Saal *et al.*, Supplemental Material

Materials and Methods

Samples. DNA was prepared using several methods following manufacturer protocols depending on the date and location of the extraction: Wizard Genomic DNA Purification (Promega, Madison, WI), or Trizol (Invitrogen, Carlsbad, CA) followed by Wizard Purification. The list of cell lines that were analyzed are presented in Table S1 along with their mutational status.

Immunohistochemical Analysis. PTEN staining was evaluated by two independent pathologists (J. Enoksson, H. Hibshoosh), blinded to the clinical data, and prior to commencing the mutational study. PTEN positive non-neoplastic ductal epithelium (and if not present, normal endothelial cells or fibroblasts which also stain positive) within each tissue section was used as an internal positive control. A staining intensity score for invasive tumor and non-neoplastic cells, respectively, were recorded on an integer scale from 0 to 6+. Consensus PTEN status was recorded as a binary value, with PTEN negative (PTEN–) tumors having reduced to no immunostaining (a difference of 3 or more between the tumor and control scores), and PTEN positive (PTEN+) tumors having equal staining as compared to the internal control (a difference of no more than 1 between the two scores). For each of the two borderline cases with a score difference of 2 (both normal:4, tumor:2), a judgment call was made resulting in one recorded PTEN+ and the other PTEN–.

Genomic PCR and Sequence Analysis. All sequence chromatograms were analyzed with Mutation Surveyor (Softgenetics, State College, PA) and/or Sequencher (Gene Codes, Ann Arbor, MI) software. For *PTEN*, all mutations were verified by repeating the PCR and bi-directional sequencing. For *PIK3CA*, the infrequent ambiguous calls and all novel mutations not reported in the literature were re-amplified and sequence verified in both directions. For the *PIK3CA* novel mutations, DNA from the corresponding normal patient tissue, when available, was also sequenced to confirm the mutation as a somatic change, and for a subset of tumors with mutations previously described, the corresponding normal DNA was also sequenced. The primers and reaction conditions are as follows:

For *PTEN* exons 1 through 9, PCR was performed in 25 µl volumes for all 9 exons. The reactions contained 2.5 µl 10x PCR buffer, 2.5 µl 25 mM $MgCl_2$ (2.2 µl for exon 6), 0.5 µl 40 mM dNTP, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.1 µl Taq/Pfu (1/10 Pfu), 1 µl genomic DNA, and ddH_2O up to 25 µl. PCR was performed in a Hybaid MultiBlock System (Thermo, Waltham, MA), beginning with a denaturation at 94° for 7 min followed by 35-40 cycles of 94° for 30 sec, 55° for 30 sec and 72° for 30 sec, and a final extension for 7 min at 72°. The PCR products were analysed on polyacrylamide gels. The PCR products were purified by MultiScreen PCR (Millipore, Billerica, MA), and sequenced using BigDye Terminator chemistry and an ABI PRISM 3100 (Applied Biosystems, Foster City, CA) instrument.

PTEN PCR & sequencing primers:

1F 5'-TGTAAAACGACGGCCAGTAGTCGCTGCAACCATCCA-3'
1R 5'-CTAAGAGAGTGACAGAAAGGTA-3'

2F 5'-TGTAAAACGACGGCCAGTTGACCACCTTTTATTACTCCA-3'
2R 5'-AGTATCTTTTTCTGTGGCTTA-3'

3F 5'-TGTAAAACGACGGCCAGTATAGAAGGGGTATTTGTTGGA-3'
3R 5'-CCTCACTCTAACAAGCAGATA-3'

4F 5'-TGTAAAACGACGGCCAGTTTCAGGCAATGTTTGTTA-3'
4R 5'-TCGATAATCTGGATGACTCA-3'

5F 5'-TGTAAAACGACGGCCAGTGCAACATTTCTAAAGTTACCTA-3'
5R 5'-CTGTTTTCCAATAAATTCTCA-3'

6F 5'-CATAGCAATTTAGTGAAATAACT-3'
6R 5'-TGTAAAACGACGGCCAGTGATATGGTTAAGAAAACTGTTC-3'

7F 5'-TGTAAAACGACGGCCAGTCAGTTAAAGGCATTTCCTGTG-3'
7R 5'-GGATATTTCTCCCAATGAAAG-3'

8F 5'-CTCAGATTGCCTTATAATAGTC-3'
8R 5'-TGTAAAACGACGGCCAGTAACTTGTCAAGCAAGTTCTTC-3'

