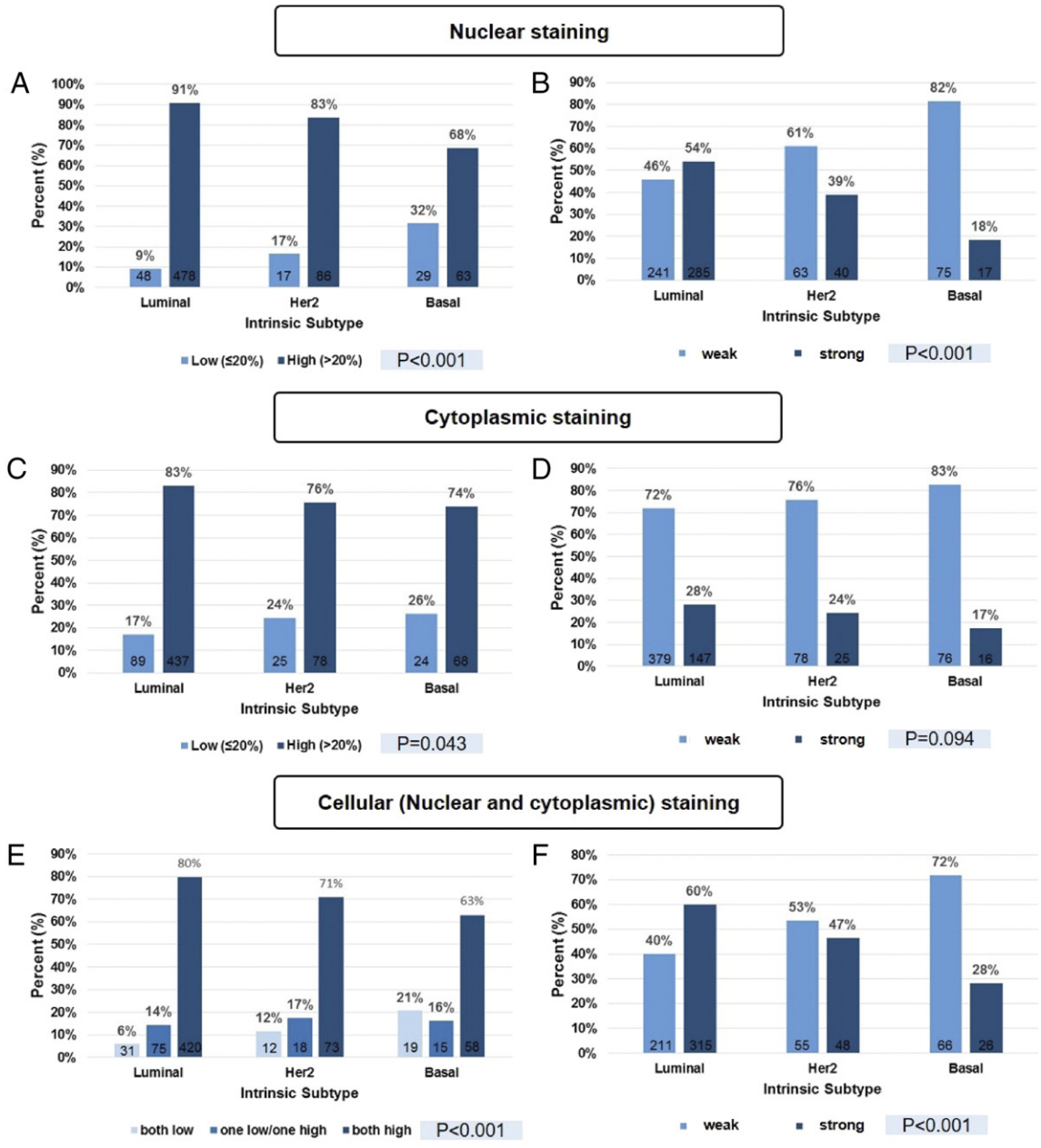

Figure 6. MAPK1/3 phosphorylation in relation with intrinsic subtypes analyzed in 721 primary breast tumors. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 6, *A* and *B*), cytoplasmic (Figure 6, *C* and *D*) and cellular staining (Figure 6, *E* and *F*) defined by proportion (Figure 6, *A*, *C*, and *E*) and by intensity (Figure 6, *B*, *D*, and *F*). The intrinsic subtypes were defined by ER, PgR, HER2/neu, EGFR and Cytokeratin 5 staining and classified as described previously by us (PMID: 27186402) as luminal (positive for ER and/or PgR, negative for HER2/neu), Her2 (positive for HER2/neu) and basal (positive for EGFR and/or Cytokeratin 5, negative for ER, and HER2/neu). *P*-values are based on chi-square test.

