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Amplification and Overexpression of the L-*MYC* Proto-Oncogene in Ovarian Carcinomas

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Gene amplification is an important mechanism of oncogene activation in various human cancers, including ovarian carcinomas (OvCas). We used restriction landmark genomic scanning (RLGS) to detect amplified DNA fragments in the genomes of 47 primary OvCas. Visual analysis of the RLGS gel images revealed several OvCa samples with spots of greater intensity than corresponding spots from normal tissues, indicating possible DNA amplification in specific tumors. Two primary tumors (E1 and S12) shared four high-intensity spots. A recently developed informatics tool termed Virtual Genome Scans was used to compare the RLGS patterns in these tumors with patterns predicted from the human genome sequence. Virtual Genome Scans determined that three of the four fragments localized to chromosome 1p34-35, a region containing the proto-oncogene L-*MYC*. Sixty-eight primary OvCas, including 40 analyzed by RLGS, were screened by quantitative polymerase chain reaction (PCR) for possible amplification of L-*MYC*. Ten tumors with increased L-*MYC* copy number were identified, including tumor E1, which showed an ~24-fold increase in copy number compared to normal DNA. Southern analysis of several tumors confirmed the quantitative PCR results. Using sequence tagged site (STS) markers flanking L-*MYC*, increased DNA copy number in tumor E1 was found to span the region flanking L-*MYC* between D1S432 and D1S463 (≈3.1 Mb). Other tumors showed amplification only at the L-*MYC* locus. Using oligonucleotide microarrays, L-*MYC* was found to be more frequently overexpressed in OvCas than either c-*MYC* or N-*MYC* relative to ovarian surface epithelium. Quantitative reverse transcriptase-PCR analysis confirmed elevated L-*MYC* expression in a substantial fraction of OvCas, including nine of nine tumors with increased L-*MYC* copy number. The data implicate L-*MYC* gene amplification and/or overexpression in human OvCa pathogenesis. *(Am J Pathol 2003, 162:1603–1610)*

Gene amplification is a major mechanism underlying activation of human proto-oncogenes in tumor cells.[1] Amplification of a variety of oncogenes, such as *HER-2/NEU* and c-*MYC*, has been reported in many types of primary human cancers, including those of the colorectum,[2] prostate,[3] breast,[4,5] lung,[6,7] cervix,[8] and ovary.[9] Although oncogene amplification can be a relatively early event in tumorigenesis, it is more often observed in advanced stages of cancer.[10–14]

Ovarian carcinoma (OvCa) is the leading cause of death from gynecological malignancy in the United States, with ~23,000 new cases diagnosed and 14,000 deaths annually.[15] A number of genetic alterations have been described in OvCas, with many observed preferentially in specific histological types.[16,17] These alterations can be hereditary (eg, inactivation of *BRCA1* and *BRCA2*) or somatic.[18] A few oncogenes have been shown to be amplified in a subset of OvCas. For example, somatic amplification of the *HER-2/NEU* gene on chromosome 17q has been observed in primary OvCas, although the frequency with which amplification is reported to occur is quite variable (0 to 66%).[4,19–21] This wide range likely reflects, at least in part, methodological differences and sample size and tumor specimen variability from study to study. Similarly, amplification of c-*MYC* on chromosome 8 has been observed in up to 40% of primary OvCas.[22–25]

Restriction landmark genome scanning (RLGS) has been widely used for comparison of genomic DNA copy number,[11,26–31] and to detect changes in DNA methylation among related tissues of interest.[32–35] For RLGS, genomic DNA is cleaved with a rare-cutting restriction endonuclease and the cleavage sites are radioactively labeled before first-dimension electrophoresis. The separated DNA fragments are then digested *in situ* with a frequent-cutting restriction endonuclease before second-dimension electrophoresis. The use of the methylation-sensitive endonuclease *Not*I to digest genomic DNA before labeling allows visualization of DNA fragments

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(landmarks) that occur preferentially in CpG islands, and hence near the vicinity of coding sequences.[36] Approximately 3000 to 4000 spots, each representing a specific chromosomal segment, can be visualized on a two-dimensional gel image. Gel spot images of labeled DNA fragments can be quantitatively compared between tissues of interest. At least two factors have hindered the ability to rapidly identify genes within amplified DNA fragments identified by RLGS: difficulty cloning the extremely small amount of DNA from two-dimensional spots of interest, and difficulty deriving sequence information for displayed fragments. The Virtual Genome Scans (VGS) informatics tool recently developed by Rouillard and colleagues,[37] applies the same set of restriction enzymes used in actual two-dimensional gel experiments to the genome sequence databases and computationally overlays the predicted fragment locations on actual RLGS images. This permits tentative identification of fragments of interest, and can lead to confident predictions if additional information is known, such as a fragment's chromosomal location or origin or knowledge that a set of fragments originates from a single, perhaps unknown, chromosome.[30,38] We used a combination of RLGS and VGS to identify additional genes frequently amplified in ovarian cancer. DNA samples from 47 primary OvCas were compared to normal controls. We show that the L-*MYC* gene on chromosome 1p34 is amplified and overexpressed in a subset of OvCas.

