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Clinical Importance of Myc Family Oncogene Aberrations in Epithelial Ovarian Cancer

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Abstract

Background: The Myc oncogene family has been implicated in many human malignancies and is often associated with particularly aggressive disease, suggesting Myc as an attractive prognostic marker and therapeutic target. However, for epithelial ovarian cancer (EOC), there is little consensus on the incidence and clinical relevance of Myc aberrations. Here we comprehensively investigated alterations in gene copy number, expression, and activity for Myc and evaluated their clinical significance in EOC.
Methods: To address inconsistencies in the literature regarding the definition of copy number variations, we developed a novel approach using quantitative polymerase chain reaction (qPCR) coupled with a statistical algorithm to estimate objective thresholds for detecting Myc gain/amplification in large cohorts of serous (n = 150) and endometrioid (n = 80) EOC. *MYC*, *MYCN*, and *MYCL1* mRNA expression and Myc activity score for each case were examined by qPCR. Kaplan–Meier and Cox-regression analyses were conducted to assess clinical significance of Myc aberrations.
Results: Using a large panel of cancer cell lines (n = 34), we validated the statistical algorithm for determining clear thresholds for Myc gain/amplification. *MYC* was the most predominantly amplified of the Myc oncogene family members, and high *MYC* mRNA expression levels were associated with amplification in EOC. However, there was no association between prognosis and increased copy number or gene expression of *MYC/MYCN/MYCL1* or with a pan-Myc transcriptional activity score, in EOC, although *MYC* amplification was associated with late stage and high grade in endometrioid EOC.
Conclusion: A systematic and comprehensive analysis of Myc genes, transcripts, and activity levels using qPCR revealed that although such aberrations commonly occur in EOC, overall they have limited impact on outcome, suggesting that the biological relevance of Myc oncogene family members is limited to certain subsets of this disease.

Epithelial ovarian cancer (EOC) is a major contributor to human cancer mortality and is molecularly and clinically diverse (1–3). Recent integrated genomic analyses of EOC revealed one of the most common focal amplifications to be in the 8q24 region containing *MYC* (2), which belongs to the Myc proto-oncogene family that encodes basic helix-loop-helix/leucine zipper transcription factors, including also *MYCN* and *MYCL1*, and plays a key role in many human malignancies (4,5). However, the reported frequency of *MYC* deregulation ranges from 15% to 76% of EOC cases (6–9), limiting an appropriate assessment of the prognostic value of these aberrations in EOC. In addition to *MYC*, amplification and/or overexpression of *MYCL1* (10) and *MYCN* (11) have been reported in this disease, which suggests that more than one Myc family member may play a biological

role in this malignancy. Moreover, subsequent gene expression profiling studies identified molecular subtypes of this disease that were characterized by activation of MYC signaling (1,3), and our previous study demonstrated that high pan-Myc transcriptional activity defined by signature score using a quantitative polymerase chain reaction (qPCR)-based 18-gene Myc activity signature had association with clinical outcome only in a discrete "high-MYCN" molecular subtype of serous tumors (12), highlighting the molecular complexity of EOC. Therefore, a systematic analysis of Myc aberrations is urgently needed and would assist in understanding the importance of each Myc family member in tumor progression and clinical outcome in this heterogeneous disease.

The application of different analytical methodologies and criteria for defining Myc aberrations, especially for copy number elevations, is a likely cause of the inconsistent findings on their clinical significance. For gene amplification, the most widely used techniques include fluorescence in situ hybridization (FISH) (8,9), microarray-based comparative genomic hybridization (aCGH) (2,13,14), and, to a lesser extent, qPCR (8,15). Although MYC amplification or gain has largely been studied using FISH, thresholds are often arbitrarily chosen and this will inevitably affect the ability to reliably determine associations with clinical outcome. Recently developed high-resolution microarray techniques coupled with computational analysis tools allow simultaneous analysis of global copy number variations or expression profiling of thousands of genes, but verification of the results is essential (16). Due to its high sensitivity, accuracy, and specificity, qPCR is widely considered the "gold standard" for validation of such data (17). Hence, here we aimed to comprehensively assess and document the incidence and prognostic impact of increased gene copy number, as determined by a novel, robust, qPCR-based methodology, mRNA expression levels, and a subsequent change in transcriptional activity for MYC, MYCN, and MYCL1 in EOC.

Materials and Methods

Patient Cohorts

Representative cohorts of 150 patients with serous and 61 patients with endometrioid EOC were obtained from the Australian Ovarian Cancer Study, a population-based, case-controlled study undertaken between 2002 and 2006. An additional 19 endometrioid samples were separately obtained from the Gynecological Oncology Biobank at Westmead Hospital, Sydney, Australia. Fresh-frozen tumor specimens of 150 serous and 80 endometrioid tumors were obtained at the time of surgical debulking, prior to chemotherapeutic exposure. Samples were verified as containing at least 70% tumor tissue. Both serous and endometrioid EOC cohorts consist of tumors from all FIGO stages with median clinical follow-up of 46.4 months. Individual cases were subject to review by either light microscopy assessment of representative formalin-fixed tissue taken adjacent to arrayed tissue material or diagnostic slides, or by extraction of information from the original pathology reports when slides were not available. Clinicopathological characteristics for the serous and endometrioid EOCs are summarized in Supplementary Tables 1 and 2 (available online), respectively. Matched genomic DNA and RNA samples were extracted as previously described for EOC cohorts (11). This project was approved by the University of New South Wales Human Research Ethics Committee (HC12551), and all methods were performed

in accordance with relevant guidelines and regulations. Written informed consent was obtained from all patients enrolled in the study.

