Phosphatidylinositol-3-OH Kinase or RAS Pathway Mutations in Human Breast Cancer Cell Lines

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Abstract

Constitutive activation of the phosphatidylinositol-3-OH kinase (PI3K) and RAS signaling pathways are important events in tumor formation. This is illustrated by the frequent genetic alteration of several key players from these pathways in a wide variety of human cancers. Here, we report a detailed sequence analysis of the *PTEN, PIK3CA, KRAS, HRAS, NRAS,* and *BRAF* genes in a collection of 40 human breast cancer cell lines. We identified a surprisingly large proportion of cell lines with mutations in the PI3K or RAS pathways (54% and 25%, respectively), with mutants for each of the six genes. The *PIK3CA, KRAS*, and *BRAF* mutation spectra of the breast cancer cell lines were similar to those of colorectal cancers. Unlike in colorectal cancers, however, mutational activation of the PI3K pathway was mutually exclusive with mutational activation of the RAS pathway in all but 1 of 30 mutant breast cancer cell lines ($P = 0.001$). These results suggest that there is a fine distinction between the signaling activators and downstream effectors of the oncogenic PI3K and RAS pathways in breast epithelium and those in other tissues.
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Introduction

The phosphatidylinositol-3-OH kinase (PI3K) and RAS signaling pathways are pivotal to the transduction of extracellular signals to intracellular targets. Both signaling pathways may be activated by growth factors or nutrients in the cell environment. The subsequent signaling events regulate cell metabolism, cell survival, cell cycle progression, and cell growth. Upon activation, usually via receptor tyrosine kinases, PI3K converts phosphatidylinositol-4,5-diphosphate to its active form, phosphatidylinositol-3,4,5-triphosphate. This lipid second messenger then transduces the activation signal to downstream targets, most notably members of the AKT family of serine/threonine kinases. The phosphatidylinositol-4,5-diphosphate to phosphatidylinositol-3,4,5-triphosphate conversion is counteracted by PTEN phosphatase, thus serving a negative feedback for PI3K signaling (reviewed in refs. 1-4). The RAS proteins are also major effectors of growth factor signaling through receptor tyrosine kinases. Ligand-induced activation of receptor tyrosine kinases generates a cascade of signaling events, during which the RAS GTPase proteins are converted from the inactive GDP-bound state to the active GTP-bound state. Activated RAS proteins confer signals to downstream effectors, including members of the RAF family protein kinases, through interaction with their RAS binding domain. RAF kinases, in turn, further transduce the signals upon the mitogen-activated protein kinase pathway or a number of other possible effectors (reviewed in refs. 5-7).

Cross-talk between the PI3K and RAS signaling pathways may occur at several stages. GTP-bound RAS proteins may directly activate PI3K (8). Further downstream, activation of the AKT pathway, through PI3K signaling, may converge with signals from the mitogen-activated protein kinase pathway, through RAS signaling, on mammalian target of rapamycin kinase (5, 9). There are ample downstream effectors of the PI3K and/or RAS pathways, with a variety of signaling routes. Specificity of the signal transduction is determined by the activating extracellular signaling molecules, with an apparent additional specificity related to cell type and cell activation status. Particularly, the unraveling of the regulation of this specificity within the PI3K and RAS signaling pathways is currently a major research challenge.

The importance of the PI3K and RAS signaling pathways for cellular processes is shown by their frequent mutational activation in human cancers. Cancer is a genetic disease driven by the accumulation of genetic abrogations in pathways that regulate the growth of cells, their survival, and their integrity. After the *p53* tumor suppressor, members of the PI3K pathway are most frequently mutated in human cancers. Most prevalent are activating mutations in the *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, and inactivating mutations in the *PTEN* tumor-suppressor gene. *PIK3CA* amplification is found in ovarian, cervical, and thyroid carcinoma (10-12), whereas mutations are found predominantly in liver, colon, and breast tumors (13-15). Most *PIK3CA* mutations are located in three mutational hotspot regions in the gene sequence, which result in increased kinase activity of PI3K (13, 16). The *PTEN*

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tumor-suppressor gene was originally identified by genetic screens of breast cancers and glioblastomas (17, 18), but it soon became apparent that its mutational involvement also includes many other tumor types (2). Importantly, germ line *PTEN* mutations were identified in patients with Cowden disease (19) and in patients with Bannayan-Zonana syndrome (ref. 20; OMIM 158350 and 153480), two cancer predisposition syndromes that share clinical symptoms such as benign hamartomatous lesions. Similar symptoms are characteristic for the tuberous sclerosis and Peutz-Jeghers syndromes, which have been associated with germ line mutations in the *TSC1, TSC2*, and *LKB1* genes (21-24). Each of these genes encodes downstream effectors from the PI3K signaling pathway, showing both the ubiquitous involvement of this pathway and its tissue specificity.

