Mutation of ERBB2 Provides a Novel Alternative Mechanism for the Ubiquitous Activation of RAS-MAPK in Ovarian Serous Low Malignant Potential Tumors

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Abstract

Approximately, 10% to 15% of serous ovarian tumors fall into the category designated as tumors of low malignant potential (LMP). Like their invasive counterparts, LMP tumors may be associated with extraovarian disease, for example, in the peritoneal cavity and regional lymph nodes. However, unlike typical invasive carcinomas, patients generally have a favorable prognosis. The mutational profile also differs markedly from that seen in most serous carcinomas. Typically, LMP tumors are associated with *KRAS* and *BRAF* mutations. Interrogation of expression profiles in serous LMP tumors suggested overall redundancy of RAS-MAPK pathway mutations and a distinct mechanism of oncogenesis compared with high-grade ovarian carcinomas. Our findings indicate that activating mutation of the RAS-MAPK pathway in serous LMP may be present in >70% of cases compared with ~12.5% in serous ovarian carcinomas. In addition to mutations of *KRAS* (18%) and *BRAF* (48%) mutations, *ERBB2* mutations (6%), but not *EGFR*, are prevalent among serous LMP tumors. Based on the expression profile signature observed throughout our serous LMP cohort, we propose that RAS-MAPK pathway activation is a requirement of serous LMP tumor development and that other activators of this pathway are yet to be defined. Importantly, as few nonsurgical options exist for treatment of recurrent LMP tumors, therapeutic targeting of this pathway may prove beneficial, especially in younger patients where maintaining fertility is important. (Mol Cancer Res 2008;6(11):1678–90)

Introduction

Malignant ovarian epithelial tumors account for ~90% of all ovarian cancers, and these, in turn, represent the fifth most common cause of cancer deaths in women in Western countries (1). The high mortality rate associated with such cancers is due, in part, to their frequent diagnosis in advanced clinical stage, with spread beyond the ovaries. Ovarian epithelial tumors are a heterogeneous group of neoplasms, comprising benign adenomas, LMP, and, frankly, malignant tumors with several major histologic subtypes, including serous, clear cell, endometrioid, and mucinous cancers. Of these, the most common are serous carcinomas, accounting for >50% of diagnosed cases and generally thought to arise from the single epithelial cell layer that lines the ovary or from inclusion cysts lined with these same cells within superficial ovarian cortex (2).

Approximately, 10% to 15% of serous tumors are classified as "proliferating," "borderline," or of low malignant potential (LMP; ref. 3). LMP tumors display atypical epithelial proliferation with nuclear atypia and frequent extraovarian lesions (so-called "implants") in the peritoneal cavity and lymph nodes yet lack (by definition) destructive stromal invasion in the notional primary neoplasm. They are considerably less aggressive than high-grade serous carcinomas (4), and progressive disease is dependent on the presence and features of the extraovarian lesions—the true nature of which, therefore,

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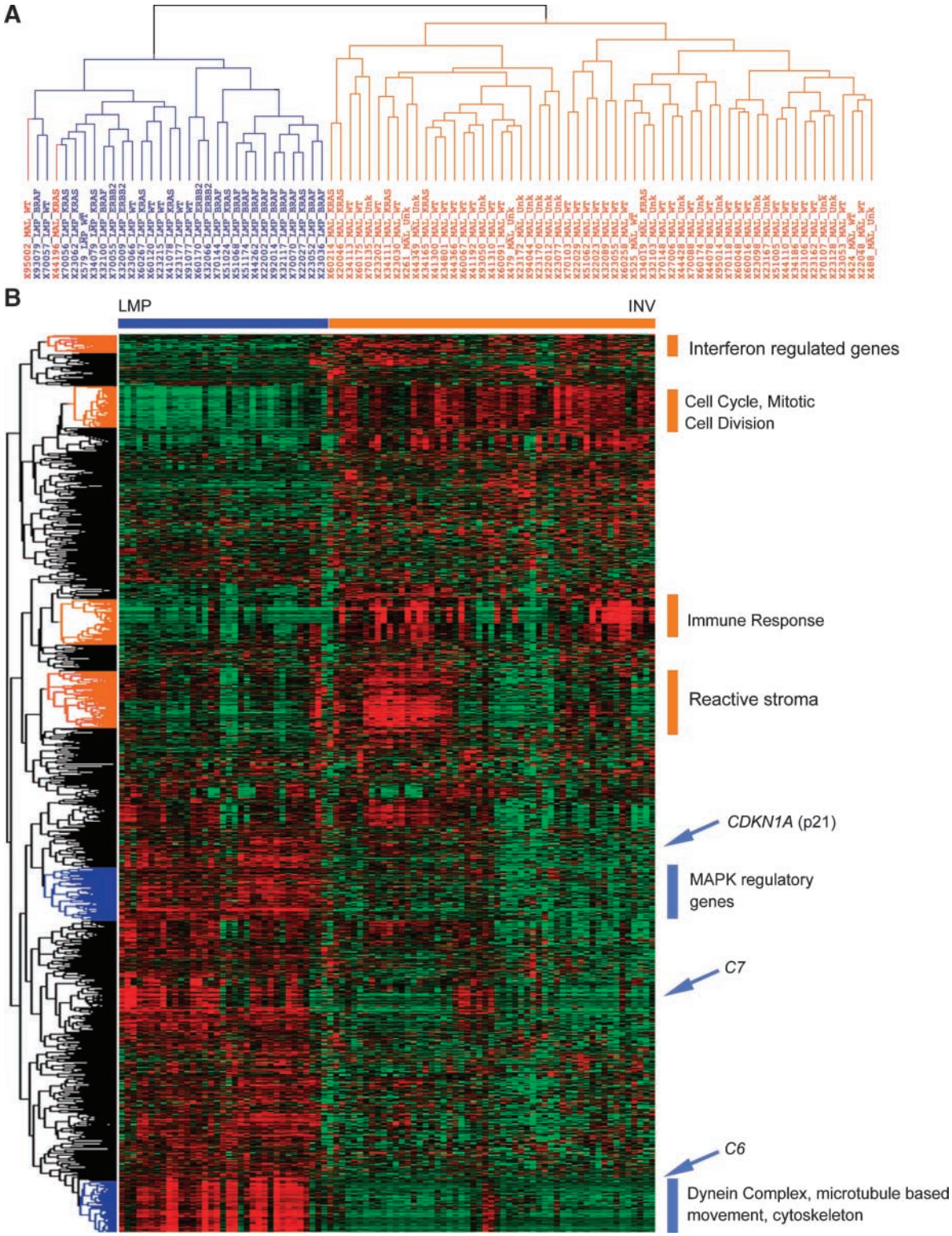

FIGURE 1. Invasive serous ovarian carcinomas and serous LMP tumors readily segregate by gene expression patterns. **A.** Experimental samples from 90 tumors were sorted by unsupervised hierarchical clustering of expression measurements made on 2,947 probes and depicted as a dendrogram with all experiment ID numbers. Samples are color coded for their corresponding pathologic diagnosis: LMP, blue; Invasive (*INV*), orange. **B.** A gene expression matrix, heat map. Coregulated gene clusters enriched for particular gene ontologies (as defined by ontology classification tools DAVID and Panther) are highlighted and labeled along the vertical axis.

remains controversial. Whereas the 5-year survival for patients diagnosed with advanced high-grade serous ovarian carcinoma is <45%, women with "high-stage" LMP tumors have a more favorable prognosis, with some reports describing 5-year survival as high as 95% (3). Serous LMP tumors typically respond poorly to chemotherapy and are primarily managed surgically. Women affected by LMP tumors are, on average, younger than those affected by high-grade ovarian carcinomas (5). Pathologic evaluation of serous LMP tumors has identified some predictive features for the recurrence of disease, poor survival, and progression. Specifically, the presence of (micro)-invasive versus noninvasive peritoneal implants suggests an aggressive phenotype (6). Similarly, the so-called "micropapillary" pattern within the primary tumor equates with high-grade change in the spectrum of serous LMP tumors and increases the likelihood of an invasive extraovarian disease and a worse clinical course. However, some debates still exist regarding the clinical significance of such histopathologic features (5).

Recently, our group and others have begun investigating the molecular profile of serous LMP tumors compared with the more common higher-grade serous carcinomas (7-10). Overall, the results have suggested that LMP tumors follow a different process of cellular transformation compared with the majority of their invasive counterparts. One striking feature of LMP tumors is the high rate of *KRAS* and *BRAF* mutations, both of which are thought to act primarily through the canonical RAS-MAPK (RAS-RAF-MEK-ERK-MAP kinase) pathway (3, 7, 10-13). Furthermore, many serous LMP tumors have been reported to have wild-type p53 and functional p53 signaling (8, 14). It has been proposed that some serous LMP tumors progress to noninvasive or invasive micropapillary serous carcinomas, which may or may not progress further to become a high-grade invasive serous carcinoma (reviewed in refs. 13, 15). However, most high-grade serous carcinomas are thought to arise without a morphologically recognizable precursor lesion, apparently evolving *de novo* and metastasizing early in their course. In addition, some controversies also exist as to the cell of origin of high-grade ovarian serous carcinomas, with several recent studies suggesting that some tumors, designated as primary ovarian, may arise from the epithelium of the fimbria of the fallopian tube (16, 17).

