RESULTS
IV. RESULTS

(A) Characterization of BS cells

(B) Status and regulation of pyruvate kinase (PK) in BS and normal (GA3) cells

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      a) Optimization of PCR conditions and amplification of active site region.
      b) Cloning, SSCP and sequencing of active site region.

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      a) Optimization of PCR conditions and amplification of ISCD region.
      b) Cloning, SSCP and sequencing of ISCD region.

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      a) Optimization of PCR conditions and amplification of IDSB region.
      b) Cloning, SSCP and sequencing of IDSB region.

(C) Structural analysis of pyruvate kinase protein

(D) Amino acid sequence alignment

(E) Accessible surface area calculations
RESULTS

A) Characterization of BS cells:

One of the characteristic feature of Bloom syndrome cells, which distinguishes it from other human syndromes especially, chromosomal instability syndromes, is the presence of a very high rate of sister chromatid exchanges (SCEs) in the chromosomes. Cytogenetic studies were carried out to establish that the cells used in this study belonged to BS patients. The rate of SCEs in normal cells (GA3) used as control, (Fig 7a) was 2.79 with standard error value of 0.55. Cytogenetic analysis in the two cell lines BS1 and BS3 and freshly isolated lymphocytes of the BS patient showed high SCEs (Fig 7b) with mean SCE per cell being 68.2, 77.0 and 72.7, respectively (Tab 11). The standard error calculated from Sigma Stat program was 2.15, 2.25 and 3.25, respectively. The SCE values ranged from 45-80 in case of BS cell lines and 27 per plate to 125 per plate in the BS patient. Chromosomal abnormalities like breaks; deletions, triradials, quadriradials and dicentric chromosomes were also observed in the BS samples. The high SCE character of the cells established the Bloom syndrome source of the cells used in this study (Tab 11).

B) Status and regulation of pyruvate kinase (PK) in BS and normal cells:

The status and regulation of pyruvate kinase (PK) was analysed in two B-lymphoblastoid cell lines: BS1 and BS3, established from two different BS patients; and in freshly obtained lymphocytes from a patient diagnosed for the first time in India. Normal lymphoblastoid cell line(s) (GA3) was used as a control. Experiments were carried out to study the status of PK in BS cells and to find out if PK could be considered, in addition to the known blm (RecQ helicase) gene in BS, playing a role in causing the disease in this syndrome.
Fig 7: a) Chromosome plate showing normal SCEs from a healthy individual.

b) Chromosome plate showing high SCEs in Bloom syndrome patient. Dark and light colored chromatids are unifiliarly and bifiliarly substituted with bromodeoxyuridine.
Fig 7
# Table 11: Sister Chromatid Exchange (SCE) Analysis of Normal and BS Samples

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>CHROMOSOME PLATES</th>
<th>SCE±S.E</th>
<th>ADDITIONAL ABNORMALITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1</td>
<td>54</td>
<td>68.2±2.15</td>
<td>Quadriradials and breaks were seen</td>
</tr>
<tr>
<td>BS3</td>
<td>48</td>
<td>77.0±2.25</td>
<td>Quadriradials and breaks were seen</td>
</tr>
<tr>
<td>BS patient</td>
<td>45</td>
<td>72.7 ±3.25</td>
<td>Quadriradials, triradials and breaks were seen.</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>2.79±0.55</td>
<td>No abnormalities seen</td>
</tr>
</tbody>
</table>
i) Status of pyruvate kinase in BS and normal cells

The pyruvate kinase enzyme assays were carried out with whole cell extracts of the two BS cell lines: BS1, BS3; the freshly obtained lymphocytes from a BS patient; and the normal cells. Results showed that the specific activity of pyruvate kinase was low in both the BS cell lines and also in the patient when compared to its specific activity in normal (GA3) cells. The specific activity was low in Bloom syndrome cells by about 50% in BS1, 90% in BS3 and 75% in the BS patient when compared to the specific activity observed in the normal cell line (Fig 8, Tab 12).

To find out if the down regulated enzyme activity in BS cells was due to cell cycle stage differences among Bloom syndrome and normal cell lines, cells from one of the Bloom syndrome cell line (BS1) and normal cell line (GA3) were serum deprived and replenished after 30 hrs. Enzyme assay studies were carried out in serum deprived and serum replenished cells at different times. A three times increase in PK activity was observed in normal cells after 24hrs of serum replenishment. However, the Bloom syndrome, BS1, cells showed lower activity at all times of measurement. In serum-deprived state, the activity in BS1 cells was low by 63%, and after 1hr, 3hr, 24hrs of serum replenishment it was low by 25%, 33% and 60%, respectively (Fig 9, Tab 13).

