Increased *MYC* gene copy number correlates with increased mRNA levels in diffuse large B-cell lymphoma

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ABSTRACT

Background
Translocations involving the *MYC* gene and increased *MYC* mRNA levels are associated with poor outcome in diffuse large B-cell lymphoma. However, the presence of increased *MYC* gene copy number and/or polysomy of chromosome 8 have not been previously described.

Design and Methods
Utilizing dual color chromogenic *in situ* hybridization, we investigated *MYC* gene copy and chromosome 8 centromere numbers in 52 cases of diffuse large B-cell lymphoma. Cases were divided into those with "increased" or "not increased" *MYC* gene copy number for comparison with *MYC* mRNA levels, Ki-67 values, and survival.

Results
Increased *MYC* gene copy number was present in 38% of cases. Overall, the average *MYC* mRNA level was 2398 (range, 342 - 9783) and the percentage of nuclei positive for Ki-67 was 57.5% (range, 20-87%). Within the group with increased *MYC* copy number, the *MYC* mRNA values ranged from 816 to 5912 (average, 2843) and the Ki-67 values ranged from 23% to 83% (average, 57%). Within the group with not increased *MYC* copy number, *MYC* mRNA values ranged from 342 to 9783 (average, 2118) and the Ki-67 values ranged from 20% to 87% (average, 58%). There was a statistically significant relationship between increased *MYC* gene copy number and increased *MYC* mRNA (P=0.034) and a trend toward a relationship between increased mRNA and higher Ki-67 values.

Conclusions
This is the first report that low level copy number increases are common in diffuse large B-cell lymphoma and that these changes correlate with *MYC* mRNA in a statistically significant manner. *MYC* copy number changes are an additional possible molecular mechanism that may result in increased mRNA and, likely, high proliferation and poor outcome.

Key words: *MYC* gene, large B cell lymphoma, mRNA.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease with multiple subtypes and varying clinical outcomes, which together account for up to 30% of adult non-Hodgkin's lymphomas.[1] Gene expression profiling has been used by various groups to predict outcome in DLBCL patients. In one study by the Lymphoma and Leukemia Molecular Profiling Project, a model for outcome prediction based on 17 genes was developed which incorporated a "proliferation signature" that included the *MYC* gene.[2] Other studies identified additional sets of genes predictive for survival in DLBCL, with at least one study also identifying the *MYC* gene as being significantly correlated with outcome.[3-5] The patients examined in the aforementioned studies were all treated with CHOP-like regimens [cyclophosphamide, hydroxydaunorubicin, oncovorine (vincristine), and prednisone]. More recently, our group sought to exploit a novel quantitative nuclease protection assay (qNPA), which can be used in formalin-fixed paraffin-embedded tissue, to examine the expression of 36 genes with previously identified prognostic value in order to determine whether or not these genes retained significance in patients treated with rituximab and CHOP (R-CHOP).[6] These data led to a two-gene model, based on mRNA levels of *MYC* and *HLADR*, for predicting outcome of DLBCL patients. Specifically, over-expression of *MYC* combined with under-expression of *HLADRA* predicted for a group of patients with an only 14% 2-year overall survival. Other studies have upheld the prognostic relevance of *MYC* expression in DLBCL patients treated with R-CHOP.[7]

MYC regulation, function, and dysfunction are highly complicated processes; however aberrations involving *MYC* (Ig-related and non-Ig related translocations, dual translocations, increased mRNA, altered microRNA) are a recurring finding in non-Hodgkin's lymphomas as well as in other malignancies. One mechanism for *MYC* gene over-expression that has not been previously investigated is gene amplification. Gene amplification via increased gene copy number is routinely detected in clinical practice by fluorescence *in situ* hybridization (FISH), most notably for *HER2/neu* and *EGFR* in breast and lung carcinoma, respectively. Recently, a technique utilizing dual-color chromogenic *in situ* hybridization (CISH) for the *HER2* gene and chromosome 17 centromere (CEN17) was validated in comparison to manual dual-color FISH for the detection of *HER2* gene copy number in breast carcinoma.[8] CISH allows the direct measurement of gene copy number in relation to chromosome number using a method adaptable to the routine histopathology laboratory and interpretable by light microscopy. This technology also provides permanent staining, overcoming the fading that occurs with FISH probes.

