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Advances in Brief

Mutation of the *PIK3CA* Gene in Ovarian and Breast Cancer

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Abstract

Phosphatidylinositol 3′-kinases are lipid kinases with important roles in neoplasia. Recently, a very high frequency of somatic mutations in *PIK3CA* has been reported among a large series of colorectal cancers. However, the relevance of *PIK3CA* mutation in other cancer types remains unclear because of the limited number of tumors investigated. We have screened a total of 284 primary human tumors for mutations in all coding exons of *PIK3CA* using a combination of single stranded conformational polymorphism and denaturing high-performance liquid chromatography analysis. Among 70 primary breast cancers, 40% (28 of 70) harbored mutations in *PIK3CA*, making it the most common mutation described to date in this cancer type. Mutations were not associated with histologic subtype, estrogen receptor status, grade or presence of tumor in lymph nodes. Among the primary epithelial ovarian cancers only 11 of 167 (6.6%) contain somatic mutations, but there was a clear histologic subtype bias in their distribution. Only 2 of 88 (2.3%) of serous carcinomas had *PIK3CA* mutations compared with 8 of 40 (20.0%) endometrioid and clear cell cancers, which was highly significant ($P = 0.001$). In contrast, *PIK3CA* gene amplification (>7-fold) was common among all histologic subtypes (24.5%) and was inversely associated with the presence of mutations. Overall, *PIK3CA* mutation or gene amplification was detected in 30.5% of all ovarian cancers and 45% of the endometrioid and clear cell subtypes. Our study is the first direct evidence that *PIK3CA* is an oncogene in ovarian cancer and greatly extends recent findings in breast cancer.

Introduction

The class IA phosphatidylinositol 3′-kinases (PI3K) are a family of widely expressed lipid kinases that exist as heterodimers consisting of a unique catalytic subunit (p110α, β, or δ) along with one of a number of shared regulatory subunits (p85α, p85β, or p55γ; ref. 1). Numerous genetic and functional studies have clearly established a fundamental role for the PI3K pathway in the development of neoplasia. As a tumor suppressor, the role of PTEN, a negative regulator of the PI3K pathway, has been well-documented whereas Akt, a major downstream target of PI3K, has been shown to be activated and/or overexpressed in a number of human cancers (2, 3). PI3K itself has also been shown to be dysregulated in human cancers. Amplification of the *PIK3CA* gene (which codes for the p110α catalytic subunit of PI3K) has been reported in a number of different tumor types (4, 5). However, we were the first to provide direct evidence for an oncogenic role for PI3K in human cancer by showing the presence of activating somatic mutations in the p85α regulatory subunit of PI3K (*PIK3R1*) in primary ovarian and colon tumors (6). Although the mutation frequency was low, it raised the possibility that other members of the PI3K family might be activated by somatic mutations. This has been borne out by the recent report of a very high frequency of somatic mutations in *PIK3CA* among a large series of colorectal cancers (7). However, the relevance of *PIK3CA* mutation in other cancer types remains unclear because few tumors of other types were investigated. In particular, no ovarian cancers and only 12 breast cancers were included in that study. Therefore, we undertook a search for somatic mutations in a large series of ovarian and breast cancers to establish the role that *PIK3CA* mutation plays in the etiology of these important malignancies.

Materials and Methods

Tumor Samples. Matching normal and tumor DNA from 70 primary breast cancers was provided by the Peter MacCallum Cancer Centre tissue bank or by Dr. Nick Hayward (Queensland Institute for Medical Research, Brisbane, Australia). One hundred and eighty-two primary ovarian tumor samples were obtained from hospitals in the south of England. DNA was extracted from fresh cancer tissue and matching peripheral blood samples were used as a source of normal DNA. Thirty-two colon carcinomas were obtained from patients undergoing elective surgery at Western Hospital (Victoria, Australia). Normal DNA was extracted from normal-appearing mucosa >5 cm from the margins of the carcinoma. The collection and use of tissues for this study were approved by the appropriate institutional ethics committees.

