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Molecular genetic analysis of ovarian serous cystadenomas

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Ovarian serous cystadenomas are common ovarian lesions that may be precursors of serous borderline tumors, which can in turn progress to low-grade serous carcinomas. It has been shown that low-grade serous carcinoma and serous borderline tumors are characterized by frequent mutations in *BRAF* or *KRAS* genes, but the mutational status of these genes in serous cystadenomas and the clonal nature of serous cystadenomas have not been fully investigated. We isolated cyst-lining epithelium from 30 consecutive serous cystadenomas, and analyzed their *BRAF* and *KRAS* mutational status. Wild-type sequences of *BRAF* and *KRAS* were detected in all specimens. Using the human androgen receptor gene as a polymorphic marker, we also examined the clonal status of epithelial cells in all of the serous cystadenomas. Four of 29 (14%) informative specimens were monoclonal based on the methylation pattern. These monoclonal cystadenomas were significantly ($P<0.01$) larger in size (>8 cm) than the nonclonal cystadenomas. These data indicate that serous cystadenomas do not contain mutations in either *BRAF* or *KRAS* genes and that most serous cystadenomas are polyclonal. Accordingly, it appears that serous cystadenomas develop as a hyperplastic expansion from epithelial inclusions with a clonal/neoplastic transformation occurring in a subset of them.
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Ovarian carcinoma is believed to arise either from the surface epithelium covering the ovary, benign epithelial inclusions or cystic tumors termed 'cystadenomas'. The World Health Organization classification divides ovarian surface epithelial tumors into three groups: benign cystadenomas, invasive carcinomas and an intermediate group designated 'borderline' tumors.[1,2] Ovarian cystadenomas are further subclassified according to the cell type into serous cystadenomas, mucinous cystadenomas and endometriomas.[3–7] Cystadenomas occur in 5–15% of postmenopausal women in the general population.[8,9] Serous cystadenomas are the most common followed by mucinous cystadenomas and endometriomas.[10] Previous studies have demonstrated that mucinous cystadenomas[11,12] and endometriomas[13] are clonal and therefore are benign neoplasms, but similar studies of serous cystadenomas have not been performed to our knowledge.

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Based on our previous morphological and molecular studies, we have proposed that serous borderline tumors (atypical proliferative tumors and noninvasive micropapillary serous carcinomas) can progress to low-grade serous carcinomas (invasive micropapillary serous carcinomas).[14,15] These low-grade serous carcinomas and borderline tumors exhibit frequent *BRAF* or *KRAS* mutations but rare p53 mutations.[6,14,16] In contrast, high-grade serous carcinomas have wild-type *KRAS* and *BRAF* but frequent p53 mutations even when they are small and confined to the ovary.[17,18] It has therefore been proposed that high-grade serous carcinomas in contrast to low-grade (invasive micropapillary) serous carcinomas develop directly from epithelial inclusions or ovarian surface epithelium, so-called, '*de novo*' development.[14,19] In order to elucidate the mechanisms of serous carcinogenesis, specifically the molecular genetic alterations in early tumorigenesis, we undertook a study of serous cystadenomas aimed at assessing whether mutations of *BRAF* and *KRAS*, which are common in serous borderline tumors and low-grade serous carcinomas are present in serous cystadenomas.[6,19–21] In addition, we determined the clonality of serous cystadenomas by analyzing the patterns of X-chromosome

inactivation of the X-linked androgen receptor (HUMARA) gene. For molecular genetic analyses, we have applied a new method to isolate pure and abundant cyst-lining epithelial cells from fresh serous cystadenomas. Finally, we compared the proliferative and apoptotic activity of the epithelium of the cystadenomas to that of ovarian surface epithelium and serous borderline tumors.

