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Research Article

Early Genetic Divergence of High-Grade Carcinomas Originating from Low-Grade Serous Ovarian Neoplasms

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ABSTRACT

The current paradigm implicates a fallopian tube precursor as the origin of most ovarian high-grade serous carcinomas (HGSCs). However, a rare subset of HGSCs develop via a distinct pathway from low-grade serous ovarian neoplasms (namely, serous borderline tumors and low-grade serous carcinoma). This alternate pathway for the development of HGSC and other poorly differentiated carcinomas of the ovary is not well understood. To elucidate the molecular pathogenesis and evolutionary trajectory of histologic transformation of low-grade serous neoplasms, we performed whole exome sequencing on microdissected low-grade and higher-grade components from 7 cases of serous borderline tumor or low-grade serous carcinoma associated with a synchronous or metachronous indeterminate/high-grade carcinoma. In most cases, there were relatively few somatic mutations shared between matched low-grade and higher-grade tumors compared with private mutations specific to each component (ie, phylogenetic trees with short trunks and long branches). Truncal mutations, present across all tumor samples from a given patient, included known drivers of low-grade serous neoplasms: KRAS (G12D, n = 4), BRAF (G469A, n = 1), NF2 (n = 1), and USP9X (n = 1). Transformation to HGSC was associated with a TP53 mutation with bi-allelic inactivation in 3 cases, all with severe nuclear atypia, and associated with genome-wide copy number alterations and allelic imbalances. TP53-wildtype tumors comprised a morphologic spectrum, which included indeterminate-grade serous carcinomas with moderate nuclear atypia and high mitotic activity, although lacking extensive chromosomal instability (n = 2) and poorly differentiated carcinomas (n = 2, including a high-grade Mullerian carcinoma and an undifferentiated carcinoma with sarcomatoid features). In summary, synchronous and metachronous low-grade serous neoplasms and higher-grade carcinomas are clonally related. Early genetic divergence, most evident in cases with TP53 mutations, suggests that high-grade transformation may be a relatively early molecular event.

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Introduction

Ovarian serous neoplasms are traditionally subclassified into low-grade and high-grade subtypes, which are considered distinct diseases with differences in clinical behavior, histomorphology,

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and molecular pathogenesis.^{1,2} The much more common high-grade serous carcinoma (HGSC) is a clinically aggressive malignancy that usually originates from fallopian tube epithelium, through the acquisition of *TP53* mutations.^{3,4} Marked nuclear pleomorphism, a characteristic histomorphologic feature of HGSC, is reflective of underlying chromosome instability and genome-wide copy number alterations. In contrast, low-grade serous tumors comprise a group of pathogenically related entities ranging on a spectrum from benign cystadenoma, to serous borderline tumor (SBT), to invasive low-grade serous carcinoma (LGSC), which represent the sequential steps in the progression of the disease.^{1,2} Up to 60% of LGSCs harbor driver mutations in *BRAF*, *KRAS*, or *NRAS*, resulting in constitutive activation of downstream MAPK signaling.^{5,6}

The low-grade and high-grade pathways of serous carcinogenesis are usually independent pathogenic processes that do not overlap. However, the rare progression of low-grade serous neoplasms to high-grade carcinomas, including HGSC and poorly differentiated/sarcomatoid carcinomas, has been well documented in multiple case series.⁷⁻¹³ In some cases, both low-grade and high-grade components coexist synchronously within the primary tumor. Alternatively, high-grade carcinoma can be diagnosed at recurrence (metachronous presentation) in a patient with a prior diagnosis of SBT or LGSC. In the limited numbers of patients with clinical follow-up, high-grade transformation was generally associated with rapid disease progression and poor clinical outcome.^{7-10,12}

The pathogenesis of high-grade transformation of low-grade serous neoplasms is poorly understood, as only a couple of studies have performed molecular analyses on these tumors and only by targeted gene panel sequencing. Through *BRAF*, *KRAS*, and *TP53* mutational analyses of matched low-grade and high-grade areas isolated by microdissection, Dehari et al¹¹ reported 2 of 6 cases with identical *KRAS* mutations in the SBT and HGSC components, and none of the cases harbored *TP53* genetic alterations. By targeted next-generation sequencing with a 341 cancer gene panel, Murali et al¹² demonstrated shared mutations (including *KRAS* or *NRAS* mutations) and copy number alterations between low-grade and high-grade tumors from the same patients; *TP53* mutations were identified in 2 of the 5 high-grade tumors. These studies support a clonal relationship between the high-grade and low-grade tumor components and suggest that high-grade transformation may be driven by *TP53* mutation, in some, but not all cases.

Due to the targeted sequencing approach used in prior studies, the extent of the relatedness between synchronous/metachronous low-grade serous neoplasms and their respective high-grade carcinomas remains unknown. Moreover, in cases of *TP53*-independent high-grade transformation, the only evidence for the diagnosis of high-grade carcinoma is based on morphologic interpretation, which may be subjective. Some serous carcinomas, with moderate nuclear atypia and high mitotic activity (grade 2 by the Shimazu–Silverberg grading system¹⁴) cannot be neatly categorized by the 2-tiered grading system. In a previous study of so-called “indeterminate-grade serous carcinomas” (IGSC), most have been reported to be *TP53* wildtype, yet associated with poor survival outcomes, similar to HGSC.¹⁵

In the present study, we examined the molecular evolution of ovarian low-grade serous neoplasms that have transformed into indeterminate/high-grade carcinoma by performing histomorphologic, immunophenotypic, and whole exome sequencing analyses of morphologically distinct tumor components.

Methods

Case Selection and Histologic Review

Following institutional review board approval, we identified 7 cases of ovarian SBT or LGSC associated with a synchronous or metachronous “high-grade” carcinoma, as originally diagnosed in the pathology report, from departmental in-house and consultation archives of Johns Hopkins Hospital and Memorial Sloan Kettering Cancer Center. To enable molecular analyses of morphologically distinct components, we were restricted to only cases with spatial separation of the low-grade and higher-grade tumor components, and with formalin-fixed paraffin-embedded tissue blocks available. Slides were reviewed by 2 gynecologic pathologists (M.H.C. and I.-M.S.). For morphologically distinct tumor areas, the degree of nuclear atypia and mitotic rate (per 10 high-powered fields [HPF], $\times 40$ objective, 0.55 field diameter) were assessed separately. Nuclear atypia was graded on a 3-tier scoring system.^{14,16,17} Grade 1 (low-grade atypia) denoted uniform round or oval nuclei, whereas grade 3 (high-grade atypia) referred to large, hyperchromatic, and pleomorphic nuclei with $\geq 3:1$ size variation overall. Grade 2 (moderate atypia), was intermediate between grade 1 and grade 3, typically characterized by enlarged and overlapping nuclei with irregular borders and $<3:1$ size variation; rare high-grade nuclei (including large, bizarre forms), were allowed. In accordance with the 2-tiered grading system for ovarian serous neoplasms,¹⁸ incorporated into WHO 2020 criteria,² tumors with $\geq 3:1$ size variation (grade 3 nuclear atypia) were classified as high-grade, whereas tumors with $<3:1$ size variation (usually grade 1 nuclear atypia) and mitotic activity < 12 per 10 HPF were classified as low grade. Tumors with grade 2 nuclear atypia and ≥ 12 mitoses per 10 HPF, along with a p53 wildtype expression pattern by immunohistochemistry, were classified as IGSC.^{15,16,18} Based on these criteria, 2 recurrent tumors originally signed-out as HGSC (from cases 2 and 6) were reclassified to IGSC following central pathology review.

