SHORT COMMUNICATION

Mutational Analysis of *AKT1* and *PIK3CA* in Intraductal Papillomas of the Breast with Special Reference to Cellular Components

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The pathologic feature of intraductal papillomas is defined as a papillary structure composed of a fibrovascular stromal core lined by luminal epithelial cells and myoepithelial cells. We used droplet digital PCR for the mutational analysis of *AKT1* (E17K) and *PIK3CA* (H1047R, E542K, and E545K) in 60 papillomas. *AKT1* and *PIK3CA* mutations were detected in 12 (20%) and 17 (28%) of the papillomas, respectively. In five tumors harboring mutations, mutational analysis of *AKT1* or *PIK3CA* was performed separately using luminal epithelial cells and myoepithelial cells sorted using anti-cytokeratin 19 antibody and anti- α smooth muscle actin antibody. The two types of cells from a given papilloma had the identical mutation. Three patients with the *PIK3CA* mutation-positive papilloma developed breast cancers at the resection site of the papilloma, but none of these subsequent breast cancers had the *PIK3CA* mutation. These results indicate that a papilloma stems from a bipotent progenitor cell that contains the *AKT1* or *PIK3CA* mutation and proliferates and differentiates to form the papilloma. Papilloma can be a risk factor for developing breast cancer but is unlikely to be its obligate precursor. (*Am J Pathol* 2018; 188: 1106–1112; <https://doi.org/10.1016/j.ajpath.2018.01.005>)

Intraductal papilloma is a common disease of the breast and often occurs as a solitary tumor beneath the areola in the fifth and sixth decades of life.¹ Histologically, typical papillomas are lined by two cell types: myoepithelial cells situated along the basement membrane and the overlying luminal epithelial cells. Some tumors contain proliferated luminal epithelial cells and include florid hyperplasia, atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ*. A papilloma is also reportedly associated with an increased risk of subsequent breast cancer, especially when it occurs in conjunction with ADH or involves terminal ductal-lobular units.^{2,3} However, pure intraductal papillomas themselves are not considered to have the potential to progress to carcinoma, although their pathogenesis remains poorly understood.^{4,5}

A previous molecular study using clonal analysis demonstrated that papilloma of the breast comprises a monoclonal tumor, suggesting that luminal epithelial and myoepithelial cells originate from a common precursor.⁴ In

the mammary epithelial cell hierarchy, mammary stem cells cause bipotent restricted progenitor cells that can divide into committed mature luminal-restricted progenitors and myoepithelial-restricted progenitors, and finally differentiate into luminal epithelial cells and myoepithelial cells, respectively.^{6–9} The view that a papilloma is monoclonal in origin assumes that a certain tumorigenic change may occur at the bipotent cell stage before differentiation.

The driver genes associated with monoclonal expansion of papillomas were unknown until recently, when Troxell et al⁵ performed mutational screening using MassARRAY of papillary lesions, including papillomas, and detected frequent somatic mutations of *AKT1* (23/72, 32%) and *PIK3CA* (25/72, 35%) in papillomas. These two genes are central players in the oncogenic phosphatidylinositol 3-kinase/AKT pathway related to cell proliferation, survival,

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angiogenesis, and motility.¹⁰ *AKT1* E17K is one of the most common *AKT1*-activating mutations, with a frequency of 1.4% to 8.2% in breast cancers.^{11–14} *PIK3CA* mutations, on the other hand, occur in approximately one-third of breast cancers, and most of those are found in the hotspots within exon 9 and 20, including H1047R, E542K, and E545K.^{15–18} Although *PIK3CA* mutations in papillomas also have these hotspot mutations, their distribution is reportedly different from that in breast cancers.⁵ Jahn et al¹⁹ also reported the *AKT1* (2/7, 29%) or *PIK3CA* (2/7, 29%) mutation in papilloma with usual ductal hyperplasia using next-generation sequencing. After these reports, however, no further studies have been reported that used mutational analysis of *AKT1* and *PIK3CA* in papillomas of the breast to validate their findings.

A mutational analysis of *AKT1* and *PIK3CA* in papillomas of Japanese women was conducted, first, to validate the previous findings, and second, to investigate any ethnic differences in mutation frequency and distribution.^{5,19} Moreover, the potential of the metachronous occurrence of these two lesions in the same region of the breast to progress from papilloma to cancer was also investigated by mutation analysis. In addition, special attention was paid to examining whether *AKT1* or *PIK3CA* mutation is observed in the luminal epithelial cells, myoepithelial cells, or both cell types in papillomas. Although the monoclonal origin of papillomas indicates that both cell types can be expected to have the same mutation, this issue has never been verified.^{4,20} Evidence of such a mutation would provide an important insight for a better understanding of the pathogenesis of papillomas containing the two cell types.