Materials and methods

Epithelial Cell Isolation from Serous Cystadenomas

A total of 30 consecutive ovarian serous cystadenomas were collected from women undergoing oophorectomy at the Johns Hopkins Hospital, Baltimore, Maryland. One serous borderline tumor was also included as a control. The study protocol was approved by the local institutional review board. Frozen sections of the cystadenomas were performed to confirm the diagnosis before harvesting the epithelial cells from at least half of the sample. For multilocular cystadenomas, the largest one was used in the assays. In order to obtain abundant and pure cyst-lining epithelial cells for multiple molecular genetic analyses, we employed a method to enrich the epithelial cells by combining chemical or mechanical separation and immunosorting. After washing in phosphate buffer saline (PBS) 3 times, the cyst-lining was incubated in 0.05% trypsin and 0.53 mM EDTA (Invitrogen, Grand Island, NY, USA) at 37°C for 10 min, then mechanically separated from the cyst wall by agitating the cyst fragments and/or gentle scraping using a rubber cell scraper (Sarstedt, Newton, NC, USA). The epithelial cell fragments were collected and washed in PBS (Figure 1). The epithelial cells were enriched by Epi-CAM-conjugated Dynal Beads (Dynal, Hamburg, Germany) that specifically bound epithelial cells. The procedures were detailed in the vendor's instructions. The purity of isolated epithelial cells was assessed by immunostaining for the expression of cytokeratin 8 (an epithelial marker), using the antibody CAM5.2 (Becton Dickinson, San Jose, CA)[22] on the isolated cells after short-term culture in 24-well plates. The isotype-matched MN-4 antibody that reacted with Mel-CAM (CD146) was also used as a negative control.[23] The immunofluorescence staining and nuclear counterstaining were detailed in the previous report.[24]

Mutational Analysis

Nucleotide sequencing was used to analyze the mutational status of *BRAF* and *KRAS*. The primers that were used to amplify exon 15 of *BRAF* and exon 1 of *KRAS* containing codons 12 and 13 and the PCR protocols have been previously described.[14,25,26]