Mutational activation of the RAS signaling pathway in human cancers is mainly achieved by mutations in the *RAS* and *BRAF* genes. Although many RAS GTPases have been identified, activating oncogenic mutations have been reported for only three *RAS* isoforms: *KRAS, HRAS*, and *NRAS*. Oncogenic *RAS* mutations seem restricted to codons 12, 13, and 61 of the proteins, resulting in constitutive active RAS GTPase. *RAS* mutations have been identified in a wide variety of human tumor types and display tissue specificity (25). *KRAS* is frequently mutated in pancreatic cancers and colorectal cancers, whereas mutations in *NRAS* seem to be more pronounced in melanoma and hematologic cancers. Activating *BRAF* mutations are also found in many different tumor types, but their mutational involvement is particularly pronounced in melanoma (6, 26). Oncogenic *BRAF* mutations are restricted mainly to exons 11 and 15 of the gene, and hotspot mutations have been shown to result in increased kinase activity of BRAF (26).

Oncogenic mutations in the PI3K and RAS signaling pathways have been instrumental in deciphering the biology of these pathways. Conversely, knowledge of the functional implications of oncogenic mutations has increased our understanding of human carcinogenesis, through the commonalities as well as the differences between tumor types. Few studies, however, have addressed the mutational activation of both the PI3K pathway and the RAS pathway in a single cohort of human tumor samples. Here, we report a detailed sequence analysis of six genes (*PTEN, PIK3CA, KRAS, HRAS, NRAS*, and *BRAF*) that are of major importance for the PI3K and RAS signaling pathways in a collection of 40 human breast cancer cell lines.

Results

We analyzed 40 human breast cancer cell lines for mutations in the *PTEN, PIK3CA, RAS*, and *BRAF* genes, by direct sequencing of PCR-amplified genomic DNA fragments. Mutational analysis of all nine exons of the *PTEN* tumor-suppressor gene revealed eight mutant cell lines (Table 1). One cell line had a homozygous deletion of exons 1 to 9 of *PTEN*; three cell lines had truncating mutations (IVS4 + 1G>T, 821delG, 951delACTT); and four cell lines had missense mutations (D92H, L108R, C136Y, E307K). The IVS4 + 1G>T splice site mutation resulted in the exact deletion of exon 4 from the encoded transcript, predicting a change in the protein sequence after codon 71 with four additional amino acids followed by a stop codon. This splice site mutation has also been identified in the germ line of a patient with Cowden disease, in two endometrial carcinomas, and in a glioblastoma (27), rendering it highly likely that this mutation is relevant for tumorigenesis. The 821delG mutation is also presumed to be oncogenic, as it resulted in a premature stop at codon 275 that has been identified in eight endometrial carcinomas (27). The 951delACTT mutation resulted in a premature stop at codon 319 that was also found in the germ line of a patient with Cowden disease and in seven endometrial carcinomas, three glioblastomas, and a prostate carcinoma (27). The D92H and C136Y missense mutations are both presumed oncogenic, as a mutation at codon 92 was found in an endometrial carcinoma and C136Y was found in the germ line of a patient with Cowden disease (27). The L108R mutation has never been reported in clinical cancer samples but is likely oncogenic, as it is located in the phosphatase domain of PTEN, which is frequently mutated in Cowden disease patients, Bannayan-Zonana patients, and in endometrial carcinoma (27). The E307K mutation also has not been reported, but it is located in the C2 domain of PTEN, and neighboring codons have been found mutated in a Cowden disease patient and in two endometrial carcinomas (27). However, the functional significance of the E307K mutation in cell line MDA-MB-453 is unclear, as this mutation is heterozygous and we did not idenify additional *PTEN* sequence alterations in this cell line. All other *PTEN* mutant breast cancer cell lines had lost the other *PTEN* allele, except for cell line CAMA-1. CAMA-1 carried the D92H mutation at one allele and had a second mutation at the other allele, where an insertion of four base pairs at position 802 was followed by a deletion of four base pairs at position 834, predicting the exchange of 12 amino acids within the PTEN protein sequence (D268_F279delins12; Fig. 1). The biallelic nature of the mutations in CAMA-1 was confirmed by transcript analysis and by cloning and sequencing of transcript fragments, both only identifying the D92H mutation. We also identified a possible primer site polymorphism in cell line UACC893, as we were unable to PCR amplify exon 2 from genomic DNA, although sequence analysis revealed expression of the wild-type *PTEN* transcript. Analysis of *PTEN* transcript expression by reverse transcription-PCR revealed that cell lines HCC1937, MDA-MB-436, and SUM149PT did not express *PTEN* transcripts (Fig. 2). Whereas cell line HCC1937 had a homozygous deletion of the *PTEN* gene, both MDA-MB-436 and SUM149PT had a wild-type *PTEN* gene sequence (Table 2). We excluded transcriptional silencing through hypermethylation of the *PTEN* promoter region as a probable cause, by culturing the cell lines in the presence of the demethylating agent 5-azacytidine. As a result, neither MDA-MB-436 nor SUM149PT reexpressed *PTEN* transcripts although 5-azacytidine did induce expression of *E-cadherin* transcripts in both cell lines (Fig. 2).[1] Together, 7 of 38 (18%) breast cancer cell lines had biallelic inactivating *PTEN* mutations, one cell line had a monoallelic missense mutation, and two cell lines did not express *PTEN* transcripts for unknown reasons.