9F 5′-TGTAAAACGACGGCCAGTGTTCATCTGCAAAATGGA-3′
9R 5′-GGTAATCTGACACAATGTCCTA-3′

For *PIK3CA* exons 1, 2, 4, 5, 7, 9, 12, 13, 18, and 20, for all the Columbia samples and cell lines and the majority of the Swedish samples, PCR was performed in 96-well plates in 50 µl reactions containing 5 µl 10x PCR buffer (670 mM ammonium sulfate, 670 mM Tris pH 8.8, 67 mM $MgCl_2$, 100 mM β-ME), 2.5 µl 100 mM dNTPs, 2.5 µl 5 µM forward primer, 2.5 µl 5 µM reverse primer, 1 µl Platinum Taq (Invitrogen, Carlsbad, CA), 3.0 µl 100% DMSO, 2 µl genomic DNA (~7.5 ng/µl), and ddH_2O up to 50 µl. PCR was performed in a ThermoCycler Tetrad (MJ Research, Boston, MA), beginning with a denaturation at 95° for 3 min, followed by 40 cycles of 94° for 30 sec, annealing (temperature noted below for each exon) for 1 minute, 72° for 3 minutes, and a final extension for 15 min at 72°. PCR products were visualized and quantitated on 2% agarose gels, and purified and sequenced by Agencourt Bioscience Corp. (Beverly, PA).

PIK3CA PCR primers & annealing temperatures:

1F 5'-GTTTCTGCTTTGGGACAACCAT-3' (Samuels *et al.*, Science 2004; 304(5670):554)
1R 5'-GGTGTTAAAAATAGTTCCATAGTTCG-3' (Samuels *et al.*)
Annealing: 58°

2F 5’-TCATCAAAAATTTGTTTTAACCTAGC-3’ (Samuels *et al.*)
2R 5’-TATAAGCAGTCCCTGCCTTC-3’ (Samuels *et al.*)
Annealing: 52°

4F 5’-TCTTGTGCTTCAACGTAAATCC-3’ (Samuels *et al.*)
4R 5’-CGGAGATTTGGATGTTCTCC-3’ (Samuels *et al.*)
Annealing: 58°

5F 5’-TAGTGGATGAAGGCAGCAAC-3’ (Samuels *et al.*)
5R 5’-TTTGTAGAAATGGGGTCTTGC-3’ (Samuels *et al.*)
Annealing: 60°

7F 5’-GGGGAAAAAGGAAAGAATGG-3’ (Samuels *et al.*)
7R 5’-TGCTGAACCAGTCAAACTCC-3’ (Samuels *et al.*)
Annealing: 52°

9F 5’-GATTGGTTCTTTCCTGTCTCTG-3’ (Samuels *et al.*)
9R 5’-CCACAAATATCAATTTACAACCATTG-3’ (Samuels *et al.*)
Annealing: 58°

12F 5’-TTTATTCTAGATCCATACAACTTCCTTT-3’ (Samuels *et al.*)
12R 5’-AAAGTTGAGAAGCTCATCACTGGTAC-3’ (Samuels *et al.*)
Annealing: 58°

13F 5’-CTGAAACTCATGGTGGTTTTG-3’ (Samuels *et al.*)
13R 5’-TGGTTCCAAATCCTAATCTGC-3’ (Samuels *et al.*)
Annealing: 58°

18F 5’-TCCTTATTCGTTGTCAGTGATTG-3’ (Samuels *et al.*)
18R 5’-GTCAAAACAAATGGCACACG-3’ (Samuels *et al.*)
Annealing: 56°

20F 5’-TGACATTTGAGCAAAGACCTG-3’ (Samuels *et al.*)
20R 5’-GGGGATTTTTGTTTTGTTTTG-3’ (Samuels *et al.*)
Annealing: 58.3°

PIK3CA sequencing primers:
1SF 5’-GGACAACCATACATCTAATTCCTTA-3’
1SR 5’-GATTACGAAGGTATTGGTTTAGACAG-3’ (Samuels *et al.*)

2SF 5’-TCTACAGAGTTCCCTGTTTGC-3’ (Samuels *et al.*)
2SR 5’-AAGATATAGACACAGGTAGAAGAC-3’

4SF 5’-CACCTTTGCAGATTAATATGTAGTC-3’
4SR 5’-ACCATCTGAAAACATACTACAGGTC-3’