Materials and Methods

Tumor Samples

Seventy-five OvCas were studied by RLGS and/or quantitative polymerase chain reaction (PCR) analyses, including 6 clear cell, 7 mucinous, 32 endometrioid, and 30 serous adenocarcinomas. Eight were obtained from the University of Michigan Health System, 11 from Cornell University Hospital, 24 from the Cooperative Human Tissue Network, and 32 from the Johns Hopkins Medical Institutions. All samples were kept frozen at −80°C before microdissection and DNA extraction. Primary tumor tissues were manually microdissected before DNA extraction to ensure each tumor sample contained at least 70% neoplastic cells. Hematoxylin and eosin-stained frozen sections were used as dissection guides. Analysis of tissues from human patients was approved by the University of Michigan's Institutional Review Board (IRB-MED no. 2001-0022).

Genomic DNA Isolation, RNA Extraction, and Reverse Transcription

Genomic DNA was extracted from frozen tumor tissues by standard proteinase K digestion and phenol/chloroform extraction. Total RNA was extracted using Trizol reagent (Invitrogen, Gaithersburg, MD), according to the manufacturer's instructions. Reverse transcription was performed with 5 μg of RNA, random hexamers (Amersham Pharmacia Biotech, Piscataway, NJ), and Superscript II reverse transcriptase (Invitrogen).

Restriction Landmark Genome Scanning (RLGS)

DNA samples from 47 primary ovarian tumors (28 serous, 6 clear cell, 7 endometrioid, and 6 mucinous adenocarcinomas) were analyzed by RLGS. The enzymatic digestions, DNA labeling, and electrophoresis were performed as previously described.[39] Briefly, 5 μg of genomic DNA was digested with *Not*I and the 5′-protruding ends were end-labeled with [α-^{32}P]dCTP and [α-^{32}P]dGTP (New Life Science Products, Boston, MA). A second enzyme, *Eco*RV, was used to further digest the DNA fragments before size fractionation (1.0 to 5.0 kb) in the first dimension in 32-cm 0.9% disk-agarose gels. The separated DNA fragments were then digested *in situ* with *Hinf*I for further cleavage before second-dimension separation in large format (25 × 43 cm) 5.2% polyacrylamide slab gels. The gels were then dried and exposed to PhosphorImager screens and scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) at a resolution of 176 μm per pixel. Digitized images were translated into 1024 × 1024 pixel formats, suitable for VISAGE software (BioImage, Ann Arbor, MI), which was used to perform spot (fragment) detection and quantitation. Amplification of specific fragments was estimated by comparing spot intensities in tumor samples to spot intensities in normal (nontumor) DNA from 10 OvCa patients.

Semiquantitative Multiplex PCR

The sequences of oligonucleotides used for PCR are summarized in Table 1. DNA microsatellite markers in the 1p34 amplicon were selected by sequence tagged site (STS) database analyses. The amplified fragment was used as the anchor to select neighboring STS markers. Primers for each selected marker were chosen using DNASTAR software to ensure that the melting temperature (Tm) of the STS PCR fragments was nearly equivalent to the Tm of the co-amplified internal control (ATPase or GAPDH). Each PCR reaction contained 2 μCi of [α-^{32}P]dCTP (ICN, Costa Mesa, CA); 25 μmol/L dCTP; 200 μmol/L dATP, dGTP, and TTP; 1 μmol/L of each primer (both target gene and internal control); 1 U of *Taq* polymerase (Invitrogen) or FastStart *Taq* polymerase (Roche, Indianapolis, IN); and 20 ng of genomic DNA. After 22 to 28 cycles of PCR, products were resolved on 6% denaturing polyacrylamide gels. After drying, the gels were exposed to PhospholmagerTM screens (Molecular Dynamics). To determine the level of target gene amplification in tumors, tumor and normal samples were compared after normalizing target gene values with the values for the co-amplified internal control gene in the same samples. All reactions were performed at least twice. Tumor samples showing increased (>2 fold) DNA copy number at selected markers were further analyzed, and the minimal amplified region was determined using additional flanking markers.

