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Inactivation of RIZ1 Gene by Promoter Hypermethylation is Associated with Disease Progression and Resistance to Imatinib in Indian Chronic Myelogenous Leukemia Patients, First Study from India

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Abstract

Background: The epigenetic impact of DNA methylation in chronic myelogenous leukemia (CML) is not completely understood. RIZ1 expression and activity are reduced in many cancers. In CML, blastic transformation is associated with loss of heterozygosity in the region where RIZ1 is located. RIZ1 is a PR domain methyltransferase that methylates histone H3 lysine 9, a modification important for transcriptional repression. In CML blast crisis cell lines RIZ1 represses insulin-like growth factor-1 expression and autocrine signaling. Together these observations suggest that RIZ1 may have a role in the chronic phase to blast crisis transition in CML.

Methods: To examine whether promoter methylation is involved in the disease development and progression of CML, we investigated promoter methylation status of RIZ1 gene in 100 chronic myeloid leukemia's (CML) patients and 50 controls by MSP method.

Results: The RIZ1 methylation was studied in 100 CML patients, 9 were cases were methylation positive cases, six of nine were in blastic phase, 2 in chronic phase and one patient in accelerated phase. It was seen that RIZ1 methylation was increased significantly from early to advanced phase. The higher frequency of RIZ1 methylation was reported in haematologically resistant cases (42% vs 2%) and molecularly resistant cases (16.77% vs 1.92%) than the responders. The higher frequency of RIZ1 methylation was found in CML patients who were treated with interferon initially followed by imatinib treatment. Also RIZ1 hypermethylation was associated with faster disease progression p<0.003 than the non methylated cases. No correlation was found between RIZ1 gene methylation with age, thrombocytopenia, types of bcr/abl transcripts of CML patients.

Conclusion: We conclude that epigenetic silencing of RIZ1 gene is associated with CML progression and imatinib resistance. Early detection of RIZ1 methylation could be a predictive marker for imatinib resistance and disease progression in CML.

Keywords: Myelogenous leukemia; Methylation; Resistance; RIZ1 gene

Abbreviations: Retinoblastoma Protein: Interacting Zinc Finger gene (RIZ1); CML: Chronic Myeloid Leukemia; MS: Methylation-Specific PCR; TSGs: Tumour Suppressor Gene; CP-CML: Chronic Phase; AP- CML: Accelerated Phase & BC- CML Blast Phase

Introduction

CML is a pluripotent haematopoietic stem cell disorder. Diagnosis of chronic myeloid leukemia is based on the detection of BCR-ABL gene, a product derived from translocation of chromosome 22 to chromosome 9 [1]. It is detected in more than 95% of patients with chronic myeloid leukemia (CML), 20% to 40% in adult acute lymphoblastic leukemia (ALL) and 2% to 5% in paediatric ALL [2]. CML progresses at a varying rate from the CP to the AP and eventually to the BP [3]. However molecular mechanisms responsible for driving the transition of CML from chronic phase to blast crisis are not well characterized. Inactivation of TSGs plays an important role in cancer formation and progression. Either genetic or epigenetic mechanisms are known to inactivate TSGs. DNA methylation-induced silencing

