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REVIEW

Correlation between *MYC* gene rearrangement and MYC protein expression suggests that MYC regulation is more complex than previously known

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SUMMARY

Since its discovery in the 1970's, MYC oncoprotein has been continuing to fascinate the scientific world and there is a growing interest in the role of MYC in the genesis and prognosis of cancer.
Initially MYC was identified as the cellular homologue of the MC29 transforming avian retrovirus. Shortly hereafter, additional related sequences were identified, suggesting that MYC might be part of a larger family of genes.
The constellation of MYC effects on genes involved in proliferation has led to the concept of MYC-driven lymphomas, that include Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), and lymphomas that share morphologic features of DLBCL and BL, officially termed B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (BCLU). Other lymphomas showing MYC over-expression comprises Plasmablastic lymphoma and Plasmacytoma, Double hit/triple hit lymphomas and Anaplastic lymphomas Kinase-positive Large B-cell Lymphoma.
MYC aberrations can be detected by standard cytogenetics, interphase fluorescence in situ hybridization (FISH), comparative genomic hybridization and most recently immunohistochemistry. By comparing expression profiles of MYC gene rearrangement and MYC protein expression has came up that MYC gene rearrangements do not necessarily correlate with MYC protein expression. In fact, by applying immunoistochemistry, the frequency of MYC protein expression appears much higher than what is detected by FISH standard method. Therefore, nowadays the key problem in the hematopathology field is to define the clinical impact of the double-expressor lymphoma status. The updated World Health Organization (WHO) of tumours of hematopoietic and lymphoid tissues asses that the status of double or triple lymphoma should rely only on molecular biology findings and not on immunohistochemistry results.

Key words: MYC gene dysregulation; MYC protein expression; Burkitt lymphoma.

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INTRODUCTION

The iconic history of the MYC oncoprotein encompasses three decades of intense scientific discovery. There is no question that *MYC* has been a pioneer, advancing our insight into the

molecular basis of cancer, as well as functioning as a model of several diverse biological processes and regulatory mechanisms.
By analyzing all the published articles on MYC, two major themes emerge: first, the fine-tuned regulation and numerous activities of MYC in normal cells; second, the role of MYC as oncoprotein when its regulation is lost (1).

Identification of MYC
In the late 1970's an avian acute leukemia virus (MC29) was shown to promote a spectrum of malignancies, including myelocytomas, sarcomas and carcinomas.
This ability to induce carcinomas was of particular interest. The transforming sequence of MC29 was identified as v-myc, and named myelocytomatosis for a resultant leukemia. v-myc was found to present in the cells as a 110 kDa v-gag-myc fusion.
Consistent with the notion that oncogenes could be stolen by retroviruses, the cellular homologue was identified soon after in normal cells from many species. The discovery of MYC further reinforced the startling realization that oncogenic transformation could be caused by the activation of a cellular gene (2-5).

The MYC family of transforming oncoproteins
MYC was first identified as the cellular homologue of the MC29 transforming avian retrovirus. A schematic representation of human *MYC* is presented in *Figure 1*. Soon after, additional related sequences were identified, suggesting that *MYC* might be part of a larger family of genes. Two family members, *MYCN* and *MYCL1*, were identified as a result of their amplifications in neuroblastoma and lung cancer respectively (6-11). While there is a clear role for all family members in tumorigenesis, there are some important differences that exist between family members. Specifically, *MYCL1* consistently promotes transformation to a lesser extent than the other two family members, and to date the mechanisms underlying this remain a question in the field. *MYCN*, on the other hand, has been shown to be functionally interchangeable with *MYC* in development through the generation of a knock-in mouse model (6).

MYC: structure and functions
All the genes belonging to the *MYC* proto-oncogene family are expressed in mammals. A forth gene, B-*MYC*, encodes a protein that shows significant homology to the N-terminus, but lacks

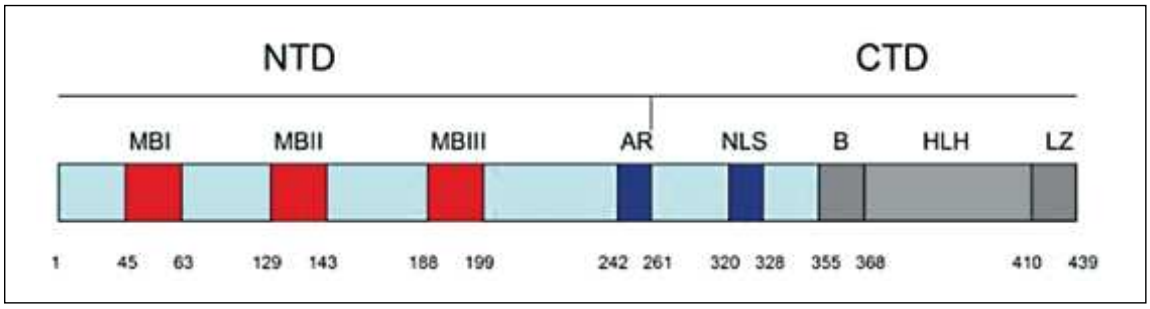

FIGURE 1 • The structural domains of human *MYC*.

essential domains in the C-terminus of the other MYC proteins, and its biology is poorly understood. The *MYC* gene is located on the human chromosome 8q24, and it consists in three exons. Translation at the AUG start codon nucleotide in the second exon produces a major 439 amino acid MYC protein (64 kDa). Alternative translation initiation start sites result in both longer and shorter forms of the protein.

A longer polypeptide of 67 kDa results from translation initiated 15 codons upstream of the AUG at a CUG codon (exon1) and the shorter one, 45-kDa polypeptide, results from an internal translation initiated.

