SHORT COMMUNICATION

Mutation Profiling of Usual Ductal Hyperplasia of the Breast Reveals Activating Mutations Predominantly at Different Levels of the PI3K/AKT/mTOR Pathway

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Usual ductal hyperplasia (UDH) of the breast is generally regarded as a nonneoplastic proliferation, albeit loss of heterozygosity has long been reported in a part of these lesions. To gain deeper insights into the molecular drivers of these lesions, an extended mutation profiling was performed. The coding regions of 409 cancer-related genes were investigated by next-generation sequencing in 16 cases of UDH, nine unassociated with neoplasia (classic) and seven arising within papillomas. Phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) activation was investigated by phosphorylated AKT, mTOR, and S6 immunohistochemistry. Of 16 lesions, 10 (63%) were mutated; 56% of classic lesions were unassociated with neoplasia, and 71% of lesions arose in papillomas. Fourteen missense mutations were detected: *PIK3CA* [6 (43%) of 14], *AKT1* [2 (14%) of 14], as well as *GNAS*, *MTOR*, *PIK3R1*, *LPHN3*, *LRP1B*, and *IGF2R* [each 1 (7%) of 14]. Phosphorylated mTOR was seen in 83% and phosphorylated S6 in 86% of evaluable lesions (phospho-AKT staining was technically uninterpretable). In conclusion, UDH displays mutations of the phosphatidylinositol 3-kinase/AKT/mTOR axis at different levels, with *PIK3R1*, *MTOR*, and *GNAS* mutations not previously described. Specifically, oncogenic G-protein activation represents a yet unrecognized route to proliferation in UDH. On the basis of evidence of activating mutations, loss of heterozygosity, and a mass forming proliferation, we propose that UDH is most appropriately viewed as an early neoplastic intraductal proliferation. (*Am J Pathol* 2016; 186: 15–23; <http://dx.doi.org/10.1016/j.ajpath.2015.09.004>)

Usual ductal hyperplasia (UDH) of the breast is morphologically characterized by a heterogeneous intraductal proliferation of mammary duct epithelial cells characteristically admixed with modified myoepithelial cells. Although atypical ductal hyperplasia (ADH) and low-grade ductal carcinoma *in situ* (DCIS) form a morphological continuum of intraductal neoplastic proliferations (ductal intraepithelial neoplasias) delineated in routine diagnostic pathology by arbitrary morphological (quantitative) criteria,^{1,2} UDH is currently denoted as a nonneoplastic epithelial proliferation in daily practice. It has long been recognized that proliferative breast lesions without atypia, such as UDH, radial scars, and complex sclerosing lesions, are associated with a mildly elevated risk for the development of

invasive breast cancer^{3,4}; nevertheless, genetic data on UDH and its link to invasive cancer are still sparse. The neoplastic nature of UDH was initially suggested on the basis of the detection of loss of heterozygosity in some of these lesions^{5–10} and divergent reports on the presence of *ESR1* mutations.^{11,12} Recent mutation analyses have demonstrated activating mutations in the phosphatidylinositol-3-kinase pathway in so-called columnar cell lesions of the mammary duct epithelium, in a subset of UDH and within radial scars.^{13–15}

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The aim of this study was to evaluate UDH of the breast for the presence of somatic mutations to gain insight into the alterations driving their neoplastic growth and to use somatic mutation status as evidence for a possible neoplastic nature of these lesions. We therefore investigated UDH unassociated with currently acknowledged neoplastic alterations (ie, intraductal papilloma, flat epithelial atypia, ADH, DCIS, and lobular intraepithelial neoplasia encompassing atypical lobular hyperplasia and lobular carcinoma *in situ*) and invasive breast cancer. In addition, UDH in intraductal papillomas in patients devoid of other neoplastic alterations, as cited above, was investigated, with the exception of one patient with a concomitant syringomatous adenoma in other areas of the specimen not used for analysis. UDH was analyzed for mutations in the coding regions of 409 cancer-related genes (oncogenes and tumor-suppressor genes) by next-generation sequencing. To assess activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling cascade, which constitutes the most important pathway active in mammary epithelial neoplasias, we performed immunohistochemistry for AKT phosphorylated at Serin 473 (p-AKT), mTOR phosphorylated at Serin 2448 (p-mTOR), and ribosomal protein S6 phosphorylated at Serin 240 (p-S6) as a downstream target of mTOR.