of gene expression is now recognized as an important contribution in all stages of carcinogenesis [4]. DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylation occurs in the sequence context 5CG3_ (also called the CpG dinucleotide), some involves CpA and CpT dinucleotides. Methylation of CpG islands within gene promoter regions can lead to silencing of gene expression [5]. DNA methylation of promoter region CpG islands may serve as an alternate mechanism to genetic defects in the inactivation of tumour suppressor genes in human malignancies [6]. Many cellular pathways are inactivated by this epigenetic event, including DNA repair, cell cycle, apoptosis, cell adherence, and detoxification [7]. Aberrant hypermethylation has been previously described in CML [8-10]. A few studies have examined the methylation status of individual TSGS in CML, with results ranging from rare or no hypermethylation (e.g., HOXA, SFRP1, RASSF1A) [11-13] to hypermethylation at progression (e.g., CALCA, CDKN2B, EBF2, ESR, HIC1, TFAP2A, and others [14-18]. RIZ is a member of the nuclear protein- methyltransferase superfamily. This family plays an important role in chromatin mediated regulation of gene expression, development and cancer. RIZ1 gene produces two protein products of different length, RIZ1 and RIZ2. RIZ1, a PR domain methyltransferase, is located at 1p36. RIZ1 contains the positive regulatory (positive regulatory domain I binding factor1) domain, but RIZ2 lacks this domain [19]. Thus, the PR domain is possibly responsible for the tumour suppressing activity of RIZ1. It methylates histone H3 lysine 9, a modification important for transcriptional repression [20]. In many human cancers, RIZ1 can induce G2-M arrest and apoptosis, hence considered as a TSG. Although RIZ1 is a putative tumour suppressor in several cancer types, for instance human hepatocellular carcinoma [21], gastric cancer [22], cholangiocarcinoma [23], thyroid carcinoma [24], pheochromocytomas and abdominal paragangliomas [25], human gastric carcinoma [26], parathyroid tumors and pheochromocytomas [27] and so on, the role of RIZ1 in chronic myeloid leukemia has not been reported yet.

Material and Method

Blood samples of 100 CML patients were collected from Maulana Azad Medical College and Associated Hospitals, New Delhi. Patients were diagnosed clinically by bone marrow hyperplasia, leukocytosis, splenomegaly, high fever and fatigue. The diagnosis was also confirmed by detecting t (9; 22) or BCR/ABL fusion gene (p210[bcr-abl]) which is further categorized into b3a2 or b2a2 subtypes on the basis of the BCR breakpoint by Reverse transcription polymerase chain reaction in the molecular oncology lab.

Molecular diagnosis

Selection criteria of patients:

- **Inclusion Criteria:** The study included newly diagnosed CML patients treated with imatinib with a dose of 400mg/day. All three stages of cases were included, Chronic phase (CP), Accelerated phase (AP) ,Blast crisis (BC). The exclusion criteria included Chronic myelomonocytic leukemia's (CMML) patients, other myeloproliferative disorder patients. The patients follow up was maintained regularly and samples were collected after every six months for imatinib response and mutation studies. The classic criteria used for imatinib mesylate responses in chronic myeloid leukemia for hematologic and molecular responses are depicted in Table 1A and B.

Sample collection: Venous blood (5ml) or 1ml bone marrow aspirate was collected in EDTA vials from CML patients. Buffy coat was isolated and washed in red cells lysis buffer. All samples were stored at -80°**C** until the RNA and genomic DNA was extracted.

RT-PCR for BCR-ABL: Total RNA was extracted from about 10^6 white cells by Trizol assay (Invitrogen). For cDNA synthesis, the concentration of RNA was measured spectrophotometrically or by gel electrophoresis and then the cDNA was synthesized using M-MuLV Reverse Transcriptase and other reaction components (Fermentas). The two μg of RNA was reverse transcribed with 200 units of M-MuLV RT in a reaction mix consisting of 1x RT buffer (50mM Tris-HCl, 50 mM, KCl, 4 mM $MgCl_2$,10 mM DTT), 0.5 g of oligo (dT)$_8$, 20 mM dNTP and 20 units RNase inhibitor, in a final volume of 20μl. The thermocycling conditions were 70°C for 5 min, 37°C for 5 min, 42 °C for 1h and 70°C for 10 min. The quality of RNA and efficiency of cDNA synthesis were analyzed by amplification of BCR gene as an internal control.