Table 1. Oligonucleotide Sequences

Markers		Sequences	Size (bp)	GenBank ID
D1S2368	Forward	CTAATCACAGTAGCACTGAACATGG	182	
	Reverse	CCTAGTGCTGGGAGTTCAGT		
D1S432	Forward	CAGAGGTTCTGGTGAGCAG	163	
	Reverse	CCCTGGTACCCCATAGTAG		
WI-15142	Forward	ATTTTTACACACTTTGACAAGGAGG	157	
	Reverse	CACATTGGCAAGGGGGAG		
stSG48383	Forward	CCATTTTCTAATTTTCTTCGCA	138	XM_044944
	Reverse	GGTCCTGGAAGCTGACGTA		
WI-12773	Forward	CGCAAATTTCAAAGTCTTTGGT	146	
	Reverse	GATTTGAGTGAATGGCAGGAA		
A002R05	Forward	GAGGTGGTTGACTTTCTCATGT	158	
	Reverse	AGTCCACCACTGTGTAATTGGT		
L-*MYC* (exon 2)	Forward	GGACATCTGGAAGAAATTCGA	191	M19720
	Reverse	CGTATGATGGAGGCGTAGTT		
L-*MYC* (exon 3)	Forward	CCATCAGCAACAGCACAACTA	178	M19720
	Reverse	CACTTTCTACAGGTGGGGGA		
D1S2743	Forward	GGTTTCACTGTAGCACCACT	176	
	Reverse	CTTGAACAGGAATGTGTGTGT		
A008Q33	Forward	TTTCCTTAGCAATTCCTTTAGTA	184	
	Reverse	GTGCTCGTTTTTTAGAGATACAT		
D1S463	Forward	TGCATTTTGTTTTCACATCTGT	164	
	Reverse	GTGTTAACCACCTCCCTTCA		
ATPase	Forward	CCCCTGCTCCAGAATTTTC	165	M30310
	Reverse	GTTCATCCATGTCCCTGTC		
GAPDH	Forward	GCCAAAAGGGTCATCATCTCT	182	XM_066753
	Reverse	GGTCATGAGTCCTTCCACGA		
TaqMan Primer/Probe Set				
L-*MYC*	Forward	TGGATCCCTGCATGAAGCA	70	M19720
	Reverse	AGGAAAACGGGCAGCATAGTT		
	TaqMan probe	6FAM-CCACATCTCCATCCATCAGCAACAGC		
ATPase	Forward	AGATCCTGGCGCGAGATG	73	M30310
	Reverse	CCGACAAAACTTGATCCATTCA		
	TaqMan probe	VIC-CCCAACGCCCTCACTCCCCCT		
HPRT1	Forward	TTGCTCGAGATGTGATGAAGGA	93	NM_000194
	Reverse	CCAGCAGGTCAGCAAAGAATT		
	TaqMan probe	VIC-CCATCACATTGTAGCCCTCTGTGTGCTC		
Probe for Southern blot				
L-*MYC*	Forward	GCTGCAGGGGAAAAGGAAGAT		M19720
	Reverse	CTAAAGGGGAGAGGGAGGTT	449	
β-actin	Forward	GGCACCACACCTTCTACAATG		M10027
	Reverse	CTCCTTAATGTCACGCACGA	780	

Quantitative PCR and RT-PCR

Primer Express (version 1.0; PE Applied Biosystems, Foster City, CA) was used to select the primer and probe sequences for quantitative PCR (Table 1). The L-*MYC* probe was labeled with the reporter dye 6-carboxy fluorescein (FAM) and the *ATPase* and *HPRT1* probes with the reporter dye VIC. Primers and probes for quantitative PCR were purchased from PE Applied Biosystems. Quantitative PCR was performed using an ABI Prism 7700 Sequence Analyzer (PE Applied Biosystems) in 25-μl reactions containing 1× Universal PCR mix, 200 nmol/L of each forward and reverse primer, 200 nmol/L of each probe, and 20 ng of genomic DNA. Each 40-cycle reaction was set up in duplicate. For quantitative RT-PCR, all RNA samples were pretreated with DNaseI to avoid amplification of contaminating genomic DNA and 10 to 20 ng of first strand cDNA was amplified in each reaction. Quantitative PCR reactions for target and internal control genes (*ATPase* on chromosome 1p13 for genomic DNA, *HPRT1* for cDNA) were performed in separate tubes. The comparative threshold cycle (C_T) method was used for the calculation of amplification fold as specified by the manufacturer.