Determination of Cutoff Points for Gain or Amplification of the Myc Family Members

To estimate objective cutoff points for gain or amplification of MYC, MYCN, or MYCL1, we created a practical algorithm that can utilize the distribution of qPCR-measured copy number values in individual cohorts. Cell lines or tumors first were ranked by level of qPCR-measured relative Myc gene copy numbers for each dataset. Both ranks and copy numbers were then normalized by dividing by their respective maximum values. The normalized copy numbers were then plotted as continuous values against normalized rank. After plotting a reference line where normalized values of copy numbers and rank are equal, the distance (d) between the reference line and the amplification curve was calculated for each sample (Equation 1). The following two steps were used to delineate amplification and gain of each Myc oncogene family member. First, the point where maximum d was measured was used as the optimal threshold for identifying the presence of amplification within a whole cohort (see Supplementary Figure 1, available online, for an example plot). Second, after filtering out samples with gene amplification, the presence of a threshold for gain was further tested using the same procedure for all remaining samples.

$$d = \frac{X \times Y_{max} - Y \times X_{max}}{X_{max} \times Y_{max}} \tag{1}$$

Where X and Y, normalized rank number, and copy number for a sample, respectively; X_{max} and Y_{max}, the maximum values of X and Y, respectively.

Additional procedures are described in the Supplementary Methods (available online).

Statistical Analysis

Statistical analysis of associations between Myc copy number gain/amplification or overexpression and prognosis was performed using SPSS software v. 22 (IBM, Sydney, Australia). Receiver operator characteristic (ROC) analysis was conducted to determine whether there was an alternative cut-point that would better differentiate between good and poor prognosis for each cohort than the statistical algorithm that was developed in this study. The area under the ROC curve (AUC), with 95% confidence intervals (CIs), was calculated for progression-free survival (PFS) or overall survival (OS). To assess for Myc family gene expression, the statistical methodology described in detail in London et al. (18) was used to determine the optimal cutoff points between low and high expression in each cohort in this study. Briefly, tumors were repeatedly divided into either "high" or "low" groups with the cutoff at lower quartile, median, or upper quartile of Myc family gene expression and signature score. For each cutoff, a Cox proportional-hazards regression model was used to produce a P value, hazard ratio (HR), and 95% CIs. The cutoff with the highest HR was selected as the optimal cutoff. Kaplan–Meier survival analysis was conducted at this cutoff and compared by two-sided log-rank tests. Comparisons of gene expression values between tumor groups were made using either Welch's t test or one-way analysis of variance (ANOVA), as appropriate. Fisher's exact test was used to study the association between clinicopathological

characteristics and Myc family gene aberrations. Data visualization of the subgroup tests and correlations were performed with GraphPad Prism v. 6.01 for Windows (GraphPad software, Sydney, Australia). Statistical tests were two-sided unless noted otherwise.

Results

qPCR-Measured Myc Family Oncogene Copy Number Determination in a Panel of Cancer Cell Lines

For robust identification of tumor samples with Myc copy-number elevations using qPCR-measured copy numbers, we developed a statistical algorithm to determine an optimal threshold for Myc amplification (Supplementary Figure 1, available online) and validated the algorithm using a large panel of cancer cell lines that included cells for which the status of individual Myc genes had already been established. To do this, we measured MYC, MYCN, and MYCL1 copy numbers by qPCR for each cell line followed by the application of the algorithm to the cell line dataset (Supplementary Table 3, available online). MYCN copy number elevation was identified in cells derived from neuroblastoma, a well-known MYCN-driven cancer, among which all cell lines were independently validated as carrying MYCN amplification, including SK-N-DZ (19), BE(2)-C (20), IMR-32 (20), CHP-134 (21), NBL-W-S (22), and NBL-W-N (22), were classified to a MYCN-amplified group using the algorithm (Figure 1A). The method was also capable of distinguishing cell lines known to carry MYC amplification, including cell lines derived from cancers of brain [SJ-G2 and HTB-187 (23,24)] and colon [SW-480 (15)], from nonamplified cell lines, including EOC cell line, A2780 (11), and prostate cancer cell line, LNCaP (18) (Figure 1B). Two small cell lung cancer cell lines known to harbor MYCL1 amplification, H378 and H889 (15), were also correctly classified with this aberration using our method (Figure 1C), indicating that the algorithm can readily and accurately identify samples with known amplification of Myc family genes.

