Pre-Invasive Ovarian Mucinous Tumors Are Characterized by CDKN2A and RAS Pathway Aberrations

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Abstract

Introduction: Mucinous tumors are the second most common form of epithelial ovarian tumor, yet the cell of origin for this histologic subtype remains undetermined. Although these tumors are thought to arise through a stepwise progression from benign cystadenoma to borderline tumor to invasive carcinoma, few studies have attempted to comprehensively characterize the genetic changes specific to this subtype or its precursors.

Methods: To explore the spectrum of genomic alterations common to mucinous tumors we carried out high-resolution genome-wide copy number analysis, mutation screening by Sanger sequencing and immunohistochemistry on a series of primary ovarian mucinous cystadenomas (n = 20) and borderline tumors (n = 22).

Results: Integration of copy number data, targeted mutation screening of RAS/RAF pathway members and immunohistochemistry reveals that p16 loss and RAS/RAF pathway alterations are highly recurrent events that occur early during mucinous tumor development. The frequency of concurrence of these events was observed in 40% of benign cystadenomas and 68% of borderline tumors.

Conclusions: This study is the largest and highest resolution analysis of mucinous benign and borderline tumors carried out to date and provides strong support for these lesions being precursors of primary ovarian mucinous adenocarcinoma. The high level of uniformity in the molecular events underlying the pathogenesis of mucinous ovarian tumors provides an opportunity for treatments targeting specific mutations and pathways. Clin Cancer Res; 18(19); 1–11. ©2012 AACR.
Mucinous tumors are the second most common form of epithelial ovarian tumor, accounting for 36% of all epithelial ovarian tumors, 81% of which are benign cystadenomas, 14% borderline (atypical proliferative), and 5% malignant (18). Current treatments for epithelial ovarian cancers are typical across all histologies of EOC despite the known differences in characteristic molecular events between histologic groups. MOCs have been consistently reported to have lower platinum sensitivity and poorer response rates relative to other histologic types. Molecular characterization of mucinous ovarian tumors (MOT) has been limited. This study carried out high-resolution molecular characterization of putative precursors to primary MOCs, namely mucinous cystadenomas and borderline tumors, as a means of providing a deeper understanding of the origins and key genetic events in initiation and progression of mucinous ovarian neoplasms. These data may offer opportunities for improvement of treatment options, particularly if it can be shown that MOTs share molecular signatures with mucinous cancers from other organs.

Translational Relevance
Current treatments for epithelial ovarian cancers are uniform across all histologies of epithelial ovarian carcinomas despite the known molecular differences between histologic groups. Mucinous ovarian carcinomas (MOC) have been consistently reported to have lower platinum sensitivity and poorer response rates relative to other histologic types. Molecular characterization of mucinous ovarian tumors (MOT) has been limited. This study carried out high-resolution molecular characterization of putative precursors to primary MOCs, namely mucinous cystadenomas and borderline tumors, as a means of providing a deeper understanding of the origins and key genetic events in initiation and progression of mucinous ovarian neoplasms. These data may offer opportunities for improvement of treatment options, particularly if it can be shown that MOTs share molecular signatures with mucinous cancers from other organs.

Materials and Methods
Tissue samples
Fresh frozen tissue samples were used for copy number and mutation analyses. All samples were collected with the patient’s informed consent and the study was approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre. Patients with ovarian tumors were identified through 2 primary sources: (a) hospitals in the Wessex Region, UK (n = 18; ref. 37); (b) the Australian Ovarian Cancer Study (AOCS; n = 24; ref. 38). The AOCS (www.aocsstudy.org) was approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre, Queensland Institute of Medical Research, University of Melbourne and all participating hospitals. Pathology review was conducted independently by an anatomical pathologist (MC) for this study, and assessed the histology and likelihood of primary ovarian status (39). Pathology review was carried out on cryosections adjacent to the tissue from which DNA was extracted. Formalin-fixed paraffin embedded samples for tissue microarray (TMA) analyses were obtained through AOCS.

