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Emerging Molecular Networks in Burkitt's Lymphoma

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ABSTRACT

Burkitt's lymphoma (BL), one of the most aggressive tumors affecting humans, characterized by the constitutive activation of the Myc oncogene together with the alteration of many other genetic and epigenetic factors. Among them, the INK4a/ARF locus has been well documented to play a central role in BL. Recently, we have discovered that simultaneous deregulation of both DNA methylation patterns and the ubiquitin-dependent proteolysis system is required to completely inactive the INK4/ARF locus, opening new possibilities for treating Burkitt's lymphoma. In this review, we integrate our discovery with the general view of BL and propose a new comprehensive approach to analyze and manage this aggressive disease. J. Cell. Biochem. 114: 35–38, 2013.

KEY WORDS: BURKITT'S LYMPHOMA; PROTEASOME; MIRNA

Burkitt's lymphoma (BL) is one of the most rapidly growing malignancies that affect humans. It is a highly aggressive non-Hodgkin's B-cell lymphoma (B-NHL) with a germinal-center B-cell phenotype [Gromminger et al., 2012].

BL is subdivided into three different subtypes: endemic BL (eBL), sporadic BL (sBL), and HIV-associated BL. About 95% of eBLs are associated with Epstein–Barr virus (EBV) and are commonly found in Africa and Papua New Guinea. In contrast, only 5–15% of sBLs and 40% of HIV-associated BLs are EBV-positive worldwide [God and Haque, 2010].

BL is a malignancy characterized by intermediately sized B cells that infiltrate nodal and extranodal tissues in a diffuse pattern. The high rate of cell turnover leads to the accumulation of apoptotic debris inside macrophages, interspersed in a malignant population of round monomorphic B cells, conferring to the tumor the typical "starry sky" pattern.

The common characteristic of virtually all BLs is the translocation of the *MYC* proto-oncogene to an immunoglobulin (Ig) locus [God and Haque, 2010]. *MYC* encodes for the c-myc transcription factor [Littlewood et al., 1992], which was first discovered as a homologue of an avian retroviral oncogene [Vennstrom et al., 1982; God and Haque, 2010]. Since the first discovery, *MYC* has been recognized as one of the most commonly overactivated oncogenes in human cancers. As a transcription factor, c-myc regulates the expression of several genes involved in cell cycle progression, proliferation, differentiation, and apoptosis [Meyer and Penn, 2008].

The invariable translocation that improperly activates the *MYC* oncogene can be considered an early event in lymphomagenesis; subsequent tumor progression requires additional genetic and epigenetic changes, which confer a further growth advantage and protection against apoptosis (Ferry, 2006; Lindstrom et al., 2001).

In this review we present an in-depth analysis of the latest discoveries regarding BL. Understanding the molecular signature of BL may improve the diagnosis and ultimately the treatment of this aggressive malignancy. High-throughput technologies are emerging as new, powerful tools in personalized medicine.

THE UBIQUITIN–PROTEASOME PATHWAY IN BURKITT'S LYMPHOMA

The activity of the c-myc oncogene on cell cycle control converges mainly on the Retinoblastoma pathway. The members of the Retinoblastoma gene family (Rb, pRb2/p130, and p107) control the

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correct progression of the cell in the cell cycle. Rb binds E2F transcription factors, whose activity is necessary for the expression of S-phase entry genes, and keeps E2F-responsive promoters inactive. Under mitogenic stimulation, accumulation of D-type cyclins allows the assembly of CDK4/6-cyclinD active complexes, which in turn phosphorylate and inactivate Rb, thus promoting E2F-mediated transcription of S-phase entry genes [Rizzolio et al., 2010, 2012ab]. On the other hand, CDK inhibitors (CDKIs) such as the $p16^{ink4a}$ protein bind and inactivate CDK complexes, thus preventing the phosphorylation of the Rb protein and consequently the inhibition of cell cycle progression. Recently, it has been shown that the *RBL2/p130* gene is mutated in BL cell lines and primary tumors. This study supports the evidence that pRb2/p130 controls the expression of several genes involved in cell growth and furthermore suggests that it plays an important role in BL [De Falco et al., 2007].

It has been recognized that the c-myc protein drives cell cycle progression by activating cyclin D1 and cyclin E expression. Recently, our group has found a new mechanism that cooperates with c-myc overactivation in BL progression. In accordance with the Eu-myc murine model, our data suggest how c-Myc overactivation could be considered just the initial prerequisite for tumorigenesis and that the loss of the INK4/ARF locus is necessary for tumor progression.

