MDM2 T309G has a Synergistic Effect with P21 ser31arg Single Nucleotide Polymorphisms on the Risk of Acute Myeloid Leukemia

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Abstract

Background: The P53 tumor suppressor gene plays a pivotal role in maintaining cellular homeostasis by preventing the propagation of genome mutations. P53 in its transcriptionally active form is capable of activating distinct target genes that contribute to either apoptosis or growth arrest, like P21. However, the MDM2 gene is a major negative regulator of P53. Single nucleotide polymorphisms (SNP) in codon Arg72Pro of P53 results in impairment of the tumor suppressor activity of the gene. A similar effect is caused by a SNP in codon 31 of P21. In contrast, a SNP in position 309 of MDM2 results in increased expression due to substitution of thymine by guanine. All three polymorphisms have been associated with increased risk of tumorigenesis. Aim of the study: We aimed to study the prevalence of SNPs in the P53 pathway involving the three genes, P53, P21 and MDM2, among acute myeloid leukemia (AML) patients and to compare it to apparently normal healthy controls for assessment of impact on risk. Results: We found that the P21 ser31arg heterozygous polymorphism increases the risk of AML (P value=0.017, OR=2.946, 95% CI=1.216-7.134). Although the MDM2 309G allele was itself without affect, it showed a synergistic effect with P21 ser/arg polymorphism (P value=0.003, OR= 6.807, 95% CI= 1.909-24.629). However, the MDM2 309T allele abolish risk effect of the P21 polymorphic allele (P value= 0.71). There is no significant association of P53 arg72pro polymorphism on the risk of AML. Conclusion: We suggest that SNPs in the P53 pathway, especially the P21 ser31arg polymorphism and combined polymorphisms especially the P21/ MDM2 might be genetic susceptibility factors in the pathogenesis of AML.

Keywords: Single nucleotide polymorphism - acute myeloid leukemia - P53 - P21 - MDM2

Introduction

The term acute myeloid leukemia (AML) refers to a group of marrow-based neoplasms that have clinical similarities and distinct morphologic, immunophenotypic, cytogenetic and molecular features. It is characterized by acquired genetic alterations which disturb the normal mechanisms of cell growth and result in the accumulation of leukemic cells in the bone marrow, ultimately replacing most of the normal hematopoietic cells and their functions (Bozzone, 2009).

P53 is a tumor suppressor gene, often referred to as the “guardian of the genome”. Under various cellular stress conditions, such as DNA damage, oncogenic insult, oxidant stress and hypoxia, P53 is activated inducing a variety of cellular protective responses (Miliani and Zhang, 2011). P53 induces cell cycle arrest, through the activation of its downstream effector P21, which gives the “stop signal”. Such stop signal gives enough time for DNA repair to take place. If DNA damage is beyond repair, P53 activates the apoptosis pathway, or the cell remains arrested until senescence (Read and Strachan, 1999).

P21 is a cyclin dependent kinase inhibitor, which causes arrest of the cell cycle in the G1/S checkpoint, through binding to proliferating cell nuclear antigen (PCNA). Induction of increased levels of P21 is caused by P53 up-regulation (Levine, 1997).

Under normal conditions, the level of P53 is kept constant through its major negative regulator the MDM2, which is a proto-oncogene. MDM2 binds directly to P53, inhibiting its transactivation activity (Haupt et al., 1997) and also ligates P53 to ubiquitin, making it a target for proteosomal degradation (Bond et al., 2005).

The P53 pathway is an important response to oncogenic stress, and p53 regulates its own intracellular levels through an autoregulatory feedback pathway with MDM2 (Bond et al., 2005). Loss of P53 function is caused by MDM2 overexpression, mutations, and other mechanisms, resulting in malignant transformation or carcinogenesis (Xiong et al., 2009). In the P53 pathway, P53, P21, and MDM2 play a crucial role together. Polymorphisms in P53-DM2 (Ara et al., 1990) and P53-P21 (Onel and Cordon-Cardo, 2004) have been reported to be associated with a variety of solid tumors, such as lung, esophageal,
colorectal, breast, and gastric cancer.

