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Role of Epigenetics in Modifying Clinical Outcome in CP-CML Patients Treated with Imatinib

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ABSTRACT

Introduction: hOCT1 (Human Organic Cation Transporter 1) solute carrier transporter of SLC22 gene family, mediates influx of Imatinib into cells. It is seen that intracellular Imatinib uptake correlates with hOCT1 expression and may alter clinical outcome in CML. DNA methylation in promoter associated CpG islands is a powerful mechanism of gene silencing. Role of promoter hypermethylation of hOCT1 gene in CML and its effect on response to treatment with Imatinib has not been studied much.

Methods: 30 newly diagnosed CML patients aged 18 to 80 years were included in the study before initiation of Imatinib. 30 healthy volunteers participated as controls. Patients were followed up after initiation of Imatinib for 6 to 12 months, hematological and molecular responses were assessed. hOCT1 gene expression was studied by Real Time quantitative PCR. Promoter Methylation of hOCT1 gene was studied by Methylation Specific PCR.

Results: Cases were divided into 2 groups, high expression (n=15) and low expression(n=15) (median fold change= 5.6). Methylation was seen in 83.33% of CML cases whereas in only 23.33% of controls.(p=0.001) Methylation was observed in all the 15 patients(100%) with low expression whereas amongst the high expression group,10 out of 15 patients(66.66%)were methylation positive.(p=0.042) No significant association was seen between methylation status and achievement of CHR or optimal molecular response to Imatinib.

Conclusions: Promoter hypermethylation of hOCT1 gene leads to silencing of gene expression, however, it does not alter the hematological and molecular response to Imatinib.

Key words: Epigenetics, CP-CML patients, Imatinib, Clinical outcome

INTRODUCTION

Chronic myeloid leukemia (CML) is the commonest adult leukaemia in India, with the annual incidence ranging from 0.8-2.2/100,000 population in males and 0.6-1.6/100,000 population in females.[1] Tyrosine-kinase inhibitors (TKIs) constitute the major form of therapy for CML patients. Imatinib is a first generation TKI which has been used as the standard therapy for patients with CP-CML.[2] Clinical outcome to Imatinib treatment is monitored in terms of hematological, cytogenetic, and molecular parameters, as proposed by guidelines given by the European Leukemia Net (ELN).[3] Resistance to Imatinib has been seen in some patients which arises due to a number of proposed mechanisms.[4]

hOCT1 (human organic cationic transporter 1) is an influx transporter responsible for transport of Imatinib into leukemia cells. hOCT1 expression has been suggested to be a useful biomarker to predict the response to Imatinib.[5]

DNA methylation in promoter-associated CpG islands is a powerful mechanism of gene silencing. There is already clinical value of methylation markers for classification, prognosis and prediction of therapeutic response in different types of malignancies.DNA hypermethylation is commonly seen in various types of leukemia including AML,

ALL, CLL and also in some myelodysplastic syndromes. In context with CML, the role of hypermethylation has been studied in many genes viz. *ABL1, CDH13, CDKN28, DPYS, NPM2, OSCP1, PDLIM4*, etc.[6] With respect to OCTs, there is evidence that kidney-specific expression of *SLC22A2* is regulated by DNA methylation[7] However, not much work has been done to study the role of hypermethylation in hOCT1 gene and its effect on response to treatment to imatinib. Therefore to assess a possible epigenetic component of imatinib resistance, comparison of promoter hypermethylation in patients responsive, intolerant and resistant to imatinib is required.[8-9]

The present study aimed to study, promoter hypermethylation of the hOCT1 (*SLC22A1*) gene in peripheral blood leukocytes of Chronic Myeloid Leukemia patients and to correlate it with mRNA expression of the gene. We also assessed association of promoter hypermethylation of hOCT1 (*SLC22A1*) gene with the hematological and molecular response to Imatinib of Chronic Myeloid Leukemia patients.

