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The *DLEU2/miR-15a/16-1* Cluster Controls B Cell Proliferation and Its Deletion Leads to Chronic Lymphocytic Leukemia

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SUMMARY

Chronic lymphocytic leukemia (CLL) is a malignancy of B cells of unknown etiology. Deletions of the chromosomal region 13q14 are commonly associated with CLL, with monoclonal B cell lymphocytosis (MBL), which occasionally precedes CLL, and with aggressive lymphoma, suggesting that this region contains a tumor-suppressor gene. Here, we demonstrate that deletion in mice of the 13q14-minimal deleted region (MDR), which encodes the *DLEU2/miR-15a/16-1* cluster, causes development of indolent B cell-autonomous, clonal lymphoproliferative disorders, recapitulating the spectrum of CLL-associated phenotypes observed in humans. miR-15a/16-1-deletion accelerates the proliferation of both human and mouse B cells by modulating the expression of genes controlling cell-cycle progression. These results define the role of 13q14 deletions in the pathogenesis of CLL.

INTRODUCTION

B cell chronic lymphocytic leukemia (CLL) represents the most common B cell-derived malignancy of adults with an incidence of around 1 in 100,000 per year. The disease is characterized by the clonal expansion of B cells that express the CD5 cell surface antigen and that are thought to derive from antigen-experienced marginal zone B cells (Chiorazzi and Ferrarini, 2003; Klein et al., 2001). Within this morphologically homogeneous tumor, two subtypes of CLL have been identified: one that expresses somatically mutated immunoglobulin variable region (IgV) genes and has a good prognosis, and one with unmutated IgV genes and a less favorable prognosis (Chiorazzi and Ferrarini, 2003; Damle et al., 1999; Hamblin et al., 1999). Recent work suggests that CLL may be preceded by $CD5^+$ monoclonal B cell lymphocytosis (MBL), which is detectable in ~5% of the healthy elderly population and that can progress to CLL in ~1% of cases (Landgren et al., 2009; Rawstron et al., 2008). A small fraction of CLL progresses toward a more aggressive malignancy diagnosed as diffuse large B cell lymphoma (DLBCL).

The pathogenesis of CLL remains obscure because no genetic alteration has been conclusively demonstrated to contribute to its pathogenesis. Although CLL only rarely shows reciprocal

SIGNIFICANCE

Emerging evidence has suggested the presence of a tumor-suppressor locus in the chromosomal region 13q14 commonly deleted in CLL. These results show that this region has a major role in controlling the pool of mature B-lymphocytes in vivo. Its deletion causes a heterogeneous B-cell-autonomous phenotype that recapitulates the spectrum of CLL-associated syndromes including MBL and lymphoma. Within the 13q14-region, the *miR-15a/16-1* cluster plays a role in controlling the proliferation of B cells, but additional genetic elements invariably deleted in human CLL contribute to the aggressiveness of the phenotype. These results elucidate the mechanism by which miR-15a/16-1 function in vivo, provide a faithful mouse model of human CLL, and provide a paradigm for the tumor-suppressor role of sterile RNA transcripts.

balanced chromosomal translocations (Döhner et al., 2000; Mayr et al., 2006), the spectrum of genomic aberrations in CLL includes trisomy of chromosome 12 (16%) and deletion of chromosomal regions 17p (p53; 7%), 11q (18%), and 13q14.3 (further on designated as 13q14), which represents the most common genomic aberration in CLL (55%) (Döhner et al., 2000; Kalachikov et al., 1997). Inactivation of p53 is observed in a fraction of cases and is associated with disease progression (Gaidano et al., 1991).

Deletion of 13q14 is mostly monoallelic (76% of cases), but can be biallelic (24%) (Döhner et al., 2000). This deletion occurs in CLL with somatically mutated as well as unmutated IgV genes, although it is more prevalent in the former subtype (~80% versus 20%). 13q14 deletions are found at high frequency (>50%) also in MBL (Rawstron et al., 2008) and at lower frequency in CD5-negative B cell-derived malignancies (Avet-Loiseau et al., 1999; Cigudosa et al., 1998; Liu et al., 1995; Stilgenbauer et al., 1998), including DLBCL and multiple myeloma, as well as mature T cell lymphomas (Rosenwald et al., 1999), and in a variety of solid tumors.

Because 13q14 deletions suggest the presence of a tumor-suppressor gene, this region has been extensively characterized (Bullrich et al., 2001; Corcoran et al., 1998; Liu et al., 1997; Migliazza et al., 2001; Rondeau et al., 2001; Stilgenbauer et al., 1998). A minimal deleted region (MDR) has been identified (Liu et al., 1997; Migliazza et al., 2001) that comprises the *deleted in leukemia* (*DLEU*) *2* gene, encoding a sterile transcript, parts of the *DLEU1* (1st exon) sterile gene, as well as the microRNA (miR)-15a/16-1 cluster (Calin et al., 2002; Lagos-Quintana et al., 2001) that is located intronic to *DLEU2* (see Figure 1A). Although *DLEU2* and the *miRs* are evolutionary conserved and expressed in B cells (Cimmino et al., 2005; Mertens et al., 2002; Migliazza et al., 2001), a role of *DLEU1* as well as the adjacent *DLEU5* and *KCNRG* genes (Ivanov et al., 2003) in CLL pathogenesis is considered unlikely based on lack of evolutionary conservation (*DLEU1*), inclusion in only a subset of 13q14 deletions (*DLEU5*, *KCNRG*), and low to absent expression in mature B cells (*KCNRG*). Taken together, these observations pointed toward *DLEU2* and/or *miR-15a/16-1* as the candidate tumor suppressors targeted by 13q14 deletions in CLL.