The aim of this study was to investigate the frequency of increased *MYC* gene copy number in DLBCL using the novel CISH technique and to relate the findings to the *MYC* mRNA levels measured by qNPA, the presence of translocations involving the *MYC* locus, proliferation rates, and the patients' survival. We hypothesized that increased *MYC* gene copy number is a mechanism of *MYC* gene over-expression in DLBCL and would be related to both proliferation and survival.

Design and Methods

Case selection

Biopsies from 52 patients, representing cases from the University of Arizona, were used for this study. All patients received either CHOP (n=39) or R-CHOP (n=13) chemotherapy after diagnosis. *MYC* mRNA levels were determined by qNPA as a part of prior studies.[6,9] Samples from the cases were assembled into a tissue microarray consisting of three punches of 1 mm in diameter for each case. These 52 cases were all the available cases with sufficient material for construction of a tissue microarray remaining from our previous study on gene expression profiling from matched frozen and paraffin-embedded tissues.[6]

MYC *mRNA measurement*

As noted above, MYC mRNA expression levels were evaluated in all cases by qNPA, a method for measuring mRNA levels in formalin-fixed paraffin-embedded tissues which has shown excellent consistency with results from frozen tissues.[9] In the qNPA technique, *MYC* mRNA is bound to a specific probe *in situ* for chemiluminescent quantification. The light emitted is proportional to the amount of bound probe and a ratio is generated.

The overall mean mRNA level was calculated and cases with values above the mean were considered to have increased *MYC* mRNA while cases with values below the mean were considered to have not increased levels of *MYC* mRNA. While somewhat arbitrary, in our previous study analysis of variable cut-points, this cut-off was proven to be meaningful and the same cut-off was, therefore, used in the current study.[6]

Fluorescence in situ *hybridization analysis*

Fluorescence *in situ* hybridization studies were performed on intact formalin-fixed paraffin-embedded tissue microarray sections using a dual color, break-apart probe for *MYC* (Abbott Molecular, Abbott Park, IL, USA) as previously described.[10] Signals were visualized on an Axioskop photomicroscope (Zeiss, Oberkochen, Germany). Based on analysis of normal lymph node controls, a threshold of 10% abnormal nuclei was established for interpretation as a positive result. Cases were initially screened by scanning in a semi-quantitative fashion. Cases estimated to have more than 20% break-apart nuclei on scanning were scored as positive, and cases with no or only rare break-apart signals (estimated at <2% of nuclei) were classified as negative. For cases estimated to have between 2% and 20% abnormal nuclei, 200 nuclei were scored to give a final quantification.

MYC *and chromosome 8 analysis by chromogenic* in situ *hybridization*

The MYC probe was designed to cover a region on chromosome 8q24, spanning 500 kb 5 prime and 280 kb 3 prime of the MYC gene (see the map in Figure 1). Bioinformatic tools (Human Genome Browser and Repeat Masker) were used to deplete the sequence of repetitive elements. Unique sequences comprised about half of the coverage. Primer3 and NetPrimer programs were used to design primers to non-repetitive sequence with strict criteria to maximize the efficiency of the polymerase chain reaction (PCR). The designed PCR fragments and primers were analyzed for homology to human genome and transcripts (by Human BLAT and Blastnt programs) and resulted in a 505 kb probe. Fragments that showed high homology to the other regions were excluded. A FISH assay with Vysis CEP8 probe and Ventana's *MYC* probe was performed on metaphase spread slides. The results showed that there were only two *MYC* signals in each cell and that each *MYC* signal co-localized with the Vysis CEP8 signal (*data not shown*).

Dual color CISH for the *MYC* gene and CEN8 was conducted on a Ventana BenchMark XT (Ventana Medical Systems, Inc., Tucson, AZ, USA). Two DNA targets were detected using an established sequential *in situ* hybridization method[8] with a modification. First, the *MYC* gene was detected using a dinitrophenyl (DNP)-labeled, nick-translated, repeat deleted *MYC* DNA probe (in product development phase) and an alkaline phosphatase (AP)-based blue detection system (Ventana property chemistry in the discovery phase process). CEN8 was visualized with a DNP-labeled CEN8 oligoprobe (in product development) and a fast red and naphthol phosphate based ultraView Red ISH Detection Kit (Ventana Medical Systems, Inc.). All slides were counterstained with hematoxylin II (Ventana Medical Systems, Inc.) and bluing reagent (Ventana Medical Systems, Inc.). After rinsing the slides with DAWN® (Proctor & Gamble Company, Cincinnati, OH, USA) to remove Liquid Coverslip™ (Ventana Medical Systems, Inc.), the slides were blotted gently with a paper towel and completely dried at 65°C for 15 min. The dried slides were covered using Tissue-Tek® film cover-slipper (Sakura Finetek Japan, Tokyo, Japan).