ii) Kinetics of pyruvate kinase

The nature of inhibition of the enzyme activity of pyruvate kinase in Bloom syndrome B-lymphoblastoid cells was analyzed by kinetic studies. Kinetics of the enzyme carried out with different concentrations of substrate phosphoenol pyruvate (PEP) showed low enzyme activity in BS1 and BS3 cells at all PEP concentrations used (Tab 14). The activity was lower in BS3 cells at all the substrate concentrations than BS1 cells. Vmax values were calculated from the intercepts on Y-axis from double reciprocal plots (Fig 10). The value was highest in normal cells followed by BS1 and then BS3 cells. Km values calculated from Linewaever-Burk plot showed lower values for BS3 and BS1 cells when compared to normal cell Km value. The Km value of BS1 was lower than BS3 cells. Both the Km and Vmax
8: Comparison of pyruvate kinase activity in normal and Bloom syndrome cells. The specific activity is the mean of three separate experiments.
TABLE 12: COMPARISON OF PYRUVATE KINASE ACTIVITY IN NORMAL AND IN DIFFERENT BLOOM SYNDROME CELLS

<table>
<thead>
<tr>
<th>CELL LINES / CELLS</th>
<th>SPECIFIC ACTIVITY (mu/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>112.15±13.8</td>
</tr>
<tr>
<td>BS1</td>
<td>62.78±10.07</td>
</tr>
<tr>
<td>BS3</td>
<td>16.31±6.33</td>
</tr>
<tr>
<td>BS patient</td>
<td>27.65±8.53</td>
</tr>
</tbody>
</table>
Fig 9: Pyruvate kinase activity in serum deprived and at different times after serum replenishment. 0hr represents activity measured after 30hrs of serum deprivation and 1, 3, 24hr represents activities measured after serum replenishment.
TABLE 13: PYRUVATE KINASE ACTIVITY IN BS AND NORMAL CELLS DURING CELL CYCLE PROGRESSION

<table>
<thead>
<tr>
<th>TIME</th>
<th>PK specific activity (mu/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BS1</td>
</tr>
<tr>
<td>30hrs SD</td>
<td>27.55</td>
</tr>
<tr>
<td>1hr SR</td>
<td>34.57</td>
</tr>
<tr>
<td>3hr SR</td>
<td>44.12</td>
</tr>
<tr>
<td>24hr SR</td>
<td>84.83</td>
</tr>
</tbody>
</table>

'SD' represents specific activity measured after 30hrs of serum deprivation and 'SR' represents specific activity measured after 1, 3 and 24hrs after serum supplementation.
### TABLE 14: KINETICS OF PK IN BS AND NORMAL CELLS WITH DIFFERENT PEP CONCENTRATIONS

<table>
<thead>
<tr>
<th>PEP (mM)</th>
<th>ENZYME ACTIVITY (µu/mg) (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BS1</td>
</tr>
<tr>
<td>0.0125</td>
<td>4.75</td>
</tr>
<tr>
<td>0.0625</td>
<td>6.89</td>
</tr>
<tr>
<td>0.125</td>
<td>10.34</td>
</tr>
<tr>
<td>0.25</td>
<td>9.65</td>
</tr>
<tr>
<td>0.3125</td>
<td>12.7</td>
</tr>
<tr>
<td>0.625</td>
<td>30.34</td>
</tr>
<tr>
<td>0.9375</td>
<td>24.82</td>
</tr>
<tr>
<td>1.25</td>
<td>30.3</td>
</tr>
<tr>
<td>2.5</td>
<td>24.13</td>
</tr>
</tbody>
</table>
Fig 10: Kinetics of pyruvate kinase with different substrate concentrations. Reciprocals of substrate concentration is taken on X-axis (1/S) and reciprocals of enzyme activity is taken on Y-axis (1/V). Km and Vmax values are calculated from the intercepts on X-axis and Y-axis (in box).
TABLE

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>Vmax (mM/min)</th>
<th>Km (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA3</td>
<td>22.2</td>
<td>0.073</td>
</tr>
<tr>
<td>BS1</td>
<td>20.0</td>
<td>0.033</td>
</tr>
<tr>
<td>BS3</td>
<td>17.3</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Fig 10
values of pyruvate kinase were different for BS1, BS3 cells when compared with normal cell pyruvate kinase. This indicated that enzyme in BS cells was uncompetitively inhibited.

Possibility of allosteric factors down regulating the enzyme was ruled out as these factors were added to saturation during the enzyme assays. Other possibilities of down regulation due to single amino acid substitutions was analyzed and important regions of PK-M gene were screened.

iii) Molecular analysis of the active site (Exon-6) region of the PK-M gene

Primers were designed for active site region located in exon-6 of PK-M gene. The specificity was checked by blast analysis. The amplicons generated through PCR for the active site region of PK-M gene were subjected to single stranded conformational polymorphism (SSCP) to detect single base changes (mutations) after cloning and sequencing of the variant DNA bands.

a) Optimization of PCR conditions and amplification of the active site (exon-6) region

The specificity of the primers designed for the active site region of PK-M gene checked by blast analysis was used for amplifying, using different magnesium chloride (1.0mM, 1.5mM and 2.0mM) and target DNA concentrations (25ng and 50ng). The results showed that out of the various possible combinations, 1.0mM magnesium chloride and 25ng DNA was optimal in obtaining required efficiency and expected product size (Fig 11a).