High throughput methods, such as cDNA and oligonucleotide microarrays, are increasingly being used to systematically compare molecular features of individual cancers to key clinical variables. Here, we used Affymetrix HG-U133 2.0plus expression arrays to interrogate the expression profile of LMP and invasive serous ovarian carcinomas. Expression profiles were compared with tumor class, stage, grade, and mutation status. Our data highlight distinct pathways that serve to differentiate invasive and LMP tumors and confirm previously published findings. We suggest that the loss of p53 functionality is crucial for the progression of high-grade serous carcinomas. Furthermore, we have explored, in detail, the similarity of expression profiles between various RAS pathway mutations within serous LMP tumors. These profiles suggest an overall redundancy between these mutations and a distinct mechanism of oncogenesis from more common high-grade serous ovarian carcinomas. We propose that RAS pathway activation is a major contributor to LMP tumor development, and, in addition to *KRAS* and *BRAF* mutations, *ERBB2* and other yet to be described mutations or perturbations of the RAS-MAPK pathway occur in all LMP tumors.

Results

Comparison of Expression Profiles between LMP and Invasive

Gene expression patterns were measured in a cohort of 30 serous LMP tumors and 60 high-grade invasive serous ovarian carcinomas using Affymetrix HG-U133 2.0plus arrays. Data were normalized and filtered, resulting in 2,947 informative probes (Supplementary Table S1). Expression levels of the filtered genes were used for unsupervised clustering and yielded a dendrogram with two distinct arms segregating LMP and invasive tumor almost perfectly (Fig. 1A). Visual inspection of the overall clustered data was combined with ontology analysis, using DAVID/EASE (18), Panther Ontology (19), and Gene Set Enrichment Analysis (GSEA; ref. 20) classification tools, to identify functional groupings (see also Supplementary Materials and Methods).

Distinct patterns of gene expression were evident between LMP and invasive tumors when using hierarchical clustering (Fig. 1B) and performing an analysis of gene ontologies. Consistent with previous reports (9, 21), a group of cell cycle–related genes (defined by Panther and DAVID/EASE) were overrepresented in a region of the cluster heatmap associated with higher gene expression in invasive carcinomas compared with LMPs (Fig. 1B and Supplementary Fig. S1A). These patterns of differential expression and group enrichment were also noted in the heatmap for immune and IFN response-related genes. GSEA analysis also showed overrepresentation of several cell cycle and DNA replication–related pathways (see Supplementary Table S2 for gene sets, enrichment scores, and *P* values). Similar with our recent analysis of a large cohort of serous invasive cancers (22), we also noted that a subset of invasive cancers had higher expression of markers associated with reactive myofibroblasts (reactive stroma; Fig. 1B and Supplementary Fig. S1B). These makers were largely absent from LMP samples, despite them frequently containing abundant stromal material. By contrast, LMP tumors differentially expressed higher levels of genes associated with MAPK signaling and feedback regulation, including *DUSP4, DUSP6, SPRY2, SERPINA1*, and the p38/c-Jun NH_2 kinase activating kinase (JNK) *MAP3K5* (Fig. 1 and Supplementary Fig. S2; GSEA analysis in Supplementary Table S3). To verify that the enrichment and overexpression of RAS-MAPK–related transcripts were, in fact, up-regulation in LMP tumors rather than a loss of these genes in invasive carcinomas, we compared our LMP dataset to normal ovarian surface epithelium data from Bonome et al. (8). We found that RAS-MAPK pathway genes (enriched gene lists derived from Panther, DAVID, and GSEA) were highly overrepresented among genes that were overexpressed (2-fold greater expression, $q < 0.05$) in serous LMP compared with ovarian surface epithelium, with a statistical significance of $P < 2.2e{-}16$ (Fisher exact; see also Supplementary Fig. S3).

In addition, components of the alternative Complement pathway, including C6 and C7 (Supplementary Table S3), and genes associated with Dynein motor complex/microtubule-based movement/cytoskeleton were abundant (Fig. 1 and Supplementary

TABLE 1.

A. LMP cases in expression array analysis by mutation status

LMP cohort	All cases		BRAF		KRAS		ERBB2		WT	
	$n = 30$	%	$n = 12$	%	$n = 7$	%	$n = 4$	%	$n = 7$	%
Grade										
Low	12	40.0	4	33.3	3	42.9	1	25.0	4	57.1
High	13	43.3	6	50.0	1	14.3	3	75.0	3	42.9
NK	5	16.7	2	16.7	3	42.9	0	0.0	0	0.0
Stage										
I	14	46.7	8	66.7	2	28.6	2	50.0	2	28.6
II	5	16.7	1	8.3	2	28.6	1	25.0	1	14.3
III	10	33.3	3	25.0	2	28.6	1	25.0	4	57.1
IV	1	3.3	0	0.0	1	14.3	0	0.0	0	0.0
Implants										
Microinvasive	5	16.7	2	16.7	0	0.0	3	75.0	0	0.0
Noninvasive	11	36.7	2	16.7	4	57.1	0	0.0	5	71.4
Negative	8	26.7	5	41.7	0	0.0	1	25.0	2	28.6
NK	6	20.0	3	25.0	3	42.9	0	0.0	0	0.0
Micropapillary										
Positive	10	33.3	3	25.0	0	0.0	3	75.0	4	57.1
Negative	14	46.7	7	58.3	3	42.9	1	25.0	3	42.9

B. Invasive cases by expression array clustering and mutation

Invasive cohort	All cases		Primary Cohort		LLI		BRAF/KRAS mutation positive	
	$n = 210$	%	$n = 60$	%	$n = 8$	%	$n = 7$	%
Grade								
1	4	1.9	0	0.0	1	12.5	0	0.0
2	57	27.1	13	21.7	4	50.0	4	57.1
3	145	69.1	45	75.0	1	12.5	2	28.6
NK	4	1.9	2	3.3	2	25.0	1	14.3
Stage								
I	9	4.3	2	3.3	2	25.0	1	14.3
II	9	4.3	2	3.3	0	0.0	0	0.0
III	174	82.9	47	78.3	6	75.0	6	85.7
IV	17	8.1	9	15.0	0	0.0	0	0.0
NK	1	0.5	0	0.0	0	0.0	0	0.0
Area of LMP noted								
Positive	11	5.2	4	6.7	8	100.0	2	28.6
Negative	22	10.5	11	18.3	0	0.0	1	14.3
NK	177	84.3	45	75.0	0	0.0	4	57.1

NOTE: NK, not known or information is not available.

Fig. S2). The complete SAM analysis (23) of differentially expressed genes is provided in Supplementary Table S4A (see also Supplementary Materials and Methods). GSEA "leading edge" analysis of enriched ontologies failed to reveal genes common between overrepresented gene sets (not shown), suggesting that the different gene sets are functionally independent.

Analysis of gene ontologies also identified an overrepresentation of p53-related genes differentiating invasive and LMP tumors, as defined by Panther ontology ($P = 0.00205$; see also Supplementary Fig. S5A). Although, p53 mutation status was not directly tested, a p53-responsive gene, such as *CDKN1A* ($p21^{CIP1}$), and proapoptotic genes, such as *FAS* and *TNFR10B*, were strongly expressed in LMP tumors. In contrast, invasive tumors differentially expressed higher levels of p53-related prosurvival and cell cycle–driving genes (Fig. 1 and Supplementary Fig. S5A; Supplementary Table S4A). Consistent with these findings, a 32–probe gene expression signature, associated with p53 mutation status and outcome in breast cancer (24), was able to clearly segregate LMPs from invasive tumors in our cohort through unsupervised clustering (Supplementary Fig. S5B). Statistical significance for this association was established using a Monte Carlo simulation, wherein we randomly selected 32 gene sets and clustered the samples using the same method. Of 100,000 random gene set selections, only 20 were able to obtain an association as significant as or better than that obtained when using the Miller et al. set ($P = 0.0002$). Overall, these findings strongly suggest that p53 function is preserved in LMP tumors and that disruption of this pathway plays a major role in the pathology of invasive tumors.