The MYC protein is O-linked glycosylated and phosphorylated and these modifications may alter the protein half-life. As such, it is important to note that *MYC* mRNA and protein have a very short half-life (20-30 min) and are tightly regulated. *MYC* gene encodes for a transcription factor of the basic helix-loop-helix leucine zipper (b-HLH-LZ) superfamily. Traditionally the MYC protein is functionally referred to a N-terminal domain (NTD) and a C-terminal domain. The N-terminal, which is defined as amino acids 1-262, and the C-terminal, defined as residues 263-439, match respectively to the N-terminal transactivation domain (TAD) and C-terminal DNA-binding and basic-region/helix-loop-helix/leucine-zipper (BR/HLH/LZ) domain (1).

The N-terminus of *MYC* is the major regulatory region responsible for assembly of the transcriptional machinery. Within the N-terminus there are three highly conserved elements, known as *MYC* boxes I-III which, together with the C-terminal b-HLH-LZ, define the MYC family of proteins**.** Of these, MYC box I (MBI, from approximately amino acids 45-63) and MYC box II (MBII, from approximately amino acids 128-143) contain sequences highly conserved among the different MYC family of proteins throughout evolution. These two regions appear to be particularly important for the transcription activity of MYC; in fact, deletion of either MBI or MBII, diminishes the transcriptional activation potential 10-50 fold, respectively.

Specifically, although MBI is required for gene activation, deletion of this region only partially abolishes the transforming ability of MYC while an MBII deletion, which is essential for the ability of MYC to transform, drive cell proliferation and activate certain target genes, completely abolished it. MYC box II is not involved in the binding of MYC to Max or to DNA, but is required for activation and repression of most, but not all, MYC target genes. Recently, a third conserved region of MYC has been described, MYC box III (MBIII), that plays a role in transformation, lymphomagenesis and apoptosis.

MYC biological activities

Cell cycle and differentiation

There were several lines of evidence to suggest that *MYC* might play a role in cell cycle progression. Ectopic expression of *MYC* promotes growth factor independent proliferation (1, 12). Not unrelated to the ability to promote cell cycle progression, *MYC* expression also blocks differentiation. It was demonstrated by multiple groups and through a number of models that MYC down-regulation is essential for cells to exit the cell cycle and undergo differentiation. These abilities to promote cell proliferation and block differenti-

ation have natural associations with tumorigenesis and are features of aggressive disease (1).

Apoptosis

The ability of MYC to promote apoptosis provides a built-in safety mechanism to protect against inappropriate proliferation as a consequence of deregulated MYC (13-16). This finding also shed considerable light on the model of oncogene cooperation and how an anti-apoptotic protein such as BCL2 could cooperate with MYC to promote oncogenesis (17-19). Moreover, deregulation of MYC was shown to activate the tumor suppressor p53 through the upregulation of ARF (20-22). A loss of either of these tumor suppressors accelerated tumorigenesis in mouse models of oncogenic *MYC* (23-27). Further, MYC is also able to promote apoptosis through p53-independent mechanisms by influencing the balance between pro- and anti- apoptotic proteins in the cell (20, 28-34).

Transcriptional activation

The C-terminus of MYC was shown to contain both a helix-loop-helix (HLH) (35) and a leucine-zipper (LZ) domain (36) to which a MYC partner can binds, MAX (MYC associated factor X) (39). MAX was shown to be a constant and obligate partner for MYC, with consistent and abundant expression in both proliferating and quiescent cells, which was not altered in response to extracellular stimuli. It is, however, important to note that MAX also forms heterodimers with members of the MXD family, which thereby provides an additional mechanism to regulate MYC activity in the cell (40, 41). In non-transformed cells, the MYC protein appears to integrate environmental signals, in order to modulate a diverse, and sometimes opposing, group of biological activities, including proliferation, growth, apoptosis, energy metabolism and differentiation. MYC protein levels are induced or suppressed by virtually all signalling cascade bearing proliferative and anti-proliferative cues, respectively. Mitogen stimulation induces *MYC* as an immediate-early response gene, whose expression is essential and sufficient for the G1/S progression. *MYC* also plays a role in G2/M transition, making it one of the key players in cell cycle regulation. Abnormal or ectopic over-expression of MYC in primary cells activates a protective pathway through the induction of p16/p14ARF and the p53-dependent cell death pathway. Hence, normal cells that overexpress MYC are eliminated from the host organism through apoptosis, thereby protecting the organism from lethal neoplastic changes.

Transcriptional regulation

MYC can both activate and repress transcription of its target genes. MYC-Max heterodimers activate transcription by binding to E-box elements. The DNA binding of MYC and Max complexes is mediated by amino terminal 143 amino acids of c-MYC (TAD). Deletions within the N-terminal TAD, can greatly affect or abrogate the biological function of MYC, as its transactivation potential. The activation involves the recruitment of multiple coactivators and protein complexes to E-box elements. Coactivators include the Mediator complex, Positive Transcription Elongation Factor b (P-TEFb), the ATPases TIP48 and TIP49, and histone acetyltransferases such as CREB-bind-

ing protein (CBP) and p300, GCN5 and TIP60. Of note, GCN5 and TIP60 are bound to MYC indirectly through the TRRAP adaptor protein that interacts with MYC box II, TRRAP also recruits the p400 histone-exchange protein to MYC. Another way for MYC to activate target genes is by interaction with E3 ubiquitin ligase SCF^{SKP2} (SKP2) which recruits components of the APIS complex to E-box sequences. Transactivation by MYC is antagonized by Mad-Max and Mnt-Max heterodimers, which repress transcription by recruiting histone deacetylase complexes (HDACs) through the adaptor protein SIN3 (33,34) RNA pol II and SAP.

