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ORIGINAL RESEARCH ARTICLE

MOLECULAR LANDSCAPE OF ACUTE MYELOID LEUKEMIA BASED ON NUCLEOPHOSMIN1 GENE: BANGLADESH PERSPECTIVE

Md. Abdullah-Al-Sayeef,[1] Devjani Banik,[2] Zinnat Ara Yesmin,[3] Md. Mohiuddin Masum,[4] Suneetha Susan Cleave Adam,[5] and Sk. Tasnuva Alam[6]

[1]MBBS (Dhaka), BCS (Health), MPhil (Anatomy-BSMMU), MACP (USA), Assistant Professor and head of the department, Department of Anatomy, Magura Medical College, Magura, Bangladesh; [2]MBBS, BCS (Health), MS (Anatomy), Lecturer, Department of Anatomy, Shaheed Suhrawardy Medical College, Dhaka, Bangladesh; [3]MBBS, MS (Anatomy), Associate Professor, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh; [4]MBBS, MS (Anatomy), Assistant Professor, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh; [5]MSc, PhD (Medical genetics), Researcher, Department of Medical Genetics, Cristian Medical College, Vellore, India; [6]MBBS, BCS (Health), MS (Gynaecology and Obstetrics), Medical Officer, Department of Gynaecology and Obstetrics, Khulna Medical College Hospital, Khulna, Bangladesh.

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Corresponding Author:
Md. Abdullah-Al-Sayeef

ABSTRACT

Background: Acute myeloid leukemia (AML) is a disorder that exhibits a high degree of heterogeneity on both a clinical and molecular level. Nucleophosmin (*NPM1*) is a nuclear phosphoprotein that contributes to the progression of acute myeloid leukemia (AML). *NPM1* exon 12 is the most common site of genetic mutation in AML. Studies have been done to determine the diagnostic and prognostic levels of AML worldwide. The study of *NPM1* gene mutation in adult AML patients has not been reported so far in Bangladesh. ***Objectives:*** The objective of the study is to detect the mutation in exon 12 of the *NPM1* gene in adult AML patients of the Bangladeshi population. ***Methods:*** This research was a cross-sectional descriptive study. For detection of mutation in *NPM1*, genomic DNA was isolated and quantified. The target sequence was amplified by short-range PCR. To detect a mutation in exon 12 of the *NPM1* gene, the amplicons were validated by gel electrophoresis and the sequence was performed using a Sanger sequencer. Data analysis was done using different databases and software. ***Results:*** In this research, the most frequent type of mutation identified was the frame-shift variant (57.11%). CATG/CCT was the most frequent allele change found in this study (16.16%). In this study, 7.21% of mutations were newly identified mutation. ***Conclusions:*** The sample size was small to decide and furthermore study is recommended. Despite of limitations this study emphasizes the importance of exploring the genetic makeup of the Bangladeshi population to develop a database.

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INTRODUCTION

Acute myeloid leukemia (AML) is a highly heterogeneous disease on both a clinical as well as molecular level (1). AML is also referred to as acute myelogenous leukemia or non-lymphocytic acute leukemia. It is a rapidly progressing malignant disease in which the blood and bone marrow contain an abnormally high number of immature blood-forming cells, and the cells in question are those that are destined to differentiate into granulocytes or monocytes (2). Bangladesh currently has an estimated incidence of new cancer cases of 12.7 million, which is expected to increase to 21.4 million by 2030 (1). Cancer is the sixth leading cause of death in Bangladesh. International Agency for Research on Cancer has estimated cancer-related death rates in Bangladesh to be 7.5% in 2005 and that will be rising to 13% by 2030 (1).

Haematological malignancies occupy an important part of the issue. AML is the most frequent haemolytic malignancy (28.3%) with a median age of 35 years in Bangladesh (3). Hossain *et al*. observed that the incidences are followed by chronic myeloid leukemia (18.2%), non-Hodgkin's lymphoma (16.9%), acute lymphatic leukemia (14.1%), multiple myeloma (10.5%) and Hodgkin's lymphoma (3.9%) (3). Mostly the diagnosis of *NPM1, CEBPA and FLT3* mutations has been introduced into clinical practice to date, involving diagnosis, risk assessment, and also treatment guidance (4). Certain genetic conditions predispose a person to AML. Familial AML transmitted in a dominant manner on a non-sex chromosome (5). The pathogenesis of AML is associated with the accumulation of somatically acquired genetic modifications in hematopoietic stem cells that impair their normal self-renewal, proliferation, and differentiation mechanisms (6).