MATERIALS & METHODS

The present study was a hospital based prospective study and was approved by the college Ethics Committee. Thirty Chronic myeloid leukemia patients who attended Medicine OPD or were admitted in Medicine ward, Lok Nayak Hospital, New Delhi, India and who met the inclusion criteria were recruited in the study. All the thirty cases were in chronic phase (CP-CML). Thirty age and sex matched healthy volunteers were also recruited in the study as controls. The inclusion and exclusion criteria are depicted in Table 1. Figure1 illustrates the study plan used in brief.

INCLUSION CRITERIA
• Newly diagnosed CML patients, in the age group 18-80 years, with diagnosis confirmed by qualitative PCR for BCR-ABL1 fusion gene , who were to be initiated on Imatinib therapy.
EXCLUSION CRITERIA
• Chronic MyeloMonocytic Leukemia (CMML) • BCR-ABL1 positive adult ALL patients. • Other myeloproliferative disorders • Patients who had previously undergone any treatment for chronic myeloid leukemia.

TABLE 1: Inclusion and exclusion criteria for selection of cases

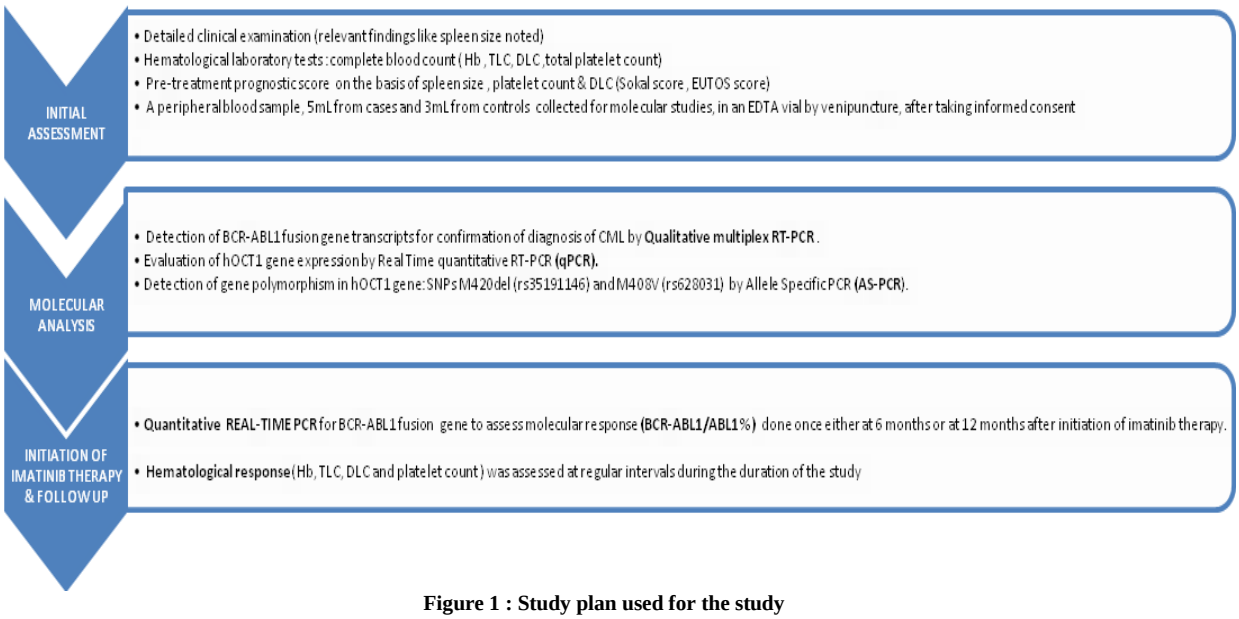


Figure 1 : Study plan used for the study

Isolation of peripheral blood leucocytes from whole blood

Blood was collected in EDTA vial and kept in standing position. After 15-20 minutes buffy coat appeared at the top of the vial and all the red cells were in the lower layer. This buffy coat was carefully pipetted out in two 1.5mL Eppendorf tubes and the remaining blood sample was stored at -80 ^{o} C. Out of the two Eppendorf tubes one tube which contained 200µL of buffy coat was immediately used for RNA extraction and other tube was stored at -80 ^{o} C for subsequent use.

