SUPPLEMENTARY MATERIAL

Supplementary Methods

Study Design

Patients were randomized (1:1 ratio) to either N+C (neratinib 240 mg orally once daily continuously in 21-day cycles with no break between cycles, plus capecitabine 750 mg/m^2 orally twice daily on days 1-14 of the 21-day cycle) or L+C (lapatinib 1250 mg orally once daily continuously, in 21-day cycles with no break between cycles, plus capecitabine 1000 mg/m^2 orally twice daily on days 1-14 of the 21-day cycle).

The randomization sequence was stratified by: hormone receptor status (hormone receptor-positive (estrogen receptor- or progesterone receptor-positive, or both; hormone-receptor positivity defined per Dako test kit guidelines [1]) *v* hormone receptor-negative [estrogen and progesterone receptor-negative]); number of previous HER2-directed therapies for metastatic breast cancer (2 or ≥3); geographic region (North America or Europe (including Israel) or rest of the world); and visceral disease (yes or no).

Outcomes

Co-primary endpoints were: independently adjudicated PFS (date of randomization until first progression per Response Evaluation Criteria in Solid Tumors [version 1.1] or death due to any cause was documented, censored at last assessable evaluation or initiation of new anti-cancer therapy); and OS (time from randomization to death due to any cause). Tumor assessments were performed every 6 weeks using computed tomography and magnetic resonance imaging.

Co-primary endpoints were analyzed using an overall Type I error rate of 0.01 for PFS and 0.04 for OS. For centrally assessed PFS, 419 events (progressive disease or death) were required for 85% power to detect an HR (control *v* treatment) of 0.70. For OS, 378 events (deaths) were required for 85% power to detect an HR of 0.725. The trial was considered positive if either PFS or OS were statistically significant at the split alpha level. Approximately 600 patients were to be enrolled and randomized equally between the two groups.

Assays

A quality check of the raw data was performed by FastQC. Reads were filtered first by quality using FASTX-Toolkit (version 0.0.14). Primer sequence was trimmed and reads were further filtered by length using HOMER (version 4.7) [2]. The remaining reads were re-synced by Pairfq and mapped to human reference genome hg19 by BWA (version 0.7.12) with default settings. The resulting BAM (binary alignment map) files were processed using SAMtools (version 1.2) and the Genome Analysis ToolKit (GATK version 3.4.0). In brief, BAM files were binary compressed, sorted, and indexed by SAMtools (SAMtools view, sort and index tools) and base quality score recalibration and local realignment around insertions and deletions followed the best practices of the GATK toolkit (RealignerTargetCreator, IndelRealigner, BaseRecalibrator and PrintReads).

Somatic variants were called using VarScan2 (version 2.3.9 [3]) with the following parameters: minimum variant allele frequency 3%, total coverage ≥10 reads; variant coverage ≥7 reads, and a *P*-value <0.05. Variant annotation was performed with ANNOVAR [4]. Variants were filtered successively: variant positions could not be listed as a single nucleotide polymorphism (SNP) in the 1,000 Genome project with a minor allele

frequency >0.05; variant position had to be annotated as exonic in the US National Center for Biotechnology Information Reference Sequence (RefSeq) collection; and synonymous/nonsynonymous calls were made and the synonymous calls excluded for further analysis. Filtering was performed using in-house parsers and all candidate mutations were reviewed manually using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

For the somatic mutation analysis, 20 µL final volume of TaqMan polymerase chain reaction (PCR) reaction mixture was assembled with 1× droplet digital PCR Supermix for Probes (no dUTP), 900 nM of each primer, 250 nM of each probe, and 5-10 ng of genomic DNA. Each assay was performed in triplicate in separate mixes and loaded in different wells for amplification. The thermal cycling program was performed according to manufacturer specifications. After PCR, droplets were read in the Droplet Reader and analyzed with QuantaSoft (version 1.7.4, Bio-Rad Laboratories, Hercules, CA, USA). Human reference genomic DNA was included as negative control and used to determine the cutoff for allele calling in each assay.

SUPPLEMENTARY REFERENCES

1. Dako. ER/PR pharmDxTM Interpretation Manual. (Available at: https://www.agilent.com/cs/library/usermanuals/public/28252_er-pr_pharmdx_interpretation_manual.pdf). Date accessed: November, 2019

2. Heinz S, Benner C, Spann N et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010;38:576-589.

3. Koboldt DC, Chen K, Wylie T et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics 2009;25:2283-2285.

4. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.

