Ovarian Brenner tumors are relatively rare, comprising 1% to 3% of all ovarian neoplasms. 1 Although most are benign, approximately 2% are atypical proliferative (borderline) and malignant Brenner tumors. 1,2 Benign Brenner tumors are biphasic, composed of nests of transitional cell epithelium, resembling urothelium, within a fibrous stroma. It is well recognized that many (up to 30%) Brenner tumors arise in association with an ovarian mucinous neoplasm and are hypothesized to share a histogenic origin and progression, however, limited
molecular characterization has been conducted that might support this.

Several theories have been proposed to explain the histogenesis of these tumors. It currently is suggested that Brenner tumors have a tubal origin, arising from the tuboperitoneal junction cells (the junction between the tubal fimbria and the mesothelial serosa). It is postulated that these cells undergo transitional cell metaplasia and invaginate into the paratubal or ovarian surface, forming nests termed a Walthard cell nest. These Walthard cell nests are frequently the origin of paratubal cysts and it is theorized that when they implant and seed the ovary, they may develop into Brenner tumors.

Mucinous epithelium is not native to the ovary and mucinous epithelial ovarian tumors do not currently have a known cell of origin in the ovary. It is not unusual to find mucinous metaplasia and cystic change in Brenner nests and one hypothesis is that mucinous ovarian tumors arise from these changes in Brenner tumors. However, evidence of transitional cell nests is not evident within a majority of mucinous neoplasms, although the Brenner component of mixed tumors typically is significantly smaller than the mucinous component and without extensive sampling it simply may be missed.

Morphologic and immunohistochemistry studies have been used to explore the relationship between Brenner tumors and mucinous cystic neoplasms of the ovary. More recently, molecular studies evaluating a limited number of genes have been performed in a few cases of Brenner, atypical proliferative (borderline) Brenner, and ovarian mucinous tumors independently. Our aim was to investigate the genetic profiles of Brenner tumors and the association with paired ipsilateral mucinous tumors using a 358-gene targeted next-generation sequencing panel. We believe that there is an important molecular relationship between Brenner tumors and ovarian mucinous epithelial tumors that may provide insight into the pathogenesis and progression of these tumors.

Materials and Methods

This study was approved by the Committee for the Protection of Human Subjects at the Dartmouth-Hitchcock Medical Center. The Department of Pathology (Dartmouth-Hitchcock Medical Center) archive was searched from 2004 to 2014 to identify all ovarian benign and mucinous cystic neoplasms of the ovary. Six Brenner tumors with paired mucinous cystadenomas (MCA; n = 4) or mucinous borderline tumor (MBT, n = 2), two Brenner tumors not associated with a mucinous neoplasm, and two atypical proliferative (borderline) Brenner tumors that had an adequate amount of tissue to complete the genetic analysis were included in this study.

Sequencing

The histopathology was reviewed by an attending pathologist (L.J.T.) and tumor blocks for each case were selected for analysis. The tissue areas of interest were marked on a hematoxylin and eosin-stained slide, and 10 unstained formalin-fixed paraffin-embedded tissue sections (4 μm) were macrodissected and used for DNA extraction. Macrodisssection allowed for separation of the Brenner and mucinous components in the six paired samples (for the Brenner components, stroma and epithelium were dissected together). Sequencing was performed as previously described. Briefly, DNA was extracted with the QIAamp DNA Formalin-Fixed Paraffin-Embedded Tissue Kit (Qiagen, Valencia, CA). The DNA quality was evaluated using the NanoDrop 2000 (Thermo Scientific, Waltham, MA). The DNA quantity was analyzed with the Qubit Fluorometer (Life Technologies, Carlsbad, CA). The sample libraries were constructed using the Agilent (Santa Clara, CA) 1-μg DNA sample preparation method, the SureSelectXT Target Enrichment System. Next-generation sequencing was performed at the Jackson Laboratory’s Clinical Genomics facility (Farmington, CT) using the JAX Cancer Treatment Profile assay consisting of a 358-gene targeted panel on a HiSeq 2500 (Illumina, San Diego, CA) with 150-bp paired end sequencing. The assay has complete exon coverage of the 358 genes and covers 10 bp of intragenic sequence on either side of each exon.

