Clinical and Biologic Significance of MYC Genetic Mutations in De Novo Diffuse Large B-cell Lymphoma

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Abstract

Purpose: MYC is a critical driver oncogene in many cancers, and its deregulation in the forms of translocation and overexpression has been implicated in lymphomagenesis and progression of diffuse large B-cell lymphoma (DLBCL). The MYC mutational profile and its roles in DLBCL are unknown. This study aims to determine the spectrum of MYC mutations in a large group of patients with DLBCL, and to evaluate the clinical significance of MYC mutations in patients with DLBCL treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) immunochemotherapy.

Experimental Design: We identified MYC mutations in 750 patients with DLBCL using Sanger sequencing and evaluated the prognostic significance in 602 R-CHOP-treated patients.

Results: The frequency of MYC mutations was 33.3% at the DNA level (mutations in either the coding sequence or the untranslated regions) and 16.1% at the protein level (non-synonymous mutations). Most of the nonsynonymous mutations correlated with better survival outcomes; in contrast, T58 and F138 mutations (which were associated with MYC rearrangements), as well as several mutations occurred at the 3' untranslated region, correlated with significantly worse survival outcomes. However, these mutations occurred infrequently (only in approximately 2% of DLBCL). A germline SNP encoding the Myc-N11S variant (observed in 6.5% of the study cohort) was associated with significantly better patient survival, and resulted in reduced tumorigenicity in mouse xenografts.

Conclusions: Various types of MYC gene mutations are present in DLBCL and show different impact on Myc function and clinical outcomes. Unlike MYC gene translocations and overexpression, most MYC gene mutations may not have a role in driving lymphomagenesis. Clin Cancer Res; 1–13. ©2016 AACR.

Introduction

MYC is a proto-oncogene encoding the Myc protein, a transcription factor critical for cell proliferation, metabolism, differentiation, apoptosis, microenvironment remodeling, and immune responses. MYC-IGH chromosomal rearrangement, resulted from aberrant class-switch recombination during germlinal center (GC) reaction and leading to Myc overexpression, underlies the pathogenesis of Burkitt lymphoma, and the poorer prognosis of approximately 10% of diffuse large B-cell lymphoma (DLBCL) associated with MYC translocation (1).

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

MYC mutations in diffuse large B-cell lymphoma (DLBCL) are not as well studied as MYC translocations, another form of MYC genetic aberrations. This study fills this knowledge gap by profiling MYC gene mutations and germline variations in a large group of patients with DLBCL and attempts to understand their impact on MYC function and clinical outcomes. We found a wide range of single-nucleotide variations of MYC genes in DLBCL, which correlated with different clinical outcomes. The results suggested that most MYC mutations in DLBCL were probably passenger mutations instead of driver mutations during lymphomagenesis. This study showed, for the first time, the clinical significance of MYC mutations in DLBCL, and supported the oncogenic role of MYC.

Paradoxically, Myc overexpression is also a potent inducer of apoptosis through the modulation of both p53-dependent and p53-independent pathways, including the activation of TP53, ARF, CD95/LFAS, and BAX, and the inhibition of BCL2, BCLX, and CFLAR/FLIP (2). Therefore, in tumors, deregulation of MYC is often concomitant with other abnormalities (e.g., Bcl-2 overexpression) that cooperate with Myc during tumor onset, progression, and chemoresistance (3–5).

In addition to MYC rearrangement, MYC mutation is another form of genetic abnormality found in Burkitt lymphoma. Multiple nonsynonymous mutations in the coding sequence (CDS) of the MYC gene have been found in approximately 40% to 70% of Burkitt lymphoma leading to a mutated Myc protein with amino acid changes (6–9). These Myc mutations cluster in the Myc transactivation domain with hotspots in the Myc box I (MBI) motif (44-63aa; Fig. 1A), and have been proposed to have a role in lymphomagenesis by enhancing the oncogenicity of Myc (9–12). Functional studies indicated that Myc T58 mutants had increased apoptotic ability, owing to alterations in posttranslational modifications during lymphomagenesis. This study showed, for the first time, the clinical significance of MYC mutations in DLBCL, and supported the oncogenic role of MYC.

