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Original Article

Molecular Evaluation of Low-grade Low-stage Endometrial Cancer With and Without Recurrence

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Summary: Low-grade, low-stage endometrioid carcinomas (LGLS EC) demonstrate 5-yr survival rates up to 95%. However, a small subset of these tumors recur, and little is known about prognostic markers or established mutation profiles associated with recurrence. The goal of the current study was to identify the molecular profiles of the primary carcinomas and the genomic differences between primary tumors and subsequent recurrences. Four cases of LGLS EC with recurrence and 8 cases without recurrence were evaluated via whole-exome sequencing. Three of the 4 recurrent tumors were evaluated via Oncomine Comprehensive Assay. The resulting molecular profiles of the primary and recurrent tumors were compared. Two of the 3 recurrent cases showed additional mutations in the recurrence. One recurrent tumor included an additional *TP53* mutation and the other recurrent tumor showed *POLE* and *DDR2* kinase gene mutation. The *POLE* mutation occurred outside the exonuclease domain. *PIK3CA* mutations were detected in 4 of 4 primary LGLS EC with recurrence and in 3 of 8 disease-free cases. LGLS EC with recurrence showed higher MSIsensor scores compared with LGLS without recurrence. The level of copy number gains in LGLS EC with recurrence was larger than LGLS EC without recurrence. This pilot study showed 1 of 3 recurrent cases gained a mutation associated with genetic instability (*TP53*) and 1 of them also acquired a mutation in the DDR2 kinase, a potential therapeutic target. We also noted a higher level of copy number gains, MSIsensor scores and *PIK3CA* mutations in the primary tumors that later recurred. **Key Words:** Low-grade low-stage endometrioid carcinoma—Recurrence—Whole exome sequencing—Microsatellite instability score—Copy number analysis.

Uterine cancer is the most common gynecologic cancer in developed countries and was the second most prevalent cancer among females in 2019 with over 60,000 new cases in the United States (1,2). It is estimated that by 2030, uterine cancer will be the third most common cancer among women, with a 2.9% average increase in incidence per year (3). Further, mortality rates have been increasing since 2000 (4). Endometrial cancer (EC) is the most common type of uterine cancer, accounting for > 90% of cases (1).

Despite these alarming statistics, the clinical approach to EC has remained largely stagnant, relying on a dualistic model developed over 30 yr ago (5). In this model, there are 2 defined subtypes of EC: type I, which is low grade, with endometrioid histology, hormone receptor positive and good prognosis, and type II, which is high grade, with nonendometrioid histology, often hormone receptor negative and has a poor prognosis (6). Most cases of EC are type 1 and can be defined as low-grade, low-stage endometrioid carcinomas (LGLS EC). These are treated primarily with surgery and the 5-yr survival rate is 95% (2). Current risk stratification systems utilize clinicopathologic information such as age, depth of myometrial invasion, histologic grade, and lymphovascular space involvement to define subgroups of women who are at high risk for recurrence (7–10). However, despite the development of multiple different stratification methods, predicting which patients with LGLS EC will eventually progress remains inaccurate and elusive (11,12). It is therefore imperative to determine the factors that predispose the small subgroup of patients with LGLS EC to behave more aggressively, which will inform the need for adjuvant therapy or more extensive initial intervention.

In the evolving age of molecular and genomic tumor characterization, there is promise in more precisely defining prognostic subtypes of EC. Data analyzed from the Cancer Genome Atlas (TCGA) revealed 4 distinct molecular subtypes of EC with different prognostic outcomes: microsatellite-instability, POLE ultra-mutated, copy-number low, and copy-number high (13). These findings have been replicated and demonstrate the immense potential for the use of molecular data in clinical risk stratification. Recent studies have suggested that the use of both molecular and clinicopathologic factors results in improved risk assessment and ability to predict outcomes (14–18).

Molecular analysis has uncovered a number of possible prognostic markers to help distinguish women in the LGLS subgroup who are at high risk of recurrence. It has been found that, among women with LGLS EC, mutations in *CTNNB1* are associated with worse recurrence-free survival and overall survival (19–22). Further, women with *CTNNB1*-mutated tumors tend to have otherwise favorable characteristics such as young age, low tumor grade, less myometrial invasion and lower incidence of lymphatic and vascular invasion (21). In addition, a combination of high expression of NF-YA, a transcription factor involved in activating genes associated with growth promotion, and low expression of lamin-A, a protein associated with cell differentiation, have been noted as potential markers of tumor aggressiveness in LGLS EC (23). Low expression of ASRGL1 protein has also been found to have prognostic impact on survival in EC (24). Finally, while mutated in only a very small proportion of LGLS EC, aberrations in p53 are associated with worse recurrence-free survival (21), though this is a more complex issue. Among heterogeneous populations of EC with both low and high-grade tumors, it has been noted that *TP53*, along with *PIK3CA*, *PTEN*, and *PPP2R1A*, are likely early drivers of primary oncogenesis. However, among LGLS EC specifically, it remains unclear if mutations in *TP53* are driving events in recurrence and metastasis or just synergistic with other molecular events underlying this process (25). Thus, the pathways involved in the process of tumor progression and recurrence in LGLS EC remain incompletely understood.

In this study, we analyzed the primary tumor and subsequent recurrence of LGLS ECs with the goal of determining molecular and genetic profiles of each, in the hopes of illuminating potential genetic risk factors for recurrence as well as pathways of progression.

MATERIALS AND METHODS

Case Selection and Pathologic Examination

The study was performed under an institutional review board—approved protocol (WCM IRB# 1007011157). Twelve cases of LGLS endometrial endometrioid carcinoma cases were identified in our surgical pathology archives, including 4 cases with recurrence and 8 controls without recurrence matched for age, histology, and body mass index. Tumor morphology was reviewed by 2 pathologists with expertise in gynecologic pathology (L.H.E. and C.E.M.). For this study, low grade was defined as grade 1 and low stage was defined as stage 1a at diagnosis with comprehensive staging. Clinicopathologic data can be found in Table 1.

