LRP1B Deletion in High-Grade Serous Ovarian Cancers Is Associated with Acquired Chemotherapy Resistance to Liposomal Doxorubicin

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Abstract

High-grade serous cancer (HGSC), the most common subtype of ovarian cancer, often becomes resistant to chemotherapy, leading to poor patient outcomes. Intratumoral heterogeneity occurs in nearly all solid cancers, including ovarian cancer, contributing to the development of resistance mechanisms. In this study, we examined the spatial and temporal genomic variation in HGSC using high-resolution single-nucleotide polymorphism arrays. Multiple metastatic lesions from individual patients were analyzed along with 22 paired pretreatment and posttreatment samples. We documented regions of differential DNA copy number between multiple tumor biopsies that correlated with altered expression of genes involved in cell polarity and adhesion. In the paired primary and relapse cohort, we observed a greater degree of genomic change in tumors from patients that were initially sensitive to chemotherapy and had longer progression-free interval compared with tumors from patients that were resistant to primary chemotherapy. Notably, deletion or downregulation of the lipid transporter LRP1B emerged as a significant correlate of acquired resistance in our analysis. Functional studies showed that reducing *LRP1B* expression was sufficient to reduce the sensitivity of HGSC cell lines to liposomal doxorubicin, but not to doxorubicin, whereas *LRP1B* overexpression was sufficient to increase sensitivity to liposomal doxorubicin. Together, our findings underscore the large degree of variation in DNA copy number in spatially and temporally separated tumors in HGSC patients, and they define *LRP1B* as a potential contributor to the emergence of chemotherapy resistance in these patients. *Cancer Res; 72(16); 4060–73. ©2012 AACR.*

Introduction

Genomic change is a hallmark of tumor progression, associated with defects in checkpoint control and DNA repair mechanisms (1, 2). Somatic mutations arising during tumor development generate intratumoral heterogeneity, which underlie within-patient differences in tumor morphology, growth, and therapeutic response. The degree of intratumoral heterogeneity is a reflection of mutation rate, emergence of clones with a selection benefit, and genetic drift in the population (3, 4).

Intratumoral heterogeneity has been reported in a number of solid cancers, leukemia and lymphoma (5, 6), and has been identified by analysis of individual genes such as *TP53* and *ERBB2* (7), using single-nucleotide polymorphism (SNP; ref. 8) or microsatellite analysis (7) of a small number of loci. More recently, complex DNA copy number microarrays and next-generation sequencing techniques have provided powerful techniques for exploring clonal diversity, tumor evolution, and development of treatment resistance. For example, chromosomal structural information from next-generation sequencing has been used to track dispersal of pancreatic metastases to different organs and estimate time course of disease (9, 10). Genomic variation has been observed between samples collected at presentation and recurrence in breast cancer (11),

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acute lymphoblastic leukemia (ALL; refs. 12, 13), and cervical cancer (14). Analysis of individual sectors of solid tumors, flow cytometry sorting (8, 15, 16), and single-cell sequencing (17) have identified distinct populations of tumor cells within individual patient samples. These studies provide insight into rates of genomic change in an evolving cancer and, importantly, help define genes associated with tumor progression and treatment failure (18).

Ovarian cancer is a histologically diverse disease, with high-grade serous cancer (HGSC) accounting for approximately 65% of deaths. Most women with advanced HGSC are initially responsive to treatment, but majority relapse with disease that becomes progressively less responsive to chemotherapy (5). HGSC show considerable DNA copy number change (19), with high rates of genomic instability (20) and somatic *TP53* mutation (21, 22). Intratumoral heterogeneity has been observed in HGSC using microsatellite probes and SNP markers in cancer-associated genes (7). A study comparing loss of heterozygosity patterns in primary and metastatic HGSC found a complex rather than sequential pattern of clonal evolution (23). Selection of preexisting minor genetic subpopulations has been associated with emergence of drug-resistant disease (5).

Several molecular events associated with emergence of treatment resistance in ovarian cancer have recently been identified. Intragenic *BRCA1/2* mutations, resulting in partial restoration of defective germ line alleles, have been observed in tumors following platinum-based chemotherapy and emergence of resistance (24). Cells lines derived from pre- and post-treatment patient samples have identified HDAC-4–regulated STAT1 activation (25) and DNA-PK mediated activation of AKT (26) as influencing sensitivity of HGSC to platins. Whether other mechanisms exist to influence development of treatment resistance and how intratumoral heterogeneity relates to clinical outcome in ovarian cancer remains to be explored.

Here we examine spatial and temporal diversity in HGSC using high-resolution SNP arrays, multiple metastatic lesions from individual patients, and paired pre- and posttreatment samples. We find considerable spatial variation in DNA copy number changes in multiple tumor biopsies, associated with differential gene expression. In the paired primary and relapse cohort, we observe a greater degree of genomic change in tumors from patients that were initially responsive compared with tumors from patients resistant to primary chemotherapy. We identify deletion and downregulation of *LRP1B* associated with acquired resistance in both a discovery and independent validation data set. Functional *in vitro* studies support the conclusion that *LRP1B*, a member of the lipid transporter family, is involved in emergence of resistance to liposomal doxorubicin in HGSC.

Patients and Methods

Patient cohorts

Human tissue use had Institutional Ethics Review Board approval from all participating centers and informed consent was obtained from all study participants.

Patients in this study were recruited from the Australian Ovarian Cancer Study (www.aocstudy.org), a population-based study, between 2002 and 2011 (27). Details of patient accrual, collection of clinical data, pathologic review, and biospecimen processing have been described previously (27). Details of primary surgical and paired pretreatment and relapse cohorts are provided in Supplementary Methods.

Genomic analysis

DNA and RNA samples were processed according to manufacture's protocol for Affymetrix SNP6.0 Mapping arrays and Affymetrix GeneST 1.0 arrays, respectively. DNA copy number was obtained as described previously (19). Information on normalization and analysis is available in Supplementary Methods. DNA copy number and gene expression data are available at National Center for Biotechnology Information (NCBI), Gene Expression Omnibus (GEO) accession numbers GSE38787 and GSE38734, respectively.

Genomic distances between 2 tumor samples from the same patient were determined by identifying regions of copy number change ($Log_2 0.3$ and -0.3 for gains and losses, respectively), where a contiguous genomic region was considered a genome-altering event. The number of distinct genomic events was considered the distance between any pair of genomes. Two genomic regions were considered distinct if, and only if, at least one breakpoint was greater than a threshold (100,000 bps). Pairwise distance between samples were used to construct a phylogenetic tree for each individual using the "neighbor-joining" tree estimation (28).

Quantitative real-time PCR

Measurement of expression of coding genes was carried out using SYBR green quantitative real-time reverse transcriptase PCR (qRT-PCR) assay (Applied Biosystems) as previously described (29). qRT-PCR conditions are provided in Supplementary Methods.

Cell culture, transfection, and drug sensitivity assays

IGROV1 and OVCAR4 cells were obtained from the National Cancer Institute Repository. Kuramochi cells from Japan Health Science Research Bank, and JHOS3 cells from Riken BioResource Centre. Cell lines authenticated by short tandem repeat (STR) loci profiling (November 2011). Culture conditions are described in Supplementary Methods.