Patients and Methods

Patients

The study cohort consists of 750 patients with de novo DLBCL between 2000 and 2010 according to the World Health Organization classification criteria as a part of the International DLBCL R-CHOP Consortium Program. Patients with transformed DLBCL, primary mediastinal, cutaneous, or central nervous system large B-cell lymphomas, or human immunodeficiency virus infection were excluded. Cell-of-origin classification by either gene expression profiling or immunohistochemical algorithms has been described previously (1, 28). Survival analysis was performed for 602 patients treated with standard rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (i.e., R-CHOP) chemotherapy whose follow-up data were available, randomly divided into a training set (n = 368) and a validation set (n = 234). At last follow-up, 208 of the 602 patients had died. The rest (394) patients were censored and had a median follow-up time of 54 months (range, 3–187 months). This study was conducted in accordance with the Declaration of Helsinki and was approved either as minimal to no risk or as exempt from review by the Institutional Review Boards of all participating centers.

The clinicopathologic features of the patients with or without mutations at the time of presentation were compared using the Fisher’s exact test. Overall survival (OS) was calculated from the date of diagnosis to the date of death from any cause or last follow-up. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of disease progression, disease relapse, or death from any cause. Patients who were alive or had no disease progression were censored at the last follow-up. Survival analysis was performed using the Kaplan–Meier method with GraphPad Prism 6, and survival was compared between groups using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards regression model with SPSS statistics software (version 19.0; IBM Corporation). All differences with P ≤ 0.05 were considered statistically significant (4, 28–30).

Gene expression profiling

For patients in the training set, total RNAs extracted from formalin-fixed, paraffin-embedded tissues were subjected to gene expression profiling (GEP) using the Affymetrix GeneChip Human Genome U133 Plus 2.0 as previously described (28). Totally 350 patients in the training sets have GEP achieved, and the CEL files have been deposited in the National Center for...
Biotechnology Information Gene Expression Omnibus repository (GSE31312). Normalized microarray data underwent univariate analysis using a t test to identify genes that were differentially expressed between various groups. The P values obtained by multiple t tests were corrected for false discovery rates (FDRs) using the beta-uniform mixture method.

The mRNA expression levels of selected genes of interest were also compared between DLBCL groups by unpaired t tests using GraphPad Software.

Detection of MYC mutations and rearrangements, assessment of Myc expression, and functional studies of Myc mutants in vitro and in vivo

Details of Sanger sequencing for MYC gene (in all patients), functional studies of Myc mutants in vitro and in vivo, FISH for MYC rearrangement detection (successful in 455 patients), and Myc expression evaluation by immunohistochemistry (successful in 556 patients) performed on tissue microarrays using formalin-fixed, paraffin-embedded samples are in the Supplementary Documents or have been described previously (1, 4, 29, 31).

Results

MYC gene resequencing results overview

The MYC gene variants found in the 750 patients were predominantly single-nucleotide substitutions of the canonical MYC sequence. The single-nucleotide variations (SNVs) from the MYC reference sequence (NG_007161.1) [wild-type (WT) MYC] were herein referred as either germline SNPs [variations in the dbSNP database (Build 132)] or somatic mutations (MUT; the rest of SNVs). Fourteen SNPs were found in the MYC CDS. Of these, two SNPs were most prevalent: rs4645959 (32A>G) which results in Myc-11S protein, and rs2070582 (693G>A) which is synonymous (Fig. 1B). After exclusion of SNPs, MYC gene mutations

![Figure 1](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-15-2296)

**Figure 1.**
Schematic illustration of the structure of MYC gene and Myc protein, and the composition and occurrence of MYC mutations. A, three MYC exons (top) are transcribed into an mRNA (middle) with untranslated regions (UTRs) and the coding sequence (CDS), and then translated into the Myc protein with MYC box I (MBI, 44-63 aa) and MYC box II (MBII, 128-143 aa) in the N-terminal domain (NTD), MYC box III (MBIII including A and B), nuclear localization sequence (NLS), and the basic helix-loop-helix leucine zipper motif (B-HLH-LZ, 355-439 aa, involved in the dimerization with MAX and interacting with other HLH proteins) motif in the C-terminal domain (CTD). TAD indicates transactivation domain. B, occurrence of the SNPs (indicated in parentheses) in the 5'UTR, CDS, and 3'UTR found in the DLBCL cohort. The SNP nucleotide positions are according to the translation start site resulting in the canonical Myc protein (439 aa). C, comparison of the mutation rate of 10 genes we sequenced for the DLBCL cohort. D, patterns of the MYC mutations and rearrangements, assessment of Myc expression, and functional studies of Myc mutants found in the DLBCL cohort. E, proportions of silent, missense, nonsense, frame-shift, and splicing mutations in the MYC CDS found in the DLBCL cohort. F, frequencies of missense and nonsense Myc mutations. Numbers in parentheses indicate occurrence in the DLBCL cohort.

