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Deregulation of DNMT1, DNMT3B and miR-29s in Burkitt lymphoma suggests novel contribution for disease pathogenesis

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ABSTRACT

Methylation of CpG islands in promoter gene regions is frequently observed in lymphomas. DNA methylation is established by DNA methyltransferases (DNMTs). DNMT1 maintains methylation patterns, while DNMT3A and DNMT3B are critical for de novo DNA methylation. Little is known about the expression of DNMTs in lymphomas. *DNMT3A* and *3B* genes can be regulated post-transcriptionally by miR-29 family. Here, we demonstrated for the first time the overexpression of DNMT1 and DNMT3B in Burkitt lymphoma (BL) tumor samples (69% and 86%, respectively). Specifically, the treatment of two BL cell lines with the DNMT inhibitor 5-aza-dC decreased DNMT1 and DNMT3B protein levels and inhibited cell growth. Additionally, miR-29a, miR-29b and miR-29c levels were significantly decreased in the BL tumor samples. Besides, the ectopic expression of miR-29a, miR-29b and miR-29c reduced the DNMT3B expression and miR-29a and miR-29b lead to increase of $p16^{INK4a}$ mRNA expression. Altogether, our data suggest that deregulation of DNMT1, DNMT3B and miR29 may be involved in BL pathogenesis.

1. Introduction

Burkitt lymphoma (BL) is an aggressive non-Hodgkin lymphoma derived from germinal center B-cells (Dave et al., 2006). It is characterized by a high degree of cell proliferation, a shortened cell cycle and a high mitotic index. The molecular signature of BL is activation of the *c-MYC* oncogene that has been translocated to the immunoglobulin gene loci (heavy and light chains) (Dalla-Favera et al., 1982; Taub et al., 1982). As a result, c-MYC becomes constitutively expressed, activating genes related to various cellular processes, such as cellular division, metabolism, apoptosis and telomerase activity, among others (Hecht and Aster, 2000). Recent evidence suggests that the c-MYC transcription factor accumulates in the promoter regions of active genes and induces transcriptional amplification. Therefore, apart from binding and regulating a particular set of genes, c-MYC also amplifies the existing gene expression program (Dang, 2012; Lin et al., 2012). Additional genetic and epigenetic events contribute to BL pathogenesis, including *TP53* mutations, Epstein–Barr virus (EBV), the silencing of tumor suppressor genes by promoter methylation, and mutations of the TCF-3 transcription factor or its regulator, ID3 (Klumb et al., 2003, 2004b; Lindstrom and Wiman, 2002; Sander et al., 2012; Schmitz et al., 2012).

The most widely studied epigenetic modification is DNA methylation, which has an important role in the control of gene expression in mammalian cells. This process is catalyzed by the DNA methyltransferase (DNMT) enzymes 1, 3A and 3B. DNMT1 preferentially methylates the unmethylated strand of hemimethylated DNA during DNA replication (Hermann et al., 2004). Its inactivation produces global demethylation, which is consistent with the finding that DNMT1 is required for maintenance of methylation (Suzuki et al., 2004). DNMT3A and DNMT3B catalyze de novo methylation of both strands and are highly expressed in embryonic cells, where most methylation events occur, but are down-regulated in adult somatic cells and overexpressed in several tumors (Bestor, 2000; Okano et al., 1999). Recently, it was shown that DNA methylation patterns play a key role in hematopoietic development. Remarkably, DNMT1 is significantly up-regulated in B cells from germinal centers (GCs), mediating the GC phenotype (Shaknovich et al., 2011). Moreover, patterns of DNA methylation can predict disease severity in some types of lymphomas (De et al., 2013).

In recent years, lymphoma studies have focused on the role of microRNAs (miRNAs), which are small non-coding RNAs 21 to 25 nucleotides long that regulate post-transcriptional gene expression by mRNA target cleavage and degradation or translational repression and deadenylation, depending on the complementarity between the miRNA and the targeted mRNA transcript (Kasinski and Slack, 2011).

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miRNAs may also be involved in normal hematopoiesis and in the pathogenesis of different types of lymphoma (Di Lisio et al., 2012; Garzon and Croce, 2008; Jardin and Figeac, 2013; Tagawa et al., 2013; Vasilatou et al., 2010). BL is characterized by the deregulated expression of c-MYC, which in turn regulates a large set of miRNAs. The MYC-regulated miRNA profile was recently described in BL (Robertus et al., 2010). Robertus et al. evaluated the expression of miRNAs in BL and demonstrated induction and repression of groups of miRNAs induced and repressed compared to other lymphoid neoplasms. Among the down-regulated miRNAs, mir29a, 29b and 29c were 9-, 8- and 16-fold decreased, respectively (Robertus et al., 2010). Decreased levels of miR-29 family members have been observed in several tumors, including leukemia, melanoma, and liver, colon, and lung cancers, and are generally associated with more aggressive forms of the disease and leukemia relapse (Cummins et al., 2006; Garzon et al., 2009a; Nguyen et al., 2011; Xiong et al., 2010; Yanaihara et al., 2006). Among the suggested mechanisms of the tumor-suppressing effect of the miR-29 family is DNMT regulation. Members of the miR-29 family have complementary 3′ untranslated regions (UTRs) to *DNMT3A* and *3B* genes (mRNAs), which may impact methylation status (Fabbri et al., 2007; Garzon et al., 2009b; Wang et al., 2013). Based on the report that DNMTs can be regulated post-transcriptionally by miRNAs, the reduction of the miR-29 family in BL might lead to deregulation of DNMTs (Denis et al., 2011), contributing to lymphomagenesis. To determine whether DNMT1, DNMT3A and DNMT3B expressions are deregulated in BL tumor cells and to explore the relationship of miR-29 family with DNMT3A and DNMT3B, we investigated DNMT and miR-29 expression profiles in tumor samples from BL patients.