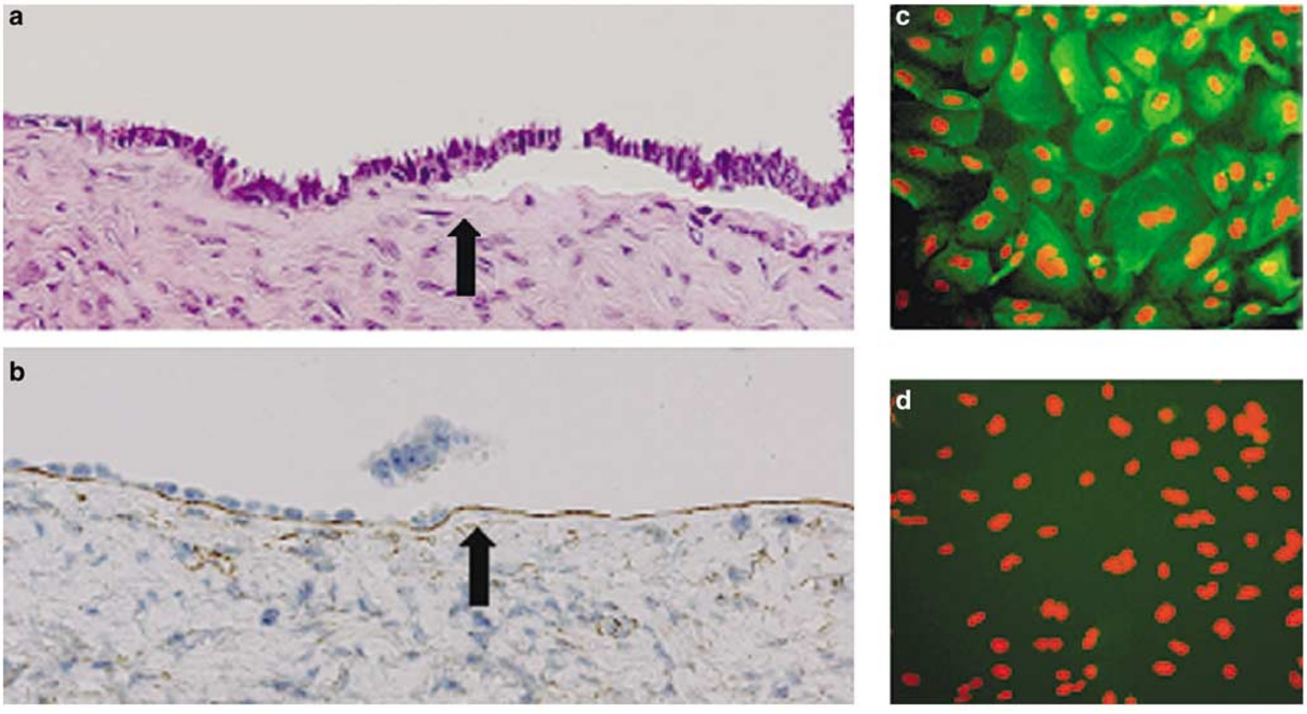

Figure 1 Isolation of cyst-lining epithelium. (**a**) The lining epithelium was gently scraped from the cyst wall to the right of the arrow; the epithelium to the left of the arrow was undisturbed (hematoxylin and eosin stain). (**b**): Immunostains for collagen IV demonstrate the basement membrane. The epithelium is present to the left of the arrow and absent to the right of the arrow that has been harvested. (**c**) Immunostaining for CK8 antibody shows green fluorescence in the cytoplasm of isolated epithelial cells in all the cells. Nuclei are counterstained red. (**d**) Immunostaining for CD146 antibody as a negative control. There is no green fluorescence present.

PCR products were purified using a MiniElute™ PCR purification kit (Qiagen, Valencia, CA, USA). Nucleotide sequencing was performed using fluorescent labeled Applied Biosystems Big Dye terminators and an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Analysis of the 1796T/A status in *BRAF* was also performed using a PCR-based restriction fragment length polymorphism (RFLP) technique as previously described.[27,28] For this method, the *BRAF* PCR product of exon 15 containing the relevant nucleotide at position 1796 was digested with TspR1 (New England BioLabs, Beverly, MA, USA) at 65°C for 3 h. The samples were electrophoresed on a 10% polyacrylamide gel.

Clonality Assay

The clonal status of the cystadenomas was evaluated by analyzing the patterns of X-chromosome inactivation in exon 1 of the X-linked androgen receptor gene (HUMARA), which contains a highly polymorphic trinucleotide repeat.[29,30] The QiaQuick PCR purification kit (Qiagen, Valencia, CA, USA) was used to isolate genomic DNA from the purified cyst epithelial cells from all the 30 specimens. In addition, a serous borderline tumor served as a positive control. Predigestion of DNA (2 μg) with 20 U of methylation-sensitive restriction endonuclease *Hha*I or *Hpa*II (New England BioLabs, Beverly, MA, USA) resulted in selective PCR amplification from the methylated (uncleaved) allele. For PCR, 3 μl of the enzyme digest was mixed with primers and all essential PCR reagents as previously reported.[30] The PCR product was separated in 6% polyacrylamide gels and visualized by ethidium bromide.

Assessment of Proliferation and Apoptotic Activity

The proliferative and apoptotic activity of the cyst-lining epithelial cells was assessed by immunohistochemistry using the MIB1 antibody to detect Ki-67[31,32] and the M30 antibody[33] to detect the cytokeratin epitope after apoptosis, respectively. The immunohistochemistry methodology has been previously described.[34] Paraffin sections from 10 normal ovaries, 50 serous cystadenomas and 15 serous borderline tumors were stained with the MIB1 and the M30 antibody. At least 4000 epithelial cells were randomly selected from different regions and analyzed using the Spotlight morphometric program under a Nikon inverted light microscope (Image System, Columbia, MD, USA). The Ki-67 and M30 labeling index was expressed as the percentage of Ki-67 or M30 labeled epithelial cells from among the total number of epithelial cells counted. Two-sided Student's *t*-test was used to compare the difference in the Ki-67 and M30 labeling index between the serous cystadenomas, normal surface ovarian epithelium and serous borderline tumors.

Results

The 30 serous cystadenomas ranged from 1.2 to 10 cm in greatest dimension with a median of 5 cm (Table 1). Of the 30 cystadenomas, 26 were unilocular and the remaining four cystadenomas were multilocular (specimen no. 15, 17, 21 and 29). The epithelial cells lining the cystadenomas ranged from flattened to columnar (Figure 1). The latter cells frequently contained cilia (serous differentiation) at least in focal areas. The nuclei were bland and none displayed cytologic atypia (so-called dysplasia). Immunostaining revealed that more than 98% of the cells isolated from cystadenomas were positive for cytokeratin 8, confirming the purity of the epithelial cells isolated by our technique (Figure 1). Mutational analysis with direct nucleotide sequencing demonstrated that none of the samples harbored mutations in either *BRAF* (exon 15) or *KRAS* (exon 1) gene. As a control, the epithelial cells isolated from a serous borderline tumor revealed a missense mutation at the codon 12 of *KRAS* (GGT to GAT). The presence of wild-type *BRAF* gene in all of the serous cystadenomas was confirmed using a restriction fragment length polymorphism assay that detects rare mutations at codon 599 of *BRAF*.[27,28]