Screening for Mutations. Exons 1–20 of *PIK3CA* were individually amplified from genomic DNA with primers complementary to surrounding intronic sequences. Details of these primers are available on request. PCR was carried out with 10 ng of genomic DNA in a reaction volume of 10 μL, with the inclusion of 0.5μCi of [α-^{32}P]dATP and 0.1 units Hot Star *Taq*DNA polymerase (Qiagen, Valencia, CA). After an initial denaturation step of 95°C for 10 minutes, a "touch-down" program was used consisting of 2 cycles of amplification at annealing temperatures of 63°C to 59°C; followed by 30 amplification cycles at an annealing temperature of 58°C and a final extension cycle of 72°C for 5 minutes. Samples were prepared for single-strand conformational polymorphism (SSCP) analysis and separated on 0.5× mutation detection enhancement gel matrix (BioWhittaker Molecular Applications Inc., Rockland, ME) as described previously (8).

In addition to SSCP analysis, exons 9 and 20 were screened for mutations by denaturing high performance liquid chromatography (DHPLC) with the Varian-Helix DHPLC system (Varian, Inc., Palo Alto, CA). To enhance heteroduplex formation, PCR products were subjected to an additional 95°C denaturation step for 5 minutes followed by gradual reannealing from 95°C to 60°C over a 40 minute period before analysis. PCR products were then introduced into the mobile phase in an injection volume of 3 μL. The products were eluted from the column with a 100 mmol/L triethylammonium acetate buffer (pH 7.0) containing 25% acetonitrile gradient at a flow rate of 0.45 mL/minutes. Temperatures required for the successful resolution of heteroduplex molecules were determined by the use of the DHPLC melting algorithm.[4]

Cases showing aberrant band shifts by SSCP or DHPLC were repeated and compared with the matching normal DNA (where available) to determine whether the change was germline or somatic. Tumors showing somatic changes or harboring rare germline variants were reamplified and sequenced directly with the BigDye terminator method (Applied Biosystems; Warrington,

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[4] http://intersion.Standford.edu/melt.html.

United Kingdom or Forster City, CA) on an autosequencer (ABI PRISM 3100).

Screening for Gene Amplification. *PIK3CA* gene amplification was assessed by SYBR Green quantitative PCR with primers to genomic sequences (*PIK3CA* forward, 5′-TATGGTTGTCTGTCAATCGGTGA-3′; *PIK3CA* reverse, 5′-GCCTTTGCAGTGAATTTGCAT) and compared with the signal obtained from two control genes *KRAS2* (*KRAS* forward, 5′-CTGAAGATGTACCTATGGTCCTAGTAGG-3′; *KRAS* reverse, 5′-AAGTCCTGAGCCTGTTTTGTGTC-3′) and *BARD1* (*BARD1* forward, 5′-TTCTGTAGCCAACCATCTGTTATCTC-3′; *BARD1* reverse, 5′-TCAGATTCTGTCAAGGAGCCACT-3′). *KRAS2* and *BARD1* were selected as controls based on a scan of the literature indicating that they are located in regions not frequently showing loss of heterozygosity or amplification in breast, ovarian, or colorectal cancers. PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7000 sequence detection system. We analyzed all results on a standard curve derived from a known sample concentration and used on each assay. Cycle threshold values obtained were then compared with the standard curve, and nanogram values were used in the subsequent analysis. Three normal samples were also used on each assay, and the mean value of these was used to normalize the data and correct for inter-assay variation. The *PIK3CA* gene copy number was calculated by dividing its value by the mean of the *KRAS2* and *BARD1* values. High-level DNA amplification was arbitrarily and conservatively defined as values >7.