TABLE S1. VHIO-Card amplicon-seq panel v4: regions included

Gene Name	RefSeq	Regions
ABL1	NM_005157	E238-Y257; T315-L324; S349-I360; T389-F401
AKT1	NM_005163	E17-R23; V167-K183
AKT2	NM_001626	E17-I19; D293-G304; F369-L381
ALK	NM_004304	G1121-V1135; K1173-C1182; V1185-L1198; Q1238-H1247
APC	NM_000038	L204-A214; T281-V291; S295-H307; V557-A571; P865-L878; T1112-N1124; S1275-T1380; R1386-S1398; S1403-I1418; D1422-A1475; A1485-D1498; E1530-S1539; D1571-P1584
BRAF	NM_004333	K439-D445; R462-H477; I582-F610; Q612-M620
CDH1	NM_004360	S337-F338; Y380-Q383
CDKN2A	NM_000077	A13-R22; G45-P70; H83-L104; L121-A134
CSF1R	NM_005211	S298-E306
CTNNB1	NM_001098210	D6-S45
EGFR	NM_005228	I107-N115; E282-V292; P589-G601; T710-V726; G729-L798; N808-W817; R831-V843; Q849-G863
HER2	NM_004448	N302-P316; G462-P481; G668-Q680; K753-G787; R840-L852; A890-S903; L313-D326
HER3	NM_001982	Y53-L67; E82-S95; N101-D112; F294-P306; C331-Q341; F351-T366; W373-K383; I454-H465; G526-L536; L775-L796; K926-I938; Q1045-G1053; T1169-L1177; Y1260-P1272
ESR1	NM_000125	S301-D313; K520-E561
FBXW7	NM_001013415	F158-I163; T345-R355; V378-V389; Q463-I475
FGFR1	NM_023110	R250-A263
FGFR2	NM_000141	R251-L262; P363-V385; K543-T555
FGFR3	NM_000142	E362-S408; Y648-N653; L690-E702
FLT3	NM_004119	L601-A620; D829-S840
GATA1	NM_002049	G7-V19; A68-P73
GNA11	NM_002067	G207-H218
GNAQ	NM_002072	G207-H218
GNAS	NM_000516	D196-L203; F208-F219
HRAS	NM_176795	V7-L19; A59-S65
IDH1	NM_005896	G123-Y135
IDH2	NM_001289910	T138-C154; P162-Q178
JAK1	NM_002227	Q134-T147
JAK3	NM_000215	L129-Q140; S568-S574; S720-A728
KIT	NM_000222	V50-D60; K550-F591; E640-T661; L667-Y672; A795-I808; I817-N828; W835-T847
KRAS	NM_033360	Y4-G15; S17-F28; I55-S65; Y137-T148
MAG	NM_080600	Q198-C217

Gene Name	RefSeq	Regions
MAP2K1	NM_002755	Q116-V127
MET	NM_001127500	K368-K380; I942-I953; S984-V993; S1006-E1017; H1106-K1128; R1245-H1256; V1265-Q1276
MLH1	NM_000249	S374-T386
MPL	NM_005373	A506-Q516
MSH6	NM_000179	P1077-L1089
MYC	NM_002467	Q48-P60; P72-G83
NF2	NM_000268	W191-A199; Y207-G218; I254-I264; E335-E348; L458-K469
NOTCH1	NM_017617	V1676-S1689
NOTCH4	NM_004557	C1971-A1989
NRAS	NM_002524	V9-T20; D57-D69
PDGFRA	NM_006206	P124-L137; R560-P577; P653-K666; P669-D681; A840-K852; S866-L871; E1065-I1078
PIK3CA	NM_006218	L58-F70; E81-C90; N107-N114; A400-G411; P449-I459; R537-L551; R899-L911; F1016-L1028; Y1038-G1049
PIK3R1	NM_181523	L449-L466; E558-L581; D643-A658
PIK3R5	NM_014308	C20-T31
PTEN	NM_000314	M1-T26; C71-C83; A86-E99; E106-D153; G165-S179; P213-V216; V222-K254; K260-K267; S287-D301; E315-A328; K332-K342
RB1	NM_000321	H129-N146; F351-K359; L452-E458; L569-E580; A658-L670; L743-S758
RET	NM_020975	G69-E82; I551-T562; P613-I625; P628-A640; D874-E884; D892-Y905; K916-H926; A1106-*1115
RUNX1	NM_001001890	S73-P86; R130-S140; A160-R177
SMAD4	NM_005359	I240-N251; S343-S368; I383-G395
SMARCB1	NM_003073	N34-R52
SRC	NM_198291	S525-G533
STK11	NM_000455	T32-K44; G47-G58; K83-A93; V133-P144; L164-H174; G188-A198; Y253-E265; S271-S283; P323-S334
TP53	NM_000546	A83-A307
VHL	NM_000551	R58-R108; H115-L135; P146-R167; K171-Y185

