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The pathogenesis of atypical proliferative Brenner tumor: an immunohistochemical and molecular genetic analysis

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Abstract

Brenner tumors are ovarian tumors, usually benign, containing epithelium that resembles transitional epithelium. As with other epithelial tumors there exist frankly malignant tumors and tumors that display greater proliferation than the benign Brenner tumors but lack destructive infiltrative growth, and these have been designated ‘atypical proliferative’ (borderline) Brenner tumors. There have been no well-documented cases of atypical proliferative Brenner tumors that have exhibited malignant behavior. Based on shared morphologic features it is generally believed that atypical proliferative Brenner tumors develop from benign Brenner tumors. The aim of the present study was to confirm this impression by investigating the immunohistochemical and molecular genetic features of benign and atypical proliferative Brenner tumors.

Immunohistochemical staining for p16, fluorescence *in-situ* hybridization (FISH) for *CDKN2A* (p16-encoding gene) and mutational analysis of the genes commonly mutated in ovarian tumors were performed. p16 immunostaining was positive in the epithelial component of 12 (92%) of 13 benign Brenner tumors, but completely negative in all 7 atypical proliferative Brenner tumors. FISH identified homozygous deletion of *CDKN2A* in the epithelial component of all atypical proliferative Brenner tumors, but *CDKN2A* was retained in all benign Brenner tumors. Two *PIK3CA* somatic mutations were detected in the stromal component in 1 (5%) of 20 Brenner tumors and 3 somatic mutations (1 in *KRAS* and 2 in *PIK3CA*) were identified in the atypical epithelial component of 2 (29%) of 7 atypical proliferative Brenner tumors. In summary, our findings suggest that loss of *CDKN2A* and, to a lesser extent, *KRAS* and *PIK3CA* somatic mutations have a role in the progression of a benign to an atypical proliferative Brenner tumor.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

Keywords

atypical proliferative; Brenner tumor; CDKN2A; mutation; pathogenesis; p16; progression

The World Health Organization classification divides Brenner tumors into three categories: benign, borderline (atypical proliferative), and malignant.¹ Benign Brenner tumors are composed of nests of mature transitional-like epithelial cells, which are surrounded by an abundant fibromatous stroma. Atypical proliferative Brenner tumors are characterized by large, irregularly shaped nests, and masses of similar appearing epithelium surrounded by dense fibromatous tissue. Some are composed of complex fibrovascular papillae, protruding into cystic spaces. Atypical proliferative Brenner tumors are usually associated with a benign Brenner tumor. Malignant Brenner tumors are characterized by cytologic atypia and stromal invasion in a context of a benign or atypical proliferative Brenner tumor.

We recently investigated the morphologic features and the immunohistochemical profile of the 'tuboperitoneal junction', which is the junction between the epithelium of the fimbria and the tubal mesothelial serosa, and proposed that it might be a 'hot spot' for ovarian carcinogenesis, and a possible site of origin of Brenner tumors.² A subsequent study provided evidence, suggesting a tubal origin of Brenner tumors through transitional metaplasia and Walthard cell nests, based on their anatomical proximity, similar immunohistochemical profile, and the presence of cilia. In addition, we showed that luteinized stromal cells immediately adjacent to the epithelial nests, as well as the epithelial nests themselves, are involved in androgen biosynthesis in view of the presence of AKR1C3, a key enzyme in the production of androgens, in these cells and that hormone stimulation therefore probably has a role in the development of Brenner tumors as the epithelial component contains androgen receptor.³ The aim of the present study was to evaluate the immunohistochemical and molecular genetic features of benign and atypical proliferative Brenner tumors, and to elucidate their relationship and identify molecular alterations that underlie progression to atypical proliferative Brenner tumors.

Materials and methods

Case Selection

A total of 20 benign Brenner tumors and 7 atypical proliferative Brenner tumors were retrieved from the pathology files of the Johns Hopkins Hospital (Baltimore, MD), Washington Hospital Center (Washington, DC), and Seirei Mikatahara Hospital (Hamamatsu, Japan). All available slides were reviewed and clinicopathological data collected. This study was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions.