Transfection of stable cell lines. LRP1B domain IV–containing minireceptor (mLRP1B) with HA-tag (30) was provided by Guojun Bu (Washington University, St. Louis, MO) and transfected into IGROV1 and OVCAR4 cells. Stable transfectants were selected and maintained with 400 and 200 μg/mL G418, respectively.

siRNA transfection. ON-Target plus individual siRNAs, siRNA pools, and transfection reagents were obtained from Dharmacon (ThermoFisher Scientific) and transfections carried out as previously described (29) and detailed in Supplementary Methods. Cell viability was determined using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) as previously described (29).

Proliferation assay. *LRP1B* overexpressing cells were seeded, 1×10^5 cells/well in 6-well plates, and cell numbers determined daily for 4 days by Countess (Invitrogen). Each experiment was carried out in triplicate, at least 3 times.

Drug sensitivity assay. *LRP1B* overexpressing or siRNA-transfected cells were seeded, 1×10^5 cells per well in 96-well plates, and treated with doxorubicin or CAELYX (liposomal doxorubicin) for 72 hours. IC_{50} dose was approximated by fitting a 4-parameter dose–response curve (Hill equation) and all parameters used in curve comparisons using Prism 5 (GraphPad) as previously described (29).

Results

Variation in DNA copy number among primary ovarian tumors and associated abdominal deposits

We and others have reported extensive DNA copy number change in primary HGSC (31, 32). Copy number change influences tumor biology by altering gene expression and can be used to infer clonal relationships (10). We therefore sought to explore the extent of DNA copy number changes between different metastatic sites from the same patient collected at primary surgery. Affymetrix SNP6.0 arrays were used to obtain high-resolution estimates of DNA copy number change in primary and multiple metastatic sites from 4 patients (Supplementary Methods). Data were normalized and segmented (Supplementary Methods) to define discrete regions of DNA copy number change and allow estimation of genetic heterogeneity between tumor deposits.

Although the majority of regions of DNA copy number change was shared among different tumor deposits within a given patient, none of the primary and metastatic tumor genetic profiles was identical (Fig. 1A; Supplementary Fig. S1).

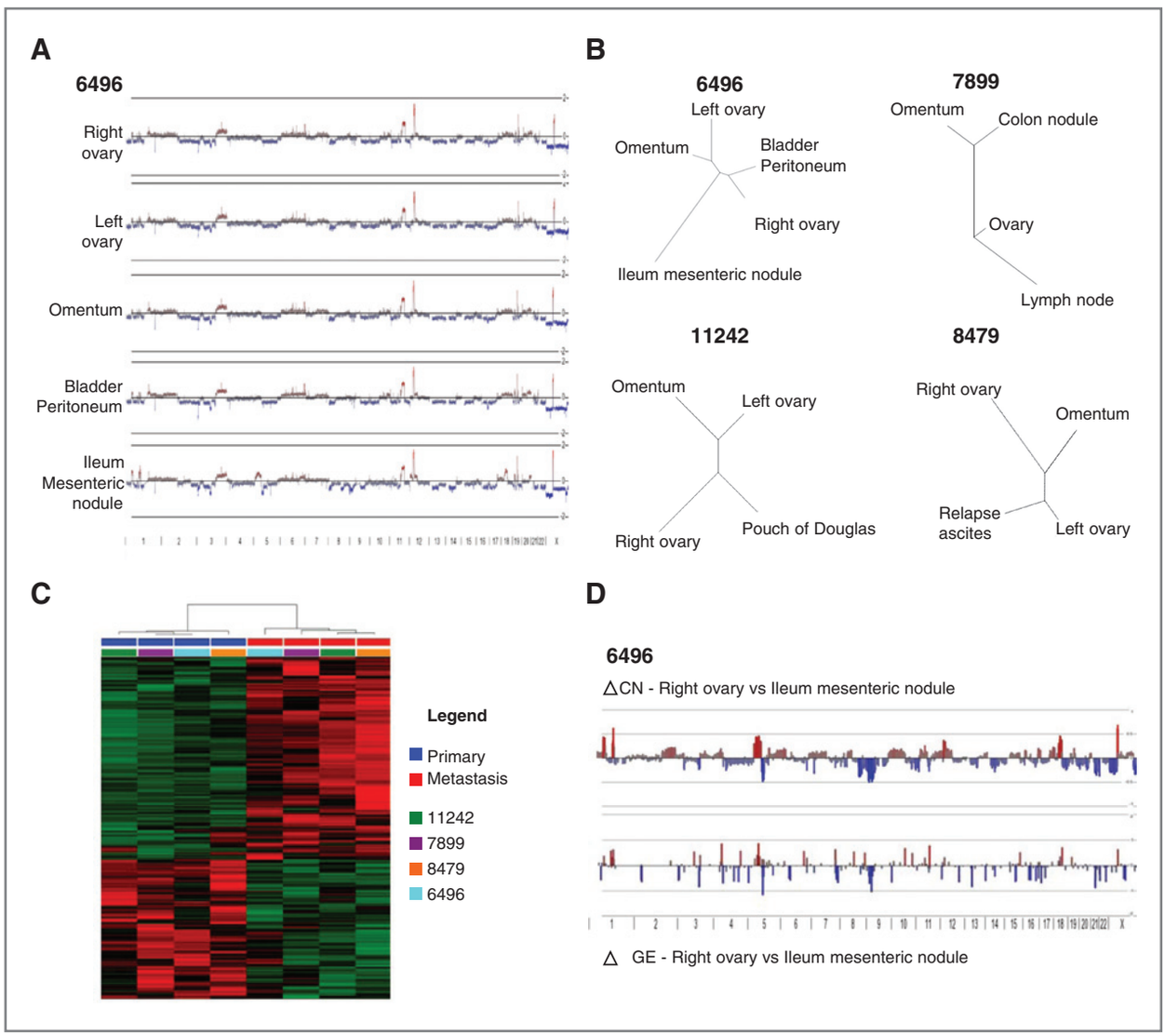

Figure 1. Variation in DNA copy number among primary ovarian tumors and associated abdominal deposits. A, copy number genome in line view of patient 6496 primary ovarian tumor (right ovary) and 4 associated abdominal deposits. B, phylogenetic trees of patients 6496, 7899, 11242, and 8479 based on the number of different genomic aberrations between the copy number profiles. Branch length is proportional to evolutionary distance. C, unsupervised clustering of gene expression data from patient primary tumors and the most genomically distant abdominal deposit. D, differential copy number and gene expression plots of the primary tumor and most distant abdominal deposit for patient 6496.

We used phylogenetic tree analysis to calculate the most parsimonious relationship between tumor deposits from a patient (Fig. 1B). The number of new genomic events between most disparate samples within each patient and fraction of all focal copy number changes was 117 (29%; patient 11242), 334 (63%; patient 8479), 114 (25%; patient 6496), and 93 (26%; patient 7899), showing that even when small subsets of metastatic deposits are analyzed, substantial copy number variation is apparent.

Many regions of differential copy number change within a patient coincide with known regions of copy number change in HGSC (Supplementary Table S1; refs. 19, 32) and therefore may affect oncogenic drivers allowing tumor cells to adapt to tissue-specific requirements. To investigate further, we obtained RNA expression profiles using Affymetrix Gene ST1.0 arrays of the most genomically distinct samples within a patient (Fig. 1C). Regions of differential copy number change between tumor deposits were associated with correspondingly large-scale changes in gene expression (Fig. 1D; Supplementary Fig. S1A and B).

