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Aberrant DNA Methylation in P1 Promoter Region of *Tp53* Gene in Acute Leukemia

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Abstract

The present study was undertaken to investigate the aberrant DNA ethymlation in P1 promoter region of *p53* gene in acute leukemia patients. The aberrant DNA methylation of *Tp53* gene in fresh leukemia cells obtained from 31 newly diagnosed patients as well as monocyte leukemia U937 cells were detected by using polymerase chain reaction (PCR). The results revealed that aberrant DNA methylation in P1 promoter region of *Tp53* was detectable in 12 cases of 31 leukemia patients (38.7%) as well as in U937 cells, while no aberrant DNA methylation of this gene was detected in normal control group (11 healthy volunteers), indicating that there was significant difference between acute leukemia patients and healthy donors (P=0.0183, Fisher's exact test). Furthermore, no significant difference was found in acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) patients (35.2% *vs* 42.8%, P=0.7241, Fisher's exact test). Our results, for the first time to our knowledge, provide laboratory evidence that aberrant DNA methylation in P1 promoter region of *Tp53* gene is an common phenomenon in both AML and ALL patients, and that the significance of this special DNA methylation in acute leukemia needs further and profound investigation.

Keywords: *p53* gene; DNA methylation; Leukemia

Introduction

Tp53 gene, located on the short arm (17P13.1) of human chromosome 17 with its 20kb's length, contains 11 extrons and 10 introns, encoding for the intranuclear phosphoprotein *p53*, which consists of 393 amino acids and combines proteins with proteins or DNA [1]. It is one of the oncogenes that being studied most widely and most comprehensively. More than 50% of human tumors are related to the structural changes or inactivation of *p53* gene. It is well known that *p53* gene acts as negative accommodation factor in the cell life cycle and plays important roles in cell-cycle regulation, DNA repair, cellular differentiation, apoptosis, aging, vascularization, etc. So *p53* gene is also called "the defender of genes" [2]. However, it is believed that the function of *p53* gene is often altered in cancer. It loses its biological activity when mutation, deletion or modification in epigenetic inheritance occurs [3,4]. Epigenetic inheritance is a phenomenon that cells transmit non-DNA information during the process of mitosis or meiosis. Through this way, gene expression can be affected while DNA sequences remain unchanged. It involves DNA methylation and histone modification. Alterations of these effects may result in at least three abnormalities, such as anti-oncogene inactivation, proto-oncogene activation, imprinted gene loss, etc. In mammals, methylation mainly affects the 5'-carbon atom of cytosine in 5'-CpG-3'structure. CpG islands, a 200-bp stretch of DNA with a C+G content of 50% and an observed CpG/expected CpG in excess of 0.6, are usually markers of the so-called "house-keeping genes" and the regulative sequences in 5' region of organism specific genes [4-7].

Although *TP53* does not have a canonical CpG island [8] in the 5' region of *p53* gene, it has a promoter region with 16 CpG dinucleotides (nucleotides (nt) 631–950,provided the major transcriptional site as no. 843; NCBI GeneBank database accession no. X54156) [8], which contains a basal promoter region (85 bp; nt 760–844) that is essential for full promoter activity [7]. Schroeder and Mass [9] have shown that methylation in the promoter region of the *p53* gene reduces reporter gene activity. They found down-regulation of *p53* in cultured cells transfected with a plasmid incorporating a *p53* promoter containing methylated CpG dinucleotides. Pogribny et al. [10] found that alterations in single-site CpG methylation in the *p53* promoter region were associated with reduced *p53* expression. It is indicated that methylation of the CpG dinucleotides in *p53* gene's promoter region may probably lead to gene inactivation. Malone CS found that methylation on mC C (A/T) GG sites may represent a new type of epigenetic modification on mammalian DNA and in mature B cell lymphoma, the B cell specific *B29* gene silencing may be related to the methylation of these sites [11,12]. There are three CCWGC sequences (nt 756, 858, and 940, NCBI ID is X54156) in P1 promoter region of *p53* gene. Xabier Agirre [13,14] and his co-workers proved in their research that in the progression of ALL, CCWGC methylation may induce *p53* gene inactivation. Few studies have hitherto dealt with hypermethylation in the *p53* gene in haematological malignancies. Nevertheless, the subject might be important in leukaemogenesis. Till now, methylation of *p53* gene's promoter has been detected only in a few malignant tumors, such as glioma [15] and ALL [13,14]. It is still unclear whether this methylation exists in AML. Our study is aimed to find out the occurrence of *p53* gene promoter methylation in acute leukemia and compare the difference between AML and ALL.