Interestingly, only a minority of the sites to which MYC and Max are bound *in vivo* have a consensus CACGTG sequence. Indeed, MYC-Max heterodimers are able to recognize non canonical sites, variations of the canonical E-box containing core TG or CG di-nucleotides. In addition, nucleotides immediately flanking the E-box, as well as methylation of CpG within the E-box, can affect MYC-Max binding. In those cases, is possible that MYC and Max are recruited to non-consensus binding sites through the interaction with other transcription factors. One example is offer by Miz1, which can recruit MYC and Max to core promoter sequence that lack a CACGTG sequence. The association Max-MYC allows the interactions with a number of additional transcription factors and co-factors that modulate transcriptional activation. One is TRAAP, which is a core subunit of the TIP60 and GCN5 histone acetyltransferase (HAT) complexes; MYC recruits HAT activity to its target genes and the recruitment depend on the integrity of MYC box II. Inhibition of TRAAP synthesis blocks MYC-mediated oncogenesis, establishing an essential role for TRAAP in MYC activity. TRAAP is also part of a complex containing the p400 E1A-binding protein, which is devoid of HAT activity, suggesting that MYC-TRAAP interaction has other roles in additional roles in addition to recruiting of HAT activity. Two other proteins bind to MYC box II independently of TRAAP; these are TIP48 and TIP49, two highly conserved hexameric ATPases, both involved in several chromatin remodelling complexes. Both proteins have ATP hydrolysing activity, as well as suspected helicase activity and have been shown to be required for the foci formation by MYC and Ras in a primary co-transformation assay. MYC box II is required for interaction with SKP2, of the E3 ubiquitin ligase, SCF^{SKP2}, and MYC recruits SKP2 to its target genes *in vivo*. Recruitment of SPK2 is required for the transactivation of several MYC target genes. Interestingly, not all MYC target genes require the integrity of MYC box II for activation, suggesting that there are other mechanisms of MYC-dependent activation. In the past *MYC* has been considered a "permissive factor", stimulating gene activity by creating a chromatin environment that is conducive for gene induction. It has been recently shown that MYC-driven transcriptional repression is critical for its oncogenic activity. However, less is known about how MYC represses transcription. Recent findings suggest the involvement of DNA methylatransferases enzymes (DNMTs) and histone deacetylase (HDAC) as possible cofactors in the *MYC*-mediated transcription repression. DNA methylation at CpG dinucleotides is the major epigenetic modification in mammals

and is known to be associated with transcriptional repression. The three active DNA CpG methylatransferases identified in mammals are DNMT1, DNMT3a and 3b. Whereas DNMT3a and 3b have been shown to be required for *de novo* methylation, DNMT1 appears to function primarily as a maintenance methylatransferase, restoring methylated cytosine following DNA replication. Several studies have shown that DNMTs can act as corepressors to silence gene expression that maintain chromatin in a compacted and silent state. Indeed, it was recently demonstrated the ability of MYC to repress the transcription through recruitment of DNMT3a, to the MYC-Miz1 complex, indicating that MYC-dependent gene repression could at least partly be mediated by methylation of its target promoters (1).

Novel biological activities

In recent years, additional biological activities of MYC have been characterized. The renewed interest in the Warburg effect and tumor cell metabolism has highlighted a new role for MYC. In addition to stimulating mitochondrial biogenesis, oncogenic levels of MYC have been shown to promote glutaminolysis (42-58). This increased glutamine metabolism fuels cell growth and proliferation, which are essential for tumor cells to thrive. It has been suggested that tumor cells become addicted to glutamine, which may provide opportunities for therapeutic intervention (44). Moreover, *MYC* gene can induce senescence in the context of the loss of other genes such as *WRN* or *CDK2* (59-63). The ability of *MYC* to block differentiation perhaps foreshadowed the recently uncovered role of *MYC* in regulating "stemness". Conditional knock-out mice have demonstrated an essential role for MYC in the normal developmental control of haematopoietic and neuronal stem cells (64,65). *MYC* has recently been identified as one of the four genes whose overexpression could re-program normal terminally differentiated fibroblasts into induced pluripotent stem (iPS) cells (66, 67). While it was later shown that *MYC* was dispensable for this process (68), it did underscore important implications for deregulated *MYC* in initiating and maintaining tumor stem cells. In fact the stem cell signature of undifferentiated and aggressive tumours has great similarity to the phenotypes of MYC-activated tumours (1).

Genetic mechanisms of MYC targets deregulation

MYC transcription factor is considered one of the most potent oncoproteins in human cancer. The consequence of MYC dysregulation is the alteration of important biological activities. Capable of acting as both a transcriptional activator and repressor, MYC controls the expression of a vast array of genes, together accounting for at least 10% of the human genome (69, 70). In general, genes targeted by MYC include mediators of metabolism, biosynthesis, DNA replication, apoptosis, and cell cycle progression (71) such that aberrant MYC expression is associated with uncontrolled cell growth, division, and metastasis (72) whereas loss or inhibition of MYC expression reduces growth, promotes differentiation, and sensitizes cells to DNA damage (73). Some of the most biologically important targets are thought to be cyclins and cyclin-dependent kinases (CDKs),

resulting in accelerated cell cycling (74) down-regulation of phosphatase and tensin homolog (*PTEN*) with consequent up-regulation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway (75) and stabilization of the proapoptotic protein and tumor suppressor p53 (76) which can bypass the apoptotic BCL2 program (77). Consistent with its potent growth promoting properties, MYC can drive oncogenic transformation, and deregulated MYC expression and activity is a hallmark of many human cancers. Indeed, by contrast to the highly regulated state of MYC and the absence of N-MYC and L-MYC expression in normal cells, cancer cells often harbour dysregulated expression of any one of these three *MYC* oncogenes.