Immunohistochemistry

Immunohistochemistry was performed using the following primary antibodies: PAX8 (BC12; Cell Signaling, 1:50), WT1 (WT49; Leica, prediluted), ER (SP1; Ventana, prediluted), PR (1E2; Ventana, prediluted), p16 (BC42; Biocare, prediluted), p53 (DO-7; Dako, prediluted), GATA3 (L50-823; Cell Marque, 1:500), and TTF-1 (SPT24; Novocastra, 1:400). All immunohistochemical stains were performed on the BOND RX platform (Leica), using the standard protocol, with BOND Epitope Retrieval Solution ER2 (Leica) for 30 minutes (25 minutes for GATA3 only), incubation of primary antibody for 30 minutes at room temperature and BOND Polymer Refine Detection (Leica).

Tissue Microdissection

Unstained sections (10-micron thickness) of tumor and matched normal tissue were microdissected under a stereomicroscope to ensure tumor purity $> 80\%$. For Case 1 only, laser capture microdissection was performed to isolate the simple epithelium lining the cystadenomatous portion of the tumor, for analysis of the *KRAS*^{G12D} mutation by droplet digital PCR, as previously described.¹⁹

Whole Exome Sequencing

Following genomic DNA extraction, samples were subjected to whole exome sequencing. Details on library preparation and data processing, including read alignment and mutation calling, have been previously described.⁴ Copy number analysis was performed using FACETS.²⁰ Based on genetic alterations including somatic mutations and loss-of-heterozygosity (LOH) events, phylogenetic analysis was performed to elucidate the clonal relationships between multiple samples from the same patient.⁴

Statistical Analysis

Comparisons of the mitotic index, fraction of genome altered (FGA), and a number of private mutations between matched low-grade and higher-grade tumor components were performed by 2-tailed paired *t* test. For cases with multiple low-grade or higher-grade tumor samples, the average value across all samples within each respective category was used for statistical comparisons.

Results

Summary of Clinical, Histomorphologic, and Immunophenotypic Features

The median age at initial diagnosis was 35 years (mean 45, range: 27–70; Table 1). In 2 cases, the low-grade and high-grade components were synchronous, coexisting in the primary ovarian neoplasm (cases 1 and 4). For the remaining cases, the diagnosis of higher-grade carcinoma was made on a recurrent tumor sample. Time from primary diagnosis of an ovarian low-grade serous neoplasm to higher-grade recurrence ranged from 2 to 25 years (median, 4 years, *n* = 5). Of the 5 patients with clinical follow-up following diagnosis of indeterminate/high-grade carcinoma, there were 3 deaths (cases 3, 5, and 7, all with high-grade carcinoma); median survival of 23 months from time of histologic transformation).

Histologically, the low-grade serous tumor components consisted of either SBT only (*n* = 4), LGSC only (*n* = 1), or SBT with associated LGSC (*n* = 2). SBTs were of conventional (*n* = 4) or micropapillary type (miSBT, *n* = 2). Nuclei were round and

uniform with mild, although occasionally moderate, irregularities (grade 1, *n* = 6; grade 2, *n* = 1); mitotic figures were rare (<5 per 10 HPF). Viewed in isolation, there were no morphologic differences in the low-grade serous tumor components compared with conventional pure SBTs or LGSCs. In all cases, the higher-grade carcinoma components showed increased nuclear atypia (grade 2, *n* = 2; grade 3, *n* = 5) compared with the corresponding low-grade components. Mitotic activity was elevated in the higher-grade (median: 14, mean: 14, range: 3–23, per 10 HPF) relative to low-grade tumor components (median: 2, mean: 1.5, range: 0–3, per 10 HPF; *P* = .001, paired *t* test).

By immunohistochemistry, the low-grade serous tumors generally showed diffuse expression of PAX8 and WT1, with the exception of Case 7, which showed only focal, weak WT1 expression (Table 2). They were positive for ER and PR, although the degree of expression varied across cases, with generally lower levels of PR expression relative to ER. Intact p16, characterized by a heterogeneous pattern of staining, was observed in low-grade serous tumors with the exception of the miSBT from Case 5 and a recurrent LGSC from Case 6, both of which showed complete absence of expression (null pattern).

For the higher-grade carcinomas, 5 (cases 1, 2, 4, 5, and 6) were diffusely positive for both PAX8 and WT1, consistent with serous differentiation (Table 2). Lower PR expression was observed in the higher-grade carcinoma compared with the primary low-grade serous tumor in 6 of 7 cases. Complete loss of both ER and PR expression was observed in high-grade carcinomas from cases 3, 5, and 7. The high-grade carcinoma from Case 3 was positive for PAX8 but negative for WT1, ER, and PR, as well as GATA3 and TTF1 (ruling out mesonephric differentiation), and hence best classified as high-grade Mullerian carcinoma, not otherwise specified. Case 7, which was morphologically an undifferentiated carcinoma with sarcomatoid features, was negative for PAX8, WT1, ER, and PR. p16 showed diffuse, strong expression in the IGSC and HGSC from cases 2 and 4, respectively, a null pattern in all samples from Case 5 and intact heterogeneous expression in the higher-grade carcinomas in the remaining cases.

Immunohistochemistry for p53 showed a wildtype expression pattern for all low-grade serous tumor components, except the SBT from Case 4, and an aberrant expression pattern in HGSCs from cases 1 and 4 (see subsequent section, “Bi-allelic TP53 inactivation is associated with transformation to HGSC” for details).

Table 1
Summary of clinical information

Case no.	Age (y)	Initial diagnosis	Stage	Clinical follow-up (from time of initial diagnosis)
1	27	SBT with HGSC	I	--Lost to follow-up
2	33	SBT	I	--IGSC, peritoneal rec. (25 y) --Lost to follow-up
3	59	LGSC	III	--High-grade Mullerian carcinoma, pelvic lymph node rec. (44 mo) --Died (74 mo)
4	55	SBT with HGSC	IV	--HGSC, abdominal wall rec. (8 mo), --Alive with disease (40 mo)
5	35	miSBT (ovary) with IGSC (diaphragm)	IV	--HGSC, axillary lymph node rec. (33 mo) --Died (49 mo)
6	35	miSBT with LGSC	III	--IGSC (55 mo), peritoneal rec. --LGSC, chest wall rec. (78 mo) --Alive with disease (11 y)
7	70	SBT with LGSC	IV	--Undifferentiated sarcomatoid carcinoma rec. (37 mo) --Died (37 mo)

HGSC, high-grade serous carcinoma; IGSC, indeterminate-grade serous carcinoma; LGSC, low-grade serous carcinoma; miSBT, micropapillary serous borderline tumor; rec., recurrence; SBT, serous borderline tumor.

