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Original contribution

Frequent epigenetic inactivation of *Rb1* in addition to *p15* and *p16* in mantle cell and follicular lymphoma☆

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Summary Dysregulation of cell cycle control is an important mechanism in carcinogenesis. Gene promoter hypermethylation is an alternative mechanism of gene inactivation. We analyzed the methylation status of the tumor suppressor components of the INK4/Rb pathway in mantle cell lymphoma and follicular lymphoma by methylation-specific polymerase chain reaction for *p15*, *p16*, *p18*, and *Rb1* in 23 mantle cell lymphoma and 30 follicular lymphoma cases and lymphoma cell lines. The methylation-specific polymerase chain reaction results showed that in mantle cell lymphoma, frequent *p16* (82%) but infrequent *p15* (8.7%) or *Rb1* (17.4%) hypermethylation occurred, with *p16* and *Rb1* hypermethylation being mutually exclusive ($P = .01$). In follicular lymphoma, frequent hypermethylation of *p15* (36.7%), *p16* (56.7%), and *Rb1* (43.3%) occurred, with *p15* and *Rb1* hypermethylation being mutually exclusive ($P = .05$). Concurrent methylation of *p15* and *p16* occurred in 26.7% of patients with follicular lymphoma and 8.7% of patients with mantle cell lymphoma. Compared with mantle cell lymphoma, there was more frequent *p15* ($P = .025$) hypermethylation but comparable *Rb1* ($P = .07$) and *p16* ($P = .07$) hypermethylation in follicular lymphoma. In a patient with follicular lymphoma with sequential biopsies, *Rb1* was unmethylated and expressed at diagnosis but became methylated and down-regulated at relapse. Moreover, methylation analysis of these 4 genes in an additional 8 patients with grade I follicular lymphoma showed that *Rb*, but not the other genes, was preferentially methylated in grade II ($P = .03$). In summary, most patients with mantle cell lymphoma and follicular lymphoma had epigenetic aberrations targeting the INK4/Rb pathway. There is more frequent *p16* hypermethylation in mantle cell lymphoma and *p15* or *Rb1* hypermethylation in follicular lymphoma. The role of *Rb* methylation in disease or histologic transformation in follicular lymphoma warrants further study.

1. Introduction

Mantle cell lymphoma (MCL) and follicular center cell lymphoma (FL) are 2 forms of small B-cell lymphomas derived from neoplastic transformation of their normal counterparts in the mantle zone and germinal center, respectively [1]. MCL is characterized in virtually all cases

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by *t*(11;14), with dysregulation of the cell cycle by up-regulation of the cyclin D1 gene located at 11q13 [1,2]. In contrast, FL is characterized by *t*(14;18), with up-regulation of BCL-2 located at 18q21, which confers resistance to apoptosis on the malignant lymphoid cells [1-3].

Cellular proliferation is mediated by progression through the cell cycle, with 2 major cell cycle checkpoints located at G_1S and G_2M [4-6]. Rb1 is a nucleoprotein important in the suppression of G_1S cell cycle progression. This is mediated by the sequestration of E2F by Rb1 in quiescent cells, a transcription factor for genes important in G_1S progression [7]. Quiescent cells in G_0 phase contain hypophosphorylated Rb1, which sequesters the transcription factor E2F. Upon activation of the cell by mitogens, up-regulation of D-type cyclins results in activation of cyclin-dependent kinases (CDKs) 4 and 6 and the resulting hyperphosphorylation of Rb1. E2F is then released, inducing the transcription of S1-specific genes and an irreversible commitment to cell cycle progression [5,6]. Regulatory elements inhibitory to cell cycle progression include the INK4 (p15, p16, p18, and p19) and the CIP/KIP ($p21^{CIP}$, $p27^{KIP1}$, $p57^{KIP2}$) families of proteins that give rise to cell cycle arrest [8,9]. The INK4 family are CDK inhibitors (CKIs) that share similar functional domains (ankyrin repeats for protein-protein interactions). These domains allow the INK4 family to compete with cyclin D for binding to CDKs 4 and 6, thus preventing CDKs 4 and 6 activation and cell cycle progression [8]. Therefore, various mechanisms exist to inhibit or inactivate Rb1 function, thereby resulting in unchecked cellular proliferation, in cancers. These include Rb1 inactivation by genetic and epigenetic mechanisms [7], overexpression of cyclin D1, and activating mutations of CDK 4.