RNA Extraction and c-DNA synthesis

Modified acid guanidinium thiocyanate-phenol-chloroform (TRIZOL) RNA extraction method was used for RNA extraction from PBLs of CML cases. (PaulD.Siebert and Alex Chenchik 1993). RNA from control samples was extracted by using Total RNA Mini Kit (Blood/Cultured Cell) from Gene Aid Biotech Ltd., Taiwan using protocol as per manufacturer's instructions. The concentration of extracted RNA was checked by using Nanodrop and quality was checked by running 2 µL of each sample on 2 % agarose gel.Extracted RNA was reverse transcribed to c-DNA by RT-PCR.cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific, EU) using protocol as per the manufacturer's instructions. A parallel PCR was performed on each sample using primers specific for the constitutively expressed β- actin gene which was used as endogenous control.

DNA isolation from whole blood and Bisulfite treatment of isolated DNA

Genomic DNA was isolated from whole blood by using Genomic DNA Mini Kit (Blood/ Cultured Cell) from Gene Aid Biotech Ltd., Taiwan as per the manufacturer's protocol. Sodium bisufite conversion of genomic DNA was done by using BisulFlash™ DNA Modification Kit obtained from Epigentek as per manufacturer's instructions. Modified DNA was stored at –20°C for further analysis

MOLECULAR ANALYSIS

Multiplex RT –PCR for detection of BCR-ABL1 fusion gene transcripts:

Diagnosis of CML was confirmed by Multiplex RT-PCR which allows simultaneous detection of all the BCR-ABL1 fusion gene transcripts in addition to normal BCR gene as an internal control. cDNA synthesized from the total RNA was used in multiplex PCR .The sequence of oligonucleotide primer sequences used for this multiplex PCR are shown in Table 2. The expected band size for different BCR-ABL1 fusion transcripts were: 808bp - normal BCR,481 bp - e1a2, 385 bp - b3a2 ,310 bp - b2a2, 103bp – b2a3 & 209bp – b3a3.

Expression of hOCT1 gene in CML patients and controls

After cDNA quality was checked it was used to study the expression of hOCT1 gene in patient and control samples by quantitative Real Time PCR using Rotor Gene Q (Qiagen) analyzer. Thermo Scientific Maxima SYBR Green qPCR master mix was used for quantative real-time PCR which contains Hot start Taq polymerase, SYBR Green qPCR buffer, SYBR Green I dye, reference dye and dNTP. βactin gene was used as internal control. In this PCR, amplification of hOCT1 gene was compared with the amplification of β actin gene and relative expression was calculated. Melting curve analysis was done in the temperature range 35°C to 95°C for assessment of homogeneity of the qPCR products. Gene expression levels were calculated based on the $\Delta\Delta C_t$ method.

Study of promoter hypermethylation of hOCT1 gene by Methylation Specific PCR (MSP)

Promoter hypermethylation in hOCT1 gene was examined by MSP. Forward and

reverse primers were designed using MethPrimer primer designing software. (Table 2)These primers corresponded to the predicted sequence of methylated or unmethylated genomic sequences in hOCT1 promoter region after sodium bisulfite treatment. Modified DNA was subjected to MSP reaction according to the standardized protocol.