Mutations in genes associated with global chromatin modifications, such as DNA methylation, histone modification (acetylation/deacetylation, methylation). and chromatin looping, occur early in the pathogenesis of AML in normal functioning hematopoietic stem cells (7). Several genes are identified with their incidence, treatment outcome and prognosis. Falini *et al*. documented that *NPM1* was mutated at exon 12 resulting in an abnormal cytoplasmic localization of NPM (NPMc+). In the leukemic ddNTs in around 35% of adult cases of AML, making *NPM1* one of the most commonly mutated genes in AML (8). These exon 12 mutations are particular to AML, as all other neoplasms studied have just shown nucleus-restricted NPM (9). Multilineage involvement and dysplastic characteristics are recurrent findings in NPMc+ AML, rendering differentiation from *NPM1*-mutated myeloproliferative disorders difficult (10). Additionally, NPMc+ is strongly associated with de novo AML, as AML secondary to myeloproliferative disorders and therapy-related AML are usually not associated with mutations in *NPM1* exon 12 (11).

These evidences indicated that *NPM1* mutations and subsequent cytoplasmic dislocation of *NPM1* was an event restricted to AML. AML with *NPM1* mutation has been recognized as a significant provisional entity in the classification of AML based on its distinctive clinical, pathologic, and biologic characteristics (12). World Health Organization (WHO). classification use a variety of factors to classify AML as poor-risk, intermediate-risk, and better-risk disease. Recent large-scale sequencing of AML genomes now enables patients to select appropriate treatments based on specific mutation categories (13). The human *NPM1* gene (24,022 bases) is located on 5q35. Exon 12 mutations in the *NPM1* gene are the most frequently occurring gene mutations in AML patients (14-16). *NPM1* mutations cause the *NPM1* protein to localize abnormally to the cytoplasm (17). So it was very important for us to find out the cytogenetic and molecular genetic findings of *the NPM1* gene for a better prognosis and outcome of AML patients as well as genetic basis of diagnosis. These mutations are found in approximately 25%–35% of all AML patients and normal karyotype was found in up to two-thirds of patients (18, 19). The high prevalence of *NPM1* mutations in AML patients, combined with their favourable prognostic benefit, emphasizes the significance of screening for these mutations (20, 21). So the detection of mutational variations is becoming a necessary step for the diagnosis, treatment, prognosis and relapse of AML (22). The objective of the research was to identify the mutation in exon 12 of the *NPM1* gene in adult AML patients of Bangladesh.

MATERIALS AND METHODS

The cross-sectional descriptive study was conducted at the Genomic Research Laboratory of the Department of Anatomy, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, with approval from the BSMMU Institutional Review Board (IRB). After obtaining permission through the proper channels, a total of fifty (50) adult Bangladeshi acute myeloid leukemia (AML) patients were selected using a selection checklist based on exclusion and inclusion criteria. Blood was collected from each patient. Later DNA was extracted and the target gene sequence was amplified by polymerase chain reaction (PCR) followed by Sanger sequencing. Obtained sequence data were analyzed to detect mutation in the exon 12 of *NPM1* gene in adult AML patients in Bangladesh.

Isolation of DNA: Three (3) millilitres of peripheral venous blood was carefully collected from each patient. Genomic DNA was extracted from the patient's blood according to the manufacturer's instructions using the commercial DNA extraction kit ReliaPrep^{TM} (Promega, USA). Two hundred (200) ml blood was combined with proteinase K and cell lysis buffer in a microcentrifuge tube. To obtain the extracted DNA, binding buffer, wash buffer, and elution buffer was used sequentially. The reagents were measured following the SOP (Standard Operating Procedure). for DNA extraction from blood. NanoDrop spectrophotometer was used to determine the quantity and quality of extracted DNA.