Correlation of Clinical Variables to LMP Expression Profiles

Tumor stage and grade, as well as the presence of microinvasive implants and micropapillary features, were used to group the LMP tumors (Table 1A) and compare expression profiles to find candidate genes related to various aspects of serous LMP tumor pathobiology. These features have been used to predict a more aggressive course of disease (6, 25). However, statistical analysis using a stringent cutoff of 2-fold change and a q value of <0.05 was unable to identify any candidate

gene expression changes when classifying based on stage, invasive implants, or micropapillary features (see also Supplementary Materials and Methods). When the analysis criteria were relaxed ($q < 0.1$), there were a small number of genes correlated with grade, but not for other clinical variables (see Supplementary Table S4C). Our serous LMP dataset was approximately equally split into high-grade ($n = 13$) and low-grade ($n = 12$) samples. Eight genes that were overexpressed in high-grade serous LMP tumors were identified (Supplementary Table S4C), including *LST1*, which is involved in the enhancement of MEK and ERK1/2 signaling through targeting of ERK-MAPK pathway components to the actin cytoskeleton (26); *PDZK1IP1* (MAP17), which may act to bypass tumor necrosis factor (TNF)–induced G_1 arrest (27); and *GPNMB* (Osteoactivin). Osteoactivin is expressed in advanced malignancies of varying origins (28) and enhances metastatic behavior in human glioma cell line models (29).

Identification of LMP-like Invasive Tumors

Although unsupervised clustering of samples yielded an almost perfect segregation of LMP and malignant carcinoma (invasive tumors), we observed two serous carcinoma samples with significant similarity to LMP tumors (Figs. 1A and 2A). To determine whether additional serous invasive samples with

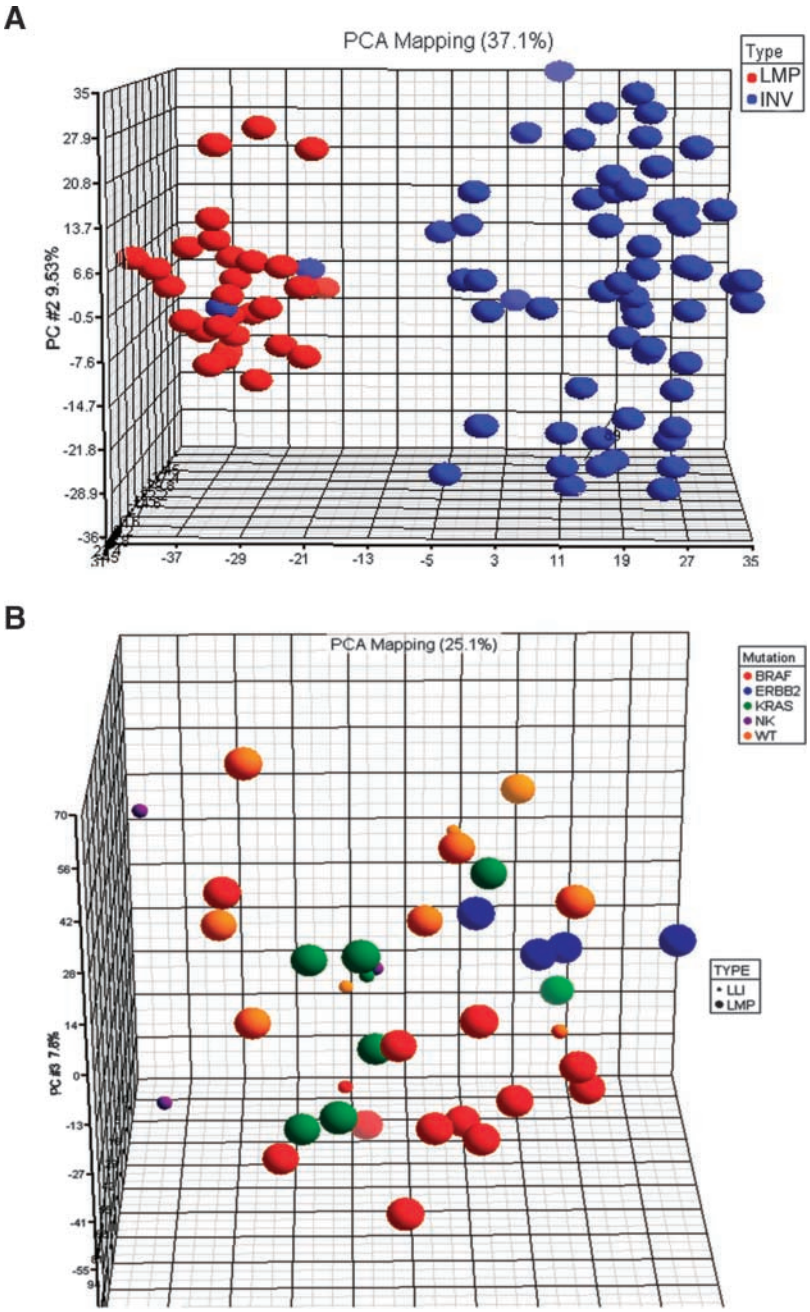

FIGURE 2. Principal component analysis (*PCA*) of the expression dataset. **A.** Three-dimensional visualization of principal components suggests a distinct separation between LMP and invasive (*INV*) serous ovarian tumors. Two LLI tumors are visible within the cluster of LMPs. **B.** Principal component analysis (*PCA*) plot focusing on LMP (30 larger spheres) and LLI (8 smaller spheres) tumors, color coded by mutation status. A great deal of overlap between mutants and wild-type tumors is observed; no clear segregation is identifiable. NK, unknown/untested; WT, wild-type for all tested genes. Specific gene expression patterns contributing to the variation are further discussed in the text.

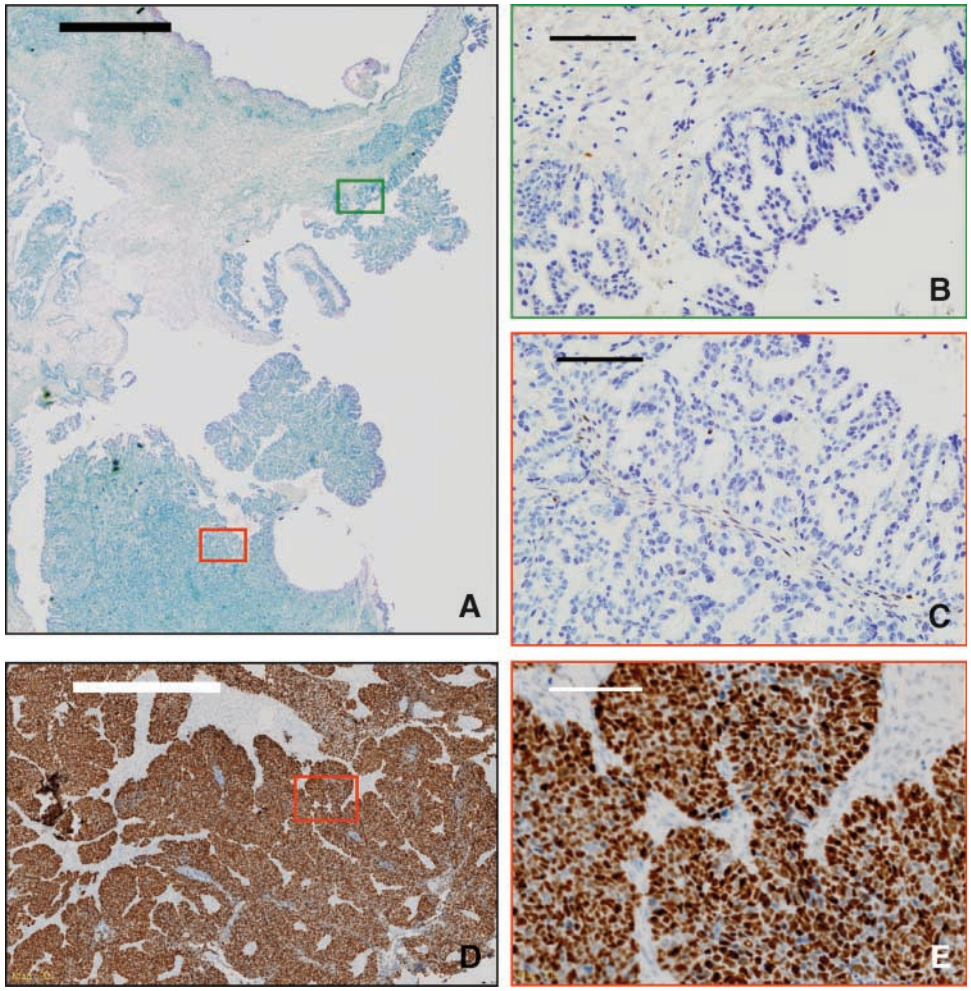

FIGURE 3. Tissue sections from a representative LLI tumor and an invasive ovarian serous carcinoma were immunostained for p53. **A.** Low power magnification of LLI tumor 32054 with regions characteristic of both LMP and more invasive histology. Staining seems to be negative overall, with no suggestion of focal positivity or high-intensity staining across the whole section. **B.** High-power magnification of the green box-marked region from **A**, representing LMP-like histology in this sample. **C.** High-power magnification of the red box-marked region from **A**, representing invasive histology in this sample. **D** and **E.** Low-power and high-power magnification of an invasive serous ovarian carcinoma (70054 positive control) tumor section illustrating high-level positive immunostaining for p53. *Scale bars,* 1 mm in low power or 100 μm in high power.

an LMP-like molecular expression signature existed in other datasets, we developed a classifier using our cohort of 30 LMP and 60 invasive tumors. Applying the classifier to an independent validation set of 150 invasive serous tumors (22) identified a further six invasive tumors with an expression profile highly similar to that of the LMP tumors (see also Supplementary Materials and Methods; Fig. 2B). Consistent with the gene expression data, detailed pathology review of the tumor specimens revealed that all identified LMP-like invasive (LLI) tumors contained regions of noninvasive LMP tumor (Table 1B). In addition, for five of the eight LLI samples, frozen sections adjacent to the array-sampled material was available for detailed examination. All of the adjacent sample material showed varying amounts of characteristic LMP histology: 70% LMP or more in four of five cases and ~40% in the remaining case. The majority of LLI tumors were of moderate to high histologic grade (five of eight, 63%), and although the overall clinical behavior of these cases was more typical of invasive serous ovarian cancer than LMP, patients with LLI tumors had a more favorable progression-free survival (log-rank test, $P = 0.1$) and overall survival ($P = 0.095$). However, there were very few patients in the LLI group, and this association was not statistically significant.