Table 2 : Primers used in different PCR reactions

Primer sequences for βactin PCR	
Primer	Sequence
βactin Forward primer	5′CGACAACGGCTCCGGCATGTGC3′
βactin Reverse primer	5′CGTCACCGGAGTCCATCACGATC3′
Primer Sequences used for Multiplex RT-PCR for BCR-ABL1 fusion gene	
Primer code	Primer Sequence
C5e	5'-ATAGGATCCTTTGCAACCGGGTCTGAA-3'
B2B	5'-ACAGAATTCCGCTGACCATCAATAAG-3'
BCR-C	5'-ACCGCATGTTCCGGGACAAAAG-3'
CA3	5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'
Primers used for hOCT1 gene expression	
Primer	Sequence
Forward primer	5′-GGGCAGCCTGCCTCGTCAT-3′
Reverse primer	5′-ACCTCCCTCAGCCTGAAGAC-3′
Primer sequences used in MS-PCR	
(Amplicon size : 124bp)	
Unmethylated Forward	5′-TTGCTCCTGGGCAAAGCAAATGATT-3′
Methylated Forward	5′-TCGCTCCCGGGCAAAGCAAACGATT-3′
Common Reverse	5′-CCAAGTGTGAAACATCTGGAAGGCAACC-3′

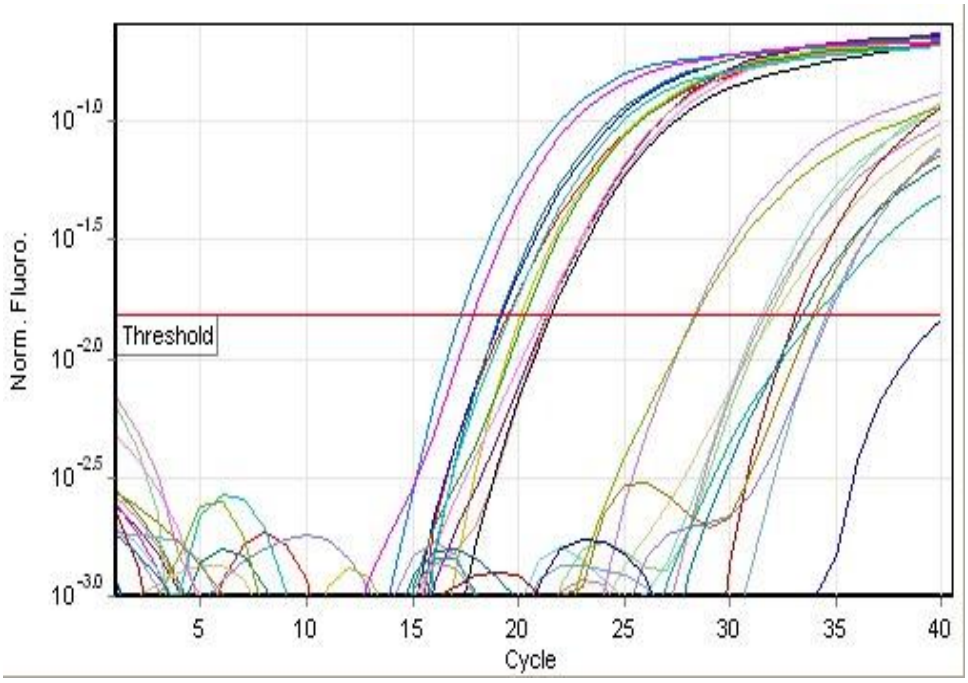
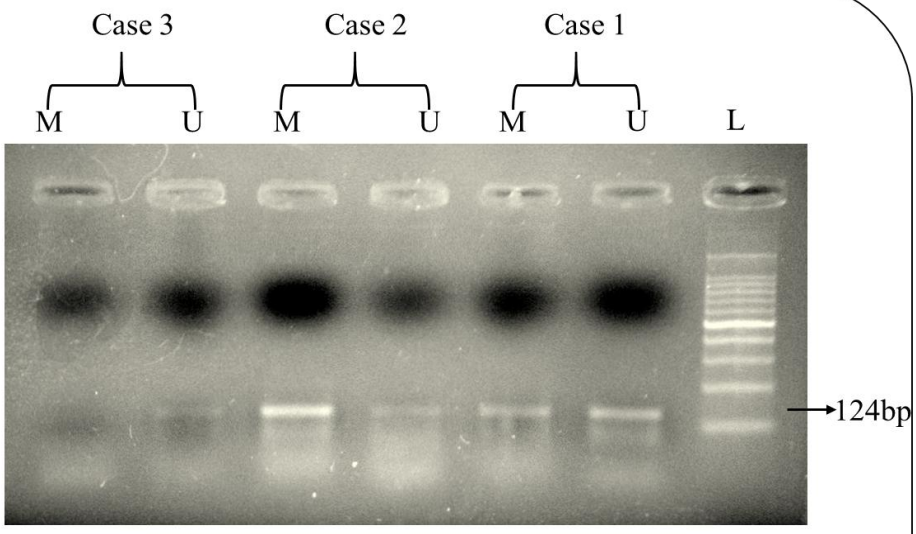