To the best of our knowledge, no previous studies investigated the possible role of these three gene polymorphisms and their gene-gene interaction in the pathogenesis of AML. In this work, we investigated the frequency of P53, P21, and MDM2 polymorphisms among AML patients referring to the National Cancer Institute (NCI) and in apparently normal healthy controls, aiming to describe the prevalence of such polymorphisms and detect whether or not they have implications for pathogenesis.

Materials and Methods

This study was carried out on 77 newly diagnosed AML patients who presented to the Medical and Pediatric Oncology Departments, NCI, Cairo University. Diagnosis was performed according to clinical, morphological, cytochemical and immunophenotypic examination. In addition, 72 apparently healthy individuals were included in the study as a control group. Informed written consent was obtained from all participants involved in the study or from their parents and approval of the IRB, NCI, Cairo University was obtained.

Cases were subjected to the following routine investigation

1-Thorough history taking. 2-Full clinical examination. 3-Complete blood picture. 4-Bone marrow aspiration and morphological examination using Romanowsky stain, supplemented with cytochemical stains such as Myeloperoxidase (MPO), Sudan Black B Stain (SBB), Esterases and Acid Phosphatase when indicated. 5-Immunophenotyping by Flow cytometry: to confirm the diagnosis of AML with a wide panel of myeloid markers (MPO, CD13, CD33, CD117, CD14 and CD15), lymphoid markers (CD10, CD19, CD22, CD79a, CD20, Cyto µ, Kappa and Lambda for B lymphoid series, and CD3, CD2, CD4, CD8, CD7 and CD5 for T lymphoid series) and the stem cell marker CD34 as well as CD56 and HLADR on routine basis.

Genotyping

DNA was isolated from peripheral blood and its concentration was measured as described by (Gupta et al., 1988). Genotyping for all studied loci was performed by PCR-RFLP method. Primers sequences, restriction enzymes and fragments obtained are presented in Table 1.

P53 arg72pro Polymorphism

PCR was performed in 25 µL containing 100 ng of genomic DNA, 0.5 µmol/L of primers (Table 1), 200 µmol/L dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 50 mmol/L KCl, and 1 U of Hot Start Taq DNA polymerase (Quiagen). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 45 seconds at 95°C, 45 seconds at 58°C, and 1 minute at 72°C. The last elongation step was extended to 7 minutes. The Arg→Pro substitution abolishes a restriction site on digestion with BstUI restriction enzyme (10 U). The resulting fragments were evaluated on a 3.5 % agarose gel at 100 volt for 30 min (Onel and Cordon-Cardo, 2004), showing 113bp and 86bp bands for the wild type and 199 bp, 113bp and 86 bp bands for heterozygous variant and the homozygous variant remains undigested showing 199 bp (Figure 1).

P21 ser31arg Polymorphism

P21 codon 31 ser/arg Polymorphism was characterized by the PCR-RFLP (Onel and Cordon-Cardo, 2004). DNA fragment of 225 bp was amplified in 25 µL containing 100 ng of genomic DNA, 0.5 µmol/L of primers (Table 1), 200 µmol/L dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 50 mmol/L KCl, and 1 U of Hot Start Taq DNA polymerase (Quiagen). After denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 95°C, 1 minute at 58°C, and 2 minutes at 72°C. The last elongation step was extended to 7 minutes. The presence of polymorphic variant arg results in abolishing the restriction site of Bsp I enzyme. The PCR product (5-10 µL) was digested with Bsp I (10 U, 37°C), and subjected to electrophoresis on a 2.5 % agarose gel at 100 volt for 30 min. The wild type (ser/ser) resulted in two 113bp and 86bp bands for the wild type and 199 bp, 113bp and 86 bp bands for heterozygous variant and the homozygous variant remains undigested showing 199 bp (Figure 1).