MAPK1/3 phosphorylation was performed, since it was the most significant protein alteration seen in *PIK3CA* exon 9 mutant luminal A and B tumors and in exon 20 mutant luminal A tumors (Figure 3).

Our TMA biopsies showed heterogeneity in staining patterns for MAPK1/3 phosphorylation as illustrated by the examples of Figure 4 and quantified in Figure 5. Moreover, MAPK1/3 phosphorylation was especially seen in the luminal and HER2 intrinsic subtypes, whereas tumors of the basal subtype had somewhat less staining of especially nuclei (Figure 6).

The MAPK1/3 phosphorylation staining revealed besides tumors with no and >80% positive tumor cells, also tumors with 11% to 80% tumor cells with MAPK1/3 phosphorylation in their cytoplasm (18% in Figure 5*D*) and in their nuclei (32% in Figure 5*B*). These findings suggest heterogeneity in a subset of specimens containing both tumor cell populations positive as well as negative for MAPK1/3 phosphorylation. Additionally, Figure 4 indicates heterogeneity in subcellular phosphorylation of MAPK1/3, which is confirmed by the staining distributions for proportions and intensities between nuclei and cytoplasm, respectively, as illustrated in Figure 5, *E* and *F*. For example, cases with no cytoplasmic MAPK1/3 had weak to strong nuclear MAPK1/3 staining (Figure 5*F*) in up to 100% of the nuclei (Figure 5*E*). Overall, the staining proportion and intensity for MAPK1/3 phosphorylation was significantly heterogeneous (both chi-square test $P < .001$) when compared between nuclei and cytoplasm.