Immunohistochemistry

Immunohistochemistry was performed using antibody against p16 (Ventana, cat# 705–4713, Ventana Medical Systems, Tucson, AZ, USA). Formalin-fixed paraffin-embedded sections were immunostained automatically in Benchmark XT^{MD} (Ventana Medical Systems) using a previously described protocol.^{4–6} In summary, 4- μ m-thick tissue sections were

deparaffinized in xylene, and rehydrated in graded alcohols. Antigen unmasking was carried out by incubating the slides in Target retrieval solution (cat# S1699, Dako, Carpinteria, CA, USA) in a steamer for 30 min. EnVision™+/HRP, Rabbit polymer (cat# K4003, Dako) was applied for 30 min followed by 5 min of incubation with streptavidin peroxidase (Dako Liquid DAB+ cat# K3468, Dako). The slides were counterstained with hematoxylin for 3 min (cat# HHS32, Sigma-Aldrich, St Louis, MO, USA), dehydrated in graded ethanols and xylene. For a negative control, the primary antibody was replaced with TBS-t.

A positive reaction was defined as diffuse staining in at least 70% of the cells (diffuse), multifocal positivity in at least 20% of the cells (patchy), or scattered cells in less than 20% of the cells (focal). In addition, subcellular localization (nuclear or cytoplasmic) was recorded, and all observations were made separately for both epithelial and stromal components.

Fluorescence *In-Situ* Hybridization (FISH)

CDKN2A copy number per cell was evaluated using a two-color FISH assay. Briefly, 4- μ m-thick sections were incubated for 30 min at 62 °C, deparaffinized in xylene, hydrated through graded ethanols, and placed in deionized water. The sections were incubated with proteinase K for 30 min at 37 °C and then washed in 2 \times Aniera saline-sodium citrate (SSC). The slides were then placed in a denaturation solution (70% formamide/2 \times SSC) for 5 min at 75 °C, rinsed in 2 \times SSC, and allowed to cool at room temperature for 5 min. The slides were then dehydrated through graded ethanols and dried in an oven at 62 °C for 2 min. Vysis LSI *CDKN2A* SpectrumOrange/CEP9 SpectrumGreen probes (cat# 05J51-001, Abbott Molecular, IL, USA) were applied to the slides according to the vendor's instructions and then coverslipped. Denaturation was accomplished by incubating the slides for 10 min at 80 °C. Hybridization was performed at 37 °C for 20–24 h. Coverslips were carefully removed and the slides were washed for 20 min in 1.5 mol/l urea in 0.2 \times SSC, followed by washing in 2 \times SSC for 2 min at room temperature. The slides were drained, dehydrated through graded ethanols, air-dried, mounted with ProLong® Gold Antifade Reagent with DAPI (cat# P-36931, Invitrogen, Eugene, OR, USA) and imaged.⁷ The *CDKN2A* copy number was assessed in at least 100 nuclei. Cases lacking both *CDKN2A* signals in the presence of CEP9 probe signal in >20% nuclei were considered homozygously deleted.

Tissue Microdissection and DNA Extraction

In order to extract DNA from the epithelial and stromal components separately, serial 10- μ m formalin-fixed paraffin-embedded sections were placed onto UV-treated PALM membrane slides (Carl Zeiss MicroImaging GmbH, München, Germany) and stained with hematoxylin. Approximately 1000–2000 cells from the nests of transitional-type epithelium from Brenner tumors and atypical proliferative Brenner tumors were laser-capture micro-dissected using PALM Micro Beam System (Carl Zeiss MicroImaging). The stromal tissue surrounding the nests of epithelium was manually micro-dissected as previously described.⁵ Genomic DNA was extracted for sequencing using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA) according to the vendor's protocol.

PCR Amplification and Mutation Analysis

Mutation analysis was performed for *BRAF* (exon 15), *CTNNB1* (β -catenin-encoding gene, exon 3), *ERBB2* (exon 20), *FOXL2* (exon 1), *KRAS* (exon 2), *PIK3CA* (exons 1, 9, and 20), and *PPP2R1A* (exons 5 and 6), which are commonly mutated in ovarian tumors.^{8,9} These exons were PCR amplified using primers shown in Table 1. All PCR amplification products were visualized with ethidium bromide under ultraviolet light by electrophoresis in 2.5% agarose gel. All amplified PCR products were sequenced at Agencourt Biosciences (Beverly, MA, USA) and all sequence variants were confirmed on at least three independent rounds.

