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PRECLINICAL STUDY

Hotspot mutations in *PIK3CA* associate with first-line treatment outcome for aromatase inhibitors but not for tamoxifen

Diana E. Ramirez-Ardila · Jean C. Helmijr · Maxime P. Look · Irene Lurkin · Kirsten Ruigrok-Ritstier · Steven van Laere · Luc Dirix · Fred C. Sweep · Paul N. Span · Sabine C. Linn · John A. Foekens · Stefan Sleijfer · Els M. J. J. Berns · Maurice P. H. M. Jansen

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Abstract *PIK3CA* mutations occur frequently in breast cancer, predominantly in exons 9 and 20. The aim of this retrospective study is to evaluate the *PIK3CA* mutation status for its relationship with prognosis and first-line endocrine therapy outcome. *PIK3CA* exon 9 and 20 were evaluated for mutations in 1,352 primary breast cancer specimens by SnaPshot multiplex analyses. The mutation status was studied for their relationship with metastasis-free survival (MFS) in 342 untreated lymph node-negative (LNN) patients and to time to progression (TTP) in estrogen receptor (ER)-positive patients with metastatic disease treated with first-line tamoxifen ($N = 447$) or aromatase inhibitors (AIs; $N = 84$). We detected in 423 patients hotspot mutations for *PIK3CA* (31 %). Mutations in exon 20 were detected in 251 patients (59 %), with H1047L and H1047R mutations in 37 (15 %) and 214 (85 %) cases, respectively. Mutations in *PIK3CA* exon 9 were discovered in 173 patients (41 %), with E542K and E545K mutations in 57 (32 %) and 104 (60 %) cases as most prevalent ones. Evaluation of the untreated LNN patients for prognosis showed no relationship between MFS and *PIK3CA* mutations, neither for exon 9 [HR = 1.04 (95 % CI 0.57–1.89), $P = 0.90$] nor for exon 20 [HR = 0.98 (95 % CI 0.63–1.54); $P = 0.94$] when compared to wild-type. The *PIK3CA* mutation status was also not associated with treatment outcome after first-line tamoxifen. On the other hand, patients treated with first-line AIs showed a longer TTP when having a *PIK3CA* mutation in exon 9 [HR = 0.40 (95 % CI 0.17–0.95); $P = 0.038$] or exon 20 [HR = 0.50 (95 % CI 0.27–0.91); $P = 0.024$] compared to wild-types, both significant in uni- and multivariate

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D. E. Ramirez-Ardila · J. C. Helmijr · M. P. Look · K. Ruigrok-Ritstier · J. A. Foekens · S. Sleijfer · E. M. J. J. Berns (✉) · M. P. H. M. Jansen
Department of Medical Oncology, Erasmus MC Cancer Institute, Room Be424, PO Box 2040, 3000 CA Rotterdam, The Netherlands
e-mail: p.berns@erasmusmc.nl

I. Lurkin
Department of Pathology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands

S. van Laere
Department Oncology, Catholic University Leuven, Leuven, Belgium

S. van Laere · L. Dirix
Translational Cancer Research Unit, Laboratory of Pathology, Oncology Centre, Antwerp University, GZA Hospitals St-Augustinus, Antwerp, Belgium

F. C. Sweep
Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

P. N. Span
Department of Radiation Oncology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

S. C. Linn
Department of Medical Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands

analysis including traditional predictive factors. All results remained when only HER2-negative patients were evaluated for each cohort. *PIK3CA* mutations in ER-positive tumors were significantly associated with a favorable outcome after first-line AIs, which needs further confirmation in other datasets. Mutations were not associated with prognosis in untreated LNN patients nor predictive outcome after first-line tamoxifen therapy in advanced disease patients.

Keywords *PIK3CA* mutations · Breast cancer · First-line endocrine therapy · Prognosis

Introduction

The phosphatidylinositol-3-kinase (PI3K) pathway regulates several cellular processes critical for cancer progression, i.e., protein synthesis, growth, metabolism, proliferation, cell survival, apoptosis, motility and angiogenesis [1]. Since this pathway is frequently deregulated in breast cancer, it is an attractive pharmacologic target to investigate. The PI3K is a complex of regulatory and catalytic proteins and one of the mechanisms of abnormal PI3K pathway activation is through mutations in the 110 kDa catalytic protein encoded by *PIK3CA* or in the 85 kDa regulatory protein encoded by *PIK3R1*.*PIK3CA* is the most frequent (30 %) mutated oncogene in breast cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic), especially in ER-positive tumors [2, 3]. Approximately 90 % of *PIK3CA* mutations [4], are clustered in two hotspot regions: exon 9 (E542K and E545K) encoding the helical domain and exon 20 (H1047R and H1047L) encoding the kinase domain [2, 5].

