BMI-1 Gene Amplification and Overexpression in Hematological Malignancies Occur Mainly in Mantle Cell Lymphomas

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Abstract

The BMI-1 gene is a putative oncogene belonging to the Polycomb group family that cooperates with c-myc in the generation of mouse lymphomas and seems to participate in cell cycle regulation and senescence by acting as a transcriptional repressor of the INK4a/ARF locus. The BMI-1 gene has been located on chromosome 10p13, a region involved in chromosomal translocations in infant leukemias, and amplified in occasional non-Hodgkin’s lymphomas (NHLs) and solid tumors. To determine the possible alterations of this gene in human malignancies, we have examined 160 lymphoproliferative disorders, 13 myeloid leukemias, and 89 carcinomas by Southern blot analysis and detected BMI-1 gene amplification (3- to 7-fold) in 4 of 36 (11%) mantle cell lymphomas (MCLs) with no alterations in the INK4a/ARF locus. BMI-1 and p16INK4a mRNA and protein expression were also studied by real-time quantitative reverse transcription-PCR and Western blot, respectively, in a subset of NHLs. BMI-1 expression was significantly higher in chronic lymphocytic leukemia and MCL than in follicular lymphoma and large B cell lymphoma. The four tumors with gene amplification showed significantly higher mRNA levels than other MCLs and NHLs with the BMI-1 gene in germline configuration. Five additional MCLs also showed very high mRNA levels without gene amplification. A good correlation between BMI-1 mRNA levels and protein expression was observed in all types of lymphomas. No relationship was detected between BMI-1 and p16INK4a mRNA levels. These findings suggest that BMI-1 gene alterations in human neoplasms are uncommon, but they may contribute to the pathogenesis in a subset of malignant lymphomas, particularly of mantle cell type.

Introduction

The BMI-11 gene is a putative oncogene of the Polycomb group originally identified by retroviral insertional mutagenesis in Eμ-c-myc transgenic mice infected with the Moloney murine leukemia virus (1, 2). These animals had a rapid development of pre-B cell lymphomas and seem to participate in cell cycle control and senescence by acting as a transcriptional repressor of the INK4a/ARF locus. The BMI-1 gene has been located on chromosome 10p13, a region involved in chromosomal translocations in infant leukemias, and amplified in occasional non-Hodgkin’s lymphomas (NHLs) and solid tumors. To determine the possible alterations of this gene in human malignancies, we have examined 160 lymphoproliferative disorders, 13 myeloid leukemias, and 89 carcinomas by Southern blot analysis and detected BMI-1 gene amplification (3- to 7-fold) in 4 of 36 (11%) mantle cell lymphomas (MCLs) with no alterations in the INK4a/ARF locus. BMI-1 and p16INK4a mRNA and protein expression were also studied by real-time quantitative reverse transcription-PCR and Western blot, respectively, in a subset of NHLs. BMI-1 expression was significantly higher in chronic lymphocytic leukemia and MCL than in follicular lymphoma and large B cell lymphoma. The four tumors with gene amplification showed significantly higher mRNA levels than other MCLs and NHLs with the BMI-1 gene in germline configuration. Five additional MCLs also showed very high mRNA levels without gene amplification. A good correlation between BMI-1 mRNA levels and protein expression was observed in all types of lymphomas. No relationship was detected between BMI-1 and p16INK4a mRNA levels. These findings suggest that BMI-1 gene alterations in human neoplasms are uncommon, but they may contribute to the pathogenesis in a subset of malignant lymphomas, particularly of mantle cell type.

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3 The abbreviations used are: BMI-1, B cell-specific Moloney murine leukemia virus integration site 1; NHL, non-Hodgkin’s lymphoma; CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; LCL, large B cell lymphoma; MCL, mantle cell lymphoma; RT-PCR, reverse-transcription-PCR; RU, relative units.

