Stem Cell Gene Mutation and MTHFR C677T Variants Increased “Risk” in Acute Myeloid Leukemia Patients

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Abstract

Acute Myeloid Leukemia (AML) is a genetic disorder designated as t(4;11) (q21;23) showing interaction between gene and environment. Stem cell markers (Oct4, Nanog3 & Sox2) are the transcription factors regulating pluripotency during early differentiation of embryo. The role of stem cells interaction with severity of disease has been poorly described. Hence, the curiosity has been developed to determine the frequency of stem cell gene mutation and their correlation with MTHFR C677T gene. Present findings reveals the variable frequency of Nanog3 i.e. up (55.0%), down (27.3%) regulation and 18.20 % complete disappearance (null) of band consist of 151bp. Oct4 (91.00%) and Sox2(64.00%) shows high prevalence of stem cell gene mutation. Similarly, Sox2 also showing variable frequency of over expression & regression (9.10%) in AML cases with respect to controls (13.33%) and showing lack of significant differences (p<0.05) using x² test. MTHFR (C677T) gene polymorphism showing variable frequency of genotypes CC(45.50%), CT(55.00%) between homozygous and heterozygous conditions, respectively. The study was further extended to confirm by chromosomal analysis, revealed 81.00% cases showing Philadelphia chromosome +ve t(4;11) (q21-q23) with 19.00 % cases involving chromosome breakage. Present study concluded that mutation of stem cell gene (s) may be consider as genetic marker for cellular heterogeneity, require to maintain pluripotency and confirming association due to heterozygous condition of MTHFR genotypes are responsible to increase “risk factor” in AML.

Keywords: Nanog3; Oct4; Sox2; Acute myeloid leukemia; MTHFR

Introduction

Since the discovery of Nanog3, Oct4 and Sox2 as stem cell markers a special attention has been drawn to maintain the pluripotency during differentiation of germ layers into different cell lineages in early embryonic life [1,2]. Acute Myeloid Leukemia (AML) is characterized by chromosome translocation t(4;11)(q21;23) and act as model of multi cell lineage system. The bone-marrow cells are characterized by accumulation of early myeloid cells which fail to further differentiate and mature. AML is the foremost human malignancy for which experimental support is required with clinical significance of stem cell gene mutation. In human, Oct4, Nanog and Sox2 are prime transcriptional factor for cellular proliferation, differentiation and maintenance of pluripotency in embryonic stem cells. The Oct4 gene primarily expressed, bind to Sox2 gene in immature cells or progenitor cells of bone marrow and mesenchymal cells [3-5]. The role of Sox2 as stem cell marker has been well documented in tumor and over expression in the combination with Oct4 play an important role in Glioma [6,7]. The role of Nanog3 is required to maintain pluripotency in embryonic cell, embryonic carcinoma, seminoma and germ cell neoplasia including cultured cells [8,9].

Stem cell regulation is an early event of cellular differentiation act as “genetic marker” for predicting diagnosis of multi cell lineage system. Hence, the present study becomes imperative with aim to determine the prevalence of Oct4, Nanog3 and Sox2 gene mutation to explore the etiopathology of AML patients. Although, little is known about the mechanism of interaction with other gene(s) such as MTHFR, the important candidate gene to regulate folate metabolism through irreversible conversion of 5,10-methylentetrahydrofolate to 5-methyltetrahydrofolate. Folate is an essential component of dietary supplement required for cell- proliferation. The mutant genotype (TT) variants of MTHFR lead to increase homocysteine are known to cause several malignancies [10-13]. Cytogenetic investigations required firmly for diagnosis with some favorable finding are involved in 50% of patients having normal karyotypes [14,15]. However, stem cells (SCs) gene might have influence cell proliferative and self renewal capacity to increase curiosity to establish the association between stem cell mutations associated “risk factor” in AML. Hence, the present study has been designed first time with the aims to determine stem cell gene regulation and their association with MTHFR gene required for the development of new therapeutics in medicine.

Materials and Methods

Collection of blood sample

Blood samples (1.0ml) of AML (n=11) were collected for cytogenetic and molecular genetic analysis from the O.P.D of S.S Hospital, I.M.S, B.H.U, with different age groups 7 to 65yrs along with their respective controls (n=15), after written consent of patients/attendant. The criteria for inclusion of an individual were based on clinically diagnosed AML. This study was approved by ethical committee of the Institute and samples were kept at -20°C, till further study.