Interpretation of chromogenic in situ *hybridization results; classification into cases with increased or not increased* MYC *gene copy number*

CISH staining was evaluated systematically for all cases. Cases were included if they contained both well-stained areas of lymphoma and appropriately stained internal control cells (usually endothelial cells, histiocytes, or benign lymphocytes). Neoplastic cells were expected to demonstrate *MYC* and CEN8 signals consistent in size and strength with those identified in the internal control cells. Since the technique is applied to paraffin-embedded tissue, there is an expected variation between nuclear signals based on the limitations imposed by the two-dimensional evaluation of three-dimensional structures. Fifty representative nuclei were evaluated and the numbers of *MYC* and CEN8 signals were recorded. The decision to evaluate 50 cells was based on analogy to consensus guidelines for the evaluation of *HER2/neu* gene amplification, which indicated that the acceptable minimum is 40 cells.[11] A *MYC*/CEN8 ratio was then calculated. A ratio of 1.0 indicates that the number of *MYC* and CEN8 signals is approximately equal within each nucleus. A ratio greater than 1.0 indicates that the number of *MYC* signals exceeds that of CEN8, consistent with an increase in *MYC* copy number relative to CEN8. The *MYC* gene copy number or CEN8 number was defined as being increased when it was more than 10% above normal (i.e. >110 signals/50 cells or >2.2 average signals/nucleus). This definition was based on the convention for describing amplifications in the *HER2/neu* oncogene system.[11]

Immunohistochemistry

In order to evaluate whether or not cell proliferation correlated with *MYC* gene copy number increase by CISH, immunohistochemical staining for Ki-67 was performed on all cases. A manual 200 cell count was performed within areas of highest staining on each sample and the percentage of nuclei positive for Ki-67 was recorded. Ki-67 was also correlated to *MYC* mRNA levels by qNPA, using the overall mean qNPA value to define increased *MYC* mRNA *versus* not increased *MYC* mRNA.

Statistical analysis

Cases were divided into two groups, one with "increased *MYC*" (greater than 10% above expected) and the other with "not increased *MYC*", based on the CISH and CEN8 results as defined above. Relations of the two groups with *MYC* mRNA levels and Ki-67 values were then examined using a Mann-Whitney test. The Ki-67 values were also directly compared to *MYC* mRNA levels using a Mann-Whitney test. Overall survival was defined as the time from diagnosis until death from any cause, and was estimated by the method of Kaplan and Meier. Two-year estimates were generated for the groups with increased *MYC* and not increased *MYC* and statistical differences were estimated using a log-rank test.[12]

Results

MYC *structural alterations*

Fifty-two cases of DLBCL were analyzed for structural alterations involving the *MYC* gene using a FISH break-apart probe. The results of all cases could be interpreted. Only one case had abnormal signals (2.0% of the total) and these did not involve the immunoglobulin heavy chain gene as assessed using a *MYC-IGH* fusion probe.

Total and average MYC *copy number by chromogenic* in situ *hybridization*

The entire slide was scanned at medium power for obvious areas of variation. CISH for the *MYC* gene was interpretable in 47 cases. The five cases excluded showed weak or absent staining in the malignant lymphocytes as compared to the internal control (normal endothelial cells, histiocytes, or benign lymphocytes). Examples of staining are shown in Figure 1 A-B. In the background endothelial cells and histiocytes, we observed the expected 0-2 CISH signals of *MYC* per cell (some signals are not present because of the sectioning of a three-dimensional cell onto a glass slide). No split signals were noted. Eighteen cases