MDM2 T309G polymorphism

The MDM2 T309G polymorphism was determined by the PCR-RFLP (Onel and Cordon-Cardo, 2004). DNA fragment of 225 bp was amplified in 25 µL containing 100 ng of genomic DNA, 0.5 µmol/L of primers (Table 1), 200 µmol/L dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 50 mmol/L KCl, and 1 U of Hot Start Taq DNA polymerase (Quiagen). After denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 95°C, 1 minute at 58°C, and 2 minutes at 72°C. The last elongation step was extended to 7 minutes. The Arg→Pro substitution abolishes a restriction site on digestion with BstUI restriction enzyme (10 U). The resulting fragments were evaluated on a 3.5 % agarose gel at 100 volt for 30 min (Onel and Cordon-Cardo, 2004), showing 113bp and 86bp bands for the wild type and 199 bp, 113bp and 86 bp bands for heterozygous variant and the homozygous variant remains undigested showing 199 bp (Figure 1).

Table 1. Primer Sequence and PCR, PCR/RFLP Fragment Size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Restriction enzyme</th>
<th>Fragment sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2 309 P2</td>
<td>P1: 5'-CGCGGGAGTTCAGGGTAAAG-3</td>
<td>MspAI (10U)</td>
<td>158 bp (T) wild</td>
</tr>
<tr>
<td></td>
<td>P2: 5'-CTGAGTCACCTGCACCTG-3</td>
<td></td>
<td>158,112,46 bp (TG) heterozygous</td>
</tr>
<tr>
<td>P53 codon 72</td>
<td>P1:5'-TTGGCGGCTCCCAAGCAAATGGATGA-3'</td>
<td>BstUI (10U)</td>
<td>113,86 bp (GG) wild</td>
</tr>
<tr>
<td></td>
<td>P2:5'-TCCTGGGAGGGACAGAAGATGAC-3'</td>
<td></td>
<td>199,113,86 bp (GC) heterozygous</td>
</tr>
<tr>
<td>P21 codon 31</td>
<td>P1:5'-ACCAGGCGCTCTCTCCTTATC-3</td>
<td>BspI (10U)</td>
<td>122,103 bp (ser/ser) wild</td>
</tr>
<tr>
<td></td>
<td>P2:5'-GTACACCTCCCCAGGTGGTCT-3</td>
<td></td>
<td>225, 122,103 bp (ser/arg) heterozygous</td>
</tr>
</tbody>
</table>

Figure 1. P53 arg72pro Polymorphsim after BstUI Digestion, Lane 1: 50 bp Ladder, Lane 2, 5, 8: Heterozygous (Arg/Pro) Showing Bands at 199, 113, 86 bp, Lane 3, 4, 6, 7: Wild Type (Arg/Arg) Showing Bands at 113, 86 bp.
Each PCR assay was performed using 100 ng of genomic DNA, 0.2 μM of each primer, 1 U of Hot Start Taq DNA polymerase (Quiagen), 200 μM dNTP, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.4), and 50 mM KCl. After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 45 seconds at 95°C, 45 seconds at 59°C, and 1 minute at 72°C. The last elongation step was extended to 7 minutes. The amplified fragments targeted the site of polymorphism: the 158-bp fragment for MDM2 T309G contained the T→G bp substitution at nucleotide 309 that creates a MspA1I restriction site. The digestion products were visualized with ethidium bromide after electrophoresis on 3.5 % agarose gel at 100 volts for 30 min. The MDM2 309TT wild type homozygous was identified by the presence of only a 158 bp fragment. 309TG heterozygous was identified by 158, 112, and 46 bp fragments, and 309GG homozygous variant was identified by 112 and 46 bp fragments (Figure 3).

**Statistical Methods**

Data was analyzed using SPSS®win statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher’s exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Logistic regression was used for calculation of odds ratio (OR) with 95% confidence interval (CI) for risk estimation. A p-value <0.05 was considered significant.