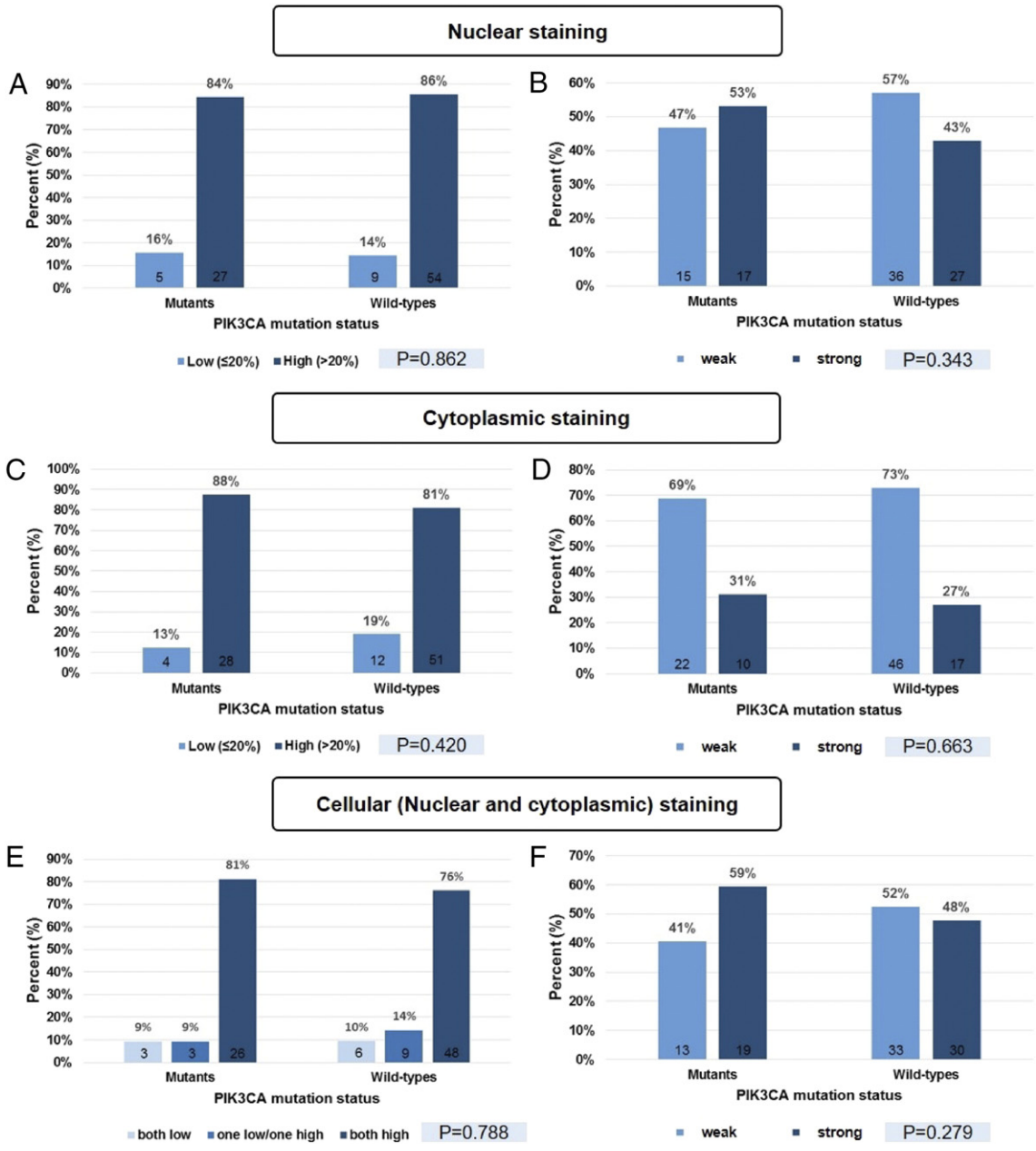

Figure 7. MAPK1/3 phosphorylation in relation to *PIK3CA* mutation status analyzed in 95 available luminal breast tumors. The figures present the percentage (above each bar) and number of cases (bottom values) for nuclear (Figure 7, *A* and *B*), cytoplasmic (Figure 7, *C* and *D*) and cellular staining (Figure 7, *E* and *F*) defined by proportion (Figure 7, *A*, *C*, and *E*) and by intensity (Figure 7, *B*, *D*, and *F*). No association was found for subcellular localization of MAKP1/3 phosphorylation with *PIK3CA* mutation status. *P*-values are based on chi-square test.

Table 2. Staining Intensity and Proportion for MAPK1/3 Phosphorylation in Nuclei and Cytoplasm of Tumor Cells and its Relationship with Metastasis-Free Survival: *P*-Value Log-rank Test for Trends

Staining Intensity and Proportion for MAPK1/3 Phosphorylation in Nuclei and Cytoplasm of Tumor Cells and its Relationship with Metastasis Free Survival in Breast Cancer: *P*-Values for Log-Rank Test for Trends

		Primary Breast Cancer (N = 721)		Luminal Breast Cancer (N = 526)		LNN Luminals (N = 290)	
Staining	Categories	Number of Cases per Category	*P*-Value	Number of Cases per Category	*P*-Value	Number of Cases per Category	*P*-Value
Nuclei							
	Negative versus Positive	72; 649	0.0225	34; 492	0.0163	18; 272	0.0376
Intensity:	Weak versus Strong	379; 342	0.0532	241; 285	0.0032	131; 159	0.0463
Proportion:	Low (≤20%) versus High (> 20%)	94; 627	0.0928	48; 478	0.0167	23; 267	0.0049
Cytoplasm							
	Negative versus Positive	133; 588	0.0957	87; 439	0.0198	42; 248	0.1008
Intensity:	Weak versus Strong	533; 188	0.0912	379; 147	0.0728	205; 85	0.2085
Proportion:	Low (≤20%) versus High (> 20%)	138; 583	0.1545	89; 437	0.0178	43; 247	0.0556
Tumor Cells (Nuclei and Cytoplasm Combined)							
	Negative versus Positive	57; 664	0.0106	28; 498	0.0003	14; 276	0.0058
Intensity:	Weak versus Strong	332; 389	0.0396	211; 315	0.0013	110; 180	0.0283
Proportion:	Both Low versus Low/High versus Both High	62; 108; 551	0.0694	31; 75; 420	0.0039	15; 36; 239	0.0058

