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Molecular Analysis of Luminal Androgen Receptor Reveals Activated Pathways and Potential Therapeutic Targets in Breast Cancer

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Abstract. *Background/Aim: Triple-negative breast cancers represent 15% of all mammary malignancies and encompass several entities with different genomic characteristics. Among these, luminal androgen receptor (LAR) tumors express the androgen receptor (AR) and are characterized by a genomic profile which resembles luminal breast cancers. Moreover, LAR malignancies are usually enriched in PIK3CA, KMTC, CDH, NF1, and AKT1 alterations. Still, molecular features, clinical behavior and prognosis of this variant remain controversial, while identification of effective treatments represents an unmet medical need. Additionally, the predictive role of the AR is unclear. Materials and Methods: We performed an extensive next generation sequencing analysis using a commercially available panel in a cohort of patients with LAR breast cancer followed at two local Institutions. We next employed bioinformatic tools to identify signaling pathways involved in LAR pathogenesis and looked for potentially targetable alterations. Results: Eight patients were included in the study. In our cohort we found 26 known genetic alterations (KGAs) in 15 genes and 64 variants of unknown significance (VUS) in 59 genes. The most frequent KGAs were single nucleotide variants in PIK3CA, HER2, PTEN and TP53. Among VUS, CBFB, EP300, GRP124, MAP3K1, RANBP2 and TSC2 represented recurrently altered genes. We identified five signaling pathways (MAPK, PI3K/AKT, TP53, apoptosis and angiogenesis) involved in the pathogenesis of LAR breast cancer. Several alterations, including those in PIK3CA, ERBB2 and PI3K/AKT/mTOR signaling, were potentially targetable. Conclusion: Our findings confirm a role for PI3K/AKT/mTOR signaling in the pathogenesis of LAR breast cancers and indicate that targeting this pathway, along with ERBB2 mutations, may represent an additional therapeutic strategy which deserves further exploration in larger studies.*

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Triple-negative breast cancers (TNBC) are characterized by the lack of estrogen (ER) and progesterone receptors (PgR) in the absence of epidermal growth factor receptor 2 (HER2) amplification (1). They account for 15% of all breast cancers

and are usually associated with an aggressive clinical behavior and a poor prognosis (2, 3). These unfavorable features, along with the absence of viable therapeutic targets, fostered increasing efforts aimed at understanding the molecular characteristics of this disease. In the last few years, massive parallel sequencing and "omics" technologies have partially clarified the biological bases of this breast cancer variant revealing an unexpected heterogeneity (4, 5). Indeed, while some TNBC harbor a limited number of somatic mutations, others display a high number of genetic alterations affecting different signaling pathways (6, 7).

Based on their molecular profile, their chemosensitivity and on the presence of potential therapeutic targets, six TNBC subtypes have been identified: basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor (LAR). The latter tumors are characterized by the expression of the androgen receptor (AR) and by an apocrine histological appearance. The gene expression profile of these tumors resembles ER-positive breast cancers (e.g. *FOXA1*, *GATA3*, *SPDEF* and *XBP1* hyperexpression) (8). Additionally, LAR breast malignancies are usually enriched in *PIK3CA*, *KMTC*, *CDH*, *NF1*, and *AKT1* mutations (9, 10). Still, the prognosis and clinical behavior of LAR breast tumors remain undefined, with conflicting outcomes emerging from the available literature (11-14). Similarly, the predictive role of the AR is unclear. Several clinical trials tested anti-androgen compounds in LAR breast cancer patients (15, 16). Given the frequent presence of *PIK3CA* mutations among these tumors, ongoing studies are also exploring PI3K inhibitors or combining anti-androgen therapies with cyclin-dependent kinase 4/6 inhibitors (17, 18). However, to date none of these targeted approaches has shown significant efficacy in LAR tumors.

Herein we describe the molecular findings emerging from a comprehensive next-generation sequencing (NGS) analysis in a cohort of LAR breast cancer patients. We also report the results of *in silico* analyses performed to identify the activated pathways in these tumors and the relative potentially actionable alterations.

Materials and Methods

Patient samples. Patients diagnosed between 2014 and 2019 with TNBC expressing AR (*i.e.*, LAR breast cancers) were considered eligible for the study if formalin-fixed paraffine-embedded (FFPE) samples from the primary tumor were available. Subjects were followed either in the Oncology Unit of the Policlinico "G. Rodolico - San Marco" or at the "Humanitas Medical Care" Center in Catania. All patients gave written informed consent in accordance with the Declaration of Helsinki.

Next-generation sequencing. Nucleic acids were isolated from FFPE samples containing ≥50% tumor cells. Comprehensive genomic profiling was performed using a hybrid capture-based (7) platform (FoundationOne™, Foundation Medicine Inc., Cambridge, MA, USA) which identifies single nucleotide substitutions (SNV), insertions and deletions (indels), copy number alterations (CNAs), and rearrangements. The platform interrogates the coding sequence of 315 cancer-related genes and introns from 28 genes often rearranged in solid tumors to a median depth of coverage greater than 500× (19).

Immunohistochemistry for the Androgen Receptor. Immunohistochemical staining was performed on representative 5-micron thick sections using antibodies against the androgen receptor (DAKO AR441 clones; 1:75 dilution; pretreatment with citric buffer, pH=6.2; HRP detection; DAB chromogen). Nuclear staining for the androgen receptor in ≥1% of tumor cells was considered positive.

Bioinformatic analysis and literature search. Known genetic alterations and VUS alterations were annotated and reported by FoundationOne patient reports.