[1] Hollestelle et al., manuscript in preparation.

Table 1. Mutations Identified in the *PTEN, PIK3CA, RAS*, and *BRAF* Genes in Human Breast Cancer Cell Lines

Breast Cancer Cell Line	Affected Gene	Gene Sequence	Transcript Sequence	Predicted Protein Effect*	Oncogenic
HCC1937 †	*PTEN*	HD Ex. 1-9 ‡	Not detectable	No expression	Yes
MDA-MB-468 †	*PTEN*	IVS4+1G>T ‡	c.del210_253 (Ex. 4)	A72fsX5	Yes
BT549 †	*PTEN*	821delG ‡	821delG	V275X	Yes
EVSA-T	*PTEN*	951delACTT ‡	951delACTT	T319X	Yes
CAMA-1	*PTEN*	274G>C§	274G>C	D92H	Yes
		802insTAGG/ 834delCTTC§	Not detectable	No expression	Yes
ZR-75-1 †	*PTEN*	323T>G ‡	323T>G	L108R	Likely
MDA-MB-415 †	*PTEN*	407G>A ‡	407G>A	C136Y	Yes
MDA-MB-453	*PTEN*	919G>A	919G>A	E307K	Likely
BT474 †	*PIK3CA*	333G>C	NA	K111N	Yes
BT20 †	*PIK3CA*	1616C>G	NA	P539R	Yes
BT483 †	*PIK3CA*	1624G>A	NA	E542K	Yes
MCF-7 †	*PIK3CA*	1633G>A	NA	E545K	Yes
MDA-MB-361 †	*PIK3CA*	1633G>A	NA	E545K	Yes
MDA-MB-361	*PIK3CA*	1700A>G	NA	K567R	Likely
BT20 †	*PIK3CA*	3140A>G	NA	H1047R	Yes
MDA-MB-453 †	*PIK3CA*	3140A>G	NA	H1047R	Yes
OCUB-F	*PIK3CA*	3140A>G ‡	NA	H1047R	Yes
SK-BR-5	*PIK3CA*	3140A>G	NA	H1047R	Yes
SUM102PT †	*PIK3CA*	3140A>G	NA	H1047R	Yes
SUM185PE †	*PIK3CA*	3140A>G ‡	NA	H1047R	Yes
SUM190PT †	*PIK3CA*	3140A>G	NA	H1047R	Yes
T47D †	*PIK3CA*	3140A>G	NA	H1047R	Yes
UACC893 †	*PIK3CA*	3140A>G	NA	H1047R	Yes
SUM159PT †	*PIK3CA*	3140A>T	NA	H1047L	Yes
MDA-MB-134VI †	*KRAS*	34G>C	NA	G12R	Yes
SK-BR-7	*KRAS*	34G>T	NA	G12C	Yes
SUM229PE	*KRAS*	35G>A	NA	G12D	Yes
MPE600	*KRAS*	35G>T	NA	G12V	Yes
MDA-MB-231 †	*KRAS*	38G>A	NA	G13D	Yes
Hs578T †	*HRAS*	35G>A	NA	G12D	Yes
SUM159PT	*HRAS*	35G>A	NA	G12D	Yes
SK-BR-7	*NRAS*	182A>G	NA	Q61R	Yes
ZR-75-30 †	*BRAF*	977T>C	NA	I326T	Unknown
MDA-MB-231	*BRAF*	1391G>T	NA	G464V	Yes
DU4475	*BRAF*	1799T>A	NA	V600E	Yes
MDA-MB-435s	*BRAF*	1799T>A	NA	V600E	Yes