Microdissection and DNA extraction
A representative hematoxylin and eosin stained section was assessed and needle microdissection was done on subsequent 10-μm sections to obtain high percentage tumor epithelial cell and fibroblast cell components. DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen). Normal DNA extracted from blood lymphocytes was available for 38 patients, where this was not available matched DNA from stroma with confirmed normal copy number was used.

Copy number arrays
The Affymetrix SNP6.0 Human Mapping (1.8 M probe set) array was used for ultrahigh resolution allele-specific copy number analysis, although before its release the Affymetrix 500K array was used (samples 446, 214, and 289 only). Arrays were carried out as recommended by the manufacturer with the exception that the input was reduced from the recommended 500 to 250 ng by reducing reaction volumes by half for all processes before the SNP6.0 PCR step. Reduction in DNA input does not result in any loss in the quality of the data. MAPD scores of <0.4 were achieved for all samples run on the SNP6.0 platforms. All SNP data has been made publicly available through Gene Expression Omnibus GSE39076 (http://www.ncbi.nlm.nih.gov/geo/).

SNP array data were analyzed using Partek Genomics Suite 6.5, using paired and unpaired copy number generation, allele-specific copy number analysis and circular binary segmentation to identify regions of somatic copy number aberration and LOH (Supplementary Tables S4 and S5). The threshold for gains was 2.3 copies and losses 1.7
copies. Homozygous deletions (HD) were less than 0.75 copies. Regions of LOH were less than 0.5 copies of the minimum allele. Regions of LOH were confirmed through examination of allele-specific copy number ratios. Nexus Copy Number 6.1 Discovery Edition (BioDiscovery, Inc.) was also used for paired copy number analysis. Quadratic correction was used to smooth noise in the data, probes were recounted around the median copy number, and segmentation was based on a minimum of 3 probes/segment. Default settings were used for calling copy number variation: high gain (0.7), gain (0.1), loss (−0.15), and big loss (−1.1).

**Mutation screening**

Whole genome amplified (WGA) DNA was used for mutation screening, with 50 ng of DNA amplified using the REPLI-g Mini kit (Qiagen). DNA sequencing was done by Sanger sequencing using BDT v3.1 reagents (Applied Biosystems) and an ABI3130 sequencer. Sequencing was used to identify the most common ovarian tumor mutations: all samples were assessed at BRAF codon 600, KRAS codons 12, 13, TP53 exons 4 to 9 and CDKN2A exons 1 to 3, and a subset were also tested for KRAS codon 61, NRAS codons 12, 13, and 61, HRAS codons 12, 13, and 61 (n = 13), and ERRB2 exon 20 (n = 23). Primer sequences are detailed in Supplementary Table S1.

**Immunohistochemistry**

TMAs were constructed by AOCS from formalin-fixed, paraffin-embedded tissues from representative 1 mm cores. Three-micrometer sections of the TMA were stained using antibodies for p16 (clone E6H4, Cintec – 9511), p53 (clone DO-7, Novoceastra – NCL-p53-D07), CK7 (clone OV-TL 12/30, Dako – M7018), CK20 (clone KS20.8, Novoceastra – NCL-CK20), and p-ERK (Cell Signaling, Cat #4370, 1/200). p-ERK staining was carried out using a DAKO Autostainer, whereas the remainder were run on a Ventana Benchmark Ultra Immunostainer using Ventana Ultraview detection reagents.