Materials and Methods

Patients and Samples

Seventy-one patients with papilloma treated in Osaka University Hospital (Suita, Japan) between 1997 and 2005 were included in this study and used for the estimation of a risk for developing subsequent breast cancer. Formalin-fixed, paraffin-embedded (FFPE) tissues were available from 65 papillomas from 62 of these 71 patients. Because of poor DNA quality, five papillomas were excluded and, finally, 60 papillomas from 57 patients were subjected to the mutational analysis. Histologic diagnosis was made by the two skillful pathologists (J.-i.I. and E.M.) using the hematoxylin and eosin sections and, if necessary, immunohistochemistry for α smooth muscle actin (α -SMA), CD10, and p63. One patient had three synchronous multiple papillomas in the same upper outer quadrant of the breast, and another had two metachronous papillomas at the same site. Nine patients developed subsequent breast cancer, including one patient with bilateral breast cancer. Among them, four breast cancers developed at the resection site of the preceding papilloma. A median follow-up period for the 71 patients after the resection ($n = 62$) or core needle biopsy only ($n = 9$) of papilloma was 5.0 years

(range, 0.1 to 17.3 years). This study was approved by the Osaka University Research Ethics Committee.

DNA Extraction from FFPE Tumor Tissues

Two to four 10- μ m sections were cut from the FFPE tumor tissues and mounted onto a polyethylene naphthalate membrane slide (Leica Microsystems, Wetzlar, Germany). The tissue slides were stained with hematoxylin after deparaffinization, and the tumor area was macrodissected with a scalpel under stereoscopic guidance. Genomic DNA from the paraffin sections was then extracted and purified using the GeneRead DNA FFPE kit (Qiagen, Valencia, CA), according to the manufacturer's instructions.

ddPCR

AKT1 E17K mutation and three *PIK3CA* hotspot mutations were detected with QX100 Droplet Digital PCR system (Bio-Rad, Hercules, CA). For PCR amplification, the final 20 μ L of the reaction mix was prepared, including at least 50 ng of DNA and 10 μ L of 2 $\times$ droplet digital PCR (ddPCR) Supermix for Probes (Bio-Rad), according to the manufacturer's instructions. The PrimePCR ddPCR Mutation Detection Assay Kit, *AKT1* WT for p.E17K and *AKT1* p.E17K Human (1863118; Bio-Rad), was used to detect *AKT1* mutations. The primers and probes for the three *PIK3CA* mutations (E542K, E545K, and H1047R) were designed as previously described.¹⁶ Each mixture and 67 μ L of droplet generation oil were loaded into a droplet generator (Bio-Rad). Emulsified samples were then subjected to PCR with the following thermal profiles: initial denaturing at 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, at individual melting temperatures for 1 minute (55°C for *AKT1*, 56°C for *PIK3CA* E542K, and 60°C for *PIK3CA* H1047R and E545K), and a final extension at 98°C for 10 minutes. After PCR amplification, the droplets were read with the QX100 droplet reader (Bio-Rad) as instructed and the data were analyzed with QuantaSoft version 1.7.4. (Bio-Rad). The mutant allele frequency (MAF; %) was defined as the proportion of mutant DNA copies relative to the sum of mutant and wild-type DNA copies. The samples were defined as positive for mutations when MAF >3% and total copies >100.

Immunohistochemical Staining

Sections (3 μ m thick) were incubated overnight at 4°C with an anti-cytokeratin (CK) 19 monoclonal antibody (1:50; clone RCK108; Agilent Technologies, Santa Clara, CA) after antigen retrieval at 98°C for 20 minutes and with an anti- α -SMA monoclonal antibody (ready-to-use; clone 1A4; Nichirei Biosciences, Tokyo, Japan). Counterstaining was performed with a hematoxylin solution. Estrogen receptor and progesterone receptor status was defined as positive when 10% or more of the luminal epithelial cells

Table 1 *AKT1* and *PIK3CA* Mutational Profile of Papillomas and Their Clinicopathological Characteristics

