

Identification of the Nucleophosmin 1 (*NPM1*) Gene Mutation in Adult Bengali Bangladeshi AML Patients

*Raman MZ,¹ Sumon MS,² Asha MT,³ Uddin E,⁴ Akter F,⁵ Yesmin ZA,⁶ Banu LA,⁷ Akhter N⁸

Abstract

Background: The nucleophosmin gene (*NPM1*) has recently been described as one of the most frequent genetic lesions in acute myeloid leukemia (AML). To the best of our knowledge, no molecular genetic study on the *NPM1* gene mutation in AML patients in adult Bengali Bangladeshi has been reported. It would, therefore, be a great opportunity to modernize our medical knowledge in this field for the betterment of the patient care.

Method: A total of 23 AML patients were selected using convenience sampling technique for collection of peripheral venous blood sample from admitted adult Bengali Bangladeshi AML patients from Hematology Department, Bangabandhu Sheikh Mujib Medical University (BSMMU) and Sanger sequencing was done at the most frequent mutation-prone region for AML, the exon 12 of the *NPM1* gene, to find out target mutation. The sequencing of *NPM1* gene exon 12 region 171410527 to 171410696 is the first time such research in Bangladesh.

Result: The sequenced data from the blood sample of the study population matched with world gene data base sequence and found wild type for each sample.

Conclusion: This research will also increase awareness about utility and importance of the genetic counseling and genetic test amongst the clinicians and also in patients.

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1. *Dr. Md. Zamilur Rahman, Assistant Professor, Department of Anatomy, BashundharaAd-din Medical College. masum.mbbs@gmail.com
2. Dr. Md. Syedur Rahaman Sumon, Assistant Professor, Department of Forensic Medicine, BashundharaAd-din Medical College
3. Dr. Moushumi Taher Asha, Assistant Professor, Department of Anatomy, BashundharaAd-din Medical College
4. Dr. Ehsan Uddin, Assistant Professor, Department of Anatomy, Shahabuddin Medical College
5. Dr. Fatema Akter, Assistant Professor, Department of Physiology, Medical College for Women, Uttara, Dhaka
6. Dr. Zinnat Ara Yesmin, Assistant Professor, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University
7. Dr. Laila Anjuman Banu, Professor, Genetic and Molecular Biology, Chairman Department of Anatomy, Bangabandhu Sheikh Mujib Medical University
8. Dr. Najnin Akhter, Assistant Professor, Department of Anatomy, Brahmanbaria Medical College

*For correspondence

Introduction

Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells. Several genes are identified with their incidence and prognosis that are now providing opportunities for AML patient stratification and personalized approaches to medication that are based on individual mutation profiles. Among them the most common (50-60%) mutation affecting AML is the *NPM1* gene mutations which also serves as a powerful prognostic indicator and affects the treatment decision. The *NPM1* gene is one of the most frequent targets of chromosomal translocations in hemopoetic malignancies.¹ *NPM1* is a nuclear phosphoprotein which moves between the nucleus and cytoplasm during the cell cycle and interacts with several proteins.² This multifunctional protein is involved in the processing of ribosomal RNA, centriole duplication, response to stress stimuli such as UV irradiation and hypoxia, maintenance of genomic stability by controlling cell ploidy, participation in processes of DNA repair and transcription regulation through the modulation of events of condensation and decondensation of chromatin.³ Licínio and Silva reported that mutations are classified as A through F according to the insertion or deletion of four base pairs in exon 12 of the C-terminal region.⁴ Among different types 'the most common being types A mutations (TCTG) in 80%,⁵ 'Mutations in exon 12 are considered to be the most frequent mutations in AML (>50%) patients with normal karyotype'.⁶ The main features of acute myeloid leukemia (AML) with mutated *NPM1* in the following table (Table I).

Mutation in *NPM1* is generally showed improved survival (changes otherwise intermediate-risk patients into better-risk), if

present together with the *FLT3* mutation, this survival benefit is negated⁸. The suspected disease-causing region of exon 12 of *NPM1* gene was targeted to find out the variants and new variants if there is any in Bengali Bangladeshi population. There is no adequate reporting on the genetic basis of AML in the adult Bengali Bangladeshis. If the commonly prevalent *NPM1* gene of AML of Bengali Bangladeshis can be identified that may explore new models for diagnosis and therapy for ourselves. According to Preetesh et al. (2014), mutated *NPM1* is stable and reliable marker in AML and can be used to assess minimal residual disease (MRD)⁶ and when the patient is in remission or no symptoms and signs of disease and monitoring of *NPM1* mutant copies every 4-6 months is advisable.⁷ It is also important to assess for *NPM1* mutations in older patients to identify those individuals who are likely to benefit from intensive conventional chemotherapy.⁹ Moreover, WHO classification included *NPM1* mutation for worldwide recognition.