Scoring was done using a semiquantitative method, based on staining intensity (none = 0, weak = 1, moderate = 2, strong = 3) and percentage of cells stained (0 = 0%, 1 = <1%, 2 = 1% to 10%, 3 = 10% to 33%, 4 = 33% to 66%, 5 = >60%). These scores were added for a final score of 0 to 8 for all stains. Cytokeratins 7 and 20 were exclusively cytoplasmic stains with any staining considered positive. CK7 staining was highly homogeneous, strong and diffuse in almost all cases. CK20 staining was typically consistent between cores within a sample, but varied significantly within the cohort, with negative, strong diffuse and strong focal staining observed. p53 staining was exclusively nuclear and was considered positive when >10% of cells stained moderately strongly (overall score >5 to 6). P-ERK staining was observed to be both nuclear and/or cytoplasmic and typically displayed significant staining heterogeneity within and between tumor cores. p16 staining was predominantly cytoplasmic with some nuclear staining, and also displayed some staining heterogeneity. P-ERK and p16 were considered to positive staining if >10% of cells stained moderately strongly (overall score >5 to 6). Representative staining images can be found in the Supplementary data (Supplementary Figure S1).

**Results**

**Copy number and loss of heterozygosity analysis identify recurrent targeting of CDKN2A/2B**

To identify recurrent genomic alterations in pre-invasive MOTs, high-resolution copy number data was generated for the epithelium and stroma from 20 benign and 22 borderline mucinous tumors using Affymetrix SNP6.0 and 500K arrays. The majority of both benign and borderline mucinous tumors had detectable genomic copy number aberrations (CNAs; 14/20 benign, 18/22 borderline). The most highly recurrent feature across both benign and borderline tumors was loss of heterozygosity (LOH) targeting chromosome 9 and 9p and focal hemizygous and HDs targeting 9p21.3 (Tables 1 and 2, Fig. 1). The minimal region of loss among samples harboring 9p deletions encompassed both the CDKN2A and CDKN2B genes (Fig. 2). LOH (hemizygous deletion or copy neutral LOH) of 9p was detected in 60% of the benign tumors and in 77% of the borderline tumors. The frequency of HDs targeting CDKN2A/2B was significantly higher in borderline tumors compared with benign tumors 55% versus 20%; Fisher's exact test P = 0.03. In our previous study of invasive MOC (40), we observed 9p LOH in 10 of 12 cases (83%), and HD in 6 of 12 cases (50%).

CNA and LOH events elsewhere in the genome were less common. Gain of chromosome 7 or 7p and LOH of chromosome 21 were observed in 4 of 22 (18.2%) and 2 of 22 (9.1%) of the borderline tumors, respectively. These CNAs were not detected in benign tumors (Table 1) but have been previously observed in invasive mucinous tumors (refs. 41, 42; Fig. 1). Other recurrent aberrations observed in benign, borderline and invasive MOTs included gain of 1q and 17q, and LOH of 17p, however, only 17p LOH was present in more than 30% of samples (in invasive cases). No CNA or LOH events were present at significantly different frequencies between benign, borderline and invasive MOT, although the power to detect such differences was limited by the small number of invasive cases available.

**Stromal copy number aberrations**

Stromal copy number aberrations suggestive of synchronous stromal neoplasia were identified in 3 cases (1 benign, 2 borderline), 2 of which had chromosome 12 trisomy and the remaining case had balanced tetrasomy of chromosome 12. Chromosome 12 gain has been observed as a frequent MOTs, high-resolution copy number data was generated for the epithelium and stroma from 20 benign and 22 borderline mucinous tumors using Affymetrix SNP6.0 and 500K arrays. The majority of both benign and borderline mucinous tumors had detectable genomic copy number aberrations (CNAs; 14/20 benign, 18/22 borderline). The most highly recurrent feature across both benign and borderline tumors was loss of heterozygosity (LOH) targeting chromosome 9 and 9p and focal hemizygous and HDs targeting 9p21.3 (Tables 1 and 2, Fig. 1). The minimal region of loss among samples harboring 9p deletions encompassed both the CDKN2A and CDKN2B genes (Fig. 2). LOH (hemizygous deletion or copy neutral LOH) of 9p was detected in 60% of the benign tumors and in 77% of the borderline tumors. The frequency of HDs targeting CDKN2A/2B was significantly higher in borderline tumors compared with benign tumors 55% versus 20%; Fisher's exact test P = 0.03. In our previous study of invasive MOC (40), we observed 9p LOH in 10 of 12 cases (83%), and HD in 6 of 12 cases (50%).