We evaluated SNV, indel and frameshift variants of VUS using different online *in silico* prediction tools. Polymorphism Phenotyping v2 (PolyPhen-2), Protein Variation Effect Analyzer (PROVEAN) and Sorting Intolerant from Tolerant (SIFT) were employed to predict the potential impact of SNVs on protein structure and function (20-22). Only SNVs considered not neutral by at least 2 of the 3 prediction tools (thereafter indicated as possibly damaging) were included into further functional analyses. Insertions, deletions and frameshift variants were studied using the MutationTaster tool (23). In this case, alterations which may lead to a dysfunctional protein will be here indicated as possibly damaging. Two authors (M.M. and S.R.V) performed a literature search on PubMed looking at genes with potentially damaging VUS to confirm their possible implication in breast carcinogenesis.

To understand the integrated biological significance of known genomic alterations (KGAs) and variants of unknown significance (VUS) we interrogated two annotation tools, Database for Annotation, Visualization and Integrated Discovery (DAVID) and Protein ANalysis THrough Evolutionary Relationships (PANTHER). The DAVID bioinformatics system analyzes a gene list using functional classifications, functional annotation charts or clustering and functional annotation tables (http://david.niaid.nih.gov). The PANTHER program is part of the Gene Ontology Reference Genome Project designed to classify proteins and genes with high-throughput analysis and exploits a database containing 20851 proteins directly associated with 165 metabolic and signaling pathways (www.pantherdb.org). We used the KEGG tool for the DAVID analysis and the CellDesigner tool in PANTHER to generate a pathway alteration status from the list of mutated genes.

Results

Population characteristics. Eleven patients diagnosed with LAR breast cancer satisfied the eligibility criteria for the study. Tumor tissue was obtained at the moment of primary surgery in all subjects. Three samples failed NGS analysis due to inadequate quality and were therefore excluded. Table I summarizes the main clinical and pathological features of the included cases. Median age at diagnosis was 74 years (range=61-81 years). All patients were female and displayed localized disease at diagnosis. Tumor size was ≤20 mm (*i.e.*, pT1) in 7 cases and nodal status was negative in 4 patients.

Table I. *Population characteristics*.

Patient characteristics (n=8)	
Age, median (range)	74 (61-81)
Sex, n (%)	
Female	8 (100%)
Pathological T, n (%)	
T1b	1 (12.5)
T1c	6 (75.0)
T2	1 (12.5)
Pathological N, n (%)	
N0*	4 (50.0)
N1	2 (25.0)
N2	2 (25.0)
Tumor stage according to AJCC TNM 8th edition	
IB	4 (50.0)
IIA	1 (12.5)
IIB	1 (12.5)
IIIB	1 (12.5)
IIIC	1 (12.5)
Tumor grading, n (%)	
2	6 (75.0)
3	2 (25.0)
Proliferation index Ki67%	
Median (range)	16 (<1-30)
Androgen receptor expression (%)	
Median (range)	35 (18-80)

*One N0 patient had isolated tumor cells in the sentinel lymph node. T: Tumor; N: lymph nodes; M: metastasis; AJCC: American Joint Committee on Cancer.

Cancer stages according to the *AJCC* TNM 8th edition were as follows: 4 IB, 1 IIA, 1 IIB, 1 IIIB and 1 IIIC. Tumor grading, defined according to the Elston-Ellis modified Scarff-Bloom-Richardson system, was intermediate (G2) in 6 cases and high (G3) in 2 subjects. Median Ki67% proliferation index was 16% (range=<1-30%), with 5 patients below the 20% threshold defined by the Sant Gallen criteria (24, 25). Median androgen receptor expression was 35% (range=18-80%).

Study workflow. We retrospectively collected tumor specimens to perform NGS analysis as detailed above. Twenty-six KGAs in 15 genes and 64 VUS in 59 genes emerged from sequencing. We then sought the potential biological significance of the above-mentioned VUS using *in silico* prediction tools (PolyPhen2, PROVEAN, SIFT, MutationTaster). This analysis identified 28 not repetitive genes with possibly damaging mutations (Table II). A literature search confirmed that all 28 genes had been previously correlated with breast cancer. Next, two functional annotation tools (DAVID and PANTHER) were used to pair biological alterations with signaling pathways considering the 15 genes with KGAs, the 28 genes with possibly damaging VUS, or their combination which totaled 38 genes as 5 were common between KGA and VUS. We then analyzed the results to identify potentially actionable therapeutic targets in our population (Figure 1).

Identification of somatic molecular alterations by NGS. Detailed NGS findings are illustrated in Figure 2. Among the 15 genes presenting KGAs, *PIK3CA* was altered in 4 patients, *ERBB2*, *PTEN* and *TP53* in 3 patients, *AKT1*, *CDH1* and *KTM2C* in 2 patients and the remaining genes in only one individual. Two alterations were recurrent, namely PIK3CA H1047R (patients 01, 08 and 09) and AKT E17K (patients 02 and 079). Of note, patient 08 harbored a double SNV in *ERBB2* (I767M, S310F), while subject 09 presented two alterations in *PTEN* (C136R, S10fs*14) (Figure 2A). In the VUS dataset 6 (*CBFB*, *EP300*, *GRP124*, *MAP3K1, RANBP2* and *TSC2*) of the 59 included genes were altered in more than one patient. Only one VUS was recurrent (TSC2 F1510del in subjects 03 and 10), while 2 individuals presented a double SNV in the same gene, namely FANCA A1132V and C1142F in patient 02 and RUNBP2 F3085L and R176C in patient 08.