The products when run on 1% agarose gels gave 309bp band with the DNA of Bloom syndrome cell lines BS1 and BS3, patient and normal cell line (Fig 11b). There was no difference in the product size between the two BS cell lines: (BS1 and BS3), the BS patient cells and the normal cell line studied. In order to resolve single base changes (mutations) in the amplified active site region of PK-M gene, single stranded conformational polymorphism (SSCP) analysis was carried out after cloning the PCR products.
Fig 11: a) Amplification of the active site region of PK-M gene using different magnesium chloride and target DNA concentrations. 1 and 2 represents amplifications using 25ng and 50ng target DNA concentrations. 1.0mM, 1.5mM and 2.0mM magnesium chloride concentrations are used for standardization. Lane 'M' on the left hand corner represents 100bp ladder used as marker.

b) Amplification of the active site region of PK-M. Lanes represent amplicons from Bloom syndrome cell lines (BS1, BS3), patient (PT) and normal (N) cells respectively. Lane M on the left hand side represents 100bp ladder used as marker. The amplified product of 309 bp is shown on the right hand side with an arrow.
AMPLIFICATION OF EXON-6 OF PYRUVATE KINASE GENE

Intron-5
Forward primer →
ANI-1U (2400-2419)
TTT ATA CAG GTG CCG ACT TC

Exon-6

Intron-6
Reverse primer ←
ANI-3L (2690-2708)
GCA GTA GAC CAG GGA CAA C

PCR amplification

309 bp PCR product

Ligated PCR product to pMOS-T vector

Hind III

EcoR I

LIGATED PRODUCT

Fig 11
b) Cloning, SSCP and sequencing analysis of the active site products

The amplified active site products from the DNA of BS1, BS3, the BS patient and normal cells were eluted from 1% agarose gels and ligated using pMOS blue T-vector kit. The ligated products were transformed in DH5α and XL-blue host cells. Colonies obtained were checked for inserts by PCR analysis of the single colony DNA using active site primers. SSCP analysis of the positive (in which insert was present) colonies showed no difference in mobility of single strand bands in BS cells when compared to normal cells (Fig 12).

Sequencing carried out with the BS1, BS3, BS patient and the normal-cloned PCR products, using T7 primer, showed no mutations in the active site region of the two BS (BS1 and BS3) cell lines and in the BS patient studied (Fig 13). The sequence of 'AAA' (complementary sequence is 'TTT') representing Lysine-269 was intact in both the BS cell lines and in the BS patient.

iv) Molecular analysis of inter sub-unit contact domain (ISCD) (Exon-10) region of the PK-M gene

Primers were designed for ISCD region located in exon-10 of PK-M gene. The specificity was checked by blast analysis. The amplicons generated through PCR for the ISCD region of PK-M gene were subjected to single stranded conformational polymorphism (SSCP) to detect single base changes (mutations) after cloning and sequencing the variant DNA bands.

a) Optimization of PCR conditions and amplification of ISCD (exon-10) region

The specificity of the primers designed for the ISCD region of PK-M gene checked by blast analysis was used for amplifying by using different
SSCP analysis of the active site region of PK-M gene. Lanes represent BS1, BS3, BS Patient (PT) and normal (N) clones used for analyses. 'SS' represents single strand bands in each case.
Fig 13: A partial sequence (automated) showing the active site region of BS1, BS3 and the BS patient. The sequence of 'TTT' representing Lys-269 is intact in all the three BS cells. The sequences are in antisense orientation.
Fig 13
magnesium chloride (1.0mM, 1.5mM and 2.0mM) and target DNA concentrations (25ng and 50ng). The results showed that out of the various possible combinations, 1.0mM magnesium chloride and 25ng DNA was optimal in obtaining required efficiency and expected product size (Fig 14a). The products gave 240bp band with the DNA from BS1, BS3, BS patient and the normal cells when run on 1% agarose gels (Fig 14b). There was no difference in the product size between the two BS cell lines (BS1 and BS3), BS patient and normal cell line studied. In order to resolve single base changes (mutations) in the amplified ISCD region of PK-M2 gene, SSCP analysis was carried out after cloning the PCR products.

b) Cloning, SSCP and sequencing analysis of ISCD region

The amplified ISCD product of the PK-M gene from the DNA of BS1, BS3, BS patient and normal cells were eluted form 1% agarose gels and ligated using pMOS blue T-vector kit. The ligated products were transformed in DH5α and XL-blue host cells. Colonies obtained were checked for inserts by PCR analysis of the single colony DNA using ISCD primers. SSCP analysis of positive (in which insert was present) colonies showed differences in mobilities between the two single strands of DNA in some colonies within BS1, BS3, the BS patient and the normal (Fig 15).