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Deleterious CDKN2A mutations

Mutation screening was done for exons 1a-3 of CDKN2A and 4 truncating mutations and 2 frameshift mutations were identified (Tables 1 and 2); these mutations were confirmed as somatic by sequencing the matching germline DNA. These mutations were truncating only in the p16 ORF and not the ARF ORF, or targeted exon 1a (p16-specific exon), suggesting p16 is the primary target. The majority of the mutations (5/6) occurred in cases with LOH of 9p21.3 and were thus homozygous.

Oncogene activation

Mutation screening was done using Sanger sequencing for the hotspot activating mutations of KRAS, known to be frequently mutated in MOTs. Mutation screening was also done for BRAF, NRAS, HRA5, ERBB2 and TP53 (Tables 1 and 2). Consistent with previous reports, KRAS was the most commonly mutated gene in both benign and borderline MOTs, with mutation rates of 60% and 64%, respectively (Tables 1 and 2). Low rates of NRAS, BRAF and TP53 mutation were identified and no ERBB2 mutations were detected. A single case harbored a high level amplification of RRAS2, showing both an alternative oncogenic target and alternative mechanism of increasing activity. A high degree of overlap was noted between activating mutations and LOH of 9p; 50% of benign tumors with KRAS mutations also had 9p LOH, whereas 83% of borderline tumors with oncogenic mutations (KRAS, BRAF, or NRAS) also had 9p LOH (Table 3).

Immunohistochemistry analysis of p16, p53, phospho-ERK (p-ERK), CK7, and CK20

We carried out immunohistochemistry for p16, p53, p-ERK, CK7, and CK20 on a tissue microarray of 95 borderline tumors with scorable cores (Table S3) including 8 of the borderline tumors analyzed for somatic mutations described above. All 8 of these tumors were strongly positive for CK7 and negative or focally positive for CK20, supportive of their primary ovarian status. The only tumor that was TP53 mutant stained strongly for p53. Three of 4 samples with a HD of CDKN2A/2B were negative for p16 and a fourth stained weakly in 1 out of the 4 cores on the TMA.
possibly representative of tumor heterogeneity. One sample with LOH of 9p stained weakly for p16. Of the samples without LOH at CDKN2A/2B 1 core stained positive for p16 and the other stained negative. All 8 borderline cases had KRAS mutations, however, only 4 of 8 cases were scored strongly positive for p-ERK (1 case was strongly positive in 2/4 cores), indicative of activation of the RAS/MEK/ERK pathway.

Overall, across the 95 borderline tumors on the TMA, the rate of p16 negative tumors was 65%, similar to the rates of p16 HD (55%) and p16 LOH (77%) observed in the copy number data. The rate of positive p-ERK staining across the 95 borderline cases on the TMA was 59% and was notably lower than the rates of RAS/RAF activating mutation (82%) in the 22 borderline cases screened by Sanger sequencing. P-ERK staining was found to be predominantly focal, with some heterogeneity between cores from the same tumor.

Discussion
Genetic alterations of MOTs
Activating KRAS mutations are recognized as one of the dominant features of MOTs but we have showed that other