Isolation of DNA and PCR based RFLP analysis

Genomic DNA was isolated from the whole blood using Bioner Kit (Korea). The details of forward & reverse primers for Nanog3, Oct4, Sox2 and MTHFR C677T and their sequences were documented in (Table 1). We have developed PCR specific strategies in total volume of 25 µl contain 50-100 ng of DNA , 20 pmole of each primer, 200 µM of each dNTPs mix with Taq buffer (10 mM Tris HCl pH 8.3, 50 mM KCl), 3.0 mM MgCl₂ and 3 unit of Taq polymerase (New England Biolab). RFLP analysis was carried out for MTHFR polymorphism. PCR

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product (6 µl) were digested at 37°C for 3 hr in reaction volume of 25 µl containing 1U of Hind-1 restriction enzyme (New England, Biolabs) and NEB buffer (2.5 µl). The digested product of RFLP was separated on 3% agarose gel, stained with ethidium bromide and bands were visualized and characterized on Gel Doc system (SR Biosystem).

**Cytogenetics study**

Short term lymphocytes cultures were setup for 72hrs at 37°C using RPMI-1640 supplement with 10% Serum (FBS), L-glutamine (0.5 µg/ml), phytohemagglutamin-M and antibiotics in triplicate under sterile conditions. These cultures were harvesting after adding colchicines (0.01 µg/ml) before 30 min to arrest the metaphase. Cells were fixed in Carony's fluid, air dried slides were used for GTG banding using trypsin (0.001%) in PBS. Chromosomal analysis were carried out by standard laboratory procedure, after staining with Giemsa (5.0%) and observed under 1000x magnification on Cytovision (Olympus, Japan).

**Statistical analysis**

The significance (p<0.05) differences between cases and controls group were evaluated using x² test. Statistical analysis was further carried out to compare the expected and observed value by using Hardy Weinberg equilibrium to determine the allele frequency (C/T). The odd-ratio was computed at 95% confidence interval to evaluate the “risk factor” between cases and controls.

**Results**

PCR based DNA analysis was carried out to screen mutational spectra of stem cell markers in terms of intensity of band and compare with controls. The present study was repeated three times to confirm the above findings. The optimal condition for amplification of DNA coding region of exon having length of the amplicons 577bp, 151bp and 236 for the exploration of the Oct4, Nanog3 and Sox2, gene respectively in human. Figure 1 showing up, down regulation and complete disappearance of the above findings. The value of calculated odd ratio in (heterozygote) CT genotype was O.R=3.3 at 95% C.I. (0.48-24.55) between cases and controls increase three folds. However, there was slight variation in individual allele frequency (C/T) between cases & control was also calculated using Hardy Weinberg equilibrium.

**Discussion**

In the present study we convincingly shows a district pattern of Oct4, Nanog3 and Sox2 gene(s) in AML cases as a pluripotent stem cell markers. Present, study showing interesting finding that highest frequency of upregulation (over expression) of Nanog3 was observed in most of the AML cases, whereas similar findings were also observed by Jeter et al. [16]. In few cases (18.20%) showing complete disappearance (null) of amplicon of 151bp (Nanog3), suggesting such extreme variation probably may be either due to the severity of the disease or unknown environmental factor. Thus Nanog3 gene regulation was highly variable in nature i.e. up-regulation (over expression) and down regulation in leukemia cases parallel finding as also reported by earlier workers [17], suggesting this mimicry of stem cell may be either due to different cell lineages in AML or interaction with other stem cell markers such as Sox2 or Oct4. However, it is still ambiguous that how these stem cell markers transcript "fusion protein", a characteristic feature of signal transduction mechanism might have influence by chromosomal translocation mutation. Interestingly, the Oct4 & Sox2 gene and over expression of Sox2 has also been required to maintain pluripotency of stem cell as suggested earlier [9]. The present findings are an evident of earlier scientist what they documented during early differentiation of embryonic life, suggested it is quite possible that 30% of the total leukemia cases cover from pediatric group associated early onset of such disease [18].

Present study is small but promising because showing first time inter relationship between stem cell gene mutation in acute myeloid leukemia patients. Schmitz et al. [19] also observed expression of Sox2 in glioma, while minimal expression in normal tissue. The variable expression with O.R=0.65 at 95% C.I. (0.20-11.602) in cases. The high frequency of complete disappearance (null) of Oct4 (91.00%) and Sox2 (64.00%) gene were observed in AML cases with calculated odd ratio for Oct4 (3.63) and Sox2 (2.05) with confidence interval (C.I.) at 95% (0.280-101.483) and (0.406-18.180), respectively as details were documented in (Table 2). Chromosomal analysis were also carried out in clinically diagnosed AML cases showing prevalence of positive Philadelphia chromosome (82.00%) in t(4;11) (q21-q23) and 18.00% cases showing chromosomal breakage in short arm of chromosome 3 and 6, although, in controls groups showing lack of such anomalies.