Table 2

Histologic, immunophenotypic, and molecular features

Case no.	Tumor component	Nuclear grade	Mitotic rate (per 10 HPF)	Immunophenotype						Mutated cancer driver genes	Fraction of genome altered (%)	Proportion of somatic variants shared/truncal (%)
				Pax8	WT1	ER	PR	p16	p53			
1	SBT (ovary)	1	2	+	+	+	+/-	Het	WT	KRAS	0.62	26
	HGSC (ovary)	3	13	+	+	+	+/-	Het	Abn (cyto)	KRAS, TP53	100 ^c	25
2	SBT (ovary)	1	2	+	+	+/-	+/-	Het	WT	KRAS	14	33
	IGSC (rec)	2	14	+	+	+/-	-	Diff	WT	KRAS	30	26
3	LGSC (ovary)	2	3	+	+	+	+/-	Het	WT	KRAS	100 ^c	18
	LGSC (lymph node)	2	0	+	+	+	+/-	Het	WT	KRAS	37	32
	HG. Ca. (rec) ^a	3	17	+	-	-	-	Het	WT	KRAS	100 ^c	28
4	SBT (ovary)	1	3	+	+	+/-	+	Het	WT ^b	BRAF, TP53	5.9	38
	HGSC (ovary)	3	12	+	+	+/-	+/-	Diff	Abn (Diff.)	BRAF, TP53	28	21
	HGSC (lymph node)	3	14	+	+	+/-	+/-	Diff	Abn (Diff.)	BRAF, TP53	22	29
5	miSBT (ovary)	1	2	+	+	+	+/-	Null	WT	NF1, NF2, ATRX	8.9	33
	IGSC (diaphragm)	2	13	+	+	+	+/-	Null	WT	NF2, ATRX	14	18
	HGSC (rec)	3	22	+	+	-	-	Null	WT	NF2, ATRX, TP53	21	9.4
6	miSBT (ovary)	1	0	+	+	+	+	Het	WT	None	0.10	43
	LGSC (ovary)	1	0	+	+	+	+	Het	WT	None	25	58
	IGSC (rec)	2	23	+	+	+	-	Het	WT	None	32	39
	LGSC (rec)	1	3	+	+	+	-	Null	WT	CDKN2A (del)	27	29
7	LGSC (ovary)	1	0	+	+/-	+	+/-	Het	WT	KRAS, USP9X	82	50
	Undiff. Ca. (rec)	3	3	-	-	-	-	Het	WT	KRAS, USP9X	97 ^c	41

Abn, abnormal expression pattern, (cyto, cytoplasmic; Diff, diffuse); Het, heterogeneous expression; HGSC, high-grade serous carcinoma; IGSC, indeterminate-grade serous carcinoma; HG. Ca., high-grade (Mullerian) carcinoma; LGSC, low-grade serous carcinoma; miSBT, micropapillary serous borderline tumor; rec, recurrence; SBT, serous borderline tumor; Undiff. Ca., undifferentiated (sarcomatoid) carcinoma; WT, wildtype expression pattern. +, positive staining (patchy-to-diffuse, moderate-to-strong intensity); +/-, focal staining (weak; up to moderate intensity in occasional cells); -, negative staining.

^a Additional immunohistochemical stains for GATA3 and TTF1 are negative, ruling out mesonephric differentiation.

^b Scattered foci with diffuse p53 overexpression present.

^c Whole genome duplication present.

Genomic Profiling of Low-Grade Serous Neoplasms and Their Associated Higher-Grade Carcinomas Reveals Relatively Few Shared Mutations

Whole exome sequencing was performed on 19 tumor samples with matched normal tissue from 7 patients. Histologic transformation was typically associated with an increase in genome-wide allelic imbalances (Fig. 1) and copy number alterations (mean FGA of low-grade vs higher-grade: 28% vs 57%, $P = .046$, paired t test; medians: 13% vs 32%, Supplementary Fig. S1). Considering only cases with metachronous presentation of a higher-grade carcinoma (cases 2, 3, 5, 6, and 7), the mean FGA of the tumor at initial diagnosis (calculated from the sample with the highest FGA from each case) was 47% (median: 25%, range: 14%-100%).

Interestingly, there were generally fewer shared mutations between low-grade and higher-grade components than private mutations specific to each component. A median of 26% (range: 13.7%-41%) of mutations in the higher-grade components were also identified in the low-grade components; these truncal mutations included known oncogenic drivers of low-grade serous neoplasms: KRAS (G12D, $n = 4$), BRAF (G469A, $n = 1$), NF2 ($n = 1$), and USP9X ($n = 1$). Accordingly, phylogenetic trees, constructed from somatic mutations and LOH events of each tumor component, had relatively short trunks and long branches, particularly for those cases harboring TP53 genetic alterations (Figs. 2C and 3B, D). Higher-grade tumor components had slightly more private mutations compared with their corresponding low-grade tumor components, although the differences were not significant (median: 28 vs 15, mean: 46 vs 35, $P = .19$, paired t test).

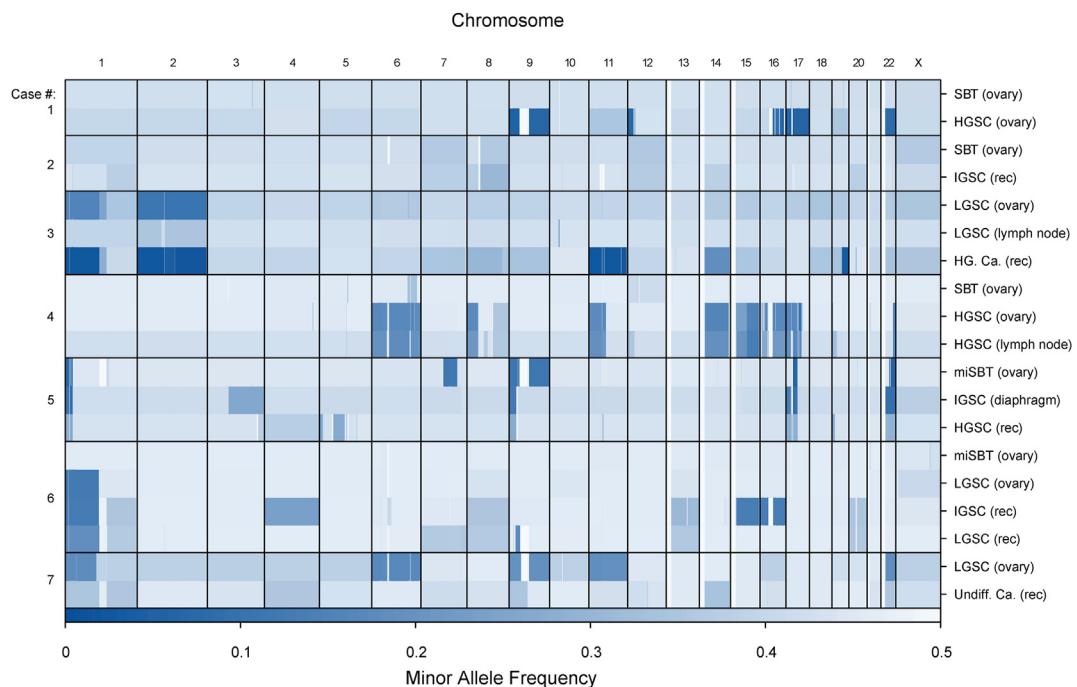
Bi-Allelic TP53 Inactivation is Associated with Transformation to HGSC