The MYC transcriptional network also includes the direct regulation of a large number of microRNAs (miRs) with oncogenic or tumor suppressor function (78-80). MYC up-regulates the oncogenic miR 17-92 cluster that is commonly amplified in several subtypes of aggressive lymphomas (81, 82) and its oncogenic function is mediated in part by the down-regulation of PTEN, TP53, and E2F1, facilitating the activation of the PI3K/AKT pathway and the inhibition of apoptosis (83). MYC represses several miRs with tumor suppressor function by the recruitment of HDACs (84). These miRs include miR15a/16-1, miR26a, miR29, and miR34, which regulate crucial functions in neoplastic development such as apoptosis (miR15a/16-1 and miR34 targeting BCL2 and TP53, respectively), proliferation (miR29a targeting CDK6), or cell differentiation (miR26a targeting EZH2) (83, 85, 86). *MYC* itself is also negatively regulated by some miRs, such as miR34 and miR494 (85, 86). miR494 is in turn repressed by EZH2, creating a positive autoregulatory loop (MYC/miR26a/EZH2/miR494) that sustains the persistent expression of MYC and EZH2, promoting the malignant phenotype of cells (85). The interactions of the networks regulated by MYC and its target miRs are complex and suggest fine-tuning of different processes that may be targeted by new therapies (84, 87). Given the emerging pathogenetic role of miRNAs in the methylation status of cancer cells, this link between the regulatory function of *MYC* over miRNAs is particularly noteworthy.

The transformative capacity of *MYC* is often in concert with other oncogenes and viruses, including the rat sarcoma (*RAS*) oncogene (88) and Epstein-Barr virus (EBV) (89). The correlation between EBV and *MYC* is complex, because EBV-associated proteins in turn potentiate *MYC* activity (90).

Intriguingly, the gene profile transcriptionally regulated by MYC varies in different cell types with relatively little overlap (91). Two recent studies shed light on this puzzling observation, showing that, instead of activating a particular gene signature, MYC acts as an amplifier of the transcribed genes in a given cell by uploading to the promoters of active genes and enhancing their transcription (92, 93). MYC does not bind to promoters of silent genes and therefore acts as an activator of the preexisting transcription program. This function of MYC may be relevant to understanding the increased aggressiveness of tumors associated with other oncogenic events carrying *MYC* alterations and may offer perspectives for new therapies (92, 93).

Although the transcriptional role of *MYC* has been well described, there are also non-transcriptional functions that only recently have been appreciated, including regulation of mRNA translation and direct regulation of DNA replication. For instance, *MYC* directly promotes methyl cap formation on the 5' end of pre-mRNA for many genes, including cyclin D1 and CDK-9, and resulting in enhanced mRNA translation (94-100).

MECHANISMS OF MYC DEREGULATION IN CANCER

Building on the awareness that, unlike other proto-oncogenes, *MYC* activation was not a consequence of mutations in the coding sequence, research focused on identifying and understanding other modes of oncogenic activation. This led to the discovery of three mechanisms through which *MYC*, and in turn other oncogenes, could be deregulated and promote transformation: insertional mutagenesis, chromosomal translocation, and gene amplification. Combined, these findings led the way for the discovery and understanding of oncogenes and provided new paradigms for the genetic basis of cancers (1, 101-104).

Non-random chromosomal translocations had been observed in a number of malignancies, including Burkitt's lymphoma (BL) and Chronic Myeloid Leukemia. It was tempting to speculate that these translocations resulted in aberrant expression of the same proto-oncogenes identified in the acutely transforming retroviruses. The mapping of *MYC* to the long arm of chromosome 8 gave credence to this hypothesis (105, 106). Specifically, Burkitt's lymphomas had been characterized to contain reciprocal translocations between chromosome 8 and chromosomes 14, 2 or 22, which harbour immunoglobulin (Ig) heavy and light chain genes (107). It was then discovered that the cancer is driven by activated expression of *MYC* resulting from the translocation. One of the first MYC transgenic mice, Eμ-MYC, was developed to model Burkitt's lymphoma. Activated *MYC* expression was driven from the heavy chain enhancer (Eμ), leading to clonal B-cell lymphomas. Mouse plasmacytomas were similarly found to be a consequence of *MYC* translocation with the Ig heavy chain locus (108, 109).

It was well established that cancer cells contained a number of chromosomal abnormalities and contributions of these aberrations to cellular transformation were largely appreciated through the study of MYC (110-112).

Amplification of *MYC* and/or dysregulated expression is evident in many tumors including melanomas and carcinomas of the breast, prostate and colon. The deregulation of *MYC* plays a decisive role in lymphomagenesis, by driving the cells through the cell cycle. In fact, as a result of the translocation, the normal control mechanisms of *MYC* expression are disrupted, leading to constitutive expression of the protein throughout the cell cycle. Briefly, MYC protein is not only a potent inducer of proliferation, it also induces as a fail-safe mechanism, a large number of pro-apoptotic and inhibits expression of anti-apoptotic genes, thereby inducing apoptosis or predisposing cells to apoptosis. As a consequence, *MYC*-driven tumors usually have acquired additional genetic mutations or epigenetic modifications

that promote cell survival and shift the balance between proliferation and apoptosis towards proliferation. Importantly, a major development within the past decade has been the realization that *MYC* dysregulation is not restricted to gross genetic changes at the *MYC* locus, such as chromosomal translocation, insertional mutagenesis and gene amplification, but *MYC* can be deregulated by one of several mechanisms that target its expression and/or activity either directly or indirectly.

Deregulation of MYC in human lymphomas

The constellation of *MYC* effects on genes involved in proliferation has led to the concept of *MYC*-driven lymphomas (113). The classic *MYC*-driven lymphoma is BL, in which balanced rearrangements between chromosome 8 and either chromosome 14 (immunoglobulin (Ig) heavy chain), chromosome 22 (IgG lambda light chain), or chromosome 2 (IgG kappa light chain), lead to a highly proliferative lymphoid malignancy with a propensity for extranodal involvement, particularly in immunocompromised patients. According to the last WHO classification, other lymphomas commonly associated with *MYC* deregulation are diffuse large B-cell lymphoma (DLBCL), B-cell lymphoma, unclassifiable, with features intermediate between DLB-

TABLE 1 • Lymphoid neoplasms characterized by MYC deregulation.