Co-analysis of KGAs and VUS identifies novel pathways in LAR breast cancer. We next evaluated whether the detected alterations cluster in known signaling pathways, using two different classification systems (DAVID and PANTHER) to analyze KGAs, possibly damaging VUS or their combination (Figure 3). Clustering genes with KGAs, similar pathways emerged from the DAVID and PANTHER analysis (Figure 3A and D). Additionally, several retrieved pathways can be traced back to broader networks. For example, HIF-1 and Hypoxia response via HIF are both involved in angiogenesis and apoptosis while FoxO also contributes to cell death. Functional analysis of genes with possibly damaging VUS provided a smaller number of activated pathways, with different results from the DAVID and the PANTHER tools (Figure 3B and E). However, the retrieved pathways were partly superimposable with those observed for gene with KGAs. Lastly, we ran a combined analysis for genes with KGAs and possibly damaging VUS (Figure 3C and F).

To compare the results from the DAVID and PANTHER analyses, we arbitrarily set a threshold defined as the median rate of pathway involvement and considered significant pathways only whose contribution was above the threshold. Among them, PI3K-Akt, p53, apoptosis, angiogenesis and MAPK signaling were the most represented. Additional activated pathways emerged from the combined analysis (Figure 3C and F, white bars). Although these pathways were mainly below our defined threshold, they were all implicated in breast cancer biology according to pre-existing evidence (2, 50). These results suggest that genes with potentially damaging VUS might provide additional information on the mutational landscape of LAR breast cancer.

Table II. *Potential damaging variants of unknown significance*.

Gene	SNV	Prediction tools		
		POLYPHEN	PROVEAN	SIFT
ABL1	A1110V	Possibly damaging	Neutral	Damaging
AR	P392S	Benign	Deleterious	Damaging
BRD4	A879V	Possibly damaging	Neutral	Damaging
FANCA	C1142F	Possibly damaging	Deleterious	Damaging
FAT1	G2653S	Possibly damaging	Deleterious	Damaging
GPR124	E453K	Probably damaging	Neutral	Damaging
KDM6A	P1007S	Probably damaging	Deleterious	Tolerated
MAP3K1	W1243S	Possibly damaging	Deleterious	Damaging
MAP3K1	R45Q	Possibly damaging	Neutral	Damaging
MLL3	R2481S	Probably damaging	Deleterious	Damaging
mTOR	K1993T	Possibly damaging	Deleterious	Tolerated
NF1	R1412G	Probably damaging	Deleterious	Damaging
NOTCH1	R2549C	Possibly damaging	Neutral	Damaging
NOTCH2	Q466K	Probably damaging	Deleterious	Damaging
PBRM1	P1023L	Probably damaging	Deleterious	Damaging
PDGFRB	R604C	Probably damaging	Deleterious	Damaging
PIK3CA	L540H	Probably damaging	Deleterious	Tolerated
PIK3R1	K134N	Possibly damaging	Neutral	Damaging
PTEN	T277K	Probably damaging	Deleterious	Damaging
RANBP2	R176C	Benign	Deleterious	Damaging
RET	P1047S	Probably damaging	Deleterious	Damaging
RPTOR	S190L	Probably damaging	Deleterious	Damaging
SF3B1	S956F	Possibly damaging	Deleterious	Damaging
TOP1	N711Y	Possibly damaging	Deleterious	Damaging
Gene	INDEL/FS		MUTATIONTASTER	
CBFB	Q41*		Damaging	
FANCL	T367fs*13		Damaging	
MYST3	R1024*		Damaging	
TOP2A	L1048_N1050del		Damaging	
TSC2	F1510del		Damaging	

SNV: Single nucleotide variants; INDELS: insertions and deletions; FS: frameshift.

Evaluation of the activated pathways identifies genes involved in LAR breast tumorigenesis. We next wanted to investigate the genes involved in the retrieved pathways. To this end, we examined the list of genes with KGAs, possibly damaging VUS and their combination, included in each activated pathway (Figure 4) and calculated the percentage of pathway contribution for each gene.

As expected, the DAVID or PANTHER tools provided a superimposable set of genes involved in the activated pathways with KGAs (Figure 5A and D), while the gene sets were mostly different when considering pathways with possibly damaging VUS (Figure 5B and E). When we investigated activated pathways considering both KGAs and possibly damaging VUS, we found that *PIK3CA*, *PIK3R1*, *PTEN* and *TP53* were above the threshold according to both the DAVID and PANTHER tools (Figure 5C and F). Additional genes emerged from the combined analysis (Figure 5C and F, white columns) but only *AKT1* and *mTOR* were above our predefined threshold. These findings confirm that the TP53, PI3K-Akt and its downstream target mTOR are strongly involved in LAR breast carcinogenesis.

Evaluation of potential therapeutic approaches. Lastly, we matched genes with KGAs with potential targeted therapies and performed an online search of ongoing clinical trials that may be suitable for LAR breast cancer patients displaying the given molecular alterations (Table III). The clinical trial search was performed using the My Cancer Genome (https://www.mycancergenome.org) and ClinicalTrials.gov (https://clinicaltrials.gov) websites.