2. Patients and methods

Tumor samples from patients with BL from the Hematology Service of the National Cancer Institute, Brazil, from 1995–2011 were retrospectively selected for this study. Seventy one paraffin-embedded tumor samples were reviewed by a second pathologist (LMMR) to confirm the diagnosis according to the criteria described by the 2008 World Health Organization (WHO) classification for hematopoietic diseases based on morphological criteria; immunohistochemical expression of CD20, CD10, and BCL6; a high proliferation rate (nearly 100% for Ki-67); and the absence or weak expression of BCL-2 (Jaffe et al., 2008). The inclusion criteria for this study were the availability of a tumor block, patient age less than 18 years and available clinical and demographic data. All patients were treated using an NHL-BFM 86/90 (Berlin–Frankfurt–Munster)-based protocol (Klumb et al., 2004b). This study was approved by the local Ethics in Research Committee (CEP Registration No. 18/09).

2.1. EBV detection by ISH

The analysis of EBV status was performed by in situ hybridization (ISH) for EBV-encoded RNA (EBER) as reported previously (Klumb et al., 2004a).

2.2. Immunohistochemical analysis of DNMT1, DNMT3A and DNMT3B

DNMT1, DNMT3A and DNMT3B detection was performed using immunohistochemistry (IHC) on tissue microarray (TMA) slides. Representative areas of the tumors were selected for analysis. All TMA slides had placental tissue sections, which were used as a positive control due to the high expression of DNMTs in embryonic tissues (Bestor, 2000; Okano et al., 1999). Furthermore, immunohistochemistry for each reaction was analyzed using an additional negative control by omitting the primary antibody. Reactive lymph nodes were included in each DNMT protein reaction. Mouse monoclonal antibody DNMT3A 1:100 (clone 64B1446, Imgenex), monoclonal anti-DNMT3B 1:200 (clone 52A1018, Imgenex) and polyclonal antibody anti-DNMT1 1:400 (clone H-300, Santa Cruz Biotechnology) were used. The specificity of these antibodies has been previously characterized (Mutze et al., 2011). Antigen retrieval was performed using steamer with Tris–EDTA buffer (pH 9.0) for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min. Detection was performed using the Dako EnVision system. Tissues were stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 1 min and counterstained with Harris hematoxylin for 30 s. A pathologist manually scored the expression levels of DNMT1, DNMT3A and DNMT3B using a 3-point scoring system. The categories were: 0–10%, 11–50%, and >50% of tumor cells stained, corresponding to negative/low, intermediate and high expression levels, respectively. The cut-off levels were based on modified Choi et al. system considering only the proportion of positive cells (Choi et al., 2003). The images were made using a Nikon Eclipse E200 microscope connected to a Digital Sight scanning system and were captured in the NIS-Elements program V2.30 (Nikon, Japan).

2.3. RNA extraction and real-time PCR

Paraffin-embedded tumor tissue samples were cut into four 10-μm sections. Tumor RNA was purified using a Recover All™ Total Nucleic Acid Isolation Kit for total nucleic acid from formalin- or paraformalin-fixed, paraffin-embedded (FFPE) tissues (Ambion) according to the manufacturer's specifications. cDNA synthesis was performed on 2 μg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using the Taqman probes *DNMT1* (Hs00154749_ml), *DNMT3A* (Hs01027166_m1), *DNMT3B* (Hs00171876_m1) and *DNMT3B7* (Alkebsi et al., 2013) and, as reference, the *TBP* gene (Hs00427621_m1), all of which were purchased from Applied Biosystems. The assay was analyzed using a StepOne™ System (Applied Biosystems).

2.4. miR-29a, miR-29b, and miR-29c quantification in BL tumor samples

miRNA was isolated from FFPE using a RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion®). Five reactive lymph node tissue specimens were used as reference samples. A quantitative real-time reverse-transcription PCR (qRT-PCR) was performed using primer-specific TaqMan® Kit (Applied Biosystems) for miR-29a, miR-29b, and miR-29c. miRNA levels were normalized to the expression level of the small nuclear RNA (snRNA) RNU6B and are expressed relative to miR-29a, miR-29b, and miR-29c reference transcript levels.

2.5. Cell lines, culture and treatments

The BL cell line Raji (EBV+) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). BL cell line BL41 (EBV−) was kindly provided by Dr. Boulanger (Greehey Children's Cancer Research Institute, The University of Texas Health Science Center, San Antonio, USA). Both BL cell lines were cultured in the presence of the DNMT inhibitor 5-aza-2-deoxycytidine (5-aza-dC, decitabine, SIGMA). Initially, cells were cultured in complete RPMI 1640 medium supplemented with 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA) and 10% heat-inactivated fetal calf serum (Gibco BRL, UK) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The BL cell lines were cultured at 8×10^5 cells/ml in the presence of 1.0 μM of decitabine for 24 h. The media was then completely replaced and refreshed without 5-aza-dC. Three days later, the cells were collected for protein expression analysis by western blotting and $p16^{INK4a}$ mRNA expression levels (Hs00923894_m1) using real time PCR as described above. Cell viability was evaluated by trypan blue exclusion.

2.6. Protein expression analysis in BL cell lines

To verify protein expression levels, a Western blotting assay was performed. Briefly, 30 μg of whole-cell protein extract from each cell