Abbreviations: HD, homozygous deletion; Ex., exon; IVS, intervening sequence; del, deletion; ins, insertion; NA, not analyzed.
*Frameshift mutations are indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X.
† Cell lines were reported to be mutated in references (17, 26, 37, 45-49).
‡ Mutations were homozygous based on sequence analysis and confirmed with polymorphic markers.
§ Mutations are heterozygous but are located on different alleles.

Mutational analysis of the complete coding sequence of the *PIK3CA* oncogene revealed 16 missense mutations in 14 breast cancer cell lines (Table 1). The mutations K111N, P539R, E542K, K567R, and H1047L were each identified in one cell line; the E545K mutation was found in two cell lines; and the H1047R mutation was found in nine cell lines. All mutations except for K111N and K567R were previously identified in colon carcinomas (13), and functional analysis of the E542K, E545K, and H1047R mutations had shown that these mutations were oncogenic (16, 28). Although the K111N mutation was not previously reported in a primary cancer, this codon was found deleted in a colon carcinoma (13), suggesting that the K111N mutation is oncogenic. The K567R mutation has also not been reported, but its location in the helicase domain of PIK3CA suggests that it may have functional implications. Notably, we identified the K567R mutation in cell line MDA-MB-361, which also carried the oncogenic E545K mutation. Similarly, cell line BT20 carried both the P539R and H1047R mutations. All *PIK3CA* mutations were heterozygous, except for the H1047R mutations in cell lines OCUB-F and SUM185PE. In addition, we identified the as yet unreported synonymous 363C>T alteration in cell line MDA-MB-231, and the 1173A>G single nucleotide polymorphism in five cell lines [single nucleotide polymorphism (SNP) rs3729680; heterozygous in SUM52PE, T47D, and ZR-75-30; homozygous in MDA-MB-231 and SUM149PT]. Available SNP array data for 19 cell lines revealed a single low-level amplification of four copies at the *PIK3CA* locus for the mutant cell line T47D and no amplifications at the *AKT2* locus, suggesting that *PIK3CA* and *AKT2* amplification is uncommon in breast cancer (average intensity ratio for *PIK3CA* was 1.1, range 0.7-1.9; average intensity ratio for *AKT2* was 1.0, range 0.7-1.4).[2] Together, we identified activating *PIK3CA* mutations in 14 of 39 (36%) breast cancer cell lines.

Mutational analysis of exons 2 and 3 of the three human *RAS* oncogenes revealed eight heterozygous *RAS* mutations in 7 of 40 breast cancer cell lines (18%; Table 1). We identified five cell lines with each a different *KRAS* mutation (G12C,

