Human Cancer Biology

Clinical Cancer Research

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

Sally M. Hunter[1], Kylie L. Gorringe[1,2], Michael Christie[3,4], Simone M. Rowley[1], David D. Bowtell[1,2], on behalf of the Australian Ovarian Cancer Study Group[1,5], and Ian G. Campbell[1,2]

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); 5267–77. ©2012 AACR.*

Introduction

Despite significant progress in recent years in identifying and defining the molecular pathogenesis of precursor lesions for individual histologic types of epithelial ovarian carcinoma (EOC), 1 question remains to be definitively answered: is there such a thing as a primary EOC? Endometrioid and clear cell ovarian carcinomas have been shown to arise from the endometrium via endometriosis (1–4). Growing evidence suggests high grade serous carcinomas (HGSC) arise from precursors in the fallopian tube, particularly the fimbriae (5–8), and more recently endometrial intraepithelial carcinoma has also been proposed as a precursor lesion to HGSC (9). Preliminary work has suggested that the epithelium of ovarian epithelial inclusions and ovarian borderline serous tumors, which are the presumed precursors to low grade ovarian serous carcinomas, may transit from the fallopian tube and undergo neoplastic changes once in the ovarian milieu (10–12).

Mucinous ovarian carcinomas (MOC) have historically generated the greatest doubt over their site of origin, with some suggestions that there may be no such thing as a primary MOC and that all carcinomas of this type represent metastases to the ovary from extra-ovarian sites such as the colon or pancreas. Risk factors associated with MOCs are distinct from those recognized for the serous and endometrioid subtypes (13–15), further suggestive of a unique aetiology. The cell of origin for primary ovarian mucinous tumors remains unknown. Co-occurrence of mucinous ovarian tumors (MOT) with Brenner (transitional cell) components is thought to suggest a common origin and has implicated para-ovarian transitional cells as potential cells of origin (16), however, the evidence remains circumstantial. More recent reports of mucinous metaplasia of fallopian tube epithelium in the presence of chronic inflammation and without evidence of co-occurring malignancy has provided an alternative and compelling potential origin for MOTs (17).

Authors' Affiliations: [1]Centre for Cancer Genomics and Predictive Medicine, Peter MacCallum Cancer Centre, East Melbourne; [2]The Department of Pathology, University of Melbourne, Parkville; [3]Ludwig Institute of Cancer Research, Royal Parade; [4]Department of Anatomical Pathology, Royal Melbourne Hospital, Parkville, Australia; and [5]The full Australian Ovarian Cancer Study Group can be found at www.aocstudy.org

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Ian Campbell, VBCRC Cancer Genetics Research Laboratory, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett Street, Melbourne, VIC 8006, Australia. Phone: 613-9656-1803; Fax: 613-9656-1411; E-mail: Ian.Campbell@petermac.org

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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.

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 been reported to have lower platinum sensitivity and poorer response rates relative to other histologic types (19–22). Thorough molecular characterization of mucinous ovarian cancers may provide opportunities for improvement of treatment options particularly if it can be shown that MOC share molecular signatures with mucinous cancers from other organs, consistent with the overlapping histologic and clinicopathological features of mucinous cystic tumors of the ovary, pancreas, liver and retroperitoneum (23–26).

Previous studies have identified *KRAS* mutations as a common defect across ovarian mucinous cystadenomas, borderline tumors and carcinomas. In contrast to serous cystadenomas and cystadenofibromas in which such mutations are not observed (27–29), the rate of *KRAS* mutation in mucinous cystadenomas has been reported at 46% to 55% (30–32). This rate increases to 63% to 73% in borderline tumors (31, 33) and 75% to 85% in mucinous carcinomas (31, 33). This molecular evidence, combined with the presence of benign and borderline epithelium in 70% to 90% of MOCs (34–36), has provided some basis for a linear progression model for these tumors. Identification and characterization of precursor lesions can be highly informative, allowing the determination of initiating molecular events and establishing a benchmark for molecular progression models, with the potential to identify biomarkers for invasive potential. This study aimed to carry out high-resolution molecular characterization of putative precursor lesions to primary MOCs, namely mucinous cystadenomas and borderline tumors, using genome-wide copy number analysis, mutation screening and immunohistochemistry.