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were found in 250 patients with DLBCL (33.3% of the DLBCL cohort), mainly in the 5'UTR and CDS regions (Supplementary Table S1); mutations in the 3'UTR were much less frequent. Mutations at the splicing sites were rare (n = 2). Most (71.4%) of the mutations were heterozygous.

Compared with the other nine genes we sequenced by Sanger sequencing method, MYC showed an elevated mutation rate in the 5'UTR, although the mutation rate was significantly lower than that of BCL6 5'UTR (Fig. 1C). Compared with MYC SNP variants, MYC mutations were predominated by C>T and C>A transitions, and thus had a higher transition/transversion ratio than the MYC SNP variants (10.1 vs. 4.9; Fig. 1D).

MUTations in the MYC coding sequence

Mutation profile. Among the 750 patients with DLBCL, 254 point mutation events (Supplementary Table S1) were found in the MYC CDS region harbored by 161 patients (21.5% of the DLBCL cohort). However, 39% of these CDS mutations were synonymous mutations, and nonsynonymous mutations resulting in mutated Myc proteins (MUT-Myc) were found in only 121 patients (16.1% of the DLBCL cohort), 75% of which were heterozygous.

Most of these nonsynonymous mutations were missense mutations (Fig. 1E). According to the in silico functional prediction models, 77% of the missense and nonsense mutations had the potential to affect Myc function.

These nonsynonymous mutations were scattered throughout the 439 codons of Myc with one to four occurrences of each mutation (Fig. 1F). The frequency of hotspot mutations within or near MBII, for example, T58 mutations found in four DLBCL patients, was much lower than that found in Burkitt lymphoma (8, 14), and there was another mutation cluster near MBII extending to residue 185. F138 mutations were found in four patients with DLBCL, including two patients carrying concurrent T58A mutations.

### Impact of nonsynonymous MYC mutations and SNPs on patient survival

No clinical parameters significantly differed between the MUT-Myc and WT-Myc groups of the training set, except that MUT-Myc patients with germinal center B-cell–like (GCB) DLBCL had significantly higher frequency of primary nodal (vs. extranodal) origin (Table 1, Supplementary Table S2). Molecularly, the MUT-Myc group compared with the WT-Myc group had

### Table 1. Clinical characteristics of the 368 patients with DLBCL (the training set) with WT or mutated MYC

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<th>MUT-5'UTR</th>
<th>WT-5'UTR</th>
<th>P</th>
<th>MUT-3'UTR</th>
<th>WT-3'UTR</th>
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Abbreviations: CR, complete remission; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; LDH, lactate dehydrogenase; PD, progressive disease; PR, partial response; SD, stable disease. For therapy response, we calculated P values as CR versus other responses. Some data for certain cases were not available.
significantly higher frequencies of MYC 5’UTR mutations (P < 0.0001), CD10 (P = 0.0052) and PI3K expression (P = 0.048), but less frequently nuclear p52 expression (P = 0.0044). Moreover, MUT-Myc patients with GCB-DLBCL more frequently had MYC rearrangements (36.8% vs. 11.7% in the WT-Myc group, P = 0.011) and less frequently expressed CD30, whereas MUT-Myc patients with activated B-cell–like (ABC) DLBCL more frequently had BCL6 rearrangements (68.8% vs. 36.8%, P = 0.015) and p63 expression (Supplementary Table S2).