[2] http://www.sanger.ac.uk/cgi

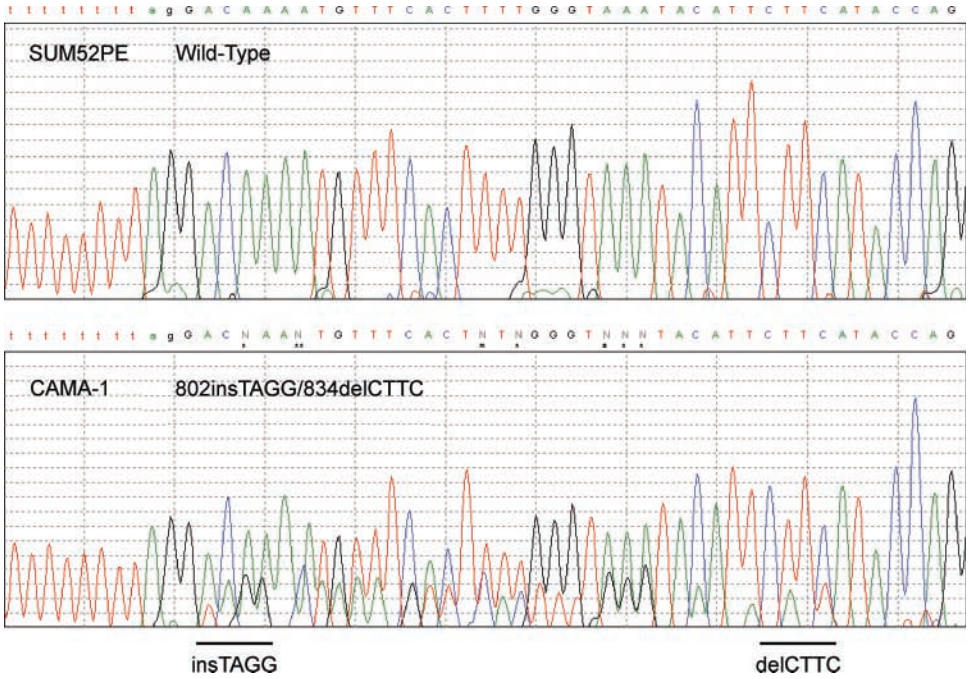

FIGURE 1. Identification of the *PTEN* 802insTAGG/834delCTTC mutation in cell line CAMA-1 by PCR amplification and sequencing of genomic DNA (*bottom electropherogram*). The wild-type *PTEN* gene sequence is shown for comparison (*top electropherogram*).

G12D, G12R, G12V, and G13D). The *HRAS* G12D mutation was found in two cell lines, and the *NRAS* Q61R mutation was found once. The latter mutation was identified in cell line SK-BR-7, which also carried the *KRAS* G12C mutation. In addition to these well-described oncogenic *RAS* mutations, we identified the synonymous *HRAS* 81T>C SNP in 15 cell lines (SNP rs12628; heterozygous in BT483, MDA-MB-175VII, MDA-MB-415, and SK-BR-3; homozygous in BT20, BT474, CAMA-1, HCC1937, MDA-MB-453, MPE600, SK-BR-5, SK-BR-7, SUM149PT, SUM159PT, and T47D).

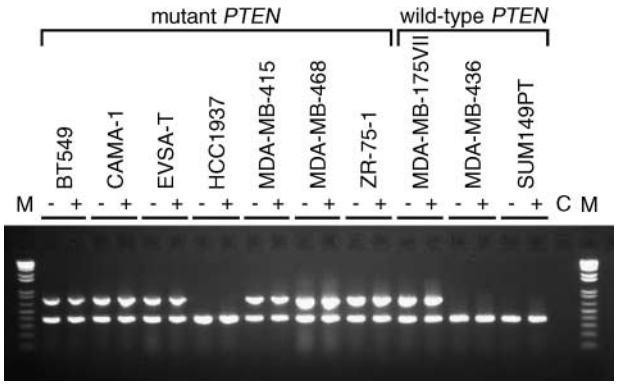

FIGURE 2. Analysis of *PTEN* transcriptional silencing through promoter methylation by cell culture in the presence (+) or absence (–) of 5-azacytidine. Reverse transcription-PCR amplification products are shown from seven mutant and three wild-type *PTEN* breast cancer cell lines, using primers specific for *PTEN* (*top fragments*) and the *HPRT* housekeeper (*bottom fragments*). These cell lines included the three cell lines without detectable *PTEN* expression, but there was no indication of *PTEN* promoter methylation.