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.aocstudy.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 done independently by an anatomical pathologist (MC) for this study, and assessed the histology and likelihood of primary ovarian status (39). Pathology review was conducted 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

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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 CN aberration and 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 recentred 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 *ERBB2* 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, Novocastra – NCL-p53-DO7), CK7 (clone OV-TL 12/30, Dako – M7018), CK20 (clone Ks20.8, Novocastra – 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 CNA in the stroma of benign serous cystadenomas and cystadenofibromas, at lower rates in serous borderline ovarian tumors (27), and commonly in pure fibromas (43). HD of the *CDKN2A/2B* locus was observed in the adjacent epithelial DNA in all 3 cases, indicating that these tumors are molecularly very similar to the other tumors in the cohort despite the presence of co-existing stromal neoplasia.

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Table 1. Genetic alterations in benign mucinous tumors

Sample	9p LOH	*CDKN2A* HD/mutations	*KRAS*	*BRAF*	*NRAS*	*TP53*	Other
8055	CNLOH	HD	G12D	WT	—	WT	None
907[b]	LOH	HD	WT	WT	—	WT	LOH 1p22.1, 7q36.1, 16p12.1-21.3, 9q
2408	CNLOH	HD	WT	WT	WT	WT	None
IC512	CNLOH	HD	WT	WT	WT	WT	LOH 9p23
11142	LOH	WT	G12R	WT	—	WT	Gain 1q, 20
8676	CNLOH	WT	G12V	WT	—	WT	None
IC446	CNLOH	WT	G12V	WT	—	WT	None
IC94	LOH	WT	G12V	WT	—	WT	None
2845	LOH	R58*	G12V/G12D[a]	WT	—	WT	None
IC214	LOH	WT	WT	WT	—	WT	None
IC276	LOH	A21-L32del	WT	WT	WT	V157F	Amp 11p15.2 (*RRAS2*), LOH 11p, 17p
IC294	LOH	WT	WT	WT	WT	WT	Gain 1q, 6p, 8p11.23-p12, 9q, 11q13.1-q13.3, 11q13.4-11q14.1, q14.1, 12q13.3, LOH 6q, 8p12-pter, 11q12.1-q13.1, 11q13.3-q13.4, 11q14.1-qter, 17p, Xp22.33
2362	WT	P75*	G12A	WT	—	WT	None
3788	WT	WT	G12D	WT	—	WT	None
1249	WT	WT	G12R	WT		R175H	LOH 14, 17p
1735	WT	WT	G12V	WT	—	WT	None
IC156	WT	WT	G12V	WT	—	WT	None
3045	WT	WT	G13E	WT	—	WT	Gain 1q
IC335	WT	WT	WT	WT	WT	WT	None
IC372	WT	WT	WT	WT	WT	WT	None
$n = 20$	12 (60%)	7 (35%)	12 (60%)	0	0	2 (10%)	7 (35%)

LOH, loss of heterozygosity = hemizygous deletion, CNLOH = copy neutral LOH. WT, wild-type.

[a]Likely to be a result of 2 distinct tumor cell population with $KRAS^{G12V/WT}$, $KRAS^{G12D/WT}$ cells, as previously described in mucinous ovarian tumors (67).

[b]Co-occurring stromal copy number aberrations; gain chromosome 12.