Data Analysis

Data analysis was performed with the Clinical Genomics Analytical bioinformatics pipeline as follows. FASTQ read files from the Illumina HiSeq instrument were quality-filtered to remove low-quality reads (reads with an average Phred quality score <70) and/or to trim out low-quality nucleotides (with an average Phred quality score <70). The resulting high-quality reads were aligned to the human reference genome [hg19; Genome Reference Consortium Human Reference 37 (GRCh37; http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13)] using the Burrows-Wheeler Aligner version 0.7.9a and alignments were processed afterward to remove duplicates, realign around insertions and deletions, and recalibrate base quality scores using Picard software version 1.95 (http://broadinstitute.github.io/picard) and the GATK tool suite version 3.1-1. Single-nucleotide variants and insertions and deletions (up to 50 bp in length) were called using the GATK Unified Genotyper tool and Pindel version 0.2.5a. Copy number variations were called with CONTRA (Copy Number Analysis for Targeted Resequencing) software version 2.0.4. Finally, genomic and functional annotations (gene name, transcript, type of change at the nucleotide and protein levels, Catalogue of Somatic Mutation in Cancer (COSMIC) identification numbers, population frequencies, conservation scores, and so forth) were assigned to variants using the SnpEff and SnpSift tool suite version 4.0.e. SnpEff/SnpSift variant call files were filtered to retain only high/medium-impact variants (including missense, nonsense, frameshifts, codon insertions and deletions, stop
codon loss or gain, and start codon loss) that met a number of filtering criteria as outlined later. Variants not passing the established quality control metrics (coverage, ≤140×; allelic frequency, ≤5%) were removed from the analysis. Amplification was defined as >6 copies. Germline variants (defined as variants with an allele frequency between 40% and 60% and a 1000 Genomes Population frequency >1%, or an allele frequency >90% and a 1000 Genomes Population frequency >1%) and noncanonical transcripts were filtered out. The resulting high-quality, high-/medium-impact somatic variants were compared within tumor pairs and among tumors to assess variant overlap.

Statistical Analysis

All called variants in paired samples sets were compared for statistical significance using the binomial exact test. Statistical analysis was performed in R version 3.0.2 (http://www.R-project.org). Results were considered statistically significant at \( P < 0.05 \).

Results

Clinicopathologic Features

The patients were 28 to 88 years of age (median age, 60 years) at the time of diagnosis. The ovarian tumors all were unilateral and ranged in size from 9.0 to 32.0 cm. The mixed Brenner and mucinous tumors contained variable amounts of cystic and solid components. Associated findings included a contralateral cystadenofibroma in one patient. The two patients with atypical proliferative (borderline) Brenner tumors both had clinical evidence of virilization.

Histologic Review

The benign Brenner tumors were composed of nests of transitional epithelium within a dense stroma. At times, the Brenner tumors showed dilatation of the epithelial nests and were associated with mucinous epithelial cells in the center of the nests as well as spiculated calcifications. The MCAs were multicystic and lined by intestinal-type mucinous epithelium containing goblet cells and lacked significant cytologic atypia. The MBTs consisted of similar mucinous epithelium with a more complex architecture, increased cytologic atypia, hyperchromasia, nuclear stratification, and mitotic activity. Only cases in which the Brenner and mucinous components could be macroscopically dissected separately were included in this study. The two atypical proliferative (borderline) Brenner tumors both were associated with a benign Brenner component and showed confluent nests and papillae of transitional cells with intraepithelial microcystic spaces (Figure 1).