PIK3CA mutations may play an important role in the carcinogenesis and development of breast cancer and has been correlated with clinical and treatment outcome [6]. Several studies presented, however, contradicting results for the relationship between *PIK3CA* mutation status and clinical outcome. Some studies showed no correlation between *PIK3CA* mutations and clinicopathologic variables [4, 7], while others have shown a worse prognosis after treatment with *HER2*-inhibitors for patients with *HER2*+ tumors and *PIK3CA* mutations [8, 9]. Moreover, *PIK3CA* alterations are mainly present in invasive lobular (46 %) and ductal (22 %) breast carcinoma [10]. In lobular tumors, *PIK3CA* mutations have been found to be associated with tumor size, ER+ tumors and poor survival [6, 10], whereas other histological types with a low incidence of *PIK3CA* mutations have shown to be associated with a favorable prognosis [10]. Especially tumors bearing exon 20 *PIK3CA* mutations are related with poor prognosis compared to those having wild type *PIK3CA* and other *PIK3CA* mutations [11]. Finally, whole genome DNA analysis of a cohort of breast cancers revealed that *PIK3CA* mutations occur predominantly in ER-positive breast cancers of the molecular luminal subtype, whereas PI3K-pathway activation was especially observed in ER-negative basal breast cancers [3]. In this study, however, relationships with clinical and treatment outcome were not investigated.

Breast cancer patients with tumors expressing the ER are treated with endocrine therapies, i.e., with tamoxifen or AIs. Unfortunately, not all patients respond (de novo resistance) while in the metastatic setting, patients who do initially respond will eventually relapse (acquired resistance). Endocrine therapy resistance may occur through activation of the *PI3K*- and *MAPK*-pathways and/or their downstream targets *AKT* and *mTOR* [12–16]. In this respect it is of interest to note that phase III clinical trials showed substantial benefit when mTOR-inhibitors were added to AI treatment [17]. Also for survival after adjuvant tamoxifen therapy contradicting results have been published. Lai et al. [11] showed in a cohort of 152 patients that invasive ductal carcinoma with exon 20 *PIK3CA* mutation had a significant shorter survival after adjuvant tamoxifen therapy compared to wild type tumors. On the other hand, Loi et al. [18] observed no relation with *PIK3CA* mutation status but a beneficial outcome after adjuvant tamoxifen therapy when applying a PI3K exon 20 gene expression signature.

To address above contradicting findings, we examined retrospectively the *PIK3CA* mutations in a cohort of 1,352 breast cancer patients to establish the prognostic and predictive significance of these mutations in tumors of 342 untreated LNN patients as well as 532 ER-positive patients with advanced disease treated with first-line endocrine therapy, i.e., tamoxifen ($N = 447$) or aromatase inhibitors ($N = 84$). Moreover, we correlated patient and tumor characteristics and clinical outcome with the *PIK3CA* mutation status, stratified for helical (exon 9) or kinase (exon 20) domain hotspot mutations, respectively.

Patients

The Erasmus University Medical Center (EMC; $N = 1{,}031$), at Rotterdam, the Netherlands Cancer Institute (NKI; $N = 159$), at Amsterdam, and the Radboud University Nijmegen Medical Centre at Nijmegen ($N = 77$) all located in the Netherlands, and the Sint Augustinus Hospital at Antwerpen ($N = 85$) in Belgium participated in this study. Primary breast cancer tissue specimens were collected from 1,352 female patients with primary or advanced breast cancer that entered the hospitals between 1978 and 2007. Patients and tumor characteristics are

Table 1 *PIK3CA* mutation status and clinicopathological characteristics in all evaluated breast cancer patients and in 3 sub-cohorts for prognosis and treatment outcome

Factor analyzed	Total	All available data				342 untreated lymph node-negative patients				447 ER-positive patients treated with first-line tamoxifen				84 ER-positive patients treated with first-line aromatase inhibitors			
		Wild-type	Exon 9	Exon 20	*P*	Wild-type	Exon 9	Exon 20	*P*	Wild-type	Exon 9	Exon 20	*P*	Wild-type	Exon 9	Exon 20	*P*
PIK3CA status	1,352	928	173	251		259	28	55		291	62	94		59	8	17	
Age (years)[a]	1,134				0.231				0.127				0.304				0.079
≤55	498	350	67	81		132	18	25		102	18	24		8	4	6	
56–70	398	273	45	80		88	4	17		93	26	34		24	1	5	
>70	238	152	35	51		39	6	13		96	18	36		27	3	6	
Tumor size (cm)	1,073				0.009				0.261				0.108[b]				0.184
≤2	369	233	62	74		112	16	28		81	24	27	(373)	13	4	6	
>2	704	500	76	128		147	12	27		162	25	54		46	4	11	
Nodal status	1,109				0.537								0.616[b]				0.450[b]
LNN	500	349	61	90						132	26	39	(427)	13	3	6	(79)
LNP	609	406	83	120						144	33	53		41	5	11	
Disease-free interval (months)	1,146				0.722				0.97				0.704				0.105[b]
0–24	352	244	42	66		49	5	9		107	21	38		24	1	3	(83)
>24	794	536	109	149		210	23	46		184	41	56		34	7	14	
Dominant site of relapse	696				0.071				0.399[b]				0.419[b]				0.272
Bone	280	169	52	59		36	2	7	(114)	117	28	40	(443)	32	6	8	
LRR	76	54	6	16		13	1	1		25	3	12		2	1	1	
Other	340	233	43	64		37	8	9		147	31	40		25	1	8	
ER protein	1,201				<0.001				0.007								
ER-negative	209	176	14	19		71	3	6									
ER-positive	992	649	142	201		188	25	49									
PR protein	1,085				<0.001				<0.001[b]				0.442[b]				0.004[b]
PR-negative	329	267	23	39		90	4	5	(332)	57	8	21	(367)	22	0	1	(82)
PR-positive	756	488	115	153		164	23	46		183	40	58		35	8	16	
HER2 amplification	995				0.071				0.041[b]				0.08[b]				0.107[b]
HER2 negative	860	594	108	158		204	23	48	(312)	121	18	43	(204)	38	7	8	(60)
HER2 positive	135	106	10	19		34	0	3		19	2	1		3	1	3	