Five altered genes (*AKT1, CDK12, ERBB2*, *FANCC* and *PIK3CA*) were potentially actionable, of which 4 (*CDK12, ERBB2*, *FANCC* and *PIK3CA*) with commercially available drugs. Among these, *ERBB2* and *PIK3CA* were the only

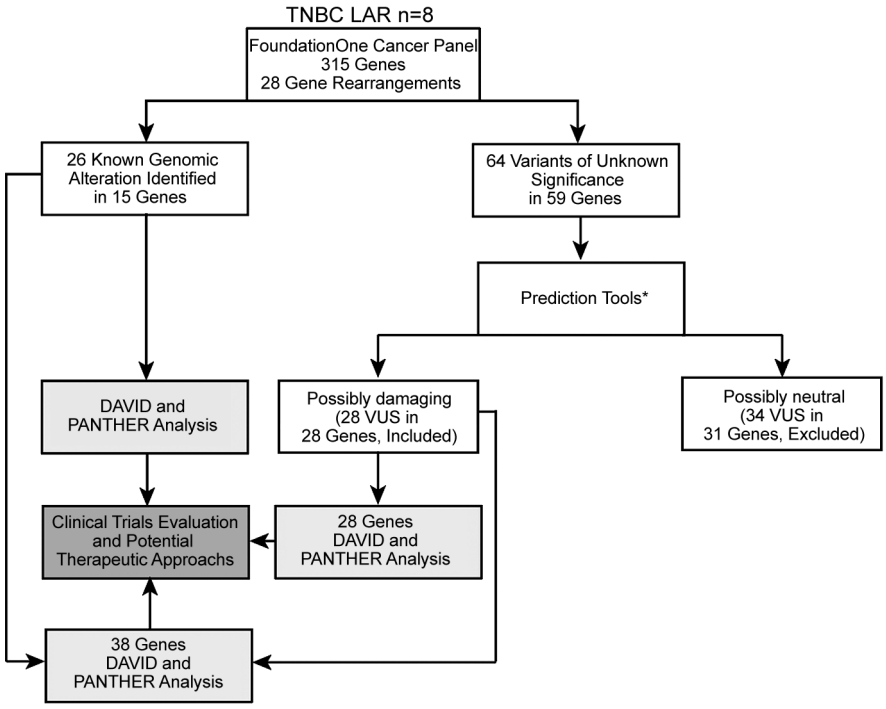

Figure 1. *Flow diagram of the study. Tumor samples from 8 luminal androgen receptor breast cancers underwent Next Generation Sequencing using the FoundationOne Cancer Panel, which interrogates 315 genes as well as introns of 28 genes involved in rearrangements. Twenty-six known genomic alterations (KGAs) and 64 variants of unknown significance (VUS) were identified. Variants of unknown significance were stratified according to their possibly damaging role as indicated by in silico prediction tools. Genes with KGAs, possibly damaging VUS and their combination, which totaled 38 genes as 5 were common between KGA and VUS, were analyzed with functional annotation tools (DAVID and PANTHER) and the results were matched with potential therapeutic targets. *Confirmed implication in breast cancer according to a literature search.*

genes retrieved in activated pathways. Alterations in all 5 genes may represent eligibility criteria for clinical trials. In addition, *CDH1, GATA3*, *MAP3K1, PIK3R1*, *PTEN* and *TP53* presented KGAs which cannot be targeted with available molecules but may candidate LAR breast cancer patients to ongoing studies. With the lone exception of *GATA3*, all these genes were found in activated pathways.

Discussion

Pursuing the goal of precision medicine for the treatment of cancer patients represents an imperative, especially for malignancies with unfavorable outcomes and limited therapeutic options (26-29). TNBC displays the worse prognosis among all breast cancer variants and the search for actionable targets is an urgent medical need. The dissection of TNBC molecular features unraveled a considerable heterogeneity encompassing at least 6 distinct pathological entities (10, 30). Among these, the LAR subtype is characterized by expression of the androgen receptor, which represents an appealing therapeutic target (31). However, the use of anti-androgen compounds has generated inconsistent and often suboptimal results in LAR breast cancer patients, while alternative targeted treatments have yet to be identified (15, 16, 32). In the present study we performed an extensive genomic sequencing in a small cohort of LAR breast cancer patients, searching for molecular alterations and activated pathways to leverage as therapeutic targets.

Clinical-pathological features of our cohort are in line with those previously reported. Indeed, while median age (*i.e.*, 74 years) is higher compared to that observed in TNBC (33), it mirrors the results of previous reports on LAR breast tumors (10, 34). Older age at diagnosis is usually associated with endocrine-sensitive breast cancer and our results reinforce the correlation between LAR and ER-positive tumors (35). Likewise, in our population median Ki67 proliferation index was below the 20% threshold, as expected from pre-existing evidence on LAR cancers. Of note, AR seems to play an anti-proliferative role that may explain the low proliferation rate observed in most of these tumors (36, 37).

Results of our NGS analysis are in line with previous characterizations of LAR tumors, which typically harbor SNVs rather than amplifications and indels (10). According to