Compared with the WT-Myc group, the MUT-Myc group showed trends toward better OS (P = 0.08) and PFS (P = 0.05), and patients with nonsynonymous SNPs had significantly better OS (P = 0.015; Fig. 2A) and PFS (P = 0.01). When analyzed in GCB-DLBCL and ABC-DLBCL separately, the better survival of MUT-Myc and SNP-Myc groups than the WT-Myc group remained significant or with border-line P values, except that MUT-Myc ABC-DLBCL versus WT-Myc ABC-DLBCL had only slightly better OS (P = 0.43) and PFS (P = 0.44; Supplementary Fig. S1A and S1B). Trends for better survival rates were also associated with Myc SNVs (mutations or SNPs) in the validation set (Fig. 2B). Between homozygous and heterozygous mutations or SNPs, no significant difference in patient survival was observed (Supplementary Fig. S1C and S1D). Patients with the Myc-11S germline variant had significantly better survival than those with the canonical WT-Myc-11N in the entire (combined training and validation) cohort (Fig. 2C).

Lists of discovered Myc mutations and SNPs and the associated GEP accession codes and clinical outcomes are shown in Supplementary Table S3.

However, multivariate survival analysis, including clinical parameters and Myc mutation and expression status, indicated that Myc protein expression levels but not Myc mutation status independently predicted poorer OS and PFS, although the presence of Myc mutations trended toward conferring better OS (HR, 0.61; P = 0.11) and PFS (HR, 0.57; P = 0.057; Supplementary Table S4).

Prognostic impact and heterogeneity of Myc mutations. Among the R-CHOP–treated patients for survival analysis (n = 602), missense mutations at T58, S62, S67, P79, R83, F138, A141, S175, and A185 occurred in at least two patients. We found these recurrent Myc mutations (defined as n/C21 occurred at a same AA) were associated with differential patient survival independently of Myc expression. Mutations at T58 and F138, which have been correlated with increased Myc stability, gain-of-function, and reduced response to apoptosis in vitro (13, 14, 18), had relative high occurrence in our cohort compared with other mutations, were all overexpressed, and were associated with significantly poor survival (Fig. 2C). In contrast, group of other recurrent mutations (S62, S67, P79, R83, A141, S175, and A185 mutations) was associated with significantly better survival than WT-Myc (Fig. 2C). Among these mutations, S62
mutations have been associated with impaired transforming ability and normal apoptosis function in vitro and in vivo (15, 16). According to the in silico functional prediction models, all the mutations at these recurrent spots except those at P79 had functional impact.

Nonsense, frame-shift and splicing mutations leading to a truncated Myc protein or substantial amino acid changes were also found associated with significantly better survival than WT-Myc (Fig. 2C). The rest of MUT-Myc, which have not been functionally characterized in the literature, were still associated with significantly better OS in combined training and validation sets (Fig. 2D) but not PFS (P = 0.15) compared with the WT-Myc cases.

Prognostic impact of WT and mutated Myc overexpression. Myc expression levels were significantly lower in the SNP-Myc group compared with the WT-Myc and MUT-Myc groups (Fig. 2E). There was no significant difference in Myc levels between the overall MUT-Myc and WT-Myc groups, but we did observe a higher mean level of Myc expression in the MUT-Myc GCB-DLBCL group compared with the WT-Myc GCB-DLBCL group in the training set only (P = 0.047).

High expression level of the canonical Myc (i.e., WT-Myc-11N) correlated with significantly poorer patient survival; Fig. 2F shows the OS curve in overall DLBCL using a 70% cutoff for Mycexp, i.e., ≥70% of tumor cells staining positive on immunohistochemistry analysis (30). This adverse prognostic effect was significant in both GCB-DLBCL (P = 0.0019) and ABC-DLBCL (P = 0.039; figures not shown). In contrast, high level of the Myc-11S germline variant showed trends toward conferring better survival (Fig. 2G). Myc overexpression did not have significant prognostic effect in the overall MUT-Myc group (for OS, P = 0.22). After the exclusion of patients with T58 and F138 mutants, which were all expressed at high levels and correlated with significantly poorer survival (Fig. 2C), patients with high expression levels of non-T58/F138 MUT-Myc did not have significantly poorer survival with those with low MUT-Myc expression levels (P = 0.62 in overall DLBCL, Fig. 2H; P = 0.97 in GCB-DLBCL and P = 0.99 in ABC-DLBCL; Supplementary Fig. S1E and S1F), but significantly better OS than patients with overexpressed WT-Myc (P = 0.031 in overall DLBCL). Breaking down into different types of MUT-Myc in Fig. 2C and D, analysis showed similar results: expression levels of MUT-Myc with recurrent non-T58/F138 mutations (Fig. 2I), nonsense, frame-shift, or splicing mutations (Fig. 2I), or other uncharacterized Myc mutations (Fig. 2K) did not show prognostic effects. Patients with high expression levels of these Myc mutants showed significant trends for better survival than those with overexpressed WT-Myc (Fig. 2F, 1-L).