The analyses of relationships between *PIK3CA* mutation status and age, TNM, dominant site of relapse, and ER, PR, HER2-status in 1,352 breast cancer patients and three sub-cohorts of patients. Patient follow-up and tumor characteristics were not available for part of the tumors in which the *PIK3CA* status was determined

[a] Age was determined at time of diagnosis for the total cohort and the 342 LNN-patients and at time of therapy start for the 447 tamoxifen and 84 aromatase inhibitor patients

[b] Factors in the three sub-cohorts that lack information for part of the tumors. The numbers within brackets indicate the numbers of samples evaluated for those factors

presented in Table 1 for all patients for which information was available and for the three different patient cohorts analyzed in this study.

Follow-up, tumor staging, and response to therapy was defined by standard International Union against Cancer (Geneva, Switzerland) classification criteria [19]. This retrospective study has been approved by the local medical ethics committees, performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl) and Belgium, and reported following the REMARK recommendations [20]. DNA isolated from primary breast tumor specimens were evaluated for *PIK3CA* exon 9 and exon 20 hotspot mutation status to assess relationships with patient and tumor characteristics and with clinical and treatment outcome. ER, progesterone receptor (PR), and *HER2* status of the primary tumor tissue specimens were established as described previously [21–24].

The prognostic value of *PIK3CA* mutations was assessed in 342 LNN breast cancer patients. These patients had no metastatic disease at time of diagnosis and received no adjuvant systemic therapy. In addition, ER-positive patients with advanced disease treated with first-line tamoxifen ($N = 447$) or aromatase inhibitors ($N = 84$) were analyzed to determine the predictive value of *PIK3CA* mutations. Patients treated with endocrine therapy were selected based on the following inclusion criteria: Invasive ER-positive breast carcinoma; advanced disease deemed not curable by surgery and/or radiotherapy for which first-line tamoxifen or AI therapy had been given for at least 4 weeks; and frozen ($N = 1{,}193$) or paraffin-embedded ($N = 159$) primary tumor specimens were available. Detailed patient characteristics for the cohort of tamoxifen treated patients have been previously described [25]. The cohort of 84 metastatic breast cancer patients treated with first-line AIs received either steroidal (15 exemestane) or non-steroidal AIs (43 anastrozole, 26 letrozole). Nine of these patients presented with metastatic disease at time of diagnosis, 52 patients had modified mastectomy and 23 patients underwent breast-conserving lumpectomy. Sixty-four patients received adjuvant endocrine therapy and 17 patients were treated with adjuvant chemotherapy, however, all developed metastatic disease that was treated with first-line aromatase inhibitors.

Methods

Multiplex PCR amplification and SnaPshot analysis

For the detection of mutations, stored DNA was amplified for exons 9 and 20 of *PIK3CA* using earlier published PCR primers [26]. The amplified exons were assessed for mutations at the following nucleotide positions (with corresponding amino acid changes) G1624 (E542K, Q), G1633 (E545K, Q), A1634 (E545G, A), A3140 (H1047R, L) using the SnaPshot® multiplex system (Life Technologies) as described previously [26, 27]. All specimens with a mutation or that failed initially, were re-analyzed with the SnaPshot to validate the mutation status of the tumor.

Statistics

The relationship of mutation status with patient and tumor characteristics was investigated using nonparametric methods, i.e., Spearman rank correlations for continuous variables and Wilcoxon rank-sum or Kruskal–Wallis and χ^2 square test for ordered variables. The Cox proportional hazards model was used to compute the hazard ratio (HR) in the analysis of metastasis-free survival (MFS) and time to progression (TTP). MFS was defined as the time elapsed between the surgery and the first distant metastasis. TTP was defined as the time elapsed between initiation of endocrine therapy and the first detection of disease progression. In multivariate analysis, the predictive value of *PIK3CA* mutation status was compared to the base model of traditional clinicopathological factors for metastatic breast cancer, i.e., age at start of therapy, disease free interval, dominant site of relapse and PR and HER2 status. 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 used to test for differences. Computations were done with the STATA statistical package, release 12SE (STATA Corp., College Station, TX). All *P*-values were two-sided and $P < 0.05$ was considered statistically significant.