Whole-exome Sequencing (WES)

WES was performed on the patient tumor/matched germline DNA pair using Illumina HiSEquation 2500 Rapid Run Mode (2×101 bp; Illumina, San Diego,

TABLE 1. *Clinicopathologic and molecular characteristics*

Case	Age (yr)	Diagnosis	FIGO stage	Clinical characteristics	Treatment	Clinical follow-up	Site of recurrence	Time to recurrence	Molecular findings
1	56	G1 EC Noninvasive	1A	BMI: 27.8 No HRT Family history of ovarian cancer	Initial: robotic total hysterectomy/BSO/LND Recurrence: surgical resection, chemoradiation also s/p subsequent adjuvant chemotherapy for a second primary peritoneal cancer	NED after recurrence and second primary	Midline pelvic mass	~1 yr (12 mo)	PTEN mut, PIK3CA mut, MYC mut*, 1q gain
2	69	G1 EC 30% invasive	1A	BMI: 38.5 No HRT	Initial: total abdominal hysterectomy/BSO/LND, vaginal cuff brachytherapy 1st Recurrence: surgical resection followed by radiation and antihormonal therapy 2nd Recurrence: surgical resection	Lost to follow-up 2015 after second recurrence	(1) Supraclavicular lymph node (2) Posterior neck mass	3 yr (~37 mo) 7.5 yr (~4.5 from first recurrence)	PTEN mut, PIK3CA mut, ARID1A mut, ATM mut, TP53 mut†, 8p deletion, 8q gain, 18p gain, 18q gain
3	54	G1 EC 12% invasive	1A	BMI: 21.5 History of HRT	Initial: robotic total hysterectomy/BSO/LND, adjuvant vaginal brachytherapy Recurrence: opted for no further therapy, inpatient hospice	Died of disease 5/2013	Pelvic mass	~5 yr (58 mo)	PTEN mut, PIK3CA mut, ARID1A mut, EGFR mut
4	80	G1 EC 15% invasive	1A	Obese (BMI unknown) No HRT	Initial: total abdominal hysterectomy/BSO/LND/appendectomy Recurrence-pelvic radiation and vaginal cuff brachytherapy	NED after recurrence Died of medical causes 2019	Pelvis/vagina	~5.5 yr (67 mo)	PTEN mut, PIK3CA mut, DDR2 mut, 1q gain
5	59	G1 EC Noninvasive	1A	BMI: 34.5 No HRT	Robotic total hysterectomy/BSO/LND	NED for 9 yr (113 mo) after surgery	None	NA	KIT mut
6	59	G1 EC 6% invasive	1A	BMI: 28.3 No HRT	Robotic total hysterectomy/BSO/LND	NED at 2 yr (22 mo) then lost to follow-up	None	NA	PTEN mut, PIK3CA mut, ARID1A mut
7	63	G1 EC Noninvasive	1A	BMI: 25.8 No HRT	Robotic total hysterectomy/BSO/LND	NED for 11 yr (135 mo) after surgery	None	NA	PIK3CA mut, ARID1A mut
8	67	G1 EC 33% invasive	1A	BMI: 23 No HRT	Robotic total hysterectomy/BSO/LND; declined vaginal brachytherapy	NED for 9 yr (113 mo) after surgery	None	N/A	KIT mut
9	53	G1 EC Noninvasive	1A	BMI: 26.9 No HRT	Robotic total hysterectomy/BSO/LND	NED for 10 yr (122 mo) after surgery	None	NA	PTEN mut, 19p gain, 21q gain
10	52	G1 EC Noninvasive	1A	BMI: 30.5 No HRT	Total laparoscopic hysterectomy/BSO/LND	NED for 10 yr (120 mo) after surgery	None	NA	PIK3CA mut, ARID1A mut, POLE mut, TMB high

11	79	G1 EC 7% invasive	1A	BMI: 35 No HRT	Robotic total hysterectomy/BSO/LND	NED at 3 yr (38 mo) then lost to follow-up	NA	None	PTEN mut, ARID1A mut
12	75	G1 EC 21% invasive	1A	BMI: 20 No HRT	Total laparoscopic hysterectomy/BSO/LND	NED for 11 yr (133 mo) after surgery	NA	None	PTEN mut, ARID1A mut

*Denotes findings identified in primary only.
†Denotes findings identified in metastasis only.
BMI indicates body mass index; BSO, bilateral salpingo-oophorectomy; EC, endometrioid carcinoma; G1, grade 1; HRT, hormone replacement therapy; LND, lymph node dissection; mut, mutation; NA, not available; NED, no evidence of disease; OSH, outside hospital; PFS, progression-free survival; TMB, tumor mutational burden.

CA), as detailed in previously described protocols (26). Our clinical-grade WES test Exome Cancer Test Version 1 (EXaCT-1) has been approved by New York State Department of Health (ID# 43032), and has been described in detail in Rennert et al. (27). This approach allows for assessment of > 21,000 genes, through the development and implementation of novel computational approaches for simultaneous detection of somatic point and indel mutations, copy-number variants, tumor mutational burden, and microsatellite instability (MSI) status. WES alterations were categorized based on their actionability and their clinical or biologic relevance. Alterations in 49 actionable or clinically significant genes were reported within "Category 1," alterations in 508 known cancer-associated genes within "Category 2," and somatic alterations of unknown significance within "Category 3." Tumor mutation burden (TMB) was calculated for each sample as the number of mutations divided by the number of bases in the coverage space per million (26,28).

Calculating MSI by MSIsensor

MSI was detected by MSIsensor, a software tool that quantifies MSI in paired tumor-normal genome sequencing data and reports the somatic status of corresponding microsatellite sites in the human genome (29). MSIsensor score was calculated by dividing the number of microsatellite unstable by the total number of microsatellite stable sites detected.

Oncomine Comprehensive Assay

Previously reported tumor DNA and RNA extraction protocols and quantitation assays were followed (30). The Oncomine Comprehensive Assay version 3 (OCAv3) (ThermoFisher Scientific) was performed on the IonTorrent_S5 XL platform, following manufacturer protocols. Positive controls (Horizon Discovery) were used in each run. OCAv3 is an amplicon-based, targeted assay that enables the detection of relevant SNVs, amplifications, gene fusions, and indels from 161 unique genes (Table 2).