Methods

The study was carried out in the Department of Anatomy, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka during the period of March 2016 to February 2017 after approval from the Institutional Review Board (IRB), BSMMU. Diagnosed twenty three (23) adult Bengali Bangladeshi AML patients were recruited from Hematology Department of BSMMU for identification of *NPM1* gene mutation using convenience sampling technique. For the present research, only diagnosed and untreated AML patients admitted in BSMMU Hematology Department were selected during study period. The age of the participants were equal or above 15 years. The AML patients with relapse were not included this research.

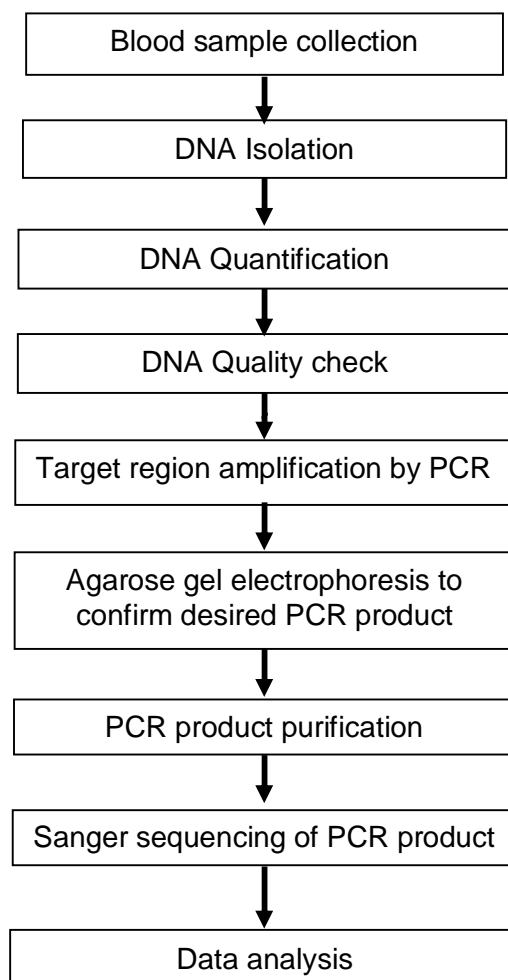
Research plan:

Figure 1. Flowchart of the method for identification of *NPM1* gene mutation

Blood sample collection and preservation

Three (3) ml of venous blood was obtained very carefully from each patient. The samples were then taken into tubes containing EDTA (1 mg/ml). For short time preservation (1-2 weeks), the blood was preserved at 4⁰C and it was stored at -30⁰C for long time preservation.

DNA isolation from blood

Genomic DNA was isolated from venous blood samples using ReliaPrep™ Blood DNA isolation kit (Promega, USA) according to the Standard Operating Procedure (SOP) of the

Genetic Research Laboratory of BSMMU (GRL, BSMMU).

Quantification of DNA and DNA Quality Check

The quantity and purity of DNA dissolved in Nuclease free water was determined using Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA) following SOP of GRL, BSMMU. The Optical Density (OD) readings were recorded at 260 and 280 nm along with the ratio between these readings. The DNA concentration was found at a time with OD readings from Nano drop measurement.

*Target region amplification by PCR***a) Primer**

The exon 12 of *NPM1* gene is 611bp in length. Of the sequence the mutation prone region covers 169 bp which was targeted for this study. This targeted region was amplified with the primers NPM-F, 5'-GATGTCTATGAAGTGTTGTGGTTCC-3' and NPM-R, 5'-GGACAGCCAGATATCAACTG-3. The primer sequence was taken from Philippe Szankasi¹⁰.

b) PCR

Table II: PCR cycle condition

Polymerase Chain Reaction of 30 μ L was performed using 100 ng of genomic DNA, 1.2 μ L NPM-F and NPM-R primers, 15 μ L GoTaq® Colorless Master Mix (Promega, USA). The reaction mixture was briefly (10 - 20 seconds) centrifuged and then placed in thermal cycler (Biometra, Germany) with the PCR profile as shown in Table 2.