Table 3 depicted the details findings of MTHFRC677T gene polymorphism, reveals variable frequency of CC genotype (45.50%) and CT (55.0%) in cases, while controls showing CC (73.50%) and CT (26.70%) between homozygous and heterozygote conditions, respectively. The homozygous mutant or rare genotype TT was completely absent in cases and their respective controls as shown in (Figure 2). The value of calculated odd ratio in (heterozygote) CT genotype was O.R=3.3 at 95% C.I. (0.48-24.55) between cases and controls increase three folds. However, there was slight variation in individual allele frequency (C/T) between cases & control was also calculated using Hardy Weinberg equilibrium.

**Stem cell markers**

<table>
<thead>
<tr>
<th>Sequences (forward &amp; reverse)</th>
<th>Base pair(bp)</th>
<th>Annealing Temperature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4 5'- CGACCTATCGCCCCCTTGGAG -3' 5'- CCCCTGCTCCCCCATCTCCA-3</td>
<td>577</td>
<td>60°C/1 min</td>
<td>Henderson JK, et.al. 2002</td>
</tr>
<tr>
<td>Sox2 5'- GGGCTACGTCACCATGATCC-3</td>
<td>236</td>
<td>60°C/30 sec</td>
<td>Bhatia B, et.al. 2011</td>
</tr>
<tr>
<td>Nanog3 5'- CTTGCTA AAG GCT CCT GTG-3</td>
<td>151</td>
<td>56°C/30sec</td>
<td>Nethersheim D, et.al. 2011</td>
</tr>
<tr>
<td>MTHFR C677T 5'- TGA AGG AGA AGG TTG CCT GTG CCG GA-3' 5'- TGA GAG TGG GGT GCA GGA AGC TT-3</td>
<td>195</td>
<td>58°C/1min</td>
<td>Frosst P, et.al. 1995</td>
</tr>
</tbody>
</table>

Table 1: Selection of primers, sequences & their annealing temperature used for PCR analysis in acute myeloid leukemia.
The increase frequency in heterozygous condition might be evident of getting high frequency of CT genotype due to heterogeneous conditions associated with severity of proband-1. Variations in the frequency of stem cell gene mutation responsible for changing expression of the "fusion protein" associated with severity of disease, and 2. Heterozygocity of MTHFR C677T genotype increased "risk factor" either as an independent or together with other stem cell gene

Table 2: Frequency of stem cell gene mutation & their odd ratio between cases & controls.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Cases</th>
<th>Controls</th>
<th>O.R at 95% C.I</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>45.50</td>
<td>73.50</td>
<td>0.303 (0.041-0.756)</td>
<td>0.149</td>
</tr>
<tr>
<td>CT</td>
<td>55.00</td>
<td>28.70</td>
<td>3.300 (0.46-24.550)</td>
<td>0.149</td>
</tr>
<tr>
<td>C</td>
<td>0.08</td>
<td>0.13</td>
<td>0.767 (0.056-2.402)</td>
<td>0.226</td>
</tr>
<tr>
<td>T</td>
<td>0.03</td>
<td>0.02</td>
<td>2.357 (0.377-15.586)</td>
<td>0.283</td>
</tr>
</tbody>
</table>

Table 3: MTHFR C677T gene showing frequency of genotypes between homozygous and heterozygous condition and their individual allele frequency between cases and controls.

frequency of Oct4, Nanog3 & Sox2 gene mutation clearly evident of gene-gene interaction and might be responsible to modulate the activity of "fusion protein" either due to multiple cell lineages system or through other stem cell gene regulation as documented in earlier, leukemia cases [18,20].

Environmental factors such as folate, an essential dietary supplement regulate MTHFR C677T gene polymorphism and their genotypic variants are responsible to increase the risk factor in neural tube defects [11]. Present findings showing lack of rare genotype in homozygous (TT) condition either due to small sample size or due to low folate levels might have responsible to increase "risk" for AML cases as Jiang et al. [4] also found similar observations. Our study is also evident of getting high frequency of CT genotype due to heterogeneous group of population belongs to different ethnic background might have responsible to decline folate supply and to increase "risk factor". Although, these variation of MTHFR genotypes between cases and control has also been worked out by Franco and Schnakenberg [21,22].

Apparently, further karyotypes, MTHFR gene variation and stem cell gene mutation clearly showing an example of gene - gene interaction suggesting, the increase frequency in heterogeneous condition might have responsible to increase "risk factor" in AML cases. These sequential changes of stem cell gene mutation lead to modulate signal transduction mechanism in different cell lineage in AML cases.

Conclusion

We concluded from the present study that there are two possible conditions associated with severity of proband-1. Variations in the frequency of stem cell gene mutation responsible for changing expression of the "fusion protein" associated with severity of disease, and 2. Heterozygocity of MTHFR C677T genotype increased "risk factor" either as an independent or together with other stem cell gene

References