Multiplex RT -PCR: The cDNA product was amplified with 1 U/μL Taq polymerase, 240 μM dNTP, 1.8 M $MgCl_2$, and 0.6 μM of the four primers (CA3, C5e, BCR-C, and B2B). Multiplex RT-PCR assay was performed on a PCR machine with the program of10 sec at 100°C, one min at 96°C, three min at 60°C, two min at 72°C, 10 sec at 100°C, 20 sec at 97°C, 25 sec at 58°C, 25 sec at 60°C, 10 sec at 78°C, 50 sec at 73°C, and 31 times to step five and 10 min at 73°C. The sequence of oligonucleotide primers used in multiplex RT-PCR for BCR-ABL fusion transcripts as the target gene and BCR transcripts as internal control are shown in Table 1. The primer combinations in multiplex RT-PCR allowed simultaneous detection of all known types of BCR-ABL and BCR transcripts in one reaction [28]. The expected bands were as follows: 808bp, normal BCR; 481 bp, e1a2; 385bp ,b3a2;310bp, b2a2; The quality of RNA and efficiency of cDNA synthesis were analyzed by amplification of BCR gene as an internal control.

Methylation analysis: Genomic DNAs from tumour tissues and cell lines were extracted using universal Genomic DNA extraction Kit (Nucleopore).The quality and integrity of the DNA was determined

Complete or major hematological response	Partial or minor hematological response	Loos or minimal hematological response
Platelet count >150×10⁹/L WBC count <10×10⁹/L Basophils :<5% Differential without immature granulocytes. Absence of blasts and promyelocytes in peripheral blood Spleen : nonpalpable spleen	Platelet count <450×10⁹/L WBC count >10×10⁹/L Basophils :>10% Presence of blasts and promyelocytes in peripheral blood Spleen : Palpable spleen	Platelet count <450×10⁹/L WBC count >20×10⁹/L Basophils :15% Presence of blasts and promyelocytes in peripheral blood Spleen : Palpable spleen

Table 1a: Hematologic responses.

Major molecular response	Minimal or No Molecular response
It indicates nonquantifiable and nondetectable bcr-abl gene transcript (BCR-ABL/ABL) ≥ 0.10³ 3 log reduction of BCR-ABL/ABL	It indicates quantifiable and detectable bcr-abl gene transcript (BCR-ABL/ABL) ≥ 0.10³ No 3 log reduction of BCR-ABL/ABL

Table 1b: Molecular response.

by the A260/280 ratios and by 2% gel electrophoresis. Genomic DNA (1μg) was modified with sodium bisulfite using EZ-DNA methylation kit (Zymo research). Bisulfite-treated DNA was used for methylation-specific PCR by using previously published primer sets 18 to distinguish between methylated and unmethylated DNA. The PCR products were electrophoresed on a 2% agarose gel.

Methylation-specific polymerase chain reaction: Methylation-specific primers were used to investigate the methylation status of samples as described by Du et al, [12]. Primers specific for methylated and unmethylated DNA alleles are depicted in the Table 2.

The thermo cycling conditions were 45 cycles of denaturation at 94°C for 30s, annealing at 68 °C for methylation-specific amplification or at 60 °C for unmethylation-specific amplification for 45s and extension at 72 °C for 60s, then a final extension at 72 °C for 10 minutes. The PCR products were then analyzed on a 2.5% agarose gel. The expected bands were as follows: 177bp for methylated DNA and 175 bp for unmethylated DNA.

Statistical analysis: Statistical analysis was performed using the SPSS 13.0 software package. Chi-square analysis and Fisher exact test were carried out to compare the difference of frequencies between groups of patients. Mann-Whitney's U-test was carried out to the difference of age, level of RIZ-1 and bcr/abl transcript between the methylated and the unmethylated groups. The correlation between the frequency of RIZ-1 promoter methylation and the clinical and hematologic parameters was analyzed with Spearman's rank correlation. For all analyses, the P-values were two-tailed, and a P-value of < 0.05 was considered statistically significant.

Results

Demographic characteristics of study population

The demographic characteristics of study population are listed in Table 3. A total of 65 males and 35 females already diagnosed with CML were included in the study. Two age groups were made, patients with age < 45 years included 36 cases, above >45 included 64 cases (Table 3).