Previous studies have sought to compare serous LMP and invasive tumors to gain insight into determinants of malignancy. However, the very substantial differences in gene expression between serous LMP and invasive tumors, observed in this study and others (8-10), confound such analyses. The identification of LLI tumors created the opportunity to examine gene expression differences between tumors that are very similar molecularly but differ phenotypically in terms of their invasiveness. However, supervised analysis failed to identify any differentially expressed genes between LLI and LMP tumors (see Supplementary Materials and Methods). A less stringent statistical analysis ($q < 0.05$, no fold change cutoff) identified a small list of genes that were differentially expressed (Supplementary Table S4), including *TIMP3*, which was more strongly expressed in LLI tumors. Previous reports have suggested that mouse embryonic fibroblasts and mouse mammary epithelial cells deficient in *TIMP3* have increased β-catenin signaling (30). In addition, TIMP3 is normally down-regulated by the activated RAS pathway and, notably, ERBB2 overexpression (31). Given our findings suggesting differential p53 pathway activation in LMP and invasive ovarian carcinomas, we examined p53 immunohistochemical staining in LLI samples, for which formalin-fixed, paraffin-embedded

TABLE 2. Mutation Frequencies in Ovarian Tumors

Gene	Mutation*	LMP			LLI	Invasive
		Serous	Mucinous	Other		
BRAF	c.1799T>A	43/95 (45%)	2/38 (5%)	0/1 (0%)	1/5 (20%)	0/40 (0%)
	c.1790T>G †	1/31 (3%)	0/22 (0%)	0/1 (0%)	N/D	N/D
KRAS	c.35G>A	11/95 (12%)	8/38 (21%)	0/1 (0%)	1/5 (20%)	0/40 (0%)
	c.35G>T	6/95 (6%)	7/38 (18%)	0/1 (0%)	0/5 (0%)	5/40 (12.5%)
	c.34G>T †	0/31 (0%)	1/22 (4.5%)	0/1 (0%)	N/D	N/D
ERBB2	Mutant ‡	5/84 (6%)	0/25 (0%)	N/D	N/D	0/39 (0%)
EGFR	Mutant	0/28 (0%)	0/21 (0%)	N/D	N/D	0/38 (0%)

Abbreviation: N/D, not done.
*Mutations are numbered from National Center for Biotechnology Information RefSeq accession NM_004333.4 (*BRAF*) and NM_033360.2 (*KRAS*).
†These cases were identified by sequencing and not by allele-specific PCR.
‡Refer to Table 3.

(FFPE) tissue was available (five of eight samples). Although all five of the available FFPE sample sections were representative, to some degree, of both LMP and invasive histology, one sample (51104) seemed to be predominantly invasive histology, whereas another (34207) was predominantly LMP. Staining for p53 was weak and sporadic or completely negative in all available LLI samples (Fig. 3 and Supplementary Fig. S4). In particular, we noted the staining to be consistent across each sample, whether in a region representative of LMP or more invasive histology (Fig. 3). These results were consistent with our molecular expression profiling and those reported in the literature for overall p53 status of serous LMP (32).

Mutation Analysis of Ovarian LMP and LLI Tumors

To allow us to determine whether the expression profiles of the LMP tumors were influenced by mutations of genes within the canonical RAS-MAPK pathway, we first screened a panel of 134 LMP tumors (Table 2) for the most common mutational sites in *BRAF* and *KRAS* (c.1799T>A *BRAF* and c.35G>A and c.35G>T *KRAS*). Overall, we found 46 cases (34%) with a *BRAF* mutation and 33 (25%) with a *KRAS* mutation. The *BRAF* mutations were found mainly in the serous LMP cases (44 of 95, 46%), but there were also two mucinous LMP cases with *BRAF* mutations (2 of 39, 5%; $P < 0.001$, Fisher exact test). *BRAF* mutations have not been previously reported in mucinous LMP cancers despite specific investigations (33, 34) and were, therefore, confirmed by resequencing of repeat sections after additional pathology review. *KRAS* mutations were more common in mucinous (15 of 38, 39%) than the serous (18 of 95, 19%) LMP cases ($P = 0.01$, Fisher exact test). There were no cases with both *BRAF* and *KRAS* mutations ($P < 0.001$, Fisher exact test).

Mutation analysis was carried out on the LLI tumors for which DNA was available (five of eight), and an additional 40 invasive tumors were selected at random to determine whether there was a higher incidence of *BRAF* and *KRAS* mutations in the LLI group compared with the other invasive serous tumors (Table 2). Overall mutations were more frequent in our small LLI tumor cohort compared with invasive serous samples (2 of 5, 40% versus 5 of 40, 12.5%). Interestingly, a single c.1799T>A *BRAF* mutation was observed in the LLI group, and this type of mutation has not previously been reported among invasive serous ovarian tumors. It must further be noted that this case did contain coexisting LMP and invasive histology and that the DNA tested for mutation was sampled from a region of primarily LMP (~70%).

LMP Tumors Share a Similar Expression Profile Regardless of the Nature of the RAS-MAPK Pathway Mutation

To further understand the significance of RAS-MAPK pathway mutations within LMP tumors, we compared the expression profiles of the 30 LMPs we had analyzed using expression arrays and on which the *BRAF* and *KRAS* mutation status was known. A number of analyses were done, including comparison of tumors that were mutant for either *BRAF* or *KRAS* versus wild-type tumors. Results are summarized in Supplementary Table S4 (see also Supplementary Materials and Methods). The number of samples in each group influenced our findings: the most significant difference in gene expression observed was between wild-type and *BRAF* mutant serous LMP cases, with 263 unique genes identified as differentially expressed. Validation of the microarray expression profiling was obtained using real-time reverse transcription–PCR analysis for selected genes that were differentially expressed between the BRAF and wild-type LMP tumors (Supplementary Fig. S6). Analysis of gene ontology indicated enrichment for genes involved in signal transduction, although without indication of a specific signaling pathway or process. A comparison was made to publicly available gene lists of differentially expressed genes in other cancers (35-38) or model systems (39-41) harboring mutant *RAS* or *BRAF*. We found little concordance between previously reported *RAS* or *BRAF* signatures and the genes we found to be differentially expressed between *BRAF, KRAS*, and wild-type LMP tumors (data not shown). Conversely, previously defined *BRAF* or *KRAS* gene signatures were unable to separate LMP tumors based on their mutation status (data not shown). Manual inspection of the gene lists and Pubmed/Pubmatrix[10] literature searches suggests that there are, in fact, some differences between the tumors with *KRAS* or *BRAF* mutations. In fact, similar investigations had been done by other groups using "metagene" analysis to separate cells based on oncogenic transformation of various oncogenes, including *HRAS, MYC*, and *ERBB2* (42). However, having applied a number of ontology classification schemes to our lists

[10] http://pubmatrix.grc.nia.nih.gov/

TABLE 3. Somatic Mutations of ERBB2 in Ovarian Tumors of Low Malignant Potential

Case	Tumor type	Nucleotide*	Amino acid*
32009	Serous LMP	c.2325dupTACGTGATGGCT	p.Try772_Ala775dup
32066	Serous LMP	c.2322dupGCATACGTGATG	p.Ala771_Met774dup
70055	Serous LMP	c.2322dupGCATACGTGATG	p.Ala771_Met774dup
92.060	Serous LMP	c.2324dupATACGTGATGGC	p.Try772_Ala775dup
60170	Serous LMP	c.2324dupATACGTGATGGC	p.Try772_Ala775dup

*Changes are given based on National Center for Biotechnology Information RefSeq accession NM_004448.2. The changes to the nucleotide sequence have been experimentally determined by nucleotide sequencing, whereas the changes to the amino acid sequence are theoretically deduced.

of genes generated from one mutation versus another, we were unable to pick up overrepresentation of RAS or alternate transforming pathways specific to *KRAS* versus *BRAF* mutations.