Analysis of the cloned ISCD-PCR product from the BS1 cell line showed 60% of the clones with normal and 40% clones with variant single strand bands. In BS3 only variant bands were obtained when compared to the single strand bands in the ISCD clones from normal cell DNA. In case of the BS patient screening of 40 clones gave one variant clone along with the remaining normal clones. The mobility shifts of single strand bands were different in the ISCD clones obtained in the BS1, BS3 and the BS patient when compared with the normal (Fig 16). Further, SSCP analysis was carried out with 28 normal DNA samples collected from the volunteers belonging to the different parts of India to establish that the mutations observed in
Fig 14: a) Amplification of the ISCD (exon-10) region of PK-M gene using different Magnesium chloride and target DNA concentrations. 1 and 2 represent amplifications using 25ng and 50ng target DNA concentrations. 1.0mM, 1.5mM and 2.0mM magnesium chloride are used for standardization. Lane 'M' on the left hand corner represents 100 bp ladder used as marker.

b) Amplification of the ISCD region of PK-M2. Lanes represent amplicons generated from Bloom syndrome cell lines (BS1, BS3), BS patient (PT) and normal (N) cells respectively. Lane M on the left hand side represents 100 bp ladder used as marker. The ISCD product of 240 bp is shown on right with an arrow.
AMPLIFICATION OF EXON-10 OF PYRUVATE KINASE GENE

Intron-9
Forward primer: ANI-5U (7365-7384)
CGT CCC TCT GGA CGG ATG TT

Exon-10
Reverse primer: ANI-5L (7585-7604)
ACC ACT GAG CAG GGC ATT CC

PCR amplification

240 bp PCR product
Ligated PCR product to pMOS-T vector

Hind III
EcoRI

LIGATED PRODUCT

Fig 14
Fig 15:  SSCP analysis of different clones obtained from BS1, BS3 and BS patient (PT) cloned and transformed PCR products. a) Clones of BS1 showing mobility shifts in single strands (ss) of the double stranded DNA in lanes 2 and 5 and normal bands in 1, 3 and 4. b) Clone of BS3 showing mobility shift in lane 3 along with normal ISCD products in lanes 1, 2 and 4. c) Clones of BS patient showing variant clone in lane 4 and normal clones in lanes 1, 2, 3 and 5.
Fig 16: SSCP analysis of the ISCD region of PK-M gene. Lanes represent variant clones obtained from BS1, BS3 the BS patient (PT) and the normal (N) cells.
BS cells are not observed in normal individuals and also do not show up as normal polymorphisms (Fig 17).

Manual as well as automated sequencing of the variant clones was performed with T7 promoter primer. The variant clones from BS1, BS3 and the BS patient showed mutations at different positions in the ISCD region. The mutated sequences were deposited in the NCBI database with the following Accession numbers: BS1-AF185280, BS3-AF157693, BS Patient-AF242584 and normal cell line; GA3-AF157692.

In BS1 there was a substitution from 'C' to 'T' at nucleotide position 7425 in the genomic sequence (Fig.18a), a silent mutation was also found in BS1 which was a 'G' to 'A' polymorphism at nucleotide position 7442 (Fig 18b). The missense mutation lead to change in amino acid residue from normal histidine to mutated tyrosine at amino acid position 391, the silent mutation found in BS1 did not lead to any change in amino acid sequence and coded for the same glutamic acid residue at 396 position (Fig 18c). The normal clones from BS1 in-turn did not show any mutation in the ISCD region.

The sequence of variant clone from BS3 showed a frame shift mutation. It was a deletion of 'C' in a repetition of four 'C's at nucleotide position 7475-7478 (Fig 19a) leading to substitution of amino acids from 408 to 427 which prematurely terminated the protein at 427 instead of normal 531 amino acids (Fig 19b).

The sequence of the variant clone from patient showed substitution at nucleotide position 7518 from 'T' to 'C' (Fig 20a). This missense mutation lead to change in amino acid residue from lysine to arginine at 421 (Fig 20b).

All the mutations were further confirmed after by automated sequencing (done commercially), from both T7 primer and M13 primer ends using AB1 Prism automated sequencer (Fig 21).
Fig 17: SSCP analyses of the ISCD (exon-10) region of PK gene of randomly chosen normal samples from different parts of India. Representative samples analyzed from: a) Uttar Pradesh, b) Andhra Pradesh, c) Punjab and d) West Bengal. Lane numbers indicate the number of samples analyzed from each state. 'SS' = single strand bands.
Fig 17
Fig 18a: a) Manual sequencing of BS1. Sequencing was carried out using T7 primer for five hours. Arrow represents $G \rightarrow A$ mutation. Lanes G, A, T and C are marked.
Fig 18a
Fig 18 b): Manual sequencing of ISCD region of PK-M2 gene in BS1 cells showing mutation and polymorphism. Sequencing was carried out using T7 primer and run was carried for 2 and half hours. Lanes G, A, T and C are marked. Arrows indicate mutation from C→T and polymorphism from G→A.
G A T C

C → T (Mutation)

G → A (Polymorphism)