INK4a/ARF locus by acting as an upstream negative regulator of p16INK4a and p14/p19ARF gene expression (5). The human BMI-1 gene has been mapped to chromosome 10p13 (6), a region involved in chromosomal translocations in infant leukemias (7) and rearrangements in malignant T cell lymphomas (8, 9). More recently, high-level DNA amplifications of this region have been found by comparative genomic hybridization in NHLs and solid tumors (10, 11). However, the possible implication of the BMI-1 gene in these alterations and its role in the pathogenesis of human tumors is not known. The aim of this study was to analyze the possible BMI-1 gene alterations and expression in a large series of human neoplasms and to determine the relationship with INK4a/ARF locus aberrations.

Materials and Methods

Case Selection. A series of 262 human tumors, including 173 hematological malignancies and 89 carcinomas (Table 1), matched normal tissues from all carcinomas. 11 samples of normal peripheral mononuclear cells, and 5 reactive lymph node tonsils, were selected based on the availability of frozen samples for molecular analysis.

DNA Extraction and Southern Blot Analysis. Genomic DNA was obtained using Protease K/RNase treatment. 15 μg were digested with EcoRI and HindIII restriction enzymes (Life Technologies, Inc., Gaithersburg, MD), for Southern blot analysis and hybridized with a 1.5-kb PstI fragment of the partial BMI-1 cDNA (6).

RNA Extraction and Real-time Quantitative RT-PCR. Total RNA was obtained from 67 lymphoid neoplasms (10 CLLs, 27 MCLs, 8 FLs, and 22 LCLs) using guanidine/isoisothiocyanate extraction and cesium/chloride gradient centrifugation. One μg of total RNA was transcribed into cDNA using MMLV-reverse transcriptase (Life Technologies, Inc.) and random hexamers, following manufacturer’s directions. Sequences of the BMI-1 and the p16 detection probes and primers were designed using the Primer Express program (Applied Biosystems, Foster City) as follows: BMI-1 sense, 5′-CTGGTGCC-CCATTGACACGC-3′; BMI-1 antisense, 5′-CAGAAATGAATGGAGGAGGAGCA-CCA-3′; p16 sense, 5′-CAACGCACCGAATAGTTACGG-3′; p16 antisense, 5′-AACTTGTCCCTCCAGATGTCGCG-3′. The probes BMI-1, 5′-CAGCTG-GCTTCAAGATGCGGCCG-3′, and p16, 5′-CGGAGGGCTAGCCCAGGTTTTG-3′, were labeled with 6-carboxy-fluorescein as the reporter dye. The TaqMan-GAPDH Control Reagents (Applied Biosystems) were used to amplify and detect the GAPDH gene, as recommended by the manufacturer. The quantitative assay amplified 1 μml of cDNA in two to four replicates using the primers and probes described above and the standard master mix (Applied Biosystems). All reactions were performed in an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). GAPDH, BMI-1, and p16INK4a expression was related to a standard curve derived from serial dilutions of Raji cDNA. The RUs of BMI-1 and p16INK4a expression were defined as the mRNA levels of these genes normalized to the GADPH expression level in each case.

Protein Analysis. Whole-cell protein extracts were obtained from additional frozen tissue available in 31 cases (7 CLLs, 12 MCLs, 8 FLs, and 4 LCLs), loaded onto a 10% SDS-polyacrylamide gel, and electroblotted to a nitrocellulose membrane (Amersham). Blocked membranes were incubated sequentially with the monoclonal antibody BMI-F6 (12), antimonocu-
gated to horseradish peroxidase (Amersham), and detected by enhanced chemiluminescence (Amersham) according to the manufacturer’s recommendations.

**Statistical Analysis.** Because of the non-normal distribution of the samples and the small size of some subsets of tumors, the statistical evaluation was performed using nonparametric tests (SPSS, version 9.0). Comparison between mRNA expression levels in the different groups of NHLs was performed using the Kruskal-Wallis Test, with a P for significance set at 0.05. For differences between particular groups, the conservative Bonferroni procedure was performed, and the P was set at 0.005. The remaining statistical analyses were carried out using the Mann-Whitney nonparametric U test, (P < 0.05). The comparison between BMI-1 and p16 INK4a quantitative mRNA levels was also performed using the Pearson’s correlation coefficient.