	Clinic	Morphology	Immunohisto-chemistry	Molecular Biology
Burkitt lymphoma (BL)	Extranodal sites of children and young adults. 3 variants: endemic, sporadic, HIV associated	Cohesive medium-sized cells with coarse chromatin, in a starry sky pattern	The neoplastic cells express CD20, CD19, CD22, CD79a, CD10, BCL6 but are negative for BCL2, proliferative index (Ki 67): >95%	t(8;14) or variant t(2;8) or t(8;22). >90%
Diffuse large B-cell lymphoma (DLBCL)	Adult and elderly with enlarging mass in nodal and extranodal sites	Diffuse growth pattern with centroblastic, immunoblastic, anaplastic or mixed morphology	Pan-B cell antigens positive with expression of germinal center markers in a subset of cases; proliferative index (Ki 67): 30-40%	t(14;18); translocation involving 3q27; two groups identified by gene expression profile, the ABC and GC type
B cell lymphoma, unclassifiable	Older patients presenting with nodal and extranodal disease, usually in an advanced clinical stage	Diffuse growth pattern with intermediate-sized cells, some admixed larger cells, irregular nuclei with single prominent nucleoli	Expression of CD19, CD20, CD22, CD79a, CD10, BCL2, variably BCL6	t(8;14) and other t(qq24), complex karyotype, *MYC* rearrangement to IG and non-IG partner, often accompanied by *BCL2* or *BCL6* rearrangement

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	Clinic	Morphology	Immunohisto-chemistry	Molecular Biology
Plasmacytoma	Middle-aged patients, bone pain at site of involvement or pathological fracture. Soft tissue extension may produce a palpable mass	Poorly differentiated plasma cells (plasmablastic or anaplastic) with eccentric nuclei, and dispersed chromatin	Neoplastic plasma cells express CD79A, CD38, CD138 and monotypic cytoplasmatic immunoglobulins (Ig), they lack surface Ig	Immunoglobulin heavy and light chain genes rearrangement
Plasmablastic lymphoma	Aggressive neoplasm usually present in extranodal sites and frequently in the head and neck region in patients with different immunodeficiency states	Diffuse proliferation of large B cells with immunoblastic morphology, admix with small to intermediate-sized cells	Neoplastic cells express CD38, CD138, MUM1 and they are negative for CD20, PAX5, proliferative index (Ki-67): >90%.	*MYC* translocations are encountered in 40-50% of cases, usually with Igh loci, EBER positive in 40-70% of cases.
ALK-positive DLBCL	Young patients (30% occur in the pediatric age group) commonly with nodal disease	The tumor cells have an immunoblastic or plasmablastic appearance, sinusoidal infiltration is common	Neoplastic cells lack mature B-cell markers but express EMA, kappa or lambda light chain, (most often IgA); CD138, ALK	Up-regulation of the *ALK* gene is mainly due to the presence of t(2;17) (p23;q23)*CLTC* (clathrin)/*ALK*. Rare cases with t(2;5)(p23;q35) (*NPM-ALK*) translocation have also been reported

CL and BL (BCLU), Plasmablastic lymphoma (PBL), Plasmacytoma (PC) and Anaplastic lymphomas Kinase-positive Large B-cell Lymphoma. *Table 1* and *Figure 2* summarize the clinical, morphological, immunophenotypical and molecular biology features of the lymphoid neoplasms characterized by *MYC* deregulation. Apart from the typical characteristics of the above mentioned lymphomas, genetic abnormalities involving *MYC* results in a more aggressive phenotype of tumours cells and a poor prognosis, largely independent of other clinical and molecular risk factors (113).

Novel concept in MYC-related B-cell lymphomas

MYC aberrations can be detected by standard cytogenetics, interphase fluorescence *in situ* hybridization (FISH), comparative genomic hybridization and most recently immunohistochemistry (IHC) (114-116). In recent years, it has been well established that patients