Mutational analysis of exons 7, 11, and 15 of the *BRAF* oncogene revealed 4 of 40 breast cancer cell lines with a heterozygous *BRAF* mutation (10%; Table 1). We identified the I326T and G464V mutations each in a single cell line, and the V600E mutation was found in two cell lines. The V600E mutation is the most frequently identified oncogenic mutation in the *BRAF* gene. The G464V mutation is less frequently identified, but also considered to be oncogenic as it is located within the highly conserved G-loop region (26). Importantly, the G464V and V600E mutations both resulted in an increased activity of BRAF kinase (26). Thus far, the I326T variant has only been identified in the ZR-75-30 breast cancer cell line and its functional effect is yet unknown (26). It is important to note that the *BRAF* mutant MDA-MB-435s cell line was recently shown to be genetically identical to the M14 melanoma cell line, although it had not conclusively been investigated which of the two cell lines was correct (ref. 29 and references therein). Because *BRAF* mutations typically associate with melanoma, one could perhaps also wonder on the origin of the other three *BRAF* mutant breast cancer cell lines. Based on gene expression and methylation profiles, there is no reason to doubt the breast origin of MDA-MB-231 (30-32). No profiles have been reported for ZR-75-30 and DU4475, but our recent identification of a truncating *E-cadherin* mutation in cell line ZR75-30 renders it likely that this cell line is indeed of breast origin.[1] We cannot be certain on DU4475, as we have as yet not identified breast-specific mutations in this cell line. But then, one can never be sure about the origin of a cancer cell line. Even so, we identified four *BRAF* mutant breast cancer cell lines or, when MDA-MB-435s and DU4475 would turn out not to be of breast origin, two *BRAF* mutants were identified.

Discussion

We did a mutational analysis of six major cancer genes from the PI3K and RAS signaling pathways in a collection of 40 human breast cancer cell lines. We identified 26 unique mutations: nine mutations in *PTEN*, seven mutations in *PIK3CA*, five in *KRAS*, one each in *HRAS* and *NRAS*, and three in *BRAF*. Four of these mutations have not yet been described in the literature (Table 1). In total, 30 of the 40 breast cancer cell lines had mutations in any of these six genes, 40% of which had not yet been reported (Table 1). This detailed mutational analysis of the PI3K and RAS pathway genes is complemented by our previously reported mutational analyses of the *E-cadherin, MKK4, p53*, and *BRCA1* genes, rendering this collection of breast cancer cell lines a valuable model for functional and pharmacologic studies (33-36).

Mutational activation of the PI3K signaling pathway was detected in 21 breast cancer cell lines (Table 2). Two cell lines were *PIK3CA* double mutants. Cell line BT20 carried the P539R and H1047R mutations, for which kinase assays had shown that the H1047R mutation resulted in a substantially higher PI3K activity (13, 16). Cell line MDA-MB-361 carried the E545K and K567R mutations, of which only the E545K mutation had been previously identified and had been shown to increase PI3K activity (16). It is conceivable that these *PIK3CA* double mutants reflect a progression of tumorigenesis through further mutational activation of PI3K. In this scenario, the more oncogenic H1047R and E545K mutations would have been the second hit of the PI3K pathway in the original breast cancers. Indeed, *PIK3CA* double-mutant tumors have previously been reported for three primary breast cancers and a gastric cancer (37, 38), suggesting that a two-hit mutational activation of the PI3K pathway may not be uncommon. Similarly, we identified the highly oncogenic *PIK3CA* H1047R mutation together with the *PTEN* E307K mutation in cell line MDA-MB-453. Importantly, MDA-MB-453 had retained a wild-type *PTEN* allele. As the *PTEN* E307K mutation is located in a mutational hotspot domain (27), it seems that *PTEN* is haploinsufficient in cell line MDA-MB-453. Mutation of *PIK3CA* at its critical H1047 residue would then have been the second hit to full activation of the PI3K pathway in cell line MDA-MB-453. Of course, a two-hit activation of the PI3K signaling pathway awaits further confirmation in primary cancer specimens, allowing dissection of tumor progression by mutational analysis of the earlier premalignant tumor

Table 2. Mutational Activation of the PI3K and RAS Pathways Is Mutually Exclusive in Human Breast Cancer Cell Lines

