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LETTER TO THE EDITOR

Next-Generation Sequencing Identifies Novel RTK VUSs in Breast Cancer with an Emphasis on *ROS1*, *ERBB4*, *ALK* and *NTRK3*

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Gain-of-function mutations and alterations in RTKs contribute to cancer development and investigation of targeted therapies against these abnormalities is expanding. Comprising 20 subfamilies, 58 human RTKs each have an extracellular domain (ECD), single transmembrane helix (TM), and cytoplasmic tyrosine kinase domain (TKD) located between juxtamembrane (JM) and C-terminal (CT) regulatory regions. Utilizing NGS, we sought to classify test-defined VUSs throughout RTKs' conserved topology in breast cancer.

We assessed a breast cancer patient database at West Cancer Center (Memphis, Tennessee) from 2013 to 2015. All patients received tumor profiling with a 592-gene NGS panel from Caris Life Sciences (Phoenix, Arizona); ER, PR and HER2 status was reviewed. The entire coding sequence was interrogated of 29 cancer-implicated RTKs: *ALK*, *AXL*, *cKIT*, *cMET*, *CSF1R*, *DDR2*, *EGFR*, *EPHA3*, *EPHA5*, *EPHB1*, *ERBB2–4*, *FGFR1–4*, *FLT1*, *FLT3*, *FLT4*, *IGF1R*, *KDR*, *NTRK1–3*, *PDGFRA*, *PDGFRB*, *RET* and *ROS1*. Single nucleotide variants test-defined as pathogenic (PATH) or variants of undetermined significance (VUS) were reported. In silico analysis with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was completed to predict the pathogenicity of VUS. Any VUS predicted-damaging we designate VUSp. Variants were then catalogued as ECD, TM, JM, TKD or CT.

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78 patients were found with median age of 58 years (range 32–83). 99% were female; 59% were Caucasian, 38% African-American. 71 were invasive ductal carcinoma, 4 invasive lobular and 1 each were invasive mammary, inflammatory and not-specified. 77 samples had ER/PR/HER2 status: 10% were triple-positive, 46% ER/PR+, 13% HER2+ and 31% triple-negative. 24% were PIK3CA+, 6% AKT1+. 12 patients (15%) harbored 14 *BRCA1/2* aberrations (5 PATHs in 4 patients and 9 VUS in 8 patients).

75 VUS and 1 PATH (*ERBB3*, TKD S846I) were found. 51/78 (65%) patients had ≥1 RTK VUS (range 0–4) and VUS were seen in 97% RTKs (excluding *FLT3*) with a median 2 (range 0–15). 34/75 (45%) VUS were VUSp and found in 27 patients (35%). 17/29 (59%) RTKs had a VUSp, with median 1. RTKs most frequently mutated were *ROS1* (12/15 VUS were VUSp), *EPHA5* (3/3; all VUSp were ECD E528Q), *FLT4* (2/5; both VUSp were TKD P1008L), *cKIT* (2/4), *ALK* (2/3), *ERBB4* (2/3) and *NTRK3* (2/2). RTK VUS were seen in 100% triple-positive patients (6/8 were VUSp), 69% ER/PR+ (17/32), 60% HER2 (2/10) and 58% triple-negative (3/24). RTKs contained VUS distributed among their domains: 57% were ECD (20/43 were VUSp), 17% TKD (9/13), 9% TM (1/7), 8% CT (2/6), and 8% JM (2/6). Public databases dbSNP and ExAC documented 21/31 unique VUSp; all ExAC-identified minor allele frequencies (MAF) were < 0.0003. 5/31 VUSp were reported on COSMIC and found in *ROS1* (2; Table 1), *NTRK3* (JM T490 M; Table 1), *cMET* (ECD R359Q; COSM1286164) and *RET* (TM S649 L; COSM4170226).

Interestingly, 15/71 (21%) unique VUS and 12/31 (39%) VUSp were found in *ROS1*. While *ROS1* single nucleotide variants are observed in solid tumors, the only published breast cancer case we found was a metastatic, triple-negative patient with a lesion (TKD Y2092C) not sensitive to crizotinib [1]. In our cohort, *ROS1* VUS were more frequently VUSp (80%) than the 28 other RTKs

Table 1 *ROS1*, *ERBB4*, *ALK* and *NTRK3* VUS in breast cancer patients predicted-damaging with in silico analysis