MAPK1/3 Phosphorylation Staining and PIK3CA Mutation

MAPK1/3 phosphorylation was also evaluated in a subset 95 luminal breast cancers with known *PIK3CA* genotype, including 32 tumors with *PIK3CA* exon 9 (n = 9) or exon 20 mutations (n = 23). No significant differences between *PIK3CA* mutants and wild-types were observed for staining proportion and intensity of nuclei and cytoplasm, when analyzed separately or combined (Figure 7).

Clinical Relevance of Subcellular Localization of MAPK1/3 Phosphorylation in Breast Cancer Tissue Specimens

Log-rank tests for trend were evaluated to determine whether MAPK1/3 phosphorylation staining intensity and proportion were related with MFS (Table 2). These tests demonstrated a relationship with MFS in especially luminal tumors for nuclear and tumor cell MAPK1/3 phosphorylation for both staining intensity and proportion. In contrast, cytoplasmic staining was associated with MFS in only 2 of 9 analyses.

MAPK1/3 phosphorylation staining patterns were evaluated for their prognostic value in a cohort of luminal primary breast tumors from 290 LNN patients who did not receive (neo)adjuvant systemic therapy. Kaplan–Meier survival curve analyses and the log-rank tests for trend showed that only nuclear and tumor cell staining were related with MFS in this cohort (Table 2 and Figure 8). The Kaplan–Meier analyses demonstrated that high (>20% in Figure 8*A*) as well as strong (Figure 8*D*) nuclear MAPK1/3 phosphorylation associate with favorable survival. Similar results were obtained for tumor cell staining (Figure 8, *C* and *F*) but not for cytoplasmic staining (Figure 8, *B* and *E*).

Uni- and multivariate analyses for MAPK1/3 phosphorylation staining patterns were performed for this cohort of 290 LNN patients (Table 3). Staining patterns in multivariate analyses were compared to the base model of traditional prognostic factors which included age, menopausal status, tumor size, Bloom-Richardson differentiation grade and progesterone receptor status. Only high nuclear and tumor cell stain staining proportions were independent from the traditional factors and associated with favorable survival. MAPK1/3 phosphorylation in more than 20% of nuclei was associated with longer MFS compared to no or less than 20% of positive nuclei (HR = 0.49; 95% CI: 0.25–0.95;*P* = .036). Similarly, in specimens with staining in more than 20% of both nuclei and cytoplasm, MAPK1/3 phosphorylation was also related with favorable MFS (HR = 0.37; 95% CI: 0.18–0.79; *P* = .010).

Discussion

This study aimed to reveal proteins downstream PI3K with altered phosphorylation or expression levels in luminal breast cancer with *PIK3CA* exon 9 and/or exon 20 mutations. Our in silico analyses of publically available RPPA data showed in luminal A and B tumors with *PIK3CA* mutations an increased phosphorylation of MAPK1/3, p38 and YAP and decreased expression of p70S6K and 4E–BP1. We showed that MAPK1/3 phosphorylation was the most significant protein up-regulated in *PIK3CA* exon 9 mutated luminal A and B tumors and in *PIK3CA* exon 20 mutated luminal A tumors. Our immunohistochemical staining of MAPK1/3 phosphorylation on TMAs demonstrated tumor cell heterogeneity in subcellular location, staining intensity and proportion. We demonstrated that high MAPK phosphorylation in especially the nuclei of tumor cells correlated with favorable prognosis.