Abbreviation: RefSeq, US National Center for Biotechnology Information Reference Sequence.

TABLE S2. Patient characteristics according to availability of NGS and HER2 test results

Characteristic, n (%)	All patients (*N*=621)	NGS result		HERmark result		p95 result	
		No (*N*=201)	Yes (*N*=420)	No (*N*=95)	Yes (*N*=526)	No (*N*=170)	Yes (*N*=451)
Age, years							
<65	492 (79.2)	164 (81.6)	328 (78.1)	79 (83.2)	413 (78.5)	136 (80.0)	356 (78.9)
≥65	129 (20.8)	37 (18.4)	92 (21.9)	16 (16.8)	113 (21.5)	34 (20.0)	95 (21.1)
Region							
Europe	244 (39.3)	85 (42.3)	159 (37.9)	37 (38.9)	207 (39.4)	67 (39.4)	177 (39.2)
North America	124 (20.0)	43 (21.4)	81 (19.3)	33 (34.7)	91 (17.3)	50 (29.4)	74 (16.4)
Rest of world	253 (40.7)	73 (36.3)	180 (42.9)	25 (26.3)	228 (43.3)	53 (31.2)	200 (44.3)
ECOG PS							
0	338 (54.4)	120 (59.7)	218 (51.9)	52 (54.7)	286 (54.4)	94 (55.3)	244 (54.1)
1	283 (45.6)	81 (40.3)	202 (48.1)	43 (45.3)	240 (45.6)	76 (44.7)	207 (45.9)
Disease location							
Nonvisceral	121 (19.5)	36 (17.9)	85 (20.2)	16 (16.8)	105 (20.0)	31 (18.2)	90 (20.0)
Visceral	500 (80.5)	165 (82.1)	335 (79.8)	79 (83.2)	421 (80.0)	139 (81.8)	361 (80.0)
Hormone receptor status (Targos)							
Negative	264 (42.5)	84 (41.8)	180 (42.9)	42 (44.2)	222 (42.2)	67 (39.4)	197 (43.7)
Positive	357 (57.5)	117 (58.2)	240 (57.1)	53 (55.8)	304 (57.8)	103 (60.6)	254 (56.3)
Prior HER2 therapy							
Trastuzumab only	237 (38.2)	74 (36.8)	163 (38.8)	32 (33.7)	205 (39.0)	63 (37.1)	174 (38.6)
Trastuzumab + pertuzumab	47 (7.6)	15 (7.5)	32 (7.6)	8 (8.4)	39 (7.4)	13 (7.6)	34 (7.5)
Trastuzumab + T-DM1	215 (34.6)	74 (36.8)	141 (33.6)	35 (36.8)	180 (34.2)	66 (38.8)	149 (33.0)
Trastuzumab + pertuzumab + T-DM1	122 (19.6)	38 (18.9)	84 (20.0)	20 (21.1)	102 (19.4)	28 (16.5)	94 (20.8)
No. of prior HER2-directed therapies							
2	430 (69.2)	141 (70.1)	289 (68.8)	65 (68.4)	365 (69.4)	108 (63.5)	322 (71.4)
3+	191 (30.8)	60 (29.9)	131 (31.2)	30 (31.6)	161 (30.6)	62 (36.5)	129 (28.6)

Differences in sample sizes due to differences in technology performance using small amounts of tissue. Because of rounding, not all percentages add up to 100%.

Abbreviations: ECOG, Eastern Cooperative Oncology Group; NGS, next-generation sequencing; PS, performance status; T-DM1, trastuzumab emtansine.