5SF 5’-TTTGAGTCTATCGAGTGTGTGC-3’ (Samuels *et al.*)
5SR 5’-GGATCATACTGCTAAACACTAA-3’

7SF 5’-TGAATTTTCCTTTTGGGGAAG-3’ (Samuels *et al.*)
7SR 5’-GAGAGAAGGTTTGACTGCCA-3’

9SF 5’-TTGCTTTTTCTGTAAATCATCTGTG-3’ (Samuels *et al.*)
9SR 5’-GATCAGCCAAATTCAGTTAT-3’

12SF 5’-ACCAGTAATATCCACTTTCTTTCTG-3’ (Samuels *et al.*)
12SR 5’-AACATAAACAAAAGTATATAAGTAA-3’

13SF 5’-TTTATTGGATTTCAAAAATGAGTG-3’ (Samuels *et al.*)
13SR 5’-CTAAACAACTCTGCCCCACT-3’

18SF 5’-TGCACCCTGTTTTCTTTTCTC-3’ (Samuels *et al.*)
18SR 5’-GCAGATACAAAATGTCTTGA-3’

20SF 5’-ATTTGCTCCAAACTGACCAA-3’
20SR 5’-GGACTTAAGGCATAACATGAA-3’

For some of the Swedish samples, *PIK3CA* PCR for exons 1, 2, 4, 5, 7, 9, 12, 13, 18, and 20 was performed in 25 µl reactions containing 2.5 µl 10x PCR buffer, 2.5 µl 25 mM $MgCl_2$, 0.25 µl 40 mM dNTPs, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.1 µl Taq, 3.0 µl 50% DMSO, 1 µl genomic DNA, and ddH_2O up to 25 µl. PCR was performed in a Hybaid MultiBlock System (Thermo, Waltham, MA), beginning with a denaturation at 95° for 3 min followed by 40 cycles of 95° for 30 sec, 58° for 1 minute and 72° for 3 minutes, and a final extension for 10 min at 72°. The PCR products were analysed on polyacrylamide gels. PCR products were purified by MultiScreen PCR (Millipore, Billerica, MA), and sequenced using BigDye Terminator chemistry and an ABI PRISM 3100 (Applied Biosystems, Foster City, CA) instrument. Primers were used as above, except as noted below. Additionally, for all exons, the PCR primers were used as sequencing primers.

PIK3CA alternate PCR/sequencing primers:
A9F 5’-GAGGGGAAAAATATGACAAAG-3’
A9R 5’-GAGATCAGCCAAATTCAGTTA-3’

A12F 5’-GCAGAAACTGACCCTGATTTG-3’
A12R 5’-TGTACCTTAAGAATTTAATGGGAAAATA-3’

A20F1 5’-TGGGGTAAAGGGAATCAAAAG-3’ (Samuels *et al.*)
A20R1 5’-CCTATGCAATCGGTCTTTGC-3’ (Samuels *et al.*)

A20F2 5’-TTGCATACATTCGAAAGACC-3’ (Samuels *et al.*)

A20R2 5’-GGGGATTTTTGTTTTGTTTTG-3’ (Samuels *et al.*)

***PIK3CA* cDNA Sequencing.** Complementary DNA was prepared by taking 0.5 µl anchored primer (1µg/µl) with 2 µg total RNA in 11.5 µl nuclease free water, and incubated at 70º for 10 min for primer annealing. 4 µl 5X First Strand Buffer (Invitrogen, Carlsbad, CA), 2 µl 0.1 M DTT and 1 µl 40 mM dNTP was added and the mixture was incubated for 2 minutes at 42º. 1 µl Superscript II Reverse Transcriptase (Invitrogen, Calsbad, CA) was added and the samples were incubated for another 50 minutes at 42º. To inactivate the enzyme the samples were heated to 70º for 15 minutes. PCR reaction conditions, verification of PCR products, purification, and sequencing was performed as described earlier for genomic PCR and sequencing of *PIK3CA* of some Swedish cases, except different primers specific to PIK3CA cDNA sequences were used:

PIK3CA cDNA PCR/sequencing primers
cDNA ex9F 5’-TGTATCCCGAGAAGCAGGAT-3’
cDNA ex9R 5’-TGGGTAGAATTTCGGGGATA-3’

cDNA ex13F 5’-TTCTTTTGGCATTTAAAATCTGAG-3’
cDNA ex13R 5’-AAAGCCCTGTAGAGCATCCA-3’