Step	Temperature °C	Time	No of cycles
Initial Denaturation	94	2 minutes	1 (First)
Final Denaturation	94	20 seconds	30
Annealing	55	20 seconds	30
Initial Extension	72	20 seconds	30
Final Extension	72	5 minutes	1
Holding Temperature	4	Till removal of the amplicon	

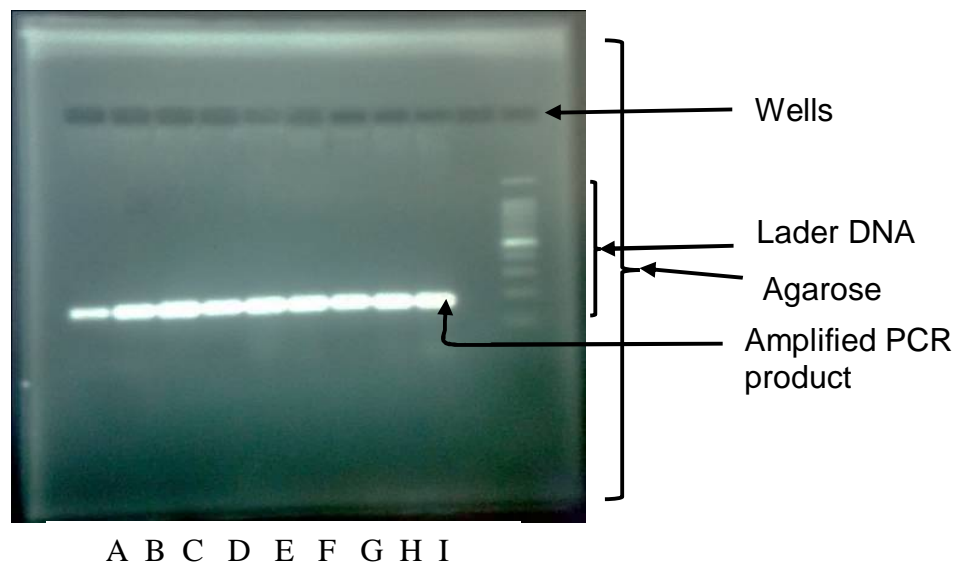


Figure 2. Photographs of the gel electrophoresis. It shows that a 169 bp fragment on *NPM1* exon 12 was successfully amplified using conventional primers. Capital letters (A, B, C, D, E, F, G, H & I) indicates AML patients sample

Agarose gel electrophoresis for confirmation of desired amplicon

PCR amplified products were checked in ethidium bromide stained 2% agarose gel. 100bp DNA step ladder (Promega, USA) was used as molecular weight marker for electrophoresis. After electrophoresis the gel was soaked in ethidium bromide solution for 10 minutes. Following completion of staining using SOP of GRL, BSMMU, the gel was visualized under UV light and subsequently photographed by gel documentation system (Fig 2).

Purification of PCR Product

After confirmation of desired band in agarose gel electrophoresis, the PCR product were then purified using the Wizard[®] SVA 9281 and PCR Clean-Up System (Promega, USA) using SOP of GRL, BSMMU.

Sequencing of PCR product

The amplicons were sent to the 1st base, Malaysia to run in Sanger sequencer (ABI Sanger Sequencer 3500).

Data analysis

The raw chromatogram data obtained for the sequence file was analyzed using the Geneious Software version R10.

Result

The whole blood sample of 23 AML patients were used to detect the presence of any mutations present in the *NPM1* exon 12. For this purpose, the genomic DNAs extracted from these samples were used to sequence the exon 12 of the *NPM1* gene using the Sanger sequencing method. Among the 23 AML patients aged between 18 to 70 years (mean age: 34 years), eleven (48%) were males and twelve patients were females (52%).

Polymerase chain reaction method successfully amplified the desired 169 bp fragment of *NPM1* exon 12 using appropriate primer and PCR conditions. Gel electrophoresis showed the presence of the amplified fragment of the hot spot region of the *NPM1* exon 12 shown in Figure 3.