<table>
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<th>Sample</th>
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<th>NRAS</th>
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LOH, loss of heterozygosity = hemizygous deletion, CNLOH = copy neutral LOH. (Note: Large LOH events with co-existing focal HD was frequently observed). WT, wildtype. *BRAF c.1799G>A, 1800_1803inv, 1804-1815del; predicted to be activating based on similarity to previously reported mutation (68). **Co-occurring stromal copy number aberrations; gain chromosome 12.
members of the RAS family and pathways are also activated in these tumors. We identified mutations in **BRAF** and **NRAS**, whereas an alternative mechanism via high-level amplification was identified for **RRAS2**. As previously reported, all oncogenic events within the RAS/RAF pathway were mutually exclusive (44–46). Over 80% of the borderline mucinous tumors had an oncogenic event, although this was not an exhaustive investigation and additional mechanisms of RAS/RAF pathway activation may occur in these tumors. Our study has also established a clear continuity of the alterations observed in benign and borderline MOTs consistent with the former being a precursor to the latter. Perhaps surprisingly, benign MOTs harbor a similar frequency of certain alterations to the borderline tumors, including numerous copy number, LOH changes and **KRAS** and **TP53** somatic mutations indicating that many benign MOTs are poised for progression.

LOH or HD targeting 9p and 9p21.3 are early events in MOTs, occurring in 60% of benign tumors. Interestingly, the proportion of HD events relative to LOH events is significantly higher in borderline tumors (0.71) compared with benign tumors (0.33), suggesting that silencing all 3 protein products (p16INK4A and ARF, and p15INK4B) in this region offers a significant selective advantage. These 3 proteins have functions central to cell cycle regulation, cellular senescence, p53 regulation and apoptosis. This is consistent with the observation that elimination of the entirety of CDK2NA (p16INK4A and ARF) is more oncogenic in mouse models compared with loss of either p16INK4A or ARF functions alone (47), whereas loss of both CDKN2A and CDKN2B is more oncogenic in mouse models than loss of CDKN2A (p16INK4A and ARF) alone (48).

It is unclear from these data whether oncogene activation or **CDKN2A/2B** deficiency occurs first, as we observed similar numbers of benign tumors with either RAS pathway activation or p16 deficiency. In other tumor types, p16INK4A expression has been reported to increase from benign to invasive neoplasms in an attempt to downregulate an upregulated cell-cycle program, suggesting selection for p16INK4A inactivation may occur subsequent to oncogene activation to allow escape from oncogene-induced senescence (49–51). However, it was notable that the co-occurrence of RAS activation and homozygous CDKN2A inactivation was higher in the borderline tumors (P = 0.06, Fisher’s exact test) with only 3 of 20 benign tumors harboring both aberrations compared with 13 of 22 borderline tumors (P = 0.005, Fisher’s exact test). Interestingly, in our previous study of serous benign and borderline tumors (27), deletion of **CDKN2A/2B** was not observed, despite a high incidence of RAS pathway activation in serous borderline tumors (~60%).

Mouse modelling of co-existing **KRAS**<sup>G12D</sup> mutations with loss of p16INK4A activity in the mouse pancreas has showed that this combination results in aggressive...
metastatic neoplasms, compared with no neoplasms or localized neoplasms with individual mutations alone (52). Intriguingly, although in the current study the vast majority of borderline MOTs carry both an oncogenic mutation and inactivated CDKN2A/2B, the low incidence of MOCs must make tumor progression even in this context quite infrequent. In the ovarian context, additional genetic or epigenetic events may be required for invasiveness. Our previous study of invasive MOC indentified additional copy number changes on several chromosomes, including 7 gain,

Figure 2. CN LOH and HDs at CDKN2A are frequently observed in mucinous ovarian precursors. A, frequency plot comparing copy number (CN) loss and LOH (allele-specific copy number loss, [ASCN], including copy neutral LOH as well as HD) in benign (top) and borderline (bottom) tumors. B, zoomed view of HDs at 9p21.3 in benign and borderline tumors.