**Results**

**BMI-1 Gene Amplification.** The BMI-1 gene was examined by Southern blot in a large series of human tumors and normal samples (Table 1). The cDNA probe used in the study detected three EcoRI fragments of 7.3, 3.8, and 2.6 kb and three HindIII fragments of 6.2, 4, and 3.5 kb. BMI-1 gene amplification (3- to 7-fold) was detected in 4 of 36 (11%) MCLs (Fig. 1). The amplifications were confirmed with both restriction enzymes. The amplified MCLs were two blasticoid and two typical variants. No amplifications were observed in any of the solid tumors when compared with their respective matched non-neoplastic mucosa. No BMI-1 gene rearrangements were observed in any of the samples examined.

**BMI-1 mRNA Expression.** To determine the BMI-1 expression pattern in NHL, we analyzed BMI-1 mRNA levels by real-time quantitative RT-PCR in 67 lymphomas (10 CLLs, 27 MCLs, 8 FLs, and 22 LCLs), including the four tumors with gene amplification. A distinct BMI-1 mRNA expression pattern was observed in the different types of lymphomas (Fig. 2; Kruskal-Wallis Test; P < 0.001). The BMI mRNA levels in CLLs (mean, 2.2 RU; SD, 1.3) and MCLs with no BMI-1 gene amplification (mean, 2.5 RU; SD, 2.3) were significantly higher than in FLs (mean, 0.9 RU; SD, 0.8) and LCLs (mean, 0.6 RU; SD, 0.4; Mann-Whitney nonparametric U test; P < 0.01). The four MCLs with BMI-1 gene amplification showed significantly higher levels of expression than all other groups of tumors (mean, 5.1 RU; SD, 1.6; P < 0.005). In addition, five typical MCLs with no structural alterations of the gene also showed very high levels of BMI-1 mRNA expression ranging from 4 to 9.8 RU, similar to cases with gene amplification (Fig. 2A).

**BMI-1 Protein Expression.** BMI-1 protein expression was examined by Western blot in 31 tumors (7 CLLs; 12 MCLs, including two cases with BMI-1 gene amplification and 4 cases with mRNA overexpression and no structural alteration of the gene; 8 FLs, and 4 LCLs) in which additional frozen tissue was available. The monoclonal antibody against BMI-1 detected three closely migrating proteins of M, 45,000–48,000 (2). The two more slowly migrating bands probably represent phosphorylated isoforms of the protein (12). The two MCLs with gene amplification and three of four cases with mRNA overexpression without amplification of the gene showed very high levels of protein expression. The remaining MCLs and CLLs showed intermediate levels of protein expression, whereas low- or no-expression signals were detected in the LCLs and FLs included in the study (Fig. 3). These results indicate that BMI-1 protein expression in NHL is concordant with the mRNA levels observed by real-time quantitative RT-PCR.

**Relationship between BMI-1 and p16 INK4a Gene Alterations.** The INK4a/ARF locus has been recently identified as a downstream target of the transcriptional repressing activity of the BMI-1 gene, suggesting that this gene may contribute to human neoplasias with wild type INK4a/ARF (5). Most of the lymphoproliferative disorders analyzed in the present study, including the four cases with BMI-1 gene amplification, had been previously examined for p53 gene mutations and INK4a/ARF locus alterations, including gene deletions, mutations, hypermethylation, and expression (13, 14). The four MCLs with BMI-1 gene amplification and mRNA overexpression and the five tumors with BMI-1 mRNA overexpression with no structural alterations of the gene showed a wild-type configuration of the INK4a/ARF locus (13). However, one case with BMI-1 gene amplification and one case with mRNA overexpression with no alteration of the gene showed p53 gene mutations associated with allelic deletions.

To determine the possible relationship between BMI-1 and p16 INK4a mRNA expression, p16 INK4a mRNA levels were evaluated by real-time quantitative RT-PCR in 50 tumors (10 CLLs, 27 MCLs, and 13 LCLs), including 6 cases with alterations in the INK4a/ARF locus (2 MCLs and 1 LCL with p16 INK4a gene deletion, 2 LCLs with p16 promoter hypermethylation, and 1 LCL with p16 INK4a gene mutation), and the 4 lymphomas with BMI-1 amplification. Negative or negligible levels of p16 INK4a were observed in the 6 tumors with INK4a/ARF locus alterations. These cases were not included in the comparisons between BMI-1 and p16 INK4a mRNA expression. The p16 INK4a expression levels were relatively similar in the different types of tumors. Only LCLs tended to have lower levels of expression, but the differences did not reach statistical significance (Fig. 2B). No differences were observed in the p16 INK4a mRNA levels between tumors with BMI-1 gene amplification and overexpression and lymphomas with germline configuration of the gene.