We investigated protein changes detected by RPPA to identify alterations specific for luminal breast cancer specimens with a *PIK3CA* exon 9 or exon 20 mutation. Mutations in *PIK3CA*, next to TP53, occur most frequently in breast cancer. TP53 mutations and PI3K/AKT/mTOR pathway activation are particularly observed in breast cancer of the basal subtype [1]. The *PIK3CA* mutations, however, are predominantly observed in ER-positive tumors and have been shown to be present in approximately 27% of luminal B up to 45% in luminal A specimens [1]. Above considerations were taken into account in our in silico analyses using a multi-regression model combining *PIK3CA* status and molecular subtype. These analyses helped to identify alterations related to the *PIK3CA* mutation status in a (luminal) subtype independent way. Although 15 protein changes had a significant relation with *PIK3CA* mutation status, only 9 of these were not correlated with luminal A and/or B subtypes. These 9 proteins were further explored within the subset of 236 luminal specimens for the relation with exon-specific *PIK3CA* mutations. This subsequent in silico evaluation demonstrated just 5 of these 9 protein alterations to be *PIK3CA* exon mutation specific in

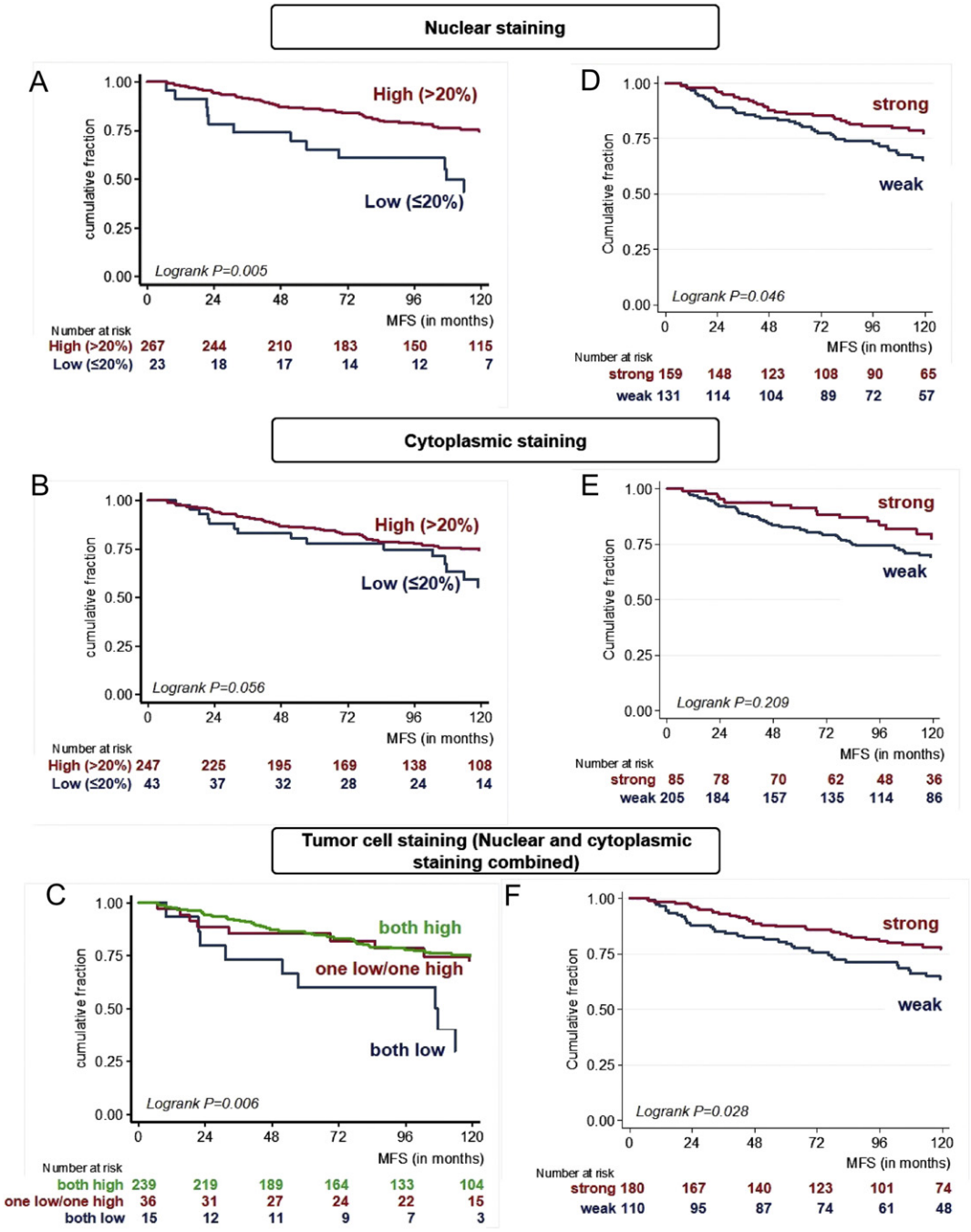

Figure 8. Subcellular localization of phosphorylated MAPK1/3 and its prognostic significance. Kaplan Meier curve analyses for MFS in luminal primary breast tumors of 290 (neo) adjuvant systemic therapy naïve LLN patients shows that high (Figure 8*A*) and strong (Figure 8*D*) nuclear MAPK1/3 phosphorylation correlates with favorable prognosis. No correlation was observed for cytoplasmic phosphorylated MAPK1/3 regarding staining proportion (Figure 8*B*) and intensity (Figure 8*E*). Finally, nuclear and cytoplasmic staining combined (cellular staining) was associated with favorable prognosis when defined by proportion (Figure 8*C*) as well as by intensity (Figure 8*F*). Abbreviations: MFS: Metastasis-free survival; LNN: Lymph Node Negative.

luminal A and/or B tumors, including increased phosphorylation of MAPK1/3, p38 and YAP and decreased expression of p70S6K and 4E–BP1. Future investigations are needed to validate our in silico results in other subsets.

Differences in protein phosphorylation patterns in relation to *PIK3CA* mutations have been reported for human cancer cell line models. It was shown that somatic knock-in of both KRAS and *PIK3CA* mutations in human breast epithelial cells was accompanied by increased MAPK1/3 phosphorylation, MAPK pathway activation, and tumor formation in immune-comprised mice [12]. Moreover, it was shown that phosphorylation of MAPK1/3 resulted in an activation of the protein and translocation from the cytoplasm to the nucleus [13]. We could not evaluate this translocation of MAPK1/3 upon phosphorylation with the available RPPA data from