Southern Blot Analysis

For Southern blotting, 8 μg of genomic DNA from each sample was digested with *Eco*RI at 37°C overnight, separated in 0.8% agarose gels, and transferred to Zeta-probe membranes with 0.4 N NaOH as described by the supplier (Bio-Rad, Richmond, CA). Membranes were prehybridized in Rapid-hyb buffer (Amersham Pharmacia Biotech) at 65°C for 1 hour and hybridized overnight with a 449-bp genomic [α-^{32}P]dCTP-labeled L-*MYC* probe (3000 Ci/mmol, Amersham Pharmacia Biotech). Blots were stripped and rehybridized with a β-actin probe as a loading control. The membranes were exposed to Kodak X-ray film overnight at −80°C.

*Evaluation of c-*MYC*, L-*MYC*, and N-*MYC *Expression Using Affymetrix Oligonucleotide Microarrays*

The results of global gene expression profiling of 113 primary OvCas using Affymetrix HuGeneFL oligonucleotide microarrays have recently been reported in detail.[40] These 113 tumors included 29 of the 75 used for studies described here. Briefly, the preparation of cRNA, hybridization, and scanning of the microarrays were performed according to the manufacturer's protocols. To obtain expression measures for each of the 7069 noncontrol probe sets on the chip, mismatch hybridization values were subtracted from the perfect match values and the average of the middle 50% of these differences was used as the expression measure. A quantile normalization procedure was performed to adjust for differences in the probe intensity distribution across different chips. These data are available at *http://dot.ped.med.umich.edu:2000/pub/Ovary/index.html*. Expression data for L-*MYC*, c-*MYC*, and N-*MYC* in the 113 primary OvCas were extracted from the larger data set. Six samples of ovarian surface epithelium (OSE) were also included in the microarray-based gene expression analysis, including two immortalized with SV40 LgTAg (IOSE-144 and IOSE-80, gift of N. Auersperg, University of British Columbia), two immortalized with HPV16 E6/E7 (96.1.24 and 96.9.18, gift of W. Lancaster, Wayne State University) and two short-term OSE cultures (D Schwartz and KR Cho, unpublished).

Results

Combined RLGS and VGS Identifies an Amplified Chromosome 1p34 Sequence in OvCas

RLGS was applied to 47 primary OvCas using the restriction enzyme combination of *Not*I/*Eco*RV/*Hin*fI. Most of the very intense spots visible in the two-dimensional gels represent ribosomal DNA sequences, which are present in multiple copies.[41] On visual scanning of the gel images, four restriction fragments with increased image intensity as compared to the corresponding RLGS fragments from normal tissue DNA were identified in tumor E1 (Figure 1A, left and middle). Using VGS, three of these four fragments (Figure 1A, right), namely fragment 1 (1059, 476), fragment 2 (1984, 470), and fragment 3 (2952,856) were matched to respective predicted fragments thought to be from chromosome 1.[30] Specifically, the sequences of matched predicted fragments 1, 2, and 3 are present in BAC clones AL606484, AL139015, and AL139158, respectively (NCBI GenBank database). A predicted fragment of similar RLGS size for fragment 4 (320,950) was not found, perhaps because this specific genomic sequence was not present in the databases. Using a combination of bioinformatics tools from the NCBI (eg, Electronic PCR, Map Viewer, UniGene Resources) all three BAC clones were localized to chromosomal band 1p34 (Figure 1B). The L-*MYC* gene resides in