RAS-MAPK Activating ERBB2 Mutations in Serous LMP Tumors

Whereas LMP tumors overexpressed genes related to RAS-MAPK pathway compared with the majority of invasive tumors, the limited ability to detect a differential RAS signature between wild-type and mutant LMP tumors suggested that, apparently, wild-type LMP tumors may harbor other RAS-MAPK pathway–activating events. Regulators of RAS-MAPK signaling have been well described. In particular, the epidermal growth factor (EGFR) family of receptor tyrosine kinases represents one of the most studied groups of upstream activators (43, 44). We chose, specifically, to examine the *ERBB2* and *EGFR* genes. EGFR (ERBB1) and its homologue ERBB2 (HER2-neu) activate RAS; hence, the RAS-MAPK pathway via the adaptor protein growth factor receptor binding protein 2 (GRB2; refs. 44, 45). Mutations in the tyrosine kinase domain of *EGFR* have been found in 8% of non–small cell lung cancers (46, 47) and 3.5% of invasive ovarian cancers (48). Similar mutations have been found in *ERBB2* in 1.6% to 4% of non–small cell lung cancers (49, 50) and a small number of ovarian tumors (50-52), including 2 of 21 serous LMP tumors (52). We therefore screened for mutations in EGFR family genes. The *ERBB2* kinase domain was screened by DHPLC in a subset of 109 LMP tumors and 39 invasive ovarian carcinomas, for which sufficient DNA was available. Heterozygous somatic mutations were discovered in 5 of 84 (6%) serous LMP ovarian tumors [or 5 of 109 (5%) of all screened LMP tumors (Tables 2 and 3)]. These mutations were tumor specific and were not present in the corresponding germline DNA. All *ERBB2* mutations were found to be mutually exclusive of the *BRAF* and *KRAS* mutations ($P = 0.0117$, Fisher exact test), as has been reported previously (52). Furthermore, all *ERBB2* mutations were in-frame insertion/duplications of 12 nucleotides in exon 20. There were two cases with identical duplication at position 2322, two cases with identical duplication at position 2324, and one case with a duplication at position 2325. All insertions/duplications overlapped a central core of nine nucleotides TACGTGATG, and all have previously been reported to occur in non–small cell lung cancers (COSMIC[11]).

The tyrosine kinase domain of the *EGFR* gene was also screened by DHPLC in 49 LMP ovarian tumors and 38 invasive ovarian carcinomas, but no somatic mutations were found (data not shown).

In keeping with our expression profile analysis of LMP tumors, *ERBB2* mutant tumors also illustrated a very similar expression profile to other LMP tumors (Figs. 1 and 2). Furthermore, principal component analysis plots of expression profile data between the various mutation groups of LMP and LLI tumors show a very tight grouping without obvious separation between classes (Fig. 2B).

Discussion

This study provides additional insight to our current understanding of ovarian serous LMP tumors. We have confirmed many previous observations but have also showed unique features of LMP tumors that have not previously been discussed and provide further support for the differential oncogenic evolution of serous LMP and high-grade serous carcinomas. We have shown the enrichment of specific clusters of coregulated genes (such as Dynein motor complex–related genes) in LMP tumors and also the enrichment of p53 pathway members in molecular differentiation of LMP from high-grade carcinomas. The loss of function of p53 in high-grade serous ovarian tumors seems to be critical and contrasts its apparent retention in serous LMP tumors. Immunostaining of p53 (as a proxy of mutation) has previously shown to differ between LMP and invasive serous ovarian tumors (32) and is consistent with the evidence of functional p53 signaling observed in LMP tumors in this study and in a study by Bonome et al. (8). Sherman et al. (5) have suggested that this could be explained, at least in part, through a progressive model for LMP development into a more invasive form. However, it may equally support the argument for a differential pathway of development between serous LMP and high-grade serous ovarian carcinomas, as has been suggested by other groups (13).

Other groups of functionally related and enriched gene sets included Complement pathway components C6 and C7. Relatively high-level expression of C7 has been previously reported in LMP tumors (53). Of note, both LMP and invasive tumors in our dataset seem to express different negative regulators of the Complement pathway membrane attack complex assembly, including *CD55* in LMP and *FHL1* in invasive tumors; both of these have been observed in other tumor systems (54). A particularly strong group of coregulated genes in LMP tumors included a number of Dynein motor complex components. It is known that Dynein motor complex genes take part in axonemal movement, as well as intracellular retrograde transport (reviewed in refs. 55, 56). Cilia are present in normal adult ovarian surface epithelium (57) and commonly observed in benign and LMP tumor cells (58). Previous literature has suggested retention of some basic expression pattern from the cell of origin to tumor tissue and even cell lines (59, 60); this may represent the gene expression pattern from a relatively well-differentiated ovarian surface epithelial cell. Although invasive serous tumors are also thought to arise from the ovarian surface epithelium, most lack expression of this Dynein cluster. It has been suggested that high-grade serous

[11] www.sanger.ac.uk/genetics.CGP/cosmic

carcinomas may, in fact, originate from a secretory cell within the fimbria of the fallopian tube, and not the ovarian epithelium. This is based on the localization of tubal carcinomas to the fimbria (17), the association of many peritoneal and ovarian serous carcinomas with the fimbriated end (61) and expression of (mutant) p53 in a secretory cell of the fimbria (16). Dynein motor complex involvement in retrograde transport should also be considered in the biology of LMP tumors as Dynein retrograde transport has been linked to MAPK [stress-activated protein kinase/c-Jun NH_2 kinase (SAPK/JNK)] signaling (62, 63). Although a number of key molecules implicated in control of this vesicle transport chain, including *DLK1* and *MAP3K5*, seem to be up-regulated in LMP tumors (versus high-grade invasive), the pathway has been mostly studied in the context of neuronal transport and axonal damage repair (64, 65), and its relation to tumor pathobiology has not been explored.

A high incidence of activating mutations in the RAS-MAPK pathway has commonly been found in LMP tumors (3, 7, 8, 11, 12), and we found either *BRAF* or *KRAS* mutations in the majority of LMP cases tested (59%, 79 of 134). We found *BRAF* mutations in 46% of serous LMP. This is higher than previous reports of ~30% of serous LMP tumors (33, 34, 66) and is probably explained by the high sensitivity of the allele-specific PCR we used. We found a much lower proportion of mucinous LMP cases with *BRAF* mutations (5%). *BRAF* mutations have not previously been reported in mucinous LMP tumors, although there is one report of a *BRAF* mutation in a mucinous invasive case (33). Previous studies of *KRAS* in LMP tumors have reported mutations in ~25% of serous and 50% to 80% of mucinous cases (34, 66, 67), which are comparable with our findings of *KRAS* mutations in 19% of serous LMP and 39% of mucinous LMP tumors. As we only screened the tumors for the most common mutations (*KRAS* c.35G>A and c.35G>T), it is possible that there may have been additional tumors with other less common mutations. As noted in other tumor types with common activating mutations along the RAS-MAPK pathway (35, 38, 68-70), these mutations are mutually exclusive in ovarian cancers. Overall these studies further support the notion that activating mutations of either *KRAS* or *BRAF* are sufficient to deregulate a common effector pathway, such as the MEK-ERK cascade.

We found that expression profiles within the whole group of LMP tumors were highly similar. Specific examination of the relatively few differentially expressed genes between known *BRAF* or *KRAS* mutants and the so-called wild-type LMP tumors could not identify any characteristics, pathways, or molecular functions to differentiate them. These differentially expressed genes probably represent bystander effects rather than transforming drivers. Taken together, we propose that perturbation of the RAS-MAPK pathway is common among all LMP tumors. However, the mechanism of the activation has not yet been completely defined and our confirmation of *ERBB2* mutations provides evidence of alternative yet functionally equivalent mechanism of activating this pathway in LMP tumors. *EGFR* and *ERBB2* have commonly been cited as amplified and overexpressed in cancer. However, the finding of activating mutations in these genes is more recent (reviewed in ref. 71). Functional studies by Wang et al. have shown that the $HER2^{YVMA}$ mutant protein has higher transforming ability than the wild-type HER2 (72). $HER2^{YVMA}$ protected cells from apoptosis has stronger catalytic ability and is more potent at associating with and activating EGFR in the absence of ligands. Furthermore, a majority of the *ERBB2* mutations in cancer have affected the αC-β4 loop (73), including those observed in this study: p.Try772_Ala775dup ($HER2^{YVMA}$) and the similar p.Ala771_Met774dup ($HER2^{AYVM}$). Fan et al. have investigated point mutation, truncations, and chimeric constructs of *ERBB2* and other HER family members to explore the function of the αC-β4 loop in regulation and autoinhibition of ERBB2 (74). Their results, along with the frequency and location of cancer-associated *ERBB2* mutations, strongly suggest that this domain is critical for the regulation (enhancement) of ERBB2 activity. In the case of ovarian serous LMP tumors, activation of the RAS-MAPK pathway is evident not only through activation of mutant RAS, BRAF, and ERBB2 but also in downstream activity of (phosphorylated) ERK (75).