Pairwise Comparisons and Reportable Mutations

After filtering variants, 122 different high- or medium-impact variants were detected in 70 genes across Brenner, atypical proliferative (borderline) Brenner, and mucinous tumors. Individual tumors had 6 to 18 variants. No characteristic genetic profile across all Brenner or all mucinous tumors was identified. However, pairwise comparisons of filtered genetic variants within the six sets of paired Brenner and mucinous neoplasm samples showed 40% to 75% overlap of variants between paired samples. Concordance of variants was found to be significant \( (P < 0.0001) \) for each pair using the binomial exact test. The reportable mutations by tumor are listed in Table 1. Four of the six tumor pairs showed KRAS hotspot driver mutations (KRAS p.G12X, 2; KRAS p.Q61H, 2) specifically in the mucinous tumor, and the KRAS hotspot variant allele frequencies for two of these pairs was less than 10%, suggesting a potential early driver event. In the two paired samples that lacked KRAS activating mutations, MYC amplification was detected in both of the MCA and the Brenner components [one Brenner had a low-level amplification with a MYC (alias c-myc) copy number of 3.6] and a PIK3CA mutation in one of the MCAs (Figure 2). MYC amplification also was detected in a third Brenner tumor (Brenner 7). Of the five tumors
with MYC amplification, three also had amplification of CDK4 and two had amplification of CCND1 (Figure 2). Five of the Brenner tumors had no reportable potential driver alterations. The two atypical proliferative (borderline) Brenner tumors both had RAS mutations (HRAS p.G12S and KRAS p.G12V), and one also contained a common PIK3CA mutation (p.H1047R). None of the benign Brenner tumors (0 of 7) had RAS mutations. These distinct results also

<p>| Table 1 Reportable Potential Driver Alterations Identified in All Tumor Types |
|-----------------------------|-------------------------------|-------------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th><strong>Brenner tumors</strong></th>
<th><strong>Genetic alterations</strong> (allelic frequency)</th>
<th><strong>Mucinous tumors</strong></th>
<th><strong>Genetic alterations</strong> (allelic frequency)</th>
<th><strong>Percentage of variant overlap of paired tumors (%)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Brenner 1</td>
<td>None detected</td>
<td>MBT 1</td>
<td>KRAS p.G12C (18%)</td>
<td>46</td>
</tr>
<tr>
<td>Brenner 2</td>
<td>c-MYC amplification</td>
<td>MCA 1</td>
<td>PIK3CA p.C420R (16%)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>CCND1 amplification</td>
<td></td>
<td>c-MYC amplification</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCND1 amplification (4.6 copies)*</td>
<td></td>
</tr>
<tr>
<td>Brenner 3</td>
<td>c-MYC amplification (3.6 copies)*</td>
<td>MCA 2</td>
<td>c-MYC amplification</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>CDK4 amplification (5.0 copies)*</td>
<td></td>
<td>CDK4 amplification</td>
<td></td>
</tr>
<tr>
<td>Brenner 4</td>
<td>None detected</td>
<td>MCA 3</td>
<td>KRAS p.G12R (8%)</td>
<td>75</td>
</tr>
<tr>
<td>Brenner 5</td>
<td>None detected</td>
<td>MCA 4</td>
<td>KRAS p.Q61H (6%)</td>
<td>57</td>
</tr>
<tr>
<td>Brenner 6</td>
<td>None detected</td>
<td>MBT 2</td>
<td>KRAS p.Q61H (43%)</td>
<td>47</td>
</tr>
<tr>
<td>Brenner 7</td>
<td>c-MYC amplification</td>
<td>MCA 2</td>
<td>c-MYC amplification</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>CDK4 amplification</td>
<td></td>
<td>CDK4 amplification</td>
<td></td>
</tr>
<tr>
<td>Brenner 8</td>
<td>None detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP Brenner 1</td>
<td>HRA S p.G12S (31%)</td>
<td>MCA 1</td>
<td>PIK3CA p.H1047R (46%)</td>
<td></td>
</tr>
<tr>
<td>AP Brenner 2</td>
<td>KRAS p.G12V (43%)</td>
<td>MCA 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Brenner tumors 1 to 6 are paired with ipsilateral mucinous tumors. The percentage of variant overlap was calculated based on a comparison of the variants remaining after data filtration.

*Low-level amplification below the data analysis pipeline cut-off value of ≥6 copies.

AP Brenner, atypical proliferative Brenner; MBT, mucinous borderline tumor; MCA, mucinous cystadenoma.