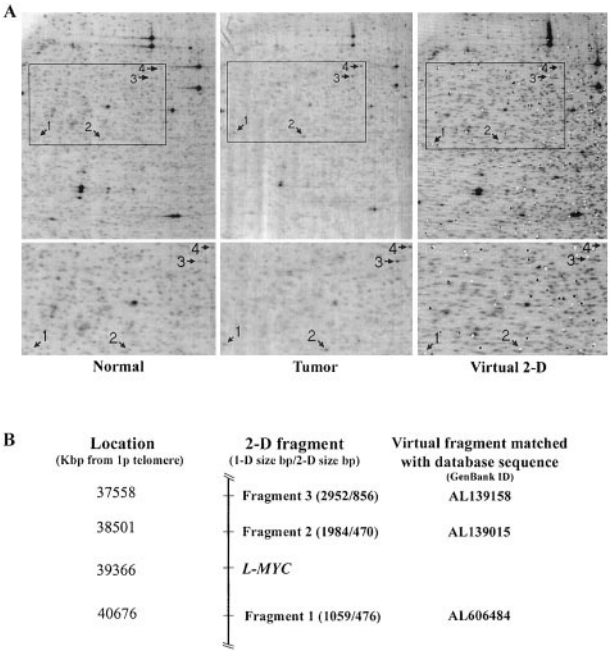

Figure 1. A: Two-dimensional RLGS and VGS analyses. Four *Not*I/*EcoR*V/*Hin*fI fragments 1 through 4 with increased image intensity are observed in E1 tumor DNA (**middle**) compared to normal tissue DNA (**left**). **White** and **black dots** in the VGS image superimposed on the RLGS image (**right**) represent final and draft sequences, respectively, in the genome sequence databases. **B:** Schematic map of the three VGS-matched fragments with increased intensity in tumors E1 and S12. L-*MYC* resides ~1 Mb centromeric to fragment 2. VGS was unable to match fragment 4 to a database sequence.

the same region of chromosome arm 1p, ~40 Mb from the telomere, and 1 Mb centromeric to fragment 2. By comparing RLGS images of all 47 primary tumors, tumor S12 was also found to display high-intensity spots corresponding to the same four RLGS fragments observed in tumor E1 (data not shown). Although none of the remaining tumors displayed the same pattern of high-intensity spots by RLGS, a small amplicon containing the specific target of the 1p34 amplification cannot be excluded based on the RLGS analysis.

*The Amplified Region of 1p34 Includes L-*MYC*, Which Is at the Center of the Amplicon in Tumor E1*

L-*MYC* gene copy number was evaluated by quantitative (Taqman or real-time) PCR in 68 primary OvCas, including 39 of the 47 analyzed by RLGS. The reference gene *ATPase* was used as an internal control because it is located in a more centromeric position on the same chromosomal arm as the presumptive amplicon. Ten primary tumors showed increased copy numbers of L-*MYC* (more than twofold) relative to DNA from normal tissue, including E1, the tumor with presumed high-copy number amplification by RLGS.

To further characterize and map the minimal critical region (core-amplified domain) of this amplicon, DNA samples from five OvCas with extra copies of L-*MYC* based on the quantitative PCR results were further screened using a STS-amplification mapping approach

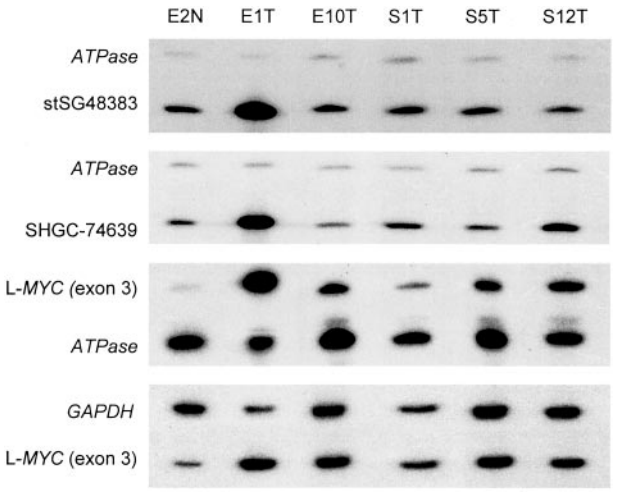

Figure 2. Semiquantitative multiplex PCR for L-*MYC* and representative STS markers in primary OvCas. Representative multiplex PCR gel images from five tumors with increased L-*MYC* copy number compared to DNA from normal tissue (E2N) using chromosome 1 STS markers in and flanking the L-*MYC* gene. *ATPase* (chromosome 1) or *GAPDH* (chromosome 12) co-amplified in each reaction served as internal controls for DNA copy number.

based on competitive multiplex PCR as previously described.[42] The ratios of internal control (*ATPase* and *GAPDH*, co-amplified in the same reaction) and tested marker between normal DNA and tumor DNA were compared for each of 10 STS or gene markers spanning more than 4 Mb along chromosome 1p34-1p35 and centered around L-*MYC* (Figures 2 and 3). The markers include one STS marker within BAC clone AL139015 and one within the *PPIE* gene (stSG48383). Based on STS mapping, tumor E1 possesses the largest amplicon (≈1.4 Mb) and the highest copy number, ≈24-fold increase, of L-*MYC* among the tumors examined (ie, ≈48 copies relative to the two copies present in DNA from normal tissue). Nine additional tumors showed more modest increases of DNA copy number at the L-*MYC* locus (twofold to fivefold) using these methods. Hence, 10 of 68 (15%) primary OvCas showed gains in L-*MYC* copy number without associated genomic amplification of chromosome 1p STS markers flanking L-*MYC*. *L-MYC* amplification in a subset of tumors was confirmed by Southern blot analysis (Figure 4).