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**Table 1 Hematological malignancies and solid tumor samples analyzed for BMI-1 gene alterations**

<table>
<thead>
<tr>
<th>Tissue samples</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematological malignancies</td>
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</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>2</td>
</tr>
<tr>
<td>B cell lymphoproliferative disorders</td>
<td>14</td>
</tr>
<tr>
<td>CLL</td>
<td>29</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
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</tr>
<tr>
<td>FL</td>
<td>15</td>
</tr>
<tr>
<td>MCL</td>
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</tr>
<tr>
<td>LCL</td>
<td>40</td>
</tr>
<tr>
<td>T cell lymphoproliferative disorders</td>
<td>8</td>
</tr>
<tr>
<td>T-Acute lymphoblastic leukemia</td>
<td>8</td>
</tr>
<tr>
<td>Large granular cell leukemia</td>
<td>4</td>
</tr>
<tr>
<td>Peripheral T-cell lymphoma</td>
<td>8</td>
</tr>
<tr>
<td>Myeloproliferative disorders</td>
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<tr>
<td>Acute myeloid leukemia</td>
<td>7</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>6</td>
</tr>
<tr>
<td>Solid tumors</td>
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<tr>
<td>Colon carcinoma</td>
<td>26</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>29</td>
</tr>
<tr>
<td>Laryngeal squamous cell carcinoma</td>
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</tr>
<tr>
<td>Total</td>
<td>262</td>
</tr>
</tbody>
</table>

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![Fig. 1. Southern blot analysis of BMI-1 gene. Four MCLs (MCL*) showed BMI-1 gene amplification (3- to 7-fold) compared with non-neoplastic tissues (N) and other NHLs. No amplifications or gene rearrangements were detected in the remaining NHLs and carcinomas included in the study.](image-url)
Discussion

In the present study, we have examined a large series of human tumors for the presence of gene alterations and mRNA expression of the BMI-1 gene. Gene amplification was identified in four MCLs. These tumors showed significantly higher levels of mRNA and protein expression compared with other lymphomas with BMI-1 in germ-line configuration. BMI-1 expression levels were also highly upregulated in a subset of MCLs with no apparent structural alterations of the gene. No alterations were detected in any of the different types of carcinomas included in the study. BMI-1 is considered an oncogene belonging to the Polycomb group family of genes. These proteins mainly act as transcriptional regulators, controlling specific target genes involved in development, cell differentiation, proliferation, and senescence. Different studies have shown the implication of BMI-1 overexpression in the development of lymphomas in murine and feline animal models (3, 4).

The findings of the present study indicate for the first time that BMI-1 gene alterations in human neoplasms are an uncommon phenomenon, but they seem to occur mainly in a subset of NHLs, particularly of mantle cell type.

The human BMI-1 gene has been mapped to chromosome 10p13. High-level DNA amplifications and gains in this region have been identified by comparative genomic hybridization in occasional solid tumors and NHLs (10, 11). Different chromosomal translocations involving the 10p13 region have also been identified in infant leukemias and T cell lymphoproliferative disorders (7, 8, 15). Most acute leukemias with this chromosomal alteration occur in children <12 months of age, whereas it seems to be extremely rare in adults. 10p translocations in T-cell lymphoproliferative disorders have been observed mainly in adult T cell leukemia/lymphomas and occasional cutaneous T cell lymphomas. In our study, we did not observe BMI-1 rearrangements or amplifications in any of the acute leukemias or T cell lymphomas. However, all of the acute leukemias in this study were diagnosed in patients over 16 years, and no adult T cell leukemia/lymphomas could be included in the series. Similarly, high-level DNA amplifications at the 10p13 region have been detected in head and neck carcinomas and other solid tumors. Although we found no evidence for BMI-1 gene rearrangements or amplifications in a substantial set of carcinomas, this does not exclude the possibility of increased gene expression or protein levels in these tumors. Additional studies are required to elucidate the possible involvement of BMI-1 in these particular groups of human neoplasms.