DNA methylation, catalyzed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in a CpG dinucleotide, leading to a conversion to methylcytosine [10,11]. In many hemic cancers, the CpG islands of selected genes are aberrantly hypermethylated, resulting in repression of transcription of these genes. Hypermethylation thus serves, in addition to mutation and deletion, as an alternative mechanism of gene inactivation [10,11].

The role of Rb1 in carcinogenesis has been illustrated by familial retinoblastoma, in which patients inheriting loss of 1 *Rb1* allele develop retinoblastoma at an early age by inactivating mutation of the other *Rb1* allele. Moreover, sporadic mutation of *Rb1* has been demonstrated in cancers other than retinoblastoma [7], indicating that the tumor suppressor role of *Rb1* is not restricted to the retina [12]. Moreover, G_1S cell cycle progression may also be dysregulated by inactivation of the members of the INK4 family of CKI [13]. In this study, we investigated if methylation of the *INK4* family of CKIs and *Rb1* might contribute to lymphomagenesis of MCL and FL. *p19* was not included in the analysis because tumor suppressor activity has not been demonstrated in transgenic mice deficient in *p19* [14].

2. Materials and methods

2.1. Patients and diagnosis

Diagnosis of MCL and FL was made according to standard criteria [1,2]. Patients were staged according to the Ann Arbor system. All patients underwent complete staging including full blood count, serum biochemistry, serum lactate dehydrogenase level, computerized tomographic scan of thorax and abdomen, and bilateral bone marrow trephine biopsies. Immunophenotyping was performed on cryostat sections and paraffin sections with standard immunoperoxidase technique. Paraffin sections of formalin- or B5-fixed tissue were stained with hematoxylin-eosin to confirm the diagnosis of lymphoma and examined for the expression of B- and T-cell markers. The panel of antibodies used included CD3 (Leu4; Becton Dickinson, San Jose, CA), CD3 (polyclonal; Dako, Glostrup, Denmark), CD5 (Dako), CD10 (J5; Coulter, Hialeah, FL), CD19 (Leu 12; Becton Dickinson), CD20 (L26; Dako), CD22 (Dako), CD23 (Dako), and cyclin D1 (Zymed, San Francisco, CA). The clinicopathologic features of the patients with MCL have been previously reported [2]. All 23 cases of MCL were classic-variant, with the phenotype of CD5+, CD10−, and CD23−, and immunoreactive for cyclin D1. Five patients were positive for nested polymerase chain reaction (PCR) for *BCL-1* gene rearrangement [2]. The 30 patients with FL were FL grade II according to the World Health Organization classification [1]. There were 23 patients (18 male and 5 female) with MCL, with a median age of 66 years (range, 49-75 years), and 30 patients (21 male and 9 female) with FL, with a median age of 57 years (range, 33-88 years).

2.2. Samples and DNA preparation

DNA was extracted from frozen lymph node biopsies of 30 patients with grade II FL and 23 patients with MCL. To study the role of methylation of these genes in histologic transformation of FL, DNA was obtained from an additional 8 patients with grade I FL. We also isolated DNA from the mononuclear cells of peripheral blood (PBMC)s of 10 healthy volunteers. Normal lymph node biopsies from 6 patients were used as negative control, whereas methylated control DNA (CpGenome Universal Methylated DNA; Intergen, New York, NY) was used as positive control in all the experiments. We also examined 3 MCL cell lines (Granta-519, Jeko-1, Mino) and 2 FL cell lines (SUDHL6, DHL16). MCL cell lines Granta-519, Jeko-1, and Mino were provided by Raymond Lai (Department of Laboratory Medicine and Pathology, University of Alberta and Cross