Unsupervised hierarchical clustering resulted in primary tumors and abdominal deposits segregating. GeneGo pathway analysis of differentially expressed genes between primary tumors and deposits (Fig. 1C) revealed 4 of the top 10 pathways related to cytoskeleton remodeling and cell adhesion (Supplementary Table S2).

Genomic change between paired primary and recurrent ovarian tumors is associated with initial treatment response

Abdominal distention, due to accumulation of fluid (ascites) and suspended tumor cells, often develops in women with HGSC following relapse. Paracentesis of ascites provided a source of tumor material to explore the variation that occurs between primary and relapse samples. We obtained paired HGSC samples from 22 patients who had previously been treated with platinum-based and, in many cases, other chemotherapy regimens (Table 1; Supplementary Table S3). Patients were divided into 3 cohorts (Supplementary Methods), primary–relapse sensitive (S-S cohort, $N = 2$, 3–5 lines of chemotherapy), primary sensitive–relapse resistant (S-R cohort, $N = 11$, 2–8 lines of chemotherapy), and primary resistant–relapse resistant (R-R cohort, $N = 9$, 2–6 lines of chemotherapy). Clinical characteristics of the cohorts, including patient age, International Federation of Gynecology and Obstetrics stage, histopathologic grade, and extent of residual disease following debulking surgery were well-balanced, with no statistically significant difference between any tested variable except for progression-free survival (PFS; $P < 0.0001$, Log-rank test) and overall survival (OS; $P < 0.001$, Log-rank test; Table 1).

Extensive differential DNA copy number change was observed between paired primary and relapse ovarian tumors, however, this seemed to be independent of time between sample collection (Fig. 2A), suggesting that degree of change is not simply a function of a constant rate of genomic drift. We then divided cases based on response to initial treatment and chemotherapy at the time of sampling. Although there was a trend toward a greater degree of genomic change in the S-R cohort compared with R-R patient tumors, this was not significant ($P < 0.11$, Fig. 2B). Germ line *BRCA1* or *BRCA2* mutation has been shown to strongly influence treatment response and OS and can confound analysis of outcomes in sporadic ovarian cancer patients (33). When we excluded cases in our series known to harbor pathogenic germ line ($N = 4$) or somatic ($N = 1$) *BRCA1* mutations (Supplementary Table S4; ref. 34), we observed significantly more differential DNA copy number change between S-R versus R-R pairs ($P < 0.005$). This did not seem to be due to a greater exposure to cytotoxic therapy, as we found no relationship between number of lines of chemotherapy and extent of genomic change (Fig. 2C).

Recurrent chromosomal aberrations at 10q25.2, Xp11.4, and 2q21.2 are associated with acquired resistance

The availability of paired samples allowed us to explore recurrent genomic changes specifically associated with emergence of chemotherapy resistance. We began by examining 19 known cancer oncogenes and tumor suppressor genes associated with common regions of gain or loss in HGSC, including *MYC*, *PAX8*, and *CDKN2A*. In addition, we tested DNA copy number change and gene expression of 19 literature-derived candidate genes either identified through induction of chemotherapy resistance *in vitro* but have not been analyzed using human samples or not validated with independent clinical data sets (Supplementary Fig. S2). These genes included *ANXA3* (35), *ANXA11* (36), *ATP7A* and *ATP7B* (37, 38), *ERCC1* (39), and *ERCC5* (40). Candidate genes were also tested in an independent unmatched cohort of 92 primary tumor and 43 relapse ascites samples (Table 2). We found no association between any known oncogenes, tumor suppressors, or candidate genes and emergence of resistance (Supplementary Fig. S3).

We then analyzed genome-wide copy number change data, seeking regions specifically amplified or deleted in S-R versus the R-R cohort (Supplementary Figs. S4–S5). Analysis of 11 S-R pairs identified the fraction of new genomic events in the recurrent samples ranged from 6% to 65% with a total of 85 discrete regions of amplification and 125 deletions in the recurrent samples not present in the primary tumors (Supplementary Tables S5 and S6). Three regions were recurrently present in 3 or more samples in the S-R but not R-R samples, including copy number gain at 10q25.2 (27%, 3 of 11 samples, containing *ACSL5*, *GPAM*, *GUCY2G*, *HABP2*, *LOC143488*, *NRAP*, *TCFL2*, *TECTB*, *VTI1A*, and *ZDHHC6*) and loss at 2q21.2 (36%, 4 of 11, containing *LRP1B*) and Xp11.4 (27%, 3 of 11, containing *BCOR*).

We considered whether recurrently altered regions could be a function of the different tissue types used for primary (solid tumor) and relapse (ascites) samples. It is unlikely any recurrent regions of copy number change seen in S-R samples reflected genomic alterations specifically required by suspended ascites cells, however, as R-R samples also involved paired solid and suspended tumor material. As a further control, we compared copy number change in 4 tumor and ascites pairs, in which both were collected at primary surgery. Discrete regions of copy number change were observed between paired samples (Supplementary Fig.

Table 1. Clinicopathologic characteristics of paired primary-relapse cases

	SS Primary sensitive – Relapse sensitive	RR Primary resistant – Relapse resistant	SR Primary sensitive – Relapse resistant	*P*
Age at diagnosis				
Mean	57	66	58	0.17[a]
SD	10	6	13	
Range	50–64	55–74	39–79	
Primary site				
Ovary	2	4	7	
Peritoneum		3	2	
Not specified		2	2	
FIGO stage				
III	2 (100%)	8 (89%)	10 (90%)	0.34[b]
IV	0 (0%)	1 (11%)	1 (10%)	
Grade				
1	0 (0%)	0 (0%)	0 (0%)	0.16[b]
2	1 (50%)	3 (33%)	1 (10%)	
3	1 (50%)	6 (66%)	10 (90%)	
Residual disease				
Nil	0 (0%)	1 (11%)	2 (18%)	0.35[b]
<1 cm	1 (50%)	3 (33%)	5 (44%)	
>1 and <2 cm	1 (50%)	1 (11%)	1 (10%)	
>2 cm	0 (0%)	3 (33%)	1 (10%)	
Unknown	0 (0%)	1 (11%)	2 (18%)	
PFS				
Median	12.53	2.99	8.65	<0.0001[c]
95% CI	6.19	1.20	2.03	
OS				
Median	39.91	17.13	39.65	<0.001[c]
95% CI	5.67	4.33	8.10	
Total cases	2	9	11	

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; PFS, progression-free survival, the interval between diagnosis and disease progression or last follow-up; OS, overall survival, the time interval between date of diagnosis and date of death from any cause or date last seen alive.

[a]Kruskal-Wallis test.

[b]Fisher exact test.

[c]Log-rank test.

S6), as seen in analysis of different metastatic deposits; however, none were associated with 10q25.2, Xp11.4, or 2q21.2.