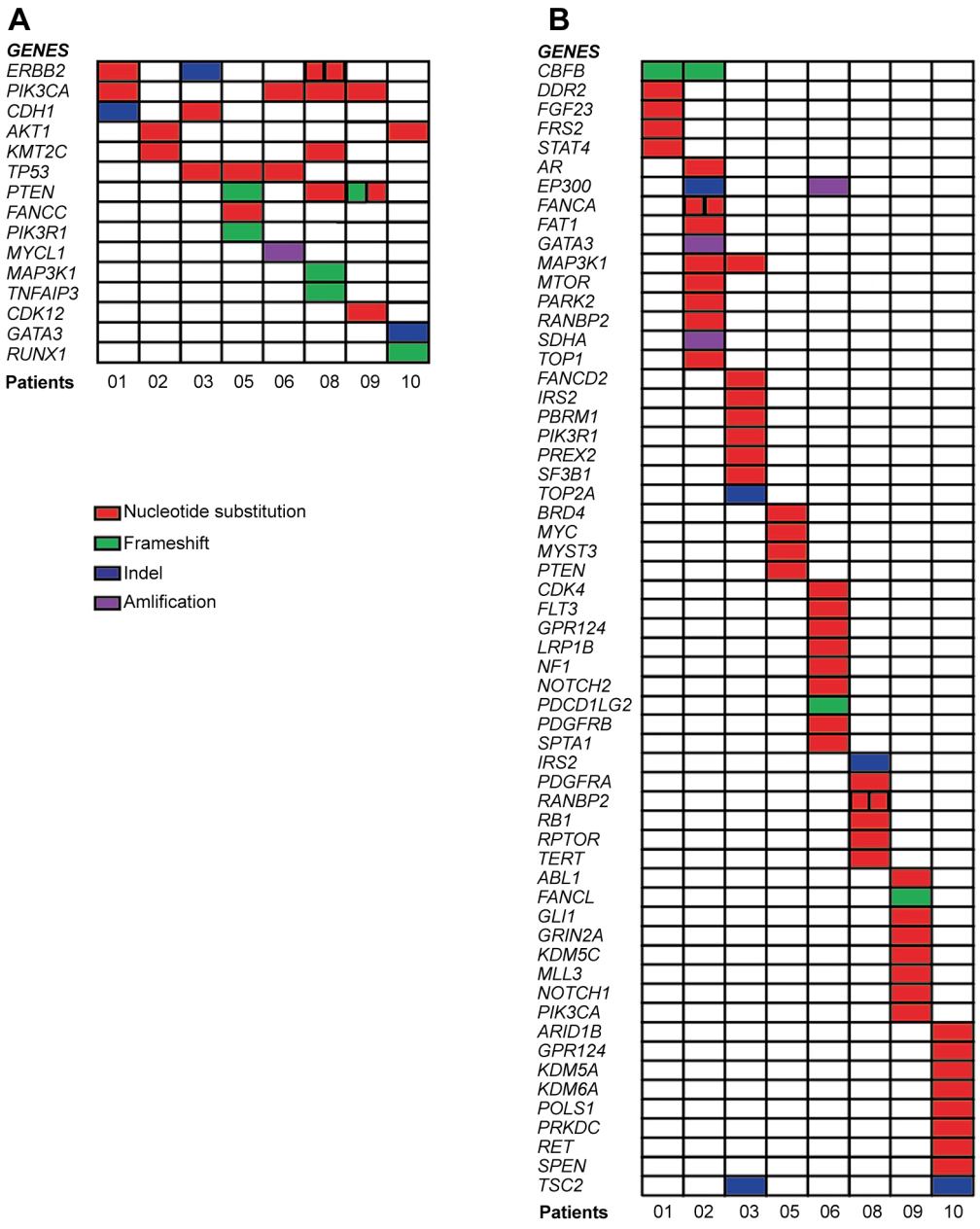

Figure 2. *Tile plot of the identified genomic alterations. Every colored square represents a specific gene alteration in each patient, indicated with a progressive number. Panel A displays known genomic alterations (KGAs) while panel B shows the variants of unknown significance (VUS) (B).*

TCGA, *TP53* is by far the most altered gene in TNBC (80%), followed by *PIK3CA* (9%). However, a specific analysis for LAR tumors was not included in TCGA (4). More recently, Bareche *et al.* carried out an integrative analysis combining somatic SNVs, CNV and gene expression profiles 550 TNBC derived from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC), specifically addressing at the different TNBC subtypes. According to their results, LAR breast tumors display a distinct molecular profile compared to the other molecular subtypes, with *PIK3CA*, *KMT2C*, *CDH1*, *NF1* and *AKT1* being the most frequently mutated genes (4, 10). Consistently, all these SNVs, except for NF1, were present in our cohort. However, we found a higher incidence of *TP53* as well as *PTEN* alterations. This discrepancy is also concordant with the results of Weismann *et al.* showing that apocrine TNBC displays a lower *TP53*