Prognostic analysis for Myc mutation in the presence or absence of MYC rearrangement. Since approximately 27.3% of the MUT-Myc group had MYC rearrangements (significantly higher compared with the 10% of the WT-Myc group, P = 0.0094; Supplementary Table S2) which has been shown as a significant adverse prognostic factor, we compared the survival outcomes of the WT-Myc and MUT-Myc groups within the MYC rearranged (MYC-R+) and MYC nonrearranged (MYC-R−) DLBCL patients separately. In both the training and validation sets, the MUT-Myc group showed trends toward better survival outcomes compared with the WT-Myc group only in the absence of MYC rearrangements (i.e., MUT-Myc/MYC-R− vs. WT-Myc/MYC-R− but not MUT-Myc/MYC-R+ vs. WT-Myc/MYC-R+; Supplementary Fig. S1G–S1J). MYC rearrangements correlated with significant poorer prognosis in both WT-Myc and MUT-Myc GCB-DLBCL groups (Supplementary Fig. S1K and S1L; MYC-R− vs. MYC-R+). Among the 13 MUT-Myc/MYC-R− cases, 4 cases had T58 and/or F138 mutations (total only five T58/F138-MUT-Myc cases had MYC rearrangement status available) with significantly poorer survival. After excluding these 4 cases from the MUT-Myc/MYC-R− group, there were still no significant difference in survival outcomes between the MUT-Myc/MYC-R− and WT-Myc/MYC-R− groups. Comparison of Myc expression levels between the MUT-Myc, WT-Myc, and SNP-Myc groups within the MYC-R− and MYC-R+ subsets is shown in Supplementary Fig. S2A–S2C.

Mutations in the untranslated regions

5′UTR mutations. Compared with the MYC CDS and 3′UTR, the MYC 5′UTR had a higher mutation rate in our cohort (Supplementary Table S1), with the mutations distributed widely starting from the ninth nucleotide of the first exon (Fig. 3A).

MYC-5′UTR mutations harbored by 139 (19.8%) of the DLBCL cohort were associated with MYC-CDS mutations, 8cl-6 expression, a lower complete remission rate (Supplementary Table S5; Table 1), and differential prognostic impact in the training (no impact) and validation (significantly poorer PFS) cohorts (Fig. 3B and C). Multivariate survival analysis indicated that MYC-5′UTR mutation was not a significant prognostic factor. However, in patients without MYC rearrangements, MYC-5′UTR mutations tended toward conferring poorer OS in the training set and poorer PFS in the validation cohort (Fig. 3D and E).

3′UTR mutations. Compared with mutations in the MYC CDS and 5′UTR, MYC-3′UTR mutations (Fig. 3F) were less frequently (Supplementary Table S1) occurring in 5.8% of patients with DLBCL. Half of these mutations occurred in the miRNA-targeting sites according to TargetScan. However, MYC-3′UTR mutation status did not correlate with Myc expression levels (Supplementary Table S5). These mutations, the affected miRNA targeting sites, and associated clinical outcomes are listed in Supplementary Table S6.

The MUT-MYC-3′UTR group had a higher proportion of men than the WT-MYC-3′UTR group (Table 1). The overall MUT-MYC-3′UTR group did not have significantly poorer survival than patients with WT-MYC-3′UTR in the training and validation sets (Fig. 3G and H). However, multivariate survival analysis adjusting clinical parameters indicated that MYC-3′UTR mutation was an independent prognostic factor for poorer OS (HR, 2.23; P = 0.024) but not PFS (HR, 1.85; P = 0.079; Supplementary Table S4). MYC-3′UTR mutations were found recurrently at ‘2G’, ‘22C’, ‘83C’, ‘345C’, and ‘368C’, which were associated with significant poorer survival than WT-MYC-3′UTR (Fig. 3I and J), although these mutations were not concurrent with MYC rearrangements.