Loss of *LRP1B* gene expression is associated with acquired treatment failure

We next sought to understand the implications of genomic changes associated with acquired resistance, focusing on the most common region of change, 2q21.2. Comparison of tumors showed a minimum common region of deletion of 0.19Mb, encompassing exons 12–22 of the low-density lipoprotein (LDL) receptor–related protein 1B (*LRP1B*) gene (Fig. 3A; Supplementary Fig. S7). Intragenic deletion of *LRP1B* is common in solid cancers (41), resulting in frameshift and translation of truncated, inactive protein (42–44). More recently, missense mutations have been reported (32) and, consistent with a role as a tumor suppressor, these do not seem to cluster to particular regions of the protein.

qRT-PCR was carried out to measure *LRP1B* mRNA expression in the 22 paired samples from the discovery set (Fig. 3B). Pair-wise comparisons of gene expression were made between R-R and S-R pairs. Primary tumor–primary ascites were included as additional controls for differences in tissue composition. Within the S-R cohort, a significant reduction in *LRP1B* expression was observed in relapse-resistant ascites compared with matched primary sensitive tumors, as well as to R-R pairs ($P < 0.0001$, paired t test; Fig. 3B). No significant reduction in expression was observed in relapse-resistant ascites compared with matched primary resistant tumors ($P = 0.61$, paired t test; Fig. 3B).

An independent unpaired data set of 92 primary tumor and 43 recurrent ascites HGSC samples (Table 2) were used to

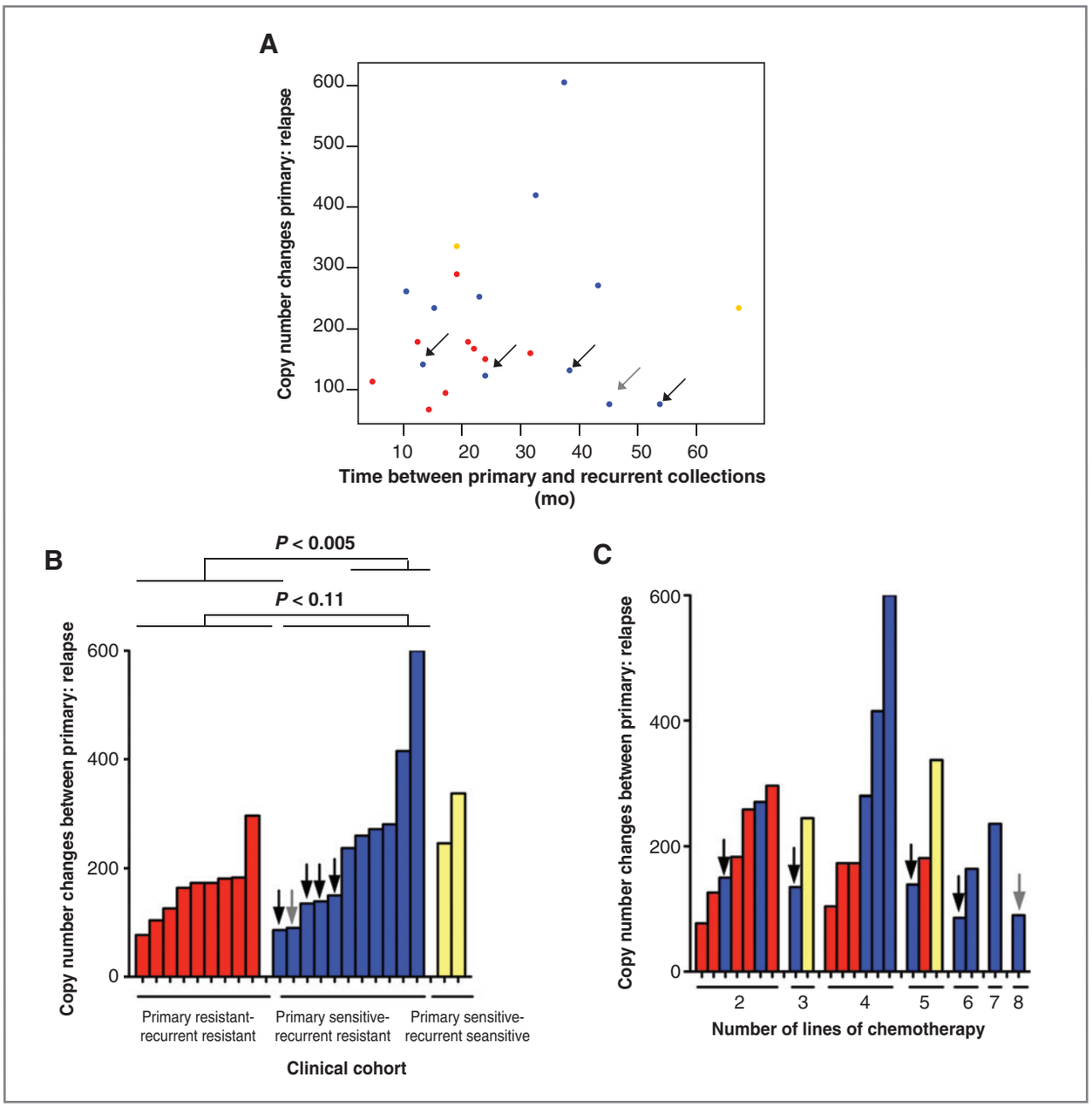

Figure 2. Genomic change between paired primary and recurrent ovarian tumors is associated with initial treatment response. A, dot plot illustrating number of genomic aberrations as a function of time between primary and relapse tumor collections. B, bar chart showing the difference in number of genomic aberrations between primary resistant and primary sensitive-relapse resistant cohorts. A significant difference was observed when *BRCA* mutation carriers were excluded from analysis. C, relationship between number of genomic aberrations and number of lines of chemotherapy received (red, primary resistant–relapse-resistant cohort; blue, primary sensitive–relapse-resistant cohort; yellow, primary sensitive–relapse-sensitive cohort; black arrows, germ line *BRCA* mutation carriers; gray arrow, somatic *BRCA* mutation carrier).

validate an association of downregulation of *LRP1B* with acquired chemoresistance. Samples were categorized into 6 groups; (i) primary sensitive tumors, (ii) primary resistant tumors, (iii) primary ascites, (iv) relapse-sensitive ascites, (v) relapse-resistant ascites from women with less than 6 months PFI (primary resistant), and (vi) relapse-resistant ascites from women with more than 6 months PFI (acquired resistant). *LRP1B* downregulation was significantly associated ($P < 0.0001$) with acquisition of resistance (Fig. 3C).