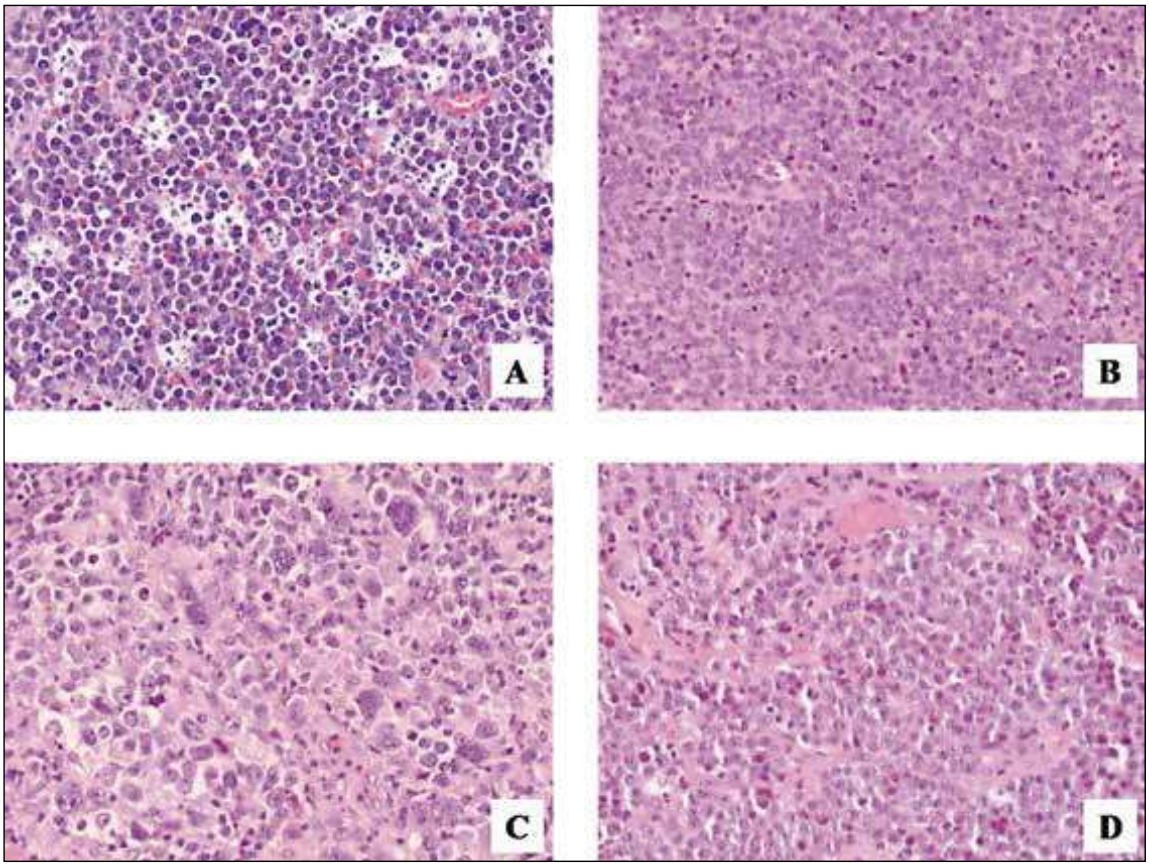

FIGURE 2 • Lymphoid neoplasms characterized by *MYC* deregulation (A: Burkitt lymphoma-BL; B: diffuse large B-cell lymphoma-DLBCL; C: B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL; D: plasmablastic lymphoma; haematoxylin and eosin, original magnification, O.M.: 20x).

harbouring FISH-detected gene-activating breaks in both *MYC* and *BCL-2*, suffer from poor response to standard therapy and have an adverse prognosis (117). Conventional cytogenetics or molecular biology method could be considered the gold standard for the identification of *MYC* rearrangements, because they identify the translocation patterns. From a technical stand-point, to test for the *MYC* rearrangement, break-apart probes and not dual-fusion are typically used to account for the non-*IGH* translocation partners of *MYC*. For *BCL-6* either break-apart or *IGH/BCL-2* dual fusion testing strategy could be used, whereas for *BCL-2* translocations break-apart probes are recommended (118). Cytogenetic analysis, however, is cumbersome, time-consuming and not routinely performed in the work-up of lymphomas at many centers. Moreover, FISH technology is generally not designed to detect genetic deregulation that affects gene expression on the transcriptional and translational levels. The availability of anti-MYC antibody (clone Ab 32072, dilution 1:200, Abcam-Cambridge, United Kingdom), amenable for use in formalin-fixed paraffin-embedded tissue offers a less costly and less laborious means of de-

tecting MYC over-expression and represents a key step forward in studying MYC-associated lymphomas (115).
By comparing expression profiles of *MYC* gene rearrangement and MYC protein expression emerged that *MYC* gene rearrangements do not necessarily correlate with MYC protein expression. In fact by applying immunohistochemistry, the frequency of MYC protein expression appears much higher than what is detected by FISH standard method (116). Therefore, nowadays the key problem in the hematopathology field is to define the clinical impact of the double/triple expressor status apart from the presence of genetic alterations characterizing the so-called double/triple hit (DH/TH) lymphomas. Although genotype controls phenotype because genes direct the products of proteins, there are proteins, in turn, that dictate virtually every reactions in the cells and thus are directly responsible for observable characteristics and effects. From a biological point of view, phenotype as the results of multiple cross-talks between genes, proteins and environment, is more relevant than genotype, proteins expression levels likely representing a more direct measure of the activity of a particular gene. Accordingly, assessing the expression of MYC, BCL-2 and BCL-6 by IHC is attractive because this should identify double/triple-expressing lymphoma patients whose disease are driven by increased MYC, and/or BCL-2, and/or BCL-6 activity secondary to a variety of mechanisms, not solely translocations (115-118).
However, there is a significative interpretative challenge to define the score of positivity for MYC, BCL-2, BCL-6 although Authors have used the threshold of greater than 40% for MYC and greater than 70% for BCL-2, respectively. Moreover, MYC staining may be heterogeneous, rendering accurate quantification wondering. In addition, many double-expressor lymphomas reflect DLBCL, for instance, activated B-cell (ABC) type of DLBCL, in which the MYC and BCL-2 are over-expressed by other mechanisms than translocations (119, 120).
For all these reasons, the updated WHO classification of tumours of hematopoietic and lymphoid tissues that will be published in the next future, suggests to limit the definition of DH/TH lymphoma only in the case in which the "hit" could be demonstrated by cytogenetic or molecular biology. Accordingly, it proposes a novel classification for high grade B-cell lymphomas, including: diffuse large B-cell lymphoma, not otherwise specified; high grade B-cell lymphomas, not otherwise specified; high grade B-cell lymphomas with *BCL-2* and/or *BCL-6* and *MYC* rearrangements. *Table 2* summarizes the clinical, morphological, immunophenotypical and molecular biology features of these novel subtypes.

MYC-TARGETED THERAPY

Because of its oncogenic properties in neoplastic cells, *MYC* has become an interesting and feasible target for novel therapies of a variety of human malignancies. However, MYC protein itself has generally been considered "undruggable" and the potential approaches have been directed at reducing its expression (121). It has been shown that several small molecules target the transcription of *MYC* gene directly or the MYC downstream path-

ways. Especially, G-rich region of *MYC* promoter has become a promising target. Many reports demonstrate that low-molecular weight compounds have a potential to be developed into therapeutic drugs in individualized cancer therapy. Even with the advances in the field of drug design and

TABLE 2 • Novel subtypes of high grade B-cell lymphomas included in the updated WHO classification of tumours of hematopoietic and lymphoid tissues.