Variable	Mutation-positive			Mutation-negative	P value
	<i>AKT1</i> mt	<i>PIK3CA</i> mt	Total		
Tumors, <i>n</i>	12 (20)	17 (28)	29 (48)	31 (52)	
MAF, %*	28.0 (10.8–80.6)	31.8 (4.7–47.0)			
Age, years*	50 (25–80)	48 (32–65)	48 (25–80)	50 (29–83)	0.401
Tumor size, mm*	6 (3–18)	7 (3–25)	7 (3–25)	7 (2–20)	0.231
Histologic subtype					0.270
Pure	9 (15)	11 (18)	20 (33)	24 (39)	
With UDH	3 (5)	4 (7)	7 (11)	3 (5)	
With ADH	0 (0)	2 (3)	2 (3)	4 (7)	
ER					0.434
Positive	11 (18)	16 (26)	27 (45)	27 (45)	
Negative	1 (2)	1 (2)	2 (3)	4 (7)	
PR					0.844
Positive	7 (12)	11 (18)	18 (30)	20 (33)	
Negative	5 (8)	6 (10)	11 (18)	11 (18)	

Data are expressed as *n* (%) unless otherwise indicated. Total *n* = 60.
*Data are expressed as median (range).
ADH, atypical ductal hyperplasia; ER, estrogen receptor; MAF, mutant allele frequency; mt, mutant; PR, progesterone receptor; UDH, usual ductal hyperplasia.

were stained by immunohistochemistry (estrogen receptor, clone SP1, Nichirei Biosciences; progesterone receptor, clone PgR 636, Agilent Technologies).¹⁶

Tumor Cell Dissociation and Cell Sorting

Dual staining of CK19 and α -SMA was performed for the separation of luminal epithelial and myoepithelial cells by fluorescence-activated cell sorting (FACS). A total of 20 to 30 sections (50 μ m thick) of a macrodissected tumor were collected in microtubes, dewaxed in xylene, and then rehydrated in ethanol and phosphate-buffered saline. For antigen retrieval, the sections were placed in Dako Target Retrieval Solution (pH 9.0; Agilent Technologies) and heated for 40 minutes at 90°C. After cooling and washing with phosphate-buffered saline, sections were incubated with 0.1% collagenase type 3 (Worthington Biochemical Corp., Lakewood, NJ) and 0.1% dispase II (Wako, Osaka, Japan) in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) at 37°C for 60 minutes. Cells were filtered through a 35- μ m nylon mesh (BD Biosciences, Franklin Lakes, NJ), washed twice in FACS buffer (phosphate-buffered saline containing 3% fetal bovine serum and 5 mmol/L EDTA), and incubated with 2.5 μ L of normal mouse serum (Jackson ImmunoResearch, Westgrove, PA) and 2.5 μ L of Human TruStain FcX (BioLegend, San Diego, CA) for 15 minutes. Cells were then stained overnight at room temperature in 100 μ L of FACS buffer containing 2 μ L of anti-CK19 (clone A53-B/A2; fluorescein isothiocyanate conjugated; Abcam plc, Cambridge, UK) and 10 μ L of anti- α -SMA (clone 1A4; allophycocyanin conjugated; R&D Systems, Minneapolis, MN). Mouse IgG2a (fluorescein isothiocyanate conjugated, BD Biosciences; allophycocyanin

conjugated, R&D Systems) was used as an isotype control for each antibody. Cells were finally stained with DAPI solution (Dojindo Laboratories, Kumamoto, Japan). FACS Aria II (BD Biosciences) was used for sorting CK19^{high}/ α -SMA^{low} and CK19^{low}/ α -SMA^{high} cells from a DAPI-positive single-cell population. A total of 3.0 to 7.7 $\times 10^6$ events were collected in FACS buffer, and DNA was extracted from the cells using the GeneRead DNA FFPE kit (Qiagen), according to the manufacturer’s instructions. All data were analyzed with FlowJo 7.6.5 software (TOMY Digital Biology Co., Ltd., Tokyo, Japan).

Statistical Analysis

Associations between clinicopathological characteristics and mutational status were evaluated by the χ^2 test or *t*-test. All statistical analyses were two sided, and *P* < 0.05 was considered significant. The JMP statistical software package version 12.2.0 (SAS Institute, Cary, NC) was used for statistical analysis.