Notably, although several cell lines were determined to have higher MYC, MYCN, or MYCL1 copy numbers than normal bone marrow DNA, which was used as a reference sample carrying a normal copy number of each Myc gene in this analysis, they were not classified to the amplified group (Figure 1A–C), suggesting that of these cells, some may have low-level amplification (gain) of Myc. To examine whether the algorithm was able to distinguish cells with Myc gain from those with normal copy, the algorithm was applied only to these remaining cell lines. Although no line was found to carry MYCN gain (Figure 1A), MYC gain was identified in cell lines derived from tumors of the colon (HT-29), brain (HTB-185), and breast (MCF7) (Figure 1B), each of which had previously been determined to carry MYC gain using FISH and aCGH (24–27), thus corroborating the findings with our method. Additional FISH analysis was performed in some cancer cell lines to validate their MYC alteration status as determined using the qPCR-algorithm method (Supplementary Table 4, available online). In the MCF-7 cell line with MYC gain (qPCR-measured MYC copy number = 2.0), the MYC:CEP8 ratio was 1.7 (Figure 1D), as distinct from a MYC-nonamplified line, LNCaP (qPCR-measured MYC copy number = 1; MYC:CEP8 ratio = 1) (Figure 1E), and from a known MYC-amplified line, SW-480, with multiple red signals for the MYC probe in FISH analysis (Figure 1F). The HT-29 cell line was estimated to have a qPCR measured copy number of 2.9, which

was determined by the algorithm to be the upper threshold for gain, and this copy number was in agreement with the MYC:CEP8 ratio of 2.7 determined by FISH (Supplementary Table 4, available online). Additionally, the PCR-algorithm method identified MYC gain in a neuroblastoma cell line, NB69, for which MYC status had not been previously ascertained. FISH analysis of NB69 (qPCR-measured MYC copy number = 1.7) showed that the MYC:CEP8 ratio (1.8) was very similar to that in MCF7, carrying MYC gain (1.7) (Figure 1G), confirming the ability of the PCR-algorithm method to distinguish MYC gain from single copy in samples for which this had not been previously detected. In addition, although it was difficult to quantitate the MYC:CEP8 ratio in MYC amplified cells due to the multiple signals, in those cell lines for which the MYC:CEP8 ratio could be accurately determined by FISH, the numerical values corresponded well to the qPCR-measured copy number values (Supplementary Table 4, available online). Using the method, we also observed MYC gain in H1299, a lung cancer cell line, and MYCL1 gain in cell lines derived from neuroblastoma (SK-N-FI) and colorectal (HT-29), prostate (LNCaP), and ovarian (HEY) tumors (Figure 1C).

Myc Gain/Amplification and Clinical Outcome in EOC

We next applied this method to determine gain and/or amplification of MYC, MYCN, and MYCL1 in cohorts of serous and endometrioid EOC and examined their associations with the clinical outcome. Of 150 serous EOC, 24 (16%) and 11 (7.3%) tumors exhibited MYC amplification and gain, respectively. Ten of 80 endometrioid EOC cases (12.5%) exhibited MYC amplification but none had gain (Table 1). MYCL1 amplification occurred in nine cases of serous and a single case of endometrioid EOC. Neither MYCN amplification nor gain was detected in either histological subtype of EOC.

When the clinical impact of MYC or MYCL1 gain/amplification was investigated in serous EOC, no association with clinico-pathological parameters, including stage, grade, or residual disease, was found (Table 2). Survival analysis indicated that neither MYC amplification nor gain was associated with PFS or OS (Table 1). When tumors with either gain or amplification of MYC were pooled, still no association with clinical outcome was found (P = .48 and P = .7 for PFS and OS, respectively; data not shown). Moreover, MYCL1 amplification was not associated with prognosis in this histological subtype (Table 1). Upon combining cases with amplification or gain of either MYC or MYCL1 into a single group, no relationship between these gene aberrations and prognosis was observed in serous EOC (Table 1). In endometrioid tumors, MYC amplification was found to be associated with late stage and high grade (Table 2), although no impact on patient outcome was observed (Table 1). Neither MYC nor MYCL1 alteration, nor combined MYC/MYCL1 alteration, were predictive of patient outcome according to multivariable analysis that took into account potential confounding effects of debulking status and FIGO stage (Supplementary Table 5, available online).