Breast Cancer Cell Lines	*PTEN*	*PIK3CA*	*KRAS*	*BRAF*	*HRAS*	*NRAS*
BT549	V275X					
CAMA-1	D92H					
EVSA-T	T319X					
HCC1937	no protein					
MDA-MB-415	C136Y					
MDA-MB-468	A72fsX5					
ZR-75-1	L108R					
MDA-MB-453	E307K	H1047R				
BT20		P539R/H1047R				
MDA-MB-361		E545K/K567R				
BT474		K111N				
BT483		E542K				
MCF-7		E545K				
OCUB-F		H1047R				
SK-BR-5		H1047R				
SUM102PT		H1047R				
SUM185PE		H1047R				
SUM190PT	NA	H1047R				
T47D		H1047R				
UACC893		H1047R				
SUM159PT		H1047L			G12D	
Hs578T					G12D	
SK-BR-7			G12C			Q61R
MDA-MB-134VI			G12R			
MPE600			G12V			
SUM229PE			G12D			
MDA-MB-231			G13D	G464V		
MDA-MB-435s				V600E		
DU4475				V600E		
ZR-75-30				I326T		
MDA-MB-157						
MDA-MB-175VII						
MDA-MB-330						
MDA-MB-436						
SK-BR-3						
SUM149PT						
SUM225CWN	NA	NA				
SUM1315MO2						
SUM52PE						
UACC812						
Mutation rate	8 of 38 (21%)	14 of 39 (36%)	5 of 40 (13%)	4 of 40 (10%)	2 of 40 (5%)	1 of 40 (3%)

NOTE: Overview of mutations that were identified in 40 human breast cancer cell lines. The mutations are detailed in Table 1.
Abbreviation: NA, not analyzed.

lesions. Either way, our observation of mutational activation of the PI3K pathway in half of human breast cancer cell lines suggests that this signaling pathway may be more important for breast carcinogenesis than currently perceived.

Mutational activation of the RAS signaling pathway was detected in 10 breast cancer cell lines (Table 2). We were somewhat surprised by the 13% *KRAS* mutation frequency among the breast cancer cell lines, given the general conviction that *KRAS* mutations are relatively rare in human breast cancers (25). Two *RAS* double-mutant cell lines were identified. Cell line SK-BR-7 carried the *KRAS* G12C mutation and the *NRAS* Q61R mutation, whereas cell line MDA-MB-231 carried the *KRAS* G13D mutation and the *BRAF* G464V mutation. The *BRAF* G464V mutation was shown to be a less potent activator of BRAF kinase than the more prevalent *BRAF* V600E mutation (2 and 10 times wild-type kinase activity, respectively; ref. 26). One again can conceive a two-hit activation of the RAS pathway, through the *BRAF* G464V mutation and subsequent mutation of *KRAS* G13D. In agreement, only *KRAS* and *BRAF* V600E mutations were reportedly mutually exclusive in colorectal cancers, and one of the four reported double mutants harbored the same combination of *KRAS* G13D with *BRAF* G464V (26, 39).

We identified an unexpected large proportion of breast cancer cell lines with mutational activation of the PI3K and RAS signaling pathways (54% and 25%, respectively). Perhaps even more surprising was that only 1 of the 30 mutant cell lines had mutations in both pathways (*PIK3CA* H1047L and *HRAS* G12D; Table 2), suggesting that mutational activation of the PI3K pathway is essentially mutually exclusive with mutational activation of the RAS pathway in breast cancer (χ^2 $P = 0.0012$, with exclusion of SUM225CWN from the analysis, and $P = 0.0043$ when MDA-MB-435s and DU4475 were also excluded; Table 2). This could imply that signals critical for breast carcinogenesis converge through the PI3K and RAS pathways, targeting a single downstream effector. Concurrent mutational activation of both the PI3K and RAS pathways would then not be observed, as double mutants would not have a selective growth advantage over single mutants. In this respect, it is of interest that the mutation spectra of genes from the PI3K and RAS pathway may differ among tumor types. For example, the *BRAF* mutation spectra of breast cancers, colorectal cancers, and melanomas are each dominated by the V600E mutation. However, these three tumor types differ in that activating *RAS* mutations occur predominantly in the *NRAS* gene in melanomas and in the *KRAS* gene in breast cancers and colorectal cancers (25). Similarly, breast cancers and colorectal cancers share a *PIK3CA* mutation spectrum that is dominated by the H1047R, E545K, and E542K mutations, whereas *PIK3CA* mutations are rare in melanomas (40). Breast cancers and colorectal cancers thus have similar *PIK3CA, BRAF*, and *KRAS* mutation spectra. Yet, *PIK3CA* mutations are coincident with RAS pathway mutations in colorectal cancers (41), whereas we found that in breast cancers, mutational activation of the PI3K pathway was mutually exclusive with mutational activation of the RAS pathway. In melanoma, on the other hand, *PTEN* mutations are coincident with *BRAF* mutations, but not with mutations of *NRAS* (42, 43). Such differences in PI3K and RAS pathway mutations among human tumor types suggest that there is a tissue-specific distinction in the activation and transduction of signals through these oncogenic pathways, at the very least for the skin and epithelia of the colon and breast.