The lack of mutations in *BRAF* and *KRAS* raises the possibility that serous cystadenomas may not be clonal. Therefore, we determined the clonality of all serous cystadenomas by analyzing patterns of X-chromosome inactivation of the X-linked androgen receptor (HUMARA). All the samples except one were heterozygous and therefore informative for clonal analysis (Table 1). The positive control specimen, a serous borderline tumor, demonstrated a monoclonal composition, as one of the alleles was absent after enzyme digestion. Based on HUMARA assay, we found that only four of the 29 (14%) of the serous cystadenomas showed a clonal pattern (Figure 2 and Table 1). The clonal serous cystadenomas tended to be larger in size (>8 cm) than the polyclonal serous cystadenomas ($P<0.01$; two-tailed *t*-test).

The Ki-67 labeled epithelial cells were scattered randomly, not clustered, as single positive cells in the serous cystadenoma with a frequency of $0.84\pm0.33\%$. This proliferative index was significantly higher than the $0.10\pm0.028\%$ in normal ovarian surface epithelium ($P<0.001$) and significantly lower than the $8.15\pm2.80\%$ in serous borderline tumors ($P<0.001$) (Figure 3). The apoptotic index as determined by the M30 immunoreactivity was extremely low ($<0.01\%$) in serous cystadenomas, as well as in ovarian surface epithelium and serous borderline tumors. There was no statistically significant difference in Ki-67 or M30 labeling index ($P>0.1$) between clonal and polyclonal cystadenomas nor did the size of the cystadenomas correlate with the Ki-67 or M30 labeling index (data not shown).

The time for a serous cystadenomas to double in diameter was calculated by assuming that the

Table 1 *BRAF* and *KRAS* mutation and clonality assays in serous cystadenomas

Sample		*BRAF mutational status*	*KRAS mutational status*	*Clonality assay*
ID	*Size (cm)*			
1	1.2[a]	Wild type	Wild type	Polyclonal
2	2	Wild type	Wild type	Polyclonal
3	2.5	Wild type	Wild type	Polyclonal
4	3	Wild type	Wild type	Polyclonal
5	3.5	Wild type	Wild type	Polyclonal
6	3.5	Wild type	Wild type	Polyclonal
7	3.8	Wild type	Wild type	Polyclonal
8	4	Wild type	Wild type	Polyclonal
9	4	Wild type	Wild type	Polyclonal
10	4.3	Wild type	Wild type	Polyclonal
11	4.5	Wild type	Wild type	Polyclonal
12	4.5	Wild type	Wild type	Polyclonal
13	5	Wild type	Wild type	Polyclonal
14	5	Wild type	Wild type	Polyclonal
15	5	Wild type	Wild type	Polyclonal
16	5	Wild type	Wild type	Polyclonal
17	5.2	Wild type	Wild type	Polyclonal
18	5.5	Wild type	Wild type	Polyclonal
19	5.8	Wild type	Wild type	Polyclonal
20	6.5	Wild type	Wild type	Polyclonal
21	8	Wild type	Wild type	Polyclonal
22	8	Wild type	Wild type	Polyclonal
23	8.3	Wild type	Wild type	NA
24	8.5	Wild type	Wild type	Clonal
25	9	Wild type	Wild type	Polyclonal
26	9	Wild type	Wild type	Clonal
27	9.2	Wild type	Wild type	Clonal
28	9.8	Wild type	Wild type	Polyclonal
29	10	Wild type	Wild type	Polyclonal
30	10	Wild type	Wild type	Clonal
SBT	12.6	Mutated[b]	Wild type	Clonal

SBT: serous borderline tumor; NA: homozygous to the polymorphic markers of the HUMARA allele.
[a]The greatest dimension of the cystadenoma.
[b]Mutation occurs at the codon 12 of *KRAS* (GGT to GAT).