DLEU2 encodes a long noncoding RNA (1.0–1.8 kb) that is polyadenylated and spliced (Migliazza et al., 2001). Members of this class of sterile RNAs exert diverse cellular functions including X chromosome inactivation or activation, imprinting, and transcriptional coactivation or regulation of gene expression (Ponting et al., 2009). The function of DLEU2, however, is unknown, and its sequence does not display homology to any other noncoding RNA. A role for miR-15a/16-1 in CLL pathogenesis has been repeatedly proposed based on in vitro studies in nonlymphoid cell systems that suggested functions of miR-15a/16-1 in negative regulation of proliferation and apoptosis (Bandi et al., 2009; Calin et al., 2008; Cimmino et al., 2005; Linsley et al., 2007; Liu et al., 2008; Raveche et al., 2007). Also, CLL cases were identified (2%) that showed germline mutations in the primary precursor of miR-15a/16-1 that appear to affect its processing (Calin et al., 2005), and a point mutation occurring in the 3′ flanking region of *miR-16-1* in NZB mice has been associated with reduced miR-16-1 expression (Raveche et al., 2007). Downregulation of miR-15a/16-1 has also been implicated in the pathogenesis of prostate carcinoma (Bonci et al., 2008). Based on these observations, the *miR-15a/16-1* cluster has been suggested as the culprit of the deletion in CLL, but no formal proof has been obtained.

In order to identify the genetic elements targeted by the putative 13q14 tumor suppressor and to determine their contribution to CLL pathogenesis, we generated transgenic mice that carried conditional alleles that either mimicked the deletion of the *MDR* or that specifically deleted the *miR-15a/16-1* cluster without affecting the expression of DLEU2.

RESULTS

Construction of Mice Deleting the *MDR* or *miR-15a/16-1*

The 13q14 region is conserved on mouse chromosomal region 14qC3 (Figure 1A). In contrast to the human genome, *Kcnrg* and *dLeu5* are intronic of and overlap with *dLeu2*, respectively, although rare splice forms have been described for *DLEU2* in the human that also overlap with those genes (Baranova et al., 2003). Analogous to the human genome, the *miR-15a/16-1* cluster is located intronic of the *dLeu2* gene ~300 bp downstream of exon 4 (Figure 1A bottom). To investigate the consequences of the deletion of the genes encoded within the 13q14 *MDR*, we generated conditional mouse alleles that upon Cre- or Flpe-mediated deletion either mimic the human *MDR* and delete both *dLeu2* and *miR-15a/16-1* (further on referred to as conditional *MDR* allele), or the *miR-15a/16-1* cluster only.

To generate the conditional *MDR* allele, we inserted two *lox*P as well as two *frt* sites in consecutive ES cell targetings into transcriptionally silent regions that are located 110 kb apart (Figure 1B; for details of the targeting, see Figures S1A1 and S1A2, available online) with the 5′ site located approximately 20 kb centromeric to *dLeu5*, and the 3′ site about 5 kb telomeric to the alternative exon 1 (exon 1b) of *dLeu2*. To allow screening for ES cell clones where both targetings occurred on the same chromosome, an eGFP mini gene and a PGK promoter were placed in the centromeric and telomeric targeting vectors, respectively, thus enabling eGFP expression upon Adeno-Cre-mediated deletion of the *lox*P-flanked *MDR*. Correct homologous recombination of the individual targetings and correct Cre-mediated deletion of the *lox*P-flanked region were confirmed by Southern blot analysis (Figure S1A4 and Figure 1B, lower right). Mice carrying the $MDR^{loxP\text{-}frt/+}$ allele were bred with *Flp*-transgenic mice, and the deletion of the *frt*-flanked region was confirmed by Southern blotting (Figure S1A3 and Figure 1B lower left); these mice are further on referred to as $MDR^{+/-}$ mice.

The conditional *miR-15a/16-1* allele was generated by placing the first *lox*P-site centromeric to the *miR-15a/16-1* cluster and the second one within the ~400 bp region that separates the *miR* cluster and exon 4 of *dLeu2* (Figure 1C; for details of the targeting see Figure S1B1). Correct homologous recombination was confirmed by Southern blot analysis (Figure S1B1). The *neo*-resistance marker was deleted by crossing mice with the targeted allele to *Flp*-transgenic mice, further on designated as $miR\text{-}15a/16\text{-}1^{fl/+}$ (conditional) mice (Figure S1B2). Mice carrying the conditional allele were bred with *Cre*-transgenic mice, and deletion of the *lox*P-flanked region was confirmed by Southern blot (Figure 1C and Figure S1B2); these mice are further on