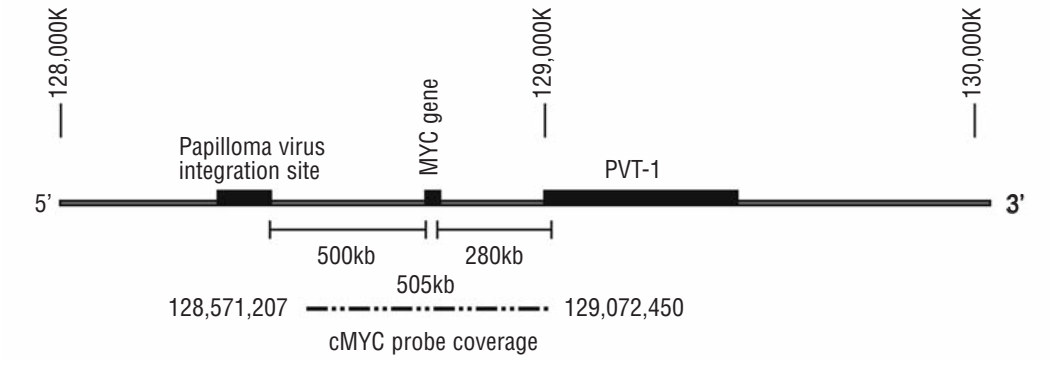

Figure 1. The *MYC* probe location on chromosome 8q24.

(38%) had more than 110 total *MYC* signals in the 50 cells evaluated (mean 138; range, 112-219). These cases with increased *MYC* showed relatively homogeneous staining with low levels of amplification ranging from two to five *MYC* signals that could be separately discerned per cell. Only one case appeared to have small possible clusters of signals and was the same case as that with the non-*IGH MYC* translocation. The same 18 cases had an average of more than 2.2 MYC signals per cell (mean, 2.76; range, 2.24-4.38) indicating that either counting method (total or average) can be used to identify cells with increased *MYC* gene copy number (Table 1).

Total and average chromosome 8 ploidy by chromogenic in situ *hybridization*

CISH for CEN8 was successful in 47 of the 52 cases studied. The five excluded cases were the same cases in which the *MYC* CISH assay was technically inadequate. No cases were identified with clearly increased CEN8 signals, most likely indicating that the cases with increased *MYC* gene copy number did not result from chromosome 8 polyploidy.

Ki-67 immunohistochemistry

Immunohistochemical staining for Ki-67 was performed on all cases and was successful in 42. The ten cases in which staining was unsuccessful comprised the five that were also uninterpretable by CISH and five additional cases that stained very weakly by immunohistochemistry. Thus, there were 42 cases for which information from all the slide-based investigations were available. The Ki-67 values ranged from 20% to 87% with an average of 57.5% (Table 1). Of the 18 cases with increased *MYC* copy number by CISH, 15 had interpretable Ki-67 immunohistochemistry. The Ki-67 values of these cases ranged from 23% to 83% with an average of 57%. Among the 27 of 29 cases without increased *MYC* by CISH in which Ki-67 could be evaluated, the Ki-67 values ranged from 20% to 87% with an average of 58%. Thus, there was no significant difference in Ki-67 results between cases with and without increased *MYC* signals. Using the median of 2400 as the cut-off value to distinguish increased and not increased *MYC* determined by qNPA, the average Ki-67 in the group with increased *MYC* by qNPA was 64.6% *versus* 53.3% in the group with not increased *MYC* by qNPA (P=0.072).

MYC *mRNA levels*

There were data for both *MYC* mRNA by qNPA as well as *MYC* gene copy number by CISH for 44 cases (Table 1). The average *MYC* mRNA level across all cases was 2398 (range, 342-9783) relative chemiluminescence units. Of the cases with increased *MYC* gene copy number (n=17), the *MYC* mRNA values ranged from 816 to 5912, with an average of 2843. Ten of these cases (59%) had qNPA values equal to or greater than the overall mean (2400). Cases without evidence of increased *MYC* gene copy number by CISH (n=27) had mRNA values ranging from 342 to 9783,

Table 1. Summary of data for patients separated into *increased MYC* and *not increased MYC* groups.