A TP53 mutation was identified in 3 cases. Case 1 consisted of separate foci of SBT and HGSC arising from a serous cystadenoma

(Fig. 2A-C). Immunohistochemistry demonstrated aberrant diffuse p53 nuclear and cytoplasmic overexpression in the HGSC, with an abrupt transition to p53-wildtype cystadenoma/borderline tumor epithelium. Molecular analyses demonstrated TP53 mutation and LOH of the remaining allele present only in the HGSC, whereas a KRAS^{G12D} mutation was present in both the SBT and HGSC. Droplet digital PCR also identified the KRAS mutation in the simple epithelium lining the cystadenomatous portion of the neoplasm (Supplementary Fig. S2).

Case 4 was composed of an SBT with areas showing invasive HGSC (Fig. 3A). Although most of the ovarian tumor comprised SBT (>70%), only the HGSC component metastasized to a pelvic lymph node. A heterozygous TP53 mutation was present even in the SBT; loss of the remaining allele was seen only in the HGSC component (Fig. 3B). The SBT exhibited a heterogeneous p53 expression pattern, with scattered foci of diffuse overexpression, whereas the HGSC showed strong, diffuse p53 overexpression throughout. All tumor components harbored a BRAF^{G469A} mutation. Despite HGSC representing only a minor component of the ovarian tumor, it acquired more private mutations (longer branch length) compared with the SBT component, suggesting an elevated mutation rate in the HGSC component. As expected, the lymph node metastasis exhibited a higher genetic resemblance to the ovarian HGSC compared with the SBT component.

In Case 5, in addition to ovarian miSBT, there was concurrent peritoneal involvement by tumor cells showing micropapillary growth (Fig. 3C, D). Compared with the ovarian tumor, metastatic tumor in the diaphragm focally showed increased nuclear atypia (grade 2), with hyperchromatic and rare bizarre nuclei, and increased mitotic activity (reaching 13 per 10 HPF). Given the focality of higher-grade nuclear features, this lesion was classified as IGSC. The patient developed overt HGSC (with grade 3 nuclear atypia diffusely) in an axillary lymph node recurrence 2 years later. Whole exome sequencing analysis of the ovarian

**Figure 1.**

Genome-wide allelic imbalance profiles of low-grade serous neoplasms and associated higher-grade carcinomas. Minor allele frequencies of heterozygous single nucleotide polymorphisms from normal samples were calculated for each case and segmented using the circular binary segmentation algorithm. The mean minor allele frequencies of chromosomal segments are depicted as a heatmap.

tumor and diaphragm lesion at the time of initial diagnosis, and the lymph node recurrence revealed shared *NF2* and *ATRX* mutations across all tumors. The IGSC in the diaphragm harbored single-copy *TP53* loss. *TP53*^{C277Y} mutation affecting the remaining *TP53* allele was only found in the HGSC nodal recurrence. Immunohistochemistry showed a wildtype p53 expression pattern in all tumor samples.

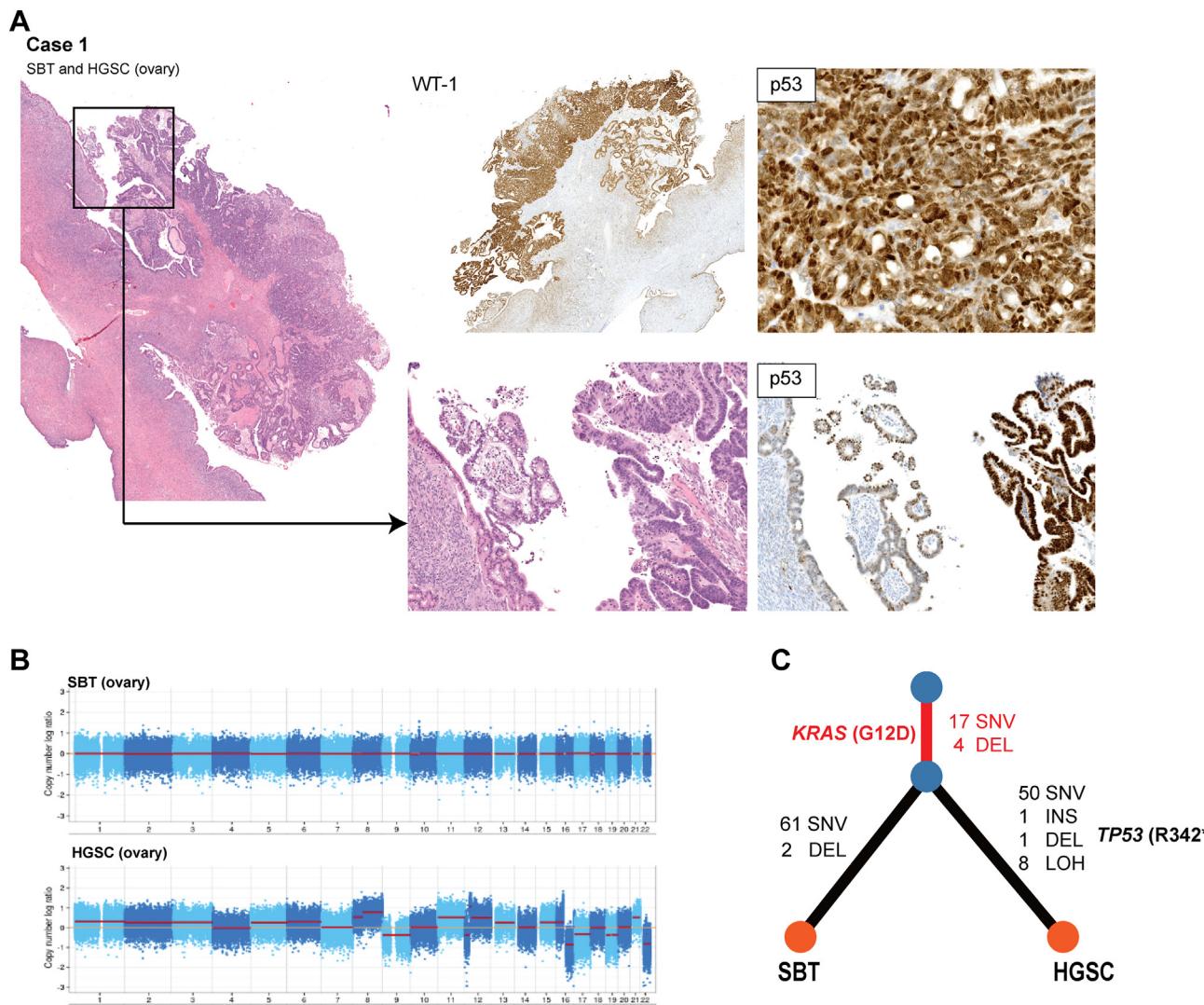
Morphologic and Molecular Heterogeneity of TP53-Wildtype Higher-Grade Carcinomas Derived from Low-Grade Serous Neoplasms