Methylation-specific polymerase chain reaction

PCR products were analyzed on 3.5% agarose gel and visualized under UV illumination. To verify successful bisulfite modification of the DNA, a sequence containing cytosines would not be amplified after bisulfite modification by using primers that would only give an

BCR-ABL primer sequences	AT
5'-ATAGGATCCTTTGCAACCGGGTCTGAA-3' 5'-ACAGAATTCCGCTGACCATCAATAAG-3' 5-ACCGCATGTTCCGGGACAAAAG-3' 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3	58°C

Table 2: Sequence of oligonucleotides used in multiplex RT-PCR for detection of BCR-ABL transcript as the target gene and BCR transcripts as the internal control.

Primer sequence of RIZ-1	Primer	AT	Product size
5'GTGGTGGTTATTGGGCGACGGC-3' 5'-GCTATTTCGCCGACCCCGACG-3'	Methylated primer Reverse primer	68°C	177 bp
5'-TGGTGGTTATTGGGTGATGGT-3' 5'-ACTATTTCACCAACCCCAAGA-3'	Unmethylated primer Reverse primer	60°C	175 bp

Table 3: Sequence of oligonucleotides used for methylated and unmethylated DNA.

	Number	Percentage
Patients	100	
AGE OF THE SUBJECTS		
Age <45	36	36%
Age >45	64	64%
GENDER OF THE SUBJECTS		
Males	65	65%
Female	35	35%
STAGE OF THE DISEASE		
CP-CML	48	48%
AP-CML	28	28%
BC-CML	24	24%
TYPE OF BCR-ABL TRANSCRIPTS		
A2b2	31	31%
A2B3	67	67%
A2b2/ A2b3	2	2%
MOLECULAR RESPONSES TO IMATINIB		
MMR	52	52%
No MR	48	48%
HEMATOLOGICAL RESPONSES TO IMATINIB		
MHR	50	50%
Minor HR	10	10%
Loss HR	40	40%
TYROSINE KINASE INHIBITOR THERAPY		
Imatinib	90	90%
Imatinib	7	7%
Interferon	3	3%
THROMBOCYTOPENIA		
Thrombocytopenia	60	60%
No Thrombocytopenia	40	40%

Table 4: Clinicopathological features of CML patients and Cancer-free healthy controls.

amplified product if the cytosines in the template sequence were not converted to uracils. Samples that did not give a band were considered completely modified and further used for MSP analysis. Those cases were defined as methylation positive if their sample showed a visual band amplified with methylated specific primers, even if the band was faint. The product size of 177 bp and 175 bp were expected for methylated (M) and unmethylated (U) DNA of RIZ1 promoter methylation respectively. All positive results were confirmed in at least two MSP analysis (Figure 1).

Frequency of RIZ-1 hypermethylation with respect to clinical parameters of the study population

Nine patients (9%) showed RIZ1 hypermethylation that was not found in any controls ($P < 0.06$) as depicted in Table 4. There were no significant correlations between the methylation status of RIZ1 hypermethylation and the clinical features such as age, sex, thrombocytopenia, chromosomal abnormalities like BCR-ABL and its transcripts.

Frequency of RIZ-1 hypermethylation with respect to type of BCR-ABL transcript

We were able to reliably detect typical p210 transcripts, such b2a2 or b3a2 transcripts in 100 patients at the presentation, b3a2 transcript was detected in 67% cases , b2a2 in 31% and both b3a2 and b2a2 in 2% patients. Among males 67.6% 29.2% and 3.07% showed b3a2, b2a2 and

both b3a2/b2a2 transcript respectively. Among females 65.7%, 34.2 % showed b3a2, b2a2 respectively. However no significant correlation was found between BCR-ABL transcript type and RIZ1 promoter methylation.