***PIK3CA* Sequencing Technical Note.** Nearly identical sequence to that of *PIK3CA* exons 9, 12, and 13 and their intron-exon boundaries can also be found at 22q11.2, and therefore care must be taken when designing primers and analyzing data. For example, in exon 9 the A at position 1634 of *PIK3CA* coding sequence (where 1 is the first base of the start codon) could be contaminated by a C substitution found in the corresponding sequence on 22q11.2, the G corresponding to G1658 is deleted on 22q11.2, and T1659 is substituted by C. Exon 12, in which we did not find any mutations, has base-pair differences between *PIK3CA* and 22q11.2 only within intronic sequence. In exon 13, G2038 is substituted by C on 22q11.2, and C2155 by G. As evidence of the specificity of our PCR, A1634C occurred only once in our data, and none of the other exon 9 changes were evident in any sample. Moreover, for 8 tumors, *PIK3CA* cDNA was sequenced to confirm their exon 9 mutational status. For exon 13, G2038C and/or C2155G was seen in 73/344 samples. For 11 of these tumors, *PIK3CA* cDNA was sequenced and no mutation corresponding to 22q11.2 sequence was found, indicating that these changes represent likely 22q11.2 sequence contamination of the genomic PCR. It is theoretically possible, in this study and in the literature, that mutations reported in exons 9, 12, and 13 could in fact reflect mutated sequence from 22q11.2, and that some “polymorphisms” that correspond to 22q11.2 sequence could in fact be under-reported *bona fide* mutations in *PIK3CA*. In addition, co-amplification of 22q11.2 sequences with *PIK3CA* exon 13 could have masked the detection of novel *PIK3CA* mutations in this exon.

***PIK3CA*/*PTEN*-double mutant verification**. Two tumors were identified as having both a mutation in *PIK3CA* and in *PTEN*. The first case harbored *PIK3CA* H701P with a *PTEN* exon 5 frameshift truncation mutation. As no normal blood DNA was available, four 10-µm paraffin sections were cut and tumor and normal cells were microdissected, and additional normal cells were obtained by taking 0.6 mm diameter punch cores from

the paraffin block using a tissue microarray needle. The mutations were confirmed to be present in the tumor compartment and not in the normal compartment by PCR amplification and direct sequencing of both strands. The second tumor had *PIK3CA* C420R with a *PTEN* exon 7 frameshift truncating mutation. Both mutations were confirmed to be somatic by sequencing normal blood DNA. For this case, the tumor and normal DNAs were genotyped by microsatellite analysis (markers BAT25, BAT26, BAT40 and BAT34C4) using standard protocols and an ABI PRISM 3100 instrument with GeneScan software (Applied Biosystems, Foster City, CA) and it was verified that the DNA samples were from the same patient.

Table S1 ***PIK3CA mutations found in breast cancer cell lines***

Cell Line	Exon 1	Exon 9	Exon 20
BT-20		C1616G:P539R	A3140G:H1047R
BT-474	G333C:K111N		
BT-483		G1624A:E542K	
BT-549			
CAMA-1			
DU-4475			
HCC-38			
HCC-70			
HCC-202		G1633A:E545K	
HCC-1008			
HCC-1143			
HCC-1187			
HCC-1395			
HCC-1419			
HCC-1428			
HCC-1500			
HCC-1599			
HCC-1806			
HCC-1937			
HCC-1954			A3140G:H1047R
HCC-2157			
HCC-2218			
HS758T			
MCF-7		G1633A:E545K	
MDA-MB-134-VI			
MDA-MB-157			
MDA-MB-175-VII			
MDA-MB-231			
MDA-MB-330			
MDA-MB-361		G1633A:E545K	
MDA-MB-415			
MDA-MB-435-S			
MDA-MB-436			
MDA-MB-453			A3140G:H1047R
MDA-MB-468			
SK-BR-3			
SUM-44-P92			
SUM-52-PE			
SUM-102			A3140G:H1047R
SUM-159			A3140T:H1047L
SUM-185			homozygous A3140G:H1047R
SUM-190			A3140G:H1047R
SUM-225			
SUM-229			
SUM-1315			
T-47D			A3140G:H1047R
UACC-812			
UACC-893			A3140G:H1047R
ZR75-1			
ZR75-30			

Nucleotide change:Amino acid change.
Novel mutation is underlined.