Fig 18 b
Fig 18 c): Sequence of normal (above) and the mutated in BS1 (below) showing the exon-10 region of PK-M gene flanked by the intron-9 and intron-10 regions where primers were designed for amplifying exon-10 region. Underlined sequences represent the regions where nucleotide substitutions were seen. Filled star represents the missense mutation observed in BS1 at amino acid residue 391 and unfilled star represents the polymorphism observed at amino acid residue 396.
CGTCCCTCTGGAGGATGGTCCCTCCCTAGATGGCCCGTGAGGCAGAGCTGCCATCT
Forward primer  I A R E A E A A I

ACCACTTGCAATTATTTGAGGAACCTCCGGCCGCTGGCGCCCATATCCAGCGACC
Y H L Q L F E E L R R L A P I T S D
391 396

CCACAGAGCCACCCCGGCTGGTTGCGGCTGGAGGCCTCTCCTTCAATGTGCCTGCAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAATCGTCCTCAACCAAGTCTGGCAGGTAAAGAGCAGCCGGAGCTCCCTGG
G A I I V L T K S G R

AATGCCCTGCTCAGTGTT
Reverse primer

CGTCCCTCTGGAGGATGGTCCCTCCCTAGATGGCCCGTGAGGCAGAGCTGCCATCT
Forward primer  I A R E A E A A I

★
★

ACTACTTGCAATTATTTGAGGAACCTCCGGCCGCTGGCGCCCATATCCAGCGACC
Y Y L Q L F E E L R R L A P I T S D
391 396

CCACAGAGCCACCCCGGCTGGTTGCGGCTGGAGGCCTCTCCTTCAATGTGCCTGCAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAATCGTCCTCAACCAAGTCTGGCAGGTAAAGAGCAGCCGGAGCTCCCTGG
G A I I V L T K S G R

AATGCCCTGCTCAGTGTT
Reverse primer

Fig 18c
Fig 19a: Manual sequencing of the ISCD region (Exon-10) of BS3 showing a deletion. Sequencing was carried out using T7 primer for 5 hours. Lanes G, A, T and C are marked. Arrow indicates deletion of G in repetition of four G's.
Deletion of G

Fig 19 a
**Fig 19b:** Sequence of normal (above) and the mutated in BS3 (below) showing the exon-10 region of PK-M gene flanked by the intron-9 and intron-10 regions where primers were designed for amplifying exon-10 region. Underlined sequence represent the regions where nucleotide deletion was seen. Filled star represents the frameshift mutation observed in BS3 at amino acid residue 407. The premature termination codon 'TAA' is underlined.
CGTCCCTCTGGACGGATGTTGCTCCCCTAGATTGCCCGTGAGGCAGAGctGCCATCT
Forward primer

ACCACCTTGCAATTATTATTTGAGGAACCCCTGCCCTGCGCCTGCCGCCATTTACCAGCGAC
Y H L Q L F E E L R R L A P I T S D

CCACAGAAAGCCACCGCCGTGGGTGCGTGAGGCTCCCTTCAAGTGTGCTGAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAAATCGTCTCTCACCAGTCTGTGCAAGTAAGAAGCAGCGCAGGGGTGCCTQG
G A I I V L T K S G R

AATGCCCTGCTCAGTGTT
Reverse primer

CGTCCCTCTGGACGGATGTTGCTCCCCTAGATTGCCCGTGAGGCAGAGCTGCCATCT
Forward primer

ACCACCTTGCAATTATTATTTGAGGAACCCCTGCCCTGCGCCTGCCGCCATTTACCAGCGAC
Y H L Q L F E E L R R L A P I T S D

CCACAGAAAGCCACCGCCGTGGGTGCGTGAGGCTCCCTTCAAGTGTGCTGAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAAATCGTCTCTCACCAGTCTGTGCAAGTAAGAAGCAGCGCAGGGGTGCCTQG
G A I I V L T K S G R

AATGCCCTGCTCAGTGTT
Reverse primer

Fig 19b
Fig 20a: Manual sequencing of the ISCD region (Exon-10) of the BS patient. Sequencing was carried out using T7 primer for 3 hours. Lanes G, A, T and C are marked. Arrow indicates mutation from T→ C.
Fig 20a

G A T C

T → C (Mutation)
Fig 20b: Sequence of normal (above) and the mutated in BS patient (below) showing the exon-10 region of PK-M gene flanked by the intron-9 and intron-10 regions where primers were designed for amplifying exon-10 region. Underlined sequence represent the regions where nucleotide substitution was seen. Filled star represents the missense mutation observed in BS patient at amino acid residue 421.
**Forward primer**