Figure 2 Brenner 2 with MYC and CCND1 amplification (A) and paired MCA 1 (B) also showing MYC amplification and 4.6 copies of CCND1. Brenner 3 with CDK4 copy number 5.0 and MYC copy number 3.6 (C) and paired MCA 2 with MYC and CDK4 (D) amplification. The blue circles indicate the areas of increased copy number; with this assay, amplification is defined as more than six copies of a gene per cell.
confirm the successful isolation of the two components from one mixed tumor (Table 1).

Discussion

In this study we performed next-generation sequencing analysis of a 358-gene panel to detect single-nucleotide variants and copy number variations in eight benign ovarian Brenner tumors, six associated mucinous neoplasms, and two atypical proliferative (borderline) Brenner tumors. The co-occurrence of a mucinous tumor with a Brenner tumor has been well recognized since the 1920s. The reported incidence of Brenner tumors with a mucinous component is 14% to 30%. This is the first study to perform such a comprehensive molecular analysis of Brenner tumors and their associated ipsilateral mucinous neoplasms as well as atypical proliferative (borderline) Brenner tumors.

Several immunohistochemical (IHC)-based studies have been performed to evaluate the association of Brenner tumors with Walthard cell nests, fallopian tube epithelium, and germ cells. The epithelial component of Brenner tumors and most Walthard cell nests are diffusely positive for GATA3, AKR1C3, androgen receptor, and lack staining for PAX8 and PAX2 (two Mullerian markers), calretinin or inhibin (these two markers are positive in the stromal component), and SALL4 (a germ cell tumor marker). This is the first study to perform such a comprehensive molecular analysis of Brenner tumors and their associated ipsilateral mucinous neoplasms as well as atypical proliferative (borderline) Brenner tumors.

Several immunohistochemistry (IHC)-based studies have been performed to evaluate the association of Brenner tumors with Walthard cell nests, fallopian tube epithelium, and germ cells. The epithelial component of Brenner tumors and most Walthard cell nests are diffusely positive for GATA3, AKR1C3, androgen receptor, and lack staining for PAX8 and PAX2 (two Mullerian markers), calretinin or inhibin (these two markers are positive in the stromal component), and SALL4 (a germ cell tumor marker). This is the first study to perform such a comprehensive molecular analysis of Brenner tumors and their associated ipsilateral mucinous neoplasms as well as atypical proliferative (borderline) Brenner tumors.

Several immunohistochemistry (IHC)-based studies have been performed to evaluate the association of Brenner tumors with Walthard cell nests, fallopian tube epithelium, and germ cells. The epithelial component of Brenner tumors and most Walthard cell nests are diffusely positive for GATA3, AKR1C3, androgen receptor, and lack staining for PAX8 and PAX2 (two Mullerian markers), calretinin or inhibin (these two markers are positive in the stromal component), and SALL4 (a germ cell tumor marker). This is the first study to perform such a comprehensive molecular analysis of Brenner tumors and their associated ipsilateral mucinous neoplasms as well as atypical proliferative (borderline) Brenner tumors.

One limitation of the JAX Cancer Treatment Profile assay and current data analysis pipeline used in this study is that it is not validated to detect gene deletions, which may be of significance. For instance, alterations in CDKN2A, the gene that encodes for p16INK4a (also known as CDKN2), have been described in atypical proliferative (borderline) Brenner tumors. Several studies have shown expression of p16 by IHC in benign Brenner tumors with loss of staining in atypical proliferative (borderline) Brenner tumors. Kuhn et al reported the identification of one KRAS and two PIK3CA somatic mutations in a study of seven atypical proliferative (borderline) Brenner tumors. Similar to prior reports, in our series four of the six (67%) MCAs/MBTs harbored KRAS mutations and the two MCAs that lacked KRAS mutations both showed MYC amplification; one also had a PIK3CA mutation. In addition, RAS mutations were identified in both atypical proliferative (borderline) Brenner tumors (a PIK3CA mutation also was identified in one Brenner tumor), however, none were detected in the benign Brenner tumors. RAS mutations were identified in both MBTs and atypical proliferative (borderline) Brenner tumors, suggesting that a more proliferative phenotype is associated with alterations in RAS genes. The two PIK3CA mutations detected (p.H1047R and p.C420R) both are considered activating mutations.