Results

Detection of AKT1 and PIK3CA Mutations in Papillomas

A ddPCR assay targeting *AKT1* E17K and three *PIK3CA* hotspot mutations (E542K, E545K, and H1047R) was performed on 60 papillomas from 57 patients. Clinicopathological characteristics of the patients are summarized in Table 1. First, six normal breast tissues adjacent to the papillomas were analyzed using ddPCR. Because their background MAF averaged 0.6% (SD = 0.7%, data not shown), we defined MAF = 3% as a threshold for the subsequent analyses of the 60 tumors. Twenty-nine

Figure 1 Immunohistochemical staining of cytokeratin (CK) 19 and α smooth muscle actin (α -SMA). Formalin-fixed, paraffin-embedded sections of three papillomas, two with *AKT1* (numbers 32 and 53) and one with *PIK3CA* (number 8) mutations, were hematoxylin and eosin (H&E) stained (left column) or immunohistochemically stained with antibodies to CK19 (middle column) and α -SMA (right column). Scale bars = 100 μ m. Original magnification, $\times 200$.

papillomas harbored mutations (48%), 12 with *AKT1* E17K mutations (20%) and 17 with *PIK3CA* mutations (28%), including 15 H1047R, 1 E542K, and 2 E545K mutations (Table 1). One tumor possessed two mutations (H1047R and E545K), and all of the others had a single mutation. The median total copy number was 1911 (range, 135 to 7406) for mutation-positive tumors, whereas the median MAF was 28.0% (range, 10.8% to 80.6%) for *AKT1* mutations and 31.8% (range, 4.7% to 47.0%) for *PIK3CA* mutations. Mutation status was not significantly associated with age, tumor size, and histologic subtypes or estrogen receptor and progesterone receptor status (Table 1).

Separate Mutational Analysis of Luminal Epithelial and Myoepithelial Cells

One tumor (number 8) with a *PIK3CA* H1047R mutation and four tumors (numbers 6, 32, 33, and 53) with *AKT1* E17K mutations were used for a separate mutational analysis of luminal epithelial and myoepithelial cells. Hematoxylin and eosin staining of these papillomas is shown in Figure 1, and immunohistochemistry of CK19 and α -SMA of these papillomas showed an exclusive expression of luminal epithelial and myoepithelial cells (Figure 1). Tumor cells obtained from the five FFPE specimens were then examined for CK19 and α -SMA by FACS.

CK19^{high}/ α -SMA^{low} cells (12% to 26%) and CK19^{low}/ α -SMA^{high} cells (7% to 22%) were identified in DAPI-positive cells and classified as luminal epithelial and myoepithelial cells, respectively (Figure 2). Mutational analysis with ddPCR showed identical findings for mutations in both CK19^{high}/ α -SMA^{low} and CK19^{low}/ α -SMA^{high} cells irrespective of the mutation types (Figure 2).

Mutational Status within Synchronous or Metachronous Tumors

The three synchronous papillomas (numbers 32, 33, and 34) exhibited the same *AKT1* E17K mutations with similar MAFs (Figure 3A), whereas the two metachronous papillomas (numbers 43 and 26) showed no mutations (Figure 3B). Four patients subsequently developed breast cancer at the resection site of preceding papilloma at 6, 8, 8, and 13 years after the resection of papilloma, respectively. These four breast cancers had no mutation, whereas three of the preceding papillomas had *PIK3CA* mutations (Figure 3C). Estrogen receptor was positive in all of papillomas and breast cancers in these four patients, and progesterone receptor was positive in two papillomas and three breast cancers (Figure 3C). Besides, the other five patients developed breast cancers separately from the resection sites of the preceding papillomas. The *PIK3CA* mutation

Figure 2 Separate mutational analysis of luminal epithelial and myoepithelial cells in papillomas. **Left column:** Fluorescence-activated cell sorting analysis of three papillomas stained with fluorescein isothiocyanate-conjugated anti-cytokeratin (CK) 19 antibody and allophycocyanin-conjugated anti- α -smooth muscle actin (α -SMA) antibody. The CK19^{high} α -SMA^{low} (red circles) and CK19^{low} α -SMA^{high} (black circles) fractions were classified as luminal epithelial and myoepithelial cell populations, respectively. **Middle and right columns:** The mutational analysis using droplet digital PCR of luminal-epithelial cells and myoepithelial cells, respectively. Mutant and wild-type alleles are plotted in blue and green, respectively, and mutant allele frequencies (MAFs) are indicated in the upper right corner.

Figure 3 Mutational analysis of multiple tumors. Mutation status of synchronous multiple tumors (#32, #33 and #34; **A**), metachronous multiple tumors (#43 and #26; **B**), and four cases with subsequent breast cancers (#12, #20, #36 and #46; **C**). Mutant allele frequencies are shown as percentages in parentheses. Green, papilloma; red, breast cancer. ER, estrogen receptor; PR, progesterone receptor.

(E545K) was found in two of these breast cancers, whereas none of the preceding papillomas had the mutation.