Results

PIK3CA mutation frequencies and clinicopathological features

We evaluated the DNA of 1,352 primary breast tumor specimens for hotspot mutations in the *PIK3CA* gene using SnaPshot multiplex analysis (Fig. 1a). No hotspot mutations for *PIK3CA* were detected in 928 tumors, here referred to as wild-type, although these tumors may harbor mutations outside the analyzed hotspots. For 423 patients a mutation in exon 9 or exon 20 (31 %) was discovered. Exon 20 mutations were detected in 251 patients (59 %), with a H1047L in 37 (15 %) and a H1047R mutation in 214 (85 %) cases. Mutations in *PIK3CA* exon 9 were detected in 173 patients (41 %), with E542K and E545K mutations in 57 (32 %) and 104 (60 %) cases, respectively, as the most prevalent ones (Fig. 1b).

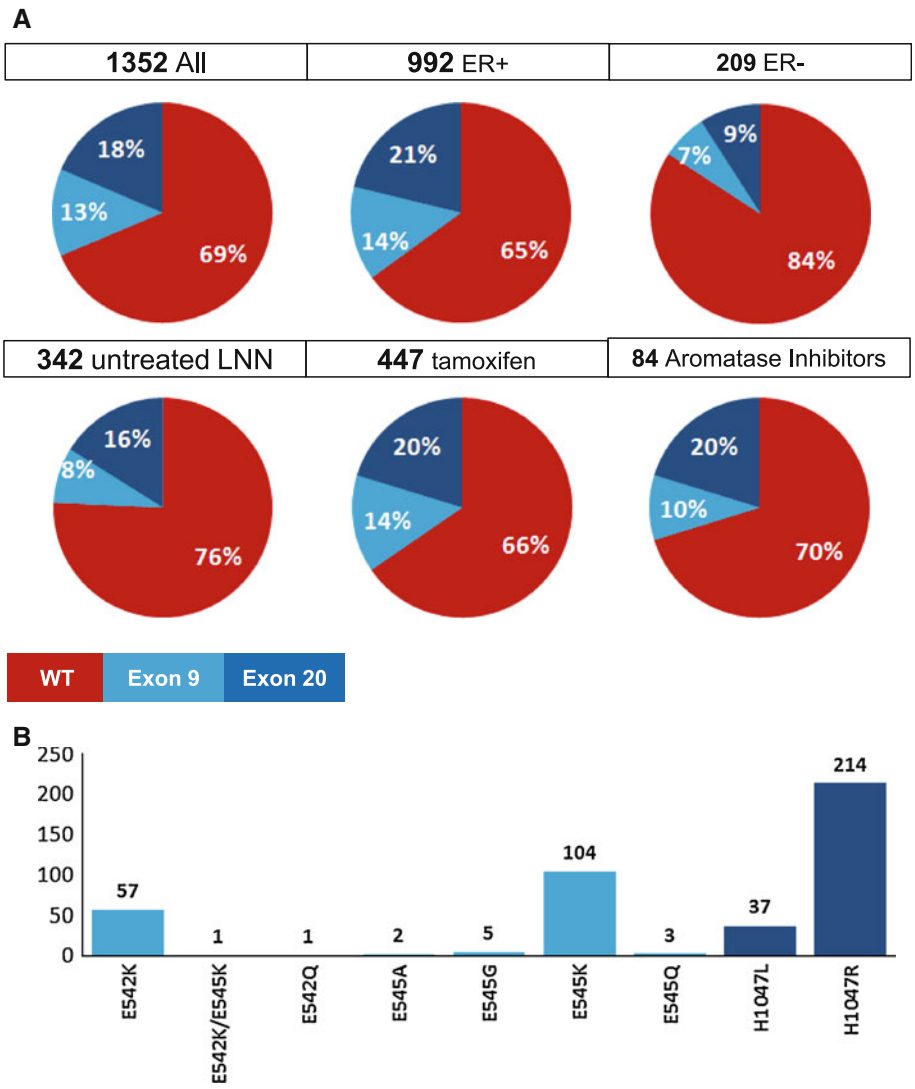

Fig. 1 *PIK3CA* mutation frequencies. **a** The frequencies of *PIK3CA* exon 9 and 20 mutations as detected in the total cohort and in subsets of breast cancer patients. Mutations occur more frequently in estrogen receptor positive (ER+) compared to ER-negative (ER−) tumors. Lymph node-negative (LNN) breast cancer patients that received no adjuvant systemic therapy ($N = 342$) were investigated for the prognostic value of the *PIK3CA* mutation status. ER-positive breast cancer patients with metastatic disease treated with first-line tamoxifen ($N = 447$) or aromatase inhibitors ($N = 84$) were evaluated for a relation between *PIK3CA* mutation status with treatment outcome. WT are patients who have no *PIK3CA* hotspot mutation in exon 9 or exon 20, defined as wild-type. **b** The type and number of mutations detected in exon 9 (all starting with E) or in exon 20 (H1047R, H1047L) for the 423 patients with a *PIK3CA* mutation in their primary tumor