TABLE S3. HER2 scores stratified by hormone receptor status

	Hormone Receptor Status		
HER2 score	**Positive (*N*=357)**	**Negative (*N*=264)**	**Total (*N*=621)**
IHC (Targos), n (%)			
2+	142 (39.8)	53 (20.1)	195 (31.4)
3+	215 (60.2)	211 (79.9)	426 (68.6)
HERmark, n (%)			
Low	88 (24.6)	38 (14.4)	126 (20.3)
Equivocal	41 (11.5)	22 (8.3)	63 (10.1)
High	175 (49.0)	162 (61.4)	337 (54.3)
Missing	53 (14.8)	42 (15.9)	95 (15.3)
HERmark, RF/mm^2			
N	304	222	526
Mean	69.3	126.0	93.2
SD	107.9	164.3	137.3
Median	22.1	72.7	39.1
Minimum, maximum	0.9, 643	1.0, 1179	0.9, 1179
H-Score, n (%)			
≥240	135 (37.8)	175 (66.3)	310 (49.9)
<240	220 (61.6)	85 (32.2)	305 (49.1)
Missing	2 (0.6)	4 (1.5)	6 (1.0)
H-Score, RF/mm^2			
N	355	260	615
Mean	212.2	242.0	224.8
SD	62.0	56.1	61.3
Median	220.0	260.0	240.0
Minimum, maximum	35.0, 300.0	35.0, 300.0	35.0, 300.0

Abbreviations: IHC, immunohistochemistry; RF, relative fluorescence; SD, standard deviation.

TABLE S4. HER2 scores stratified by *PIK3CA* mutation status

HER2 score	*PIK3CA* Mutation Status: Positive (*N*=143)	*PIK3CA* Mutation Status: Negative (*N*=277)	Total (*N*=420)
IHC (Targos), n (%)			
2+	45 (31.5)	84 (30.3)	129 (30.7)
3+	98 (68.5)	193 (69.7)	291 (69.3)
HERmark, n (%)			
Low	36 (25.2)	65 (23.5)	101 (24.0)
Equivocal	18 (12.6)	26 (9.4)	44 (10.5)
High	84 (58.7)	168 (60.6)	252 (60.0)
Missing	5 (3.5)	18 (6.5)	23 (5.5)
HERmark, RF/mm^2			
N	138	259	397
Mean	61.63	105.62	90.33
SD	112.94	142.69	134.60
Median	30.19	42.79	37.38
Minimum, maximum	0.9, 1179.0	1.2, 866.0	0.9, 1179.0
H-Score, n (%)			
≥240	65 (45.5)	137 (49.5)	202 (48.1)
<240	76 (53.1)	139 (50.2)	215 (51.2)
Missing	2 (1.4)	1 (0.4)	3 (0.7)
H-Score, RF/mm^2			
N	141	276	417
Mean	221.72	224.53	223.58
SD	59.93	59.41	59.53
Median	230.00	236.00	235.00
Minimum, maximum	35.0, 300.0	35.0, 300.0	35.0, 300.0

Abbreviations: IHC, immunohistochemistry; RF, relative fluorescence; SD, standard deviation.

Figure S1. Biomarker testing flow diagram. ddPCR, droplet digital polymerase chain reaction; ER, estrogen receptor; IHC, immunohistochemistry; NGS, next-generation sequencing; PR, progesterone receptor.