Ectopic expression of LRP1B suppresses cell growth

To identify cell lines for functional studies, we analyzed high-resolution SNP6.0 copy number and qRT-PCR gene expression data (Supplementary Fig. S8) from 30 ovarian cancer cell lines

Table 2. Clinicopathologic characteristics of unpaired validation cases

	Primary sensitive	Tumor resistant	Relapse sensitive	Ascites resistant	Primary sensitive	Ascites resistant	*P*
Age at diagnosis							
Mean	58	62	52	60	62	65	0.33[a]
SD	10	9	7	10	12	10	
Range	40–80	43–80	45–59	40–82	44–76	60–80	
Primary site							
Ovary	43	38	3	25	6	4	
Peritoneum	2	9		10			
Fallopian tube				1			
Genital Tract				2			
Not specified				2			
FIGO stage							
I	1 (2%)	0 (0%)	0 (0%)	1 (2.5%)	0 (0%)	0 (0%)	
II	0 (0%)	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
III	40 (87%)	38 (81%)	2 (66%)	34 (85%)	6 (100%)	3 (75%)	0.51[b]
IV	4 (11%)	7 (15%)	1 (33%)	2 (5%)	0 (0%)	1 (25%)	
Not staged/not known	0 (0%)	0 (0%)	0 (0%)	3 (7.5%)	0 (0%)	0 (0%)	
Grade							
1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.21[b]
2	9 (21%)	13 (27%)	1 (33%)	2 (5%)	1 (16%)	0 (0%)	
3	36 (79%)	29 (62%)	1 (33%)	28 (70%)	5 (84%)	4 (100%)	
Unknown	0 (0%)	5 (11%)	1 (33%)	10 (25%)	0 (0%)	0 (0%)	
Residual disease							
Nil	8 (16%)	6 (13%)	1 (33%)	7 (17.5%)	0 (0%)	0 (0%)	0.65[b]
<1 cm	21 (48%)	21 (44%)	0 (0%)	12 (30%)	2 (33%)	0 (0%)	
>1 and <2 cm	8 (16%)	5 (11%)	1 (33%)	3 (7.5%)	2 (33%)	1 (25%)	
>2 cm	7 (16%)	14 (30%)	1 (33%)	9 (22.5%)	1 (16%)	3 (75%)	
Unknown	1 (2%)	1 (2%)	0 (0%)	6 (15%)	1 (16%)	0 (0%)	
Tumor not resected	0 (0%)	0 (0%)	0 (0%)	3 (7.5%)	0 (0%)	0 (0%)	
PFS							
Median	15.85	3.06	10.68	6.89	8.45	2.91	<0.0001[c]
95% CI	6.43	0.49	0.25	3.05	3.09	2.64	
OS							
Median	47.70	12.99	45.50	20.70	12.18	6.18	<0.001[c]
95% CI	6.95	4.23	27.77	7.16	5.93	5.72	
Total cases	45	47	3	40	6	4	

NOTE: Grade refers to the classification of the primary tumor. Primary ascites indicates ascites that was collected at primary surgery.
Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; PFS, progression-free survival, the interval between diagnosis and disease progression or last follow-up; OS, overall survival, the time interval between date of diagnosis and date of death from any cause or date last seen alive.

[a]Kruskal-Wallis test.

[b]Fisher exact test.

[c]Log-rank test.

and 2 normal ovarian cell lines. We identified 4 cell lines of serous histology, with either low (IGROV1, OVCAR4) or high (Kuramochi, JHOS3) *LRP1B* gene expression and no evidence of copy number change involving *LRP1B*.

Due to the large open reading frame of *LRP1B* (13,797 base pairs with 91 exons), we used a previously reported fully functional minigene, *mLRP1B4* (30). IGROV1 and OVCAR4, which showed low *LRP1B* expression (Supplementary Fig. S8), were transfected with either empty vector (pcDNA3) or a HA-tagged mLRP1B4 expression vector (pcDNA3-HA-mLRP1B4). Stable transfectants were selected and *mLRP1B4* expression confirmed by qRT-PCR (Fig. 4A). Consistent with previous reports (45), cells expressing *mLRP1B4* grew significantly slower than parental and vector control in both the IGROV1 cells ($P < 0.005$, Fig. 4B) and OVCAR4 cells ($P < 0.0005$, data not shown).

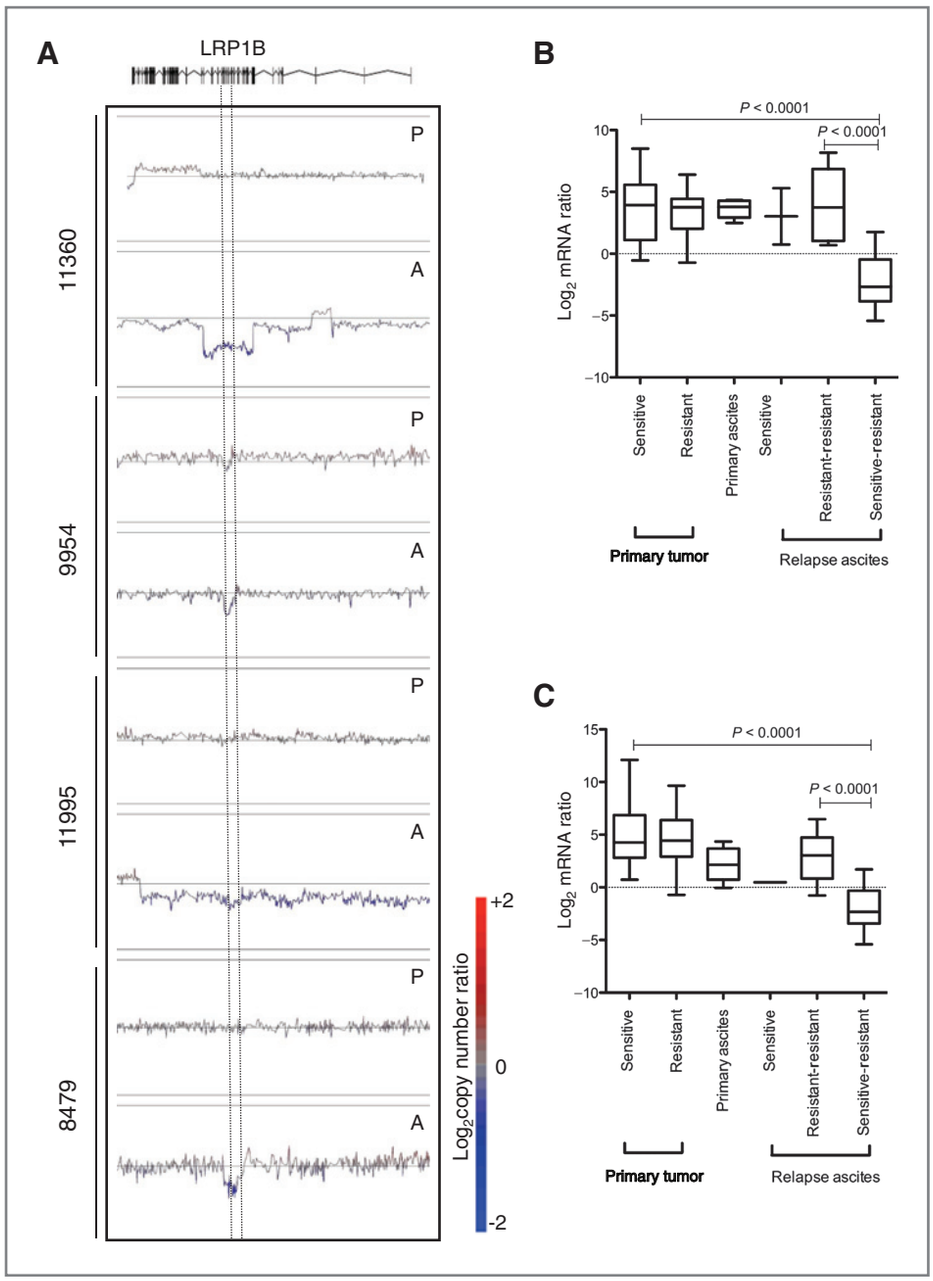

Figure 3. Loss of LRP1B is associated with acquired treatment failure. A, Affymetrix SNP6.0 copy number of 4 paired primary sensitive–relapse-resistant ascites with focal deletion of 2q21.2, containing LRP1B, in ascites samples only. Minimal region of deletion indicated by dotted line (P, primary tumor; A, ascites). B, *LRP1B* expression analysis in paired primary-relapse samples. C, expression analysis in unpaired samples.