BAC clone	Locus	Samples E1	E10	S1	S5	S12	E8	S2	S3	S8	S24	STS(kb)
	D1S2369	○	○	○	○	○	-	-	-	-	-	37321
AL139158	-	-	-	-	-	-	-	-	-	-	-	37558
	D1S432	○	○	○	○	○	-	-	-	-	-	38385
AL139015	SHGC-74636/WI-15142	●	○	○	○	○	-	-	-	-	-	38501
	stSG48383/*PPIE*	●	○	○	○	○	-	-	-	-	-	39233
	SHGC-74642/WI-12773	●	○	○	○	○	-	-	-	-	-	39310
	SHGC-74639/A002R05	●	○	○	○	○	-	-	-	-	-	39327
	L-MYC, exon 2	●	●	●	●	●	●	●	●	●	●	39366
	L-MYC, exon 3	●	●	●	●	●	●	●	●	●	●	39366
	D1S2743	●	○	○	○	○	-	-	-	-	-	39718
	SHGC-74655/A009G33	●	○	○	○	○	-	-	-	-	-	39935
AL606484	-	-	-	-	-	-	-	-	-	-	-	40676
	D1S463	○	○	○	○	○	-	-	-	-	-	41516

● ratio of amplification >2
○ ratio of amplification <2
\- not done

Figure 3. Mapping of the chromosome 1p34-1p35-amplified region in primary OvCas using multiplex PCR. The loci are listed based on distance from the telomere. DNA from tumor E1 contains the largest amplicon, spanning >1.4 Mb of genomic DNA.

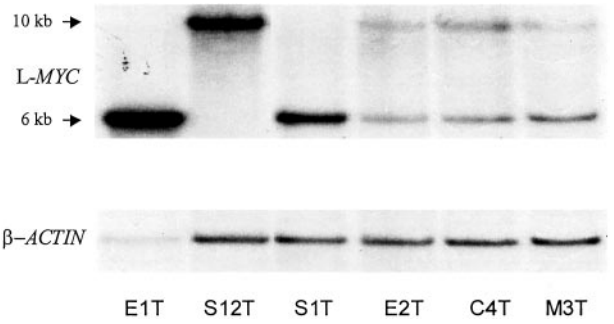

Figure 4. Southern blot showing amplification of L-*MYC* in selected primary OvCas. Three tumors with L-*MYC* amplification (E1T, S12T, and S1T) and three tumors without L-*MYC* amplification (E2T, C4T, and M3T) based on quantitative PCR were evaluated by Southern analysis. After hybridization to the L-*MYC* probe, the blot was stripped and rehybridized with a β-actin probe to control for loading. An *Eco*RI polymorphism in the region of L-*MYC* detected by the probe accounts for variability in the band pattern for L-*MYC* observed in tumor DNA from different individuals.

c-MYC, *L*-MYC, *and Less Frequently, N*-MYC, *Are Overexpressed in Primary OvCas Relative to OSE*

The relative expression of c-*MYC*, L-*MYC*, and N-*MYC* transcripts in 113 primary OvCas and 6 OSE samples based on oligonucleotide microarrays is shown in Figure 5. This set of tumors was largely independent of the group analyzed by RLGS or q-PCR for increased L-*MYC* copy number. Notably, when expression levels of each gene were compared between OvCas and OSE, a greater fraction of OvCas showed elevated expression of L-*MYC* than either c-*MYC* or N-*MYC*. Specifically, 46 of 113 (41%) of OvCas showed L-*MYC* expression values of at least 500 (compared to mean expression of −6 for OSE samples). Two tumors (E8 and E10) with 4.8-fold and 3.3-fold increased L-*MYC* copy number, respectively, were included in the analysis (marked by asterisk in Figure 5). A few tumors showed strikingly high levels of N-*MYC*, suggesting the possibility of N-*MYC* gene defects (eg, amplification) in those samples.

L-MYC *Is Overexpressed in OvCas with L*-MYC *Amplification and in Additional OvCas Lacking the 1p34 Amplicon*

To further examine whether increased copy number of L-*MYC* is associated with elevated L-*MYC* expression, we performed quantitative (Taqman) RT-PCR to measure levels of L-*MYC* transcripts in 59 of the 68 primary tumors evaluated for increased L-*MYC* copy number by q-PCR. Increased expression of L-*MYC* was defined as more than twofold L-*MYC* expression relative to *HPRT1*, primarily because this is approximately the minimum value obtained from tumors with amplifications. Based on this definition, 21 of the 59 (36%) of the OvCas examined showed increased L-*MYC* expression (Figure 6). These