To further assess the predictive impact of Myc amplification and validate our algorithm, we conducted ROC curve analysis, which can determine whether there was an alternative cutoff point for Myc amplification that would discriminate between patients with good and poor outcome in each EOC cohort. As a measure of accuracy, the AUC value with 95% CIs was calculated for each ROC curve. The ROC analysis for MYC, MYCN, and MYCL1 amplification for serous and endometrioid EOC cohorts showed that the AUC does not differ statistically from 0.5,
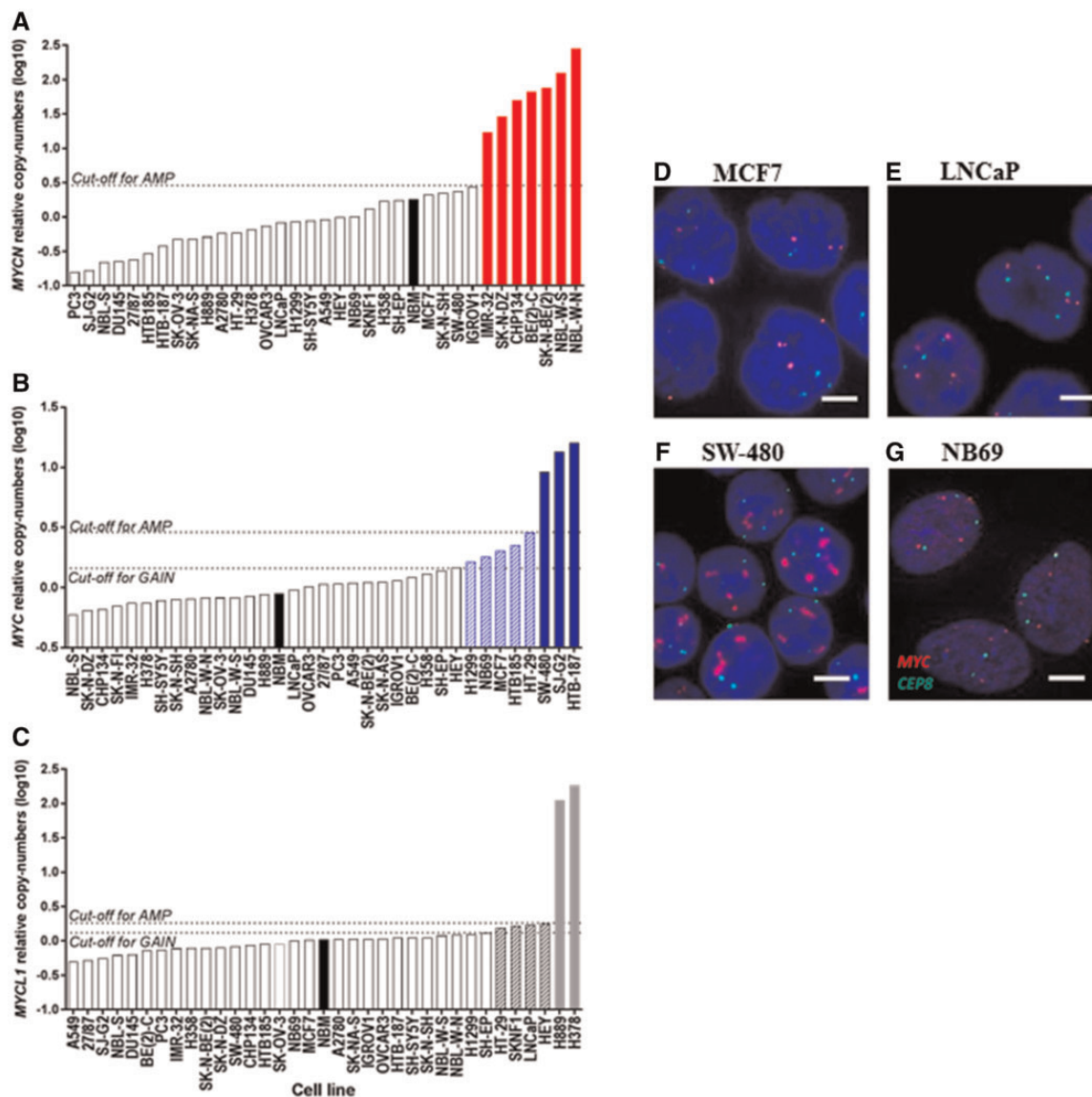
Figure 1. Elevation of MYC, MYCN, and MYCL1 copy numbers in a panel of cancer cell lines. **A–C)** MYCN (**A**), MYC (**B**), and MYCL1 (**C**) copy numbers determined using qPCR and calculated as the geometric means relative to each of three endogenous control genes (*Albumin*, *RNaseP*, and *NAGK*) as described in the Supplementary Methods (available online). The cutoff for amplification was determined using a statistical algorithm. The **dotted lines** indicate optimal cutoff point for amplification or gain and the black bars indicate DNA of normal bone marrow. The **bars with dashed lines** or **solid fill** indicate gain or amplification of Myc gene, respectively, in each graph. **D–G)** The panels show representative images for FISH performed on cell lines as a tool to validate the qPCR-based method for determining of aberrations for MYC in MCF7 (**D**), LnCaP (**E**), SW-480 (**F**), and NB69 (**G**) (63x magnification). Vysis probes were used for MYC and CEP 8. The scale bars = 5 μm.

indicating that Myc amplification is not associated with patient outcome (Supplementary Figure 2, available online). Furthermore, a common approach to identify gene copy number alterations to date is aCGH analysis coupled with computational algorithms, such as Genomic Identification of Significant Targets in Cancer (28). Using this approach, the putative amplification status of MYCN, MYC, or MYCL1 in publicly available TCGA datasets of serous EOC was determined through cBioPortal (http://www.cbioportal.org/faq.jsp). Consistent with our findings, MYC amplification was confirmed to be the most frequent aberration among the Myc family in the serous EOC dataset (n = 557) in which MYC, MYCN, or MYCL1 amplification was identified in 154 (28%), 6 (1.1%), or 50 (9%) cases, respectively. None of these Myc aberrations was associated with poor patient outcome (Supplementary Figure 3, available online).

mRNA Expression Levels and Transcriptional Activity of Myc and Clinical Outcome in EOC

In the absence of any association between Myc gain/amplification and clinical outcome, we examined the relationship of Myc gene expression to copy number and to clinical outcome in EOC. In both histological subtypes, MYC-amplified tumors expressed higher levels of mRNA than tumors without amplification (Figure 2A,B); however, MYC gain, which was observed in some serous tumors, was not associated with an increase of MYC mRNA expression levels (Figure 2A). MYCL1-amplified serous tumors expressed higher levels of MYCL1 as compared with non-amplified tumors (Figure 2C). Despite a trend for high MYCL1 gene expression to be associated with reduced PFS, this failed to achieve statistical significance and no relationship was observed