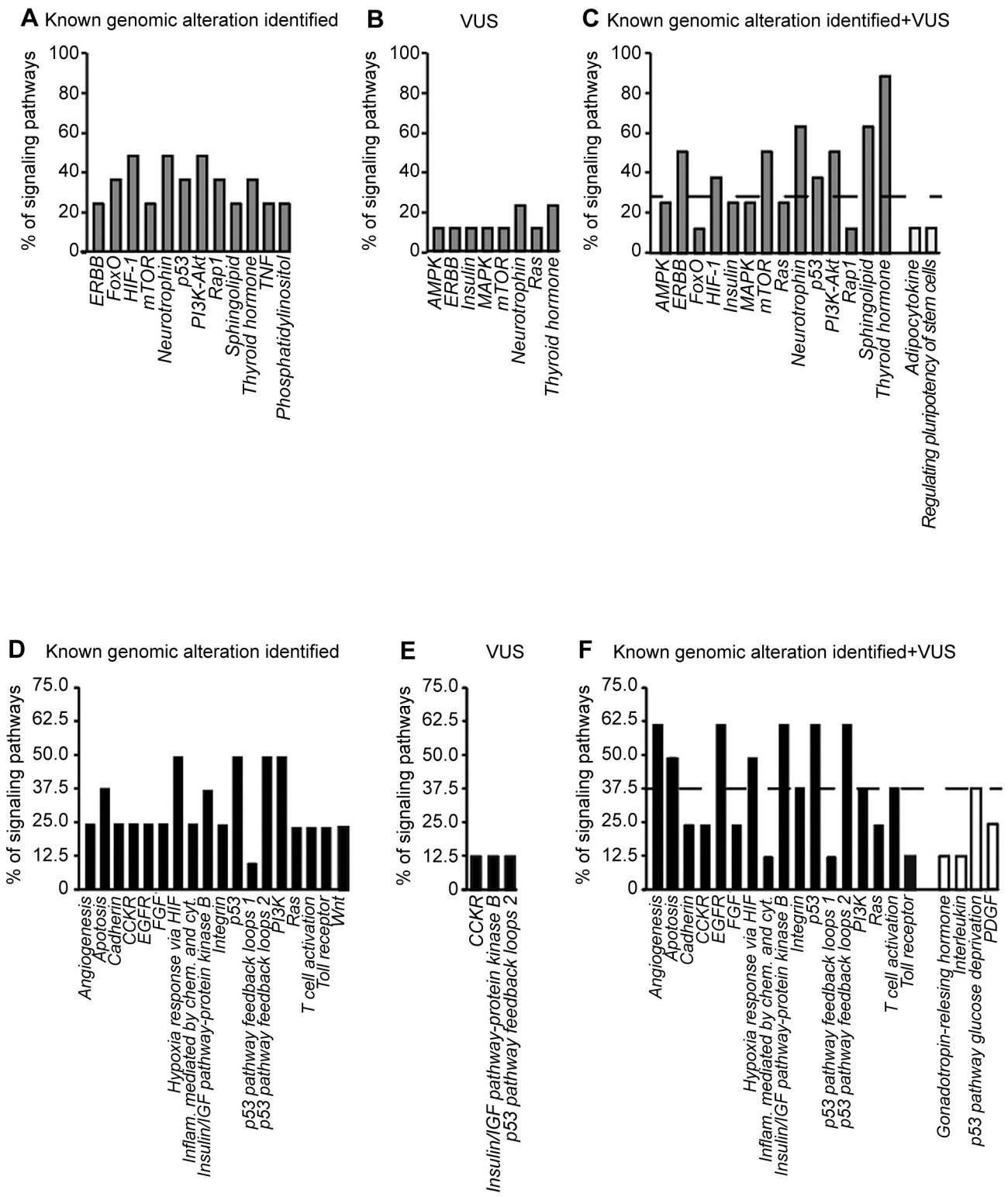

Figure 3. *Signaling pathways activated by known genomic alterations, possibly damaging variants of unknown significance (VUS), and their combinations. Bars represent the activated signaling pathways according to known genomic alterations (A-D), possibly damaging VUS (B-E) or both (C-F), analyzed using the DAVID (gray bars) or PANTHER (black bars) tools. The height of each bar varies according to the different rate of pathway involvement. White bars in the C and F panels indicate pathways identified exclusively by the combination of known genomic alterations and possibly damaging VUS. Dashed lines indicate the median rate of pathway involvement.*