Deleterious *CDKN2A* mutations

Mutation screening was done for exons 1α-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 1α (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*, *HRAS*, *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,

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Table 2. Genetic alterations in borderline mucinous tumors

Sample	9p LOH	*CDKN2A* HD/mutation	KRAS	BRAF	*NRAS*	*TP53*	Other
8566	LOH	HD	G12C	WT	—	WT	None
IC263	CNLOH	HD	G12C	WT	—	WT	Gain 7, Amp 9p21.1, CNLOH 12q,17q
IC387	CNLOH	HD	G12C	WT	—	WT	None
IC537	LOH	HD	G12C	WT	—	WT	Gain 9q
5401	LOH	HD	G12D	WT	—	WT	Gain 1q
IC289	CNLOH	HD	G12D	WT	—	WT	None
4584	CNLOH	HD	G12V	WT	—	WT	None
6181	LOH	HD	G12V	WT	—	R273C	Gain 1q21.3-q23.1, 2q33.2-qter, 6q22.1-qter, 17q, 19p13.2, LOH 6p21.1-pter, 9, 17p, 19p12-p13.11, 19p13.12-pter, X
4779[b]	LOH	HD	G12V	WT	—	WT	None
IC531	CNLOH	HD	G12V	WT	—	WT	Gain 3, 7, 5p13.1-q11.2
2895[b]	CNLOH	HD	WT	V600E + indel[a]	—	WT	None
IC92	CNLOH	HD	WT	WT	WT	WT	Gain 7p22.1-p11.2, LOH 5q11.2, 8p11.22, 20p13, CNLOH 1q
4691	CNLOH	R80*	G12D	WT	—	WT	None
4515	CNLOH	WT	G12D	WT	—	WT	Amp 17q21.3, LOH 9p23, 21, CNLOH 17q12-qter
4551	CNLOH	WT	WT	V600E	—	WT	None
4884	LOH	K62*fs*V	WT	WT	Q61R	WT	LOH 9q33.2-9q34.12, 21
IC388	LOH	R58*	WT	WT	WT	WT	Gain 1q, 2, 3, 6, 7p, 10, 12p, 15, 16, 17q, 18, 19q, 20, 22 LOH 5, 7q, 9, 14, 17p, 19p, 21
528	WT	WT	G12C	WT	—	WT	None
2063	WT	WT	G12D	WT	—	WT	None
IC186	WT	WT	WT	V600E	—	WT	Gain 7, LOH 18p11.23
11552	WT	WT	WT	WT	WT	WT	None
IC322	WT	WT	WT	WT	WT	WT	None
$n = 22$	17 (77%)	15 (68%)	14 (64%)	3 (14%)	1	1 (5%)	10 (45%)

LOH, loss of heterozygosity = hemizygous deletion, CNLOH = copy neutral LOH. (Note: Large LOH events with co-existing focal HD was frequently observed). WT, wildtype. [a]*BRAF* c.1799G>A, 1800_1803inv, 1804-1815del; predicted to be activating based on similarity to previously reported mutation (68). [b]Co-occurring stromal copy number aberrations; gain chromosome 12.

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. Staining rates for p53, CK7, and CK20 were similar for the 8 BL cases as for the entire 95 tumors included in the TMA (Supplementary Table S3).

Across the entire set of 95 borderline tumors, an inverse relationship between p16 status and p-ERK status was identified, with over half (53%) of the borderline tumors displaying negative or very weak p16 staining and strong p-ERK staining (Table 4). Fifteen cases (17%) displayed the reverse, with strong p16 staining and negative or very weak p-ERK staining. A small number of cases displayed strong staining for both p16 and p-ERK, whereas 24% of cases were negative for both.