Gene expression profiling analysis

Comparisons between WT-Myc and MUT-Myc. By supervised clustering analysis, no genes showed significant differential expression between the MUT-Myc and WT-Myc groups (overall cohort or only Mycexp subcohort), or between MUT-MYC-5′/3′UTR and WT-MYC-5′/3′UTR groups. Individual analysis of particular mutation types showed differentially expressed genes involved in proliferation, metabolism, and apoptosis.
Mutations in the MYC UTRs. A, distribution of mutations in the MYC 5'UTR. The nucleotide positions shown before the parentheses are in relation to the translation start site for canonical Myc protein (439aa). Numbers in parentheses indicate occurrence frequency in our cohort. B and C, MYC 5'UTR mutations did not correlate with survival in the training set, but did correlate with significantly poorer PFS in the validation set. D and E, in patients without MYC rearrangements, MYC 5'UTR mutations tended toward conferring poorer OS in the training set and poorer PFS in the validation sets. F, distribution of mutations in the MYC 3'UTR. Numbers in parentheses indicate occurrence. Mutations disrupting the known miRNA targeting sites (according to TargetScan) are highlighted in red. G and H, the overall MUT-MYC-3'UTR group did not show significant poorer survival in the training and validation sets. I and J, 3'UTR mutations recurrently (n ≥ 2) occurred at ‘2G, ‘22C, ‘83G, ‘345C, and ‘368C were associated with significantly poorer survival.

( Supplementary Fig. S2D–S2G; Supplementary Table S7), but the significance of these analyses was hindered by small numbers and the heterogeneity of the MUT-Myc cases likely because some patients carried multiple mutations. Notably, these signatures included genes involved in Ras/Rho GTPase signaling which interacts with the Myc T58 residue (10, 13) and can cooperate with Myc during tumorigenesis (32).

Comparisons between WT- or MUT-Myc<sup>high</sup> and Myc<sup>low</sup>. We further identified the GEP signatures of Myc overexpression (Myc<sup>high</sup> GEP signatures) in the WT-Myc and MUT-Myc groups separately, and compared these GEP signatures (Fig. 4A–D; Table 2). Differentially expressed genes were shown between WT-Myc<sup>high</sup> and WT-Myc<sup>low</sup> in overall DLBCL, GCB-DLBCL, and ABC-DLBCL, and between MUT-Myc<sup>high</sup> and MUT-Myc<sup>low</sup> in overall DLBCL and GCB-DLBCL but not in ABC-DLBCL even with a high FDR threshold of 0.50. These GEP signatures include MYC, some genes which have important oncogenic roles in transformation by Myc (for example, CDC24L, UVBL2, MKI67IP, NOP16, MINA, and DDX18), and genes which regulate MYC/Myc (for example, PRKDC, PURB, SKP2, NME2, CSNK2A2, APEX1, and AIMP2).

All the WT-Myc<sup>high</sup> and MUT-Myc<sup>high</sup> GEP signatures are characterized by strong proliferation and growth signatures (especially for WT-Myc<sup>high</sup> ABC-DLBCL) resembling those identified by previous studies (33, 34). However, MUT-Myc<sup>high</sup> GEP signatures also included downregulation of CCND2 (cyclin D2) and JUND in overall DLBCL, and downregulation of CCND1 (cyclin D1) and TAF13 RNA polymerase II, whereas upregulation of COMMD5 (which negatively regulates cell-cycle transition and proliferation) in GCB-DLBCL. In contrast, in WT-Myc<sup>high</sup> GCB-DLBCL (vs. WT-Myc<sup>low</sup> GCB-DLBCL), CDKN1B (inhibitor of cell-cycle progression) and ANKRD12 (which inhibit transactivation) were downregulated. STAT3 was significantly upregulated in WT-Myc<sup>high</sup> DLBCL but downregulated in MUT-Myc<sup>high</sup> GCB-DLBCL.

Expression of apoptotic genes also showed differences between WT-Myc<sup>high</sup> and MUT-Myc<sup>high</sup> GEP signatures. Proapoptotic HRK was significantly upregulated in MUT-Myc<sup>high</sup> but not in WT-Myc<sup>high</sup> DLBCL, which instead had upregulation of PDCD5 (which promotes p53-mediated apoptosis; in DLBCL and GCB-DLBCL), BID, and GNL3 (which stabilize MDM2; in ABC-DLBCL). Other upregulated genes having roles in regulating the p53 pathway included P16INK4A, MDM2, and TP53INP1 in WT-Myc<sup>high</sup> ABC-DLBCL, and DAPK1 in WT-Myc<sup>high</sup> GCB-DLBCL. In contrast, in MUT-Myc<sup>high</sup> GCB-DLBCL, proapoptotic RASSF4 and DAPK1 were downregulated compared with MUT-Myc<sup>low</sup> GCB-DLBCL.