CGTCCCTCTGGACGGATGTTGCTCCCTTAGATTTGCCCGTGAGGCAGAGCTGCCATCT

**Reverse primer**

AATGCCCTGCTCAGTGGT

Fig 20b
Fig 21: Mutations observed in the ISCD region (Exon-10) of PK-M gene in BS1, BS3 and the BS patient after automated sequencing. Top: Mutated sequences; Bottom: normal sequence. The underlined sequences indicate the nucleotide positions involved in mutations in each case and star indicates mutations. The sequences of BS3 and the BS patient are in antisense orientation.
Fig 21
Interestingly, the ISCD clones from normal cell line (GA3) showed polymorphism, a substitution at nucleotide position 7412 from 'G' to 'A' in the exonic region and at 7565 and 7568 in the intronic region (Fig 22). However, the ISCD clones form another normal cell line, (Jiyoye, procured from NCCS, Pune, India) when analyzed and sequenced, did not show any change. The polymorphism (single nucleotide/ SNPs) observed in the GA3 ISCD (Exon-10) region incidentally, was observed in BS1 cell line too.

v) Molecular analysis of inter domain salt bridge (IDSB) (Exon-11) region of the PK gene

Primers were designed for IDSB region located in exon-11 of PK-M gene. The specificity was checked by blast analysis. The amplicons generated through PCR for the IDSB region of PK-M gene were subjected to single stranded conformational polymorphism (SSCP) to detect single base changes (mutations) after cloning and sequencing the variant DNA bands.

a) Optimization of PCR conditions and amplification of IDSB region

The specificity of the primers designed for the IDSB region of PK-M gene was checked by blast analysis and used for amplifying with different magnesium chloride (1.0mM, 1.5mM and 2.0mM) and target DNA concentrations (25ng and 50ng). The results showed that 1.0mM magnesium chloride and 25ng DNA was optimal in obtaining required efficiency and expected product size (Fig 23a). The product was seen as a 230bp band in all: BS1, BS3, the BS patient and normal cells when run on 1% agarose gels. There were no difference in the product size between the two BS cell lines (BS1 and BS3), the BS patient and the normal cell line studied (Fig 23b). In order to resolve single base changes (mutations) in the amplified IDSB region of PK-M gene, SSCP analysis was carried out after cloning the PCR products.
Fig 22: Sequence of normal (above) and the GA3 (below) showing the exon-10 region of PK-M gene, flanked by the intron-9 and intron-10 regions where primers were designed for amplifying exon-10 region. Underlined sequence represent the regions where nucleotide substitution was seen. Star represents the polymorphisms observed in GA3 at 386 and in intronic regions.
CGTCCCTCTGGACGGATGTTGCTCCCCTAGATTGCCCGTGAGGCAGAGGCTGCCATCT
Forward primer

ACCACTTGCAATTATTTGAGGAACTCCGCCGCTGGCAGGCCAAGCATTACAGCGACC
Y H L Q L F E E L R R L A P I T S D

CCACAGAAGCCACCACCGGTGGTGGCTGGCAGGGGCTTCAAGTGCTGCAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAATCGTCCTCAACAAAGTCTGGCAGGTAGAAGCGCAGCGCTCCCTGG
G A I I V L T K S G R

AATGCCCCTGCTCATGGT
Reverse primer

CGTCCCTCTGGACGGATGTTGCTCCCCTAGATTGCCCGTGAGGCAGAACTGCCATCT
Forward primer

AccAATTTGCAATTATTTGAGGAACTCCGCCGCTGGCAGGCCAAGCATTACAGCGACC
Y H L Q L F E E L R R L A P I T S D

CCACAGAAGCCACCACCGGTGGTGGCTGGCAGGGGCTTCAAGTGCTGCAGT
P T E A T A V G A V E A S F K C C S

GGGGCCATAATCGTCCTCAACAAAGTCTGGCAGGTAGAAGCGCAGCGCTCCCTGG
G A I I V L T K S G R

AATGCCCCTGCTCATGGT
Reverse primer

Fig 22
23a: Amplification of IDSB region of PK-M gene using different magnesium chloride and target DNA concentrations. 1 and 2 represent amplifications using 25ng and 50ng target DNA concentrations. 1.0mM, 1.5mM and 2.0mM magnesium chloride was used for standardization. Lane 'M' on the left hand corner represents 100bp ladder used as marker. The product of 230bp is represented with an arrow on the right hand side.

23b: Amplification of IDSB region of PK-M gene. Lanes represent amplicons generated from Bloom syndrome cell lines (BS1, BS3), the BS patient (PT) and normal (N) cells, respectively. Lane M on the left hand side represents 100 bp ladder used as marker. The product of 230bp is indicated by an arrow on the right hand side.
AMPLIFICATION OF EXON-11 OF PYRUVATE KINASE GENE

1 2 3 4 5 6 7 8 9 10 11 12

Intron-10
Forward primer ➔ ANI-7U (9340-9359)
GTC CCC CAT CCC ATC TCA GG

Exon-11

PCR amplification

230 bp PCR product

Ligated PCR product to pMOS Blue-T vector

Hind III EcoRI

LIGATED PRODUCT

Intron-11
Reverse primer ← ANI-7L (9550-9569)
AGG CTC TAG CCC CTG CTC CA

Fig 23
b) Cloning, SSCP and sequencing analysis of IDSB region

The amplified IDSB products from BS1, BS3, the BS patient and the normal cells were eluted from 1% agarose gels and ligated using pMOS blue T-vector kit. The ligated products were transformed in DH5α and XL-blue host cells. Colonies obtained were checked for inserts by colony PCR, using exon-11 primers. SSCP analysis of the positive (in which insert was present) colonies showed no difference in mobility of single strand bands in BS cells when compared to normal cells (Fig 24). To confirm further, sequencing was carried out with the BS1, BS3, the BS patient and the normal cloned PCR products using T7 primer. No mutations were observed in the IDSB region of the two BS (BS1 and BS3) cell lines and in the BS patient. Exon-11, therefore, was intact in both the BS cell lines and in the BS patient.