Table 1. Univariate analysis of gene copy number and expression levels of MYC, MYCN and MYCL1 in EOC

| | Serous EOC (n = 150) | | | | | | Endometrioid EOC (n = 80) | | | | |
| | | PFS | | OS | | | | PFS | | OS | |
Gene	No. of cases	HR (95% CI)	P*	HR (95% CI)	P	No. of cases	HR (95% CI)	P	HR (95% CI)	P
MYC										
Gain	11	0.99 (0.70 to 1.39)	.94	0.93 (0.59 to 1.47)	.75	0	—	—	—	—
Amplification	24	1.23 (0.78 to 1.96)	.38	0.92 (0.49 to 1.70)	.79	10	1.11 (0.38 to 3.23)	.85	0.66 (0.15 to 2.87)	.58
MYCN										
Gain	0	—	—	—	—	0	—	—	—	—
Amplification	0	—	—	—	—	0	—	—	—	—
MYCL1										
Gain	0	—	—	—	—	0	—	—	—	—
Amplification	9	1.64 (0.80 to 3.36)	.18	1.56 (0.68 to 3.6)	.29	1	ND	ND	ND	ND
Any Myc gene alteration	44	1.28 (0.87 to 1.88)	.20	1.04 (0.64 to 1.69)	.88	11	0.97 (0.33 to 2.82)	.95	0.59 (0.14 to 2.57)	.48
Overexpression										
MYC	MED	1.04 (0.73 to 1.48)	.84	1.12 (0.73 to 1.74)	.60	MED	1.37 (0.63 to 2.94)	.43	1.69 (0.66 to 4.34)	.27
MYCN	LQ	0.89 (0.59 to 1.32)	.55	1.51 (0.68 to 1.95)	.60	LQ	1.92 (0.66 to 5.60)	.22	1.02 (0.34 to 3.10)	.97
MYCL1	LQ	0.82 (0.55 to 1.23)	.06	0.70 (0.42 to 1.12)	.13	MED	2.06 (0.92 to 4.64)	.07	2.22 (0.87 to 5.63)	.09

*Log-rank P value. PFS = progression-free survival; OS = overall survival; HR = hazard ratio; CI = confidence interval; ND = not determined; MED = Medium; LQ = Lower Quartile.

Table 2. The association of MYC and MYCL1 aberrations with clinicopathological parameters in 150 serous and 80 endometrioid EOC

	Serous EOC										Endometrioid EOC			
		MYC						MYCL1				MYC		
		Gain			Amplification			Amplification				Amplification		
Parameters	Total	No	Yes	P*	No	Yes	P	No	Yes	P	Total	No	Yes	P
Stage														
I/II	17	16	1	1.000	15	2	1.000	17	0	.598	53	50	3	.010
III/IV	133	123	10		111	22		124	9		25	18	7	
Grade														
1 or 2	34	32	2	1.000	31	3	.288	33	1	.685	49	46	3	.041
3	116	107	9		95	21		108	3		31	24	7	
Residual disease														
No	96	89	7	1.000	82	14	.643	91	5	.723	66	57	9	.683
Yes, >1 cm	54	50	4		44	10		50	4		14	13	1	

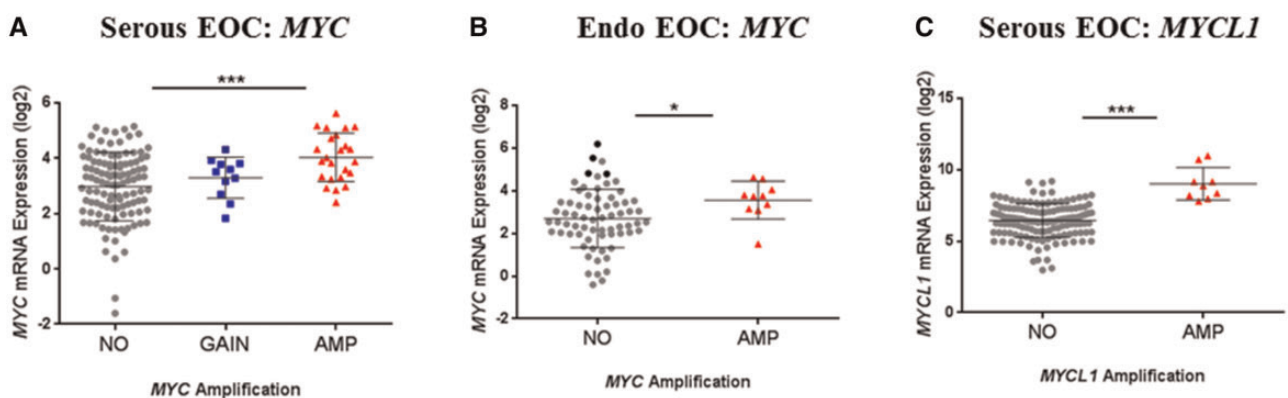*P values are obtained from Fisher's exact test. EOC = epithelial ovarian cancer.