Detection of Somatic Copy Number Alterations (SCNA)

Copy number scores for matched tumor normal pairs in this cohort were calculated using CNVseeqer (31) as implemented in the EXaCT1 V0.9 pipeline (27). Capture regions with a total coverage <100 reads in both the tumor sample and matched control sample are filtered out. For SCNA, read counts are normalized in both the tumor sample and the matched control sample by the

TABLE 2. *List of the genes covered by the oncomine comprehensive assay (OCAv3)*

Hotspot genes				Full length genes		Copy number genes		Gene fusions (intergenic and intragenic)	
AKT1	FGFR1	KRAS	PIK3CA	ARID1A	NOTCH2	AKT1	FGFR3	AKT2	MYB
AKT2	FGFR2	MAGOH	PIK3CB	ATM	NOTCH3	AKT2	FGFR4	ALK	MYBL1
AKT3	FGFR3	MAP2K1	PPP2R1A	ATR	PALB2	AKT3	FLT3	AR	NF1
ALK	FGFR4	MAP2K2	PTPN11	ATRX	PIK3R1	ALK	IGF1R	AXL	NOTCH1
ARAF	FLT3	MAP2K4	RAC1	BAP1	PMS2	AR	KIT	BRAF	NOTCH4
AXL	FOXL2	MAPK1	RAF1	BRCA1	POLE	AXL	KRAS	BRCA1	NRG
BRAF	GATA2	MAX	RET	BRCA2	PTCH1	BRAF	MDM2	BRCA2	NTRK1
BTK	GNA11	MDM4	RHEB	CDKN2A	PTEN	CCND1	MDM4	CDKN2A	NTRK2
CBL	GNAQ	MED12	RHOA	CDK12	RAD50	CCND2	MET	EGFR	NTRK3
CCND1	GNAS	MET	ROS1	CDKN1B	RAD51	CCND3	MYC	ERBB2	NUTM1
CDK4	HIST1h3B	MTOR	SF3B1	CDKN2B	RAD51B	CCNE1	MYCL	ERBB4	PDGFRA
CDK6	HNF1A	MYC	SMAD4	CHEK1	RAD51C	CDK2	MYCN	ERG	PDGFRB
CHEK2	HRAS	MYCN	SMO	CREBBP	RAD51D	CDK4	NTRK1	ESR1	PIK3CA
CSF1R	H3F3A	MYD88	SPOP	FANCA	RB1	CDK6	NTRK2	ETV1	PRKACB
CTNNB1	IDH1	NFE2L2	SRC	FANCD2	RNF43	CDKN2A	NTRK3	ETV4	PPARG
DDR2	IDH2	NRAS	STAT3	FBXW7	SETD2	CDKN2B	PDGFRA	ETV5	PTEN
EGFR	JAK1	NTRK1	TERT	MLH1	SLX4	EGFR	PDGFRB	FGFR1	RAD51B
ERBB2	JAK2	PDGFRA	TOP1	MRE11A	SMARCA4	ERB2	PIK3CA	FGFR2	RAF1
ERBB3	JAK3	PDGFRB	U2AF1	MSH2	SMARCB1	ESR1	PIK3CB	FGFR3	RB1
ERBB4	KDR		XPO1	MSH6	STK11	FGF19	PPARG	FGR	RELA
ERCC2	KIT			NBN	TP53	FGF3	RICTOR	FLT3	RET
ESR1	KNSTRN			NF1	TSC1	FGFR1	TERT	JAK2	ROS1
EZH2				NF2	TSC2	FGFR2	TSC1	KRAS	RSPO2
				NOTCH1			TSC2	MDM4	RSPO3
								MET	TERT

total number of reads aligned in the tumor sample and the matched control sample, respectively. Then the ratio of the normalized read counts in the tumor sample and the normalized read count in the control sample is calculated. These capture regions are then ordered karyotypically and sorted by genomic coordinates according to the log2 value of the ratio of normalized read counts of the tumor sample and control sample. The normalized ratios of these bins were segmented using the Circular Binary Segmentation algorithm implemented in the R package DNAcopy (32). The algorithm outputs segments where every capture region found within these segments is represented by the same log2 value. This log2 value indicates whether the segment has DNA copy number gain (amplification) or DNA copy number loss (deletion). A negative log2 would suggest a segment was deleted and a positive value would suggest a segment is amplified. Segments with a log2 value > 0.5 to are amplified and segments with a log2 value < −0.5 are categorized as deleted. We then took the segments called by the algorithm and with a custom script annotated these segments by RefSeq genes whose transcription start and end sites overlap with the genomic coordinates assigned to these segments. Copy number alteration (CNA) burden was calculated as the percentage of the cancer genome showing copy number changes according to a previously reported method (33).

Correcting SCNA for Tumor Purity

The copy number scores were then adjusted for ploidy and purity corrections using CLONET (34). In tumor samples where purity could not be estimated by CLONET, we used pathology estimates of tumor purity. Copy number scores that could not be adjusted by CLONET were adjusted by the following formula

Computing SCNA by Chromosomal Arm

We binned the hg19/b37 genome into chromosomal arms. Genomic coordinates for the p (short) arm and q (long) arm, for each chromosome is obtained using UCSC cytoband file (http://hgdownload.cse.ucsc.edu/goldenpath/hg19/database/cytoBand.txt.gz). Using the segment calls generated by CNVseeqer, log2 scores associated with each segment were corrected using the previously described method above. The corrected segmented calls were binned into the chromosomal arm based on the genomic coordinates using a custom script. To compute the SCNA burden for each arm, the fraction of bases altered was calculated based on log2 threshold (> 0.5 for amplification and < −0.5 for deletion).