Clinicopathological characteristics in relation with *PIK3CA* status for all patients and the three distinguished sub-cohorts of patients are shown in Table 1 and in more detail in Supplemental Table S1. *PIK3CA* mutations, both in exon 9 and 20, are most frequently observed in luminal ER-positive and/or in PR-positive tumors (all $P < 0.001$), in agreement with observations by others. Moreover, tumors with an exon 9 mutation appeared to be smaller (<2 cm; $P = 0.009$) and tend to metastasize preferentially to bone ($P = 0.071$) than those with a wild-type or exon 20 mutant *PIK3CA* gene. These cohorts included a cohort for prognosis of untreated LNN patients and two cohorts for treatment outcome of ER-positive patients with metastatic disease treated with endocrine therapy. For the prognostic cohort the *PIK3CA* mutations were predominantly observed in luminal ($P = 0.001$), ER-positive ($P = 0.007$), PR-positive ($P < 0.001$), and HER2-negative ($P = 0.041$) tumors. In the tamoxifen treated cohort, *PIK3CA* exon 20 mutations were especially detected in postmenopausal women ($P = 0.003$), whereas for the AI cohort exon 9 and 20 mutations were overrepresented in PR-positive tumors ($P = 0.004$). Since HER2 affects prognosis and response to endocrine therapy, survival analyses in the three sub-cohorts below were performed on all tumors (Table 1) and on HER2-negative tumors only (Supplemental Table S2).

PIK3CA mutation status and prognosis

To assess the prognostic value of *PIK3CA* mutation status, a subset of 342 LNN patients who received no adjuvant systemic therapy have been evaluated for the relation between mutation status and MFS. No significant differences were observed between wild-type and mutated *PIK3CA* tumors with regard to traditional prognostic factors age and menopausal status at diagnosis, tumor size, and grade. No association between *PIK3CA* mutation and MFS was found, neither for exon 9 [HR = 1.04 (95 % CI 0.57–1.90); $P = 0.90$] nor for exon 20 [HR = 0.98 (95 % CI 0.63–1.54); $P = 0.94$]

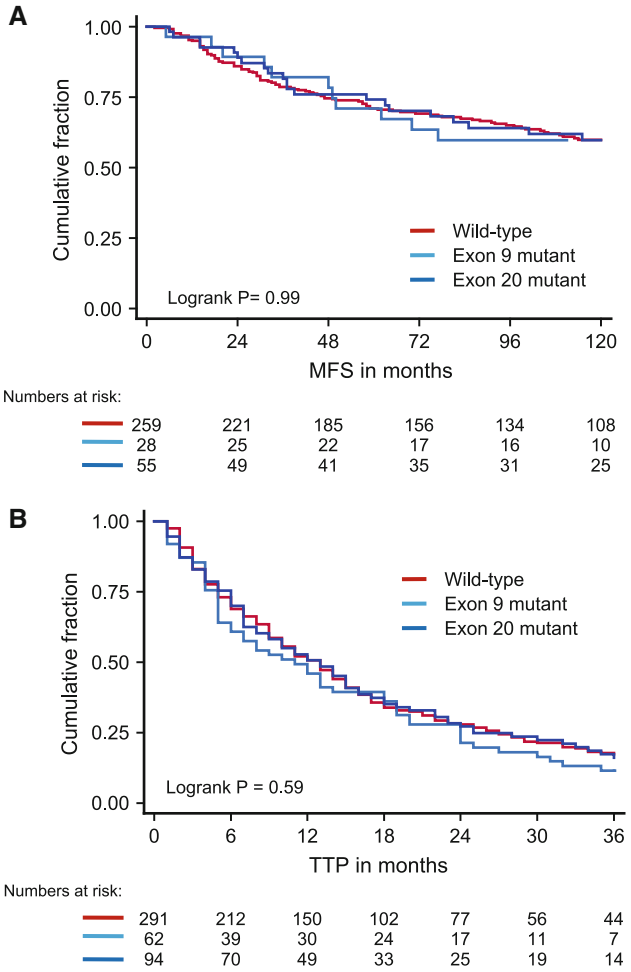

Fig. 2 *PIK3CA* mutation status: Prognosis and treatment outcome after first-line tamoxifen. **a** MFS analysis in 342 LNN breast cancer patients who had no adjuvant systemic therapy as a function of *PIK3CA* mutation status. **b** TTP analysis in 447 ER-positive breast cancer patients with advanced disease treated with first-line tamoxifen as a function of *PIK3CA* mutation status

when compared to wild-type (Fig. 2a). Since mutations occurred predominantly in luminal tumors, the prognostic value was also established in 262 ER-positive LNN patients of this sub-cohort. Again, for these 262 patients, no association between *PIK3CA* mutation and MFS was found, neither for tumors with exon 9 mutations [HR = 1.03 (95 % CI 0.55–1.93); $P = 0.94$] nor for tumors with exon 20 mutations [HR = 1.04 (95 % CI 0.65–1.66); $P = 0.88$] when compared to wild-type tumors. Mutation status and MFS showed also no relationships in the subsets of (ER-positive) HER2-negative tumors only (Supplemental Table S2).