Figure 2 : Gene expression curve

FOLLOW-UP

After initiation of Imatinib therapy, follow-up and response monitoring of patients was done for 6 months to 12 months i.e. during the duration of the study, depending on the time point at which the patient was recruited in the study. Hematological response (Hb, TLC, DLC and platelet count) was assessed at regular intervals during the duration of the study. Molecular Response (BCR-ABL1/ABL1 %) was assessed once, either at 6 months or at 12

months after beginning of imatinib therapy, depending on the time of recruitment of patient in the study.



Gel Electrophoresis band pattern for hOCT1 gene methylation as visualised under 2% agarose gel under UV transillumination yielding 124bp PCR product for unmethylated as well as methylated samples
M- Methylated U- Unmethylated L-100bp ladder

Figure 3 : Agarose gel electrophoresis picture for hOCT1 gene methylation

Quantitative Real-Time PCR for BCR-ABL1 fusion gene for monitoring molecular response

A peripheral blood sample was collected at follow-up either at 6 months or at 12 months after initiation of imatinib therapy to assess the molecular response. Molecular response was assessed by calculating BCR-ABL1/ABL1 ratio. This was done by quantification of BCR-ABL1 p210b2a2 or b3a2 transcripts. *Ipsogen* BCR-ABL1 Mbcr kit (from QIAGEN, Netherlands,) was used for this purpose and protocol followed was as per the manufacturer's instructions. For each gene (ABL1 and BCR-ABL1), raw Ct values obtained from plasmid standard dilutions were plotted according to the log copy number (3,4 and 5 for C1,C2 C3 ; and 1,2,3,4,5 for F1, F2, F3, F4, F5).The ABL1 standard curve equation was used to transform raw Ct values for the unknown samples into ABL1 copy numbers ($ABL1_{CN}$)The BCR-ABL1 standard curve equation was used to transform raw Ct values for the unknown samples into BCR-ABL1 copy numbers ($BCR\text{-}ABL1Mbcr_{CN}$). Normalized copy number (NCN) was calculated using the formula: NCN = [$BCR\text{-}ABL1Mbcr_{CN}$ / $ABL1_{CN}$] * 100

RESPONSE CRITERIA

Response to imatinib treatment was measured in terms of hematological and molecular parameters as proposed by the European leukemia Net (ELN).

TABLE 3: CRITERIA FOR HEMATOLOGICAL AND MOLECULAR RESPONSE TO IMATINIB

Complete Hematologic Response (CHR)	Platelets < 450 $x10^9$/L, AND White cells < 10 $x10^9$/L, AND No circulating immature myeloid cells, AND < 5% basophils on differential, AND No palpable splenomegaly

Duration of treatment	OPTIMAL	WARNING	FAILURE
6 months	BCR-ABL1 < 1%	BCR-ABL1 = 1-10%	BCR-ABL1 >10%
12 months	BCR-ABL1 ≤ 0.1 %	BCR-ABL1 > 0.1-1%	BCR-ABL1 > 1%

STATISTICAL ANALYSIS

All statistical analysis was performed using SPSS software22.0. Variables were presented as range, Mean ± SD and median value. Fisher exact, Mann Whitney-U and Kruskal Wallis tests were used to estimate the statistical significance of differences observed between the groups. $p<0.05$ was taken as statistically significant.