Figure 2. Expression levels of MYC, MYCN, and MYCL1 mRNA according to Myc status in ovarian tumors. In cohorts serous epithelial ovarian cancer (EOC) (A,C), and endometrioid EOC (B), relative gene expression levels of MYC (A,B) and MYCL1 (C) were calculated using the ΔΔCt, method and were compared between tumors with no alteration, gain, and amplification of each Myc gene. P values were calculated using either one-way ANOVA followed by Dunnett's post-hoc analysis (A) or Welch's t test (B,C).
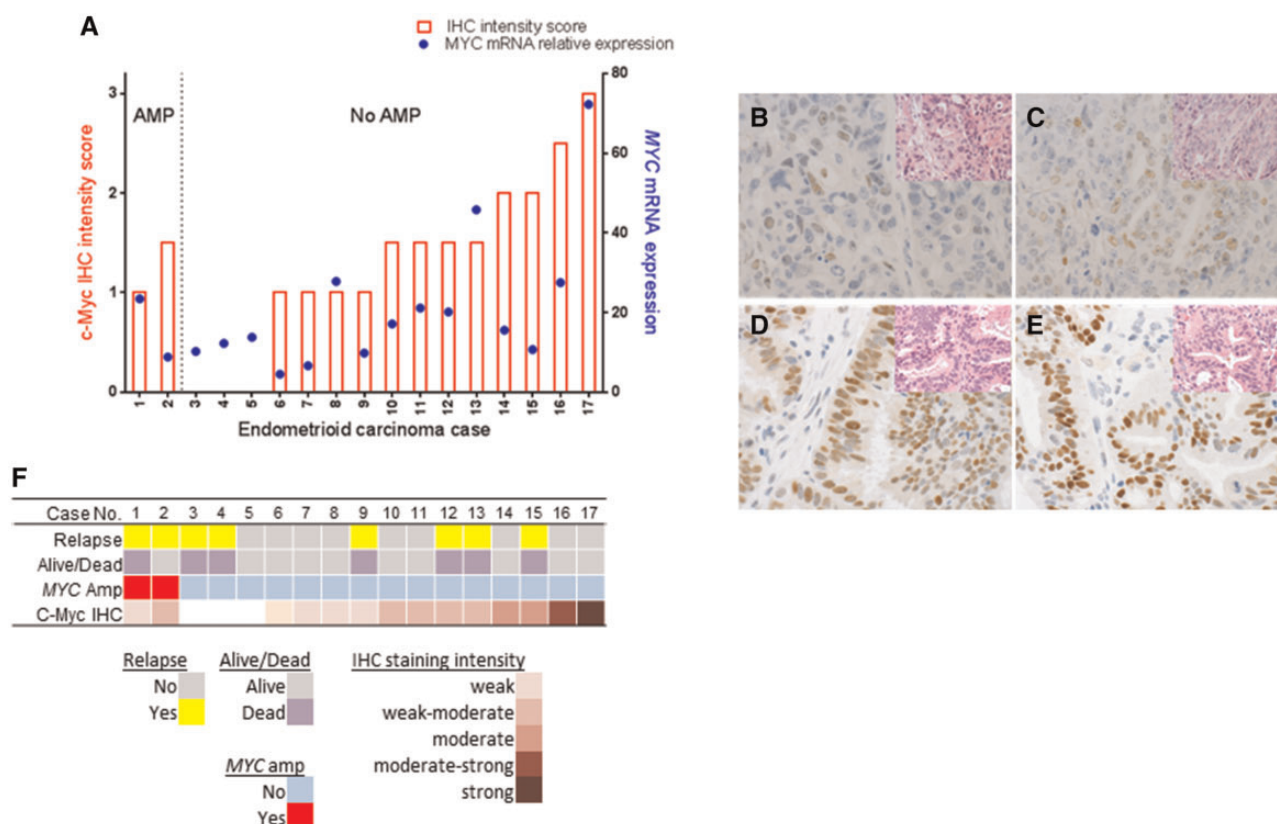
Figure 3. Relationship between genomic amplification, mRNA, and protein expression levels of *MYC* in endometrioid epithelial ovarian cancer (EOC) tumors. Seventeen paraffin-embedded endometrioid EOC tumors were stained with anti c-Myc (Antibody: 9E10). Tumors were scored as having absent, weak, moderate, or strong c-Myc protein staining, as defined in the Supplementary Methods (available online). **A)** Tumors are aligned according to *MYC* status and in order of increasing immunohistochemistry score. Also indicated are mRNA expression and clinical information for each sample. **B–E)** Examples of tumors with different levels of nuclear c-Myc protein expression and *MYC* amplification status with corresponding hemotoxylin and eosin-stained tissue in inserts are shown (600x magnification). **B,C)** Cases 1 and 2: *MYC* amplification but negative protein expression. **D)** Case 16: No *MYC* amplification but moderate-strong protein expression. **E)** Case 17: No *MYC* amplification but strong protein expression. **F)** Overview of properties of the 17 endometrioid tumor samples. Different tracks are indicated below.

for OS (Table 1). High mRNA expression of *MYC*, *MYCN*, or *MYCL1* was not predictive of outcome, following adjustment for the debulking status and FIGO stage in multivariable analysis of both serous and endometrioid EOC (data not shown).

