The Expression of the Bloom Syndrome Gene (BLM) in Two Cancer Lines

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Abstract

Bloom syndrome is an autosomal recessive disorder associated with a predisposition to a variety of cancers. The defective gene in Bloom Syndrome, BLM, is a member of the RecQ family of helicases and plays a role in DNA repair and recombination. This study focused on the expression of BLM in two cancer-derived cell lines, HeLa and LAI-5S. RT-PCR analysis revealed the lack of differential expression of the BLM mRNA within these two cell lines as compared to a control line, WI-38. However, variation in the level of the BLM protein was detected in Western blot analysis. The full-length BLM protein was absent from the HeLa and LAI-5S cell lines while a smaller, immunologically related protein was detected. Preliminary conclusions from these results suggest that alternative splicing of the BLM gene product may occur and the HeLa and LAI-5S cell lines may preferentially express the alternatively spliced protein.

Introduction

Bloom syndrome (BS) is an autosomal recessive disorder characterized by small stature, sun-sensitivity, immunodeficiency, male infertility, increased frequency of diabetes, and a predisposition to a variety of cancers at an early onset (1). Cells from affected individuals exhibit genomic instability including chromosome breakage, an excess of somatic mutations and elevated levels of sister chromatid exchanges (SCE’s)
Bloom syndrome occurs most frequently in the Ashkenazi Jewish population with patients almost exclusively homozygous for a frameshift mutation resulting from a 6 bp deletion/7 bp insertion at nucleotide 2,281 (BLM<sup>Ash</sup>). This mutation causes premature termination of the encoded gene product producing a truncated protein of 739 amino acids while the full length protein contains 1417 amino acids (2).

The mutated gene in Bloom syndrome, BLM, was localized to chromosome 15q26.1 and encodes a member of the RecQ family of DNA helicases (2). This family also contains several other genes that are associated with disease phenotypes including the Werner Syndrome protein (WRN) and the defective protein in Rothmund-Thomson syndrome (RecQL4) (3,4). Both of these diseases also feature an increased incidence of cancer. BLM, along with the rest of the members of this family, exhibits 3'-5' helicase activity and plays a role in DNA repair and recombination. BLM functions during replication stress and is required for the recruitment of several other important repair proteins including NBS1, BRCA1, Rad51 and MLH1 (5-7). In addition, the BLM helicase is involved in recombinational repair events as evidenced by its ability to promote branch migrations of Holliday junctions at stalled replication forks (8). BLM may also play a role in apoptosis since it directly interacts with p53 and helps regulate its transcriptional activity (9,10).

RecQ helicases, including BLM, facilitate the maintenance of genomic stability and influence the rate of genetic alterations, indirectly regulating the incidence of cancer. The elevated frequency of various types of cancer in individuals with Bloom syndrome prompted a study to determine if certain cancer cell lines exhibit differential expression of BLM mRNA and protein. The cell lines studied, HeLa and LAI-5S, are cancer-
derived lines that were obtained from cervical carcinoma and neuroblastoma, respectively.

**Materials and Methods**

**Cell lines**

WI-38 and HeLa cell lines were provided by Dr. Berish Rubin and the LAI-5S cell line was supplied by Dr. Robert Ross. All three cell lines were cultured in MEM with 10% fetal calf sera, 10 IU/mL penicillin and 10µg/mL streptomycin and were incubated at 37ºC with 5% CO2 and humidity.

**Antibodies**

The polyclonal anti-BLM antibody was purchased from Calbiochem. The antibody was raised in rabbits and was generated against the protein encoded in exon 22 of BLM. An anti-GAPDH antibody was obtained from Ambion.

**Primers**

Primers specific for BLM were purchased from Invitrogen and were designed to span three large introns in order to prevent genomic DNA contamination. The forward primer (BLM-F), located in exon 19, was 5’-GTCACCTCTCAAGAAGCTTGC-3’ (nucleotides 3804-3823 of Accession number NM_000057) and the reverse primer (BLM-R), located in exon 22, was 5’-AGGCTGAACTGGATCAATG-3’ (nucleotides 4221-4202 of Accession number NM_000057). The primers were expected to generate a 418bp product from the cDNA and a product greater than 10kb from genomic DNA. Primers specific for β-actin were used to ascertain whether equivalent amounts of RNA were introduced into RT-PCR reactions. For the amplification of β-actin, the following primers were employed: 5’-CTGACTGACTACCTCATGAAG-3’ (nucleotides 620-640
of Accession number BC001301) 5’-GAGGAGCAATGATCTTGA TCT-3’ (nucleotides 1064-1044 of Accession number BC001301). These primers generated a product of 423bp from the cDNA. All primers were diluted to a concentration of 10pmol/µl.

**RNA extraction**

Total RNA was extracted from the three cell lines using the RNAqueous Phenol-Free Total RNA Isolation Kit (Ambion) according to the manufacturer’s protocol. The RNA concentration was determined and RNAs were stored at -80°C.