PIK3CA mutation status and outcome after first-line tamoxifen therapy

Next, a subset of 447 ER-positive patients with advanced disease treated with first-line tamoxifen has been evaluated for the relationship between *PIK3CA* mutation status and treatment outcome. No significant differences were observed between *PIK3CA* wild-type and mutated tumors in relation to traditional predictive factors age at start of tamoxifen treatment, dominant site of relapse, disease-free interval and PR. The tumors with *PIK3CA* exon 9 and 20 mutations did not associate with TTP after tamoxifen, i.e., for exon 9 [HR = 1.17 (95 % CI 0.87–1.57); $P = 0.30$] and for exon 20 [HR = 1.01 (95 % CI 0.78–1.31); $P = 0.93$] (Fig. 2b). The relationship between *PIK3CA* mutation status and TTP was also not observed in the subset of HER2-negative tumors (Supplemental Table S2). The *PIK3CA* mutation status showed a relation with menopausal status at start of therapy in this subset ($P = 0.003$), which was also confirmed by a significant test of interaction ($P = 0.010$) for exon 20 mutation status and menopausal status. For this reason, the mutation status in relation to TTP was also evaluated independently for pre- and postmenopausal patients. No associations were observed for *PIK3CA* exon 9 mutations in premenopausal [$N = 77$; HR = 0.94 (95 % CI 0.50–1.78); $P = 0.85$] and postmenopausal [$N = 369$; HR = 1.22 (95 % CI 0.87–1.71); $P = 0.25$] women. The *PIK3CA* exon 20 mutations, on the other hand, showed a significant relation with TTP in premenopausal women [HR = 2.59 (95 % CI 1.08–6.25); $P = 0.034$] but not in postmenopausal women [HR = 1.00 (95 % CI 0.76–1.31); $P = 0.99$], although exon 20 mutations were especially detected in postmenopausal women.

PIK3CA mutation status and outcome after first-line aromatase inhibitor therapy

Finally, the *PIK3CA* mutation status was evaluated for a relationship with treatment outcome in a set of 84 ER-positive patients with advanced disease treated with first-line AIs. The PR status in this cohort was the only traditional predictive factor for metastatic disease that linked to the *PIK3CA* exon 9 and 20 mutation status ($P = 0.004$). Interestingly, patients with a *PIK3CA* mutation when compared with those being wild-type had a prolonged TTP after AI treatment, both for exon 9 [HR = 0.40 (95 % CI 0.17–0.95); $P = 0.038$] as well as for exon 20 [HR = 0.50 (95 % CI 0.27–0.91); $P = 0.024$] mutations (Fig. 3). These associations between mutation status and TTP were still significant when analyzed in the subset of HER2-negative tumors (Supplemental Table S2). An explanatory analysis in only patients treated with non-steroidal AIs ($N = 69$) demonstrated that the relation between *PIK3CA* mutation and prolonged TTP kept significant, i.e., for exon 9 [HR = 0.27 (95 % CI 0.08–0.88); $P = 0.030$] and for exon 20 [HR = 0.45 (95 % CI 0.23–0.87); $P = 0.019$]. The subset of steroidal AI treated patients ($N = 15$) was too small to draw conclusions. In multivariate analysis including age, disease-free interval, dominant site of relapse and PR and HER2 status as traditional

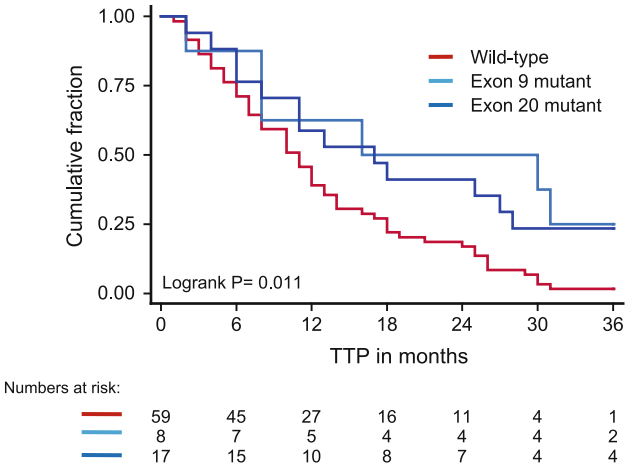

Fig. 3 *PIK3CA* mutation status and treatment outcome after first-line aromatase inhibitors. TTP analysis in 84 ER-positive breast cancer patients with advanced disease treated with first-line aromatase inhibitors as a function of *PIK3CA* mutation status

predictive factors, the association with AI treatment outcome remained significant for both exon 9 and exon 20 mutants (see Table 2). These significant preliminary findings, however, need further validation in additional larger datasets.