Statistical Analysis

Comparisons of immunohistochemistry and FISH outcomes were performed using the two-tailed unpaired Fisher's exact test. *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

p16 Immunostaining and CDKN2A FISH Assay

p16 immunohistochemical staining has been shown to be frequently negative in Brenner tumors.^{10,11} We performed p16 immunostaining and *CDKN2A*, gene encoding both p16 (INK4a) and p14 (ARF), FISH assay on 13 Brenner tumors and 7 atypical proliferative Brenner tumors in order to evaluate whether they demonstrated p16 alterations.

All benign Brenner tumors expressed p16 in the epithelial component, either in a diffuse or patchy distribution (Figure 1), except in one case which was negative (Table 2). In contrast, all seven atypical proliferative Brenner tumors were negative for p16 in the epithelial component (Table 2). Interestingly, five atypical proliferative Brenner tumors with an associated benign Brenner tumor showed loss of p16 expression in the benign epithelial component, but in two of these cases focal positivity restricted to few epithelial nests was noted (Figure 2).

CDKN2A FISH was performed to investigate *CDKN2A* copy number and its correlation with p16 immunostaining. The results are summarized in Table 2. None of the 13 Brenner tumors showed *CDKN2A* copy number alterations, in either the epithelial or stromal components (Figure 1). All seven atypical proliferative Brenner tumors showed *CDKN2A* homozygous deletion (Figure 1). Interestingly, the benign Brenner tumor component associated with five atypical proliferative Brenner tumors also showed mainly homozygous loss (Figure 2). In addition, in two of these cases, the benign Brenner tumor focally retained *CDKN2A* (Figure 2). *CDKN2A* copy number aberrations were not identified in the stromal component. Thus, there was a strong correlation between p16 immunohistochemical expression and *CDKN2A* copy number, and between *CDKN2A* copy number and tumor type (*P*-value <0.0001, two-tailed Fisher's exact test, Table 3).

Mutational Analysis

A total of 20 Brenner tumors and 7 atypical proliferative Brenner tumors were analyzed for mutations of *BRAF*, *CTNNB1* (β -catenin-encoding gene), *ERBB2*, *FOXL2*, *KRAS*, *PIK3CA*, and *PPP2R1A* genes, which are commonly mutated in ovarian tumors.^{8,9} The transitional-type epithelium and the stromal component were analyzed separately. Two *PIK3CA* mutations were identified in the stromal component in one (5%) of 20 benign Brenner tumors, but not in the epithelial component, confirming the somatic nature of the mutation (Table 4 and Figure 3). The mutations identified in this benign Brenner tumor were a point mutation in position c.1634A>C that results in an amino-acid substitution at position 545 from a glutamic acid (E) to an alanine (A), and a single-nucleotide deletion of G at position c.1658 causing a frameshift mutation. None of the other benign Brenner tumors harbored mutations in the genes investigated.

In two (29%) of seven atypical proliferative Brenner tumors (APBT2 and APBT6), three somatic mutations, one in *KRAS* and two in *PIK3CA*, were identified in the atypical epithelial component but not in the stromal component (Table 4 and Figure 3). Interestingly, the benign component associated with APBT6 did not harbor the two mutations in *KRAS* and *PIK3CA* identified in the atypical component. APBT2 harbored a point mutation in position c.310C>A causing an amino-acid substitution at position 104 from a proline (P) to threonine (T). APBT6 harbored a *KRAS* point mutation c.35G>A, leading to an amino-acid substitution at position 12 from a glycine (G) to an aspartic acid (D), and a *PIK3CA* point mutation c.35472A>G resulting in an amino-acid substitution at position 1047 from histidine (H) to arginine (R).

Discussion

Atypical proliferative Brenner tumors are composed of large, irregularly shaped masses of transitional-type epithelium surrounded by dense fibromatous tissue, which usually are associated with a benign Brenner tumor component. The morphological similarity and common association of these two tumors suggest atypical proliferative Brenner tumors develop from benign Brenner tumors.¹ The present study was undertaken in order to investigate molecular genetic characteristics of Brenner tumors focusing on the events that might underlie progression to atypical proliferative Brenner tumors.