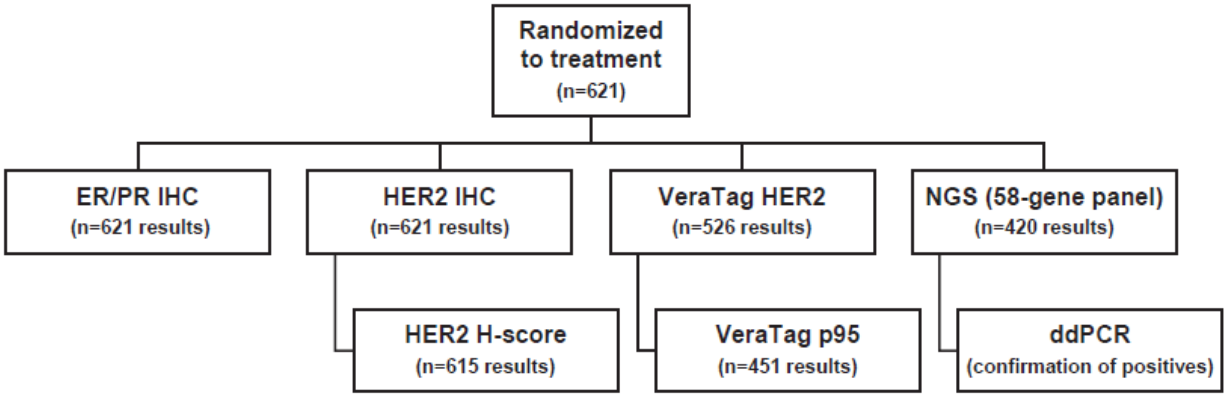
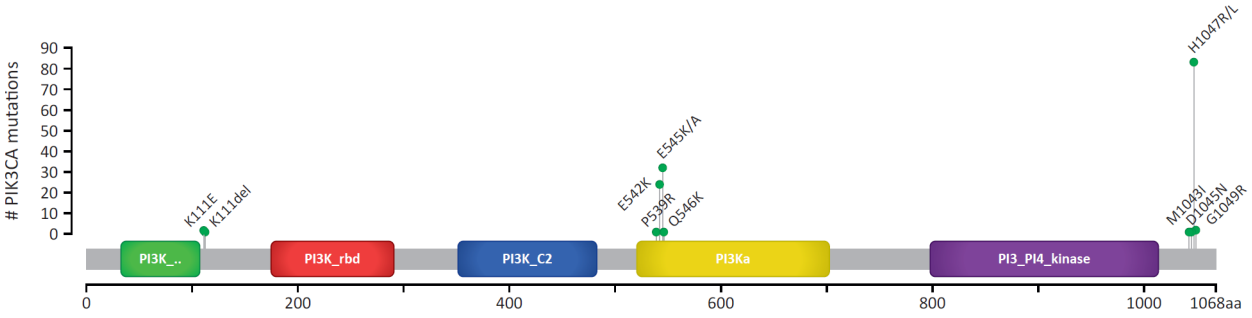
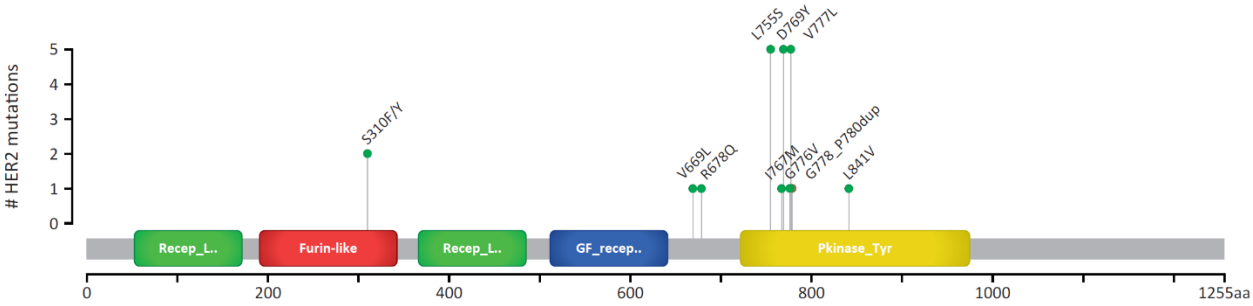


Figure S2. Spectrum of mutations detected: (A) *PIK3CA*; (B) *HER2*; (C) *HER3*; (D) *AKT1*; (E) *ESR1*; and (F) *KRAS.*

A



B



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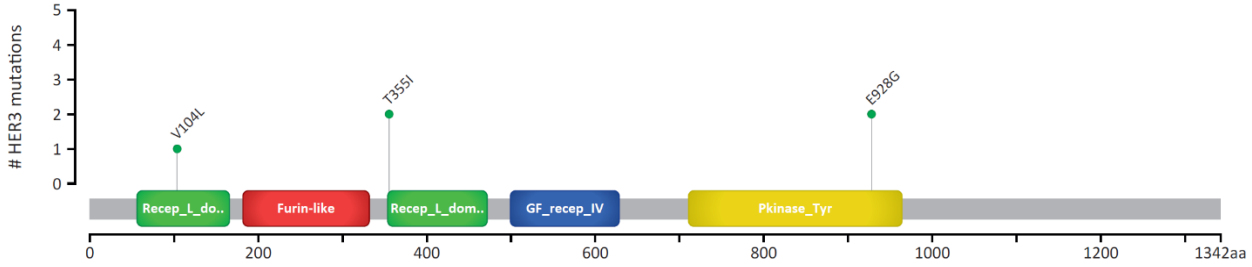

