Association between (GT)n Repeats in Heme Oxygenase-1 Gene Promoter and 3-Year Survival of Patients with Acute Leukemia: a Controlled, Cross-Sectional Study

Mohammad Kazemi1,2,3, Farinaz Khosravian3, Amir Abbas Sameti4, Alireza Moafi5, Mohammad Reza Merasi6, Mansour Salehi1,2,3, Majid Nejati7, Mohaddeseh Behjati8

1Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
2Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Non-Communicable Disease, Isfahan University of Medical Sciences, Isfahan, Iran
3Genome Medical Genetics Center, Isfahan University of Medical Sciences, Isfahan, Iran
4Isfahan Dental Student Research Committee, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran
5Department of Pediatric Hematology, School of Medicine, and Child Health Promotion Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
6Department of Epidemiology and Biostatistics, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran
7Department of Pediatric Hematology, School of Medicine, and Child Health Promotion Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
8Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran
9Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences, Tehran, Iran

Corresponding Author: Mansoor Salehi, Ph.D., Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
Tel: +98 31 36687898
Email: m_salehi@med.mui.ac.ir

ABSTRACT

Background: Acute leukemia is a common pediatric cancer. Novel strategies for treatment of acute leukemia have been developed, but treatment resistance is remained as the most problematic issue. It is hypothesized that the HO-1 gene up-regulation is responsible for tumor resistance to chemotherapy or radiotherapy-induced apoptosis. The levels of HO-1 expression are related to (GT)n microsatellite polymorphisms in the location of its promoter. This study designed to compare allelic frequencies of (GT)n microsatellite polymorphisms in HO-1 gene between acute leukemia patients and healthy controls. Indeed, 3-year disease-free survival was also evaluated.

Methods: Sixty-three patients with acute leukemia and seventy healthy infants were included in this study. We used the medical records of patients to collect information about survival after chemotherapy. The number of GT repeats in HO-1 promoter was determined by an ABI 3100 sequencer.

Results: The HO-1 GT repeats ranged from 14 to 34 with peaks at 27 repeats in both cases and controls. Children with longer alleles ((GT)n ≥ 27) had enhanced 3-year survival rate after treatment with chemotherapy or radiotherapy (P<0.05).

Conclusion: Although no significant differences were observed between leukemia patients and controls regarding allelic frequency, we found elevated frequency of “LL” genotype in leukemia patients with good prognosis and 3-year surveillance. Radiotherapy and chemotherapy might elevate the expression levels of HO-1 with subsequent increased resistance of leukemia patients to therapy.

Keywords: Acute leukemia, Survival, GT repeats, Heme-oxygenase-1 gene promoter
INTRODUCTION

Acute Leukemia is the most common pediatric cancer\textsuperscript{1,2}. Despite numerous efforts focused on leukemia treatment, this type of disease remains one of the greatest challenges in pediatric oncology\textsuperscript{3}. Current therapeutic strategies are unable to provide long-term remission for 20% of children with ALL (25-40% cure rate after relapse)\textsuperscript{4}. Some subgroups of infants are likely to be over treated and might be healed using less intensive regimens which results in reduced toxicity and fewer long-term side effects\textsuperscript{5-7}. Response to treatments is variable depending on different clinical, immunological, cytogenetic/genetic characteristics and environmental exposures, which are associated with increased risk of leukemia and their diverse response to treatment\textsuperscript{8-12}. One of the mentioned genetic factors is Heme Oxygenase-1 gene (HO-1), located on chromosome 22. This gene produces an essential enzyme in heme catabolism. It has 2 isozymes, an inducible heme oxygenase-1 and a constitutive heme oxygenase-2\textsuperscript{13}. \textit{HMOX1} and \textit{HMOX2} belong to the heme oxygenase family. Heme Oxygenase (HO) gene encodes an enzyme which catalyzes the first and rate-limiting steps in oxidative degradation of heme to the form of open-chain tetrapyrrole biliverdin-IX with final release of carbon dioxide, biliverdin and free iron. By now, three isozymes of mammalian HO have been identified, in which \textit{HO-1} variant is the only inducible isozyme with expression in different normal and neoplastic cells. \textit{HO-1} expression in malignant tissues is higher than its surrounding healthy tissues which could be up regulated by different stimuli and oxidative stresses such as radiotherapy, chemotherapy and photodynamic therapies\textsuperscript{14-20}. \textit{HO-1} is involved in pathogenesis of acute leukemia patients and their resistance to treatment\textsuperscript{8-11}. Expression of HO-1 has the protective and anti-apoptotic effects through its active biological products\textsuperscript{21,22}. As a result, it's possible that the upregulation of HO-1 is responsible for increasing tumor resistance to chemotherapy or radiotherapy\textsuperscript{23,24}. However, \textit{HO-1} expression level is dependent to its polymorphisms and fluctuates between individuals, quantitatively\textsuperscript{25,26}. GT repeats in \textit{HO-1} gene promoter modulate transcription of \textit{HO-1} gene. The dinucleotide sequence can adversely affect the basal promoter activity\textsuperscript{27-30}. GT repeats ranged between 12 - 40 base pairs. This range can be divided into short “S” and long “L” alleles although there is not an exact cut-off to separate two alleles\textsuperscript{16,31,32}. Moreover, some surveys revealed higher prevalence of “S” allele in renal cell carcinoma, pancreatic cancer, gastric cancer and gastric adenocarcinoma\textsuperscript{33}. Paradoxically, patients with squamous cell carcinoma and lung adenocarcinoma often carry “L” allele\textsuperscript{16,31,34-37}. Some studies revealed that \textit{HO-1} targeting with either pegylated zinc protoporphyrin (PEG-ZnP) or styrene maleic-acid–encapsulated ZnP (SMAZnP) results in growth inhibition in cancer cells with subsequent increased sensitivity to radiotherapy and chemotherapy\textsuperscript{22,38-40}. Despite advent of novel strategies for treatment of acute leukemia, resistance to treatment is the greatest concern until now. \textit{HO-1} over expression provides cytoprotective and antiapoptotic effects on malignant cells against radiotherapy and chemotherapy. Although the role of \textit{HO-1} in AML and CML cell lines has been studied, the distribution of allele frequency in leukemia and its association with resistance to treatment and survival remains unveiled\textsuperscript{10}. This study was designed to compare the allelic frequencies of genetic variants in the promoter of \textit{HO-1} among patients suffering from acute leukemia compared with healthy controls. The relationship between GT repeats and patient’s 3-year and 5-year survival was also assessed.