CDKN2A, the gene encoding both p16 (INK4a) and p14 (ARF), is a tumor-suppressor gene frequently inactivated in human neoplasms by promoter hypermethylation and homozygous deletion.¹² In the present study, we identified *CDKN2A* loss in all atypical proliferative Brenner tumors, whereas it was retained in all the benign Brenner tumors. Moreover, in two of the five atypical proliferative Brenner tumors that were associated with a benign Brenner tumor, *CDKN2A* was lost with just focal retention in the benign Brenner tumor. We found strong correlation between p16 immunohistochemical expression and *CDKN2A* copy number, so that p16 expression in the epithelial component of Brenner tumors was associated with retention of *CDKN2A*, whereas negative p16 immunostaining was associated with a complete loss of *CDKN2A*. Finally, there was correlation between *CDKN2A* copy number and tumor types, all benign Brenner tumors retained *CDKN2A*, whereas all atypical proliferative Brenner tumors lost this gene. Taken together, these findings suggest that loss

of *CDKN2A* is involved in the progression from a benign to an atypical proliferative Brenner tumor. Accordingly, loss of expression of p16 by immunohistochemistry can be useful in distinguishing a benign Brenner tumor from an atypical proliferative Brenner tumor, in difficult cases. Consistent with our findings, two previous studies found that there was loss of p16 expression in Brenner tumors.^{10,11} In particular, Cuatrecasas *et al.* reported low immunohistochemical expression or absence of p16 in benign, atypical proliferative, and malignant Brenner tumors.¹⁰ In addition, this study found loss of heterozygosity and promoter hypermethylation at the p16 locus in one malignant Brenner tumor. In the second study, Ali *et al.* reported p16 immunohistochemical expression in three benign Brenner tumors, and absence of p16 in three atypical proliferative and one malignant Brenner tumors.¹¹ In any case, to our knowledge, ours is the first study that consistently identified homozygous deletion of *CDKN2A*, strongly correlated with loss of immunohistochemical expression of p16, in all atypical proliferative Brenner tumors examined, and *CDKN2A* retention, with associated p16 immunopositivity, in all benign Brenner tumors. Finally, *CDKN2A* has been shown to be frequently lost in ovarian mucinous tumors by SNP array.^{13,14} The transitional epithelium of Brenner tumor frequently shows focal mucinous differentiation, and mucinous tumors (cystadenomas or atypical proliferative tumors) are occasionally identified concurrently with a Brenner tumor.^{1,15} The common loss of *CDKN2A* in both tumors provides additional molecular evidence showing their close association.

The identification of two *PIK3CA* somatic mutations in the stromal component of a single (5%) benign Brenner tumor suggests that mutations of the genes analyzed are unlikely to be common driver events underlying the development of these tumors. Nonetheless, two (29%) of seven atypical proliferative Brenner tumors harbored somatic mutation of *KRAS* and/or *PIK3CA*, neither of which was detected in the associated benign Brenner tumor, when present. Hence, these findings suggest a possible role of *KRAS* and *PIK3CA* somatic mutations in the progression from a benign to an atypical proliferative Brenner tumor. E545A and H1047R mutations of *PIK3CA* occur within the helical and kinase domain, respectively, and both have increased catalytic activity enhancing downstream signaling and oncogenic transformation *in vitro*.¹⁶ To our knowledge, the only previous study, which investigated somatic mutations in Brenner tumors, identified *PIK3CA* mutations in one (33%) of three atypical proliferative Brenner tumors, but did not interrogate the concurrent benign component.¹⁰ The mutations identified in this study have been previously described in other ovarian tumors.^{8,17} For example, *PIK3CA* is also mutated in 5–45% ovarian endometrioid, clear cell, and low-grade serous carcinomas.^{8,17} Finally, it is not surprising that *KRAS* mutation, the most common mutation identified in ovarian mucinous tumors, is identified in a minority of atypical proliferative Brenner tumors, as noted above they are frequently associated and the majority of Brenner tumors shows mucinous differentiation.^{3,13,15}

In conclusion, our current study confirms that Brenner tumor can be considered a type I ovarian tumor based on the slow biological progression and stepwise acquisition of molecular genetic alterations.^{8,18} In particular, it appears that *CDKN2A* loss, and to a lesser

extent *KRAS* and *PIK3CA* mutations, may have a role in the progression of a benign Brenner tumor to an atypical proliferative Brenner tumor.