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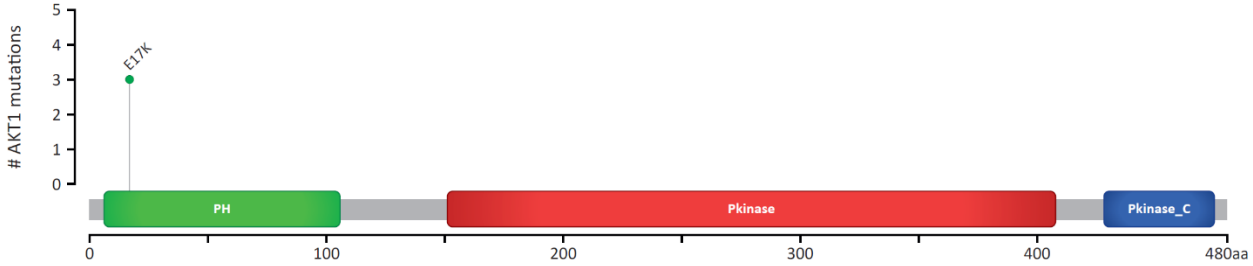

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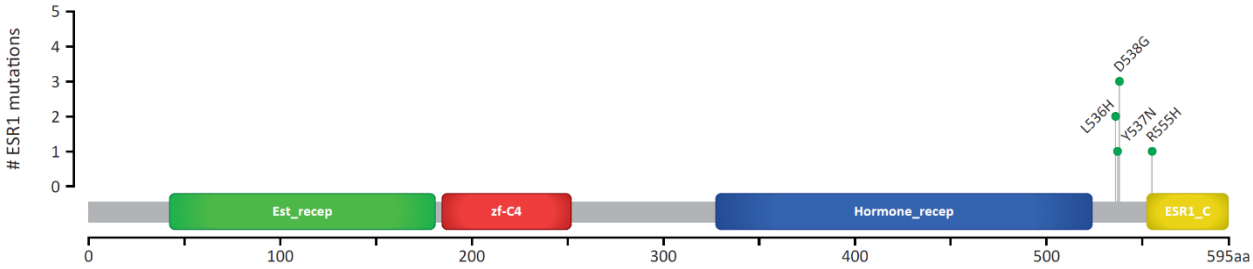

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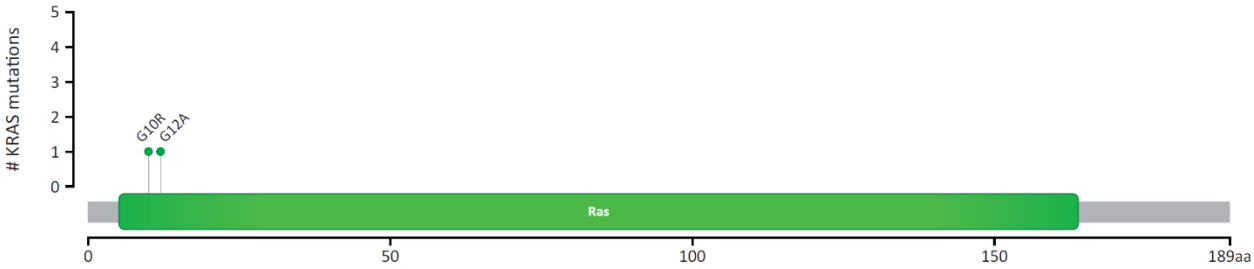

PIK3CA mutations were detected in 148 of 420 (35.0%) patient samples tested by next-generation sequencing. *HER2* mutations were detected in 23 of 420 (5.5%) patient samples tested. *HER3* mutations were detected in 1.0% (4/420) of patient samples tested (one sample had dual *HER3* mutations). Three of 420 samples (0.7%) had *AKT1* mutations. *ESR1* mutations were detected in 7 of 420 (1.7%) of patient samples tested. *KRAS* mutations were detected in 2 of 420 (0.5%) patient samples tested.