Materials and Methods

Sample Selection

Approval from the ethics committee of the Medical University of Graz (Graz, Austria) and the Institutional Review Board of the Yale University School of Medicine (New Haven, CT) was obtained for the study. Case selection was on the basis of evaluation of computerized pathological reports from the Institute of Pathology (Medical University of Graz) screened for surgical specimens comprising UDH in sufficient quantity to allow for analysis and then assigned to one of the two groups. The first group comprised 10 cases of UDH unassociated with any currently acknowledged neoplastic alterations (including papilloma, DCIS, and invasive breast cancer). Only lesions from patients without a history of any such lesions in the medical records available to us were selected. The second group consisted of seven cases of UDH within intraductal papilloma and the patient's medical history equally devoid of other neoplastic alterations up to the time of resection, with the exception of one case (papilloma with UDH, Case 6) (Table 1) with a concomitant syringomatous adenoma in other areas of the specimen not used for analysis. Slides and formalin-fixed, paraffin-embedded (FFPE) tissue were provided by the

Table 1 Clinicopathological Characteristics of Investigated Lesions and Mutations Detected

Case	Gene symbol	Mutation	Assessed biological relevance*	Size of lesion, UDH, cm	Patient age, years	Apocrine metaplasia	Size of apocrine metaplastic focus, cm
UDHs arising in papillomas							
1	<i>AKT1</i>	p.E17K	Yes	1.0	53	Yes	0.5
	<i>GNAS</i>	p.R201H	Yes				
		p.R844H [†]					
2	<i>IGF2R</i>	p.V1888L	No	0.8	61	Yes	0.1
3	<i>AKT1</i>	p.E17K	Yes	0.7	56	Yes	Multiple foci (<0.15)
	<i>MTOR</i>	p.V2406M	Yes				
4	<i>PIK3CA</i>	p.H1047R	Yes	1.4	40	No	
5	None			0.4	39	Yes	Two foci (<0.08)
6	None			0.7	63	No	
7	<i>PIK3CA</i>	p.H1047R	Yes	0.9	74	No	
	<i>LPHN3</i>	p.R465Q	No				
UDHs unassociated with papilloma							
8	<i>PIK3CA</i>	p.E542K	Yes	Max, 0.2 [‡]	73	No	
9	<i>PIK3CA</i>	p.E545K	Yes	Max, 0.2 [‡]	48	No	
	<i>LRP1B</i>	p.R3323H	No				
10	<i>PIK3CA</i>	p.H1047R	Yes	0.5	59	No	
11	None			0.5	37	No	
12	<i>PIK3CA</i>	p.H1047R	Yes	Max, 0.4 [‡]	23	No	
13	<i>PIK3R1</i>	p.K567E, p.K267E, [†] p.K297E [†]	Yes	0.3	35	Yes	Single focus (0.15)
14	None			0.4	71	No	
15	None			0.3	51	No	
16	None			0.3	48	No	

*On the basis of evaluation of Sorting Intolerant From Tolerant algorithm score and literature/database search.

[†]Alternative annotation of mutation at identical genomic location in the Cosmic database.

[‡]Maximum diameter of multiple adjacent foci.

Max, maximum; UDH, usual ductal hyperplasia.

Biobank of the Medical University of Graz (Graz, Austria) in 16 cases. In one case, tissue was provided externally by the Department of Pathology, Yale Medical School. All cases were reviewed by two pathologists (F.M. and S.W.J.), and modified, myoepithelial cells were verified by positive immunoreaction for keratin 14. The mean patient age was 49 years for patients with UDH unassociated with neoplasia/papilloma and 55 years for UDH arising in papilloma.