	Increased Total *MYC* (CISH) Range [average]	Not Increased Total *MYC* (CISH) Range [average]
Total *MYC*	112-219 [140], n=18	66-107 [91], n=29
Ki-67	23%-83% [56%], n=15	20%=87% [58%], n=27
MYC mRNA (qNPA)	816-5912 [2843], n=17	342-9783 [2118], n=27

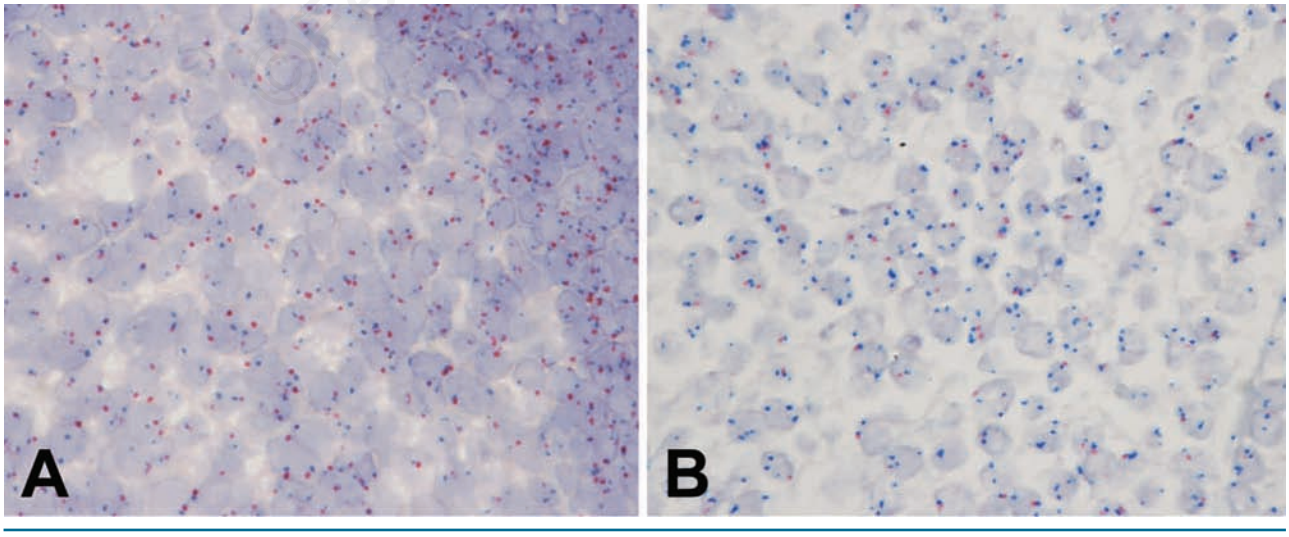

Figure 2 (**A**) Normal tonsil control with two copies each of *MYC* (blue) and chromosome 8 centromere (red) per cell (dual color CISH, Nikon Eclipse 80i, x400) Occasional cells have less than two copies due to sectioning of the tissue. (**B**) Diffuse large B-cell lymphoma with increased *MYC* gene copy number (blue) relative to chromosome 8 centromere (red) per cell (dual color CISH, Nikon Eclipse 80i, x400). In most cells, clearly separate *MYC* probes can be discerned.

with an average of 2118. Twenty-one of these cases (78%) had qNPA values less than 2400. There was a statistically significant relationship between increased *MYC* mRNA and increased *MYC* gene copy number (P=0.034). These results are shown in Figure 2. The single case with a *MYC* translocation involving a non-*IG* partner gene also had increased gene copy number and did have a *MYC* mRNA level above the mean. This value was the fifth highest of the 44 cases with qNPA results (5035).

Overall survival

Outcome data were available for 30 patients. All received CHOP-like regimens, and 13 also received rituximab. Of these 30 patients, eight had increased total *MYC* copy number and 22 did not. The patients with increased total *MYC* copy number had higher relapse rates and lower survival rates at 2 years after diagnosis with an estimated 2-year survival of 43% compared to 73% in those without increased total *MYC* copy number; however, the power of this analysis was low given the limited size of the sample, and overall survival between groups with high *versus* low total MYC copy number, stratified by treatment, was not significantly different (P=0.55; *data not shown*). We have previously reported data on the mRNA levels related to survival in two publications including the cases that were in this study and that analysis was, therefore, not repeated.[2,6]

Discussion

MYC has been identified in multiple studies as highly prognostic in DLBCL.[3,6,13-15] Possible mechanisms of *MYC* over-expression in DLBCL include t(8;14)*IGH/MYC*, increased *MYC* copy number, chromosome 8 polyploidy, and altered transcriptional or post-translational regulation. Prior studies that have analyzed the *MYC* gene with regard to DLBCL have largely focused on the incidence and prognostic relevance of *MYC* (8q24) rearrangements/translocations or, more recently, *MYC* mRNA and microRNA levels. The significance of increased *MYC* gene copy number in the absence of a translocation or the relationship of *MYC* gene copy number to mRNA levels has not been previously examined.