Discussion

Genetic alterations of MOTs

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

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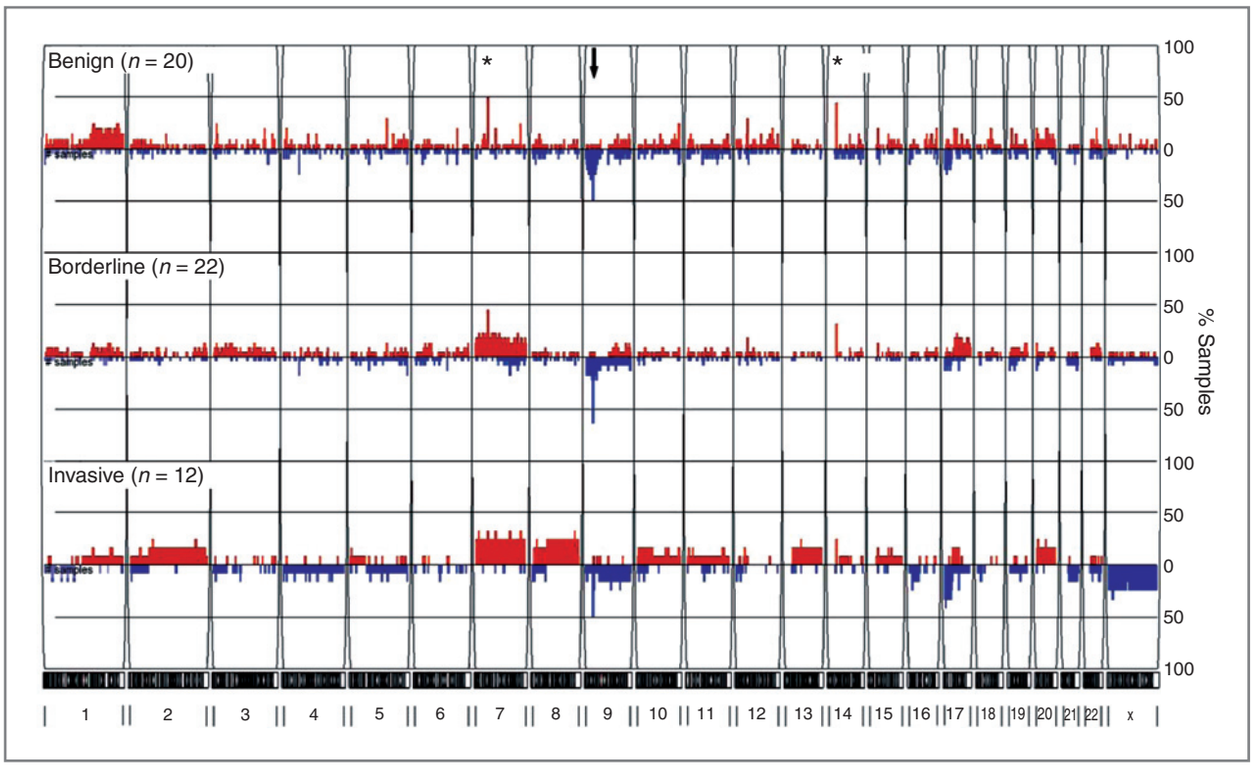

Figure 1. Frequency plot of CNA in mucinous tumors. Benign ($n = 20$), borderline ($n = 22$), and invasive ($n = 12$)[40] mucinous ovarian tumors, from chromosome 1 at left to chromosome X at right. Gains are above the line (red) and losses below (blue), with percentage of samples scaled to 100%. Arrowhead locates recurrent deletions focused at 9p21.3 (*CDKN2A/2B*). Asterisks indicate recurrently identified amplifications located at T-cell receptor gene clusters at 7p14 and 14q11.2, which are artifacts that arise from normalizing against lymphocyte DNA.

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 ($p16^{INK4A}$, ARF, and $p15^{INK4B}$) 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* ($p16^{INK4A}$ and ARF) is more oncogenic in mouse models compared with loss of either $p16^{INK4A}$ or ARF functions alone (47), whereas loss of both *CDKN2A* and *CDKN2B* is more oncogenic in mouse models than loss of *CDKN2A* ($p16^{INK4A}$ 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, $p16^{INK4A}$ expression has been reported to increase from benign to invasive neoplasms in an attempt to downregulate an upregulated cell-cycle program, suggesting selection for $p16^{INK4A}$ inactivation may occur subsequent to oncogene activation to allow escape from oncogene-induced senescence (49–51). However, it was notable that the co-incidence of RAS activation and homozygous *CDKN2A* inactivation was higher in the borderline tumors ($P = 0.06$, Fishers 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 $KRAS^{G12D}$ mutations with loss of $p16^{INK4A}$ activity in the mouse pancreas has showed that this combination results in aggressive