Discussion

The purpose of this study was to evaluate *PIK3CA* exon 9 and exon 20 hotspot mutations in 1,352 primary breast tumor tissue specimens and to associate the mutation status with clinicopathological characteristics and with clinical outcome. Using SnaPshot analyses we identified *PIK3CA* mutations in 31 % of the patients with 13 % mutations in exon 9 and 18 % mutations in exon 20. The mutations were mainly detected in the ER-positive tumors (35 %) compared to the triple-negative tumors (11 %) (Supplemental Table S1). Moreover, fewer mutated tumors were observed for patients with LRR (29 %) compared to those patients with bone metastasis (40 %; $N = 356$, $P = 0.056$) and to those patients with bone or other distant metastases (35 %: $N = 696$, $P = 0.17$). The frequency and distribution of *PIK3CA* hotspot mutations are comparable to those reported (Supplementary Figure S1) [3].

We evaluated exon 9 (helical) and exon 20 (kinase) domain *PIK3CA* mutations separately since exon 9 mutations have been reported mainly in lobular carcinomas and to associate with poor prognosis [28] and AKT-independent downstream signaling [29]. Although exon 9 mutations occur less frequent than exon 20 mutations, no significant differences were observed for almost all clinicopathological characteristics and clinical outcome in the sub-cohorts when stratified for *PIK3CA* exon 9 and 20 mutation status. We only observed in ER-positive tumors that *PIK3CA* exon 9 mutated tumors showed a trend to metastasize more often to bone and are smaller when compared to wild-type or exon 20 mutant tumors.

The *PIK3CA* mutant tumors in our analyses are mainly of the luminal subtype and PR-positive (80–83 %) in contrast to only 65 % PR-positive wild-type tumors. Other studies observed also significantly more PR-positive tumors in the *PIK3CA* mutant tumor group compared to the *PIK3CA* wild-type group, i.e., in a meta-analysis in 252 of the 333 mutant tumors (76 %) versus 374 of the 708 wild-type tumors (53 %) are PR-positive [5, 30, 31], respectively. However, presence of PR recently has shown to be only prognostic but not predictive for adjuvant tamoxifen therapy [32]. Moreover, the uni- and multivariate analyses for TTP after AI treatment in our cohort revealed no significant relationship between PR and outcome and *PIK3CA* mutation status remained independently from PR predictive for response to AI. In line with this, whole-genome analysis of breast cancers of patients treated with AI in the neoadjuvant setting showed that mutations in the PI3K-pathway mainly occur in luminal A breast tumors with low recurrence risk [33]. Additionally, invasive ductal and lobular carcinomas did not differ in *PIK3CA* mutation frequencies and distributions in all our specimens (Supplemental Table S1).

Our study shows that patients with a *PIK3CA* mutation in their tumors do have a favorable outcome on first-line AI therapy, whereas *PIK3CA* mutations are not prognostic nor related to first-line tamoxifen outcome. These findings are unexpected since somatic mutations in *PIK3CA* have been shown to activate AKT and induce oncogenic transformation of breast cancer cells [34] in vitro [35] and in vivo [36]. Additionally, in vitro studies demonstrated that resistance to endocrine therapy might be due to activation of the PI3K-pathway and/or it downstream targets AKT and mTOR [13, 15, 37]. Moreover, recent phase II and III clinical trials of metastatic breast cancer patients treated with tamoxifen or exemestane showed benefit from addition of everolimus, an mTOR-inhibitor, further stressing the role of the PI3K-pathway activation in endocrine resistance [17, 38]. On the other hand, no improve in progression free survival after first-line letrozole plus temsirolimus, also an inhibitor of mTOR, was observed in a randomized phase III trial of postmenopausal metastatic breast cancer patients [39]. Additionally, mutations in the kinase domain of *PIK3CA* were associated with favorable relapse-free survival and weakly with clinical response in a neoadjuvant endocrine therapy trial [40]. Finally, comprehensive reviews of a large number of clinical studies regarding *PIK3CA* mutations in breast carcinomas [2, 41] indicate that *PIK3CA* mutations are associated with favorable prognosis in ER-positive breast cancer. All these studies, however, included lymph node-positive and/or

Table 2 Uni- and multivariate analysis in 84 ER-positive breast cancer patients with metastatic disease treated with first-line aromatase inhibitors

Factor of base model	No. of patients	Univariate analysis			Multivariate analysis in 81 patients		
		HR	95 % CI	*P*	HR	95 % CI	*P*
Age at start therapy (years)							
≤55	18	1.00			1.00		
56–70	30	0.98	0.53–1.81	0.94	0.81	0.42–1.56	0.52
>70	36	0.95	0.52–1.73	0.86	0.87	0.45–1.66	0.66
Disease-free interval (months)							
0–24	28	1.00			1.00		
>24	55	0.66	0.41–1.06	0.088	0.74	0.45–1.22	0.25
Dominant site of relapse							
LRR	4	1.00			1.00		
Bone	46	3.50	0.84–14.53	0.085	3.28	0.77–13.96	0.11
Other	34	2.66	0.63–11.18	0.182	2.49	0.58–10.68	0.22
PR status							
Negative	23	1.00			1.00		
Positive	59	0.63	0.38–1.06	0.080	0.64	0.35–1.16	0.14
HER2 status #							
Negative	73	1.00			1.00		
Positive	11	1.09	0.56–2.13	0.79	0.78	0.33–1.82	0.57
PIK3CA mutation status					Added to the base model		
Wild-type	59	1.00			1.00		
Exon 9 mutant	8	0.40	0.17–0.95	0.038	0.40	0.16–1.00	0.051
Exon 20 mutant	17	0.50	0.27–0.91	0.024	0.50	0.26–0.98	0.045