	Clinic	Morphology	Immunohisto-chemistry	Molecular Biology
DLBCL, not otherwise specified (NOS)	Adult and elderly, nodal and extranodal sites	Large tumour cells with abundant cytoplasm, large nuclei and prominent nucleoli	Pan-B cell antigens positivity; Ki-67: 30-40%	ABC or GC type
High grade B-cell lymphoma, NOS	Elderly patients	Histologic appearance that resembles Burkitt lymphoma more than DLBCL; irregular nuclear contour; although many areas mimic DLBCL, the nuclear size remains small	Positivity for CD20, negativity for TdT, MUM1/IRF4; BCL6, CD10,Ki-67 variable; MYC expression depends on the presence of *MYC* translocation	Approximately 20-35% of the cases have a *MYC* breakpoint (with or without increased copy numbers or rarely amplification of 18q21 involving *BCL2*). The presence of *BCL2* and/or *BCL6* breakpoint in combination with *MYC* breakpoint should be excluded
High grade B-cell lymphoma, with *BCL2* and/or *BCL6* and *MYC* rearrangements/ translocations	Elderly patients, two peaks of incidence (30 and 70 years). Widespread disease, including involvement of lymph nodes with more than one extranodal site, bone marrow (59-94%) and CNS (45%-60%)	Fibrosis as well as starry sky macrophages may be (focally) present. The number of mitotic figures and apoptotic figures is highly variable. The nuclei have a variable size and contour. The cytoplasm is usually more abundant and less basophilic than in BL	Neoplastic cells are CD19, CD20, CD79a, PAX5, BCL2 positive and TdT negative. CD10 and BCL6 (75-90%) MUM1/IRF4 (20%)	In addition to the *MYC* rearrangement, all cases contain *BCL2* rearrangement at 18q21 and/or a *BCL6* rearrangement at 3q27. HGBL-DH often have complex karyotypes with many other structural and numerical abnormalities Sequencing studies reveal frequent *TP53* mutations mainly in the *MYC&BCL2* double hit cases, few *MYD88* mutations

HIV: Human Immunodeficiency Virus, ABC: Activated B-cells, GC: germinal center, EBER: Epstein-Barr encoding region, ALK: Anaplastic lymphoma kinase, CNS: central nervous system.

in the mechanisms underlying the MYC over-expression in tumor cells, it is still difficult to obtain highly specific and active anti-cancer drugs (121).

Several approaches may be employed to target MYC activities:

– *Blocking MYC activation:* one small molecule, 10058-F4 recognizes the MYC amino acid residues 402-412, which reside within the HLH-LZ domain inhibiting the MYC/MAX heterodimerization (122). Despite its success *in vitro* (cell cycle arrest and apoptosis) 10058-F4 did not prove to be effective in *in vivo* animal studies primarily because of its limiting PK/PD properties.

– *Inhibiting MYC-associated chromatin modifications:* a small molecule, named JQ1, was designed to BRD4 the first bromodomain of the BET family member BRD4. The molecule inhibits MYC pathway activation by targeting chromatin modifications associated with the process of MYC-mediated transcriptional activation. Thanks to encouraging results in *in vitro* models, further preclinical efficacy studies on BRD4 inhibition against a wider range of cancers with elevated MYC expression *via* different mechanisms are ongoing.

– *Exploiting MYC-dependent synthetic lethal interactions:* the essential role of MYC in both cancer and normal tissue development and homeostasis raises the concern that even if direct MYC inhibitors could be developed, they might be too toxic for clinical use. An alternative approach is to identify and target signalling pathways activated by MYC selectively in tumor cells but not in non-tumorigenic cells. Several Poly (ADP-ribose) polymerase (PARP) inhibitors are currently being evaluated in late phase clinical trials. PARP inhibitors thus serve as an important proof of concept that synthetic lethal approaches are clinically relevant and exploitable.

– *Targeting cell cycle kinases:* cancer cells with elevated MYC expression often exhibit highly proliferative and poorly differentiated phenotypes, suggesting that the MYC-activated cells are poised to continuously drive the cell cycle. It may also suggest that other cellular processes have had to adjust to accommodate such significant changes in cell physiology (123, 124). Based on these observations, a dinaciclib phase I trial using MYC expression and signalling as a clinical correlate biomarker of response has been initiated (ClinicalTrials.gov Identifier: NCT01676753). This is among the first trials in which a small molecule CDK inhibitor is used to determine whether MYC overexpressing cancers are selectively targeted. Among other CDKs, an interphase cell cycle kinase CDK2, was reported to be essential for the viability of neuroblastoma cells with *MYCN* amplification (125). CDK2-specific siRNAs and seliciclib (also known as roscovitine), a small molecule CDK inhibitor with higher specificity toward CDK2, 7, and 9, induced apoptosis in a panel of established neuroblastoma cell lines. The sensitivity to CDK2 inhibition was dependent on wild-type p53 and MYCN over-expression. Seliciclib was previously evaluated in phase I and II trials. The potential clinical efficacy of CDK2 inhibition has been controversial. Earlier genetics studies demonstrated that CDK2 was not essential for mammalian embryonic development *in vivo* or for the cell cycle progression of non tumorigenic as well as tumorigenic cells *in vitro* (126, 127). It was recently reported that specif-

ic small molecule inhibition of CDK2 kinase activity diminished cell cycle progression in non-transformed and MYC-transformed epithelial cells without induction of cell death (128, 129). Interestingly, CDK2 genetic depletion *via* siRNA in the same system resulted in accelerated cell proliferation, which was accompanied by the upregulation of CDK1 that has been shown to be capable of functionally compensating for any of the interphase CDKs (128). Whether CDK2 inhibitors will have a role for therapy of neuroblastomas or other MYC or MYCN-driven tumors remains to be determined. Mitosis regulators Aurora kinases A and B, which control mitotic spindle attachment and dynamics, have been targeted in MYC-deregulated cancer cells. It was reported that multiple Aurora selective small molecule inhibitors caused strong antitumorigenic effects-including cell cycle arrest, apoptosis, and autophagy-in model epithelial cells in a MYC-dependent manner (130). Small molecule Aurora kinase inhibitors were also effective in extending animal survival in multiple mouse models of MYC-induced lymphomas (130). More recently, an Aurora kinase small molecule inhibitor, alisertib, was found to increase animal survival in a mouse model of *MYCN*-driven neuroblastoma, in which Aurora kinase plays a key role in maintaining MYCN protein stability that is central to its tumorigenic activity (131). Alisertib is currently being evaluated in numerous phase I and II trials. Chk1-an essential kinase involved in DNA damage and cellular stress-responsive pathways-is another cell cycle-related kinase that has been targeted in MYC-deregulated cancer cells. The hypothesis is that highly proliferative MYC-driven cancer cells increase endogenous DNA damage from replicative stress, DNA replication fork collapse, or oxidative stress. A Chk1 checkpoint allows for repair of these insults and protects rapidly proliferating MYC-driven cells from these endogenous DNA damage insults (132).
– *Other possible target:* beyond the cell cycle, MYC has also been shown to regulate numerous additional signalling pathways critical for tumor development and maintenance. A current challenge is to identify additional synthetic lethal targets in these signalling pathways downstream of MYC. Among all the possible targets there are: small ubiquitin-related modifier (SUMO)-activating enzyme 1/2 (SAE1/2, a heterodimer complex), metabolism regulator enzymes, 5' AMP-activated kinase (AMPK)-related kinase 5 (ARK5) or AMPK itself (121).