Table 3. Univariate and Multivariate Analyses for Metastasis-Free Survival of MAPK Phosphorylation

Uni- and Multivariate Analyses of MAPK1/3 Phosphorylation: Its Relationship with Metastasis-Free Survival in 290 Luminal Lymph Node Negative (Neo)Adjuvant Systemic Therapy Naïve Breast Cancer Patients

	Univariate Analysis					Multivariate Analysis		
Factor	Number Patients	%	HR	95% CI	*P*-Value	HR	95% CI	*P*-Value
Age at diagnosis (in years):								
<40	26	9%	1.00			1.00		
41–55	116	40%	0.75	0.37–1.53	0.432	0.73	0.34–1.55	0.412
56–70	101	35%	0.56	0.26–1.20	0.135	0.26	0.09–0.79	0.018
>70	47	16%	0.81	0.34–1.92	0.634	0.31	0.09–1.03	0.057
Menopausal status:								
premenopausal	126	43%	1.00			1.00		
postmenopausal	164	57%	0.99	0.63–1.57	0.995	2.32	1.03–5.20	0.042
tumor size (cm):								
<2 cm	196	68%	1.00			1.00		
≥2 cm	94	32%	2.37	1.51–3.73	<0.001	2.34	1.47–3.74	<0.001
Differentiation grade:								
1	68	23%	1.00			1.00		
2	153	53%	2.12	1.07–4.23	0.032	1.79	0.89–3.60	0.104
3	69	24%	3.05	1.46–6.37	0.003	2.67	1.26–5.68	0.011
PgR status:								
negative	63	22%	1.00			1.00		
positive	227	78%	0.82	0.48–1.40	0.462	0.82	0.46–1.43	0.477
Staining of MAPK1/3 Phosporylation						*added to base model*		
Nuclei								
Intensity								
Negative	18	6%	1.00			1.00		
Positive	272	94%	0.49	0.24–0.97	0.042	0.58	0.27–1.25	0.165
Weak	131	45%	1.00			1.00		
Strong	159	55%	0.63	0.40–0.99	0.048	0.77	0.47–1.25	0.293
Proportion:								
Low (≤20%)	23	8%	1.00			1.00		
High (> 20%)	267	92%	0.42	0.23–0.79	0.006	0.49	0.25–0.95	0.036
Cytoplasm								
Intensity								
Negative	42	14%	1.00			1.00		
Positive	248	86%	0.63	0.36–1.10	0.104	0.69	0.39–1.22	0.203
Weak	205	71%	1.00			1.00		
Strong	85	29%	0.71	0.42–1.21	0.211	0.78	0.46–1.34	0.377
Proportion:								
Low (≤20%)	43	15%	1.00			1.00		
High (> 20%)	247	85%	0.59	0.34–1.02	0.059	0.64	0.36–1.13	0.121
Tumor cells (nuclei and cytoplasm combined)								
Intensity:								
Negative	14	5%	1.00			1.00		
Positive	276	95%	0.37	0.18–0.77	0.008	0.42	0.19–0.92	0.029
Weak	110	38%	1.00			1.00		
Strong	180	62%	0.61	0.39–0.95	0.030	0.78	0.49–1.27	0.319
Proportion:								
both low	15	5%	1.00			1.00		
low/high	36	12%	0.40	0.16–0.99	0.047	0.39	0.15–1.02	0.056
both high	239	82%	0.33	0.16–0.67	0.002	0.37	0.18–0.79	0.010