For cases lacking *TP53* mutation, the underlying genetic basis for histologic transformation was not identified. Immunohistochemistry for p53 showed a wildtype expression pattern in these cases. Case 3 was an advanced-stage LGSC at presentation, which recurred as a high-grade Mullerian carcinoma in a pelvic lymph node (Fig. 4A, B). All tumor samples harbored the driver *KRAS*^{G12D} mutation. Whole genome duplication was identified in the high-grade component and the original ovarian LGSC. Notably, a nodal metastasis from the primary resection, comprising carcinoma and adjacent endosalpingiosis, was diploid and had fewer genetic alterations than the ovarian LGSC, suggesting that nodal dissemination occurred early in the course of the disease.

Case 7 was an ovarian LGSC that recurred as a clinically aggressive undifferentiated carcinoma with sarcomatoid features (Fig. 4C, D). The ovarian tumor comprised a typical SBT with areas of LGSC exhibiting a macropapillary invasion pattern. The subsequent undifferentiated carcinoma comprised sheets of spindled and epithelioid cells with abundant eosinophilic cytoplasm, marked nuclear pleomorphism and low mitotic activity, and areas of necrosis. Despite the lack of morphologic or

immunophenotypic resemblance, these tumors shared identical *KRAS*^{G12D} and *USP9X* mutations, consistent with a clonal relationship. Although these tumors harbored relatively few private mutations, the total number of mutations was also low. The extensive differences in their genome-wide LOH (Fig. 1) and copy number profiles (Supplementary Fig. S2), however, support early genetic divergence.

In the recurrent higher-grade tumors from cases 2 and 6, the nuclei were enlarged and crowded with irregular borders but lacked the nuclear pleomorphism and bizarre forms typically encountered in HGSC (Fig. 5). Case 2 was a patient with a remote history of ovarian SBT, who developed a peritoneal recurrence 20 years later (Fig. 5A, B). The recurrent tumor was composed of a complex papillary arrangement of tumor cells with crowded vesicular and hyperchromatic nuclei, prominent nucleoli, and abundant mitoses (14 per 10 HPF). Both primary and recurrent tumors harbored an identical *KRAS*^{G12D} mutation. Genome-wide copy number and allelic imbalance profiles were relatively similar between primary and recurrent tumors (Fig. 1). Case 6 was a patient with advanced-stage LGSC, arising from miSBT, who developed multiple recurrences (Fig. 5C-E). In the first recurrence, the tumor showed nested and solid growth; the nuclei were enlarged, with moderate size variation, and mitotic activity was high (23 per 10 HPF). For both cases 2 and 6, given the moderate/equivocal level of atypia (grade 2), coupled with the high mitotic activity, the recurrent tumors were considered IGSC. Notably, for Case 6, following transformation, a subsequent recurrence developed, which showed mild nuclear atypia and low mitotic activity, consistent with LGSC. Whole exome sequencing was performed on the ovarian miSBT and LGSC in the primary resection specimen, the subsequent IGSC recurrence, and the second LGSC recurrence. There was a relatively high proportion of shared genetic alterations (39% of mutations/LOH events in the IGSC). The copy number profile of the IGSC recurrence showed a few

**Figure 2.**

High-grade serous carcinoma (HGSC) and serous borderline tumor (SBT) arising from ovarian cystadenoma. (A) Photomicrographs from Case 1 illustrate the juxtaposition between SBT and HGSC components. Immunohistochemical stains show WT1 and p53 expression patterns. (B) Copy number profiles of SBT and HGSC components. (C) Phylogenetic tree constructed based on mutations and LOH events. Truncal mutations are highlighted in red and pathogenic driver alterations are indicated. DEL, deletion; INS, insertion; LOH, loss-of-heterozygosity; SNV, single nucleotide variants.

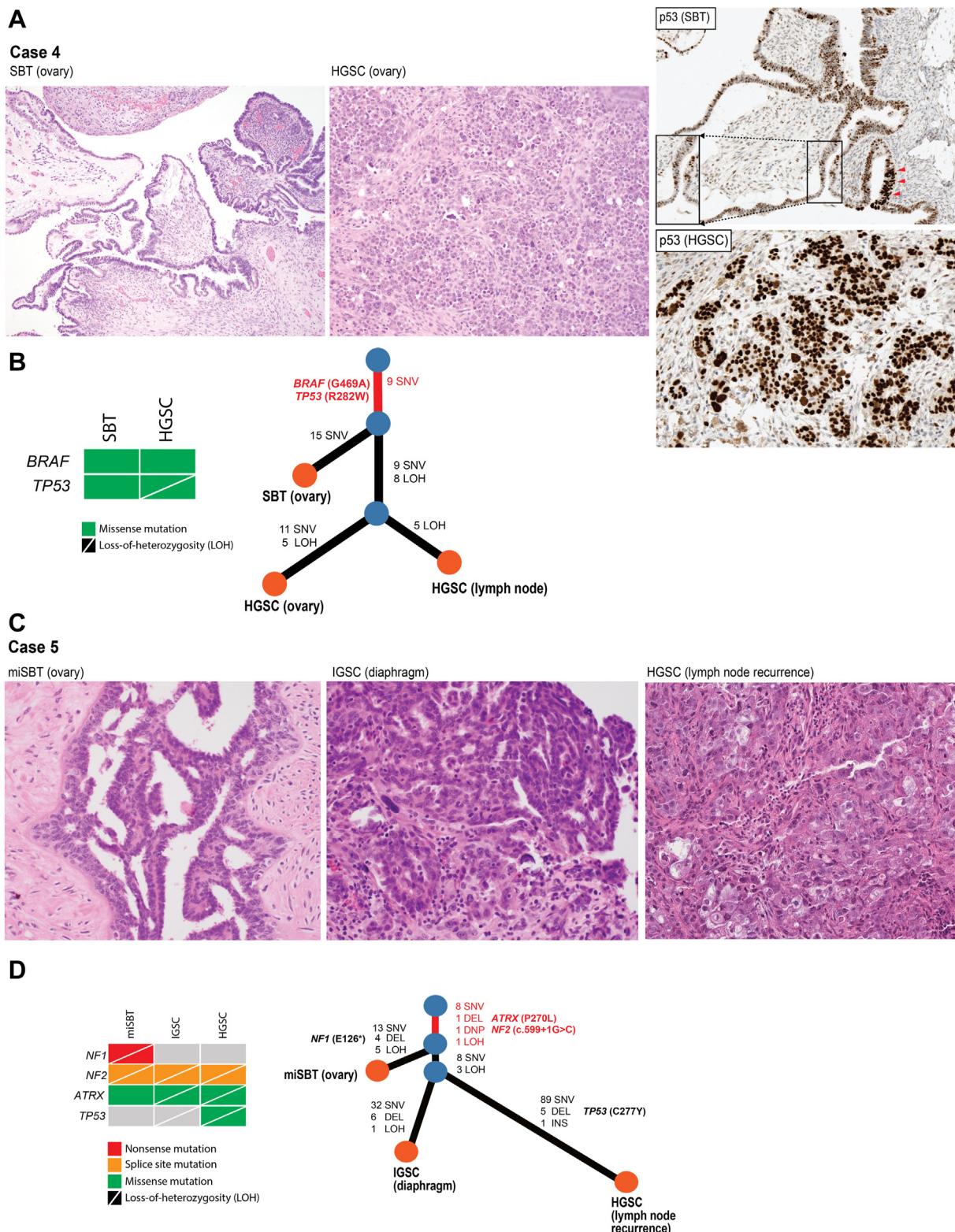
additional gains and losses compared with the LGSC tumors, however overall, the differences were not striking (Fig. 5D). The second (low grade) recurrence acquired a *CDKN2A* deletion, a known driver of progression in LGSC,^{5,6} and reflected by immunohistochemical loss of p16 expression.