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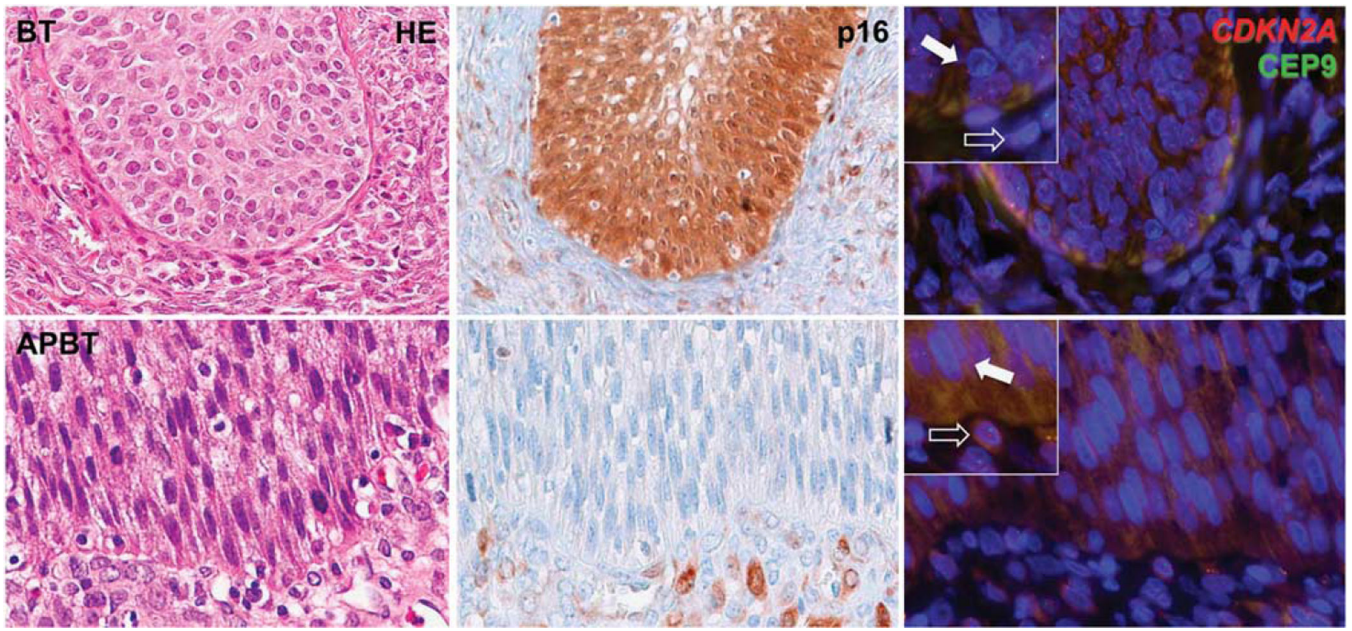


Figure 1.

Representative images of histological appearance (hematoxylin–eosin, HE), p16 immunostaining, and fluorescence *in-situ* hybridization for *CDKN2A* of benign Brenner tumor (BT) and atypical proliferative Brenner tumor (APBT). Benign BT shows intense and diffuse epithelial p16 positivity, both cytoplasmic and nuclear, and normal copy number (either 1 or 2) for *CDKN2A* (red) as compared with centromere region (CEP9, green) in both epithelial cells (filled arrow) and stromal cells (empty arrow). APBT is consistently negative for p16 and shows complete loss of *CDKN2A* (red) and retention of centromere 9 (CEP9, green) in the majority of epithelial cells (filled arrow) as compared with stromal cells, where both signals are identified in normal number (empty arrow). Higher magnification in the insets.