The *MYC* gene maps to chromosome 8q24 and was the first gene associated with the pathogenesis of non-Hodgkin's lymphoma.[16] *MYC* is a proto-oncogene that encodes a transcription factor involved in proliferation, differentiation, apoptosis, and overall regulation of hematopoietic homeostasis.[17] The MYC protein has multiple genomic targets involved in regulating normal cellular responses and its over-expression is often associated with malignant transformation.[18] *MYC* aberrations in DLBCL are a well-known phenomenon. Rearrangements of *MYC* have been reported in a variety of subtypes of non-Hodgkin's lymphoma and occur in 3-10% of *de novo* DLBCL, mostly in association with a non-immunoglobulin translocation partner.[14] *MYC* rearrangements have been associated with an aggressive disease course[13-15,19] and patients with these rearrangements may benefit from more intensive chemotherapy, such as that used for Burkitt's lymphoma.[20]

In our study, a rearrangement involving the *MYC* gene was present in only one patient (2%), who also had increased *MYC* gene signals. Based on these results, we conclude that no more than one of the cases in our series with increased *MYC* mRNA levels had a translocation as a mechanism of over-expression.

Nuclear staining for the proliferation marker Ki-67 was examined by immunohistochemistry, but there was no significant correlation between the proliferative index and *MYC* gene copy number by CISH. When the Ki-67 values were compared to *MYC* mRNA levels, higher proliferative rates tended to be associated with higher *MYC* mRNA levels although the association was not statistically significant. Future studies including higher numbers of cases may reveal a stronger correlation between Ki-67 and *MYC* mRNA. Although MYC is a known regulator of proliferation, it is a functionally complicated protein and over-expression may result in consequences other than increased proliferation. Indeed, the relatively high incidence of *MYC* alterations in DLBCL probably indicates some cellular advantage which may or may not ultimately be related to increased proliferation. One possibility is that this advantage may be mediated through effects of *MYC* on microRNA linked to other pro-tumorigenic pathways.[21-23]

Dual color CISH analysis for the *MYC* gene and the chromosome 8 centromere was technically successful in a high percentage of cases (90%), easy to interpret, and did not have the bleaching limitations common with fluorescence-based techniques. CISH was, therefore, an important technical advance in this study. CISH signals for centromere 8 were not increased in any of our cases, indicating the absence of polysomy 8. The cases of increased *MYC* gene copy number were, therefore, probably not due to polysomy 8 but, rather, due to low-level amplification of the *MYC* gene. We observed a statistically significant correlation between increased *MYC* mRNA levels, assessed by qNPA, and increased *MYC* gene copy number

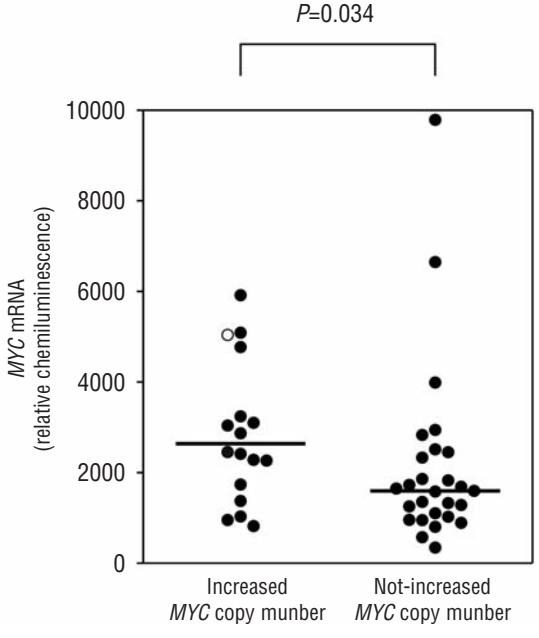

Figure 3. Cases were divided into those with increased *MYC* copy number and not increased *MYC* copy number by CISH and average *MYC* mRNA by qNPA was compared between the two groups. The open circle represents the case with a non-*IG* associated translocation.