C) STRUCTURAL ANALYSIS OF PYRUVATE KINASE MUTANTS

Placement of mutations in the derived homology human PK-M2 model showed that the mutation in BS1 from His 391 (present actually in the C-domain) to Tyr 391 is located in the interface of domain A and C. His391 in the model of human PK-M2 shows that the histidine side chain is located in the interface of domain A and C. It lies in the loop connecting helices Aα8 and Cα1. The Histidine side chain in the normal enzyme forms a hydrogen bond with the backbone O of Glu-386 (Fig. 25). This hydrogen bond links the A and C domains (inter domain salt bridge) within the enzyme subunit. The hydrogen bond distance HisNe2....Glu386O distance is 3.0 A°. This hydrogen bond in case of BS1 mutant was found to be formed by the OH of the tyrosine side chain. However the length of the hydrogen bond is shortened and the O....O distance is 2.2 A°. In the other Bloom syndrome cell line, BS3, a frameshift mutation observed in the ISCD region of PK-M gene resulted into a premature termination of protein leading to the deletion of the residues 428-531 of the C domain, and substitution of residues 408-427 (Fig 26). Among these, seven of the residues forming helix Cα1 (residues 408-422) are mutated to helix-breaking proline residues. In case of the patient, normally present Lys421 formed four hydrogen
Fig 24: SSCP analysis of the IDSB region of PK-M gene. Lanes represent BS1, BS3, the BS patient (PT) and the normal (N) clones. 'SS' = single strand bands in each case.
Fig 25: Derived structures of normal and BS1 showing the mutated region. Pink represents 'A' domain, Blue represents 'C' domain in a subunit of pyruvate kinase protein and Green represents the mutated residue. The hydrogen bond length is reduced from 3.0Å in normal (H391) to 2.2 Å in BS1 (Y391).
Fig 26: Structure of normal pyruvate kinase-M2 tetramer protein showing mutated region in BS3. Blue represents the deleted regions and green represents the substituted region in BS3.
bonds out of which two were with backbone oxygens of Ala401 (1.78Å) and Thr404 (1.77Å) of adjacent subunit; one with backbone oxygen of Glu417 (1.80Å) of the same subunit and the fourth with side chain oxygen of Glu409 (2.27Å) of the adjacent subunit. In case of patient three H-bonds were formed by the substituted arginine residue. The normally formed H-bond with Ala401 of adjacent subunit was lost and the bond lengths of the remaining H-bonds changed: the bond with Thr404 increased to 1.86Å, the bond with Glu409 decreased to 2.06Å and the H-bond with Glu417 increased to 1.82Å (Fig 27).

D) AMINO ACID SEQUENCE ALIGNMENT

Amino acid sequence alignment (Fig 28) using AMPS program of the residues in ISCD region of the two isozymes made by alternative splicing the same PK-M gene, M1 and M2 showed that the mutated histidine residue in BS1 is not only conserved between M1, M2, and the other two isozymes made from another gene PK-L/R, L and R-type but also across the mammalian species. In BS3 there was a major change in the conserved amino acid sequence of the ISCD region. The mutated residue in the BS patient also was conserved in all the isoforms and across the mammalian species.

E) ACCESSIBLE SURFACE AREA CALCULATIONS

Accessible surface area results using Lee and Richards method for normal human derived homology model of PK-M and the mutated models showed not much change in the accessible surface area because of the BS1 mutation. But, BS3 mutation changed accessible surface area of some of the important amino acid residues in PK-M2 protein. Accessible surface area of active site (Lys-269), ADP binding site (Arg-42) and loop at the entrance of the ADP binding pocket (Phe 97-Ile 102) did not change significantly. The accessible surface area of the Asp-356 and that of ISCD region changed significantly. ASA of Asp-356 in the mutated BS3 protein increased by about 13 times when compared to the normal. In the normal, ISCD region includes 45 amino acid residues. Whereas in the truncated BS3
Fig 27: Derived structures of normal and BS patient pyruvate kinase protein in the mutated region. Pink and blue indicate different subunits of tetramer. The mutated residue in normal and in BS patient is shown in green. H-bond formed with A401 is lost in the patient, and the remaining bond lengths changed.
Fig 27