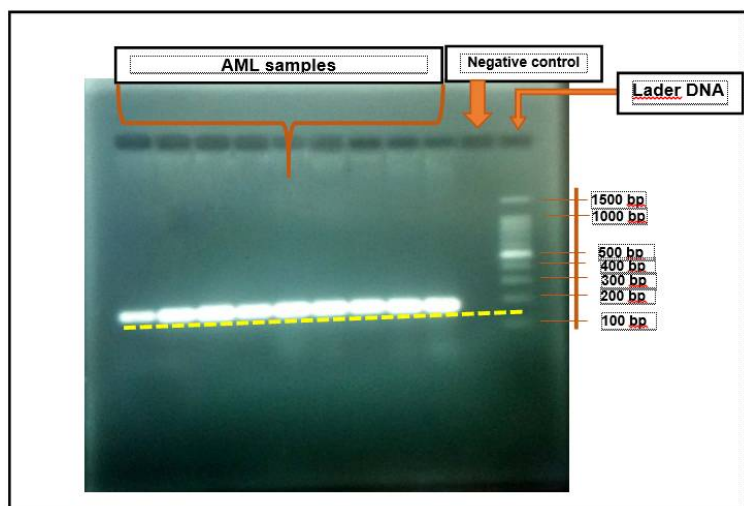


Figure 3. Photograph of the gel electrophoresis of the PCR products on 2% agarose gel. It shows that the desired 169 bp (base pair) fragment of *NPM1* exon 12 (under yellow line) was successfully amplified. There was no non-specific PCR product or contaminations in this experiment.

Human *NPM1* gene located on the long (q) arm of chromosome 5 at position 35.1, consists of 24022 bp (NCBI ID 4869) sequence. Out of this sequence, 169 bp from 171410527 to 171410696 region of exon 12 of *NPM1* gene were sequenced for this research. The Sanger sequencing was performed by capillary electrophoresis of the cycle sequencing product using the ABI 3500 genetic analyzer. The raw chromatogram data

were aligned with the *NPM1* exon 12 reference sequence downloaded from the 'Ensemble Genome Browser' using the 'Geneious sequence analysis software version R10'. The present research showed no mutation in the *NPM1* exon 12 among the AML patients. Some representative examples of the Sanger sequencing results are shown in Figure 4.

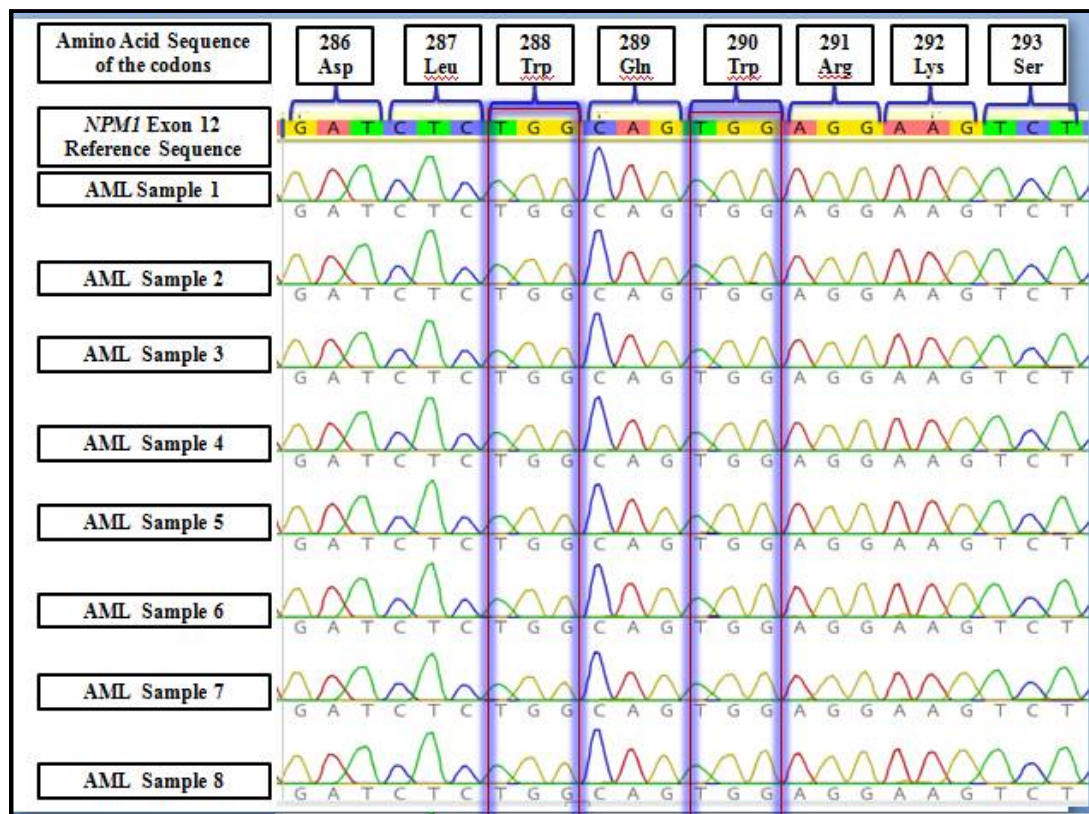


Figure 4. Sanger sequencing of the amplified region on *NPM1* exon 12 of investigated AML patients. The amplified fragment revealed wild type (no mutation) peaks. The mutation hot-spot region is located within the two tryptophan residues at 288 and 290 positions. The amino acid sequence coded by the sequence is also shown. Asp=Aspartic acid, Leu= Leucine, Trp= Tryptophan, Gln= Glutamine, Arg= Arginine, Lys= Lysine, Ser= Serine.