Moreover, in WT-Myc<sup>high</sup> ABC-DLBCL (vs. WT-Myc<sup>low</sup> ABC-DLBCL), several T-cell marker genes (CD4, GIMAP1, TRA@, and FOXP3) were downregulated but CIQBP (which inhibits the complement subcomponent C1) was upregulated. NCR3LG1 (which triggers natural killer cell activation) was upregulated in WT-Myc<sup>high</sup> (vs. WT-Myc<sup>low</sup>) GCB-DLBCL. In MUT-Myc<sup>high</sup>
Figure 4. GEP analysis, and functional studies of Myc variants in Rat1a cells. A, GEP signatures for high levels (≥70%) of Myc expression (Myc$^{\text{high}}$) in WT-Myc patients with GCB-DLBCL (FDR < 0.20). B, GEP signatures for Myc$^{\text{high}}$ in WT-Myc patients with ABC-DLBCL (FDR < 0.05) with a cutoff of 1.65 for fold change of differential expression. C, GEP signatures for Myc$^{\text{high}}$ in WT-Myc GCB-DLBCL patients (FDR < 0.20). D, GEP signatures for Myc$^{\text{high}}$ in overall WT-Myc patients (FDR < 0.01). E, Western blot analysis of expression of WT-Myc and Myc variants in Rat1a cells transduced with retroviral vector expressing WT-Myc and Myc variants. F, cell proliferation analysis of WT-Myc and Myc variants. G, cell apoptosis analysis of WT-Myc and Myc variants using serum withdrawal. H, an anchorage-independent colon formation assay of WT-Myc and Myc variants. I, Tumorigenicity of cells expressing WT-Myc or its mutants. J, schematic illustration for the possible mechanism of Myc mutation and rearrangement origin, i.e., the activities of AID, which depend on MYC transcription activation and can affect up to approximately 2 kb downstream DNA from the transcription initiation site. Abbreviations: AID, activation-induced cytidine deaminase; SHM, somatic hypermutation; P0, P1, P2, and P3 indicate multiple promoters of the MYC gene. K, a hypothetical model for origin of MYC genetic lesions and effects on Myc expression, Myc function, and clinical outcomes.

Comparisons between WT-Myc and MUT-Myc groups using unpaired t test. By unpaired t test, the MUT-Myc group compared with the WT-Myc group had significantly (P < 0.05) higher levels of MDM2, TP63, CD10/MME, and CD22, and significantly lower levels of CD44, ICAM1, JAK3, STAT3, STAT5A, TNFSF13B/BAFF, CTLA4, and ICOS mRNA, as well as suble changes in HLA, PMAP1/NOXA, TP53, CDKN2A, BCL2, BCL2L1/BIM (which mediates the proapoptotic function of Myc; refs. 17, 35), BID, CHUK/IKK1, IKBKB, NFkBA, and NFkBIZ expression, but not in MIR17HG (which mediates the oncogenic function of Myc; ref. 36), EZF1, and EZH2 levels (Supplementary Figs. S3 and S4). Genes encoding regulators of Myc degradation/stability according to the literature, such as FBXW7, SKP1/2, PIN1, GSK3, PP2A subunits (13), did not show significantly differential expression between the MUT-Myc and WT-Myc groups. Nonetheless, the MUT-Myc group had significant lower FBXW9 expression compared with both WT-Myc$^{\text{low}}$ and WT-Myc$^{\text{high}}$ groups.
Table 2. Significantly differentially expressed genes between Mycc� and Myccľ DLBCL (WT or MUT)
Comparison of protein expression levels between WT-Myc and MUT-Myc groups using unpaired t test

The MUT-Myc group compared with the WT-Myc group had significantly increased CD10 and decreased nuclear p52 levels. Expression of p53, Ki-67, pAKT, c-Rel, Bcl-2, and p63 levels may also be affected by Myc mutation status (Supplementary Fig. S5).