Amplification of DNA: To amplify the target region of exon 12 of the *NPM1* gene, we selected the primers that were used in several studies targeted for the *NPM1* gene and specifically for the exon 12 (15, 23). That selected primer was validated by 'OligoAnalyzer Tool' of Integrated DNA Technologists. Table 1 shows the sequences of oligonucleotide primers and other relevant details. By using traditional PCR, the desired region of the *NPM1* gene was amplified. Short-range PCR was used to amplify this series using a Biometra thermal cycler (Biometra, Germany). Samples were amplified using the following PCR environments: preheating at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 30 seconds. Visual confirmation of the amplicons was performed using 1% agarose gel electrophoresis. The PCR products were then purified and prepared for Sanger sequencing.

Table 1. The Sequences of oligonucleotide primers and their relevant information

Primer	Sequence (5' – 3')	Length	GC %	Tm (ºC)	Product size
F	TTAACTCTCTGGTGGTAGA ATGAA	24	37.5	61.0	256 bp
R	CAAGACTATTTGCCATTCC TAAC	23	39.1	60.8	

DNA sequencing: Sanger sequencing was performed on the PCR products using an ABI-3730 Genetic Analyzer (Thermo Fisher Scientific, USA). The cycles were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit according to the standard protocol included with it.

Data analysis: Four-colour raw chromatogram data that were generated by the Sanger sequencer represents the sequencing run's results. Chromas® software was used to analyze raw data derived from the sequence in ABI files for this study. The Chromas® software was used to edit the sequences and to align the multiple sequences to an alignment file in SCF format. Then the sequenced data were compared with the reference sequence of the EnsemblGRCh 37 database. The reference sequence was taken for comparison from the EnsemblGRCh 37 database. The specific ENST number was 00000296930.5 and it was the maximum base pair containing transcript, the detailed of Ensemble software figure is shown in appendix i. The exact location of the exon 12 of the *NPM1* gene was ENSE 00001907503. Figure 1.a shows the different variant of the known mutations with different colours. For furthermore analysis the cDNA position, allele, amino acid changes and the consequences of mutations variants were reported from the dbSNP database (see Figure 1.b).

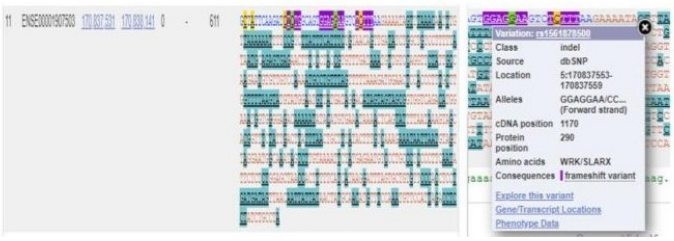

Figure 1.a ENSE number with variants of mutations

After that, the data were validated by using the UCSC BLAT software. Then the percentage of similarity with the reference sequence and the side by side alignment was observed and reported accordingly (see Figure 3). The chromatogram results of Sanger sequencing were checked for the minute details of any mutation (appendix ii). The transcripts containing the maximum number of bases were taken for comparison. By using the dbSNP database the presence and types of mutation were identified. Then allele, consequences and amino acid changes were recorded. Finally, the mutation whether new or known were reported.

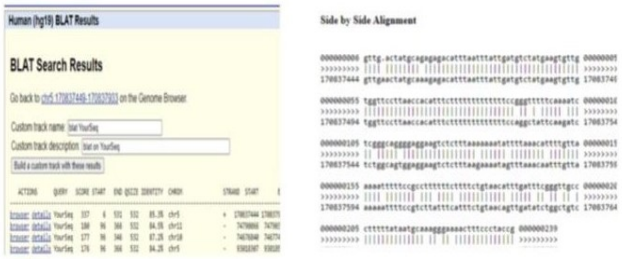

Figure 1.b Percentage of similarity with the UCSC BLAT and side by side alignment with the reference sequence

RESULTS AND DISCUSSION

Results regarding the identification of the mutation in exon 12 of the NPM1 gene: There were total 402 number of *NPM1* gene mutations were identified. Several types of mutations were observed in this study. Among them, the frameshift variant was the most prevalent (57.11%). The least frequent mutation was SNP which accounts for 0.25% of all mutations (see Figure 2).