Inactivation of $p16^{ink4a}$ is a common event in cancer and different mechanisms of inactivation have been demonstrated, including biallelic deletion, hemizygous deletion associated with mutations, allelic rearrangement, and promoter hypermethylation [Villuendas et al., 1998]. We demonstrated that although the $p16^{ink4a}$ promoter is hyper-methylated in BL cell lines, the demethylating agent 5-Aza-dC is not able to restore normal expression of the *CDKN2A* gene, suggesting that additional mechanisms may be involved in its improper inactivation. The UPS is a protein complex mediating the degradation of target proteins by a chemical reaction that breaks peptide bonds. This system has an important function in the regulation of cellular protein concentrations and degradation. By means of these features, the UPS plays a pivotal role in several crucial cellular processes such as protein quality control, signal transduction, cell cycle and cell differentiation control, and apoptosis [Bedford et al., 2011]. We showed that combining 5-Aza-dC treatment with the proteasome inhibitor MG-132 stabilizes the expression of the $p16^{ink4a}$ protein, suggesting that, in BL cell lines, $p16^{ink4a}$inactivation is concurrently achieved at both transcriptional and post-transcriptional levels [Roberti et al., 2011].

Another pathway involved in BL transformation is the p53 apoptotic pathway. After stress signals, p53 can negatively regulate cell cycle progression by the induction of p21, a CKI of the CIP/KIP family that neutralizes the kinase activity of the CDK2-cyclin E complex [El-Deiry et al., 1993]. In addition, the $p14^{ARF}$ protein [Zindy, 1998], an alternate reading frame (ARF) product of the CDKN2A locus, inhibits the interaction between p53 and its major repressor MDM2 [Kubbutat et al., 1997], thus allowing programmed cell death. We have demonstrated that, in some BL cell lines, there is no correlation between $p14^{ARF}$ mRNA and protein levels. As a consequence of c-Myc overactivation, $p14^{ARF}$ transcription is upregulated, but the corresponding protein is not expressed. Analysis of the proteasome pathway showed that $p14^{ARF}$ is a direct target of ubiquitination, leading to its improper degradation. We found that the MG-132 proteasome inhibitor stabilized the $p14^{ARF}$ protein and led to the accumulation of the polyubiquitinated form of $p14^{ARF}$, suggesting that proteasome-mediated $p14^{ARF}$ degradation is ubiquitin-dependent. In summary, we have provided evidence that the inactivation of $p14^{ARF}$ and $p16^{INK4}$ via the proteasome pathway is a common mechanism in BL, suggesting that proteasome inhibitors may be further considered in the treatment of BL [Roberti et al., 2011].

THE EMERGING ROLE OF THE miRNA PATHWAY

Most endemic BLs are EBV-positive; nonetheless, the exact role of EBV-mediated transformation is still unclear [Tao and Wasik, 2001]. EBV infection in B lymphocytes involves at least five viral glycoproteins. The binding of EBV to the complement receptor 2 (CR2) on B cells is partially mediated by the viral gp350 envelope protein, allowing the viral gp42 protein to bind HLA class II epitopes [Spear and Longnecker, 2003; McShane and Longnecker, 2004]. Following infection, the virus has three distinct latency programs, each one with a different expression pattern for latent EBV-encoded (EBNA) genes [Kuppers, 2003]. Latency I is associated with BL, and is characterized by the expression of EBNA1 and non-coding EBV small RNAs. After the expression of EBNA1, the protein EBNA2 [Kohlhof et al., 2009], together with EBNA-LP, activates cyclin D2, leading B cells to proceed from G_0 into G_1 [Sinclair et al., 1994; Kempkes et al., 1995]. Other EBNA2-associated functions in B cell transformation include the transactivation of all six EBNA virus proteins, the cellular DNA-binding element RBP-jk, the PU.1 protein [God and Haque, 2010] and control of the *MYC* gene at the transcriptional level. As happens in sBL, the cell cycle control pathway is deregulated in EBV-positive BL cells as well. A major role in this process is carried out by the EBNA3C viral protein, which promotes the degradation of the tumor suppressor Rb [Maruo et al., 2006] and consequently the transition of the cell from G_1 to S phase.

Emerging evidence has suggested a link between EBV and the endogenous miRNA machinery of B cells. miRNAs are a class of small RNAs that are able to regulate gene expression at the post-transcriptional level by targeting mRNA. These molecules are involved in cell growth, differentiation and apoptosis and so they could act as tumor suppressors or oncogenes. It has been discovered that EBV-encoded viral miRNAs (viRNAs) can interfere with the function of endogenous miRNAs, suggesting the involvement of miRNAs in the pathogenesis of BL [De Falco et al., 2009]. The complex interplay between viRNAs and miRNA machinery is important in many aspects of viral life, and in modulating the pathogenicity of the infection.

miRNA pathways are also involved in sporadic BL. Leucci et al. [2008]. have shown that the down-regulation of mir-34b is responsible for c-myc upregulation and transfection of let-7a miRNA in BL cells reverts c-myc-induced cell growth stimulation.