Results and Discussion

Two hundred and eighty-four primary breast, ovarian, and colorectal cancers were screened for mutations in all 20 exons of the *PIK3CA* gene. SSCP analysis was used as the primary means of mutation detection because it combines relatively high sensitivity with the ability to detect mutations even in the presence of a high background of normal DNA contamination. To further increase sensitivity, DHPLC was used to analyze exons 9 and 20, where the majority of mutations have thus far been reported (7). All of the exon 9 and 20 mutations detected by DHPLC were also easily visualized on SSCP gels, examples of which are shown in Fig. 1. Table 1 summarizes the 45 mutations that were identified among the primary cancers. Mutations were identified in exons 6, 7, 9, 14, and 20, with the vast majority (88.9%) occurring in exons 9 and 20. Matching normal DNA was available for all but eight tumors and in all instances the mutations were shown to be somatic. With the exception of the putative exon 14 A2198G (K733R) mutation, the alterations detected among the eight cases (three colorectal and five breast cancers) where no matching normal DNA was available have been shown to be somatic changes in primary cancers. A number of lines of evidence indicate that these represent pathologic mutations rather than benign chance somatic events. First, the frequency of somatic mutations far exceeds the expected background rate, and all 45 mutations were nonsynonymous changes. As expected for an oncogene, all of the mutations were either missense changes or in-frame deletions. Finally, *in vitro* studies have shown that the H1047R mutant, which is the most common mutation detected in our study, has increased lipid kinase activity (7).

The frequency, location, and type of mutations detected among the colorectal cancers were consistent with those reported by Samuels *et al.* (7). In addition, we found no evidence of gene amplification as assessed by quantitative real-time PCR, a result consistent with previous studies (7). Among the 70 breast cancers, 28 (40.0%) harbored somatic mutations, with 53.6 and 32.1% occurring in exon 9 or 20, respectively. Interestingly, 4 of 9 exon 20 mutations detected in the breast cancers were of the H1047L type compared with only 1 of 22 reported by Samuels *et al.* (7) among 234 colon cancers. Nevertheless, the overall proportion of exon 9 and exon 20 mutations detected in the breast cancers is almost identical to that observed by Samuels *et al.* (54% and 31%, respectively) suggesting that there is no major difference in the selection advantage of these mutations between these two

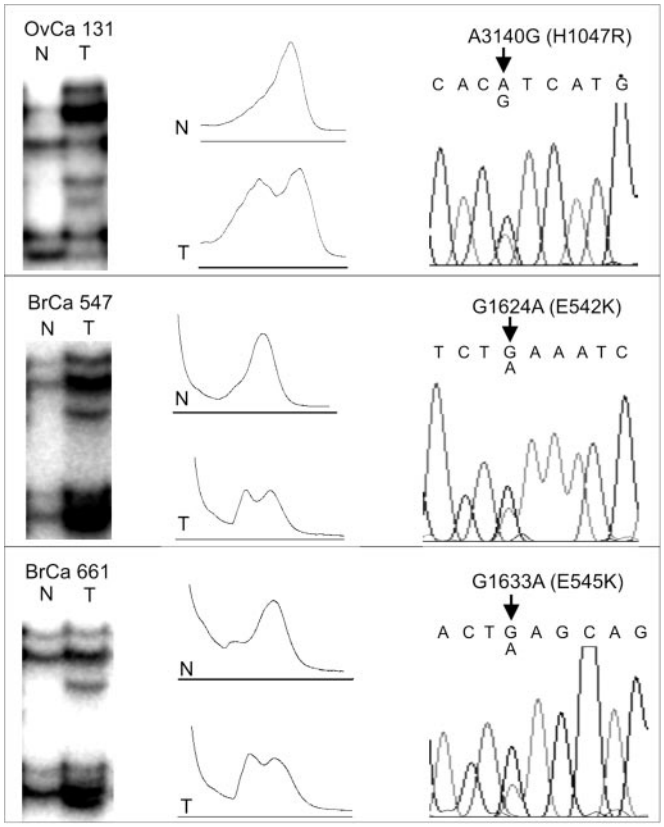

Fig. 1. Somatic mutations found in the *PIK3CA* gene in ovarian and breast cancers. Aberrant band shifts were detected by both SSCP and DHPLC in the tumor DNA (*T*) compared with the matching normal DNA (*N*). The sequence trace of the tumor DNA is shown and the mutation indicated.