Sanger Sequencing

For genes of interest, PCR was performed with appropriate human gDNA control samples using custom PCR primers designed to amplify short (~200–400 bp) regions in FFPE samples, as previously described (30).
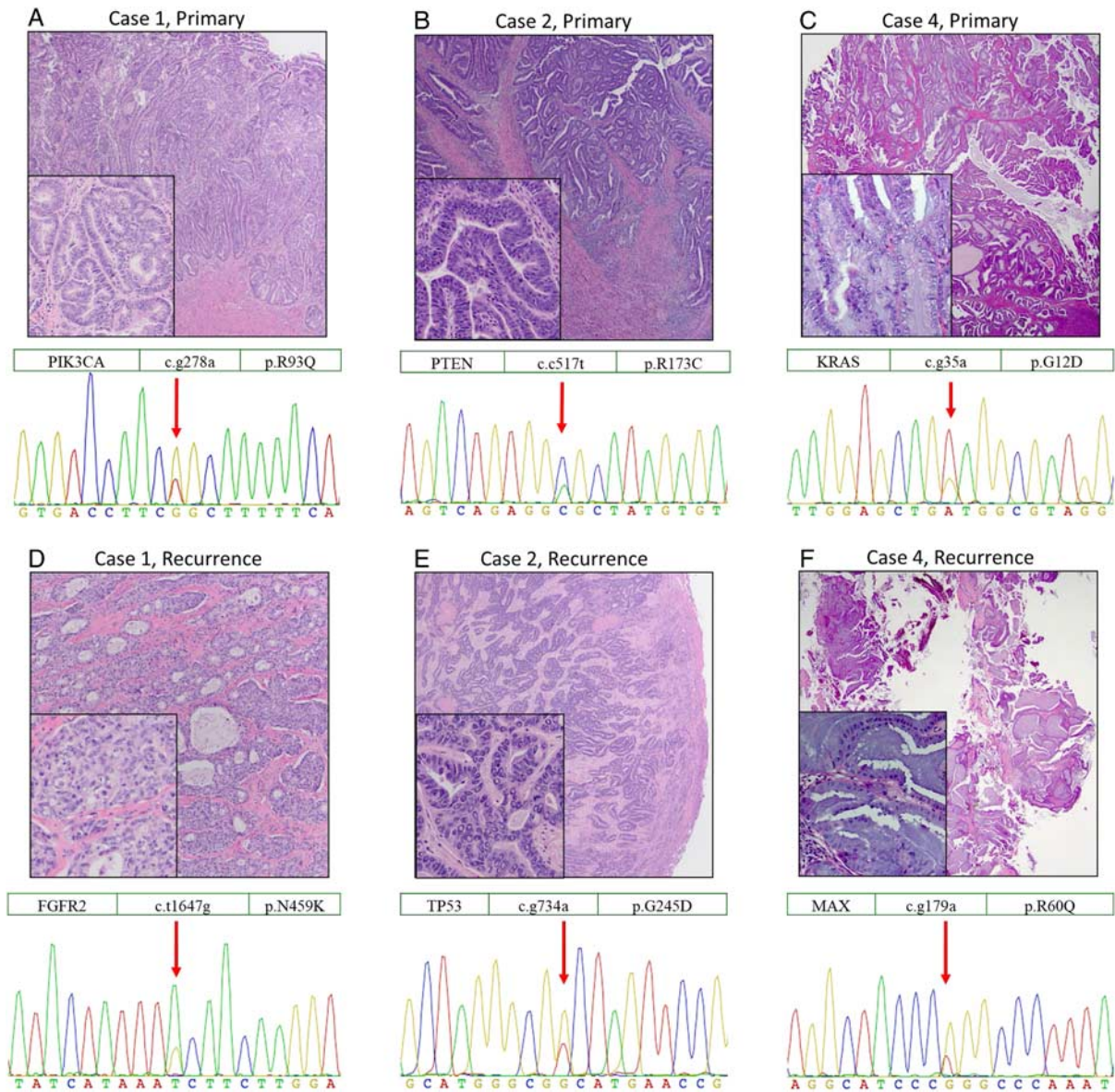
FIG. 1. Histopathology of low-grade low-stage endometrioid carcinoma with recurrence. Top row corresponds to primary tumors and bottom row corresponds to recurrences, represented by low and high (insets) magnification images. (A) Noninvasive well-differentiated tumor (case 1) and (D) recurrence in pelvis, 2 yr later. Sanger sequencing confirmed *PIK3CA* and *FGFR2* mutations. (B) Invasive (30% myometrial thickness) well-differentiated tumor (case 2) and (E) recurrence in right posterior neck (frozen material), 8 yr later. Sanger sequencing confirmed *PTEN* and *TP53* mutations. (C) Invasive (15% myometrial thickness) well-differentiated tumor with mucinous features (case 4) and (F) recurrence in vagina, 6 yr later. Sanger sequencing confirmed *KRAS* and *MAX* mutations.

Data analysis was performed with DNASTAR Laser-gene12 software and the threshold for SNP detection was set to 10%. Mutations from the reference sequence were called whenever sequence quality and coverage allowed.