Discussion

The aim of the research was to identify *NPM1* gene mutation in AML patients in adult Bengali Bangladeshis. For this reasons, to observe the frequency of the *NPM1* gene mutation, peripheral venous blood sample was collected from untreated 23 diagnosed adult Bengali Bangladeshi AML patients for Sanger sequencing.

According to John et al. AML is the 11th leading cause of cancer-related death¹¹. Due to lack of awareness on AML signs and symptoms most patients are diagnosed at an advanced stage of the disease and suffer from worse treatment outcome. Cancer screening program and genetic counseling can play a pivotal role in early diagnosis and in decreasing the rate of death from this cancer. A mutation database based on the genetic makeup of Bangladeshi population is very important for proper screening and counseling. To develop such a mutation database for AML cancer, it is essential to find out the variant pattern of relevant causal genes of AML in Bangladeshi population. Since this type of study is costly, present research designed with relatively small sample size (23 patients) focusing small genomic region (169 base pair) related to AML to check the feasibility of conducting such a study on large cohort.

In this study, total 23 wild type (normal allele) were found within 171410527 to 171410696 region of chromosome five (5). The presence of the wild type peak indicate successful DNA extraction and PCR amplification. According to Szankasi, Jama&Bahlar (2008) the most common mutation prone region is 'TGGCAGTGGAGGAAGTC' on exon 12 of the *NPM1* gene.¹⁰ According to Daniel (2012), whole-genome sequencing is likely to become the dominant platform for mutation discovery and the exon sequencing will not

detect mutations in regions outside of the exome (>98% of the genome) and will not detect most structural variants, such as chromosomal translocations with intronic breakpoints.¹² The present research do not find mutation in this hot-spot region of the exon 12 of the *NPM1* gene in AML patients in the adult Bengali Bangladeshis. In two studies from Asia the incidence of *NPM1* mutation in AML appeared to be particularly low 0-2%¹³ which is very much similar to present research. But incase of Indian population the *NPM1* mutations were observed 19.5%³ and for Chinese population it was 16.4%.¹⁴ For the target region of this study the forward primer was designed within the intron between *NPM1* exons 11 and 12, a region unique to this gene on chromosome 5 where no polymorphism seen.

The short size (169 bp) fragment and small sample size of this study was not large enough to determine nationwide incidence of the *NPM1* mutation. Since the frequency of *NPM1* exon 12 mutations are higher in cytogenetically normal AML patients; the presence of these mutations will be very low in the cytogenetically abnormal AML patients. Though, no cytogenetic study was done for the 23 patients.

In this context, *NPM1* mutation detection by using bone marrow specimens through flowcytometry that revealed high blast cells (higher proportion of myeloid cells average 63%) and cloning of individual *NPM1* allele based more advanced sensitive approach ensured during extraction of genomic DNA.¹⁰

In addition, because of small sample size and without control it was not possible to statistically compare the frequency of wild type found in the present study between AML patients and control subjects. However, findings of present research the wild-type fragment was used as an internal quality

control for test performance and highlights the importance of screening in larger cohort of Bangladeshi population in order to explore the variant pattern of these genes and to develop a mutation database for the purpose of accurate interpretation of genetic predisposition to AML.

Conclusion

Although the present research is unable to provide information about frequency of *NPM1* gene mutation in study population, it will help our medical professionals in improving their knowledge towards genetic services. It is the first time research work in Bangladeshi context for *NPM1* gene mutation detection in adult Bengali Bangladeshis AML patients and sequencing revealed wild type peaks. It is necessary to increase awareness about utility and importance of the genetic counseling as well as genetic test amongst the clinicians and also in patients. However, this finding needs to be confirmed by more studies on large scale with bigger sample size and result of genotyping further confirmed by both cytogenetics, clinical feature, bone marrow finding as well as with molecular analysis.

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