Our data add to the mechanisms by which RAS pathway activation can occur in LMP tumors. The inability of our gene expression profiling studies to separate apparently wild-type and mutant tumors suggests that additional as yet undefined mechanisms exist to activate the RAS pathway in LMP tumors. Collectively, our findings suggest that RAS pathway activation is a constant feature of all LMP tumors. Despite the mutation of known oncogenes in LMP tumors, this group is characterized by generally indolent behavior. In examining this seemingly contradictory observation, Sieben et al. suggested that two genes (*DUSP4* and *SERPINA5*) within the LMP expression profile are integral in suppressing the transforming effect of RAS-MAPK pathway mutation in LMP (10). Although our data support an enrichment of both *DUSP4* and *SERPINA5* in LMP tumors, more detailed examination of our own data suggests a much broader representation of RAS-MAPK regulators. In addition to the above-mentioned genes, this also includes enrichment of the negative regulators of RAS-MAPK function: DUSP family members *1/4/5/6* (76), *TRIB2* (77), *RPS6KA2* ($p90^{RSK}$; refs. 78, 79), *SPRY2* (80), and *SPRY4* (81). It is unclear whether activation of these genes reflects constraint of RAS-MAPK–mediated tumor growth and invasion or a cellular response aimed to down-regulate chronic RAS pathway activation. Of particular interest, *MAP3K5 (ASK1)* is one of the genes at the core of the GSEA enrichment analysis for LMP tumors. MAP3K5 is an upstream activator of the parallel MAPK pathways linked to $p38^{SAPK/JNK}$ and antagonistic to ERK signaling (82), primarily leading to apoptosis, senescence, or differentiation (reviewed in ref. 83). It is possible that partial negative regulation of the RAS-MAPK pathway may result in a delicate balance between tumor growth while allowing LMPs with functional p53 to circumvent p53-mediated senescence or apoptosis that is typically associated with high-level RAS activation (84, 85).

There are currently few nonsurgical treatment options for LMP patients, as LMP tumors are not generally thought to be responsive to chemotherapy. Small molecule inhibitors of ERBB2 (epidermal growth factor family), RAS, MEK, or ERK may prove to be viable treatment options for patients with recurrent or progressive LMP tumors and, potentially, for young patients with LMP wherein preservation of fertility is an issue. In thyroid tumors with *BRAF* or *KRAS* mutations, the

MEK inhibitor CI-1040 has shown promise both *in vitro* and in mouse models (86). However, overall clinical trials of MEK inhibitors have given mixed results (87, 88), and any potential treatment options need to be weighed against the already favorable prognosis for LMP tumors and the presence of histopathologic features.

Clinical and pathologic features of LMP tumors that stratify tumors into subgroups were further examined for molecular signatures. Overall, this analysis was difficult due to a low number of samples, but some inferences can be made. For example, Sherman et al. suggested reclassifying LMPs with substantial micropapillary growth as carcinomas (5). Here, we have shown that the global expression signature between tumors with or without micropapillary growth is not significantly different, supporting the view that these tumors are closely related at a molecular level, although still distinct from high-grade serous ovarian carcinoma. This finding is in line with the progression model relating LMP with the micropapillary serous carcinoma subtype proposed by several studies (13, 15, 66, 89). Further exploration of the differences between indolent and aggressive LMP tumors may be possible as additional clinically annotated datasets, similar to that described here, are generated.

The discovery of LLI tumors is of further interest in defining progressive LMP tumors. Unsupervised hierarchical clustering identified eight LLI tumors; all of these tumors were reviewed in detail and contained coexisting invasive carcinoma and LMP histology. Specific review of the material directly adjacent to the array-sampled frozen tissue showed that most or all LLI samples included LMP components. In such cases, microdissection would be required to determine if, and to what extent, the LMP element may have obscured the expression signature of the invasive component of the LLI. However, more detailed analysis of p53 immunohistochemistry in LLI tumors suggests some levels of molecular consistency across both LMP-like and more invasive regions, at least in our cohort. It should be noted that, among our cohorts, an additional four invasive tumors were identified with LMP histology that did not fall within the LLI/LMP cluster (see Table 1B). Again, microdissection and molecular comparison of differential histologies within the same patients would be helpful and is likely to be necessary for a complete understanding of each of these tumor classes. Following this, a previous analysis of serous ovarian cancers noted that low-grade invasive tumors selectively cosegregated with LMP tumors (8). The majority of LLI tumors in our study were Grade 2, but also included one Grade 3 and one Grade 1 tumor (Table 1B). Interestingly, three additional low-grade invasive tumors were included in our dataset but failed to segregate with the LMP tumors. These findings are in part in contrast to those of Bonome et al. (8), who concluded that most low-grade serous invasive tumors resemble their LMP counterparts. Further study is required to address whether there may be, in fact, two classes of low-grade tumors: those that progress from LMP versus others that are more closely related to high-grade invasive carcinomas. Overall, the findings of this study support the view that the molecular events underlying the development of serous ovarian tumors of LMP are quite distinct from those leading to high-grade serous ovarian carcinoma. We have defined p53 pathway activation as a major discriminator of the LMP and invasive subtypes. Finally, we have confirmed that *ERBB2* activating mutations are the third most common oncogene mutation among LMP tumors and have linked this, along with *KRAS* and *BRAF* mutations, to a ubiquitously present LMP RAS-MAPK expression signature.

Materials and Methods

Primary Tumors

Ovarian tumor samples were obtained from 194 patients undergoing surgery at the Royal Brisbane Hospital or Westmead Hospital and through the Australian Ovarian Cancer Study.[12] These included 134 LMP tumors, of which 30 were used for expression profiling, and 99 invasive ovarian tumors, 60 of which underwent expression profiling. Patients were staged at laparotomy, in accordance with the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Of the LMP tumors, there were 94 serous, 39 mucinous, and 1 tumor of mixed histology, whereas 89 of the invasive tumors were serous, 6 were endometrial, 3 were mucinous, and 1 was of clear cell histology. Constitutional DNA was available in all cases from peripheral blood, and informed consent was obtained from all patients. Expression array data from an additional 150 invasive serous ovarian carcinomas were included in the analyses; the characteristics of this cohort have previously been described (22). DNA from frozen tumor blocks and blood was extracted by the salting-out method, as described in ref. (90). This study was approved by the Human Research Ethics Committees at the Peter MacCallum Cancer Centre, Queensland Institute of Medical Research, University of Melbourne, and all participating hospitals.

Sample, Microarray, and Data Processing

Frozen tissue specimens were collected at the time of primary debulking surgery and snap frozen in liquid nitrogen. Serial tissue sections (12 × 100 μm) were cut, and RNA was extracted using TRIZOL reagent (Invitrogen) and then further purified by column chromatography using a Qiagen RNeasy spin column (Qiagen). Total RNA quality was assessed using an Agilent Bioanalyzer 2100 Pico assay (Agilent) and Nanodrop Spectrophotometer (Nanodrop Technologies). Only samples with a Bioanalyzer degradation factor of less than 8 and a Nanodrop A260/A280 ratio between 1.8 and 2.1 were used for further analysis. A single round amplification was used to generate cRNA from total RNA extracts, hybridized to Affymetrix HG-U133 2.0plus expression arrays, and scanned in accordance with standard Affymetrix protocols (Affymetrix). Image analysis and probe quantitation were done using Affymetrix Gene Chip Operating Software, using a scaling factor of 150. R packages "Simple Affy" and "Affy," available from the Bioconductor project,[13] were used for quality control and normalization, respectively. CEL files were subject to quality control before batch normalization using the robust multiarray average algorithm method (91). Expression from selected probes/genes was further validated, as described in Supplementary Fig. S6. Technical details on data processing, including normalization, filtering, differential gene expression

[12] http://www.aocstudy.org
[13] www.bioconductor.org

statistical analysis, and classifier design, can be found in Supplementary Materials and Methods. Data associated with this study can be found on the public Gene Expression Omnibus repository.[14]

Immunohistochemistry of p53

FFPE tissue from five of eight LLI tumors was available for immunohistochemical analysis (no FFPE or frozen tissue was available for the remaining three LLI samples). As FFPE material was used for immunohistochemistry and frozen tissue for gene expression profiling, we reviewed the FFPE samples to ensure they were representative of the samples used for expression analysis; all of the available samples were representative of both LMP and invasive histology and were similar in composition to the frozen material used for expression analysis. Immunohistochemical staining was done as recommended by manufacturer and has been previously described (89). Tissue sections (4 μm) on Superfrost Plus slides were subject to 20-min heat retrieval (pH 9 buffer) in a 100°C water bath. Immunostaining was done in a Dako auto-stainer: 5 min in 3% hydrogen peroxide, 30 min with anti-p53 antibody (1:400 dilution; monoclonal antibody DO-7; NovoCastra Labs). Staining was visualized with Dako EnVision/HRP FLEX polymer (30 min; Dako) and DAB FLEX (10 min; Dako). Sections were counterstained with Mayer's hematoxylin and Scott's tap water. Slides were scanned on an Olympus dotSlide automated microscope scanning system; no image postprocessing has been applied.