RESULTS
Summary of Clinical Characteristics

Detailed clinicopathologic characteristics are summarized in Table 1. Four cases of LGLS EC with recurrence (including primary tumor and recurrence) and 8 cases without recurrence were identified. Sites of recurrence included the pelvis, vagina, lymph node, and neck. One patient (case 2) received adjuvant vaginal brachytherapy following their initial surgery (performed prior to updated 2009 FIGO staging for EC). Follow-up was available for all patients with a mean of 114 mo (range: 24–180 mo). An average observational period for cases of LGLS EC without recurrence was 100 mo
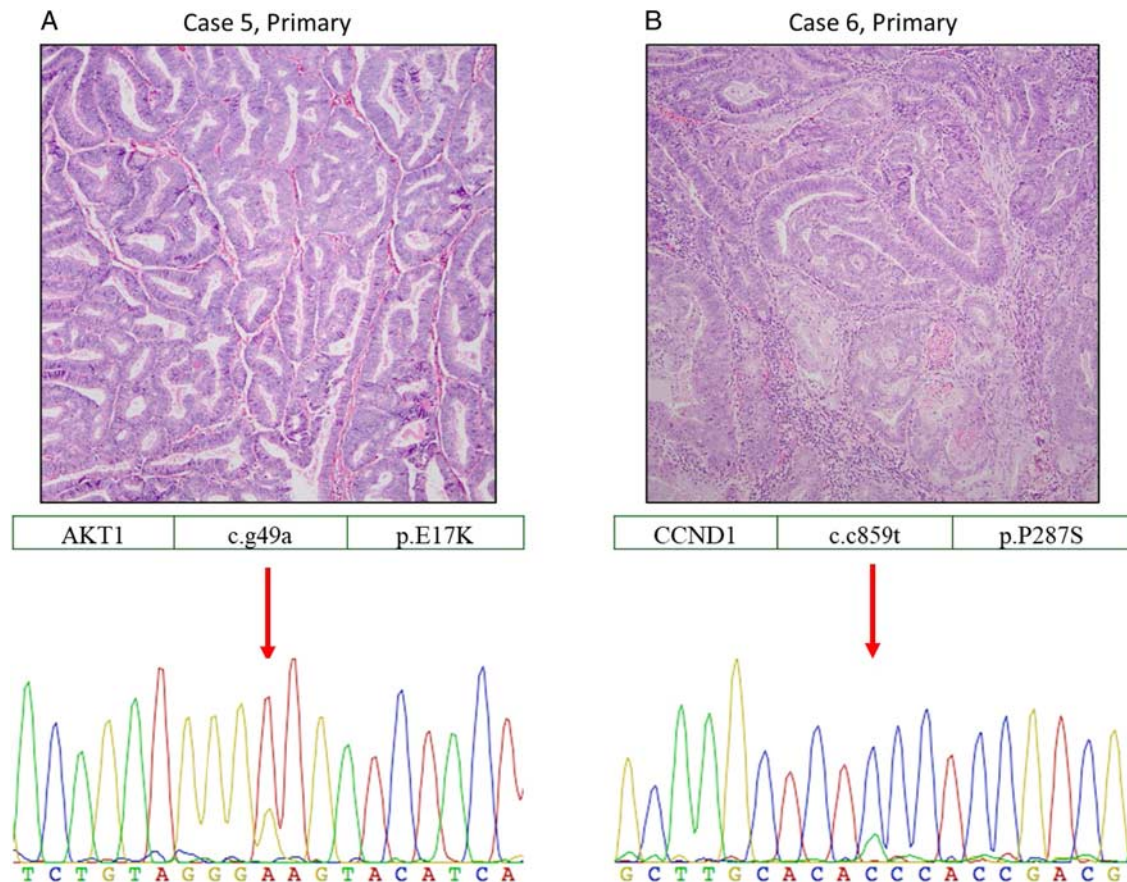
FIG. 2. Histopathology of low-grade low-stage endometrioid carcinoma without recurrence. (A) Noninvasive, well-differentiated tumor (case 5). Sanger sequencing confirmed *AKT1* mutation. (B) Invasive (33% of myometrium) well-differentiated tumor (case 8). Sanger sequencing confirmed *CCND1* mutation.

(range: 24–135 mo). All patients were postmenopausal with an average age of 67 yr.

Histopathology

All available hematoxylin and eosin (H&E) slides from each case were reviewed by 2 pathologists with expertise in Gynecologic Pathology (L.H.E. and C.E.M.). Cases were assessed for classic morphology and all diagnoses were confirmed. All cases displayed characteristic features of well-differentiated grade 1 endometrioid carcinoma, including glandular configuration, lack of significant solid growth, smooth luminal borders, fencepost nuclei, and mild to moderate nuclear atypia at most. Case 4 showed focal mucinous differentiation. Histologic features can be seen in Figures 1 and 2. Although the cytologic atypia in the recurrence for case 2 was slightly higher than that in the primary, all the recurrent tumors also displayed low-grade architectural and cytologic features.

Genomic Landscape of LGLS EC With Recurrence and Without Recurrence

The 12 primary LGLS EC underwent WES and Oncomine, and 3 of 4 recurrent tumors were interrogated by Oncomine (Fig. 3). The median coverage was 89.8× and 91.2×, respectively, by WES for tumor and germline samples and 1362× by OCAv3. Exclusion of the fourth case was based upon insufficient material. Two of the 3 recurrent cases showed additional mutations in the recurrence.