cancer types. Two mutations occurring in the breast cancers have not been reported previously; a 15 bp deletion of nucleotides 1352–1366 in exon 7 and a C1241T (S405P) substitution in exon 6. Only one invasive ductal breast tumor showed amplification (22-fold) of the *PIK3CA* gene. As summarized in Table 2, there was no evidence of an association of mutations with histologic type, estrogen receptor status, tumor grade, or nodal status. A recent study by Bachman *et al.* (9) of a smaller set of 41 breast tumors identified somatic *PIK3CA* mutations in 22% of cases, which is significantly lower than the 40% reported here. The discrepancy is explained in part by the fact that Bachman *et al.* only analyzed the mutation hot spots (based on the spectrum of mutations found in colorectal cancers) in exons 1, 9, and 20. In addition, our adoption of an SSCP-based mutation analysis is likely to have improved the detection rate because, unlike direct DNA sequencing, the sensitivity of SSCP is maintained even in the presence of high normal DNA contamination. This is particularly critical when screening for oncogenic mutations where unmasking of a mutant allele through loss of heterozygosity of the wild-type allele rarely occurs.

With respect to the spectrum of mutations identified, Bachman *et al.* (9) suggested that exon 20 mutations predominate in breast cancers in contrast to colorectal cancers where exon 9 mutations are more common. Our data does not support this and highlights the need to be cautious when defining both tumor type-specific mutations and mutational hot spots at this early stage where relatively few primary tumors have been analyzed. In fact, the proportion of exon 9 to exon 20 mutations identified in our larger cohort of breast tumors (15 *versus* 9) is almost identical to the proportion identified by Samuels *et al.* (7) among colorectal cancers (43 *versus* 27). Furthermore, 14.3% of the mutations identified in our breast cancer cohort were outside the mutation "hot spots" identified by Bachman *et al.* (9).

Table 1 *PIK3CA mutations among primary ovarian, breast, and colorectal cancers*

PIK3CA mutations *			Tumor type			
Exon	Nucleotide	Amino acid	Breast	Ovarian	Colon	Total
Exon 6	**C1241T**	**S405P**	1			1
Exon 7	T1258C	C420R	2			2
	del1352–1366	**del451–456**	1			1
Exon 9	G1624A	E542K	5	2		7
	G1633A	E545K	9	1	2	12
	A1634G	E545G			1	1
	C1636A	Q546K		1	1	2
	C1636G	**Q546E**	1			1
Exon 14	A2198G	K733R			1 †	1
Exon 20	A3140G	H1047R	5	7	1	13
	A3140T	H1047L	4			4
Total mutations (%)			28/70 (40.0%)	11/182 (6.0%)	6/32 (18.8%)	

* Exon number with nucleotide position and amino acid change. Mutations in bold have not been reported previously.
† No matching normal DNA was available for this sample and therefore the status of this nonsynonymous change as a somatic mutation should be considered preliminary.

Table 2 *PIK3CA mutations and histologic subtype among breast cancers*

Tumor classification	Mutation frequency (%)
Histologic type	
Ductal	24/55 (43.6%)
Lobular	4/9 (44.4%)
DCIS	0/3
Mucinous	0/1
Neuroendocrine	0/2
Grade	
1	2/6 (33.3%)
2	13/24 (54.2%)
3	11/29 (37.9%)
ER status	
+ve	15/32 (46.9%)
−ve	7/19 (36.8%)
Nodal status	
+ve	20/38 (52.6%)
−ve	6/19 (31.6%)

Mutations among the ovarian tumors were only identified in exons 9 and 20 (Table 1). Overall, only 6% of the tumors (6.6% of the malignant tumors) harbored *PIK3CA* mutations. However, stratifying the ovarian cancer according to histologic subtype reveals a striking bias in the distribution of the mutations. Only 2 of 88 (2.3%) of the serous and none of 24 mucinous carcinomas harbored somatic *PIK3CA* mutations compared with 8 of 40 (20%) of the endometrioid and clear cell ovarian cancers. This finding is consistent with the concept that the major histologic subtypes, serous, endometrioid, clear cell, and mucinous arise through different developmental pathways. In particular, we have shown that endometrioid and clear cell ovarian cancers probably arise through malignant transformation of endometriosis and not the ovarian surface epithelium (10, 11).