indicating that in most instances, the increased number of genes led to an increase in mRNA. Fifty-nine percent of our cases with increased *MYC* CISH signals had a *MYC* qNPA value equal to or greater than the mean (2400) while 78% of the cases without increased *MYC* CISH signals had qNPA values below the mean. These data support the use of the mean *MYC* qNPA value as a cut-off, as used here and in our prior study. The correlation between *MYC* mRNA levels and survival is not reported here, since it was previously published for two larger series of cases including all of the cases studied here.[2,6]

Despite the correlation between *MYC* copy number and mRNA, there were some cases with increased *MYC* mRNA without increased *MYC* gene copy number as well as cases with elevated *MYC* mRNA values that were not associated with increased MYC copy number. One of the latter cases can be explained by a non-*IG* associated translocation. In other cases a reason for the discrepancy between copy number and mRNA was not readily apparent but suggests that other mechanisms contribute to *MYC* over-expression. The mechanism of transcriptional control of *MYC* is complex. The human *MYC* promoter contains four promoters (P1–P4) with 75-90% of transcriptional activity initiated at the P2 promoter, and another 10-25% at the P1 promoter.[24-29] Within this region of DNA there are segments that can adopt alternative secondary DNA structures such as g-quadruplexes or i-motifs. The CT-element and the far-upstream element (FUSE) are two segments that affect *MYC* transcription, and are governed by helical stress driven by active transcription.[30-33] DNA secondary structures in these elements can contribute to enhancement or repression of *MYC* transcription.[34-37] Certain microRNA may also modulate MYC expression. A recent report described down-regulated hsa-mir-34b in classical Burkitt's lymphomas not harboring a *MYC* translocation, suggesting another possible mechanism of *MYC* deregulation.[38] Given the dynamic control of *MYC* transcription, it is evident that other mechanisms could contribute to mRNA levels in addition to gene copy number. It is possible that alterations in these mechanisms may account for the outliers, and could also provide therapeutic targets.

Of interest, the proportion of patients surviving at 2 years was much lower in those with increased *MYC* copy number. However, the small sample size in this study prevents any definitive conclusions regarding survival and copy number. We also recognize the limitation of not having a uniformly treated series of patients. This observation, however, would be consistent with the quick relapses seen in a disease that uniformly over-expresses *MYC* (Burkitt's lymphoma) when treated with CHOP-based regimens.[39,40] A larger series of patients is necessary to determine whether *MYC* copy number, in addition to the presence of translocations and/or increased mRNA levels, also correlates with patients' survival. If so, *MYC* copy number alterations could become a useful investigative and prognostic tool in DLBCL

Although the poor outcome associated with *MYC* translocations and increased mRNA levels in DLBCL was previously known, this is the first investigation into *MYC* gene copy number changes and the relationship of copy number to other variables. In this small series of cases using CISH to study the *MYC* gene and chromosome 8 centromere, we found that low level copy number increases of *MYC* are common (38%) among cases of DLBCL. These copy number increases correlated with increased *MYC* mRNA in a statistically significant manner, although fell short of correlating with overall survival in this series. There are most likely multiple mechanisms that result in the common end-point of increased *MYC* mRNA in DLBCL. We are planning imminent future studies involving larger numbers of cases and patients treated according to a standardized protocol. This additional research may further clarify the most important factors affecting *MYC* expression and, therefore, overall survival in DLBCL.

Authorship and Disclosures

CJS: generated data by interpreting CISH slides, organized data, and wrote the manuscript. HN: developed the double staining procedure and performed CISH staining; WZ: designed and created the CEN and MYC probes; CHM: generated data by interpreting immunohistochemistry slides; JRC: interpreted FISH assays, wrote parts of the *Design and Methods* section; RRT: interpreted FISH assays, wrote parts of the *Design and Methods* section. JMU: performed statistical analyses; TAB: interpreted data and wrote parts of the manuscript; DOP: interpreted data and wrote parts of the manuscript; STW: performed statistical analyses and created figures; TMG: generated data by interpreting CISH slides; LMR: designed the study, collected case material, interpreted data, and wrote the manuscript.

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