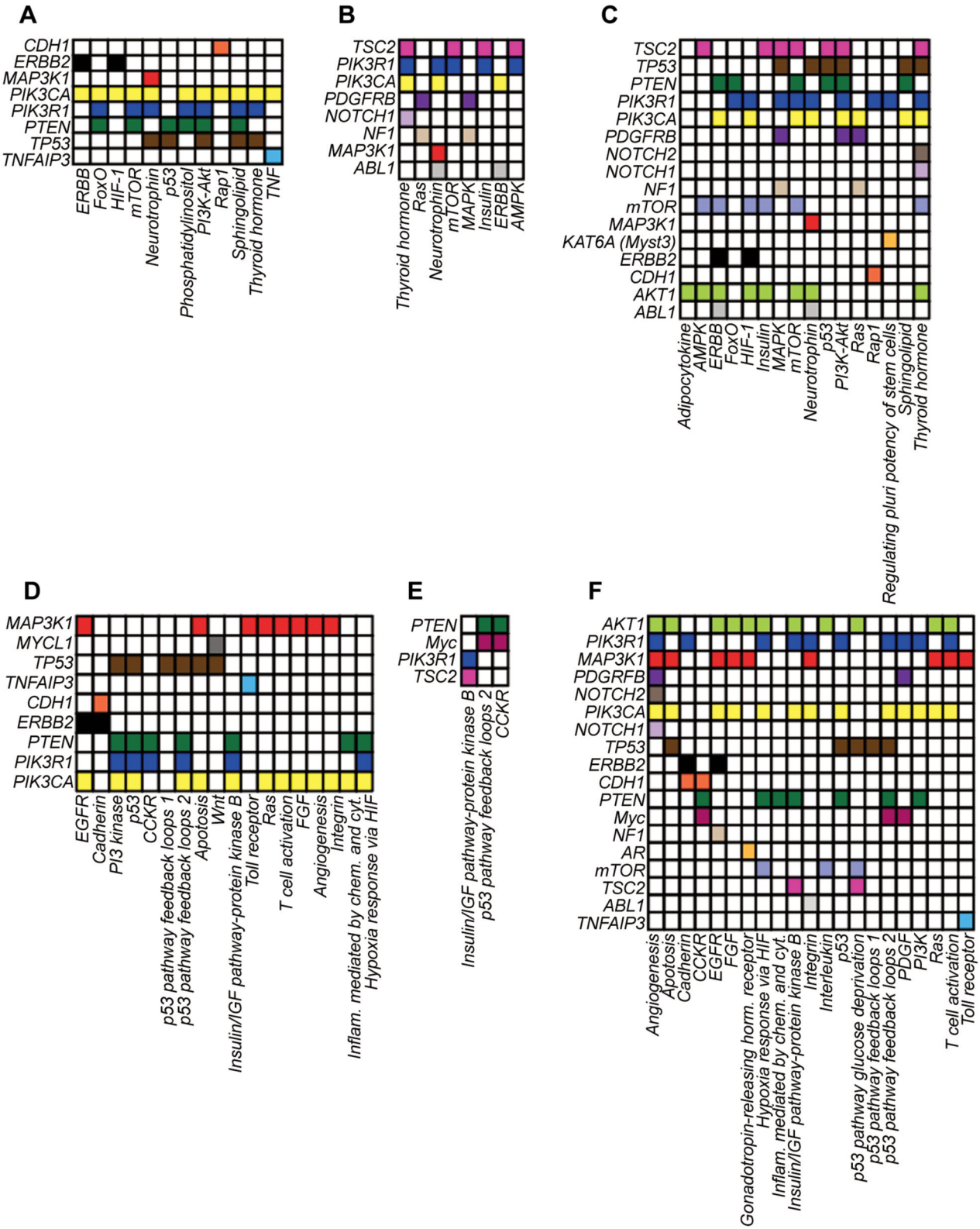

Figure 4. *Heatmap of altered genes involved in the activated signaling pathways of the eight LAR-TNBC patients. Panels display the involvement of the known genomic alterations (A-D), possibly damaging VUS (B-E) or both (C-F) in modulating the signaling pathways reported in Figures 3 and 4. Each gene is indicated with a different color.*

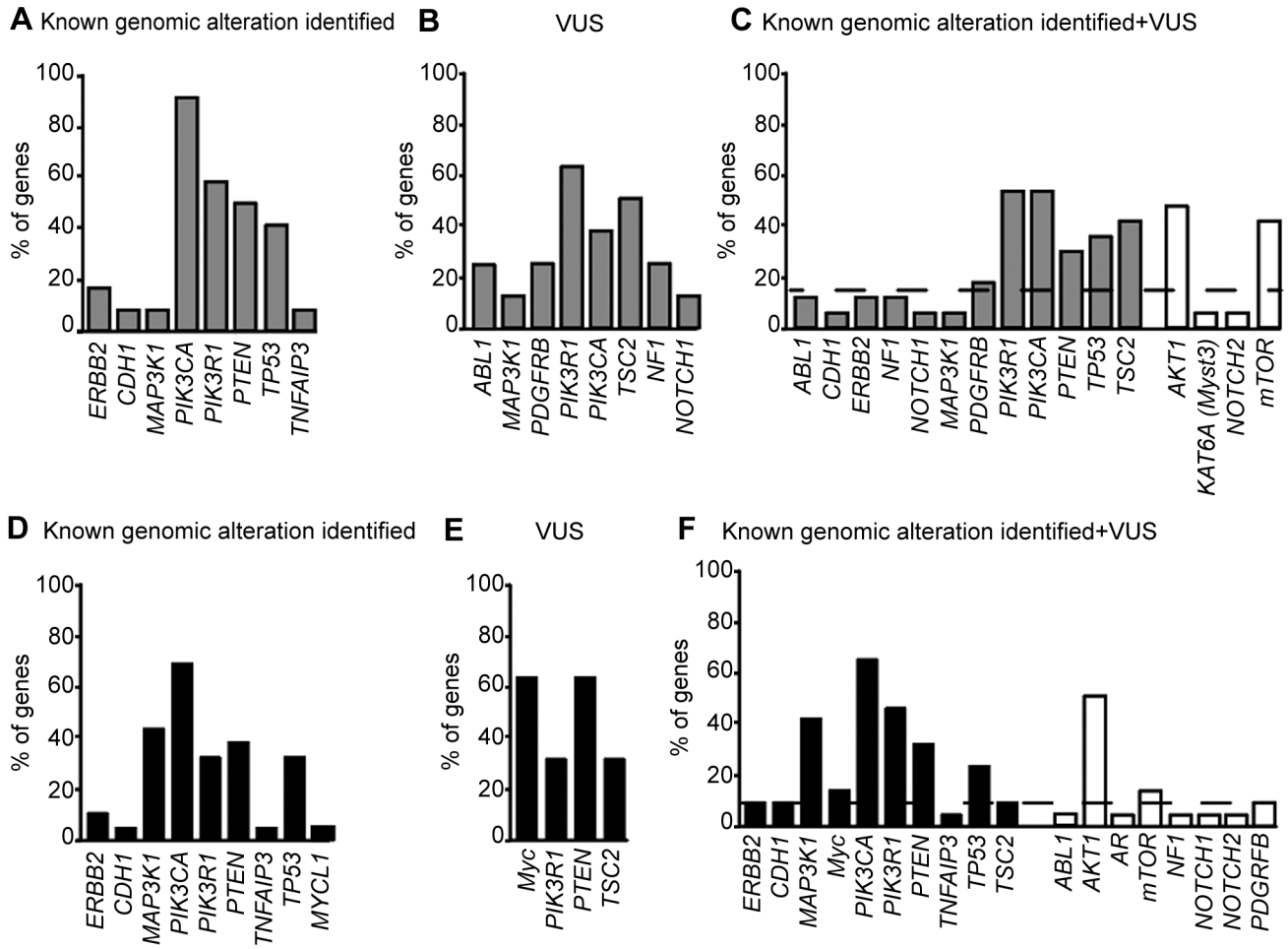

Figure 5. *Implication of known genomic alterations, variants of unknown significance (VUS), and their combination in activated pathways. Bars represent the known genomic alterations (A-D), possibly damaging VUS (B-E) or their combination (C-F) involved in the activated pathways reported in Figure 3 and generated by the DAVID (gray bars) or PANTHER (black bars) tools. The height of each bar varies according to the different rate of gene involvement in the different pathway. White columns in C and F panels indicate genes identified exclusively by the combination of known genomic alterations and possibly damaging VUS. The dashed line identifies the median rate gene involvement in the different pathways.*

mutational rate compared with other TNBCs and may be explained by the small number of patients in our study (34).

Functional analysis of VUS by *in silico* prediction tools revealed novel genes, such as *CBFB*, and *EP300* that may be implicated in LAR tumorigenesis although additional studies are needed to confirm this evidence.