Materials and Methods

Breast Cancer Cell Lines

The 40 human breast cancer cell lines used in this study are listed in Table 2. Cell lines EVSA-T, MPE600, and SK-BR-5/7 were kind gifts of Dr. N. de Vleesschouwer (Institut Jules Bordet, Brussels, Belgium), Dr. H.S. Smith (California Pacific Medical Center, San Francisco, CA), and Dr. E. Stockert (Sloan-Kettering Institute for Cancer Research, New York, NY), respectively. The SUM cell lines were generated in the Ethier laboratory.[3] Cell line OCUB-F was obtained from Riken Gene Bank (Tsukuba, Japan), and all other cell lines were obtained from American Type Culture Collection (Manassas, VA). All cell lines are unique and monoclonal as shown by extensive analysis of nearly 150 polymorphic microsatellite markers (44). We were unsuccessful in obtaining constitutional normal tissues or tumor blocks from the cell lines, precluding assessment of the somatic or germ line nature of mutations.

Mutational Analysis

The complete coding sequences and intron-exon boundaries of *PTEN* (ENSG00000171862) and *PIK3CA* (ENSG00000121879), as well as exons 2 and 3 of the *RAS* genes (ENSG00000133703, ENSG00000174775, and ENS00000168638) and exons 7, 11, and 15 of *BRAF* (ENSG00000157764) were analyzed for genetic alterations. For each of the six genes, intronic primers were used to PCR-amplify gene-specific fragments from genomic DNA. *PTEN* transcripts were amplified from total RNA, using the Qiagen (Hilden, Germany) one-step reverse transcription-PCR kit and gene-specific exonic primers (with or without inclusion of gene-specific *HPRT* primers). For sequence analysis, amplification products were incubated with Shrimp Alkaline Phosphatase and Exonuclease-I enzymes, and subsequently sequenced with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using an ABI3100 Genetic Analyzer. All sequence variants identified were validated by sequencing an independently amplified PCR product, and for *PTEN* mutants also by transcript sequencing. Allelic loss at the *PTEN* chromosomal locus was determined by microsatellite analysis, using markers D10S1765, D10S1687, and D10S1744. Forward microsatellite primers contained a M13 sequence at their 5′ end. Amplification products were obtained by using both the microsatellite primers and a FAM-labeled complementary M13 sequence in a single reaction. Product lengths were determined on an ABI3100 Genetic Analyzer. Primer sequences are available as Supplementary Table S1. Amplification of the *PIK3CA* locus at chromosome 3q and the *AKT2* locus at chromosome 19q was established from SNP array data that were available for 19 cell lines,[2] with an intensity ratio cutoff of 1.5 for low-level amplification (equivalent to three allele copies).

[3] http://www.asterand.com

Gene Cloning

PTEN transcripts of cell line CAMA-1 were amplified with the Qiagen one-step reverse transcription-PCR kit, using gene-specific primers designed to include either a *Bam*HI or *Eco*RI restriction site and to span both mutations in CAMA-1 (Supplementary Table S1). The reverse transcription-PCR products were digested with these restriction enzymes and subsequently cloned in the multiple cloning site of the pcDNA3.0 vector (Invitrogen, Paisley, Scotland). Inserts from 14 single colonies were PCR amplified and sequenced using vector-specific primers.

Methylation Analysis

Exponentially growing cells were seeded at a density of ~1 million cells per T75 flask, in RPMI 1640 with 10% FCS. On each of the following 3 days, 10 μmol/L filter-sterilized 5-aza-2′-deoxycytidine (Sigma, Steinheim, Germany) was added to the cell cultures. On the 4th day, cells were washed with PBS at 37°C, harvested by lysis in the flask, and total RNA was isolated. As a control, cultures untreated with 5-aza-2′-deoxycytidine were included.

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