Materials and Methods

Materials

Bone marrow samples were aspirated from 31 patients presenting with AML (17) including M1 (1), M2 (2), M3 (5), M4 (1), M5 (5) and chronic leukemia in blast crisis of M5 (1)M6 from M2 (1) and ALL (14), acute mixed lineage leukemia (1). Of all the patients, 18 were males and 13 were females. Their average age is 30 years (15–78). All of them were randomly taken from the Second and the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou China. 11 cases with normal bone marrow were also enrolled to form a normal control group. The control subjects, of whom 6 were males and 5 were females, were 15–60 years in age with an average age of 31 years. U937 leukemic cell strain is conserved in our own department lab. Specifically, the diagnosis of the 31 patients was made by their symptoms and signs, morphological findings of bone marrow samples, as well as histochemical stain and flow cytometry results, which were recommended in the diagnostic criterion of AML by WHO [16,17].

Reagent

DNA extraction kit contents (Omega Co. U.S.A), DNA restriction enzyme (*MspI, HapII, EcoRII, KOD-Plus Taq* enzyme (TOYOBO Co. JAPAN), DNA restriction enzyme *BstNI* (New England Bio lab), primer (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. China), DNA Marker (Tiangen Co. China), RM1640 (Gibico Co. U.S.A).

DNA extraction

Ethical approval to use residual bone marrow was obtained. DNA was extracted with salt fractionation method from the bone marrow specimens (2 ml) and stored at -80°C after being anti-coagulated by sodium citrate. DNA soluted in double distilled water is tested for concentration as well as purity by DU640 DNA/RNA/Protein purity determination (Beckman, Co. U.S.A). The specimens with purity in 1.7-1.9 were stored at -30°C for preparation (see below). U937 leukemic cell strain is sub cultured with medium containing 10% fetal bovine serum and extract DNA until the cell line is in the log phase.

Cleavage of DNA restriction enzyme

MspI and *HapII* can recognize and cleave the same sequences (C↓CGG) at the slash while *BstNI* and *EcoRII* can work at another same one ↓CC (A/T)GG. The difference lies in that the *HapII* and *EcoRII* are sensitive to methylation while *MspI* and *BstNI* are not. If the cytosine at the cleaving site is methylated, the sensitive *HapII* and *EcoRII* can cleave the sequences while *MspI* and *BstNI* cannot. 1 μg of each DNA sample with purity of 1.7-1.9 was mixed with *MspI* (2 μL), *HapII* (1μL), *BstNI* (1μL), *EcoRII* (2 μL) respectively and fresh buffer to form a mixture with a fixed volume of 50 μL. After being instantaneously centrifuged, the mixture was incubated for 16 hours at 37°C (*MspI, HapII*, and *EcoRII*) and 60°C (*BstNI*), respectively. After denaturalizing the enzymes by high temperature, we stored the mixture containing cleavage products at -30°C for preparation.

PCR amplification

We detected the *p53* gene and ß-actin gene in genome DNA and cleavage product for each specimen. Firstly, we determined the primer of *P53* gene according to the citation [13,14] and then improved *p53* and ß-actin gene (NCBI Gene Bank accession no. is BC013835) with Primer 5.0 software. Then, we found out the sequences which cannot be recognized and cleaved by the above 4 DNA restriction enzymes, and designed the corresponding primers for them respectively and specifically (Figures 1 and 2). Of all the designed primers, we chose the one with Tm index coincident with the target gene. In addition, the chosen primer's product must bear a 100-bp difference with the target gene. Finally, we determined the sense primer of *P53*: 5' GGCGGATTACTTGCCCTTAC 3'Anti-sense primer: 5' AGCCCGTGACTCAG AGAGGAC 3'; primer of ß-actin as the intra-reference gene: sense primer: 5' GTCCACCGC AAA TGCTTCTA 3' Anti-sense primer: 5' GCAATGCTATCA CCTCCCCT 3'. 50 μl of each mixture contained 3 μl cleavage product or 2 μl genomic DNA, 2 μl primer of *p53* or ß-actin (10umol/L), 5 μl 10xdNTP, Mg2+2μl (25 mmol/L), 5 μl 10X pcr buffer, 1 μl KOD-Plus *Taq* DNA polymerase and double distilled water. After being instantaneously centrifuged, PCR reaction was carried out with 33 cycles of the following thermal profile: 95°C for 10 min, 94 for 45 s, 68°C for 30 s, and 68°C for 10 min. (Gene Amp PCR System 2700, U.S.A). PCR products were stored at 4°C. We mixed 5 μl PCR products of each sample and 1 μl 6x loading buffer. Then the mixture containing PCR products were run on 2% agarose gel electrophoresis for 40 min(100V) followed by ethidium bromide staining. After staining, the gels were carefully analyzed in order to determine the main fragments generated by specific primers in comparison with the molecular size of anticipated amplification length of *P53* and ß-actin as 287 bp and 393 bp respectively by ultraviolet spectrophotometer (UVP-GDS8000 Beckman, Co. U.S.A. Corresponding chart and graph data was shown below. Parts of PCR products were also sequenced followed by being verified through Blast software on NCBI.