A novel, previously unreported finding in this study was the amplification of MYC in five samples (two pairs of Brenner and MCAs, and a separate Brenner tumor). MYC is a proto-oncogene and the encoded protein is considered a pleiotropic transcription factor that plays roles in multiple pathways that regulate cellular proliferation, metabolism, differentiation, and apoptosis. MYC amplification has been reported in up to 25% of epithelial ovarian cancers, although no Brenner tumors were studied. In our study, MYC amplification appeared to co-occur with amplification of either CDK4 (three cases) or CCND1 (two cases). MYC amplification was mutually exclusive with RAS mutations, suggesting an alternate mechanism of pathway activation. It is known that activation of the extracellular signal-regulated kinases/mitogen-activated protein kinases pathway by KRAS mutants leads to downstream MYC activation and tumor dependency. Therefore, in the tumors lacking RAS

### Table 2 Immunohistochemistry Profiles of Brenner Tumors and MCAs/MBTs

<table>
<thead>
<tr>
<th>Histology</th>
<th>GATA3</th>
<th>AKR1C3</th>
<th>AR</th>
<th>PAX8</th>
<th>PAX2</th>
<th>SALL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brenner</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mucinous metaplasia associated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with Brenner</td>
<td>-</td>
<td>-</td>
<td>UK</td>
<td>UK</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCA/MBT</td>
<td>-</td>
<td>-</td>
<td>UK</td>
<td>UK</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

AR, androgen receptor; MBT, mucinous borderline tumor; MCA, mucinous cystadenoma; UK, unknown at this time.
Hormonal, particularly androgen, signaling likely also plays a role in the development of atypical proliferative (borderline) Brenner tumors. Brenner tumors are known to express androgen receptors and the aldo-keto reductase AKR1C3 (HSD17B5), which is a key enzyme in androgen production,\textsuperscript{3,28} and excessive androgen stimulation may be responsible in part for the epithelial proliferation of these tumors. Of importance, both of our patients with atypical proliferative (borderline) Brenner tumors had clinical signs of virilization as a result of excess androgen production.

Wang et al\textsuperscript{29} recently reported another approach to evaluate the relationship between mixed Brenner and mucinous tumors. In their study, they analyzed the patterns of X chromosome inactivation (methylation) at eight loci, and microsatellite patterns at nine loci, in five paired Brenner and mucinous tumors, respectively. They showed that the two components of the combined tumors shared a clonal relationship that was statistically significant.

Taking our findings and prior literature together, we propose a model for the relationship between Brenner and mucinous tumors of the ovary (Figure 3). Transitional metaplasia in the form of Walthard rests likely derived from fallopian tube epithelium, may seed the ovary, and develop into a Brenner tumor. Walthard rests also may undergo cystic change to form paratubal cysts and possibly mucinous metaplasia to form other mucinous tumors. Brenner epithelial components may develop mucinous metaplasia with cyst formation. At this point, accumulation of genetic alterations including RAS mutations and CDKN2A aberrations or MYC and CDK4/CNND1 amplification are necessary to further drive histogenesis. Although the RAS alterations are common to both mucinous neoplasms and atypical proliferative (borderline) Brenner tumors, other alterations likely are necessary for the development of these neoplasms. Although the expression of androgen receptors and AKR1C3 have not been well characterized in mucinous tumors, Brenner tumors are known to express androgen receptors and the aldo-keto reductase AKR1C3 (HSD17B5), which is a key enzyme in androgen production,\textsuperscript{3,28} and excessive androgen stimulation may be responsible in part for the epithelial proliferation of these tumors. Of importance, both of our patients with atypical proliferative (borderline) Brenner tumors had clinical signs of virilization as a result of excess androgen production.