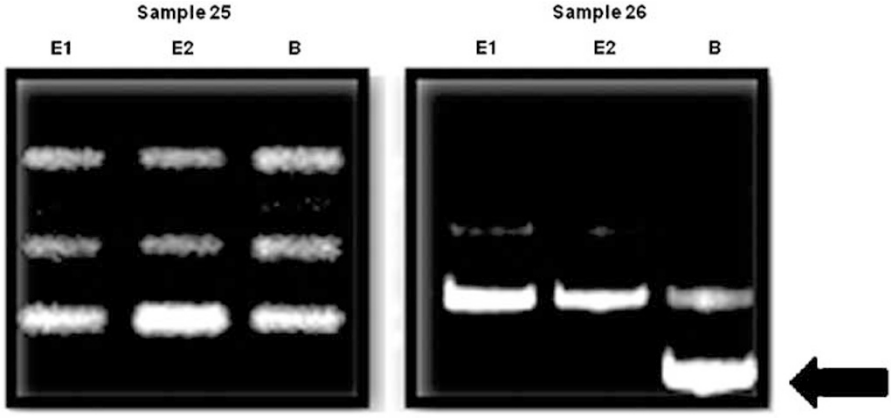

Figure 2 Clonality assay with the polymorphic markers of the HUMARA gene. Representative cystadenoma samples (25 and 26) are shown. Sample 25 retains all the bands in *Hpa*II (E1) and *Hha*I (E2) predigested samples as compared to the control with buffer only (B). In contrast, sample 26 shows an absence of the shorter band (arrow) in enzyme-digested samples, indicating a homogenous methylation pattern, that is, monoclonality, in this sample.

cystadenomas are spheres with a diameter of A cm and that the epithelium evenly lined the inner surface of the cyst. The total number of epithelial cells in a cyst (N_A) was equal to the total inner surface area of a cyst ($A^2\pi$)/the (en face) area of an individual epithelial cell which was $12 \times 12\,\mu$m based on morphometric measurements. For a cyst with a diameter of A cm to enlarge to a diameter

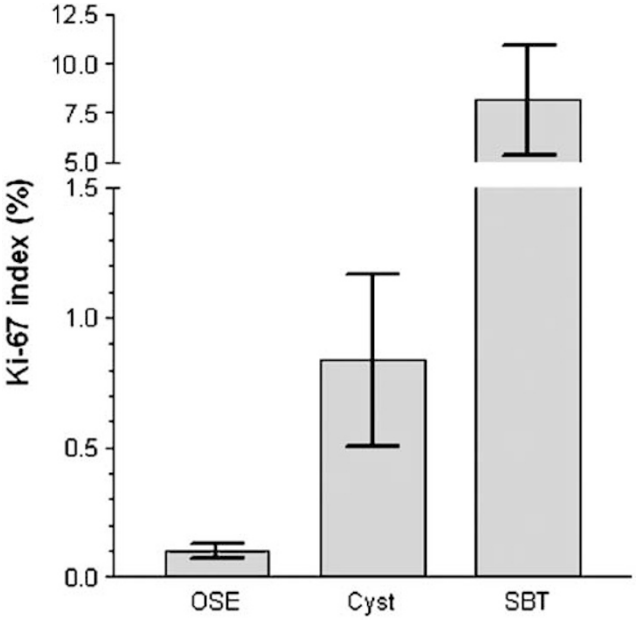

Figure 3 Proliferative activity in ovarian surface epithelium (OSE), serous cystadenomas and serous borderline tumors (SBT) as determined by the Ki-67 labeling index. Serous cystadenomas have a significantly higher Ki-67 labeling index than ovarian surface epithelium ($P<0.001$) but a lower index than serous borderline tumors ($P<0.001$).

of $2A$ cm, a four fold increase in cell number is required since $N_{2A}/N_A = 4A^2\pi/144\,\mu m^2/A^2\pi/144\,\mu m^2 = 4$. Given the net proliferation index (Ki-67 index−M30 index) of 0.84% and a presumable cell doubling time of t hours in cyst-lining epithelial cells, it was estimated that it would take $200/0.84 \times t$ hours for a cyst with an original cell number of N_A to increase to 4 N_A (N_{2A}). The cell doubling time, t, depends on the cell type and the microenvironment, and generally is between 48 and 96 h. Therefore, given $t = 72$ h, the estimated time for a serous cyst to double in size was $200/0.84 \times 72\,h = 17.143\,h = 714$ days or 23.8 months.