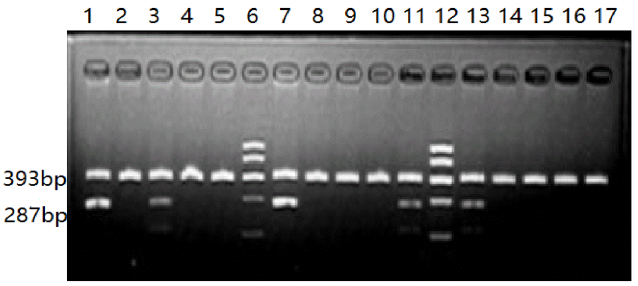

Figure 1: Electrophoresis results of PCR products. Lanes 1-5: Samples with 883 site methylation, Lane 1: PCR amplification products from DNA genome template, Lanes 2-5: PCR amplification products from cleaved DNA template by *MspI, HapII, BstNI, EcoRII* respectively, Lanes 7-11: Samples with 756, 858, 940 sites methylation, Lane 7: PCR amplification products from DNA genome template, Lanes 8-11: PCR amplification products from cleaved DNA template by *MspI, HapII, BstNI, EcoRII* respectively, Lanes 13-17: Samples from the normal controls, Lane 13: PCR amplification products from DNA genome template, Lanes 14-17: PCR amplification products from cleaved DNA template by *MspI, HapII, BstNI, EcoRII* respectively, Lanes 6 and 12: DNA marker, binds represent DNA fragments with a 600, 500, 400, 300, 200,100-bp length, respectively, in a downward order.

Statistical analysis

We compared the methylation rate between acute patients and normal people, also 5 ALL and AML. Statistical analysis was calculated using Stata software (SAS 8.0 software). Fisher's exact test was employed to compare the methylation rate between groups. An effect was considered statistically significant if the P value was 0.05 or less.

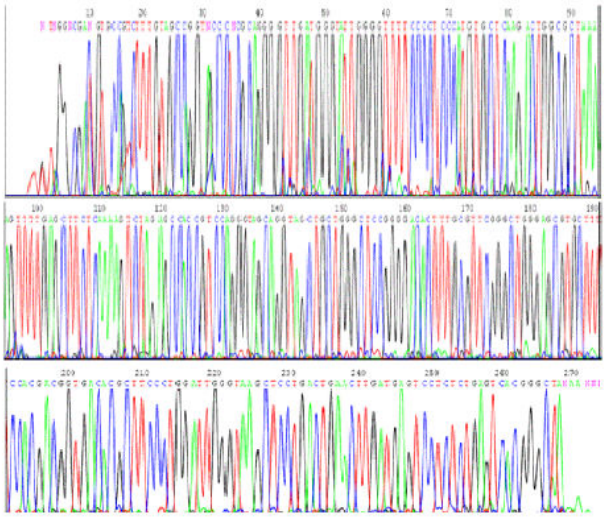

Figure 2: Partial sequencing results of *p53* gene.

Results

In detected samples, if the target gene can be amplified in the genomic DNA, we could conclude that the case had no deletion in *p53* gene. Adversely, the patient might have deletion in *p53* gene and should certify by other methods include fluorescent *in situ* hybridization. The normal template containing enzyme *HapII* cutting site could generate the target gene. If the genomic DNA containing *MspI* cutting site could not generate the target gene, this indicates that *p53* gene was methylated at the site of nt883 (NCBI ID: X54156). If the specimen with *EcoRII* cutting site could amplify target gene, while the one with *BstNI* could not, it might indicate that *p53* gene was methylated at the site of nt 756, 858, and 940 (NCBI ID: X54156). If every sample in the same group could all amplify target band, it might indicate that all the four DNA restriction enzymes could not work on their specific site because of other mutation.

	Positive	Negative	Total (n)
Acute leukemic samples	12	19	31
Normal samples	0	11	11
P-value derived from Fisher's exact test, P=0.0183<0.05			

Table 1: Methylation between acute leukemia and normal volunteers.