Intriguingly, a proportion of endometrioid tumors expressed very high levels of *MYC* mRNA in the absence of gene amplification (Figure 2B), indicating that *MYC* overexpression can occur independently from gene amplification. To further investigate whether these tumors with *MYC* overexpression but lacking *MYC* amplification also express high c-Myc protein, 17 of the 80 endometrioid EOC were analyzed for c-Myc protein levels immunohistochemically. The analysis revealed that 14 of the 17 cases were positively stained for nuclear c-Myc (Figure 3A). The discordance between amplification and expression was confirmed, such that four cases without *MYC* amplification showed high (moderate to strong) staining intensity for c-Myc (highlighted in black in the no amplification group in Figure 2B), whereas the two *MYC*-amplified tumors exhibited only weak to weak-moderate c-Myc protein staining intensity (Figure 3A). Examples of c-Myc staining characteristics are presented in Figure 3B–E. Despite the lack of correlation between *MYC* amplification and protein expression levels, a positive and statistically significant correlation between c-Myc protein expression levels (intensity score) and mRNA expression levels was found (Pearson's $r = 0.6067$, $P = .0098$), indicating that overexpression of *MYC* mRNA can be acquired without the gene amplification

and that this generally translates into high protein expression. Nonetheless, high c-Myc protein expression levels appeared to have no relationship with patient outcome (Figure 3F), as found for *MYC* amplification and mRNA expression.

We further investigated the relationship between gain/amplification or expression levels of the Myc family genes and elevated overall *MYC/MYCN/MYCL1* transcriptional activity in the endometrioid EOC cohort using a qPCR-based, 18-gene Myc activity signature that we previously developed and showed to have prognostic value in a subset of serous tumors with high *MYCN*, but not in the overall serous cohort using data available through TCGA (12). Signature score did not differ between tumors based on Myc gain or amplification status (Figure 4A) but was positively correlated with *MYC*, *MYCN*, or *MYCL1* mRNA expression levels (Figure 4B–D). High signature score was not associated with patient outcome in the endometrioid tumors (Figure 4E,F). Together these analyses indicate that although elevated expression levels of Myc transcripts and/or transcriptional activity can occur without gene amplification, there is no evidence that these factors are determinants of outcome in serous and endometrioid EOC.

Discussion

Here we undertook to provide complete analyses of aberrations in copy number, gene expression, and oncoprotein activity of
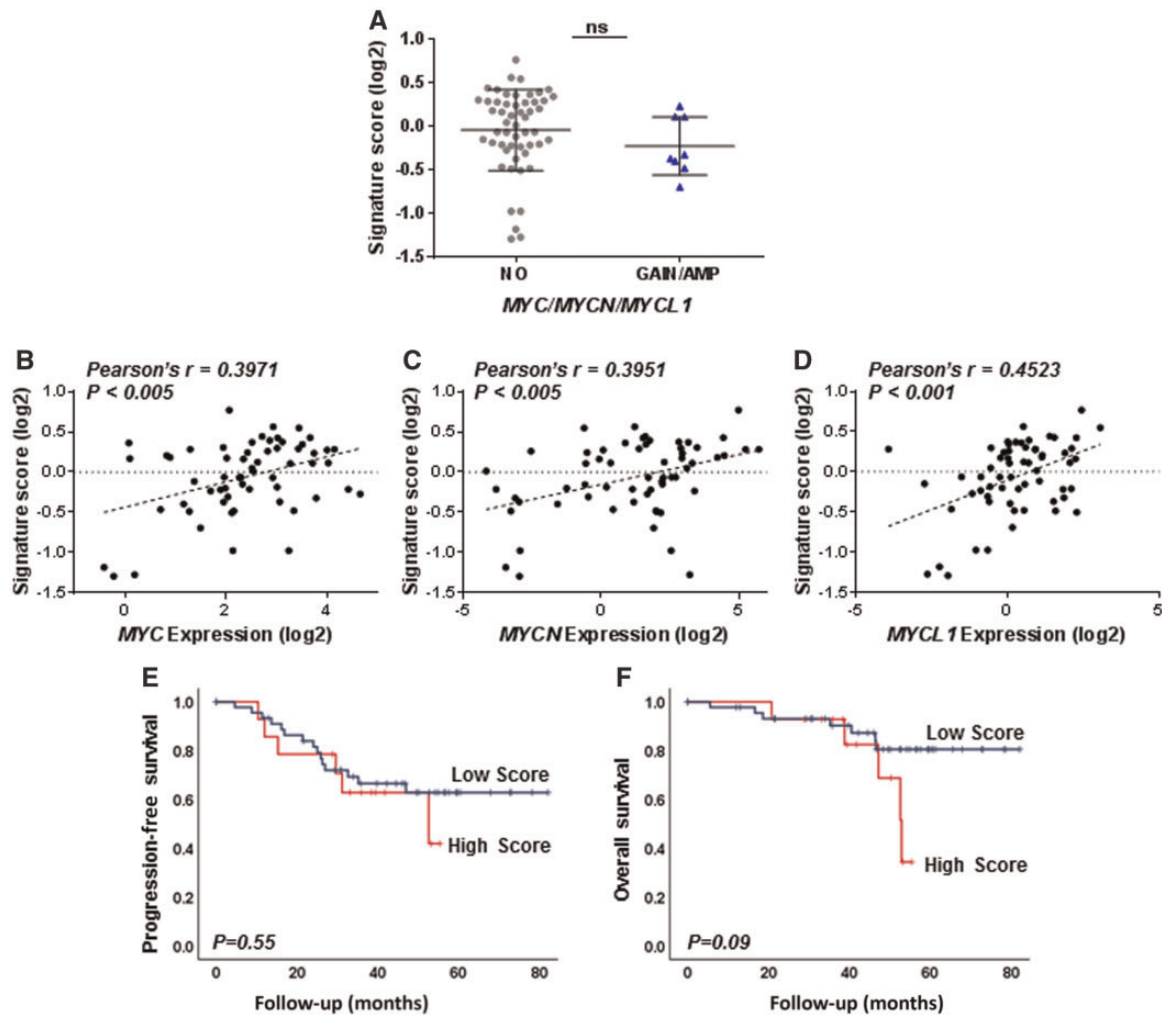
Figure 4. Relationship of Myc activity signature to molecular alterations of Myc family genes and the prognostic significance in endometrioid epithelial ovarian cancer (EOC). **A)** Myc activity levels were compared between tumors without Myc alteration (NO) and those with either gain or amplification (GAIN/AMP) in endometrioid EOC. Welch's *t* test was used to determine different levels of Myc activity. **B–D)** Expression levels of *MYC* (**B**), *MYCN* (**C**), or *MYCL1* (**D**) genes were also measured using qPCR and correlated to signature score in EOC. **E,F)** Kaplan–Meier analysis of signature scores in 61 endometrioid EOC was conducted for PFS (**E**) and OS (**F**). Signature scores were dichotomized at upper quartile in the overall cohorts of each tumor. *P* values were obtained from the log-rank test in each case.