RESULTS

An overview about age and sex distribution of cases and controls are depicted in Table 4. The clinical and hematological parameters analyzed at baseline and at follow up are summarized in Table 5

Table 4: Age and Sex distribution of cases and controls

	CML cases (n=30)	Controls (n=30)	p-value
Age distribution			
Mean± S.D. (yrs)	39.70 ± 18.04	39.27 ± 16.00	0.906
Range(yrs)	18-80	20-75	
Sex distribution			
Males(%)	22 (73.34)	19(63.34)	0.580
Females(%)	8 (26.67)	11(36.67)	

Table 5: Clinical and Hematological parameters

Parameter	Mean±S.D. at baseline (n=30)	Mean±S.D.after 6 months of imatinib therapy (n=30)	Mean±S.D.after 12 months of imatinib therapy (n= 10)
1.Hb (gm%)	11.01±2.08	10.61±1.73	10.29±1.46
2.TLC(*10^9/L)	203±176.73	6.82±1.63	6.47± 2.21
3.PLATELET COUNT (*10^9/L)	318.30±166.75	166.40±42.16	148.20±43.27
4. SPLEEN SIZE (cms below costal margin)	11.93±2.98	Not palpable	Not palpable
5.BLASTS(%)	3.73±1.20	00±0.000	0.30±0.95
6.BASOPHILS(%)	2.23±0.89	1.63±0.85	1.90±0.87
7.EOSINOPHILS(%)	4.03±1.84	2.73±0.82	1.70±0.67

hOCT1 gene expression and promoter hypermethylation

The fold change in expression of hOCT1 gene ranged from 0.02 to 96.33.On the basis of the median value for hOCT1 fold change in expression (5.60), the cases were divided into 2 groups – high expression (n=15) and low expression (n=15). Promoter hypermethylation in hOCT1 gene was studied by Methylation Specific PCR after sodium bisulfite conversion of genomic DNA. It was observed that methylation frequency in cases was 83.33% whereas in controls it was 23.33%. This difference was found to be statistically significant. (p=<0.001) [Table 6(a)]. It was also analysed whether there was any association between hOCT1 gene expression and promoter hypermethylation of the gene. As shown in table 6(b), a significant association was found between them.(p=0.042)

Table 6(a) : hOCT1 promoter methylation frequencies

	Methylation positive	Methylation negative	p-value
CASES (n=30)	25(83.33%)	5(16.67%)	<0.001*
CONTROLS(n=30)	7(23.33%)	23(76.66%)	

Table 6 (b): Association of hOCT1 gene expression with methylation

hOCT1 gene expression	Methylation positive	Methylation negative	p-value
High	10	5	0.042*
Low	15	0	

Study of association of hOCT1 gene hypermethylation with response to Imatinib

Association of gene hypermethylation with haematological response was analysed. As seen in table 7(a) &7 (b), there was no significant association between the methylation status of cases and hematological response. Moreover, it was also analysed whether there was any association between the methylation status and type of molecular response (optimal, warning or failure) achieved. No significant difference was found as shown in Table 8.

Table 7(a): Association of methylation status with achievement of CHR

Methylation status	No. of cases with CHR at 3 months present, n=	No. of cases with CHR at 3 months absent, n=	p-value
Methylation positive	21	4	1.0
Methylation negative	5	0	

Table 7(b): Association of methylation with time to HR (THR)

Methylation status	No.of patients	Mean THR± S.D. (months)	Median THR (months)	Range (months)	p-value
Positive	25	2.28± 0.99	2.0	1-4.5	.512
Negative	5	2.4 ± 0.65	2.5	1.5-3	

Table 8: Association of methylation with molecular response

Methylation status	No.of cases with Optimal response, n=	No. of cases with Warning responses, n=	No. of cases with Failure Response, n=	p-value
Methylation positive	12	6	7	0.190
Methylation negative	5	0	0	

DISCUSSION

Epigenetic modifications, particularly increased gene promoter region hypermethylation, have been shown to be associated with Imatinib meslyate resistance.[10]As per current literature, the mechanism behind the association between gene promoter hypermethylation and response to Imatinib meslyate treatment is not known. However, one possible mechanism might be an aberrant expression of genes related to the transport of Imatinib meslyate to leukemic cells.