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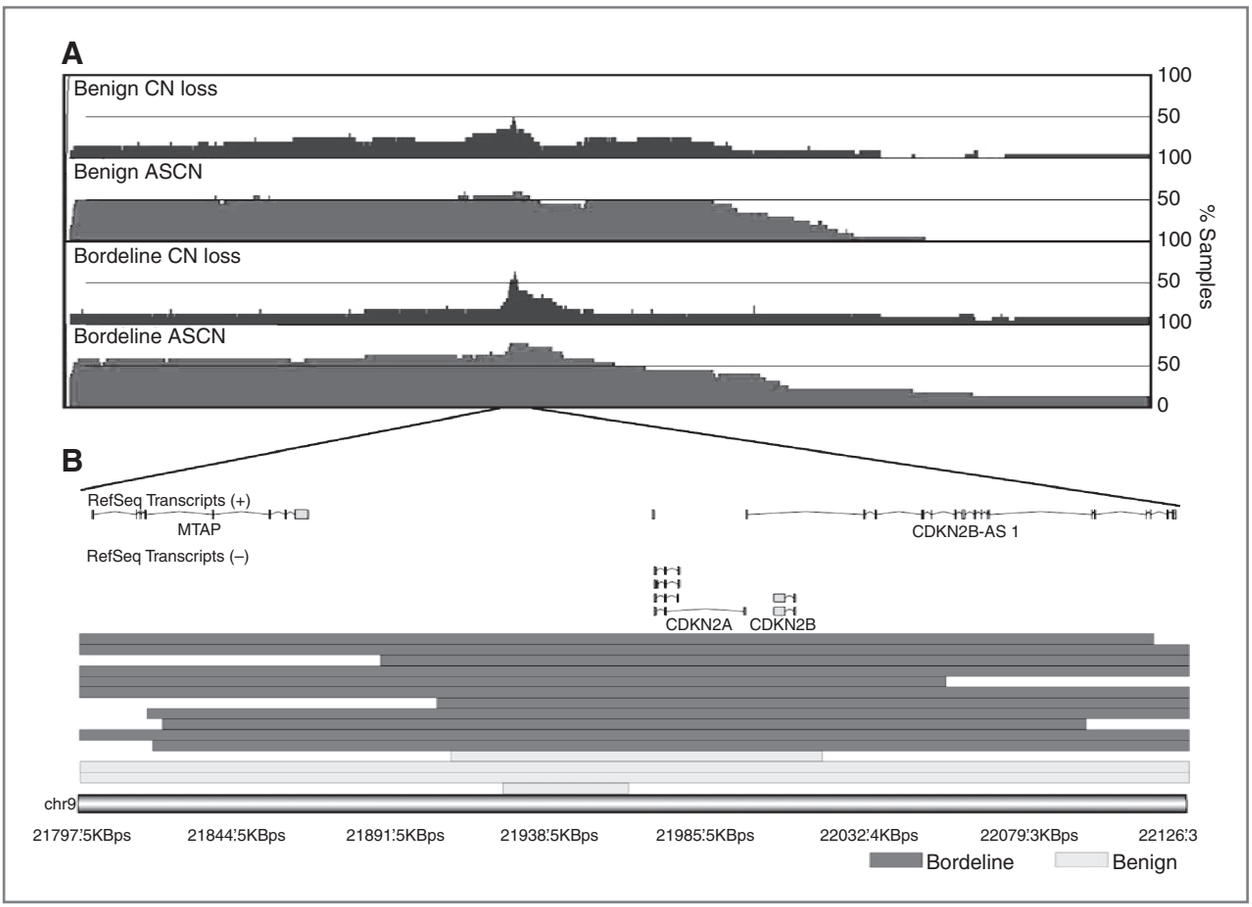

Figure 2. CN LOH and HDs at *CDKN2A* are frequently observed in mucinous ovarian precursors. A, frequency plot comparing copy number (CN) loss and LOH (allele-specific copy number loss, (ASCN), including copy neutral LOH as well as HD) in benign (top) and borderline (bottom) tumors. B, zoomed view of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

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,

Table 3. Immunohistochemistry of selected borderline samples

Sample	p16 (IHC/CN)	p-ERK (IHC/RAS)	p53 (IHC/MUT)	CK7	CK20
4691	–/LOH	+++/KRAS	–/N	+++	+
4584	–/HD	–/KRAS	–/N	+++	–
5401	–/HD	++/KRAS	–/N	+++	+
6181	–/HD	+++/KRAS	+++/Y	+++	–
4515	+/LOH	–/KRAS	–/N	+++	+
2063	–/WT	–/KRAS	–/N	+++	+
528	+++/WT	Mixed/KRAS	–/N	+++	+
4779	–/HD	–/KRAS	–/N	+++	+

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.