**RT-PCR**

RT-PCR was performed on the RNAs prepared from the normal and cancer cell lines using the Qiagen One-Step RT-PCR Kit. Added to each reaction was 3µl 5X RT buffer, 0.6µl 10mM dNTPs, 0.75µl primer (10pmol/µl), 0.6µl enzyme mix, 3µl RNA template (with a concentration of 20ng/µl) and 6.3 µl dH₂O for a final volume of 15µl. The RT-PCR amplification program used was as follows: 50°C x 30 min and 95°C x 15 min for one cycle, followed by amplification at 94°C x 30 sec, 56°C x 30 sec and 72°C x 30 sec for 28 cycles for BLM and 19 cycles for β-actin. A final extension at 72°C x 5 min was used. PCR products were analyzed on a 1% agarose gel run at 140 volts.

**PCR purification**

PCR products were purified using the Concert Rapid PCR Purification System (Marligen Biosciences, Inc) according to the manufacturer’s instructions. Products were eluted from the column in 30µL dH₂O.

**Sequencing**

Purified PCR products were sequenced by the Sanger dideoxy method using the AmpliCycle Sequencing kit (Applied Biosystems). For each reaction, 50fmol of PCR
product was mixed with 4µl of 10X cycling buffer, 2µl of primer, 0.2µl of \( \alpha^{32}P \)ATP and dH₂O to a final volume of 30µl. 6µl of this master mix was added to four tubes containing 2µl of either ddGTP, ddATP, ddTTP or dCTP. All of the reaction mixtures were overlaid with 12µl of mineral oil to prevent evaporation. These reactions were set up using both the forward and reverse BLM primers. The sequencing program employed was as follows: 94ºC x 2 min, followed by 35 cycles of 94ºC x 30 sec, 58ºC x 30 sec and 72ºC x 1 min. After the completion of the reactions, 4µl of Stop Solution was added to each tube and the samples were denatured by heating for 3 min. The products were electrophoresed on a denaturing polyacrylamide gel and the nucleotide sequence was visualized by autoradiography. The sequence obtained was aligned with the BLM mRNA sequence listed in GenBank (NM_000057).

**Protein purification**

Total cell protein was extracted from each of the cell lines by washing cell cultures twice with PBS and then detaching the cells from the flask using EDTA-trypsin. The cultures were centrifuged to pellet down the cells and the media was removed. Cell lysis buffer (25mM Tris-phosphate, 2mM DTT, 1mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 1% TritonX-100) was added to the cell pellet at a volume of 100µL per 1 x 10⁵ cells. The Bio-Rad Protein Assay Kit was used to measure the concentration of the protein in the extracts following the manufacturer’s protocol. Bovine serum albumin was used for the standard curve.

**Western blot analysis**

Proteins extracted from WI-38, HeLa and LAI-5S cells were diluted 1:1 with 2X SDS sample buffer and denatured by boiling for 2 minutes. Equivalent amounts of
protein (45µg) were fractionated by SDS-PAGE on an 8% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane for 1 hour at 100 volts at 4°C. The membrane was then cut in half to separate the regions containing the BLM protein and GAPDH. Nonspecific binding of the antibodies was prevented by incubating the nitrocellulose in a blocking buffer containing 5% non-fat dry milk in TBST for 1 hour before adding the primary antibodies. The blot containing BLM was probed overnight with a polyclonal rabbit anti-BLM antibody (0.8µg/mL; Calbiochem). The blot was then washed with TBST and probed with an anti-rabbit antibody conjugated to alkaline phosphatase (Promega) for 2 hours. The nitrocellulose was washed again with TBST and the presence of alkaline phosphatase was detected using Western Blue Substrate Solution (Promega). As a loading control, the second half of the blot was probed for GAPDH using a mouse anti-GAPDH antibody (4µg/µl; Ambion) following the above procedure.

**Results**

The high incidence of cancer in individuals affected by Bloom syndrome prompted an examination of the levels of BLM transcript in two different cancer cell lines. RT-PCR was performed on RNA isolated from HeLa and LAI-S RNA as well as RNA isolated from WI-38 cells to serve as a control for the normal expression of BLM. Primers were designed to preferentially amplify the BLM cDNA using sequences from exons, separated by large introns. RT-PCR analysis revealed no significant differences in expression among these cell lines (Fig.1A). The predicted product of 418bp was present in all three cell lines. RT-PCR was also performed on these RNA preparations using primers for β-actin to serve as a control for RNA concentration. All three cell lines appear to contain comparable amounts of RNA (Fig.1B).
Figure 1. RT-PCR analysis of BLM mRNA in WI-38, HeLa and LAI-5S cell lines. 
A.) RT-PCR products generated with primers located in exons 19 (nucleotides 3804-3823 of Accession number NM_000057) and 22 (nucleotides 4221-4202 of Accession number NM_000057) was performed using 60ng of WI-38, HeLa and LAI-5S total RNA. The first three lanes on the left show RT-PCR performed with an amplification step at 28 cycles and the last three lanes at 31 cycles. Lane (-) is a non-template control. The position and size of BLM is indicated by an arrow. A 100bp ladder was run on the gel for size comparison. 
B.) RT-PCR products generated with primers specific for β-actin (F: nucleotides 620-640 of Accession number BC001301; R: nucleotides 1064-1044 of Accession number BC001301) using 19 cycles of amplification. 60ng of RNA isolated from each of the cell lines was used. Lane (-) is a non-template control.