Mutation Analysis by Next-Generation Sequencing

Unstained, paraffinized serial sections with corresponding hematoxylin and eosin-stained levels for topographic reference were obtained. FFPE tissue from lesions (UDH and UDH arising in papilloma) and corresponding normal breast tissue were manually microdissected from unstained sections. The corresponding normal (nonlesional) reference tissue was retrieved from either a different surgical specimen of the same patient (preferred) or nonneoplastic tissue of the same resection specimen, in case further material was not available. In the one case provided externally, already mounted slides were provided for histological assessment, immunohistochemistry, and DNA retrieval. DNA was purified on a Maxwell, MDxResearch System (Promega, Fitchburg, WI). Highly multiplexed PCR was used to generate amplicon libraries covering the coding region of 409 genes encompassing >50% of the Wellcome Trust Sanger Institute Cancer Gene Census using the Comprehensive Cancer Panel primer pool (model 4477685; Thermo Fisher Scientific, Waltham, MA). The complete gene list is provided in [Supplemental Table S1](#). Libraries were prepared using the Ion AmpliSeq Library Kit 2.0 (model 4475345; Thermo Fisher Scientific). Library preparation from UDH was performed in duplicate, whereas a single library was prepared from corresponding normal tissue. Sequencing was performed on an Ion Proton Sequencer (Thermo Fisher Scientific). Emulsion PCR and sequencing runs were performed with the appropriate kits (Ion One Touch Template Kit version 2 and Ion Proton 200 Sequencing Kit; Thermo Fisher Scientific) using Ion PI chips. Sequencing length was set to 520 flows and yielded reads ranging from 70 to 150 bp, consistent with the expected size range. On average, 20 million reads were obtained for each DNA library with consistently >80% of bases at Phred scaled quality following alignment 20.

Data Analysis

Initial data analysis was performed using the Ion Torrent Suite Software version 4.1 Plug-ins (Thermo Fisher Scientific, open source, general public license). Briefly, this included base calling, alignment to the reference genome (HG19) using the Torrent Mapper and variant calling by a modified diBayes approach by taking into account the flow space information. All called variants were annotated using open source software (Annotvar,¹⁶ http://www.openbioinformatics.org/annovar/annovar_download_form.php, and SnpEff,¹⁷

<http://snpeff.sourceforge.net/download.html>; both last accessed September 28, 2015) and custom Perl scripts (available from the author upon request). Only coding, nonsynonymous mutation calls present in both duplicates from UDH and absent in nonlesional DNA were further evaluated and visually inspected in Integrative Genomics Viewer.¹⁸ Variant calls resulting from technical read errors or sequence effects were excluded from the analysis. Mutations were subsequently assessed for biological relevance by computerized prediction (*in silico* prediction). Sorting Intolerant From Tolerant algorithm (SIFT)¹⁹ was used to generate SIFT scores. Mutations with scores <0.05 were interpreted as functionally relevant (prediction damaging), scores between 0.05 and 0.1 were regarded as possibly relevant (possibly damaging), and scores >0.1 were regarded as irrelevant (functionally neutral) by computerized prediction. Final assessment of biological relevance was interpreted in conjunction with data from the Cosmic database²⁰ and literature search, as explained in the text. SPSS Statistics version 22 (IBM, New York, NY) was used for statistical comparison of mutation frequencies between classic UDH unassociated with neoplasia and UDH arising in papillomas using Fisher's exact test.

Immunohistochemistry for p-AKT, p-mTOR, and p-S6

FFPE sections (3 µm thick) were deparaffinized and stained for Ser-473 phospho-AKT [clone D9E; Cell Signaling Technology (CST), Danvers, MA], Ser-2448 phospho-mTOR (clone 49F9; CST), and Ser-240 phospho-S6 (clone DAK-S6-240; Dako, Glostrup, Denmark). Phospho-AKT immunohistochemistry was performed with Tris (pH 9) heat retrieval (microwave), and Dako + diaminobenzidine (DAB) was used as chromogen for visualization. Briefly, heat-mediated antigen retrieval (Tris, pH 9, buffer, microwave), followed by primary antibody blocking for 1 hour with 1× Tris-buffered saline Tween 20 plus 5% added normal goat serum (model 5425; CST) and overnight incubation (antibody diluted 1:25 in SignalStain antibody diluent; CST) at 4°C, followed by detection with SignalStain Boost IHC Detection Reagent (horseradish peroxidase, rabbit; CST). Subsequently, DAB (Envision DAB⁺; Dako) was used as a chromogen for visualization. Slides were counterstained with Mayer's hemalum, dehydrated, cleared, and coverslipped. For phospho-mTOR antigen, retrieval with Tris, pH 9.0 (microwave), was performed, followed by primary antibody incubation at a 1:100 dilution for 45 minutes and visualization with Dako Envision detection system, DAB (Chemmate; Dako) as chromogen, and slides were counterstained and coverslipped as stated above. For phospho-S6 immunohistochemistry, automated staining on the BenchMark ULTRA stainer (Ventana Medical Systems, Tucson, AZ) was used. Antigen retrieval (cell conditioning solution 1; Ventana Medical Systems) was used, and primary antibody was diluted 1:50 in antibody diluent (Ventana Medical Systems) and incubated for