Celik S et al[11] identified that *DAPK1* methylation was significantly higher in imatinib resistant patients and also there was no DAPK1 methylation in any of the healthy controls. Elias MH et al [12] have reported that aberrant DNA methylation of HOXA4 and HOXA5 could be an epigenetic mechanism mediating Imatinib resistance in CML patients. San José-Eneriz E et al [13]demonstrated that down-regulation of BIM (BCL-2- interacting mediator)expression was present in 36% of the patients and was significantly associated with a lack of optimal response to imatinib. Expression of BIM was found to be mediated by promoter hypermethylation. They concluded that epigenetic down-regulation of BIM expression is associated with reduced optimal responses to imatinib treatment in chronic myeloid leukaemia.As per current knowledge, very few studies regarding hOCT1 hypermethylation are available. Schaeffeler E et al[14] investigated DNA methylation, quantified by MALDI-TOF mass spectrometry and gene expression of hOCT1 using fresh-frozen Hepatocellular Carcinoma tissues and non-tumor adjacent liver tissues as well as histologically normal liver samples. They observed that DNA methylation of hOCT1 was significantly higher in HCC compared with non-tumor adjacent liver tissue and was lowest in histologically normal liver tissue. Lin R et al[15] tested the hypothesis that cisplatin resistance is associated with alteration of expression of OCTs and studied the levels of expression of OCTs in paired esophageal cancer and adjacent non-cancerous tissues by use of immunohistochemistry.This study showed that long-term exposure to cisplatin promotes methylation of the OCT1 gene in human esophageal cancer cells, which in turn results in cisplatin resistance.[19]

As per current literature,[16-18] role of promoter hypermethylation of hOCT1 has not been in studied much, in relation to Imatinib resistance.In this study methylation in the promoter region of hOCT1 gene was studied by MSP and its association was correlated with hematological and molecular response to Imatinib.Methylation was seen in 83.33% of the cases whereas it was seen in only 23.33% of the controls. This difference was found to be statistically significant.The association of methylation with hematological response was studied and it was observed that out of 25 cases who

were methylated, 21 (84%) achieved CHR by the end of 3 months whereas 4 (16%) did not. Among the unmethylated cases, all 5 (100%) achieved CHR by the end of 3 months. This observation, however, was not found to be statistically significant. Also, it was found that THR in the methylation positive cases was not significantly different from the methylation negative group. Hence, it can be concluded that hOCT1 methylation has no significant association with haematological response. The association of methylation with molecular response was also studied and it was observed that although all 5 methylation negative cases (100%) had an optimal response to imatinib, only 12 out of 25 (48%) had an optimal response in methylation positive cases. In methylation positive cases, 6 (24%) were categorized as warning and 7 (28%) were categorized as failure. This observation, however, was not found to be statistically significant. Thus, it can be concluded that hOCT1 methylation has no significant association with molecular response.

CONCLUSIONS

As per the findings of our study, the frequency of methylation was significantly more in the cases as compared to the controls. Moreover, frequency of methylation was significantly increased in patients with low gene expression. Thus, we conclude that promoter hypermethylation of hOCT1 gene causes silencing of its expression.

Role of hOCT1 gene in altering outcome in Imatinib treated patients has been established in previous studies. However, no significant association was seen between methylation (which causes gene silencing) and hematological as well as molecular response to Imatinib. Therefore, these findings need to be confirmed in a bigger sample.

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