Ectopic expression of LRP1B increases drug sensitivity to liposomal doxorubicin but not doxorubicin

Acquired drug resistance has variously been attributed to enhanced DNA repair, decreased drug influx, increased drug efflux, reduced drug activation, enhanced drug inactivation, changes in drug–target interaction, inhibition of apoptosis, and enhanced EGFR MAPK/ERK signaling (46, 47). As a member of the LDL receptor family, *LRP1B* has been suggested to play a role in endocytosis of certain drugs (48), including liposomally modified agents, such as liposomal doxorubicin (CAELYX).

Given the proposed role of LRP1B in drug endocytosis and association of *LRP1B* deletion with acquisition of resistance, we examined the effect of ectopic expression of *LRP1B* on drug sensitivity. We carried out a dose–response analysis to characterize the effect of doxorubicin and liposomal doxorubicin following transfection in IGROV1 and OVCAR4 cells (Fig. 4C–H). No difference in dose response or IC_{50} was observed between IGROV1 (Fig. 4C and G) or OVCAR4 (Fig. 4E and G) parental, pcDNA3 vector alone, and *mLRP1B4*-transfected cells following treatment with doxorubicin. However, a statistically significant shift in the dose–response curve was observed in IGROV1 and OVCAR-4 *pcDNA3-HA-mLRP1B4*–transfected cells compared with parental and vector alone following treatment with liposomal doxorubicin (Fig. 4D

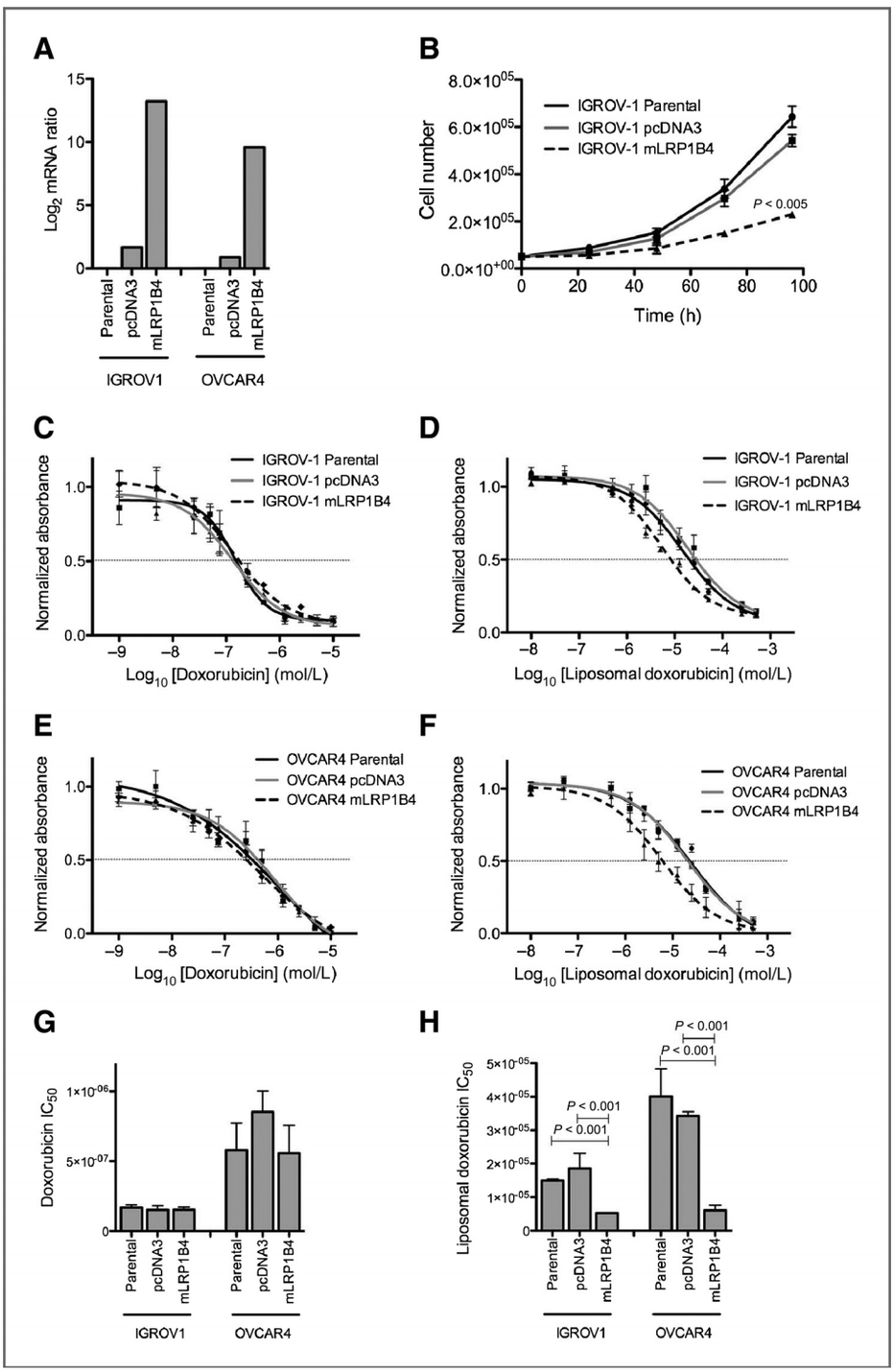

Figure 4. Ectopic expression of *LRP1B in vitro* reduces proliferation and alters drug sensitivity in ovarian cancer cell lines, IGROV1 and OVCAR4. A, ectopic expression of *mLRP1B4* in IGROV1 and OVCAR4 cell lines, compared with parental and vector only pcDNA3 cells, by qRT-PCR. B, cell proliferation and growth in IGROV1 cells transfected with *mLRP1B4* over 4 days (black line; $P < 0.05$) compared with parental (black) and pcDNA3 (gray) vector only cells. Average cell number from 3 independent assays (triplicate wells per condition) and SEM plotted ($n = 3$). Short-term cytotoxicity assays (C–H) with doxorubicin (C, E, G) and liposomal doxorubicin (D, F, H) in IGROV1 (C, D) and OVCAR4 (E, F) cells. Average normalized MTS assay absorbance to cells without drug treatment, SEM and 4-parameter fitted Hill slope plotted. Mean ± SEM plotted from 3 independent experiments.

and F), resulting in a significant reduction in IC_{50} ($P < 0.0001$; Fig. 4H).

siRNA-mediated knockdown of LRP1B decreases drug sensitivity to liposomal doxorubicin but not doxorubicin

Given that loss of *LRP1B* expression was associated with emergence of resistance in tumor samples, we examined the consequences of attenuating expression in cell lines using siRNA. Expression of *LRP1B* in ovarian cancer lines JHOS3 and Kuramochi was efficiently and specifically reduced up to 96 hours after siRNA transfection with individual and pooled siRNAs (Fig. 5A and Supplementary Fig. S10), with no effect on cell viability in either cell line (Fig. 5B). The most effective target attenuation was achieved with the *LRP1B* siRNA pool