RIZ-1 methylation is increased in blast crisis or advanced stage CML patients

Among the 100 CML patients at the time of analysis, 48 were in chronic phase, 28 in accelerated phase and 24 in blast crisis respectively. RIZ1 promoter was methylated in 9% of CML patients. Six out of 9 patients were in blastic phase, 1 in accelerated phase and 2 in chronic phase CML. A significant difference was found between the frequency of RIZ-1 methylation and CML stages ($P < 0.05$) as depicted in Table 5. Among the 2 patients in chronic phase having RIZ1 promoter methylation one patient showed transition from chronic phase to blastic phase indicating that RIZ1 inactivation might be one main factor for disease progression. RIZ1 promoter methylation was increased significantly from early to advanced phase in our CML patients .The high frequency of RIZ-1 hypermethylation in blast crisis may suggest that aberrant methylation is a non-specific, global process that is a feature of advanced phase chronic myeloid leukemia, at least in a subset of patients.

RIZ-1 methylation is increased in hematological resistant CML patients

Among 100 CML patients at the time of analysis, 50 display major HR, 10 minor HR and 40 minimal haematological responses respectively. The higher frequency of RIZ1 promoter methylation was reported in minor HR (30%), minimal HR (12.50%) followed by major (2%) hematological responders. A significant difference ($P<0.01$) was reported between RIZ1 methylation and haematological resistances in CML patients.

RIZ-1 methylation is increased in molecular resistant (BCR-ABL/ABL >0.50%) CML patients

Among the 100 CML patients at the time of analysis, 52 displayed major MR, 48 minimal or no molecular responses. The higher frequency of RIZ1 promoter methylation was reported in molecular resistant cases (16.77%) than the good molecular responders (1.92%) i.e., (16.77% vs 1.92%). The significant difference was reported between

Clinical feature	N	RIZ1 unmethylated	%	RIZ1 methylated	%	P Value
Patients	**100**	**91**	**91%**	**9**	**9%**	**0.06**
Controls	**50**	**50**	**100%**	**0**	**0%**	
Age >45	36	33	91.6%	3	8.4%	**0.98**
Age <45	64	58	90.6%	6	9.4%	
Males	65	58	89.2%	7	10.7%	**0.63**
Female	35	33	94.2%	2	5.8%	
CP-CML	48	46	95.8%	2	4.2%	
AP-CML	28	27	96.4%	1	3.6%	**0.009**
BC-CML	24	18	75%	6	25%	
b2a2	31	29	93.5%	2	6.5%	
b3a2	67	60	89.5%	7	10.5%	**0.73**
A2b2/ A2b3	2	2	100%	0	0%	
MMR	52	51	98.08%	01	1.92%	**0.03**
Loss of MR	48	40	83.33%	8	16.77%	
MHR	50	49	98%	01	2%	**0.01**
Minor HR	10	07	70%	03	30%	
Minimal HR	40	35	85%	05	12.50%	
Imatinib alone	90	84	93.33%	6	6.66%	**0.02**
Imatinib+Dasatanib	07	06	85.70%	1	14.30%	
Interferon	03	01	33.33%	2	66.66%	
Thrombocytopenia	60	54	90%	6	10%	**0.9**
No Thrombocytopenia	40	37	92.5%	3	7.5%	

MR:Molecular response, HR: Hematological response

Table 5: Correlation between RIZ-1 promoter methylation and the clinical characteristics of CML patients.

the molecular resistant cases and cases with RIZ1 hypermethyaltion (<0.03).

RIZ-1 methylation is increased in CML patients treated with interferon initially

It is established now that RIZ1 DNA methylation is increased in imatinib resistant and advanced stage or progressed cases. The higher frequency of RIZ1 promoter methylation was found in CML patients who were treated with interferon initially followed by imatinib treatment. The significant difference was reported among the three groups and cases with RIZ1 hypermethyaltion (<0.03).

RIZ1 hypermethyaltion induced faster disease progression and shorter survival

Follow-up of patients regarding disease progression in terms of survival was performed with median duration of 80 months. A total of 52 patients suffered progression with mean follow up time of 4 years. It was observed that the RIZ1 hypermethyaltion cases were significantly associated with faster disease progression p<0.003 (Figure 2).