Allele-Specific PCR and Melt Curve Analysis

Screening for the *BRAF* c.1799T>A and *KRAS* c.35G>A and c.35G>T mutations was carried out by allele-specific PCR and melt-curve analysis (92, 93). Multiple PCR primers were designed to specifically amplify either the mutant or wild-type *BRAF* or *KRAS* alleles (Supplementary Table S5). A 17-bp GC clamp was incorporated at the 5′ end of each mutant primer to increase the melting temperature of the mutant alleles by ~5°C. The PCR reaction was carried out on a Rotor-gene 3000 (Corbett Research) with 10 to 20 ng of genomic DNA in a 15-μL volume containing Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). For *BRAF*, the PCR reaction was carried out with mutant forward (200 nmol/L), wild-type forward (200 nmol/L), and reverse (600 nmol/L) primers at 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. For *KRAS*, the c.35G>A mutation was assessed with the wild-type forward (670 nmol/L), A mutant forward (670 nmol/L), and reverse (1,340 nmol/L) primers, whereas the c.35G>T mutation was assessed with the wild-type forward (177 nmol/L), T mutant forward (177 nmol/L), and reverse (533 nmol/L) primers. Cycling was carried out for both *KRAS* PCR reactions at 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 35 s. After amplification, samples were subjected to a temperature ramp from 50°C to 99°C, increasing 1°C each step. For *BRAF*, wild-type samples produced single melt peaks at 80°C, whereas mutant samples produced either single peaks at 85°C or peaks at both 80°C and 85°C (Supplementary Fig. S7). For *KRAS*, the wild-type melt peaks were observed at 80.5°C and mutant peaks at 87°C.

Sequencing of PCR products from samples that gave melt peaks at both 80°C and 85°C for *BRAF* did not reveal mutant alleles in all cases. In three cases, only wild-type alleles were detected by direct sequencing. The PCR products from these three cases were cloned into pGEM-T and sequencing of 20 clones of each found two mutant clones in each case, suggesting that these samples contain ~10% mutant *BRAF* alleles. To more precisely determine the sensitivity of the allele-specific PCR for both the *BRAF* c.1799T>A and *KRAS* c.35G>A and c.35G>T mutations, different proportions of plasmids containing the cloned mutant and wild-type *BRAF* and *KRAS* alleles were mixed together (Supplementary Fig. S8). In all three cases, we found that the allele-specific PCR could detect 14% or more mutant allele, thereby confirming the high sensitivity of this assay.

DHPLC Analysis of ERBB2 and EGFR

PCR primers were also designed to amplify the seven exons that contain the *ERBB2* kinase domain (Table 2). Exons 21 and 22 were amplified in a single PCR fragment, whereas exons 18, 19, 20, 23, and 24 were each amplified separately. PCR primers were also designed to amplify exons 18, 19, 20, and 21 of the *EGFR* gene, which encompass the tyrosine kinase domain (see Supplementary Table S5). PCR fragments were amplified from 50 ng of DNA using AmpliTaq Gold (PE Applied Biosystems) in a final volume of 20 μL. Amplicons were then denatured at 95°C for 5 min and cooled to 60°C over 30 min (1°C/min) before injection into the Varian Helix System (Varian). Initially, DHPLC was carried on all tumor samples at the recommended melt temperature, as determined by the Stanford melt algorithm.[15] Analysis was done using the Star Workstation version 5 (Varian). Samples that produced an aberrant shift in retention time and/or peak shape were reamplified along with the corresponding constitutional DNA, and both samples were analyzed on the DHPLC. Samples showing tumor-specific profiles on the DHPLC were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 377 sequencer. Samples with indel mutations were cloned into the pGEM-T vector (Promega), and individual clones were sequenced to define the mutation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[14] http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12172
[15] http://insertion.stanford.edu/melt.html

References

1. Stewart BW, Kleihues P, editors. IARC: World Cancer Report. Lyon: IARC Press; 2003.

2. Choi KC, Auersperg N. The ovarian surface epithelium: simple source of a complex disease. Minerva Ginecol 2003;55:297–314.

3. Fukumoto M, Nakayama K. Ovarian epithelial tumors of low malignant potential: are they precursors of ovarian carcinoma? Pathol Int 2006;56:233–9.

4. Trimble EL, Trimble CL. Ovarian tumors of low malignant potential. Curr Treat Options Oncol 2001;2:103–8.

5. Sherman ME, Berman J, Birrer MJ, et al. Current challenges and opportunities for research on borderline ovarian tumors. Hum Pathol 2004;35:961–70.

6. Silva EG, Kurman RJ, Russell P, Scully RE. Symposium: ovarian tumors of borderline malignancy. Int J Gynecol Pathol 1996;15:281–302.

7. Ho CL, Kurman RJ, Dehari R, Wang TL, Shih Ie M. Mutations of BRAF and KRAS precede the development of ovarian serous borderline tumors. Cancer Res 2004;64:6915–8.

8. Bonome T, Lee JY, Park DC, et al. Expression profiling of serous low malignant potential, low-grade, and high-grade tumors of the ovary. Cancer Res 2005;65:10602–12.

9. Gilks CB, Vanderhyden BC, Zhu S, van de Rijn M, Longacre TA. Distinction between serous tumors of low malignant potential and serous carcinomas based on global mRNA expression profiling. Gynecol Oncol 2005;96:684–94.

10. Sieben NL, Oosting J, Flanagan AM, et al. Differential gene expression in ovarian tumors reveals Dusp 4 and Serpina 5 as key regulators for benign behavior of serous borderline tumors. J Clin Oncol 2005;23:7257–64.

11. Haas CJ, Diebold J, Hirschmann A, Rohrbach H, Lohrs U. In serous ovarian neoplasms the frequency of Ki-ras mutations correlates with their malignant potential. Virchows Arch 1999;434:117–20.

12. Meinhold-Heerlein I, Bauerschlag D, Hilpert F, et al. Molecular and prognostic distinction between serous ovarian carcinomas of varying grade and malignant potential. Oncogene 2005;24:1053–65.

13. Shih Ie M, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511–8.

14. Ceccaroni M, Chieco P, Alboni C, et al. p53 expression, DNA ploidy and mitotic index as prognostic factors in patients with epithelial ovarian carcinoma. Tumori 2004;90:600–6.

15. Burks RT, Sherman ME, Kurman RJ. Micropapillary serous carcinoma of the ovary. A distinctive low-grade carcinoma related to serous borderline tumors. Am J Surg Pathol 1996;20:1319–30.

16. Lee Y, Miron A, Drapkin R, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. J Pathol 2007;211:26–35.

17. Medeiros F, Muto MG, Lee Y, et al. The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. Am J Surg Pathol 2006;30:230–6.

18. Dennis G, Jr., Sherman BT, Hosack DA, et al. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol 2003;4:P3.

19. Thomas PD, Campbell MJ, Kejariwal A, et al. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 2003;13:2129–41.

20. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.

21. Schaner ME, Ross DT, Ciaravino G, et al. Gene expression patterns in ovarian carcinomas. Mol Biol Cell 2003;14:4376–86.

22. Tothill RW, Tinker AV, George J, et al. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. Clin Cancer Res 2008;14:5198–208.

23. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116–21.

24. Miller LD, Smeds J, George J, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A 2005;102:13550–5.

25. Seidman JD, Kurman RJ. Subclassification of serous borderline tumors of the ovary into benign and malignant types. A clinicopathologic study of 65 advanced stage cases. Am J Surg Pathol 1996;20:1331–45.

26. Harrison RE, Sikorski BA, Jongstra J. Leukocyte-specific protein 1 targets the ERK/MAP kinase scaffold protein KSR and MEK1 and ERK2 to the actin cytoskeleton. J Cell Sci 2004;117:2151–7.

27. Guijarro MV, Castro ME, Romero L, Moneo V, Carnero A. Large scale genetic screen identifies MAP17 as protein bypassing TNF-induced growth arrest. J Cell Biochem 2007;101:112–21.

28. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol 2001;19:816–27.

29. Rich JN, Shi Q, Hjelmeland M, et al. Bone-related genes expressed in advanced malignancies induce invasion and metastasis in a genetically defined human cancer model. J Biol Chem 2003;278:15951–7.

30. Hojilla CV, Kim I, Kassiri Z, Fata JE, Fang H, Khokha R. Metalloproteinase axes increase β-catenin signaling in primary mouse mammary epithelial cells lacking TIMP3. J Cell Sci 2007;120:1050–60.