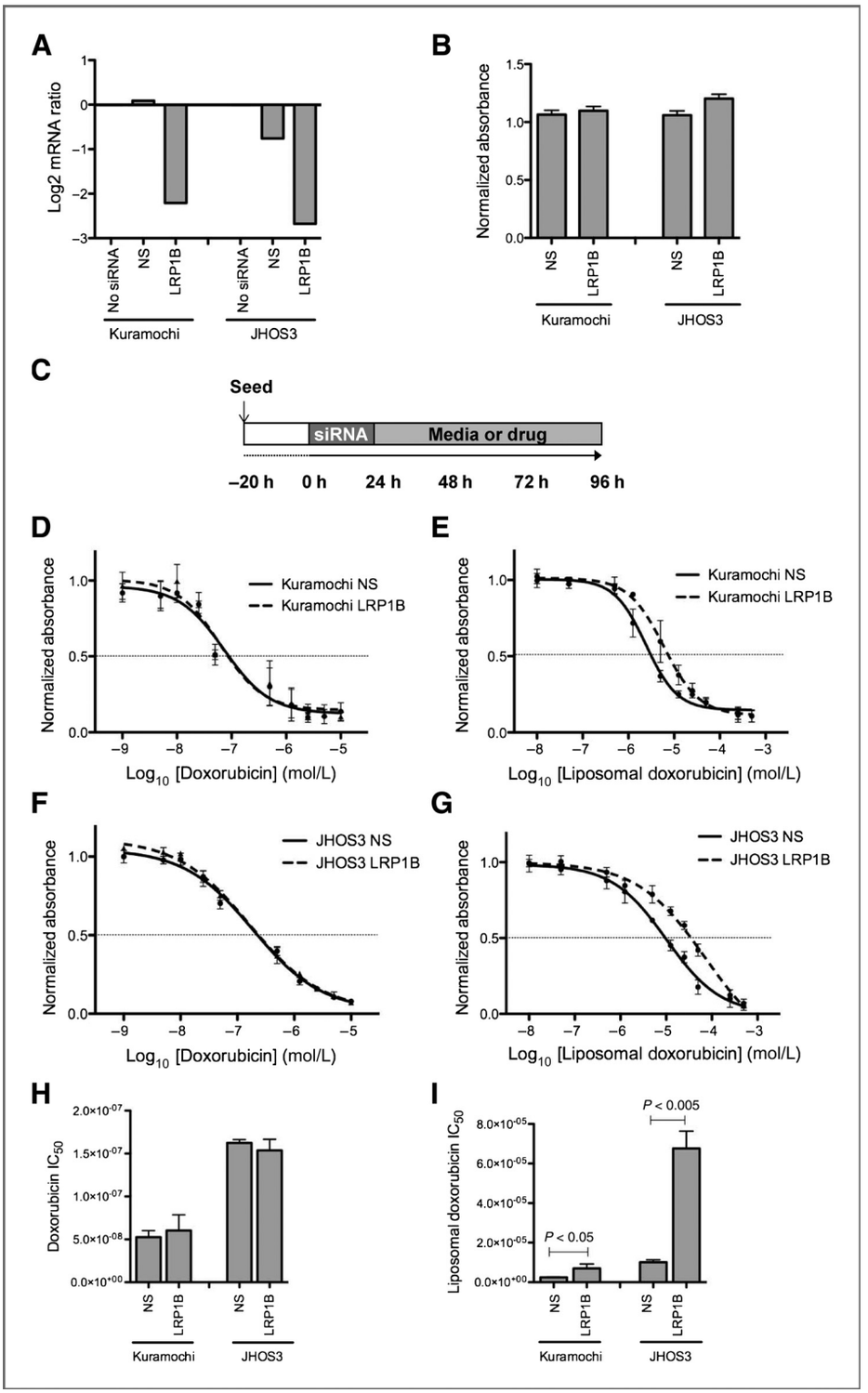

Figure 5. siRNA knockdown of *LRP1B in vitro* alters drug sensitivity in ovarian cancer cell lines, Kuramochi and JHOS3 A, siRNA knockdown of *LRP1B* in Kuramochi and JHOS3 cell lines by qRT-PCR. B, effect of *LRP1B* knockdown on cell viability in Kuramochi and JHOS3 cell lines. C, schematic representation of short-term cytotoxicity assay following siRNA knockdown. Short-term cytotoxicity assays (D–I) with doxorubicin (D, F, H) and liposomal doxorubicin (E, G, I, H) in Kuramochi (D, E) and JHOS3 (F, G) cells. Average normalized MTS assay absorbance to cells without drug treatment, SEM and 4-parameter fitted Hill slope plotted. Mean ± SEM plotted from 3 independent experiments.

(Fig. 5) and individual siRNAs #2 and #4 (Supplementary Fig. S10). Experimental design for siRNA knockdown and subsequent drug treatment protocol is shown in Fig. 5C. No significant difference in dose response or IC_{50} was observed in Kuramochi (Fig. 5D and H) or JHOS3 (Fig. 5F and H) cells following knockdown and treatment with doxorubicin. Consistent with overexpression studies, however, a statistically significant reduction in sensitivity to liposomal doxorubicin was observed in both Kuramochi ($P < 0.05$; Fig. 5E and I) and JHOS3 ($P < 0.005$; Fig. 5G and I) cell lines. As an additional

control for the specificity of siRNA targeting, cells expressing the *LRP1B* minigene, which lacked the *LRP1B* siRNA-binding sites (Supplementary Fig. S9A), were targeted. As anticipated, minigene expression suppressed the reduced sensitivity of siRNA-transfected cells to liposomal doxorubicin (Supplementary Fig. S9).

Discussion

Our work provides the most extensive high-resolution analysis to date of genomic variation in spatially and temporally separated tumor deposits recovered from individual patients with HGSC. Consistent with a previous report using microsatellite markers (23), we found considerable genomic difference between individual abdominal deposits within a patient. It is perhaps not surprising that substantial copy number differences can be found between metastatic deposits, given the high degree of DNA copy number change that commonly occurs in HGSC (19, 32, 49). Whether the variants observed were preexisting in the primary tumor or represent *de novo* genomic change arising after dissemination can be addressed by generating precise breakpoint information using next-generation DNA sequencing and highly sensitive PCR assays (12).

HGSC tumor cells disseminate rapidly through the abdomen from the ovary or fallopian tube, as the surface of these tissues are contiguous with the peritoneal space. Although shedding is unlikely to present the same biologic obstacle to spread as invasion through tissue planes, the genomic change we observed might partially reflect selection for specific growth requirements within the peritoneum. For example, it has been recently shown that adipocytes modulate growth of HGSC and seem to promote establishment on the omentum (50). The hypoxic environment of the peritoneal space may create additional selective pressures. DNA copy number is an important determinant of gene expression in HGSC (51) and consistent with this, we see global effects on gene expression associated with regions of copy number change. Analysis of regions of differential copy number change and gene expression revealed signaling pathways plausibly linked to peritoneal dissemination and establishment of metastatic foci. Additional samples are needed to determine whether the variation we see in deposits reflects selection of driver or passenger events.