MATERIALS AND METHODS

Patient selection
This is a controlled, cross-sectional study that included 63 patients with acute leukemia admitted to Seyed-al-Shohada Hospital, Isfahan from 2006 to 2009. The control group consisted of 54 gender and age-matched healthy children. Our data about survival after chemotherapy and bone graft were gathered from patients’ medical records. DNA was extracted from bone marrow samples of the patients. Genomic DNA was extracted through QIAamp DNA blood mini kit (QIAGEN, USA).
according to the manufacturer’s instruction. The study protocol was approved by the Ethics Board of Isfahan University of Medical Sciences.

**Heme oxygenase-1 genotyping**
The HO-1 microsatellite was amplified through PCR using a 5'-FAM-labeled forward primer (AGAGCCTGCAGCTTCTCAGA) and a 3'-unlabelled reverse primer (GTGCTATGGCCAGACTTTGT), in 30 cycles (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) and final extension at 72°C for 5 minutes by thermo cycler (Eppendorf Mastercycler EP Gradient, USA). Labeled PCR products were compared with a standard size marker GenoType™ TAMRA DNA ladder (size range 50–500 bp) (Gibco-BRL, Paisley, Scotland, UK). Number of GT repeats was determined with an ABI 3100 sequencer (Applied Biosystems, USA), using GeneMarker V1.97 software (Softgenetics, USA).

**Statistical analysis**
Statistical analyses were performed using SPSS 16.0. Chi-square test was used for categorical variables. Three-year survival was compared with allelic frequencies of HO-1 polymorphism. Differences in allelic frequency of HO-1 promoter were compared between leukemia patients and controls. Difference of data with $P<0.05$ was considered as a significant.

**RESULTS**
Demographic characteristics of patients including age and sex are shown in Table 1. Frequency distribution of age and sex was not significantly different between studied groups. Patients whose data were incomplete were excluded from the study.

| Group | Case | control | P-value |
|-------|------|---------|---------|
| Age (years) Mean ±SD | 5.93 ±0.63 | 4.87±0.26 | >0.05 |
| Gender (%) | | | |
| F | 59.5 | 46.2 | >0.05 |
| M | 40.5 | 53.8 | |

The HO-1 genotyping process for 63 cases and 54 healthy controls was performed successfully. The alleles frequency of (GT)$_n$ microsatellite polymorphism of HO-1 gene promoter was found in both case and control groups (Figures 1, 2 and 3). The GT repeat numbers ranged from 14 to 34, and the most prevalent allele in our population was (GT)$_{27}$. Therefore, we divided these alleles into two subgroups. (GT)$_n$ : short (S) alleles <27 repeats and long (L) alleles ≥27 repeats. Three genotypes (SS, SL and LL) were created for the study population. The subjects with genotype SS and SL were placed in a group identified as "short carrier genotype" and those with LL genotype were placed in a group identified as "long genotype".
Figure 1: Frequency distributions of (GT) repeats in all participants (n=117)

Figure 2: Frequency distributions of (GT) repeats in leukemia patient (n=56)
There was no significant difference in the frequency of genotypes between leukemia patients and controls ($P=0.629$) (Table 2). The "long genotype" was more frequent in leukemia patients with better vs. worse 3-year surveillance ($P<0.05$) (Table 3). Furthermore, there was no significant difference in hepatomegaly, splenomegaly and CSF metastasis in two genotype groups ($P \geq 0.05$) (Table 4).