Since imatinib treatment changed radically the outcome of CML, we analyzed separately the groups of patients treated before and in the imatinib era. Our results suggest that epigenetic aberrations in RIZ1 gene may result in a blockage of apoptotic and differentiation pathways, which causes expansion of the myeloid blast cell population. Hypermethylation of tumour suppressor RIZ1 may be a therapeutic target. However, more extensive pre-clinical and clinical studies incorporating demethylating agents are needed to assess their therapeutic role in chronic myeloid leukemia blast crisis.

Discussion

The frequency of hypermethylation of tumour suppressor gene RIZ1 in CML blast crisis is not known. One possible explanation is that the RIZ1 promoter CpG island is aberrantly hypermethylated. In the CML blast crisis cell line, K562, the RIZ1 promoter is hypermethylated and addition of a methylation inhibitor, 5-aza-2'-deoxycytidine, induces RIZ1 expression, Dong W et al. [20]. Epigenetic silencing has also been reported to reduce RIZ1 expression in other cancers Du Y et al. [18]. Molecular mechanisms responsible for driving the transition of CML from chronic phase to blast crisis are not well characterized. CML evolves from a chronic phase that is associated with the Philadelphia chromosome to a blast crisis phase, which is associated with additional chromosome or molecular aberrations.

Evolution to blast crisis is correlated with frequent loss of heterozygosity at chromosome region 1p36. Tumour suppressor gene silencing is important in progression of cancers. **RIZ1**, a PR domain methyltransferase, is located at 1p36. RIZ1 methylates histone H3 lysine 9, a modification important for transcriptional repression RIZ1 is a candidate tumour suppressor gene and its expression has been found to be lost in many cancers. RIZ1 expression and activity are reduced in many human cancers by genetic and epigenetic mechanisms.

RIZ1 expression is reduced in acute myeloid leukemia [20] and the **RIZ1** knockout mouse has a high incidence of diffuse large B-cell lymphoma [16]. RIZ1 also regulates IGF-1 signaling in CML blast crisis cell lines and its expression has been found to be down regulated in CML blast crisis [29]. Mechanism for decreased RIZ1 expression in CML is not known. The RIZ1 promoter has been demonstrated to have the characteristics of a CpG island, which suggests that RIZ1 is a target of inactivation by epigenetic mechanisms [19].
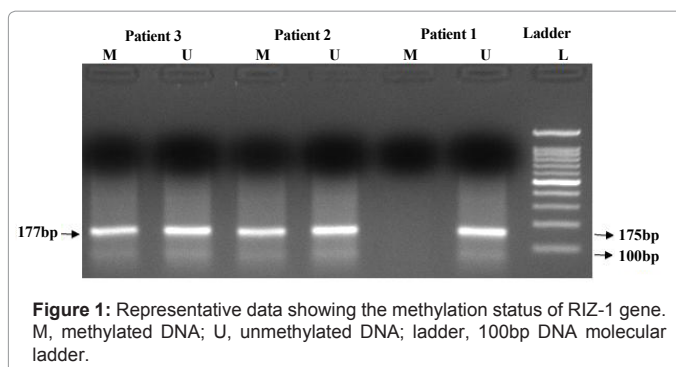

Figure 1: Representative data showing the methylation status of RIZ-1 gene. M, methylated DNA; U, unmethylated DNA; ladder, 100bp DNA molecular ladder.
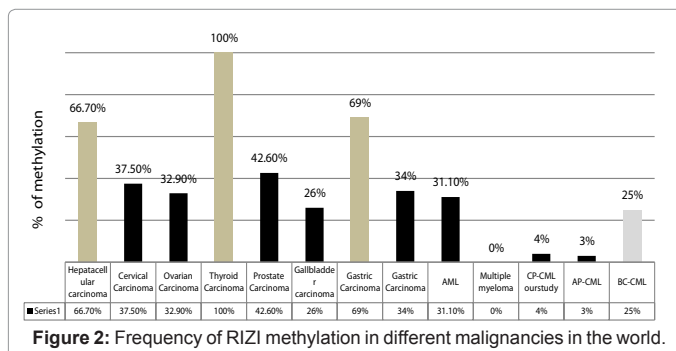