Several studies have investigated the more frequently activated pathways in LAR breast cancers, demonstrating a central role for PI3K and TP53 signaling (34, 38). Moreover, estrogen and androgen response as well as adipogenesis, fatty acid metabolism and protein secretion pathways have also been implicated in LAR carcinogenesis (14). Our analysis corroborates a significant contribution of PI3K and TP53, while also including additional pathways, such as apoptosis, angiogenesis, integrin, ERBB, hypoxia and MAPK. The information emerging from combination of KGAs, and possibly damaging VUS implies that unknown variants might also contribute to LAR carcinogenesis. In any case, we confirm a pivotal role for PI3K signaling in our cohort. This correlation has already been observed in apocrine triple-negative breast cancers (34). Indeed, a mechanistic link between the PI3K pathway and AR signaling was initially demonstrated in prostate cancer, while preclinical evidence suggests an interplay between AR and PIK3CA in supporting LAR cancer pathogenesis (39, 40).

Given the preeminent role of PI3K signaling in LAR breast cancers, targeting the components of this pathway presents a strong biological rationale. A study on patient-derived xenografts (PDX) of LAR TNBC resistant to anti-androgens showed a remarkable sensitivity towards PIK3CA

Table III. *Potential targeted treatments and clinical trials for retrieved alterations*.

Genes with actionable KGAs	Potential targeted drugs	Selected clinical trials
AKT1	Capivasertib*, Ipatasertib*	NCT03805399, NCT04551521
CDH1	None	NCT03620643
CDK12	Olaparib, Niraparib, Rucaparib, Talazoparib	NCT04983745, NCT04826341, NCT04692662, NCT04550494, NCT04123366, NCT03842228
ERBB	Lapatinib, Neratinib, Peruzumab, Poziotinib*, Pyrotinib*, Trastuzumab, Trastuzumab deruxtecan, Trastuzumab ematansine, Tucatinib	NCT04551521, NCT04209465, NCT04172597, NCT04579380,
FANCC	Olaparib, Niraparib, Rucaparib, Talazoparib	NCT02401347, NCT03742895, NCT03767075, NCT04550494, NCT04983745, NCT04826341
GATA3	None	NCT02576665
MAP3K1	None	NCT03162627, NCT03520075, NCT04528836, NCT04551521
PIK3CA	Alpelisib, Buparlisib*, Gedatolisib*	NCT04774952, NCT04632992, NCT04589845, NCT04586335, NCT03337724, NCT04317105, NCT03006172, NCT02583542
PIK3R1	None	NCT04774952, NCT04551521, NCT03673787
PTEN	None	NCT03673787, NCT04774952, NCT04632992, NCT04586270, NCT04551521, NCT04317105, NCT04251533, NCT03065062, NCT03207529
TP53	None	NCT04383938, NCT04293094

*Drugs in clinical development. Genes in bold are those retrieved in activated signaling pathways. KGAs: Known genetic alterations.

and mToR inhibitors (41). Additional preclinical evidence demonstrated that dual blockade of AR and PI3K has a synergistic effect on AR-positive TNBC cell lines and PDX (38). Multiple clinical trials are testing PI3K/mToR/AKT inhibitors in patients harboring alterations in this pathway (Table III), with one specifically addressing LAR breast cancer patients by combining the PIK3CA inhibitor alpelisib with the anti-androgen enzalutamide (NCT03207529) (42).

HER2 represents a further potentially relevant target for patients with LAR breast cancer, since four *ERBB2* mutations emerged in three subjects included in our cohort. Somatic alterations in *ERBB2* are usually considered driver events in breast cancer and a consolidated body of evidence suggests that they may be effectively targeted with anti-HER2 agents even in absence of *HER2* amplification (43, 44). Among the *ERBB2* mutations detected in our cohort, one was the 755-759 *in frame* deletion, which increases ERBB2 heterodimerization resulting in higher phosphorylation of EGFR and HER3 (44). This alteration confers sensitivity towards EGFR inhibitors and neratinib, but apparently induces resistance to lapatinib (44-47). Of the other *ERBB2* mutations retrieved, two (S310F and S653C) are activating, while one (I767M) has no functional effect (44, 48, 49). However, $HER2^{I767M}$ responds to conventional anti-HER2 therapies (trastuzumab, lapatinib, neratinib) (44, 50). Of note, while a trial is investigating combinations of AR and HER2 inhibitors in AR positive/*HER2*-amplified breast cancer (NCT02091960), no studies are available for HER2-mutated LAR TNBC.

In conclusion, despite the small number of patients included, our study defines a molecular portrait of LAR tumors which is in line with previous evidence. Functional analyses incorporating KGAs and VUS to explore activated pathways not only reinforce pre-existing knowledge, but also provide information concerning potential targeted treatments. Future studies are warranted to shed light on the clinical impact of these molecular alterations in LAR breast tumors.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: SS, SVR, MM, MC and PV; methodology: SS, MM, SVR, CF, GMV, CL, GM and FM; software: SS, SRV, CL, MM and GM; validation: SS, SRV and MM; formal analysis: SS, SVR, CL, GM and MM; investigation: SS, SVR, MM and PV; resources: CL, GM, KL, NI and RC; data curation: SS, SRV, MM, LM and PV; writing - original draft preparation: SS, SVR, LM and FM; writing - review and editing: SVR, FM, MM, PV, SS and LM; visualization: SS, SRV, MM, CL, GM, KL, FM, CF, GMV, ET, RC, NI, LM, MC and PV; supervision: PV. All Authors have read and agreed to the published version of the manuscript.

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