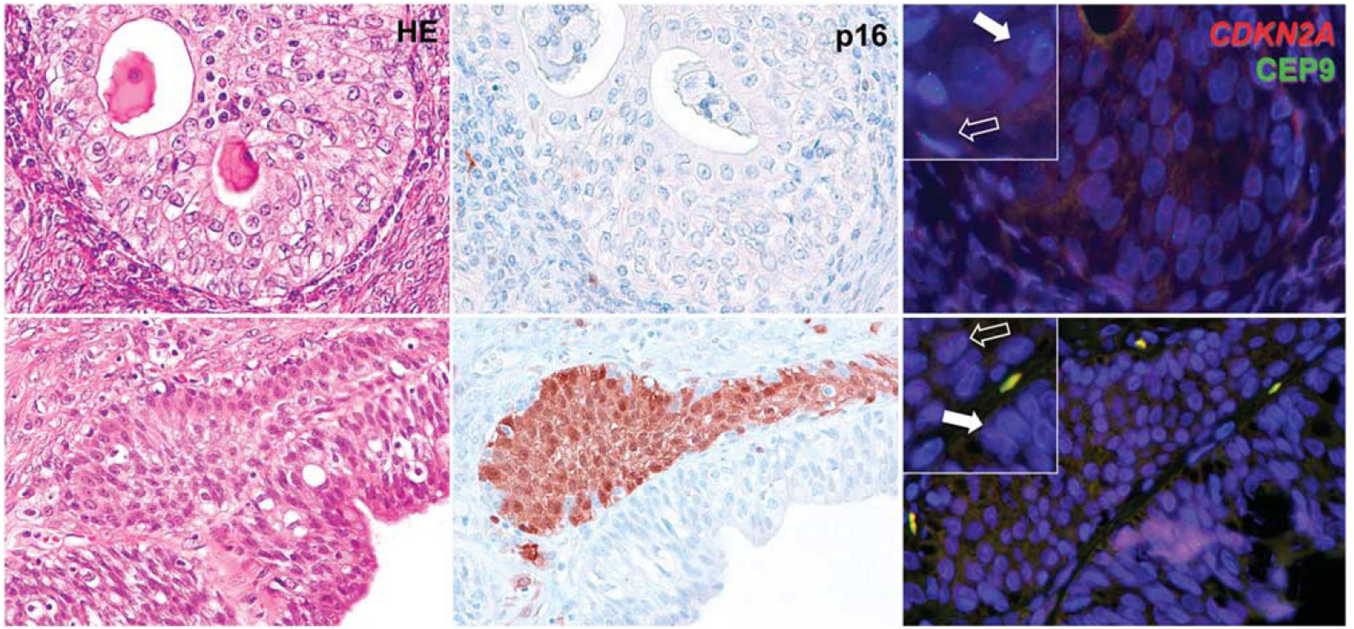


Figure 2.

Histological appearance (hematoxylin–eosin, HE), p16 immunostaining, and fluorescence *in-situ* hybridization for *CDKN2A* of benign Brenner component in the context of atypical proliferative Brenner tumor. In the upper panels, the benign Brenner tumor component does not express p16, and shows complete loss of *CDKN2A* signals (red) and retention of centromere 9 signals (CEP9, green) in the majority of epithelial cells (filled arrow) as compared with stromal cells, where both signals are identified in normal number (empty arrow). In the lower panels, the benign Brenner tumor component associated with the atypical proliferative Brenner tumor shows focal p16 positivity, limited to one epithelial nest, retention of *CDKN2A* signals (red), and centromere signals (green; empty arrow), and adjacent p16 negative atypical proliferative Brenner tumor component, with loss of *CDKN2A* signals (red; filled arrow). Higher magnification in the insets.