Gene	VUS; Caris allele frequency	Domain	Accession Number; ExAC MAF	COSMIC ID	Stage; Biopsy Site	Age, race, gender	Genomics (ER/PR/HER2, PIK3CA, AKT1, BRCA1/2)
ROS1	S85R; 14%	ECD	rs369171859; *T* = 5.0e-5	Not found	IV; Metastasis	52, C, F	ER+/PR-/HER2-, PIK3CA+
	R466Q, 49%	ECD	rs140178288; *T* = 9.1e-5	1,072,614	II	41, AA, F	ER+/PR+/HER2-, BRCA2 VUS (D551G)
	H1074P; 12%	ECD	None	Not found	IV; Metastasis	54, C, F	ER+/PR+/HER2-, BRCA2 PATH (N3124I)
	G1135E; 49%	ECD	rs142442666; *T* = 4.1e-5	Not found	IV; Metastasis	54, C, F	ER+/PR+/HER2-
	L1238R; 38%	ECD	rs765579396; C = 8.3e-6	Not found	II	48, AA, F	ER+/PR+/HER2-, BRCA2 VUS (C1960Y), PIK3CA+
	L1516F; 8%	ECD	None	Not found	IV; Metastasis	56, C, F	ER+/PR-/HER2-
	L1719I; 63%	ECD	None	Not found	IV; Metastasis	41, C, F	ER+/PR+/HER2-, PIK3CA+
	G1809E; 22%	ECD	None	5,890,218	IV; Metastasis	73, C, F	ER+/PR-/HER2-
	I1848L; 31%	ECD	rs765553432; G = 1.7e-5	Not found	IV; Metastasis	43, C, F	ER+/PR+/HER2-, BRCA2 PATH (K242X), PIK3CA+
	G1915R; 39%	JM	rs759091073; T = 4.1e-5	Not found	II	51, C, F	ER-/PR-/HER2+
	M2029 L; 95%	TKD	rs199682124; A = 3.3e-5	Not found	IV; Metastasis	41, AA, F	ER+/PR+/HER2-
	Y2274C; 84%	CT	None	Not found	IV; Metastasis	31, C, F	ER+/PR+/HER2-, BRCA2 VUS (M408I)
ERBB4	I436M; 10%	ECD	None	Not found	IV; Metastasis	59, C, F	ER+/PR+/HER2-; PIK3CA+
	E934V; 47%	TKD	None	Not found	IV; Primary	65, AA, F	ER+/PR+/HER2+
ALK	P40S; 48%	ECD	rs371679329; 8.2e-5	Not found	IV; Metastasis	37, AA, F	ER+/PR+/HER2+
	R136W; 54%	ECD	None	Not found	IV; Metastasis	59, AA, F	ER+/PR+/HER2-
NTRK3	T490 M; 67%	JM	rs761822626; A = 6.0e-5	2,014,299	IV; Metastasis	61, AA, F	ER+/PR-/HER2-
	A631T 14%	TKD	rs56300182; *T* = 0.0001	Not found	IV; Metastasis	52, C, F	ER-/PR-/HER2+

(37%; $\chi^2 = 12.1$, $p = 0.0005$). Of 12 *ROS1* VUSp, 9 were ECD, 1 JM, TKD and CT; 11/12 were ER/PR+ and 1/12 HER2+. 5/12 (42%) *ROS1* VUSp+ samples were co-mutated with *BRCA2* (2 PATH, 3 VUS) accounting for 5/8 RTK VUS found in the cohort's 12 BRCA1/2+ patients.

Other genes worth noting are *ERBB4*, *ALK*, and *NTRK3* (Table 1). While *ERBB4* single nucleotide variants are observed in solid tumors, ECD and TKD mutations located at dimerization interfaces were only recently demonstrated as activating in non-small cell lung cancer (NSCLC) [2]. In our 78-patient cohort, 2 patients had *ERBB4* VUSp (1 ECD and TKD) and TKD E934V is flanked by known activating lesions (D931Y and K935I) in NSCLC discovered by Kurppa et al. thus warranting further investigation. We also identified 2 *ALK* ECD VUSp. Although oncogenic *ALK* TKD mutations are largely detailed in neuroblastoma, activating extra-TKD missense mutations occur in other cancers [3]. Additionally, *NTRK3* JM and TKD VUSp were found. Point mutations in *NTRK3* have been recognized in breast, lung, colorectal and pancreatic tumors for over a decade and postulated to impact the kinase domain's activation loop [4, 5].

Overall, >65% breast cancer patients contained VUSs and 35% VUSp in ≥1/29 RTKs. VUSp were distributed throughout RTKs domains and present in >50% RTKs. *ROS1* VUS were the most common, more likely to be VUSp and 5/12 *ROS1* pnsSNP+ biopsies were BRCA1/2+ (2 PATH, 3 VUS). The frequency of *ROS1* mutations should be evaluated on a larger scale to see if these associations extrapolate, have prognostic significance or are targetable. Novel RTK VUSp in *ERBB4*, *ALK*, *NTRK3* and others likewise warrant further classification in breast cancer.

Authors' contributions All authors contributed equally to the data collection, analysis, writing and editing the manuscript.

Compliance with Ethical Standards

Ethics Approval This study was conducted in accordance with the Declaration of Helsinki.

Competing Interests The authors declare that they have no competing interests.

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