**Results**

The present study was conducted on 77 cases of de novo AML who presented to the Medical and Pediatric oncology departments of the National Cancer Institute, Cairo University as well as 72 age and sex comparable apparently healthy individuals serving as a control group. We investigated the relative frequency of P53 Arg72Pro, P21 Ser31Arg and MDM2 T309G SNPs, which represented a three genes in the P53 activation pathway, regarding the wild, heterozygous and homozygous types in the two studied groups.

The age of cases in our study ranged from 4-83 years with a median of 34 years and a mean of 38±17.3 years. The patient group included 43 males (55.8%) and 34 females (44.2%). The presenting total leucocytic count in the study cases ranged from 2 to 180 with a mean of 34.709±31.224 and a median of 29x10⁹/L. The platelet count ranged from 2 to 271 with a mean of 45.601±4.138 and a median of 33x10¹²/L. Hemoglobin ranged from 4-13, with a mean of 7.7±3.2, and a median of 7.7 gm/dl. Blasts in peripheral blood ranged from 0-90% with a mean of 38.3±28% and a median of 32%. The mean percentage of blasts in marrow was 69.2 ±18.5, the median was 72% and the range was 20-95%. Bone marrow megakaryocytes and platelets were reduced in 98.7% of AML cases.

**Cytocchemistry**

Myeloperoxidase was positive in 94.7% of cases while NASDA/NASDAF was positive/partially inhibited in 18.2% of cases and positive/totally inhibited in 2.6% of cases.

**Immunophenotyping**

The myeloid lineage markers including MPO were detected in 96.1% of cases, CD33 in 93.5% of cases and CD13 in 98.7% of cases. Also CD 117 was positive in 57% of cases and HLA-DR was positive in 71.4% of cases. Myeloid with monocytic markers including CD14 and CD64 were positive in 11.7% and 6.5% of cases respectively. Aberrant expression of lymphoid markers was detected in 19.5% of cases with CD7 showing the highest expression in (7.8%) of cases.

**FAB classification**

The most commonly encountered FAB subtype was M2 (32.5%), followed by M1 (29.9%), M4 (18.2%), M3 (14.3%), M5 (2.6%) and finally both M0 and M7 (1.3%).

**P53 results**

Tables (2 and 3) demonstrate the frequency of p53 pro72arg polymorphism and compare mutant versus wild types in cases versus controls. P53 results could be only obtained in 68/77 AML patients (88.3%) and in 69/72 controls (95.8%). No significant difference could be detected between the studied groups in either condition. Figure 2 demonstrates the different PCR/RFLP p53 fragments.
**P21 results**

Table 3 demonstrate the frequency of p21Ser31Arg polymorphism and compare mutant versus wild types in cases versus controls. P21 results could be only obtained in 65/77 AML (84.4%) patients and in 70/72 controls (97.2%). We found that P21 (ser/arg) heterozygous showed a statistical significant difference among AML patients compared to controls (p=0.017, OR 2.946, 95% CI 1.216-7.134), also P21 polymorphism (ser/arg plus arg/arg) versus P21 wild type was higher in AML patients compared to controls (p=0.008). Figure 2 demonstrates the different PCR/RFLP p21 fragments.

**MDM2 results**

Table 4 demonstrate the frequency of MDM2 polymorphisms and compare mutant versus wild types in cases versus controls. MDM2 results could be only obtained in 68/77 AML (88.3%) patients and in 65/72 controls (90.3%). A non significant difference between MDM2 wild, heterozygous and homozygous types was detected in AML patients compared to controls. Figure 3 demonstrates the different PCR/RFLP MDM2 fragments.

**Gene to gene interactions**

Combination of MDM2 and P21 SNPs is shown in Table 5, where a significant difference was found between the AML and the control groups as regards comparing the frequency of both mutant types versus both wild types (p=0.009) this result in increased the risk of AML by 6.8 folds (OR 6.807 and 95% CI 1.909-24.629). However the presence of MDM2 309T allele result in abolisk risk effect of P21 31arg allele (p value=0.71). Combination could be done in 64/77 AML patients (83.1%) and in 65/72 controls (90.3%).