Figure 2: Frequency of RIZI methylation in different malignancies in the world.
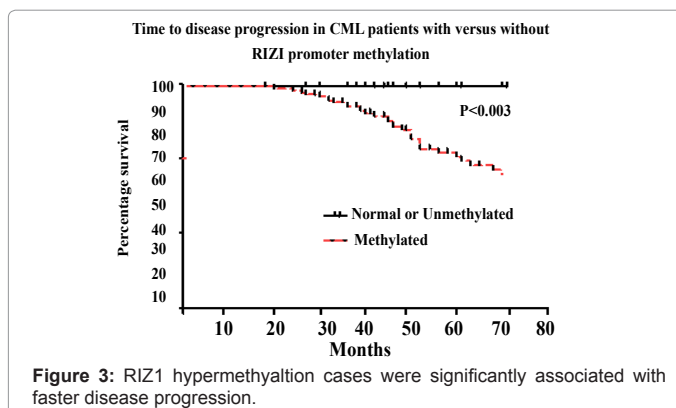

Figure 3: RIZ1 hypermethyaltion cases were significantly associated with faster disease progression.

In the CML blast crisis cell line, K562, the RIZ1 promoter is hypermethylated and addition of a methylation inhibitor, 5-aza-2'-deoxycytidine, induces RIZ1 expression [29]. In our study we found 9% of CML patients with RIZ1 promoter methylation, 25% of blastic phase cases were found methylated compared to 3.6% and 4.2% in accelerated and chronic phases respectively.

RIZ1 promoter methylation was methylated in 100% thyroid Carcinoma cases in USA, 79% Hepatocellular carcinoma cases in Japan, 66.70% hepatocellular carcinoma in China, 69% Gastric carcinoma in Japan ,30% in AML cases in Japan and 0% of multiple myeloma cases Honkong as depicted in the Figure 3.

Both genetic and epigenetic changes contribute to development of human cancer. Oncogenomics has primarily focused on understanding the genetic basis of neoplasia, with less emphasis being placed on the role of epigenetics in tumourigenesis. Genomic alterations in cancer vary between the different types and stages, tissues and individuals.

Moreover, genomic change ranges from single nucleotide mutations to gross chromosomal aneuploidy; which may or may not be associated with underlying genomic instability.

Collectively, genomic alterations result in widespread deregulation of gene expression profiles and the disruption of signalling networks that control proliferation and cellular functions. In addition to changes in DNA and chromosomes, it has become evident that oncogenomic processes can be profoundly influenced by epigenetic mechanisms. DNA methylation is one of the key epigenetic factors involved in regulation of gene expression and genomic stability, and is biologically necessary for the maintenance of many cellular functions. While there has been considerable progress in understanding the impact of genetic and epigenetic mechanisms in tumourigenesis, there has been little consideration of the importance of the interplay between these two processes. In this review we summarize current understanding of the role of genetic and epigenetic alterations in human cancer.

It is possible that gene silencing provides an alternative to BCR-ABL mutations in conferring imatinib resistance. Indeed, only about 50% of imatinib resistance can be conclusively traced to acquired mutations. It will therefore be worthwhile to study mutations and methylation simultaneously and determine whether there is an inverse correlation between the two events, and whether it is relevant to resistance to other tyrosine kinase inhibitors.

It is also interesting to consider the fact that DNA methylation can be partially reversed by treatment with decitabine or azacitidine. Decitabine has demonstrated single-agent activity in CML, [30] and a combination of decitabine and imatinib has shown a promising response rate in AP and BP. Given that many patients with blastic-phase CML continue to die of their disease, such drug combinations may be relevant even after therapy with second-generation tyrosine kinase inhibitors.

Conclusion

We conclude that aberrant methylation of RIZ1 gene is associated with disease progression and that DNA methylation could be a marker associated with imatinib resistance. Early detection of RIZ1 methylation may help identify a subset of CML patients that would benefit from treatment with demethylating agents.

Acknowledgment

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