32 minutes. The ultraView universal DAB detection kit (Ventana Medical Systems) was used as a secondary detection system. For the purpose of this study, any staining in epithelial cells of investigated lesions was scored as positive.

Results

Seventeen patients were included in the study. Sixteen cases were successfully analyzed, whereas in one case (UDH unassociated with papilloma), insufficient DNA quantity necessitated exclusion of the patient from further analysis.

UDH was manually microdissected and mutation profiled by next-generation sequencing. Both UDH unassociated with currently acknowledged neoplastic alterations and UDH arising in papillomas were investigated. In one case (Case 1, papilloma with UDH), original levels evaluated were devoid of areas suspicious for ADH, yet on keratin 14 immunohistochemistry, 2-minute foci of ADH (1.5 and <1 mm) appeared on deeper levels of the block used for tissue harvesting and DNA preparation. Because these areas only consisted of a small part of the entire lesion (whole lesion diameter, 10 mm), the case was included in the study, and focal transition to ADH was noted. [Figure 1](#), A–D, depicts exemplary cases with UDH-exhibiting mutations. [Table 1](#)

Figure 1 A and B: Usual ductal hyperplasia (UDH) arising in papilloma exhibiting *PIK3CA* p.H1047R mutation (Case 4 of [Tables 1](#) and [2](#); A) and intermixed, modified myoepithelial cells highlighted by K14 immunohistochemistry (B). C: UDH with *PIK3R1* p.K567E mutation (Case 13) and associated apocrine metaplasia. D: Higher magnification of boxed area in C. E–H: Immunohistochemical p-mammalian target of rapamycin (mTOR; E) and p-S6 (F) positivity in UDH arising in papilloma (Case 4), as well as p-mTOR (G) and p-S6 (H) positivity in UDH unassociated with neoplasia (Case 12, *PIK3CA* p.H1047R). Original magnifications: $\times 10$ (A, B, and D–F); $\times 4$ (C); $\times 20$ (G and H).

Table 2 Mutation Details

Case	Gene symbol	Mutation	Transcript	Genomic coordinate of nucleotide alteration	% mutated reads*	SIFT score
UDHs arising in papilloma						
1	<i>AKT1</i>	p.E17K	NM_001014432.1	Chr 14: g. 105246551 C>T	58	0.01
	<i>GNAS</i>	p.R201H	NM_000516.4	Chr 20: g. 57484421 G>A	44	0
2	<i>IGF2R</i>	p.V1888L	NM_000876.2	Chr 6: g.160500795 G>T	32	0.73
3	<i>AKT1</i>	p.E17K	NM_001014432.1	Chr 14: g.105246551 C>T	43	0.01
	<i>MTOR</i>	p.V2406M	NM_004958.3	Chr 1: g. 11174459 C>T	19	0.02
4	<i>PIK3CA</i>	p.H1047R	NM_006218.2	Chr 3: g.178952085 A>G	26	0.06
5	None					
6	None					
7	<i>PIK3CA</i>	p.H1047R	NM_006218.2	Chr 3: g. 178952085 A>G	42	0.06
	<i>LPHN3</i>	p.R465Q	NM_015236.4	Chr 4: g. 62758491 G>A	45	0.28
UDHs unassociated with papilloma						
8	<i>PIK3CA</i>	p.E542K	NM_006218.2	Chr 3: g. 178936082 G>A	11	0.04
9	<i>PIK3CA</i>	p.E545K	NM_006218.2	Chr 3: g. 178936091 G>A	11	0
	<i>LRP1B</i>	p.R3323H	NM_018557.2	Chr 2: g. 141208226 C>T	20	0.13
10	<i>PIK3CA</i>	p.H1047R	NM_006218.2	Chr 3: g. 178952085 A>G	39	0.06
11	None					
12	<i>PIK3CA</i>	p.H1047R	NM_006218.2	Chr 3: g. 178952085 A>G	12	0.06
13	<i>PIK3R1</i>	p.K567E	NM_181523.2	Chr 5: g. 67591106 A>G	23	0
14	None					
15	None					
16	None					