Combination of MDM2 and P53 SNPs is shown in Table 6, where a non significant difference was found between the AML and the control groups as regards comparing the frequency of both mutant types versus both wild types with a p value of 0.065. Combination could be done in 64/77 AML patients (83.1%) and in 65/72 controls (90.3%).

From the previous results, when the MDM2 and P21 polymorphisms were combined, a synergistic effect was observed concerning the risk of AML development.

Correlation of the clinical, hematological and immunophenotypic parameters with the three gene polymorphism expression in AML cases showed that their expression was not related to any of these parameters.

**Discussion**

Our study shows that the P21 ser31arg and MDM2 T309G have an implication on the pathogenesis of AML, however P53 arg72pro do not affect the risk of AML.

In our study we found that there is no additional effect from the P53 arg72pro polymorphism in the pathogenesis of AML (p=0.82) (Table 2). This is consistent with a case-control study consisting of 231 AML patients and 128 normal individuals from a northern Chinese population (P=0.25) (Xiong et al., 2009). Also these results are lending weight to the conclusion of Nakano et al. that the P53 arg72pro was not associated with risk or clinical parameters of AML (Nakano et al., 2000). Studies failed to establish a consistent association between the codon 72 polymorphism and age of tumor onset (Sotamaa et al., 2005). In a study of 25 patients with ALL and 65 patients with AML, mutation of the P53 gene was found in one patient with ALL and in five patients with AML (Wojcik et al., 2005). Another study about therapy-related acute myeloid leukemia susceptibility, showed that one polymorphism alone cannot influence the risk of t-AML, and an interactive effect was detected such that individuals carrying both an MDM2 G allele and a TP53 Pro allele, were at increased risk of t-AML (P value for interaction is 0.009) (Ellis et al., 2008).

In the present study, the p21 codon ser31arg polymorphism was significantly associated with increased risk for AML by 2.9 folds (Table 3). This result is consistent with other studies that investigated this polymorphism in various types of cancers. A study on 104 cancer cervix patients and 160 control in Chinese southern women showed that the frequencies of p21 arg/arg, ser/arg, and ser/ser genotypes among patients were significantly different from those among controls (P<0.05) (Jiang et al., 2010). In another study on 53 bladder cancer patients, 119 healthy controls in Taiwanese patients, there was a significant difference in p21 codon 31 polymorphism between the control and the cancer patients (p<0.01). The arginine form was prominent in the cancer patients (per
copy of the A allele, odds ratio=2.03, 95% confidence interval=1.23-3.37) (Chen et al., 2002).

On the contrary, another study from northeastern Iran on 126 esophageal squamous cell carcinoma cases and 100 controls showed that the frequencies of the wild type and variant genotypes for each of these SNPs were the same and equal to 78.57% for the wild type genotype and 21.43% for the variant genotype, respectively, among cases and 82% and 18%, respectively, among controls and this difference was not statistically significant (P=0.52) (Taghavi et al., 2010).

In the present study, when MDM2 T309G polymorphism was examined, a non significance was observed between AML cases and controls, with a p value of 0.07 (Table 4). Contrary to our results, Xiong and his colleagues observed a nearly 3.52-fold increase in AML risk associated with the MDM2 GG genotype compared with the MDM2 TT genotype (P=0.001) in a study consists of 231 patients with AML and 128 normal controls from a northern Chinese population (Xiong et al., 2009). We expect that with increasing the numbers of individuals incorporated in future studies, similar results could be obtained. Also, ethnic variation between Chinese and Egyptians could contribute to the difference in results.

Our result is partially consistent with the report by Ellis et al. (2008) who tested associations between 171 patients with t-AML and 2 common functional P53-pathway variants, the MDM2 SNP309 and the TP53 codon 72 polymorphism. They showed that MDM2 SNP309G allele was associated with a modest increased risk in de novo AML but not in therapy-related AML. So far there exists one published leukemia study claiming that the disease in a Singaporean Chinese population (Phang et al., 2008). The discrepancy between their studies and ours could perhaps be due to ethnic and/or geographic variations of the frequency of these alleles in different healthy populations (Bond et al., 2006). It is noteworthy that the basal frequency of the G allele in Singaporean Chinese population is much higher than that in our studied healthy population. Hence, it appears that ethnicity and/or geography bias may influence the effect of the MDM2 G allele on AL risk, perhaps in combination with genetic background, carcinogen exposure in different populations, or just simply sample sizes.