We used paired samples, collected pre- and posttreatment, to investigate genomic change over time and in response to chemotherapy, contrasting patients who were initially responsive with those with a short PFS. Paired material is a powerful means of identifying genes that may confer resistance in HGSC, especially given the difficulty of discerning driver events among the high degree of genomic change already present in primary samples. Previously, a small set of cell lines, derived from pre- and posttreatment tumors assisted in identification of HDAC4/STAT1 (25) and DNA-PK/AKT activation (26) as mechanisms associated with emergence of platinum resistance. Analysis of relapse samples has also shown that reversion of *BRCA1* and *BRCA2* germ line mutations following therapy seems to be a common mechanism associated with reduced platinum sensitivity (24) in *BRCA* mutation carriers.

Cooke and colleagues (5) found few DNA copy number changes between paired pre- and postneoadjuvant treatment (3 cycles) samples, whereas we found substantial genomic change between tumor material collected pretreatment and that obtained months to years later. We observed significantly greater genomic change in sensitive–relapse pairs than in patients who were resistant from the outset, possibly reflecting the requirement for selection of a subpopulation of resistant cells in initially sensitive cases. Less genomic copy number change was observed in *BRCA1* mutation–positive cases, perhaps because small intragenic deletions can be sufficient for emergence of resistant clones (24). Our studies made use of relapse ascites, with tumor cells isolated using anti-EpCAM–coated magnetic beads. Although most tumor cells are likely to express the EpCAM marker, we are aware that other cell populations, particularly a stem cell population, could be lost during purification and subsequent analyses. However, copy number events occurring in tumor-initiating cells should be propagated during differentiation. We also considered whether our analysis would be confounded by copy number events required for prevention of anoikis in tumor cells suspended in ascites fluid and controlled for this possibility by use of R-R and primary tumor/primary ascites cohorts. Our studies involved women who developed malignant ascites and thus may represent a subset of all patients with HGSC. Ascites is common at presentation in ovarian cancer and especially so in progressive disease (52); therefore, our analysis is likely to be broadly applicable to HGSC patients.

The most frequent genomic change we observed in S-R pairs was at 2q21.2, with recurrent deletion focused at *LRP1B*. Although the number of samples was small, several lines of evidence favor *LRP1B* as the driver of the 2q21.2 event, including interstitial deletions in some cases, loss of RNA expression, and functional data that were consistent with patient data. *LRP1B* is a member of the LDL receptor family and is among the top 10 genes mutated in human cancer (41). Its large size and location near the FRA2F fragile site (53) may contribute to a high mutation rate as a passenger event; however, several observations favor *LRP1B* being a *bona fide* driver mutation in human cancer, including a very high frequency of homozygous deletions (54), frequent point mutation in lung cancer (55, 56) and melanoma (57), and promoter methylation in esophageal (58), oral (59), thyroid (60), and gastric cancer (45). Although located adjacent to a fragile site, FRA2F is the least sensitive fragile site in the aphidicolin fragility assay and scored highest in a computational measure for homozygous deletion selection pressure (54). *In vitro* data also supports a role for *LRP1B* as a tumor suppressor with LRP1B interacting with uPAR to inhibit cell migration (61). Overexpression of LRP1B inhibits cell growth and colony formation *in vitro* and tumor formation in nude mice (45).

A mouse mammary tumor model of *Trp53/Brca1* mutation was found to have internal deletion of *Lrp1b* at exons 4–11 (62). In this study, we identified internal deletions of *LRP1B* with a minimal amplicon boundary targeting a region of 0.19Mb, encompassing exons 12–22. Similar to our findings, an analysis of a panel of human cancer cell lines revealed an internal homozygous deletion rate of 4.2%, some of which generated

in-frame fusions and others that resulted in truncated protein (62). Copy number analysis of our cases suggests a heterozygous loss; however, analysis of gene expression shows a very substantial reduction in all cases and in some, almost complete loss of *LRP1B* expression. Importantly, we validated expression loss in resistant samples from patients who were initially responsive to treatment using an independent data set. Furthermore, *LRP1B* loss does not seem to be a general feature of ascites, as it was not identified in R-R and primary tumor/primary ascites cohorts. Gain and loss of function experiments *in vitro* were consistent with *in vivo* data, with the effect specific to liposomal doxorubicin treatment. In these studies, doxorubicin acts as a valuable control, especially given the effect that alteration of *LRP1B* expression has on cell growth and division.

LRP1B binds to fibrinogen and apoE-containing ligands (63) and importantly, several studies have suggested that LDL receptor family members are involved in uptake of anionic liposomes and drugs (48, 64). Primary chemotherapy treatment was predominantly a combination of carboplatin/paclitaxel while relapse patients were exposed to a variety of agents, one of the most common being liposomal doxorubicin. Of note, all patients with *LRP1B* deletion in our series received liposomal doxorubicin treatment, with no clinical response observed. We propose that against a background of high mutation frequency, cells with *LRP1B* loss may be readily selected for during liposomal doxorubicin treatment, providing an additional opportunistic advantage to the tumor. As such, testing for *LRP1B* loss in the clinical setting may be a useful predictor of response to liposomal doxorubicin, although the assay would need to be sensitive enough to detect rare existing clones that could rapidly contribute to the emergence of resistance. The large size of the *LRP1B* gene will add additional challenges to mutation scanning. An investigation of a possible role of LRP1B in resistance to other chemotherapeutic agents commonly used in late-stage ovarian cancer treatment is warranted.

To date most studies investigating acquired chemoresistance in ovarian cancer have been undertaken *in vitro*, with few studies validated in patient material. However, a number of mechanisms of resistance have been identified recently using samples from women with recurrent disease, including HDAC4/STAT1 activation (25), DNA-PK/AKT activation (26), and *BRCA1/2* germ line mutation reversion (24). Due to high degree of intratumoral heterogeneity and the large number of chemotherapeutic agents commonly used in the relapse setting in HGSC patients, it is likely that there will be multiple mechanisms of acquired resistance. Our data indicates that *LRP1B* loss contributes to the emergence of resistance to chemotherapy, specifically to liposomal doxorubicin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.A. Cowin, S. Fereday, E. Loehrer, C. Cullinane, S. Ftouni, L. Galletta, M.S. Anglesio, J. Hendley, L. Bowes, K.E. Sheppard, E.L. Christie, The Australian Ovarian Cancer Study, R.B. Pearson, P.R. Harnett, V. Heinzelmann-Schwarz, M. Friedlander, M. Quinn, A. deFazio
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.A. Cowin, J. George, P.V. Loo, M.S. Anglesio, P. Campbell, A. deFazio, D.D.L. Bowtell
Writing, review, and/or revision of the manuscript: P.A. Cowin, S. Fereday, P.V. Loo, C. Cullinane, D. Etemadmoghadam, K.E. Sheppard, E.L. Christie, R.B. Pearson, P.R. Harnett, V. Heinzelmann-Schwarz, M. Friedlander, O. McNally, M. Quinn, P. Campbell, A. deFazio, D.D.L. Bowtell
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LRP1B Deletion in High-Grade Serous Ovarian Cancers Is Associated with Acquired Chemotherapy Resistance to Liposomal Doxorubicin

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