Functional studies of MYC mutations and SNPs

We made MYC expression constructs for three mutants (S159R, G160S, and P164L) and two germline variants, including N11S and P57S (by SNP rs28933407), and introduced them into MYC-null Rat1a fibroblasts. We first determined the expression of Myc in Rat1a cells and found that all three mutants and the germline variant N11S resulted in lower Myc protein levels, whereas P57S variant had higher Myc expression, in line with a previous report (P57S was considered as a Myc protein levels, whereas P57S variant had higher Myc expression, expression of Myc in Rat1a cells and found that all three mutants and the germline variant N11S resulted in lower Myc protein levels, whereas P57S variant had higher Myc expression, in line with a previous report). We seeded 5 × 10⁴ cells in 6-well plates, and 72 hours later, adherent cells were enumerated. Cells with Myc-P57S grew fastest, and cells with WT-Myc grew modestly faster than did the controls with the parental vector (Fig. 4F). Cells with Myc-N11S and Myc-P164L proliferated at similar rates as WT-Myc, yet cells with Myc-S159R and Myc-G160S had a significantly slower rate than WT-Myc. We next asked whether any of the mutations altered the well-known ability of Myc overexpression to sensitize cells to apoptosis induced by serum withdrawal. Cells expressing WT and N11S Myc were sensitized to serum withdrawal-induced apoptosis, whereas cells with P57S, S159R, G160S, and P164L Myc showed apoptosis resistance (Fig. 4G). In anchorage-independent colony formation assay, Myc-P57S greatly enhanced the transformation ability compared with WT-Myc, consistent with the previous report (17); however, N11S, S159R, G160S, and P164L Myc had compromised transformation ability compared with WT-Myc (Fig. 4H). To further assess the tumorigenesis of these Myc mutants in vivo, xenograft in nude mice was applied. Rat1a cells stably expressing WT-Myc or its mutants were subcutaneously implanted to 8-week-old male nude mice (ten implantations each). Eighteen days after inoculation, significant tumors were visible in mice injected with cells expressing WT-Myc (NG_007161.1), regardless of Myc expression levels. This correlation and functional study results may suggest that many Myc mutations attenuated Myc oncogenic function, potentially due to functional changes or haploinsufficiency effects (40). Attenuated proapoptosis function may play roles in tumorigenesis (17, 18, 41). Moreover, identified Myc<sup>hap</sup> GEP signatures may suggest that to a certain extent, there were differences in tumor survival, proliferation, and microenvironment between the WT-Myc and MUT-Myc groups.

In contrast, T58 mutations, which are frequent in BL and have gain-of-function in vitro and in vivo (13), were associated with significantly poorer survival than other patients with DLBCL. However, the occurrence was low in DLBCL (0.8% of the training set, and 0.5% of the combined training and validation cohorts) and associated with MYC rearrangement (an independent prognostic factor for adverse survival). In addition, the recurrent MYC-3'UTR mutations in 1.3% of patients with DLBCL were associated with significant poorer survival than WT-MYC-3'UTR. TargetScan indicated that '22C, '83G, '345C of MYC-3'UTR are targeted by miR-196b, miR-33b, and miR-429, respectively. Intriguingly, 3'UTR mutations were not associated with Myc overexpression, suggesting the presence of MYC suppression by multiple miRNAs (42) and posttranslational regulations, and that the molecular mechanisms underlying the adverse prognostic impact of these 3'UTR mutations may not be simply Myc activation due to the disruption of miRNA-mediated MYC suppression. GEP analysis showed that TNCRG6, which plays important roles in miRNA-mediated suppression, was significantly downregulated in patients harboring recurrent MYC-3'UTR mutations. In addition, MAGEA2/MAGEA2B and ZNF415, which inhibit p53 transcription activities, were upregulated, and RAD1, which plays a role in DNA repair, was downregulated (Supplementary Table S7).

In this study, MYC mutations were not associated with TP53 mutations which increase genomic instability. It has been proposed that MYC mutations originated from aberrant somatic hypermutation initiated by activation-induced cytidine
MYC mutations in DLBCL Have Variable Prognostic Impact

Disclosure of Potential Conflicts of Interest
E.D. Hsi reports receiving speakers bureau honoraria from Seattle Genetics and is a consultant/ advisory board member for HTG Molecular Diagnostics. K.H. Young reports receiving conference travel expenses from Seagen and a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

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