Since the discovery by Bond et al. that SNP309 of the MDM2 gene can accelerate the onset of leukemia at a young age in the patients, there have been quite few studies assessing the impact of the G allele on timing of leukemia onset (Bond et al., 2006). The evidence from studies of leukemia is controversial, showing an association with early onset in Caucasian and Black populations but not in Hispanic and Singaporean populations. Hence, it appears that the SNP309 has common susceptibility across populations with different ethnicity-specific effects. Their work in northern Chinese population showed that the SNP309 had no effect on the timing of acute leukemia onset, which further consolidated the idea that the SNP309 has different ethnic effects. Other studies (Ishibe et al., 1997; WrenschKelsey et al., 2005) investigating genetic polymorphisms have demonstrated similar ethnicity-specific effects, which might suggest interactions between gene polymorphisms and unidentified factors associated with ethnic status.

Another study who genotyped children (n=575) with de novo acute myeloid leukemia (AML) treated on three Children’s Oncology Group protocols for the presence of SNP309 and healthy blood donors as control population, showed that MDM2 polymorphism increased the susceptibility to childhood AML where the variant G/G genotype was associated with an increased susceptibility to AML (OR 1.5; p=0.049) (Matakidou et al., 2003). The difference in results may be due to different age groups as our patients have a mean of 38 years and a median of 35 years. This finding of increased risk of malignancy in association with the homozygous variant genotype is in agreement with a number of prior studies.

We found that the MDM2 G allele has a synergistic effect with P21 codon 31arg allele, where risk of AML increased to 6.8 instead of 2.9 with P21 31arg allele alone. on the other hand the MDM2 T wild allele abolish the risk effect of this P21 allele (Table 5). However the combination of both variant alleles of P53 and P21 or MDM2 and P53 did not affect the risk of AML.

To the best of our knowledge, no previous studies addressed the correlation of the 3 previous parameters in AML cases. Previous studies only investigated either one or two of them in AML patients. Our data are partially consistent with a study about therapy-related acute myeloid leukemia susceptibility, which showed that one polymorphism alone cannot influence the risk of t-AML and an interactive effect was detected when MDM2 TT and TP53 CC double homozygotes were at increased risk of t-AML (Ellis et al., 2008). The difference in results may be attributed to the fact that this study observed this interactive effect only in a certain type of patients with loss of chromosomes 5 and/or 7, acquired abnormalities associated with prior exposure to alkylator chemotherapy.

In conclusion, this is the first report to the best of our knowledge to show the relation between AML and three single nucleotide polymorphisms in the P53 pathway. There is a significant association between P21 polymorphism and AML and MDM2 polymorphic allele increases this association. We suggest that the p21 ser31arg polymorphism might be a genetic susceptibility factor in the pathogenesis of AML. We recommend the performance of future studies on a larger sample size to validate the effect of MDM2 polymorphism on the pathogenesis of AML, if any. Also we recommend the incorporation of P21 gene polymorphism in any study of MDM2 and/or P53 polymorphisms concerning AML.

Although CRC is a common disease in the older population, it has been evident since the largest systemic review that the disease is not infrequent in the young (O’Connell et al., 2004). In this study, CRC proportion in patients aged <40 (29.78%) is approximately 4 times hicohort. Results from this study suggested that dietary habits in this popmental agents, and the prevalent consanguinity in Cambodia justify further research, which will advance our understanding of the risk factors for the disease in young adults. These studies should investigate environmental exposures, family history,
and consanguinity as well as explore gene–environment interactions in colorectal cancer carcinogenesis in this high-risk population.

References