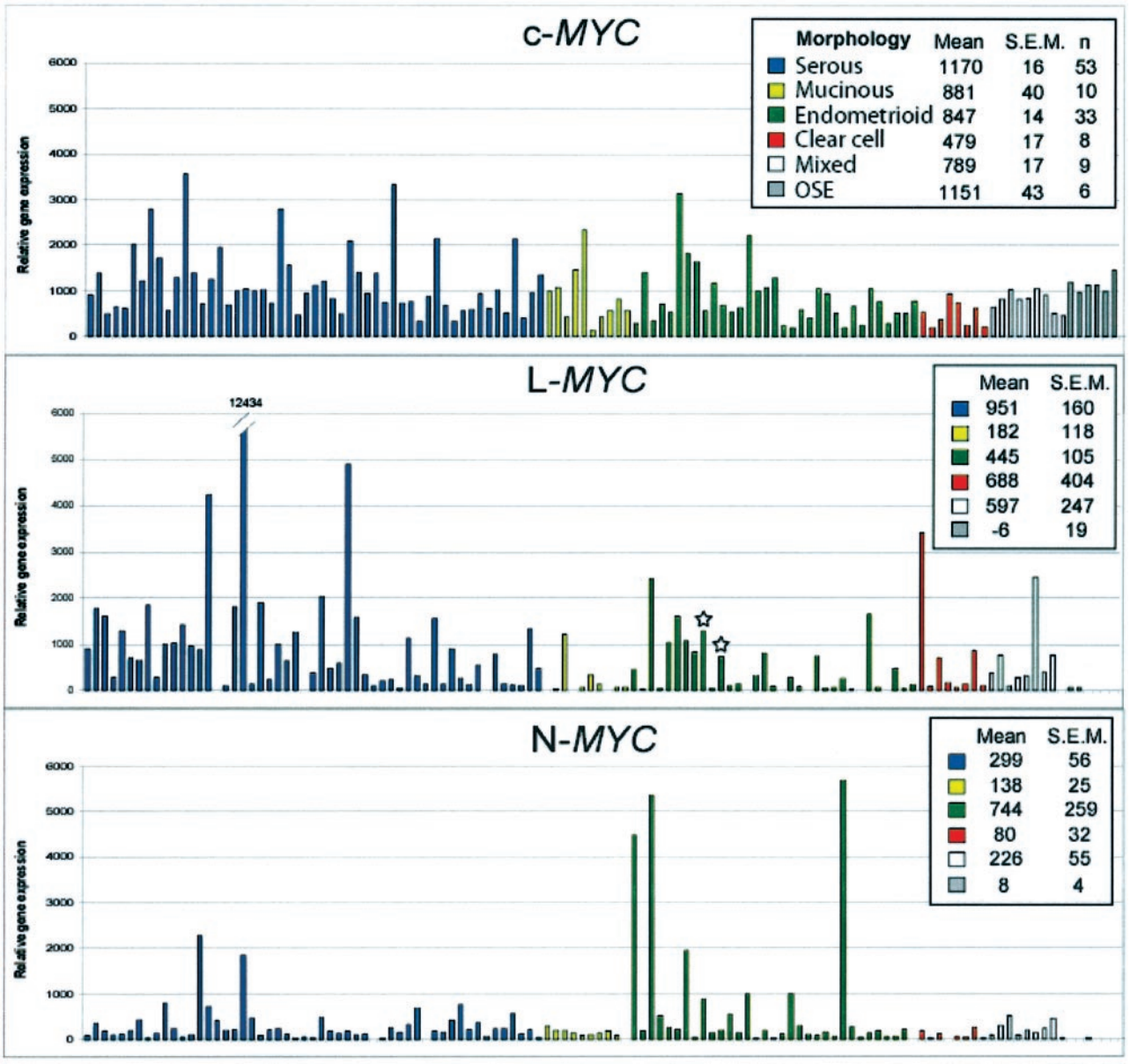

Figure 5. Comparison of C-*MYC*, L-*MYC*, and N-*MYC* gene expression in primary OvCas and OSE samples using oligonucleotide microarrays. Relative expression of each gene in individual samples is expressed in arbitrary units. Samples are color coded by histological type as indicated. The two tumors with increased L-*MYC* copy number based on quantitative PCR are indicated by **asterisks**.

data are in keeping with those based on the microarray-based analysis. As expected, nine of nine tumors containing the 1p34 amplicon overexpressed L-*MYC*. Increased expression of *L-MYC* was also observed in 12 tumors without the 1p34 amplicon, suggesting that activation of *L-MYC* can also occur via mechanisms independent of gene amplification.