*Represent mean of duplicate analyses.

Chr, chromosome; SIFT, Sorting Intolerant From Tolerant algorithm; UDH, usual ductal hyperplasia.

gives an overview of mutations detected, an assessment of their biological relevance predicted by SIFT score in conjunction with information from the Cosmic database and literature search, size of UDH, as determined by histological measurement, patient age, and the extent of any concomitant apocrine metaplasia. Table 2 summarizes mutation details [gene symbol protein change (Human Genome Variation Society nomenclature), reference transcript, genomic position and nucleotide change, percentage mutated reads given as mean value of technical duplicates, and SIFT score]. All mutations detected were missense point mutations. Mutations were detected in 5 (71%) of 7 UDHs within papilloma and in 5 (56%) of 9 UDHs unassociated with neoplastic alterations (difference not statistically significant, $P = 0.6$). The mean diameter of mutated lesions (UDH) within papilloma was 10 mm, and the mean diameter of UDH mutated and unassociated with papilloma was 3 mm. The mean percentage of mutated reads was 19% (range, 11% to 39%) for UDH unassociated with neoplasia, compared with 39% (range, 19% to 58%) for UDH arising in papilloma.

After the biological relevance of mutations was assessed by computation of SIFT scores and evaluation of data from the Cosmic database, 5 (56%) of 9 UDHs unassociated with neoplasia and 4 (57%) of 7 UDHs arising in papillomas exhibited missense mutations of presumed biological relevance, consisting of *AKT1*, *GNAS*, *MTOR*, *PIK3CA*, and *PIK3R1* mutations. In UDHs unassociated with neoplasia, four *PIK3CA* mutations and one *PIK3R1* mutation were found. In one of these *PIK3CA* mutated lesions, an

additional mutation in the *LRP1B* gene (p.R3323H) was detected (SIFT score, 0.13; no Cosmic database entry), which we regard as a functionally neutral (biologically irrelevant) passenger mutation. Two cases of UDH arising in papilloma demonstrated presumed passenger mutations, with both mutations assessed as functionally neutral by algorithmic prediction with SIFT: one case demonstrated a mutation in the *IGF2R* gene (SIFT score, 0.73; single Cosmic database entry), and a second case harbored a *LPHN3* mutation (SIFT score, 0.28; two Cosmic database entries) in addition to a *PIK3CA* mutation. Among UDHs arising in papillomas, two lesions showed a double mutation (*AKT1* and *GNAS* as well as *AKT1* and *MTOR*). Overall somatic mutations were confirmed in 63% (10 of 16) of UDHs investigated.

Immunohistochemical staining for p-AKT was unsuccessful for technical reasons. Although designated positive and negative controls [SignalSlide Phospho-Akt (Ser473) IHC Controls 8101 purchased from CST] yielded strong specific staining in paraffin-embedded LNCaP cells and acceptable background in PI3K inhibitor LY294002-treated LNCaP cells, positive controls of FFPE surgical tissue from our own archived material with known *PIK3CA* mutations and/or *PTEN* mutations and from samples of this study yielded signal at approximately background level only, a limitation not to be overcome by diverse antigen retrieval conditions and antibody dilutions. We thus had to exclude p-AKT immunohistochemistry from further evaluation because of insufficient sensitivity in our FFPE material.