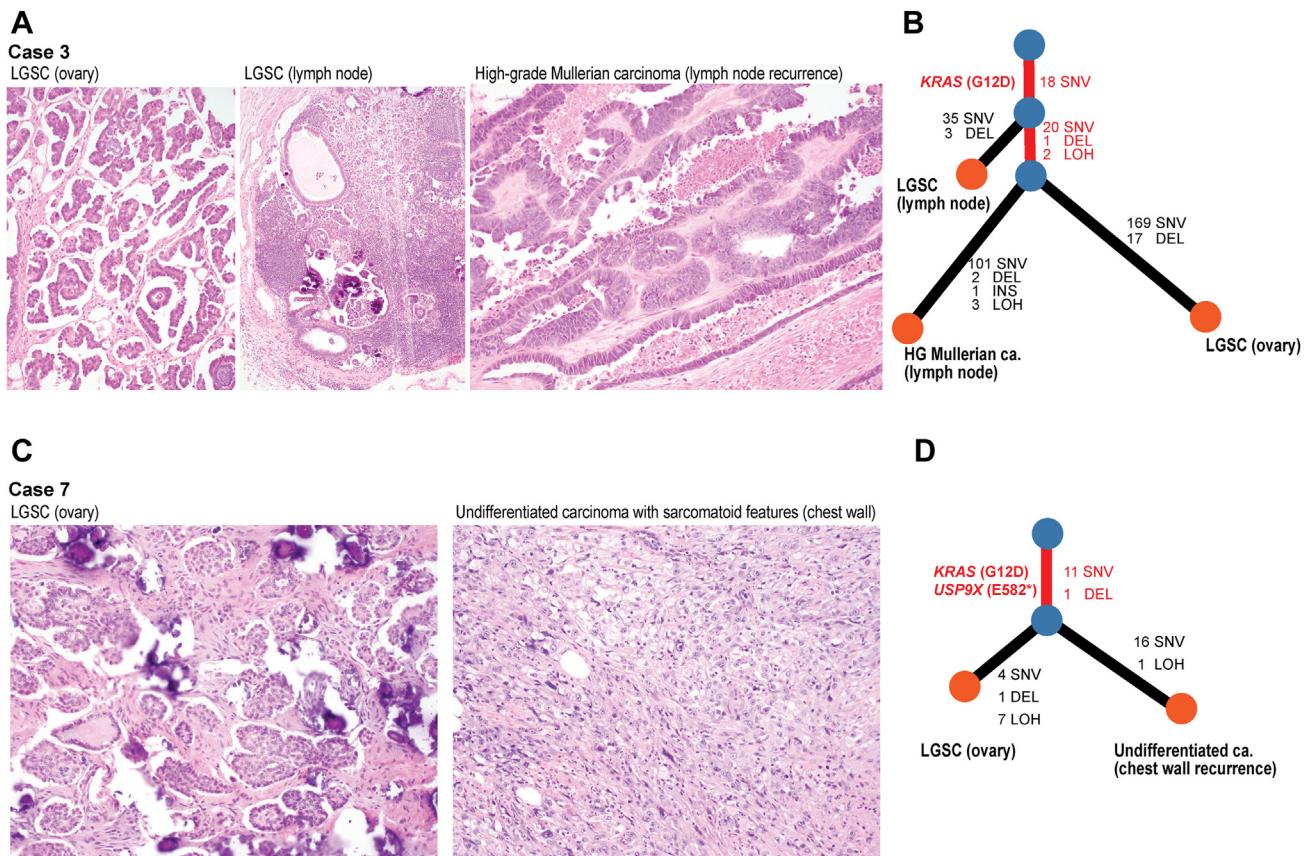
Discussion

The origin of HGSC has puzzled pathologists and researchers for decades, until the identification of fallopian tube epithelium as the site of origin for most cases.^{3,21} However, other mechanisms have also been proposed. Rarely, high-grade carcinomas, including HGSC, evolve directly from an ovarian low-grade serous neoplasm.⁷⁻¹³ This represents an exciting area of research from the perspective of tumor biology, because high-grade and low-grade serous tumors are generally considered distinct entities with independent pathways of pathogenesis.²⁰ The literature on high-grade transformation of low-grade serous neoplasms is

relatively scant, and molecular analyses on these rare cases have, to date, focused on single genes or targeted gene panels^{11,12}; hence, the evolutionary relationships of the low-grade and high-grade components have not been defined. Conventional wisdom postulates that high-grade transformation is probably a late-stage event in the natural history of low-grade serous neoplasia, through the gradual accumulation of genetic damage over time. In the present study, whole exome sequencing of tumor samples from multiple sites at different time points enabled the phylogenetic reconstruction of the evolutionary histories of low-grade serous neoplasms with histologic transformation. In so doing, we provide cogent evidence that high-grade carcinomas often diverge from low-grade serous neoplasms early on in their molecular pathogenesis.

Based primarily on morphologic observations, a stepwise sequence for the development of LGSC has been proposed and involves the progression of benign cystadenoma to SBT, miSBT, and ultimately, invasive LGSC. Although reminiscent of the stepwise model of colorectal carcinogenesis,²² the situation is



**Figure 4.**

TP53-wildtype high-grade carcinomas derived from low-grade serous neoplasms. (A, B) Case 3. (A) Photomicrographs of primary ovarian LGSC, pelvic lymph node metastasis with endosalpingiosis, and recurrent high-grade Mullerian carcinoma. (B) Phylogenetic tree based on mutations and LOH events. (C, D) Case 7. Photomicrographs of primary ovarian LGSC and recurrent undifferentiated carcinoma with sarcomatoid features. (D) Phylogenetic tree based on mutations and LOH events. DEL, deletion; INS, insertion; LGSC, low-grade serous carcinoma; LOH, loss-of-heterozygosity; miSBT, micropapillary serous borderline tumor; SNV, single nucleotide variants.