![Figure 3: Frequency distributions of (GT) repeats in control group (n=61)](image)

| Genotypes | P-value |
|-----------|---------|
| LL        | 33.3%   |
| LS        | 47.6%   |
| SS        | 19%     |

* (GT)$_n < 27$ Repeats: $S$ allele, (GT)$_n \geq 27$ Repeats: $L$ allele, SS: Homozygote for Short Allele, LL: Homozygote for Long Allele, LS: Heterozygote Person

| Genotype | P-value |
|----------|---------|
| S carrier | LL      |
| Yes      | 38.8%   |
| No       | 27.7%   |

| Genotype | P-value |
|----------|---------|
| S carrier | LL      |
| Yes      | 39.3%   |
| No       | 30.3%   |

* (GT)$_n < 27$ Repeats: $S$ allele, (GT)$_n \geq 27$ Repeats: $L$ allele, SS: Homozygote for Short Allele, LL: Homozygote for Long Allele, LS: Heterozygote Person
DISCUSSION

In this study, we investigated polymorphic (GT)ₙ repeats in HO-1 promoter region in patients suffering from leukemia. In addition, we investigated the association between allelic frequency and patients’ 3-year surveillance. We found no relationship between frequency distribution of (GT)ₙ repeats and leukemia incidence. We have observed that patients carrying “LL” allele had significantly better 3-year survival. Five-year survival was slightly better in carriers of “LL” allele although this difference was not statistically significant. Moreover, we studied genotype distribution among patients considering hepatomegaly, splenomegaly and CSF metastasis (Table 4), which revealed no relationship between (GT)ₙ repeats in HO-1 promoter and clinical features of patients.

HO-1 gene is induced in response to oxidative stress such as chemotherapy and radiotherapy. The basal and induced levels of HO-1 gene expression are different in each individual. The polymorphism length of HO-1, influences the level of gene transcription. Excessive GT repeats reduced promoter activity, while short GT alleles led to higher levels of HO-1 expression with further anti-apoptotic effects. Our results suggest that “SS” and “SL” genotypes make tumors more resistant to anticancer therapy in acute leukemia.

HO-1 considered as a “friend” which supports normal tissues against carcinogen-induced invasion. Nevertheless, during the neoplastic development, HO-1 turns into a “false friend” and facilitate tumor development. HO-1 over expression in CML tumor cells increases its viability through apoptosis inhibition.

Meyehof et al. have treated CML cells by zinc-(II)-deuteroporphyrin-IX, inhibitor of HO-1 and hemin, inducer of an HO-1. Parallel usage of HO-1 inhibitors and inducers, decreases and increases CML cells viability in stress oxidative environment, respectively. Also, it has been apparent that HO-1 reduces sensitivity of cancer cells to chemotherapy and radiotherapy.

The length polymorphism of GT repeats in HO-1 gene promoter had various impacts in different disease states. Chang et al. reported an increased risk of oral squamous cell carcinoma in patients with more than 31 GT-repeats. Moreover, some surveys revealed a higher frequency of "S" allele in renal cell carcinoma, pancreatic cancer, gastric cancer, gastric adenocarcinoma, lymphoma and Kaposi sarcoma. In this study, the frequency of “S” alleles and “L” alleles were equal in both leukemia patients and control groups.

This study is the first to investigate HO-1 gene promoter polymorphism and surveillance (3 years) in leukemia patients. Although we found no significant differences between leukemia patients and control groups regarding short (GT) n repeat alleles in HO-1 promoter, higher frequency of “LL” genotype in leukemia patients with positive 3-year surveillance was found. Indeed, LL genotype in leukemia patients was associated with slightly better 5-year surveillance.

Oxidative stress conditions such as radiotherapy and chemotherapy lead to additional expression of HO-1 gene. These data supports the idea that higher levels of HO-1 gene expression, associated with “SS” and “SL” genotype, might play an important role in elevated resistance of leukemia patients to chemotherapy or radiotherapy.

| Genotypes       | Hepatomegaly | S carrier | LL |
|-----------------|--------------|-----------|----|
| (GT)ₙ (+)       | 21.0%        | 15.85     | 0.142 |
| (GT)ₙ (-)       | 50.0%        | 13.1%     |     |
| Splenomegaly    |              |           |     |
| (GT)ₙ (+)       | 40.5%        | 16.2%     | 0.571 |
| (GT)ₙ (-)       | 29.7%        | 13.5%     |     |
| CSF Metastasis  |              |           |     |
| (GT)ₙ (+)       | 2.9%         | 0.0%      | 0.706 |
| (GT)ₙ (-)       | 67.6%        | 29.4%     |     |

* S carrier: a person who is carrier for S allele, LL: Homozygote for L allele, CSF Metastasis: Metastasis to Cerebrospinal fluids (CSF)
CONCLUSION

Modification of HO-1 expression level might have positive effect on the prognosis of leukemia patients under treatment. Further studies are required to determine the impact of HO-1 genotype in patients with acute leukemia.

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CONFLICT OF INTEREST

Authors declare any conflict of interest.

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