The most commonly observed genomic mutations were *PTEN* (73.3%), *PIK3CA* (66.7%), and *ARID1A* (53.3%), which were consistent with previously reported results from TCGA (13). We found no significant difference in the prevalence of mutations between LGLS EC with recurrence and without recurrence. Comparing the mutations between primary LGLS EC and the matched recurrent tumors, we identified an additional loss-of-function *TP53* mutation (p.Gly245Asp) in case 2 and *POLE*
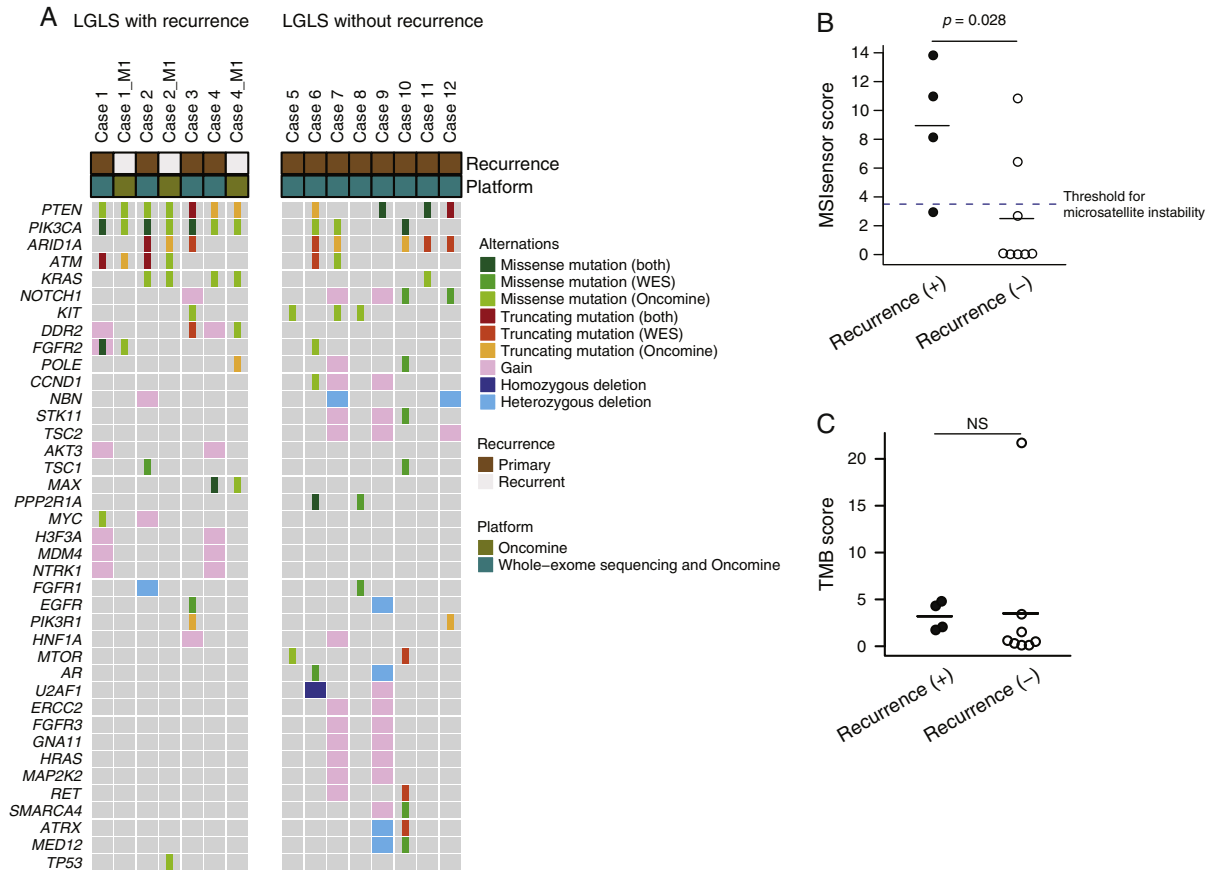
FIG. 3. Genomic landscape of low-grade low-stage endometrioid carcinoma (LGLS EC) with and without recurrence. (A) The oncoprint shows cancer genes that were altered by mutation and/or copy number change in at least 2 tumor samples, except for *TP53*. Mutations of unknown significance are not included. Each column corresponds to 1 tumor sample. (B and C) MSIsensor and TMB scores in LGLS EC with and without recurrence. A blue dashed line represents a threshold for microsatellite instability. Black lines represent averages of the scores in each group. NS indicates not significant; TMB, tumor mutational burden; WES, whole-exome sequencing.

nonsense mutation (p.Glu1951Ter) and a receptor tyrosine kinase *DDR2* mutation (p.Leu239Met) in case 4. The identified *POLE* mutation occurred outside the proofreading domain (residue 86–426). The *DDR2* mutation is predicted to be damaging by PolyPhen-2 (35). The recurrent tumor from case 1 did not accrue additional mutations. Case 3 recurrence had insufficient material for sequencing. *PIK3CA* mutations were detected in 4 of 4 primary LGLS EC with recurrence and in 3 of 8 disease-free cases.

Secondly, we examined WES data from primary tumors to determine whether MSI status and TMB of LGLS EC with recurrence differ from those without recurrence. Three LGLS EC with recurrence and 2 without recurrence were MSI high (Fig. 3B). MSIsensor scores in LGLS EC with recurrence were significantly higher than those without recurrence. TMB in one LGLS EC without recurrence, which harbored a *POLE* mutation (p.Ser1380Leu, case 10),

was notably high (Fig. 3C). There was no statistically significant difference in TMB between LGLS EC with recurrence and without recurrence.

We found no germline mutations in MSI-related or cancer-related genes in cases with LGLS EC with recurrence, while *AKT1* variants (p.Glu17Lys) were identified in case 5 and *APC* nonsense mutation (p. Arg876*) in case 10.

Somatic Copy Number Alterations in LGLS EC With Recurrence and Without Recurrence

SCNAs were assessed for all primary LGLS EC using WES data. The SCNA landscape revealed arm-level broad copy number alterations in 3 LGLS EC with recurrence (Fig. 4A). We observed 1q gain in 2 LGLS EC with recurrence (cases 1 and 4), and 8p deletion, 8q gain, 18p, and 18q gain in 1 case with recurrence (case 2). Arm-level CNA analysis confirmed these alterations and additionally revealed
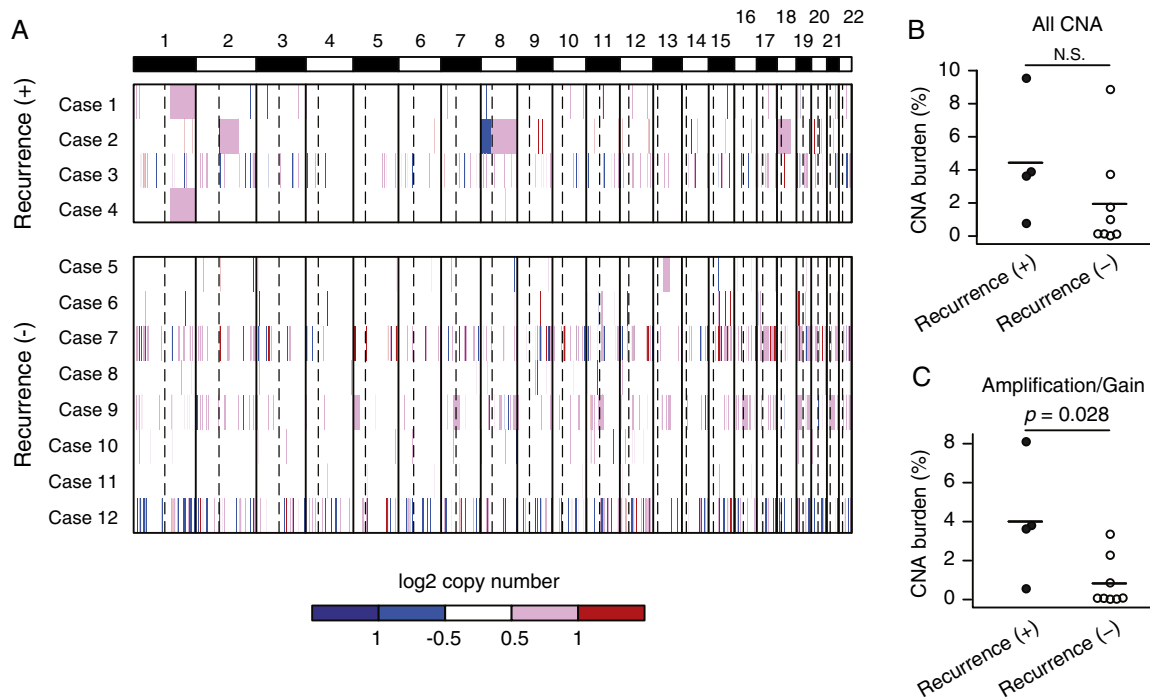
FIG. 4. Comparison of somatic copy number alterations (SCNA) between tumors with and without recurrence. (A) Overall picture of SCNA in low-grade low-stage endometrioid carcinoma (LGLS EC) with and without recurrence based on whole-exome sequencing data; dark blue, homologous deletion; light blue, heterologous deletion; pink, gain; red, amplification. Each row corresponds to 1 LGLS EC. Dashed lines represent borders between short arms and long arms in each chromosome. (B) Comparison of CNA burden. CNA burden represents the level of copy number gain and loss. Black lines represent averages of the scores in each group. (C) Comparison of CNA burden values, which are restricted to copy number gain. Black lines represent averages of the scores in each group. CAN indicates copy number alteration; NS, not significant.