MYC/MYCN/MYCL1 in order to determine the relevance of Myc deregulation to clinical outcome in serous and endometrioid tumors, the two most common EOC histological subtypes. Unlike most previous studies either reporting the prognostic significance of aberrations of one of the Myc family members in EOC or using tumor cohorts consisting of all different EOC histological subtypes (6,7,9), our detailed analysis of alterations of each Myc gene showed that *MYC* amplification was the most common Myc family gene aberration but had no impact on clinical outcome in the overall cohorts of serous and endometrioid tumors. Moreover, elevated *MYC* mRNA or protein can occur in the absence of gene amplification, highlighting the need to consider other measures of Myc functionality in addition to copy-number aberrations. These findings also suggest a fundamental difference between EOC and clearly Myc-driven malignancies like neuroblastoma, where *MYCN* amplification, occurring in ∼25% of cases, is associated with the highest levels of *MYCN* transcription and with the worst clinical outcome (29). Hence, Myc genes may not be a major contributor of EOC tumor progression, but aberrant expression and activity of Myc may represent an epiphenomenon conferred by a complex network of upstream regulators. However, together with several previous studies reporting the effects of direct or indirect targeting of *MYC* on the malignant phenotypic changes in EOC cells in vitro (30,31), significant associations between *MYC* amplification and stage or grade in endometrioid EOC, which was demonstrated in this study using a large number of samples compared to a previous study (8), suggest a potential role of this *MYC* aberration in tumor progression in certain subsets of EOC. In support of this notion, our previous study demonstrated that the association of high Myc activity with poor clinical outcome was limited to a "high-MYCN" molecular subtype of EOC (12). Therefore, given the heterogeneous nature of EOC, being composed of subtypes according to molecular characterization, clinical presentation, and response to therapy, we cannot rule out the possibility that highly activated Myc oncogenes may be confined to tumor subsets. Accordingly, further investigations into Myc oncogenes as prognostic indicators and therapeutic targets for EOC would be best conducted in the context of disease subgroups as well as other less common histological subtypes, including clear cell and mucinous EOC, subject to the availability of adequate sample numbers.

Compared with FISH or aCGH, the statistical algorithm created in this study is a relatively rapid and inexpensive approach to estimate objective cutoffs for gain or amplification using relative copy numbers measured by qPCR. Although qPCR has not been commonly used for determining copy number, a strong correlation between the results of FISH, aCGH, and qPCR in detecting the presence of *MYCN* amplification supports the utility of qPCR as a suitable rapid, cost-effective, and reliable means of detecting gene aberrations ([32]). Such methods typically calculate the copy number relative to a normal reference sample, but a robust approach is still needed to distinguish meaningful increases above normal levels and further to discriminate between gain and amplification. Therefore, when used together with the statistical method that can estimate objective thresholds for gain/amplification, qPCR might be a valuable tool in reducing the inconsistencies in this field that exist to date. Although due to the limited availability, the threshold in genomic level using FISH could not be further validated in clinical samples, validation of this method using a large panel of cancer cell lines with existing data derived from different analytical methodologies allowed robust determination of clear thresholds for Myc gain/amplification. Such methodology could be applied to future studies investigating the biological and clinical relevance of Myc gain in numerous other malignancies.

Overall, this comprehensive study could find no relationship between Myc aberrations at the levels of their genes, transcripts, or transcriptional activities and clinical outcome in either EOC histological subtype, suggesting the biological relevance of Myc oncogene family members is limited to certain subsets of this disease. These findings provide new insights into our understanding of the prognostic importance and potential therapeutic relevance of the Myc oncogene family in EOC.

Funding

Notes

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