Discussion

Previous studies have demonstrated frequent alterations of chromosome 1 in OvCas, including genomic gains/amplifications on the short arm and chromosomal translocation at distal 1p.[43–47] However, the amplified region(s) have not been well characterized nor have candidate genes targeted by the amplifications been identified. In the present study we have shown that L-*MYC* is a gene affected by chromosome 1p amplification in a subset of primary OvCas.

c-*MYC*, N-*MYC*, and L-*MYC* are the three major members of the *MYC* proto-oncogene family. These genes encode proteins that play distinct, but overlapping roles in a wide range of normal and aberrant cellular processes including cell proliferation, differentiation, apoptosis, and tumorigenesis.[48–51] All three *MYC* proteins have been shown to bind the CACGTG (E-box) motif, a function that is critical for the transforming activity of myc proteins.[52,53] Either n-*myc*, c-*myc*, or l-*myc* can cooperate with mutant

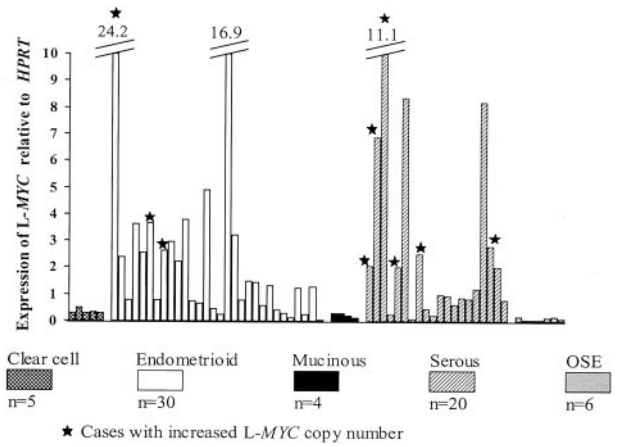

Figure 6. Quantitative (Taqman) RT-PCR analysis of L-*MYC* expression in primary OvCas and OSE samples. After normalization for *HPRT1* expression, data from individual tumors and OSE samples were plotted showing relative levels of L-*MYC* expression. The nine tumors with increased L-*MYC* copy number included in the q-RT-PCR analysis are indicated.

ras to neoplastically transform primary rodent cells. However, in such *in vitro* assays, the transforming activity of l-*myc* is only 1 to 10% that of c-*myc*.[54,55]

L-*MYC* has been much less intensively studied than c-*MYC* or N-*MYC*, and no previous studies have reported genetic alterations of L-*MYC* associated with OvCa. In contrast, *c-MYC* has been found to be amplified in a significant fraction of OvCas.[22–24] L-*MYC* was first found to be amplified in human primary small cell lung cancer and lung carcinoma cell lines.[56] Approximately one-fourth of primary small cell lung cancer tumors contain amplified copies of one of the three *MYC* proto-oncogenes,[57] although amplification of L-MYC is relatively infrequent compared to c-*MYC* or N-*MYC*.[58] Co-amplification of the cyclophilin-like gene (*PPIE*) has been reported in small cell lung cancer cell lines containing L-*MYC* gene amplification.[59] We found *PPIE* co-amplified with L-*MYC* in one tumor (E1) but not in other tumors with more modest L-*MYC* amplification, suggesting that *PPIE* is likely to be a passenger associated with L-*MYC* amplification in tumor E1.

We observed increased L-*MYC* gene copy number in ~15% of primary OvCas, and overexpression of L-*MYC* transcripts even more frequently (~40%). We note that we have compared L-*MYC* expression in primary tumors to short-term and immortalized OSE cultures, rather than uncultured or *in situ* OSE cells. Although immortalization per se does not appear to change L-*MYC* transcript levels, we cannot exclude the possibility that the process of cell culture itself may affect L-*MYC* gene expression. Certainly, *in situ* hybridization or immunohistochemical studies could provide further evidence for low L-*MYC* expression in normal OSE and high expression in a subset of OvCas.

It is interesting that L-*MYC* overexpression and/or amplification seem less prevalent in mucinous OvCas compared to the other tumor types, as L-*MYC* overexpression was observed in only 1 of 10 mucinous carcinomas based on the microarray analysis and none of the tumors with increased L-*MYC* copy number were mucinous. However, these findings may be in part because of the relatively small number of mucinous tumors studied. In addition to implicating L-*MYC* gene amplification and/or overexpression in the pathogenesis of human ovarian cancer, our findings confirm that VGS provides a powerful tool to enhance gene discovery using RLGS. Further work with RLGS and VGS approaches will likely implicate additional oncogene and tumor suppressor gene defects in cancer pathogenesis.

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