Table 3 Immunohistochemistry for Phosphorylated mTOR and Phosphorylated S6 Protein

Case	Gene symbol	Mutation	p-mTOR	p-S6
UDHs arising in papilloma				
1	<i>AKT1</i>	p.E17K	+	—
	<i>GNAS</i>	p.R201H		
2	<i>IGF2R</i>	p.V1888L	+	NE
3	<i>AKT1</i>	p.E17K	+	+
	<i>MTOR</i>	p.V2406M		
4	<i>PIK3CA</i>	p.H1047R	+	+
5	None		+	+
6	None		+	+
7	<i>PIK3CA</i>	p.H1047R	+	+
	<i>LPHN3</i>	p.R465Q		
UDHs unassociated with papilloma				
8	<i>PIK3CA</i>	p.E542K	+	+
9	<i>PIK3CA</i>	p.E545K	NL	NL
	<i>LRP1B</i>	p.R3323H		
10	<i>PIK3CA</i>	p.H1047R	NE	—
11	None		NE	+
12	<i>PIK3CA</i>	p.H1047R	+	+
13	<i>PIK3R1</i>	p.K567E	—	+
14	None		—	+
15	None		NE	+
16	None		+	+

+, positive; —, negative; NE, not evaluable because staining unsuccessful (negative internal positive control); NL, no lesion (lesional tissue exhausted); p-mTOR, phospho-mammalian target of rapamycin; UDH, usual ductal hyperplasia.

p-mTOR and p-S6, on the other hand, were evaluable in most cases. Table 3 gives an overview of p-mTOR and p-S6 staining results, and Figure 1 illustrates p-mTOR and pS6 stains in UDHs arising in papilloma (Figure 1, E and F) compared with staining in UDHs unassociated with papilloma (Figure 1, G and H). Unevaluable cases consisted of one UDH unassociated with papilloma where deeper levels were exhausted for lesional tissue and of four stains, which were not evaluable for technical reasons (negative internal positive control). Overall, 10 of 12 evaluable UDHs demonstrated positivity for p-mTOR, and 12 of 14 evaluable lesions were positive for p-S6.

Discussion

UDH has mostly been viewed as a nonneoplastic proliferation of the mammary duct epithelium, a notion still regarded as standard in terms of routine reporting. Evidence for a clonal, neoplastic nature of UDH was originally derived from loss of heterozygosity observed in 30% to 40% of lesions.^{7,21–23} Additional evidence supporting the neoplastic nature of UDH has recently emerged through the detection of *PIK3CA* mutations in UDH, radial scars, and so-called columnar cell lesions in cases largely associated with concomitant DCIS and/or invasive breast cancer, depending on the study.^{13–15,24,25} In contrast, we have analyzed UDH in patients devoid of *in situ* or invasive breast cancer and one

case associated with a syringomatous adenoma (in a different area of the specimen, Case 6), in which the investigated lesion (UDH) displayed a wild-type status on molecular analysis. Furthermore, only the hot spots of a comparably restricted number of oncogenes were investigated in the earlier mentioned studies, whereas our analysis comprised the coding sequence of >400 cancer-related genes, including almost all cardinal tumor-suppressor genes in breast cancer (*TP53*, *PTEN*, *RBI*, *ARID1A*, *NF1*, and *ATM*).