For 81 of the 84 patients information was available for *PIK3CA* and all traditional predictive factors of the base model for metastatic disease, i.e., age, disease-free interval, dominant site of relapse, PR- and HER2-status

HER2-status was based on TargetPrint HER2 mRNA classification

(neo) adjuvant treated patients, which may explain why we could not confirm this association with prognosis in our study on untreated LNN patients. The contradicting findings might also be explained by recent views that *PIK3CA* mutations not always translate into a downstream activated PI3K pathway [3]. A system biological approach was applied in this study to reveal a mechanism of action in clinical samples, which showed that while *PIK3CA* mutations are predominantly present in luminal breast tumors the PI3K pathway activation mainly occurs in basal-like tumors. Luminal A *PIK3CA* mutated tumors in this study were low in PI3K pathway activation markers pAKT, pS6 and p4EBP1 and showed less PI3K-gene signature activity when compared to basal *PIK3CA* wild-type tumors. Analyses of down-stream PI3K pathway activation might be informative in our sub-cohorts for prognosis and tamoxifen, since no relationship with *PIK3CA* mutation status was observed for these cohorts, however, a system biological approach on these two cohorts was not applicable because genome wide RNA and/or protein data were not available.

We investigated primary tumor specimens and related their *PIK3CA* status with treatment outcome for advanced disease, nevertheless, it has been shown that the *PIK3CA* status can differ between primary tumor and metastatic lesions [42]. Biopsies of metastatic lesions, however, are often difficult to obtain due to their localization. Additionally, serum/plasma-derived circulating free DNA (cfDNA) from a subset of metastatic breast cancer patients contained *PIK3CA* mutations whereas no mutations were detected in cfDNA from patients with localized breast cancer [43]. These studies also identified wild-type primary tumors with matched metastatic lesions or cfDNAs that harbored *PIK3CA* mutations [42, 43]. All this indicates that primary disease may differ sometimes from metastatic disease with regard to *PIK3CA* status. It may affect slightly our findings and might explain partially differences between our observations in metastatic disease from those obtained by others in the adjuvant setting [11, 18].

Interestingly, we found that patients with a *PIK3CA* mutation in primary tumors have longer TTP after AI therapy when compared to wild-type tumors whereas there is no association with treatment outcome after tamoxifen treatment. The association with TTP after aromatase inhibitor therapy remained significant in the subset of patients treated

with non-steroidal AI (letrozole, anastrozole) and after multivariate analysis of the *PIK3CA* mutation status together with the traditional predictive factors. The discrepancy in *PIK3CA* mutation status relationship with treatment outcome after AI and tamoxifen therapy may be of relevance for the choice of treatment of ER-positive breast cancer patients. This discrepancy needs further evaluation since the AI-cohort is relatively small and because these sub-cohorts were not controlled for differences that could affect response to tamoxifen or AI for metastatic disease due to the retrospective design of the study. Our analyses indicate, however, that HER2 status does not affect the relationships between mutation status and outcome after AI and tamoxifen. Moreover, none of metastatic breast cancer patients treated with tamoxifen whereas 64 patients treated with AI received adjuvant endocrine therapy, but Cox regression analyses for adjuvant tamoxifen in the AI-cohort revealed no significant relationship with TTP [HR = 1.66, (95 % CI 0.95–2.89); $P = 0.074$]. Based on these observations we do not believe that adjuvant endocrine therapy could explain the differences in response for metastatic disease for our analyzed cohorts of patients.

In a neoadjuvant study on ER-positive breast tumors, it has been reported that everolimus increases the efficacy of letrozole [44]. Tumors with exon 20 *PIK3CA* mutations in this study showed less proliferation based upon Ki67-measurements after letrozole therapy compared to wild-type tumors and tumors carrying exon 9 mutations. Unfortunately, for analyses of overall survival, patients were not stratified for *PIK3CA* mutation status. Endocrine therapy combined with mTOR inhibitors, such as everolimus, has also been studied in cell line models. Cell lines harboring the *PIK3CA* mutation are sensitive to this combined therapy, however, cell lines with an active PI3K pathway due to *PTEN* deletions or activating mutations in *KRAS* or *BRAF* have no additional benefit from this combined therapy [45, 46]. Further studies are needed to evaluate treatment opportunities for ER-positive breast cancer patients with and without a *PIK3CA* mutation and PI3K/AKT/mTOR pathway activation.

Conclusions

This study is the first to report that metastatic breast cancer patients with *PIK3CA* mutations in ER-positive primary tumors show favorable outcome after first-line aromatase inhibitor treatment. This significant preliminary association should be verified in randomized prospective clinical trials to establish predictive significance [47]. Moreover, *PIK3CA* mutations in luminal ER-positive tumors have no prognostic value and are not predictive for first-line tamoxifen treatment.

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

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