arm-level alterations in 19p and 21q in 1 LGLS EC without recurrence (case 9). CNA burden, which represents the level of copy number gains and losses across the genome was similar between LGLS EC with and without recurrence (Fig. 4B). When CNA burden was restricted to regions showing gains, CNA burden in LGLC EC with recurrence was significantly higher than those without recurrence (Fig. 4C).

Sanger Sequencing

Sanger sequencing confirmed a selection of mutations in representative cases: *PIK3CA* in case 1 primary, *FGFR2* in case 1 recurrence, *PTEN* in case 2 primary, *TP53* in case 2 recurrence, *KRAS* in case 4 primary and *MAX* in case 4 recurrence, *AKT1* on case 5.

DISCUSSION

Although LGLS EC has a 95% 5 yr survival, those patients who recur have a poor prognosis and limited treatment options (5,19,36,37). The underlying cause as to why certain tumors recur despite a lack of histologic or clinical evidence portending aggressive

behavior has not yet been elucidated. Our data support that a subset of LGLS EC may be genetically predisposed to recurrence. In this pilot study, 3 of 4 recurrent cases showed segments affected by copy number gain or amplification and the MSIsensor scores were significantly higher than non-recurrent cases, with the exception of 1 case with an extremely high tumor mutation rate and *POLE* mutation (801 counts of nonsynonyous mutations, case 10). Recurrent cases also showed higher TMB burden than nonrecurrent cases, again with the exception of case 10. All recurrent cases also showed *PIK3CA* mutation (Fig. 1) while only 3 of 8 controls did. In addition, 2 of 3 recurrent cases gained a mutation associated with genetic instability (*TP53* and *POLE*) and 1 case acquired a mutation in *DDR2,* a gene encoding a receptor tyrosine kinase which is a potential therapeutic target, results which may have important clinical implications. A prior *in vivo* study suggests that *DDR2* mutations are a potential therapeutic target in lung squamous cell carcinoma (33). Another recent study has shown that targeting DDR2 kinase increases the efficacy of anti-PD-L1 therapy (38).

The TCGA has noted that 25% of high-grade endometrioid tumors and a limited percentage of lower grade (1 and 2) endometrioid tumors ($<5\%$) show extensive copy number alterations. By WES analysis, the recurrent primaries in our study showed higher levels of copy number gains than controls. This finding is of clinical importance because a previous report suggested that high SCNA burden is a prognostic factor in TCGA cohorts, including EC cases (36). Further investigation into the significance of these findings in larger cohorts would be warranted to identify patients who may be at higher risk of recurrence and need alternative treatment strategies.

SCNA analysis also demonstrated that arm-level 1q gain was enriched in LGLS cases with recurrence although the trend did not reach statistical significance. 1q gain has been documented in association with worse prognosis in a subset of tumor types including multiple myeloma, Ewing sarcoma, pediatric tumors including Wilms tumor and neuroblastoma, and multiple brain tumors including ependymoma and high-grade gliomas (39–43). To our knowledge there are only a few reports in the literature addressing gains in 1q in the endometrial carcinoma. Depreeuw et al. (44) reported that 1q32.1 amplification significantly correlated with worse relapse-free survival and *MDM4* is a potential oncogenic driver in the region in a nonspecific molecular profile subgroup of EC. Of note, *MDM4* gain was detected by WES in 2 of 4 LGLS EC with recurrence (Fig. 3A). Sever et al. (45) also reported that gains in 1q had a negative impact on survival in EC patients. In addition, aberrations in 1q have also been described in Müllerian tumors of mesonephric type, though neither of our cases showed histologic features of this tumor type, despite the fact that case 4 also harbored *KRAS* mutation, a finding also documented in a high percentage of mesonephric carcinomas (46–48).

Tumor mutational burden was lower in the cases with recurrence (Fig. 3C; average total number of nonsynonymous mutations = 118 vs. 130), though not to statistical significance.

The same held true for the TMB density (3.19 vs. 3.5 mut/Mb). The difference was reversed when adjusting for nonrecurrent case 10 (TMB = 118 vs. 33, $P = 0.024$ 2-sided; TMB density = 3.19 vs. 0.9 mut/Mb, $P = 0.024$), which showed a markedly higher total number of mutations (801) and TMB density (21.7 mut/Mb) in comparison to the remaining control cases.