31. Beckers J, Herrmann F, Rieger S, et al. Identification and validation of novel ERBB2 (HER2, NEU) targets including genes involved in angiogenesis. Int J Cancer 2005;114:590–7.

32. Kupryjanczyk J, Bell DA, Yandell DW, Scully RE, Thor AD. p53 expression in ovarian borderline tumors and stage I carcinomas. Am J Clin Pathol 1994;102:671–6.

33. Mayr D, Hirschmann A, Lohrs U, Diebold J. KRAS and BRAF mutations in ovarian tumors: a comprehensive study of invasive carcinomas, borderline tumors and extraovarian implants. Gynecol Oncol 2006;103:883–7.

34. Sieben NL, Macropoulos P, Roemen GM, et al. In ovarian neoplasms, BRAF, but not KRAS, mutations are restricted to low-grade serous tumours. J Pathol 2004;202:336–40.

35. Giordano TJ, Kuick R, Thomas DG, et al. Molecular classification of papillary thyroid carcinoma: distinct BRAF, RAS, and RET/PTC mutation-specific gene expression profiles discovered by DNA microarray analysis. Oncogene 2005;24:6646–56.

36. Bloethner S, Chen B, Hemminki K, et al. Effect of common B-RAF and N-RAS mutations on global gene expression in melanoma cell lines. Carcinogenesis 2005;26:1224–32.

37. Frattini M, Ferrario C, Bressan P, et al. Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. Oncogene 2004;23:7436–40.

38. Pavey S, Johansson P, Packer L, et al. Microarray expression profiling in melanoma reveals a BRAF mutation signature. Oncogene 2004;23:4060–7.

39. Schulze A, Nicke B, Warne PH, Tomlinson S, Downward J. The transcriptional response to Raf activation is almost completely dependent on mitogen-activated protein kinase kinase activity and shows a major autocrine component. Mol Biol Cell 2004;15:3450–63.

40. Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J. Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev 2001;15:981–94.

41. Tchernitsa OI, Sers C, Zuber J, et al. Transcriptional basis of KRAS oncogene-mediated cellular transformation in ovarian epithelial cells. Oncogene 2004;23:4536–55.

42. Huang E, Ishida S, Pittman J, et al. Gene expression phenotypic models that predict the activity of oncogenic pathways. Nat Genet 2003;34:226–30.

43. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000;103:211–25.

44. Roskoski R, Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004;319:1–11.

45. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.

46. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.

47. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.

48. Schilder RJ, Sill MW, Chen X, et al. Phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma and evaluation of epidermal growth factor receptor mutations and immunohistochemical expression: a Gynecologic Oncology Group Study. Clin Cancer Res 2005;11:5539–48.

49. Shigematsu H, Takahashi T, Nomura M, et al. Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. Cancer Res 2005;65:1642–6.

50. Stephens P, Hunter C, Bignell G, et al. Lung cancer: intragenic ERBB2 kinase mutations in tumours. Nature 2004;431:525–6.

51. Lassus H, Sihto H, Leminen A, et al. Gene amplification, mutation, and protein expression of EGFR and mutations of ERBB2 in serous ovarian carcinoma. J Mol Med 2006;84:671–81.

52. Nakayama K, Nakayama N, Kurman RJ, et al. Sequence mutations and

amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer Biol Ther 2006;5:779–85.

53. Biade S, Marinucci M, Schick J, et al. Gene expression profiling of human ovarian tumours. Br J Cancer 2006;95:1092–100.

54. Jurianz K, Ziegler S, Garcia-Schuler H, et al. Complement resistance of tumor cells: basal and induced mechanisms. Mol Immunol 1999;36:929–39.

55. Huitorel P. From cilia and flagella to intracellular motility and back again: a review of a few aspects of microtubule-based motility. Biol Cell 1988;63:249–58.

56. Asai DJ, Wilkes DE. The dynein heavy chain family. J Eukaryot Microbiol 2004;51:23–9.

57. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 2001; 22:255–88.

58. Franchina M, Del Borrello E, Caruso A, Altavilla G. Serous tumors of the ovary: ultrastructural observations. Eur J Gynaecol Oncol 1992;13:268–76.

59. Tothill RW, Kowalczyk A, Rischin D, et al. An expression-based site of origin diagnostic method designed for clinical application to cancer of unknown origin. Cancer Res 2005;65:4031–40.

60. Khan J, Wei JS, Ringner M, et al. Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. Nat Med 2001;7:673–9.

61. Kindelberger DW, Lee Y, Miron A, et al. Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: evidence for a causal relationship. Am J Surg Pathol 2007;31:161–9.

62. Horiuchi D, Collins CA, Bhat P, Barkus RV, Diantonio A, Saxton WM. Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. Curr Biol 2007;17:1313–7.

63. Verhey KJ. Motor proteins: trafficking and signaling collide. Curr Biol 2007; 17:R804–6.

64. Cavalli V, Kujala P, Klumperman J, Goldstein LS. Sunday driver links axonal transport to damage signaling. J Cell Biol 2005;168:775–87.

65. Hanz S, Fainzilber M. Retrograde signaling in injured nerve-the axon reaction revisited. J Neurochem 2006;99:13–9.

66. Singer G, Oldt R III, Cohen Y, et al. Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J Natl Cancer Inst 2003;95:484–6.

67. Mok SC, Bell DA, Knapp RC, et al. Mutation of K-ras protooncogene in human ovarian epithelial tumors of borderline malignancy. Cancer Res 1993;53: 1489–92.

68. Brose MS, Volpe P, Feldman M, et al. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 2002;62:6997–7000.

69. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.

70. Gorden A, Osman I, Gai W, et al. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. Cancer Res 2003;63:3955–7.

71. Sergina NV, Moasser MM. The HER family and cancer: emerging molecular mechanisms and therapeutic targets. Trends Mol Med 2007;13: 527–34.

72. Wang SE, Narasanna A, Perez-Torres M, et al. HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer cell 2006;10: 25–38.

73. Lee JW, Soung YH, Seo SH, et al. Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas. Clin Cancer Res 2006;12: 57–61.

74. Fan YX, Wong L, Ding J, Spiridonov NA, Johnson RC, Johnson GR. Mutational activation of ErbB2 reveals a new protein kinase autoinhibition mechanism. J Biol Chem 2008;283:1588–96.

75. Hsu CY, Bristow R, Cha MS, et al. Characterization of active mitogen-activated protein kinase in ovarian serous carcinomas. Clin Cancer Res 2004;10:6432–6.

76. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene 2007;26:3203–13.

77. Kiss-Toth E, Bagstaff SM, Sung HY, et al. Human tribbles, a protein family controlling mitogen-activated protein kinase cascades. J Biol Chem 2004;279: 42703–8.

78. Douville E, Downward J. EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2. Oncogene 1997;15:373–83.

79. Kim M, Lee JH, Koh H, et al. Inhibition of ERK-MAP kinase signaling by RSK during Drosophila development. EMBO J 2006;25:3056–67.

80. Tsavachidou D, Coleman ML, Athanasiadis G, et al. SPRY2 is an inhibitor of the ras/extracellular signal-regulated kinase pathway in melanocytes and melanoma cells with wild-type BRAF but not with the V599E mutant. Cancer Res 2004;64:5556–9.

81. Sasaki A, Taketomi T, Kato R, et al. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Cell Cycle 2003;2:281–2.

82. Chen G, Hitomi M, Han J, Stacey DW. The p38 pathway provides negative feedback for Ras proliferative signaling. J Biol Chem 2000;275:38973–80.

83. Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene 2007;26:3100–12.

84. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602.

85. Nakagawa H, Opitz OG. Inducing cellular senescence using defined genetic elements. Methods Mol Biol 2007;371:167–78.

86. Liu D, Liu Z, Jiang D, Dackiw AP, Xing M. Inhibitory effects of the mitogen-activated protein kinase kinase inhibitor CI-1040 on the proliferation and tumor growth of thyroid cancer cells with BRAF or RAS mutations. J Clin Endocrinol Metab 2007;92:4686–95.

87. Arslan MA, Kutuk O, Basaga H. Protein kinases as drug targets in cancer. Curr Cancer Drug Targets 2006;6:623–34.

88. Wang D, Boerner SA, Winkler JD, LoRusso PM. Clinical experience of MEK inhibitors in cancer therapy. Biochim Biophys Acta 2007;1773:1248–55.

89. Singer G, Stohr R, Cope L, et al. Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis: a mutational analysis with immunohistochemical correlation. Am J Surg Pathol 2005;29:218–24.

90. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215.

91. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185–93.

92. Papp AC, Pinsonneault JK, Cooke G, Sadee W. Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves. Biotechniques 2003;34:1068–72.

93. Young J, Barker MA, Simms LA, et al. Evidence for BRAF mutation and variable levels of microsatellite instability in a syndrome of familial colorectal cancer. Clin Gastroenterol Hepatol 2005;3:254–63.