Wang et al\textsuperscript{29} recently reported another approach to evaluate the relationship between mixed Brenner and mucinous tumors. In their study, they analyzed the patterns of X chromosome inactivation (methylation) at eight loci, and microsatellite patterns at nine loci, in five paired Brenner and mucinous tumors, respectively. They showed that the two components of the combined tumors shared a clonal relationship that was statistically significant.

Taking our findings and prior literature together, we propose a model for the relationship between Brenner and mucinous tumors of the ovary (Figure 3). Transitional metaplasia in the form of Walthard rests likely derived from fallopian tube epithelium, may seed the ovary, and develop into a Brenner tumor. Walthard rests also may undergo cystic change to form paratubal cysts and possibly mucinous metaplasia to form other mucinous tumors. Brenner epithelial components may develop mucinous metaplasia with cyst formation. At this point, accumulation of genetic alterations including RAS mutations and CDKN2A aberrations or MYC and CDK4/CNND1 amplification are necessary to further drive histogenesis. Although the RAS alterations are common to both mucinous neoplasms and atypical proliferative (borderline) Brenner tumors, other alterations likely are necessary for the development of these neoplasms. Although the expression of androgen receptors and AKR1C3 have not been well characterized in mucinous tumors, Brenner tumors are known to express androgen receptors and the aldo-keto reductase AKR1C3 (HSD17B5), which is a key enzyme in androgen production,\textsuperscript{3,28} and excessive androgen stimulation may be responsible in part for the epithelial proliferation of these tumors. Of importance, both of our patients with atypical proliferative (borderline) Brenner tumors had clinical signs of virilization as a result of excess androgen production.

Wang et al\textsuperscript{29} recently reported another approach to evaluate the relationship between mixed Brenner and mucinous tumors. In their study, they analyzed the patterns of X chromosome inactivation (methylation) at eight loci, and microsatellite patterns at nine loci, in five paired Brenner and mucinous tumors, respectively. They showed that the two components of the combined tumors shared a clonal relationship that was statistically significant.

Taking our findings and prior literature together, we propose a model for the relationship between Brenner and mucinous tumors of the ovary (Figure 3). Transitional metaplasia in the form of Walthard rests likely derived from fallopian tube epithelium, may seed the ovary, and develop into a Brenner tumor. Walthard rests also may undergo cystic change to form paratubal cysts and possibly mucinous metaplasia to form other mucinous tumors. Brenner epithelial components may develop mucinous metaplasia with cyst formation. At this point, accumulation of genetic alterations including RAS mutations and CDKN2A aberrations or MYC and CDK4/CNND1 amplification are necessary to further drive histogenesis. Although the RAS alterations are common to both mucinous neoplasms and atypical proliferative (borderline) Brenner tumors, other alterations likely are necessary for the development of these neoplasms. Although the expression of androgen receptors and AKR1C3 have not been well characterized in mucinous tumors, Brenner tumors are known to express androgen receptors and the aldo-keto reductase AKR1C3 (HSD17B5), which is a key enzyme in androgen production,\textsuperscript{3,28} and excessive androgen stimulation may be responsible in part for the epithelial proliferation of these tumors. Of importance, both of our patients with atypical proliferative (borderline) Brenner tumors had clinical signs of virilization as a result of excess androgen production.

Wang et al\textsuperscript{29} recently reported another approach to evaluate the relationship between mixed Brenner and mucinous tumors. In their study, they analyzed the patterns of X chromosome inactivation (methylation) at eight loci, and microsatellite patterns at nine loci, in five paired Brenner and mucinous tumors, respectively. They showed that the two components of the combined tumors shared a clonal relationship that was statistically significant.
the progression and pathogenesis of ovarian MCAs/MBTs and atypical proliferative (borderline) Brenner tumors from a benign Brenner tumor.

Acknowledgments

We thank the staff of the Dartmouth-Hitchcock Medical Center Molecular Pathology Laboratory, the Translational Research Program (Dartmouth-Hitchcock Medical Center), and the Jackson Clinical Genomics Laboratory. We also thank Dr. Jiang Gui (Geisel School of Medicine at Dartmouth) for statistical advice.

References