Chromosomal amplifications are commonly associated with activation of oncogenes, and therefore we assessed the *PIK3CA* copy number by real-time quantitative PCR. *PIK3CA* gene amplification was common among the ovarian cancers (24.6%) but absent in the borderline and benign tumors (Table 3). The mean copy number increase was 35-fold with highest amplification of 226-fold recorded in an endometrioid cancer. In contrast to the distribution of somatic *PIK3CA* mutation, gene amplification was not restricted to any particular histologic subtype with the serous ovarian cancers showing a similar frequency to the endometrioid and clear cell cancers. Interestingly, there was an almost perfect reciprocal association of the presence of gene amplification and a somatic *PIK3CA* mutation suggesting that these only occur in tumors without amplification. Furthermore, in previous studies we did mutational analysis on a subset of these ovarian cancers for both p85α (*PIK3R1*), which is a regulatory binding partner to p110α and *PTEN* (6, 12). Among 66 cancers, we identified one mucinous tumor with a somatic p85α mutation and three endometrioid tumors with somatic *PTEN* mutations. None of the four cancers harbored a *PIK3CA* mutation and none showed gene amplification.

The data shows that somatic changes in *PIK3CA* through mutation or gene amplification is extremely common in ovarian cancer with 30.5% harboring one or the other alteration. Among the endometrioid and clear cell ovarian cancers, *PIK3CA* involvement is even more striking with 45% harboring one or other alteration. With respect to the gene amplification however, it should be noted that the 3q26 region harbors several putative oncogenes. Consequently, it is possible that the amplification we and others have observed in ovarian cancers (5) might not be targeting *PIK3CA* in all cases (13). Although a strong case could be made for a direct association of gene amplification with involvement of PIK3CA among the endometrioid and clear cell cancers (because they show a concomitant high frequency of somatic mutations), the argument is not as strong for the other histologic subtypes. Indeed, it is curious why *PIK3CA* amplification should be so common among the serous ovarian cancers whereas somatic mutations are quite rare and why one serous tumor showed *PIK3CA* gene amplification (15-fold) despite the coexistence of a somatic coding mutation. Although Shayesteh *et al.* (5) has shown increased *PIK3CA* transcription and p110α protein expression in many (but not all) ovarian cancer cell lines with *PIK3CA* amplification, it will be important to verify specific targeting of the *PIK3CA* gene in ovarian cancers through detailed amplicon mapping studies.

In summary, our comprehensive mutation screen has unequivocally established that *PIK3CA* mutations occur with high frequency in both ovarian and breast tumors. These data, together with other studies demonstrating the involvement of other components of the PI3K pathway in these tumors, suggests that the PI3K pathway plays a central role in breast and ovarian tumorigenesis and implies that this pathway may be a valuable target for the development of novel therapies for these cancers.

Table 3 *PIK3CA mutations and amplification among histologic subtypes of ovarian tumors*

Group	Mutation frequency (%)	Amplification *	Amplification and mutation †	Amplification or mutation ‡
Malignant	11/167 (6.6%)	41/167 (24.6%)	1/41 (2.4%)	51/167 (30.5%)
Serous	2/88 (2.3%)	26/88 (29.5%)	1/26 (3.8%)	27/88 (30.7%)
Endometrioid	7/35 (20.0%)	8/35 (22.9%)	0/8	15/35 (42.9%)
Clear cell	1/5 (20.0%)	2/5 (40.0%)	0/2	3/5 (60.0%)
Mucinous	0/24	3/24 (12.5%)	0/3	3/24 (12.5%)
Undifferentiated	1/14 (7.1%)	3/14 (21.4%)	0/3	4/14 (28.6%)
Borderline	0/7	0/7	0	
Benign	0/8	0/8	0	

* Amplification defined as >7-fold *PIK3CA* signal compared with the mean of the control genes *KRAS* and *BARD1*.
† Number of tumors with *PIK3CA* amplification that also harbour a somatic mutation.
‡ Total number of tumors with *PIK3CA* amplification and/or somatic mutation.

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