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Table 4. Staining patterns for p16 and p-ERK

	p16–/p-ERK+	p16+/p-ERK–	p16+/p-ERK+	p16–/p-ERK–	Fisher's exact test
No. cases ($N = 95$)	48	15	5	22	$P = 0.0007$
% cases	53%	17%	5.60%	24%	

NOTE: Five cases had either inverse staining patterns within the cores ($n = 3$) or ambiguous staining patterns ($n = 2$).
BL, borderline; CK, cytokeratin; CN, copy number; CNA, copy number aberrations; CN LOH, copy neutral LOH; EOC, epithelial ovarian carcinoma; HGSC, high grade serous carcinoma; IHC, immunohistochemistry; LOH, loss of heterozygosity; MOC, mucinous ovarian carcinoma; MOT, mucinous ovarian tumor; TMA, tissue microarray.

8q gain, 17p LOH, and 17q gain, however, none of these were observed at a high frequency in this small cohort.

Activation of ERK1/2 (p-ERK) has been showed in cultures of transformed cells to be induced downstream of activated RAS and RAF, and is used as a functional readout for RAS/RAF/MEK/ERK pathway activation (53, 54). In this study, however, only 50% of tumors with activating *KRAS* mutations were found to have activated ERK1/2 by immunohistochemistry. This is consistent with studies in melanoma that found activating *BRAF* and *NRAS* mutations to have no correlation with p-ERK status (55). This study also identified a clear inverse relationship between p-ERK and p16 staining ($P = 0.0007$, Fisher's Exact Test; Table 4) and a high level of overlap between activating KRAS mutations and p16 loss. Both the lack of correlation between *KRAS* mutation status and ERK phosphorylation, and the inverse relationship between p-ERK and p16 status can be explained by the negative regulation of KRAS expression and activity by p16 (56). When p16 is actively expressed KRAS activity is downregulated and ERK is not phosphorylated, therefore p-ERK status is dependent on and correlates with p16 status, even in the presence of an activitating KRAS mutation. These data reiterate in ovarian tissues the findings of Rabien and colleagues (56) in pancreatic and colon cancer cells.

Mutations in the tumor suppressor *TP53* were observed at low rates in borderline and perhaps surprisingly, in benign mucinous tumors. These mutations occurred in the context of *KRAS* mutation ($n = 1$), p16 inactivation ($n = 1$), or both ($n = 1$). In mouse models, addition of *TP53* mutation has been showed to increase tumorigenicity of cells above that of either activated KRAS or inactivated p16 alone (57). *TP53* mutations have been reported in 20% of invasive MOC ($n = 5$; ref. 58) but there is currently insufficient data to draw definitive conclusions as to whether *TP53* mutation distinguishes high and low grade mucinous tumors as clearly showed for serous ovarian carcinomas.

Amplification of *ERBB2* has been previously reported as relatively common in MOCs and has also been observed in mucinous borderline tumors (59, 60). No focal 17q amplification of *ERBB2* was observed in any of the samples in this study. A single borderline tumor was found to carry an extra copy of the entire arm of 17q, where *ERBB2* is located. Although previous reports have showed that polysomy-17 does not correlate with Her2 expression, polysomy-17 occurs 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 metastasizing 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 ovary, 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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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
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Hunter, K.L. Gorringe, M. Christie, I. G. Campbell
Writing, review, and/or revision of the manuscript: S.M. Hunter, K.L. Gorringe, M. Christie, D.D. Bowtell, I.G. Campbell
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Rowley, I.G. Campbell
Study supervision: I.G. Campbell

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