A LOOK FORWARD

To overcome the conflicting data present in the literature on the correlation among *MYC* gene aberrations and MYC protein expression, we reviewed a total of 119 clinical, morphological and immunophenotypical typical BL cases and we checked the expression of MYC at both mRNA and protein level by respectively RT-PCR and immunohistochemistry. In addition, FISH analysis for *MYC*-translocation was also performed by using the available probes (dual-color break-apart probe). Different patterns of *MYC* gene translocation/MYC protein expression were identified:
– 99 cases bearing a translocation involving *MYC* gene expressed MYC at both mRNA and protein level (positivity

in almost 80% of neoplastic cells). This finding is in line with the data reported in the literature (133, 134).

– 10 cases in which a translocation involving *MYC* gene was not detectable, expressed MYC at both mRNA and protein level. It is known that none of the techniques currently used to diagnose genetic changes can unambiguously rule out all of *MYC* translocations (134). In fact this may be due to technical failure of FISH, as these cases may present with a very small excision of *MYC* and insertion of the gene into one of the *IG loci*, which is missed by the available probes. Another option is that the breakpoint is localized far outside the region covered by the currently available FISH probes. However, some observations suggest that mechanisms other than translocation may be responsible for elevated MYC protein expression in BL even in the absence of genomic rearrangements (134). Therefore, we investigated the microRNA expression profile of *MYC* translocation-positive and *MYC* translocation-negative BL cases in order to uncover possible differences at the molecular level. We found that *MYC* translocation - positive and negative - BL cases are slightly different in terms of microRNA and gene expression, and we validated our findings at the mRNA and protein levels. Interestingly, in *MYC* translocation-negative BLs we found overexpression of DNA methyltransferase (DNMT) family members, secondary to hypo-expression of hsa-miR-29 family. This finding suggests an alternative way for the activation of lymphomagenesis in these cases, based on global changes in methylation landscape, aberrant DNA hypermethylation, lack of epigenetic control on transcription of targeted genes, and increase of genomic instability. In addition, we observed the over-expression of another *MYC* family gene member, *MYCN* that may therefore represent an additional mechanism for malignant transformation (*Figure 3*). Our finding may be helpful to explain the pathogenetic mechanisms of tumours in which over-expression of *MYC* is independent of a chromosomal translocation or a gene amplification (134);

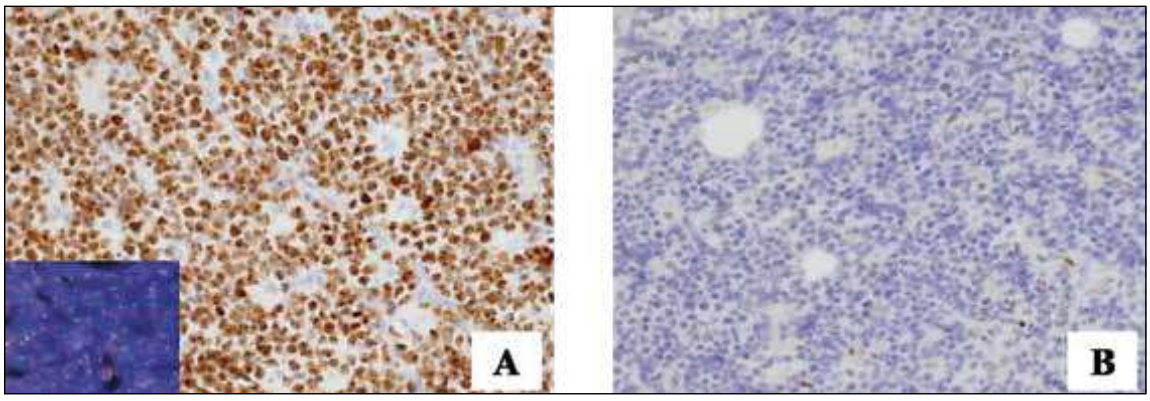

FIGURE 3 • Inverse correlation between MYC (A) and N-MYC (B) protein expression in a BL case carrying a *MYC/IGH* rearrangement (A inset) (A: MYC stain, B: N-MYC stain; A, inset: FISH analysis; A-B, O.M.: 20x).

– 10 cases showed *MYC* gene translocation but did not express MYC at protein level. To evaluate if the negativity for MYC protein was due to a defect in the transcription of *MYC* gene or in the assembling of the protein, RT-PCR for MYC mRNA was performed. We found that among the 10 cases not expressing MYC protein, 5 lacked also MYC mRNA. A study is ongoing in our laboratory to shed new light on how a *MYC* gene aberration does not result in MYC mRNA and protein over-expression.

Authorship

Wrote the paper: MRA, GLB, LL; made contributions to acquisition of histological images: AB; contributed in the field and fruitful discussion: RS; coordinated the work and gave final approval of the version to be published: MRA, LL. All authors read and approved the final manuscript.

Conflict of interest

The Authors declare that they have no competing interests.

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