Importantly, *POLE* alteration was identified in this case on WES, which explains the high TMB without associated impact on outcome. This patient was recurrence free at last follow up, which would be in keeping with the proposed improved prognosis associated with *POLE* mutated tumors (49–52). It is unclear whether the *POLE* mutation detected in case 10 (p.Ser1380Leu) is pathogenic because the mutation is not located in the exonuclease domain. However, 1 ultramutated tumor in TCGA EC cohort harbored the same *POLE* mutation as case 10 (13), which may provide evidence that the observed *POLE* mutation could lead to extensive somatic mutations.

A notable absence in our data is that of *CTNNB1* mutations, which have been shown to be associated with more aggressive behavior in carcinomas of endometrioid histology with higher rates of recurrence and decreased survival (19,21,22). Kurnit and colleagues found that 26% of LGLS endometrioid patients harbored mutation in *CTNNB1*, though they defined this as grades 1 and 2 and stages 1 and 2, while Moroney and colleagues found overall 60% of LGLS (defined as grade 1 stage 1) showed *CTNNB1* mutation (vs. 28% of controls). None of the 4 recurrent cases in our study showed alteration in *CTNNB1* while 2 of 8 nonrecurrent cases did. The small sample size in the current study may account for this discrepancy. In addition, given tumoral heterogeneity seen in LGLS cases (53), it is possible that sampling could play a role, though it would be unusual to occur in all 4 cases.

Although there have been reports of low and intermediate grade endometrioid carcinomas with *TP53* mutation and associated reduced survival (21,25), these tumors appear to be quite uncommon, particularly with respect to grade 1 tumors. Interestingly, we identified a *TP53* mutation (p.Gly245Asp) in recurrent case 2 (Fig. 1E). Kurnit and colleagues showed an 8.8% incidence of *TP53* mutations when grouping grade 1 and 2 tumors, and the TCGA study showed 0% and 11.8% for grade 1 and 2 tumors, respectively (21,49). When present in endometrioid carcinomas, *TP53* mutations are overwhelmingly associated with higher grade morphology (16). In addition, *TP53* mutations may occur in association with other markers of genetic instability (MMR/POLE), and the clinical impact of these "passenger mutations" may be minimal, though they still remain a challenge in terms of accurate classification. It is generally accepted that the incidence of *TP53* mutations in low-grade tumors is very low, a finding corroborated in this study, including in cases demonstrating subsequent recurrence.

Among nonrecurrent cases, 3 of 8 had *KIT* mutations while 1 was seen in 1 of 4 recurrent cases (Fig. 3A). In the setting of gynecologic malignancy,

the primary focus of *KIT* mutation in recent years has been in uterine sarcomas/MMMT (54–56). While data regarding other EC subtypes is limited, a few studies have looked at the incidence of *KIT* mutation/expression in endometrioid ECs, with numbers ranging from 0% (immunohistochemistry and molecular) (57) to 25% to 60% (immunohistochemistry only) (58,59). Slomovitz and colleagues showed 2 of 8 recurrent endometrioid tumors stained positively for c-kit while 0 of 33 nonrecurrent cases did. Other researchers found that EC cases with C-KIT immunohistochemical expression more frequently had metastases and shorter disease-free survival (59). Still, the majority of these studies looked at immunohistochemical expression rather than sequencing data. Kafshdooz et al. (60) proposed that the pattern and frequency of *KIT* mutation differed between tumors of different stages, but they did not comment on the specifics of incidence in the groups. Hence, data regarding *KIT* mutation in carcinomas of the endometrium remains somewhat limited. All 3 recurrences available for sequencing showed shared mutations with the primary. Two gained mutations associated with genetic instability (*TP53* and *POLE*, cases 2 and 4, respectively) and case 4 also acquired a mutation in *DDR2* kinase, a potential therapeutic target. Case 1 showed no additional mutations and lacked the *MYC* mutation seen in the primary tumor (Fig. 3A). Few studies have investigated the mutation profiles related to progression of EC from primary to metastatic lesions. Gibson and colleagues analyzed complex atypical hyperplasia, primary tumors, and paired abdominopelvic metastatic lesions of EC of both endometrioid and nonendometrioid histologies with WES and found heterogeneity among all stages, with only about 50% of mutations being shared between metastatic lesions and primary tumors. Further, no recurrent, metastasis-specific mutations were found (61). As LGLS EC is a heterogenous subgroup itself, there is a need to study this population individually to better characterize molecular events related to tumor progression.

It is unclear whether the *POLE* and *DDR2* mutations additionally detected in the recurrent tumor from Case 4 promoted tumor recurrence. *POLE*-ultramutated ECs are defined by the presence of a somatic mutation in the exonuclease domain of *POLE* gene (62). The detected nonsense mutation does not result in an amino acid change in the exonuclease domain, which suggests that the proofreading activity of POLE is preserved. In addition, targeted sequencing did not show very high mutation frequency in the recurrent tumor from case 4 (6 nonsynonymous mutations). On the other hand, the *DDR2* mutation was predicted to be functionally damaging by a computational tool for prediction of the possible impact of an amino acid substitution. While *in vitro* studies using tumor cell lines have proven DDR2 protein exerts oncogenic role by promoting cellular proliferation, migration and metastasis (63), the oncogenic role resulting from the *DDR2* mutation is unclear in EC. Further functional studies would be warranted in order to clarify whether the *DDR2* mutation promotes malignant progression of ECs.

The underlying cause as to why certain LGLS ECs recur despite a lack of histologic or clinical evidence portending aggressive behavior has not yet been elucidated. The increasing use of molecular studies in characterizing EC has resulted in notable progress in further defining these tumors. As molecular testing continues to become more commonplace in practice, it is imperative that we continue to seek out molecular identifiers for patients who are at risk for recurrence, as there is untapped potential to preemptively identify patients who are at risk for recurrent and typically untreatable disease. Although this is a small WES-based and targeted sequencing study, it supports that molecular differences may define cases of LGLS EC that recur and those that do not.

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