As shown in Table 1 and Table 2, 12 samples among 31 from patients with acute leukemia and U937 leukemic cell strain was methylated on *p53* gene. The prevalence of methylation in acute leukemia group was 38.7%, of which ALL is 42.8% (6 of 14) and AML 35.2% (6 of 17). There was no *p53* gene methylation detected in the normal control group. There is significant difference between the prevalence of methylation between the acute leukemia group and the normal control group (Fisher's exact test, P=0.0183). But the prevalence between ALL and AML was not significantly different (Fisher's exact test, P0.7241).

	Positive	Negative	Total (n)
ALL	6	8	14
AML	6	11	17
P-value derived from Fisher's exact test, P=0.0183<0.05			

Table 2: Methylation difference between ALL and AML.

Discussion

Acute leukemia is clonal malignant hematopoietic disorders that result from genetic alterations in normal hematopoietic stem cells. The malignant cells lose their ability to mature and differentiate. They proliferate in an uncontrolled fashion and replace normal bone marrow elements. Aberrant methylation patterns are a common event in human neoplasm and constitute a functionally equivalent mechanism to classical genetic alterations. It is found for many genes and occurs in almost all cancer types. Especially in hematopoietic neoplasms, DNA methylation abnormalities have emerged as one of the most frequent molecular changes and play critical roles [18,19]. The pathologic effect of epigenetic modification in the formation of tumor does not contradict with the mutational hypothesis about oncogenesis. Otherwise, it provides an alternative approach for the etiopathogenesis research without altering DNA sequences. Because pigenetic changes are potentially reversible, they make attractive targets for therapeutic intervention. Therefore, a thorough understanding of epigenetic regulation and the identification of loci involved in dysregulation are critical for the rational use of demethylating agents and histone deacetylase inhibitors in cancer patients. In addition, DNA methylation alterations can also be exploited as biomarkers for monitoring treatment efficacy and minimal residual disease. Since aberrant is a DNA methylation reversible modification, it represents an attractive therapeutic target. In clinical studies favourable results were achieved with the usage of methylation inhibitory agents (the azanucleosides: 5-azacytidine and 5-aza-2_-deoxycytidine (decitabine)) [20,21]. These compounds covalently bind DNMT1, resulting in depletion of enzymatic activity. Histone deacetylase inhibitors allow an 'open' chromatin configuration and have been shown to be synergistic when used with demethylating drugs in some systems [22,23]. Unraveling the complexities of the interactions between DNA methylation and chromatin modification is critical for understanding their roles in tumor pathogenesis and for designing rational approaches for the use of epigenetic modifiers in cancer patients. By demethylation or using histone acetylating drugs, we can modify the methylated states of target genes in order to alter their transcriptional activities. Thus, with regard to biological medicine, it also provides a theoretical basis for new therapeutic research curing cancer, which shows greater attraction than genetic therapy. Furthermore, we have gained achievements in recent clinical trials that some of the targeting methylation drugs, such as decitabine and azacitidine, have shown their abilities to restrict the progression of cancer and improve patients' prognosis, which rationalize further

researches. Without question genetic alterations underlie the pathogenesis of cancer. In recent years, however, epigenetic alterations have gained increasing recognition as important participants in tumor development and progression. Epigenetic changes include DNA methylation and histone modifications (acetylation and methylation), which influence chromatin structure or modify the DNA without altering the native nucleotide sequence. Methylation of CpG islands in 5' promoter regions of certain genes is an approach to regulate gene expression. Generally speaking, methylation of promoter region of most genes results in gene inexpression, such as *p15* and *p16* genes [24]. Otherwise, some genes may be activated and alter their biological characteristics as a result of DNA demethylation. A case in point is the multi-drug resistant gene *MDR1* [25].

We used DNA restriction enzymes and PCR technique to detect the methylation of *p53* gene and assessed its prevalence in acute leukemia. In this study, we were unable to establish a direct correlation of methylation with gene expression due to the lack of sufficient RNA. Previous studies have shown that for *p53* methylation is associated with gene silencing [13,14]. Taken together, according to our results, we draw conclusions as follows: (1) The prevalence of *p53* gene methylation in acute leukemia is significantly higher than that of normal populations. (2) There is no significant difference between ALL and AML concerning the prevalence of *p53* gene methylation. Collectively, the data presented here in conjunction with the most recent literature indicate that the involvement of *p53* hypermethylation in cancerogenesis should be studied further, especially concerning the functional significance of this gene in the biology of acute leukemia.

Conflict of Interest

All authors of this research study disclose that they do not have any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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