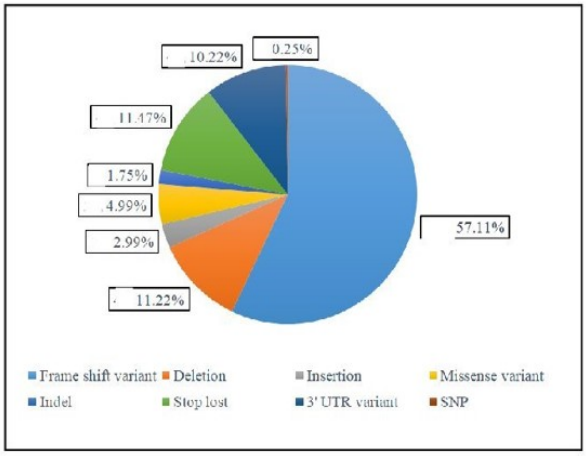

Figure 2. Types of *NPM1* mutations in the AML patients with percentage frequency (n=402)

Table 2. Allele changes of *NPM1* mutations in the AML patients with percentage frequency

Allele change	Number	Frequency (%)
CATG/CCT	65	16.16
GGAGGAA	51	12.68
TCTTT/T	46	11.44
GTTTTTAAGT	43	10.69
TCTT	40	9.95
TCTG	37	9.20
GTTTTAA	35	8.70
TCTG/TCTGT	26	6.46
CATG	20	4.97
C/T	12	2.98
C/G	7	1.74
C/A	7	1.74
Others	10	2.48
Total	402	100

The other important findings observed in this study was the allele changes in the case of AML patients. The most frequent type of allele change was CATG/CCT (16.16%). Other allele changes are shown in Table 2. The UCSC BLAT was used to compare the results of Sanger sequencing with the human gene sequences. The dbSNP summary was used for the recognition of the known and neo variant of the mutation. Among the identified 402 *NPM1* gene mutations 373 (92.79%) were known mutation in comparison to Ensembl GRCh 37 database, and 29 (7.21%) were neo mutations (see Figure 3).

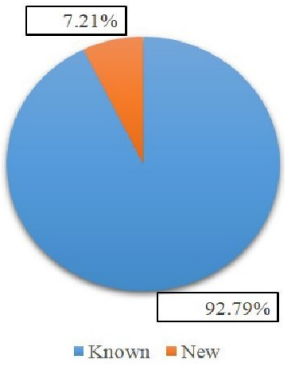

Figure 3. The variant of mutation of *NPM1* gene in AML patients with percentage frequency (n=402).

Discussion on genetic analysis the exon 12 of the NPM1 gene: Among the different variations of 402 mutations in exon 12 of the *NPM1* gene, the most frequent was the frameshift variant (57.11%). In most studies worldwide the most frequent variant was frameshift. In a study, Dohner *et al*. observed in the German population the frameshift variant of *NPM1* mutation (37.77%). (5). Study on Italian population by Falini *et al*. and Noguera *et al*. observed this variant 77% and 26.31% respectively (8, 24). In the British population the same variant was found at the rate of 53.5% (see Figure 4) (25).

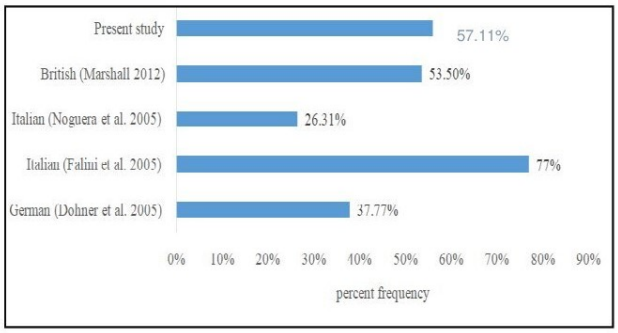

Figure 4. Comparison of the percentage frequency of mutations (frame shift variant) in AML patients in different studies

In the Italian population, deletion (1.86%) and insertion (1.86%) were observed (26). In our study we found the deletion (11.19%) and insertion (2.98%). The same deletion (63.5%) and insertion (11%) was also found in the Egyptian population (27). However, in the Korean population (28) the deletion variant was observed at the rate of 31% and in the German population (29) the insertion variant was documented in 78.3% (see Figure 5 and 6).