Table 3. Immunohistochemistry of selected borderline samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>p16 (IHC/CN)</th>
<th>p-ERK (IHC/RAS)</th>
<th>p53 (IHC/MUT)</th>
<th>CK7</th>
<th>CK20</th>
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<td>~/HD</td>
<td>~/KRAS</td>
<td>~/N</td>
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</tbody>
</table>

IHC, immunohistochemistry result; ~, negative, + weakly or focally positive, +++ strongly or diffusely positive; HD at CDKN2A/2B; WT, no CN or LOH at CDKN2A/2B; LOH, CN loss or CNN LOH at CDKN2A/2B; MUT, mutation of p53, N, no mutation, Y, p53 mutation positive; KRAS, KRAS mutation.
Although previous reports have showed that polysomy-17 clearly showed for serous ovarian carcinomas. TP53 mutations were found to have activated KRAS and inactivated p16 alone (57). These data reiterate in ovarian tissues the inverse relationship between p-ERK and p16 status, even in the presence of an activating KRAS mutation. These mutations occurred more frequently in samples with ERBB2 amplification and may be a precursor aberration (61, 62). No samples were found to have ERBB2 mutations although we only focused on exon 20, which has been previously reported as an ERBB2 mutation hotspot in serous borderline ovarian tumors (63).

### Cell of origin for MOTs

Extra-ovarian mucinous carcinomas have a long history of misdiagnosis as primary ovarian tumors after metasta-
sizing to the ovary in a form highly similar to primary MOCs. These metastases may arise from a wide variety of primary sites, including the pancreas, colon, appendix, breast and lung (64, 65). In a reassessment of archival MOC cases, the majority (80%) of MOCs were reclassified as extra-ovarian in origin with only 2.4% to 4.9% retaining the classification of primary ovarian epithelial carcinoma (21, 64). Diagnosis of metastases is confounded by the tendency of some mucinous metastases to form large, cystic masses with extensive apparent benign and borderline elements known as a “maturation” phenomenon (66). In recent decades guidelines have been established to aid the diagnosis of primary and secondary carcinomas, such as size, morphology, laterality and tissue-specific immunohistochemistry (39). The majority of tumors are able to be distinguished using these features, however, these are broad guidelines and some tumors remain difficult to classify. In the cohort analyzed here, each case was reviewed using these criteria to exclude cases arising from an extra-ovarian origin. The genetic similarities between the tumors in this study and the invasive MOC studied previously (n = 12) suggest that MOC are likely to develop through a classic adenoma–borderline–carcinoma sequence within the ova-
ry, however, a closely related pathway at an extra-ovarian site cannot be entirely excluded. None of the cases in this study had teratomatous elements reported. More MOC and extra-ovarian mucinous carcinomas need to be analyzed to evaluate these possibilities.

### Conclusions

This study is the largest and highest resolution analysis of mucinous benign and borderline tumors conducted to date and provides strong support for these precursors being the origin of primary ovarian mucinous adenocarcinomas. Although a number of nonovarian potential precursors

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<th>Table 4. Staining patterns for p16 and p-ERK</th>
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<tr>
<td>No. cases (N = 95)</td>
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NOTE: Five cases had either inverse staining patterns within the cores (n = 3) or ambiguous staining patterns (n = 2).
have been postulated, no bona fide alternative precursor has been identified for the majority of benign or borderline MOTs, and mucinous tumors may remain as the only true primary ovarian tumors. Current data does not, however, preclude the possibility that benign mucinous epithelial cells undergo migration from an unknown primary location to the ovary, which may provide an ideal niche for growth and tumorigenesis. The high level of uniformity in the molecular events underlying the pathogenesis of MOTs provides an opportunity for treatments targeting specific mutations and pathways. Further molecular characterization is required to determine whether molecular events can be identified that can distinguish between mucinous tumors of ovarian and extra-ovarian origin.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.M. Hunter, K.L. Gorringe, I.G. Campbell
Development of methodology: M. Christie, I.G. Campbell
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Hunter, D.D. Bowtell, I.G. Campbell

References


Pre-Invasive Ovarian Mucinous Tumors Are Characterized by \textit{CDKN2A} and \textit{RAS} Pathway Aberrations

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