Missense mutations were confirmed in >60% of lesions investigated. The proportion of lesions exhibiting mutations was higher in UDHs arising in papillomas (71%) than in classic UDHs not arising in papillomas (56%). Mutations in *PIK3CA*, the most common oncogene in invasive breast cancer and DCIS,²⁶ were also most frequently seen in our study. Of the 10 UDHs exhibiting mutations, 6 displayed alterations in the *PIK3CA* gene (two exon 9 and four exon 20 mutations). *PI3KCA* is one of four catalytic subunits (catalytic subunit α , β , γ , or δ), to be joined with one of five regulatory subunits (PI3K-regulatory subunit α , β , γ , 5, 6) to constitute the heterodimeric PI3K class I complex. Interestingly, apart from PI3K activation through mutation of the catalytic subunit α , PI3K activation was achieved in one case (UDHs unassociated with papilloma) through alteration of the PI3K-regulatory subunit α , by mutation of the encoding *PIK3R1* gene (p.K567E). This pathogenic mutation has been reported in esophageal carcinoma,²⁷ Burkitt lymphoma,²⁸ and endometrial carcinoma. The amino acid alteration localizes to the iSH2 domain of PI3K-regulatory subunit α and has been shown to uncouple and retain its PI3KCA stabilizing activity, while abrogating its inhibitory activity on PI3KCA.²⁹ Mutations in *PIK3R1*, other than the p.K567E mutation seen in our study, have been described in a small proportion (2%) of invasive breast cancers, according to the Cosmic database, but *PIK3R1* mutations have not been previously reported in UDH or carcinoma *in situ*. Mechanistically, PI3K activation (either by mutated *PIK3CA* or *PIK3R1*) fits well with reports on oncogenic PI3K in DCIS as an early event in neoplastic transformation of the mammary duct epithelium.³⁰ In agreement with our findings, Ang et al¹³ have recently reported a similar *PIK3CA* mutation spectrum in proliferative breast lesions without atypia, including 6 of 12 cases of UDH displaying the p.E542K, p.E545K, and p.H1047R mutation in *PIK3CA*.

Two cases of UDH arising in papilloma exhibited a well-characterized *AKT1* mutation (p.E17K). No *AKT1* mutations were described in UDH unassociated with papilloma in the study of Ang et al¹³ either, but Troxell et al³¹ have identified this *AKT1* mutation in intraductal papillomas, including cases with superimposed UDH. We observed *AKT1* mutations to be mutually exclusive, with oncogenic PI3K activation an inverse correlation also seen in invasive and preinvasive breast cancer.^{26,30,32} Generally, the percentage of *AKT1*-positive lesions in our study (13% for all lesions compared with 30% for UDHs arising in papilloma) is much

higher than observed in invasive breast cancer, which displays *AKT1* mutations in only 1.4%³³ and 2% (Cosmic database) of cases, indicating a low risk for malignant transformation in *AKT1* mutated breast lesions. Interestingly, we found both *AKT1* mutated cases to be associated with a second oncogenic mutation, namely a *GNAS* mutation in Case 1 and an *MTOR* mutation in Case 3. The lesion with a combined *AKT1* and *GNAS* mutation revealed small foci of UDH with focal transition to ADH, whereas the second case with a compound *MTOR* mutation did not. *GNAS* mutations have repeatedly been found together with a second *KRAS* mutation in gastrointestinal tract tumors,^{34,35} an analogous observation to the *AKT1* and *GNAS* compound mutation in our study. The *GNAS* gene encodes the guanine nucleotide-binding protein G(s) subunit α responsible for constitutively increased cAMP formation in the mutant form. The detected p.R201H mutation has predominantly been associated with neoplastic precursor lesions or low-grade neoplastic lesions, such as fibrous dysplasia,³⁶ intraductal papillary mucinous neoplasm of the bile duct,^{34,37} low-grade appendiceal mucinous neoplasias,³⁸ and intestinal adenomas.³⁹ Only 17% of Cosmic database entries for *GNAS* mutations are from invasive cancers, with only six confirmed somatic mutations in invasive breast cancer reported, again arguing for a low progression potential to invasive breast cancer, as with *AKT1* mutations themselves. The second compound mutation (Case 3) displayed an *MTOR* mutation (p.V2406M) unequivocally predicted as biologically relevant by algorithmic prediction (SIFT score, 0.02). The mutation is localized at the kinase domain of mTOR and was previously detected in anaplastic astrocytoma⁴⁰ and as a point mutation at identical position with a different amino acid exchange (p.V2406A) in a clear cell renal cell carcinoma.⁴¹ We thus regard this mutation as an oncogenic driver mutation, which again pertains to the PI3K/AKT/mTOR axis. Comparing the recurrent detection of *AKT1* compound mutations in our study with the literature, no compound mutations were found in UDH in the study by Troxell et al.³¹ The study detected *AKT1* mutations in 15% of papillomas with UDH, but the study design would have precluded detection of *AKT1* compound mutations because only *AKT1* and *PIK3CA* wild-type tumors were further investigated for an additional set of selected hot spot mutations not comprising *MTOR* and *GNAS*.