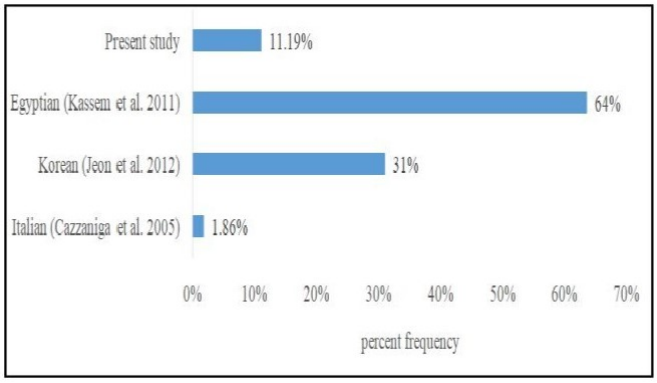

Figure 5. Comparison of the percentage frequency of mutations (deletion) in AML patients in different studies

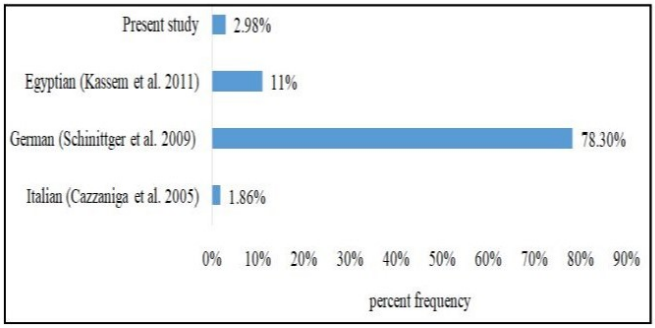

Figure 6. Comparison of the percentage frequency of mutations (insertion) in AML patients in different studies

The allele change in different studies around the world was observed and compared with our study. In the present study, we found the most frequent variant of allele change was CATG (21.13%). The second most frequent variant was identified was TCTG (15.66%). In a study on the German population, the most frequent variant of allele change like TCTG (80.3%) was observed (11). One of the other common variant CATG was found most frequently in the British population and that was 10% (25). Figure 7 shows the CATG and TCTG allele changes in different population. There were some new mutations (7.21%) found in the present study.

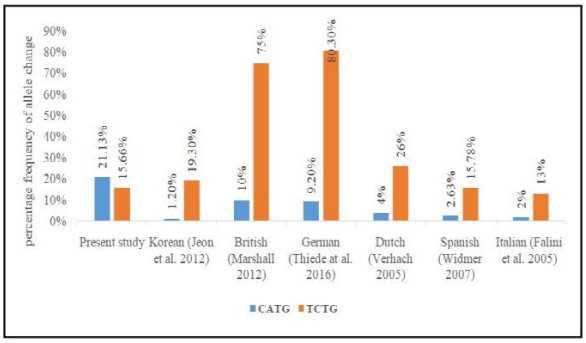

Figure 7. Comparison of the percentage frequency of allele change (CATG and TCTG) in AML patients in different population

The percentage frequency of new mutations found in other studies was similar and partly dissimilar as well. Ostgard *et al*. observed 19.8% new mutations in his study on the Danish population (19.8%) (30). However, Jeon *et al*. found 3.61 % new mutation in his study on the Korean population (28). Figure 8 shows the comparison of the percentage frequency of new mutation in AML patients in different studies.