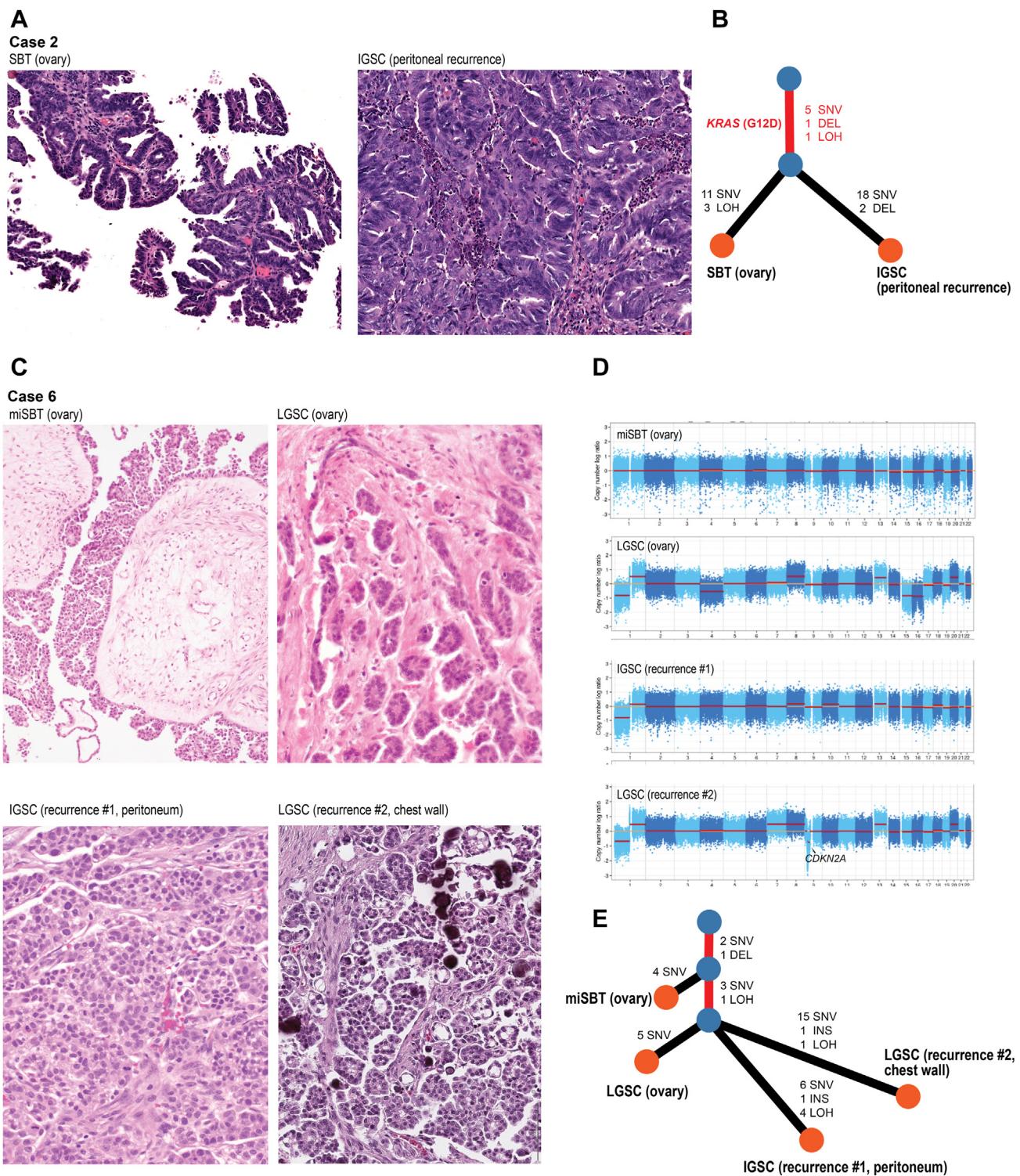
complicated by a peculiar aspect of low-grade serous neoplasia: its propensity for early dissemination, as evidenced by peritoneal implants associated with ovarian SBTs.²³ These implants share an identical *BRAF/KRAS* mutational status as the primary ovarian tumor, suggesting a common origin.²⁴ We have recently shown that even foci of endosalpingiosis, associated with low-grade serous neoplasms, often harbor concordant *BRAF/KRAS* mutations as the ovarian neoplasm, indicating that clonal populations migrate to distant sites even at a preneoplastic stage.¹⁹ In Case 3, LGSC in a lymph node, which harbored private mutations and a lower total number of mutations than the ovarian tumor, was associated with endosalpingiosis, suggesting that tumor cells in the lymph node potentially arose from an endosalpingiotic precursor, rather than representing metastasis from the ovarian LGSC.

The progression of SBT/LGSC to HGSC is a rare phenomenon. Our phylogenetic analyses indicate that this does not represent the culminating step of a linear carcinogenic sequence. Rather, a branching evolution model supports low-grade and high-grade serous carcinogenesis as distinct pathogenic processes. This is well illustrated in Case 1, in which the HGSC was derived from benign cystadenomatous epithelium, which in other areas developed into SBT. The limited number of shared mutations between the low-grade and high-grade components implies that the latter arose from an early subpopulation within the primary tumor, or at an extraovarian site, presumably from endosalpingiosis

or an implant. Even for cases in which the higher-grade carcinoma was only detected at recurrence, the initiating lesion was already present when the ovarian tumor was diagnosed. In Case 2, the time to recurrence exceeded 20 years, suggesting that (pre) neoplastic clones that disseminated early in the course of disease may undergo long periods of dormancy. The factors that trigger these cells to escape from dormancy and form a tumor mass remain an important unresolved question.

Our cases with clinical follow-up provide additional support to prior reports of high-grade transformation of low-grade serous neoplasms being associated with poor prognosis.^{7–10,12} Predicting the potential for high-grade recurrence in an SBT or LGSC at the time of initial diagnosis is therefore an unmet clinical need. We could not discern any distinguishing morphologic features in the low-grade serous tumor components in our cases compared with conventional SBTs or LGSCs, except the diaphragm lesion upgraded to IGSC in Case 5. However, some of the low-grade serous tumor components appeared to exhibit relatively high genomic instability at the copy number level (median FGA of 25%, for cases with metachronous higher-grade carcinoma) compared with conventional LGSCs (median FGA of 16% reported in a recent study).⁶ Further work should therefore evaluate the prognostic impact of FGA, or other measures of chromosomal instability, in low-grade serous neoplasia.