Discussion

Clonality and mutations are the molecular signatures of the vast majority of neoplasms.[35] Accordingly, our data demonstrating an absence of *BRAF* and *KRAS* mutations in all of the serous cystadenomas in this analysis and an absence of clonality in the vast majority of them provide cogent evidence that most so-called 'serous cystadenomas' are not neoplasms. This conclusion is supported by other studies showing a diploid chromosomal content[36] and lack of p53 mutations in serous cystadenomas.[6] The Ki-67 immunohistochemical findings in the present study indicating that the epithelial cells of these cystadenomas do proliferate, albeit at a very low rate, suggest that the development of a non-clonal serous cyst is a hyperplastic process. The mechanism underlying increased proliferative activity in the cyst-lining epithelium as compared to the ovarian surface epithelium is unknown, but it may be related to the sustained hydrostatic pressure that has been shown to induce cellular proliferation.[37–39] The mildly increased proliferation index could explain why serous cystadenomas can reach a size as large as 10 cm. Based on our estimates, the time for a cyst to double in size (diameter) is approximately 2 years. Therefore, it may take 16 years for a small inclusion cyst of 300 μm in diameter to enlarge to a 10 cm cyst. It should be noted that the doubling time of a cyst is based on estimation and in fact it depends on several factors, especially the cell cycle transit time of proliferating cyst-lining epithelium, which is difficult to measure *in vivo*. It is apparent that not all serous inclusion cysts will progress to large cysts and further studies are required to investigate the growth kinetics of cysts and molecular mechanisms underlying their development. The present study demonstrates that ovarian serous cystadenomas do not contain mutations in either *BRAF* or *KRAS* genes and that most are polyclonal. In previous studies, we reported that low-grade serous carcinomas develop in a stepwise fashion from serous borderline tumors.[14,15] Although the molecular events that lead to the development of serous borderline tumors are not known, our findings suggest that activating mutations in *BRAF* or *KRAS* may play an important role as both mutations are found in over 60% of serous borderline tumors and in low-grade serous carcinomas.[27] This view is supported by the observation that activating mutations in these genes are oncogenic in experimental cell culture systems.[25,40,41] Accordingly, the clonal nature of only a small proportion of serous cystadenomas and the absence of *KRAS* or *BRAF* mutations in them suggests that mutations in these genes may be a key early event in the transformation of clonal serous cystadenomas to serous borderline tumors.

Although the findings in this report indicate that 86% of ovarian serous cystadenomas are polyclonal and non-neoplastic, this may be an overestimate as monoclonality may have been undetected in some cases for several reasons. First, for the genetic analysis, we pooled the bulk of the cyst-lining epithelium from each specimen to avoid the bias introduced by the X-inactivation patch size in the assessment of clonality.[42] It is possible that clonal proliferation occurs only focally in some cystadenomas and that the molecular changes in these foci occur in the absence of morphologic alterations that are not detected microscopically. Second, our results do not exclude the possibility that multiple independent clonal events occur in a cyst, which would then result in the erroneous impression of polyclonality. Third, since the monoclonal cystadenomas tended to be large and the number of large cystadenomas (>8 cm) that were analyzed in this study was relatively small, it is possible that the

proportion of monoclonal serous cystadenomas would have been higher if a greater number of large cystadenomas were analyzed. Finally, very small inclusion cysts (<1 cm) were not analyzed in this study because they are too small and by definition do not qualify as cystadenomas.[43]

In conclusion, the data in this report demonstrate that mutations in *BRAF* and *KRAS* that characterize serous borderline tumors and low-grade serous carcinomas are absent in serous cystadenomas. In fact, only 14% of serous cystadenomas are clonal, suggesting that serous cystadenomas develop as a hyperplastic expansion of ovarian surface epithelial inclusions. We speculate that a small proportion of these cystadenomas become clonal and that mutations of *KRAS* or *BRAF* in some of these clonal cystadenomas lead to the development of serous borderline tumors, which are the precursors of low-grade serous carcinoma. These findings have important implications for understanding the pathogenesis of ovarian serous carcinoma, and for the screening and treatment of ovarian cancer.

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