Analyses performed in 290 patients with luminal LNN breast tumors. The 290 patients did not receive adjuvant systemic therapy. Abbreviations: LNN: Lymph Node Negative; HR: Hazard ratio; PgR: Progesterone receptor.

the TCGA consortium, since the RPPA was performed on protein lysates of cancer tissue and not on protein lysates of nuclear and cytoplasmic fractions. Therefore, we performed an immunohistochemical staining for MAPK1/3 phosphorylation on TMAs containing core biopsies of breast cancer specimens. These stainings enabled us to establish the subcellular location of MAPK1/3 phosphorylation and to examine tumor cell heterogeneity. Our results detected in 32% of cases nuclear MAPK1/3 phosphorylation for only a subset of tumor cells (ranging from 20% to 80%). This suggests that MAPK activation is not always seen in all tumor cells for a substantial subset of breast cancers.

Importantly, high MAPK1/3 phosphorylation in nuclei was related with favorable prognosis in our cohort of LNN luminal breast cancer specimens. Others have stained tissue microarrays for MAPK1/3 phosphorylation in breast cancer to determine either the relation with PI3K or with clinical outcome. Nuclear MAPK1/3 phosphorylation was investigated on 563 estrogen receptor positive primary tumors from patients participating in a randomized trial of 1 to 3 years adjuvant tamoxifen [14,15]. One study showed that at baseline both *PIK3CA* exon 9 and 20 mutated specimens have higher MAPK1/3 phosphorylation levels compared to wild-types [14]. Although this association with *PIK3CA* mutations was not confirmed in our TMA cohort, this is explained by the small number of cases for which the *PIK3CA* status could be determined. The other study demonstrated a trend between tamoxifen response and MAPK1/3 phosphorylation [15]. Additionally, nuclear and cytoplasmic MAPK1/3

phosphorylation were also studied in large well-characterized breast cancer series to investigate its biological and clinical significance [16,17]. This study showed that nuclear MAPK1/3 phosphorylation was associated with better outcome, especially in tamoxifen-treated cases. All these immunohistochemical studies in large cohorts of (luminal) breast cancer specimens confirm in part our in silico findings in relation to *PIK3CA* mutation [18] and our TMA findings that nuclear MAPK1/3 phosphorylation correlates with favorable outcome [16,17]. Future prospective studies are needed to verify above retrospective findings and to establish MAPK1/3 phosphorylation as biomarker.

In conclusion, we have explored several methods combining in silico multivariate analyses and immunocytochemistry on luminal breast cancer specimens. We have shown that *PIK3CA* mutated breast tumor specimens are characterized in an exon dependent manner by increased MAPK1/3 phosphorylation. The observed relationships in clinical specimens indicate that the phosphorylation of MAPK1/3 and its subcellular localization might associate with clinical outcome. Considering the high prevalence of *PIK3CA* mutations in breast cancer, our findings will aid in the design of future studies to evaluate and/or target downstream effectors of the *PIK3CA* mutation. Our findings indicate that MAPK1/3 phosphorylation may play an important role as a target downstream effector in luminal breast cancer patients with a *PIK3CA* mutation.

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Conflicts of Interest

None.

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