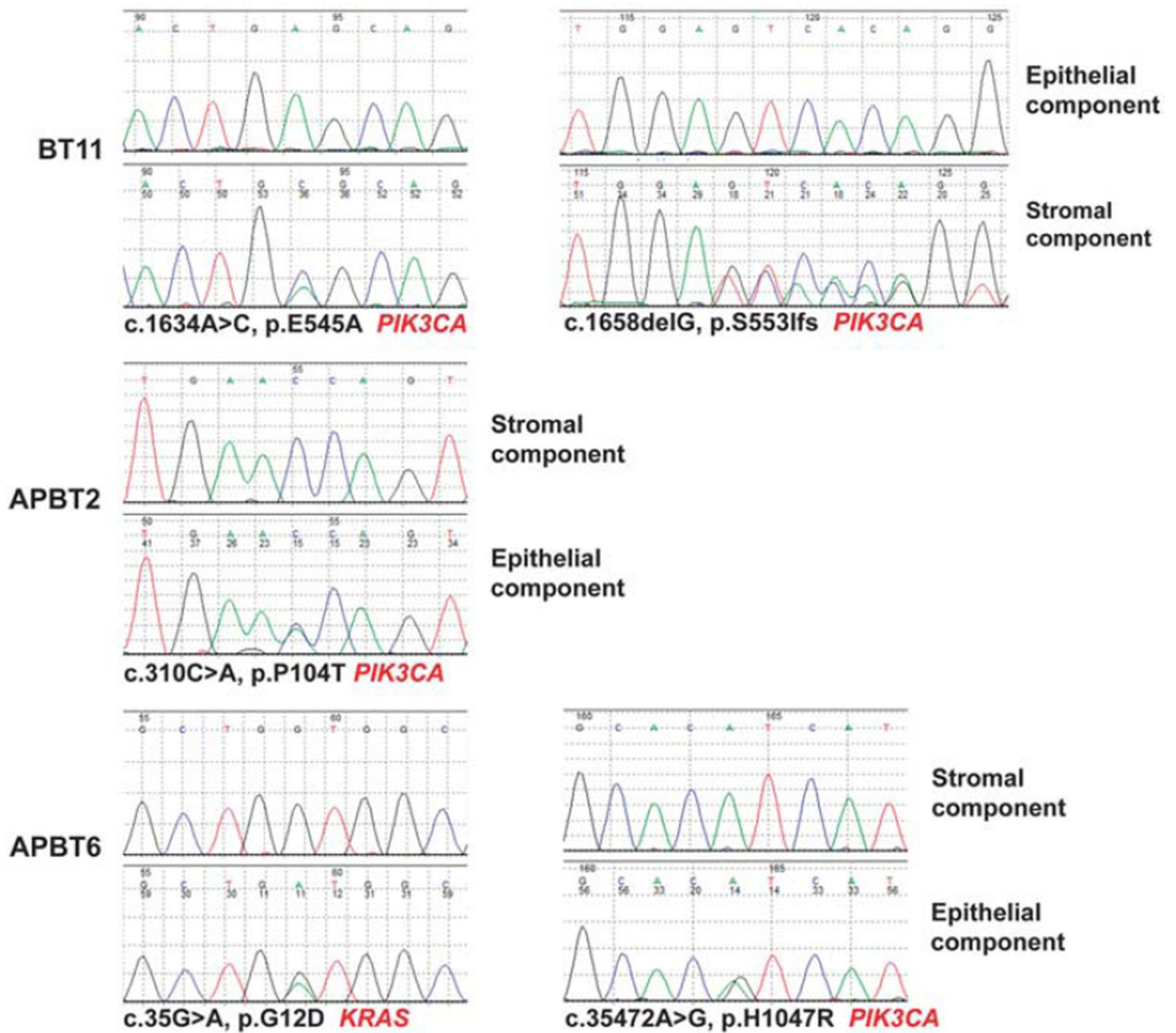


Figure 3.

Chromatograms and annotations show *PIK3CA* somatic mutations in the stromal component (below) of one benign Brenner tumor (BT11) compared with the wild-type sequences of the epithelial component (above). Chromatograms and annotations of the three somatic mutations identified in the epithelial component (below) of two atypical proliferative Brenner tumors (APBT2 and APBT6), two in *PIK3CA*, and one in *KRAS*, compared with the wild-type sequence of the stromal component (above).