Consistent with prior studies,^{11,12} high-grade transformation was associated with acquisition of *TP53* mutation in some, but not

**Figure 5.**

Recurrent indeterminate-grade serous carcinomas (IGSC) with moderate (grade 2) nuclear atypia and lacking *TP53* mutations. (A, B) Case 2 (A) Photomicrographs of the primary ovarian serous borderline tumor and recurrent IGSC. (B) Phylogenetic tree based on mutations and LOH events. (C-E) Case 6. (C) Photomicrographs of primary ovarian miSBT LGSC, and subsequent recurrent tumors, IGSC and LGSC, respectively. (D) Genome-wide copy number plots for each sample. (E) Phylogenetic tree based on mutations and LOH events. DEL, deletion; INS, insertion; LGSC, low-grade serous carcinoma; LOH, loss-of-heterozygosity; SNV, single nucleotide variants.

all tumors. Bi-allelic inactivation of *TP53*, either simultaneously or sequentially, appeared to be necessary for a complete transition to a high-grade phenotype. In Case 1, both hits must have occurred around the same time, as no intermediate lesion was identified. In

Case 4, a heterozygous *TP53* mutation was present in the SBT, with loss of the remaining allele in the HGSC, although the converse was observed in Case 5: *TP53* LOH was observed in tumor cells that showed concerning morphologic features but fell short of

HGSC, with the acquisition of *TP53* mutation in the full-blown HGSC at recurrence.

Of the 4 *TP53*-wildtype higher-grade tumors, cases 2 and 6 showed only moderate nuclear atypia with high mitotic activity, warranting classification as IGSC, despite the diagnosis of HGSC in the original pathology report. Aside from the high mitotic activity, it is notable that the nuclei, despite showing crowding and enlargement, lacked significant pleomorphism. Notably, a few copy number alterations were observed in Case 6, and there was a relatively high proportion of shared mutations between the primary LGSC and IGSC. The patient also developed subsequent recurrences in the form of LGSC and followed an indolent clinical course. Hence, this IGSC was probably more "LGSC-like" in terms of morphology, molecular features, and clinical behavior, compared with the other cases in our cohort. In contrast, in the IGSC component in Case 5, occasional bizarre nuclei were observed, and this patient subsequently developed an HGSC recurrence.

The diagnostic category of IGSC has previously been proposed by Zarei et al¹⁵ to describe serous carcinomas with morphologic features that fall between LGSC and HGSC. In this study, these tumors had enlarged nuclei with irregularities and overlapping, but lacked overt pleomorphism, and had a mean mitotic rate of 11 per 10 HPF. The majority (67%) were *TP53* wildtype by immunohistochemistry and next-generation sequencing. Although the numbers were small, IGSCs were associated with worse overall survival compared with classic LGSCs, and more similar to classic HGSC. Notably, in a cohort of serous carcinomas with grade 2 atypia reported by Ayhan et al,¹⁶ almost all cases (10/11, 91%) harbored *TP53* mutations. These contrasting studies highlight the intrinsic interobserver variability in the assessment of nuclear atypia. IGSCs likely represent a heterogeneous cohort, with some cases biologically closer to LGSC and others more toward the HGSC end of the spectrum. Further work, potentially involving machine learning approaches, is needed to define the prognostically relevant features in IGSCs.

Several caveats should be considered in the interpretation of our results. It should be clarified that comparisons of the somatic genetic alterations between low-grade and high-grade components do not allow us to make direct inferences relating to chronological time, but only, the sequence of mutational events. Accumulation of genetic alterations likely occurs more rapidly following high-grade transformation, due to increased genomic instability. The number of private mutations between low-grade and higher-grade components was comparable, however (although trending slightly higher in the latter for most cases), suggesting that the impact of high-grade transformation on mutation rate was not pronounced. In contrast, the impact at the copy number level was more appreciable, particularly for those cases with high-grade transformation driven by *TP53* mutation, in keeping with the known association between *TP53* mutation and chromosomal instability.²⁵ Although sequence-level alterations may therefore better serve as a "molecular clock," inferring chronological time would also require accounting for proliferation rate and cell doubling times, which is beyond the scope of the present study. Another caveat is that for most cases, only a single representative sample of the low-grade and/or higher-grade components was sequenced. Analysis of different tumor regions would provide a more comprehensive picture of the degree of heterogeneity within each morphologic component, as observed in cases 3 (LGSC in ovary vs lymph node), 4 (HGSC in ovary vs lymph node), and 6 (miSBT vs LGSC components in ovary). Nevertheless, similar to our observations in Case 4, prior multi-regional genomic analyses of HGSC have demonstrated that the

majority of mutations are often conserved across samples taken from different anatomical sites at the same time point.^{26,27} Samples showing the most markedly different genetic profiles were those that were morphologically distinct, as demonstrated in one of the previously reported cases with mixed HGSC and endometrioid components.²⁶ Finally, as our sample size is small and the cohort is heterogeneous, this study serves mainly as a series of illustrative examples, and a larger cohort is needed to confirm our findings. Correlating morphologic features with the molecular findings and clinical outcomes in a sizable cohort will also be necessary to refine criteria for *TP53*-independent high-grade transformation in low-grade serous neoplasms.

Our study raises important biological and clinical implications. It is widely accepted that high-grade and low-grade serous tumors are different pathologic entities characterized by distinct and independent pathways of molecular pathogenesis. The phenomenon, albeit rare, of HGSCs evolving from low-grade serous neoplasms, has therefore been particularly enigmatic, and somewhat inconvenient for the dualistic model of ovarian serous tumorigenesis.²⁰ Early genetic divergence of low-grade and high-grade tumors provides some reconciliation of this apparent contradiction. Our results refute the idea that high-grade transformation is an end-stage of LGSC progression. Perhaps only normal or "near-normal" tubal-type epithelium, as in cys-tadenomas or early SBTs, could develop into HGSC through *TP53* mutation. With the advances in sensitive molecular techniques, in the future, it may be possible to use liquid biopsy to monitor patients diagnosed with ovarian low-grade serous tumors for *TP53* mutations, enabling early detection of clones that may potentially recur as HGSC.²⁸ Presently although, a practical implication of this study is to emphasize thorough sampling and careful morphologic examination of low-grade serous tumors to exclude high-grade transformation, which may be focal. However, a high threshold for diagnosis of high-grade carcinoma in the setting of a preexisting low-grade serous neoplasm is warranted when p53 alteration cannot be identified by immunohistochemistry or sequencing analysis. Future investigation of other molecular alterations, including epigenetic and gene expression analyses, may be useful to identify drivers of *TP53*-independent high-grade transformation.

Author Contributions

M.H.C. performed study conceptualization and design, data collection, analysis and interpretation, and manuscript writing. Q.S., J.Z., and Y.J. performed bioinformatics analysis. Y.W. performed data collection and interpretation. B.W., T.L.W., R.V., and I.-M.S. performed data interpretation. All authors have read and approved the final paper.

Data Availability

Sequencing data available upon reasonable request to the corresponding author.

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Declaration of Competing Interest

None declared.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Boards of Johns Hopkins Medical Institutions and Memorial Sloan Kettering Cancer Center, waiving the requirement of informed consent. The study was performed in accordance with the Declaration of Helsinki.

Supplementary Material

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