Discussion

For this study, 60 breast papillomas from 57 patients were subjected to a mutational analysis using ddPCR to search for *AKT1* and *PIK3CA* hotspot mutations. Twenty-nine papillomas were detected with mutations (48%), including 12 with *AKT1* mutations (20%) and 17 with *PIK3CA* mutations (28%), with a frequency consistent with previous reports (namely, almost half of the papillomas of the breast harbor either *AKT1* or *PIK3CA* mutation).^{5,19} Although the *PIK3CA* mutations in exon 20 appeared to be more common in papillomas than in breast cancers in the study by Troxell et al,⁵ the distribution of *PIK3CA* mutations between the two types of tumors was not significantly different, as reported elsewhere.^{16,18} In addition, no ethnic differences were found between white and Japanese women regarding mutation types and frequencies.⁵ Although no significant associations of *AKT1* or *PIK3CA* mutation with clinicopathological features of papillomas were observed, the *PIK3CA* mutation tended to be more common in papillomas with hyperplasia and ADH (6/16, 38%) than in those without them (11/44, 25%), consistent with the one reported by Troxell et al.⁵ However, such a tendency was not observed in *AKT1* mutation [3/16 (19%) versus 9/44 (20%)].

It has been reported that papillomas of the breast are monoclonal in origin, with their clonality determined with a method based on restriction fragment length polymorphism of the X-chromosome-linked *PGK* and on random inactivation of the gene by methylation. This indicated that the

two cell components of papilloma (ie, luminal epithelial and myoepithelial cells) originate from the same bipotent progenitor cells.^{4,20} If this hypothesis is correct, it can be expected that both luminal epithelial and myoepithelial cells harbor the same mutation. To test this hypothesis, the luminal epithelial and myoepithelial cells were separated from papillomas with *AKT1* or *PIK3CA* mutation by FACS, and a separate mutational analysis of each cell component was conducted. Both luminal epithelial and myoepithelial cells possessed the same mutation with similar MAFs. Although this separate mutational analysis was performed in five but not in all papillomas, consistent results were obtained. To confirm that luminal epithelial and myoepithelial cells were clearly separated by FACS, second FACS was performed for the sorted cells in the luminal epithelial fraction and in the myoepithelial fraction by the first FACS, respectively, from three papillomas, and it was demonstrated that contamination in each fraction was low (0.2% to 0.8%) (Supplemental Figure S1). Taken together, these results provide strong evidence that papilloma arises from a common bipotent progenitor cell. It has been hypothesized that *AKT1* or *PIK3CA* mutation takes place in a bipotent progenitor cell that proliferates and differentiates into both luminal epithelial and myoepithelial cells to form a papilloma.

Four patients subsequently developed breast cancer at the resection site of preceding papilloma among the 71 patients (5.6%). This frequency is similar to the previous reports (1.4% to 4.7%), although the spatial relationship between preceding papilloma and subsequent breast cancer has rarely been described in detail.^{21,22} Papilloma with ADH, but not papilloma with usual ductal hyperplasia, is reportedly related with the risk for developing subsequent breast

cancer, but such a trend was not observed in the current study (Table 1), and the presence of ADH or usual ductal hyperplasia in papilloma did not correlate with the mutational status either.^{1–3,18,23} In the present study, both luminal epithelial cells and myoepithelial cells of the mutation (*AKT1* or *PIK3CA*)—positive papilloma had the same mutations, being consistent with the previous report that papilloma is monoclonal in origin.⁴ The *AKT1* or *PIK3CA* mutation is tumorigenic, so that it transforms a normal cell into a tumor cell, which grows monoclonally to form a papilloma. Thus, it is likely that the mutation-positive papilloma is composed of only the mutation-positive cells. Therefore, the fact that three patients with *PIK3CA* mutation—positive papilloma subsequently developed the mutation-negative breast cancer at the resection site of preceding papilloma appears to indicate that papilloma is unlikely to be an obligate precursor of breast cancer. However, the following possibility cannot be completely ruled out that a papilloma, originating from a transformed cell by the mutation other than *PIK3CA*, gains the *PIK3CA* mutation during its progression to form a papilloma composed of mixture of the *PIK3CA* mutation—positive and *PIK3CA* mutation—negative cells and that the *PIK3CA* mutation—negative cells serve as a precursor of subsequent breast cancer.

In conclusion, we demonstrated the presence of *AKT1* or *PIK3CA* mutations in approximately half of the papillomas of the breasts analyzed in this study. The fact that both the luminal epithelial and myoepithelial cells harbor the same mutation indicates that papillomas stem from a bipotent progenitor cell harboring a mutation that proliferates and differentiates into luminal epithelial and myoepithelial cells to form papillomas.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.01.005>.

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