The multigene panel used in our study also included *ESR1* encoding the estrogen receptor α . Fuqua et al¹² have reported *ESR1* mutations in 34% of UDHs activating PI3K/AKT signaling, which they showed to confer aromatase inhibitor resistance in transfected MCF7 cells. In this regard, no *ESR1* mutations were detected in our samples. In addition, our study represents the first comprehensive screen of tumor-suppressor gene mutations in UDH, and no tumor-suppressor gene mutations (eg, *TP53*, *RBI1*, and *PTEN*) were identified, indicating that tumor-suppressor gene inactivation through mutation is not a feature in UDH.

Immunohistochemistry for components of the PI3K/AKT/mTOR pathway displayed evidence for activation downstream of mutated PI3K, AKT, and mTOR, respectively, corroborating the biological relevance of the respective mutations detected. We regard the results of the phosphorylation-specific immunohistochemistry of PI3K/AKT/mTOR pathway components as a valuable, overall confirmation that this pathway is functionally activated in this type of lesion. Conversely, we would be reluctant to question well-characterized oncogenic mutations known to occur in the appropriate setting (breast), pathway (AKT pathway), and context (proliferation) on the basis of phosphorylation-specific immunohistochemistry alone. This occurred because FFPE tissue might well show decreased immunoreactivity for phosphorylation-specific antibodies, possibly because of fixation and/or tissue archiving conditions. The fact that immunohistochemical positivity for p-mTOR and p-S6 was also seen in some wild-type lesions of our analysis may point to the possibility of yet unidentified, alternative molecular driver alterations converging on a common pathway downstream.

Regarding histomorphological characteristics, we evaluated all lesions for the presence of focal apocrine metaplasia (Table 1), commonly viewed as an indication of heterogeneous differentiation of UDH in papilloma. This feature has been cited as a criterion in favor of a nonneoplastic nature of the epithelial proliferation (traditionally equated with UDH) in papilloma. Of 5 lesions, 4 (80%) with focal apocrine metaplasia exhibited somatic mutations in our study, arguing against such an interpretation and favoring focal apocrine metaplasia as a histological feature possibly associated with clonality/neoplasia.

In conclusion, our data demonstrate mutations in >60% of investigated lesions (classic UDH and those arising in papillomas combined). Assigning the pathogenic mutations detected in our study to their respective pathways, all but one (*GNAS*) are activating PI3K/AKT/mTOR signaling at different levels, with *PIK3CA* and *AKT1* most commonly mutated. In addition, our study is the first to demonstrate functionally relevant mutations in *PIK3R1*, *MTOR*, and *GNAS* in UDH. Specifically, oncogenic *GNAS* points to a yet unrecognized role of altered G-protein signaling in a subset of these lesions. With regard to the question whether UDH is, in fact, an early, clonal neoplastic proliferation, the authors certainly agree that the mere detection of a biologically relevant mutation is not proof of neoplasia without a corresponding autonomous cell proliferate. It is known that mutations in morphologically normal-appearing cells in the vicinity of neoplastic alterations⁴² and even unassociated with neoplasia have been demonstrated in various organs and have been described in seemingly normal gastric mucosa⁴³ or chronic pancreatitis.⁴⁴ On the contrary, UDH displays clonality⁷ by loss of heterozygosity in a sizable proportion of cases, is an intraductal mass-forming lesion at the microscopic level and occasionally even macroscopically, and is not dissimilar in size and appearance to small

papillomas, which are universally accepted as benign neoplastic alterations. In addition, papillomas and UDH share the same specific *AKT1* and *PIK3CA* mutations as shown in our data and those of previous reports,^{13,31} and the novel mutations detected in our study mostly map to the same pathway (PI3K/AKT/mTOR) as well. In our opinion, the triad of activating mutations, clonality, and the morphological correlate of a mass forming lesion constitute the rationale to regard and finally address UDH as a neoplastic lesion (albeit with low risk of progression to carcinoma) which according to our current knowledge, is largely driven by mutational activation at different levels of the PI3K/AKT/mTOR pathway.

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Supplemental Data

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