In human hematopoietic cells, BMI-1 is preferentially expressed in primitive CD34+ bone marrow cells, whereas it is negative or very low in more mature CD34+ cells (16). In peripheral lymphocytes, and particularly in follicular B cells, BMI-1 protein expression has been detected in resting cells of the mantle zone, whereas it is downregulated in proliferating germinal center cells (17, 18). These observations indicate that BMI-1 expression in normal hematopoietic cells is tightly regulated in relation with cell differentiation in bone marrow and antigen-specific response in peripheral lymphocytes. BMI-1 expression in human tumors has not been examined previously. In this study, we have demonstrated that BMI-1 mRNA and protein expression show a distinct pattern in different types of lymphomas. Thus, BMI-1 levels were low in LCLs and FLs and significantly higher in MCLs and CLLs. These findings suggest that BMI-1 expression patterns in B cell lymphomas maintain in part the expression profile of their normal cell counterparts; because FLs and at least a subgroup of LCLs are considered lymphomas derived from follicular germinal center cells, whereas MCLs and CLLs are tumors mainly derived from naive pregerminial center cells. However, the four MCLs with BMI-1 gene amplification expressed significantly higher mRNA levels than all other tumors. In addition, five MCLs with no structural alterations of the gene showed high mRNA levels similar to those observed in tumors with BMI-1 gene amplification, suggesting that other mechanisms may be involved in up-regulation of the gene in these lymphomas. Different studies using animal models have shown a dose-dependent effect of BMI-1 gene expression on skeletal development.

Fig. 2. A, quantitative BMI-1 mRNA transcript analysis (median and range) using real-time RT-PCR in a series of NHLs. MCLs with BMI-1 gene amplification (MCL*) revealed significantly higher overall BMI-1 mRNA levels than all other types of NHLs, including MCLs with no structural alterations of the gene (P < 0.005). MCLs and CLLs expressed significantly higher levels than FLs and LCLs (P < 0.001). Results are depicted as the ratio of absolute BMI-1:GADPH mRNA transcript numbers (RU). Bars, SD. B, quantitative p16INK4a mRNA transcript analysis (median and range) using real-time RT-PCR in a series of NHLs. Expression levels were relatively similar in the different types of tumors. Results are depicted as the ratio of absolute p16INK4a:GADPH mRNA transcript numbers (RU). Bars, SD.

Fig. 3. Western blot analysis of BMI-1 protein in NHLs. The amplified MCL (17624) showed the highest BMI-1 protein levels, whereas other MCLs and CLLs had intermediate levels of expression. Very low or negative signal was observed in FLs and LCLs.
and lymphomagenesis (1, 3). These observations suggest that the high mRNA and protein levels detected in a subset of MCLs may play a role in the pathogenesis of these neoplasms.

Recent studies have identified the INK4a/ARF locus as a downstream target of the BMI-1 transcriptional repressor activity, suggesting that BMI-1 overexpression may contribute to human neoplasias that retain the wild-type INK4a/ARF locus (5). Interestingly, in our study, BMI-1 amplification and overexpression appeared in tumors with no alterations in p16INK4a and p14ARF genes. However, we could not detect differences in the expression levels of p16INK4a in tumors with and without BMI-1 gene alterations. The reasons for this apparent discrepancy with experimental observations are not clear. One possibility may be that genes other than INK4a/ARF are the main targets of BMI-1 repressor activity in these tumors. Particularly, different genes of the HOX family are regulated by BMI-1 and may also be involved in lymphomagenesis (19, 20).

In conclusion, the findings of this study indicate that BMI-1 gene expression is differentially regulated in B cell lymphomas. Alterations of the gene seem to be an uncommon phenomenon in human neoplasms, but they may contribute to the pathogenesis in a subset of MCLs. Although, BMI-1 gene alterations occurred in tumors with wild-type INK4a/ARF locus, the possible cooperation between these genes and the oncogenic mechanisms of BMI-1 in human neoplasms require additional analysis.

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References

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