Other than specific miRNAs, a miRNome network has been identified as being essential for the normal development of B cells. Basso et al. have shown that normal B cell subpopulations are

characterized by specific miRNA signatures. These signatures are B cell stage-specific during development and deregulation of these pathways may lead to a malignant phenotype [Basso et al., 2009].

GENE EXPRESSION PROFILING IN BL CLASSIFICATION

Although BL has a peculiar clinical presentation, diagnostic dilemmas may arise due to the phenotypical overlaps with subsets of other aggressive mature B-cell lymphomas, in particular with diffuse large B-cell lymphomas (DLBCL). These two pathologies differ in the treatment and prognosis, as BL responds poorly to standard DLBCL therapy (CHOP-like regimen) [Boerman et al., 2000]. While there are substantial diagnostic differences between BL and DLBCL, daily practice shows that some aggressive B-NHLs display some (but not all) morphological, immunophenotypical, and genetic features of classical BL. In fact, although the translocation of the *MYC* gene is a hallmark feature of classical BL, 5–10% of DLBCLs also carry a *MYC* translocation.

It appears evident that the diagnostic tools that are currently available are insufficient to definitively classify BL patients and that new molecular classifications are necessary. Many gene expression profiling (GEP) studies have been published and have helped to discern BL from DLBC at the molecular level. In these studies, BL could be distinguished from other aggressive lymphomas by a distinct gene expression pattern. For instance, a signature of a defined set of mutated genes is characteristic of BL (molecular BL) and is distinct from DLBCL (non-molecular BL). These studies help to sharpen the molecular distinction between BL and DLBCL but, at the same time, extend the spectrum of molecular BL to some cases that would currently be classified as DLBC [Hummel et al., 2006; Salaverria and Siebert, 2011].

Other studies have defined a particular signature of BL characterized by increased expression of a subgroup of GC-promoter-containing gene targets of c-myc and by low expression of MHC class I genes and nuclear factor kappa B target genes [Orsborne and Byers, 2011]. This signature is however characteristic also for atypical BL and DLBCL, underlining that new studies are necessary in order to identify additional targets.

Besides providing more diagnostic information, these studies offer some insight into potential therapeutic targets and clinical outcomes. Such insight leads to a better understanding of each specific case, and could furthermore lead to a different therapeutic approach for each patient. Gene expression profiling studies can potentially offer a better classification of BL and they have already opened the way to new, more promising approaches in the field of systems biology.

FUTURE DIRECTIONS

Some of the most important problems related to GEP are the lack of standardized methods [Orsborne and Byers, 2011], differences in gene annotation, working methodology, and platforms that limit the benefits in clinical setting.

Among the technologies that are emerging, systems biology represents the state-of-art for understanding how the molecular ''players'' talk to each other; this is what we call interactomics. Proteomics, chromatin regulation and miRNA studies combined with GEP are integrated together to define the signature of either normal or pathological conditions.

Recently, a systems biology approach referred to as interactome dysregulation enrichment analysis (IDEA) was used to identify oncogenes and molecular perturbation in B-cell lymphomas. The aim of the work was the use of an informatics hybrid interactome containing protein–protein, protein–DNA, and post-translational interactions inferred by an algorithm. Starting from these three networks and by integrating the information, Mani et al. [2008] found a B-cell interactome (BCI). Comparing the BCI of normal cells with that of aberrant cells, the authors identified clusters in specific areas called ''cancer modules.'' This work is very important because it identified not only the single oncogenic lesion, but also the interaction that may occur during transformation and, unlike GEP, the IDEA system can predict the key effectors of a phenotypic transition.

Keeping in mind that the systems biology approach is not without weaknesses [Marbach et al., 2010] the global read-out offered by systems biology can create predictive models representing the cellular macrocosm. In fact, the ''goal'' of systems biology is to generate a model in which we can understand how the cell or organism is self-organized in response to a given perturbation and how the same process can evolve differently when different perturbations appear.

This avenue of research could yield new, valuable information about lymphomas and other tumors that affect humans, and could provide new tools for diagnostics through the identification of genetic risk factors for diseases, or for achieving model-based, personalized treatment regimens in which all benefits and risks are well-evaluated.

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