Table 1

Primers used in PCRs of this study

Gene	Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size, bp
<i>BRAF</i>	15	TGCTTGCTCTGATAGGAAAATGA	CCACAAAATGGATCCAGACAAC	173
<i>CTNNB1</i>	3	ATGGAACCAGACAGAAAAGCGGC	GCTACTTGTTCTTGAGTGAAG	200
<i>ERBB2</i>	20	CTCTCAGCGTACCCTTGTC	AGGTGCATACCTTGGCAATC	220
<i>FOXL2</i>	1	AGAAGGGCTGGCAAAATAGC	GCCGGTAGTTGCCCTTCT	150
<i>KRAS</i>	2	TAAGGCCTGCTGAAAATGACTG	TGGTCCTGCACCAGTAATATGC	167
<i>PIK3CA</i>	1	TGCTTTGGGACAACCATAATC	CTTGCTTCTTTAAATAGTTCATGCTTT	232
<i>PIK3CA</i>	1	CCCCTCCATCAACTTCTTCAA	ATTGTATCATACCAATTCTCGATTG	196
<i>PIK3CA</i>	9	TTTTCTGTAAATCATCTGTGAATCC	TCTCCATTTTAGCACTTACCTGTGA	223
<i>PIK3CA</i>	20	ATCATTTGCTCCAAACTGACCA	TTGTGTGGAAGATCCAATCCAT	291
<i>PPP2R1A</i>	5	TACTTCCGGAACCTGTGCTC	CCAGGAAGCAAACTCACCT	165
<i>PPP2R1A</i>	6	GTTCTGCCCATGAAAGAGA	TTATTGCTCAAACGCCCAAT	353

Table 2

CDKN2A fluorescence *in-situ* hybridization and p16 immunohistochemical results

	CDKN2A copy number		p16 IHC expression ^a	
	Epithelium	Stroma	Epithelium	Stroma
<i>Benign Brenner tumors</i>				
1	Disomy	Disomy	Diffuse	Focal
2	Disomy	Disomy	Diffuse	Focal
3	Disomy	Disomy	Diffuse	Focal
4	Disomy	Disomy	Patchy	Focal
5	Disomy	Disomy	Patchy	Focal
6	Disomy	Disomy	Patchy	Focal
7	Disomy	Disomy	Diffuse	Focal
8	Disomy	Disomy	Diffuse	Focal
9	Disomy	Disomy	Diffuse	Focal
10	Disomy	Disomy	Diffuse	Focal
11	Disomy	Disomy	Diffuse	Focal
12	Disomy	Disomy	Diffuse	Focal
13	Disomy	Disomy	Negative	Focal
<i>Atypical proliferative Brenner tumors</i>				
1	Homozygous deletion	Disomy	Negative	Focal
2	Homozygous deletion	Disomy	Negative	Focal
3	Homozygous deletion	Disomy	Negative	Focal
4	Homozygous deletion	Disomy	Negative	Focal
5	Homozygous deletion	Disomy	Negative	Focal
6	Homozygous deletion	Disomy	Negative	Focal
7	Homozygous deletion	Disomy	Negative	Focal

Abbreviation: IHC, immunohistochemical.

^a p16 IHC expression is both cytoplasmic and nuclear.

Table 3Correlation of *CDKN2A* fluorescence *in-situ* hybridization results with tumor type

Tumor type	CDKN2A copy number	
	Disomy	Homozygous deletion
Benign Brenner tumors	13	0
Atypical proliferative Brenner tumors	0	7

P-value <0.0001, two-tailed Fisher's exact test.

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Table 4

Mutational analysis results

Gene	Benign Brenner tumors		Atypical proliferative Brenner tumors	
	Epithelium n/tot (%)	Stroma n/tot (%)	Epithelium n/tot (%)	Stroma n/tot (%)
<i>BRAF</i>	0/20 (0)	0/20 (0)	0/7 (0)	0/7 (0)
<i>CTNNB1</i>	0/20 (0)	0/20 (0)	0/7 (0)	0/7 (0)
<i>ERBB2</i>	0/20 (0)	0/20 (0)	0/7 (0)	0/7 (0)
<i>FOXL2</i>	NA	0/20 (0)	0/7 (0)	0/7 (0)
<i>KRAS</i>	0/20 (0)	0/20 (0)	1/7 (14)	0/7 (0)
<i>PIK3CA</i>	0/20 (0)	1/20 (5)	2/7 (29)	0/7 (0)
<i>PPP2RIA</i>	0/20 (0)	0/20 (0)	0/7 (0)	0/7 (0)

Abbreviations: n, cases number mutated; tot, total case number analyzed; NA, not analyzed.