BLM RT-PCR products were purified and sequenced in order to confirm that the BLM primers amplified the expected BLM mRNA sequence. Sequencing revealed a portion of the BLM mRNA located in exon 21 between the BLM forward and reverse primers, matching identically to the mRNA sequence listed in GenBank (Fig. 2).
**Figure 2. Sequence alignment of BLM.** The sequenced BLM PCR product (BLM pr. BLM-R) was aligned with the BLM mRNA sequence from GenBank (nucleotides 3981-4141 of NM_000057).

To examine the level of BLM protein, Western blot analysis was performed using a polyclonal antibody that recognizes the ~170 kD BLM protein. Proteins extracted from the WI-38, HeLa and LAI-5S cell lines were analyzed. The blot reveals the presence of the full-length BLM protein in the wild-type cell line (WI-38), with the expected size of ~170 kD, but this molecular weight protein is absent in the two cancer cell lines (HeLa and LAI-5S) (Fig. 3A). In addition, all three cell lines appear to contain an immunologically related protein of ~60kD. This smaller protein produces a stronger signal in the HeLa and LAI-5S cell lines as compared to the WI-38 cell line. The blot was also probed with an anti-GAPDH antibody which confirmed that equal amounts of protein were loaded onto the gel (Fig. 3B).
Figure 3. Western Blot analysis of the BLM protein. (A) Equivalent amounts of total cell protein (45µg) was loaded onto an 8% SDS polyacrylamide gel and transferred to nitrocellulose. A polyclonal anti-BLM antibody was used to probe the nitrocellulose. The position and size of the proteins recognized by the antibody is indicated with arrows. A prestained molecular weight marker (Bio Rad) was used to determine the sizes of the products. (B) The blot was also probed with an anti-GAPDH antibody to confirm equal protein loading. The position and size of GAPDH is indicated.

Discussion

These studies revealed that the WI-38, HeLa and LAI-5S cell lines do not exhibit differential expression of the BLM mRNA; however, there appears to be variation in the expression of the protein. RT-PCR analysis showed equivalent amounts of mRNA expression in each of the three lines. DNA sequencing analysis confirmed that the RT-PCR product amplified was, in fact, a portion of the BLM mRNA sequence. Finally, Western blot analysis revealed the presence of the full-length BLM protein in the WI-38 cells but not in the HeLa or LAI-5S cells. In addition, a smaller, immunologically-related protein of ~ 60kD was observed in all three cell lines but appears to be expressed to a greater extent in the two cancer cell lines. It is possible that the smaller molecular weight protein represents an alternatively spliced product of the BLM gene.
The expression pattern of both the BLM and the smaller molecular weight protein lead us to speculate that the cancer cell lines may exhibit a higher degree of alternative splicing of the BLM gene product. The HeLa and LAI-5S cells lack expression of the full-length BLM protein, demonstrating the presence of only the immunologically-related protein at ~60kD. In addition, the WI-38 cells express both of these proteins but the smaller molecular weight protein appears to be expressed to a lesser extent as compared to the BLM protein and also relative to the HeLa and LAI-5S cells. This observation further supports the evidence for increased alternative splicing of the BLM protein in the cancer-derived cell lines. Several studies have shown that cancer-specific alternative splicing exists in the absence of genomic mutations due to alterations in the amount of transcription of splicing factors. For instance, Mathioudaki et al. recently demonstrated the overexpression of SR-A1, a member of the family of SR proteins essential for pre-mRNA splicing, in colon cancer tissues (11). In addition, He et al. documented the overexpression of two splicing factors, PTB and SRp20, in human ovarian tumors as compared to normal ovarian tissue. Significantly, the overexpression of both of these factors was associated with the occurrence of an increased number of multidrug resistance protein 1 (MRP1) splice variants in the ovarian tumor tissues (12). Because alternative splicing has a strong role in the development of cancer, splicing events can be seen as a potential target in the search for a possible therapy for cancer patients.

Additional studies should focus on characterizing the smaller molecular weight protein in order to determine if alternative splicing of the BLM gene product occurs. It is possible that this protein is unrelated to the BLM protein but it awaits further analysis using a monoclonal antibody in order to determine if it is specifically associated with
BLM. Furthermore, more detailed experiments examining other cancer-derived cell lines would help ascertain if a relationship exists between the expression of BLM and tumorigenesis.

Acknowledgements

I would like to thank Dr. Rubin for his guidance and the opportunity to pursue this project along with Brian Fox and Jinsong Qiu for their extreme patience and time. I would also like to acknowledge Dr. Robert Ross for generously providing LAI-5S cells.
References