NORMAL

BS PATIENT
Amino acid sequence alignment using AMPS program. The conserved residues are drawn in pink boxes and blue box represents the mutated histidine residue in BS1. The last residue lysine (K) which is conserved among all the forms is mutated in the BS patient. Sequence alignment is done from amino acid residue 389 to amino acid residue 421. M2, M1, L and R on left hand side represent different isoforms of pyruvate kinase and H, B, R, C and D represent human, rabbit, rat, cat and dog pyruvate kinase protein sequences.
| M2H: IYLQLFEELRRLAPITSDPTEAATAVGAVEASFK | M2B: IYLQLFEELRRLAPITSDPTEAATAVAVGAVEASFK |
| M2R: IYLQLFEELRRAAPLSRDPTEAAAVGAVEASFK | RH: VYHRQLFEELRRAAPLSRDPTEVTAINGAVEAASFK |
| LH: VYHRQLFEELRRAAPLSRDPTEVTAINGAVEAASFK | RD: VYHRRLFEELRRAAPLSRDPTEVTAINATVEAASFK |
| M1H: MFHRKLFEELVRASSHSTDLMEAMAMGSEASYK | M1C: MFHRKLFEELVRGSSSHSTKLMEEAMAMGSEASYK |
| M1B: MFHRKLFEELLARSSSHSTDLMEAMAMGSEASYK | M1R: IYLQLFEELRRASSQSTDPLEAMAMGSEASYK |

Fig 28
protein ISCD region involves only 38 residues. But, results have shown that the truncated protein has lesser number of amino acid residues and more accessible area when compared to the native protein (Tab 15). In case of BS patient, there was a significant change in the accessible surface area of the active site (Lys-269), IDSB (Asp-356) and in the area of the ISCD (388-433). The accessible surface area of the active site increased by 156%, Asp-356 increased by 960%. The accessible surface area of the ISCD region in the monomer decreased by 49% whereas the ASA of the tetramer increased by 107% (Tab 15).

Finally, the accessible solvent area lost during tetramerisation was calculated as the difference in accessible surface area between total ASA per chain in complex and isolated chain. In normal enzyme the loss in area during tetramerisation was found to be 4722.7, whereas for BS1 it was 4504.8 and for BS3 it was 3923.4 and in case of the BS patient it was 4722.7 square angstroms (Tab 16).
### TABLE 15: ACCESSIBLE SURFACE AREA CALCULATIONS OF IMPORTANT RESIDUES IN MONOMER AND TETRAMER PK-M2 FROM BS1, BS3, BS PATIENT AND NORMAL PROTEINS

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Normal Monomer</th>
<th>Normal Tetramer</th>
<th>BS1 Monomer</th>
<th>BS1 Tetramer</th>
<th>BS3 Monomer</th>
<th>BS3 Tetramer</th>
<th>BS patient Monomer</th>
<th>BS patient Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP binding site</td>
<td>33.456</td>
<td>137.735</td>
<td>35.003</td>
<td>137.573</td>
<td>48.793</td>
<td>197.632</td>
<td>30.38</td>
<td>121.52</td>
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<tr>
<td>(Arg-42)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Active site</td>
<td>22.653</td>
<td>72.314</td>
<td>20.069</td>
<td>72.323</td>
<td>20.069</td>
<td>72.323</td>
<td>28.22</td>
<td>112.88</td>
</tr>
<tr>
<td>(Lys-269)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Asp-356)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSCD</td>
<td>2328.80</td>
<td>4321.05</td>
<td>2080.82</td>
<td>4524.12</td>
<td>3031.57</td>
<td>8420.49</td>
<td>1158.4</td>
<td>46336.6</td>
</tr>
<tr>
<td>(aa 388-433)</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>23016.64</td>
<td>73175.76</td>
<td>22790.31</td>
<td>73142.18</td>
<td>21051.56</td>
<td>68512.75</td>
<td>20809.80</td>
<td>61544.21</td>
</tr>
</tbody>
</table>

* All the values are in square Angstroms
# TABLE 16: COMPARISION OF ACCESSIBLE SURFACE AREA OF PK MONOMER AND TETRAMERS IN BS1, BS3, BS PATIENT AND NORMAL PROTEINS

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>TETRAMER ASA</th>
<th>ASA/CHAIN IN COMPLEX</th>
<th>ASA OF ISOLATED CHAIN</th>
<th>DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1</td>
<td>73142.19</td>
<td>18285.54</td>
<td>22790.37</td>
<td>4504.8</td>
</tr>
<tr>
<td>BS3</td>
<td>68512.75</td>
<td>171128.18</td>
<td>21051.56</td>
<td>3923.37</td>
</tr>
<tr>
<td>BS PATIENT</td>
<td>61544.21</td>
<td>15386.05</td>
<td>20809.80</td>
<td>5423.75</td>
</tr>
<tr>
<td>NORMAL</td>
<td>73175.76</td>
<td>18293.94</td>
<td>23016.64</td>
<td>4722.7</td>
</tr>
</tbody>
</table>

* All the values are in square Angstroms