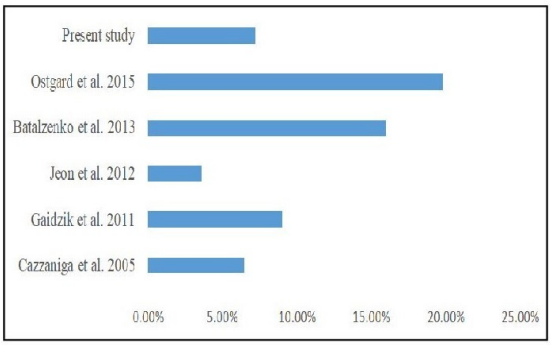

Figure 8. Comparison of the percentage frequency of new mutation in AML patients in different studies

The present study is the first to determine the frequency of *NPM1* gene mutation in adult Bengali Bangladeshi AML patients. The findings of any mutation in this study might be due to several reasons. The findings need to be analyzed up to the protein level detection. As well as the animal model might be prepared. But for the first time, this combined approach of both cytogenetic and molecular genetic study for the detection of AML will pave a great opportunity for the clinicians for diagnostic, prognostic and therapeutic indices.

Conclusion

The Conclusions section should clearly explain the main findings and implications of the work, highlighting its importance and relevance. The result of the present research has put some light on the frequencies, changes in adult Bengali Bangladeshi AML patients. It can be expected that overcoming the limitation of the current study will provide further information on the genetics of AML among the Bengali Bangladeshi population.

Ethical implication: The study's purpose and potential benefits were explained to the study population. Both verbal and written consent was taken from all AML patients. Each patient was assigned a unique identification number to maintain confidentiality and privacy. All patients who were chosen were told that their DNA samples would be used solely for research purposes. Additionally, they were told that they had the right to withdraw their consent or names from the study at any time.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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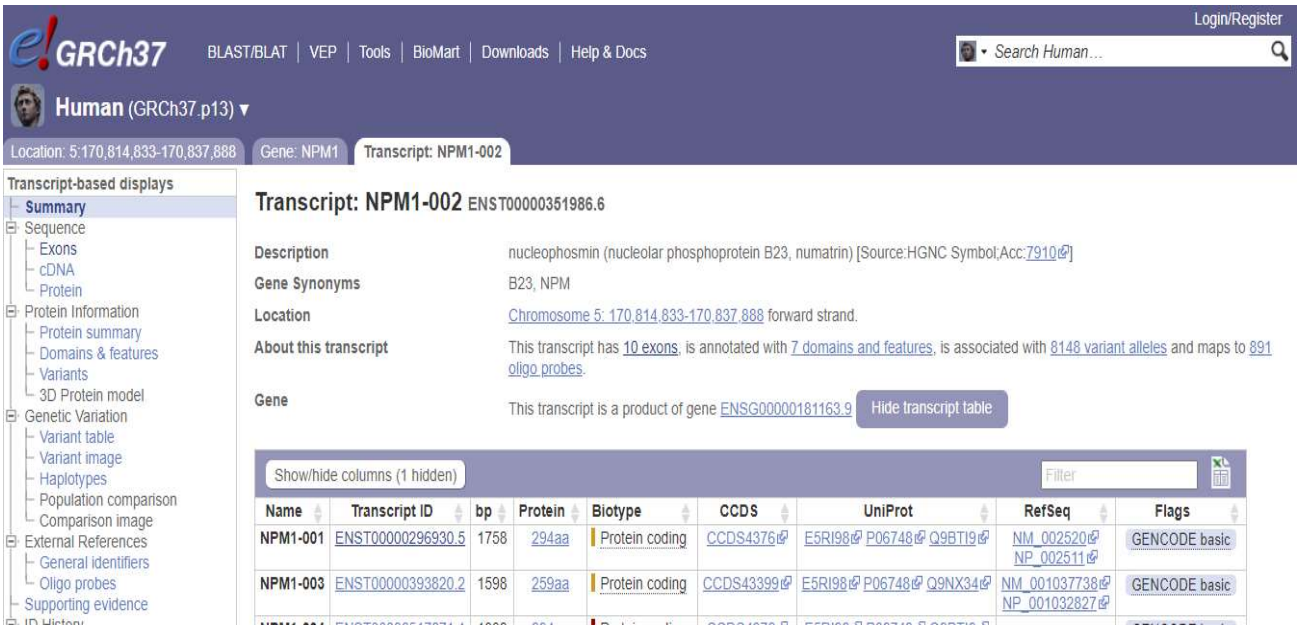
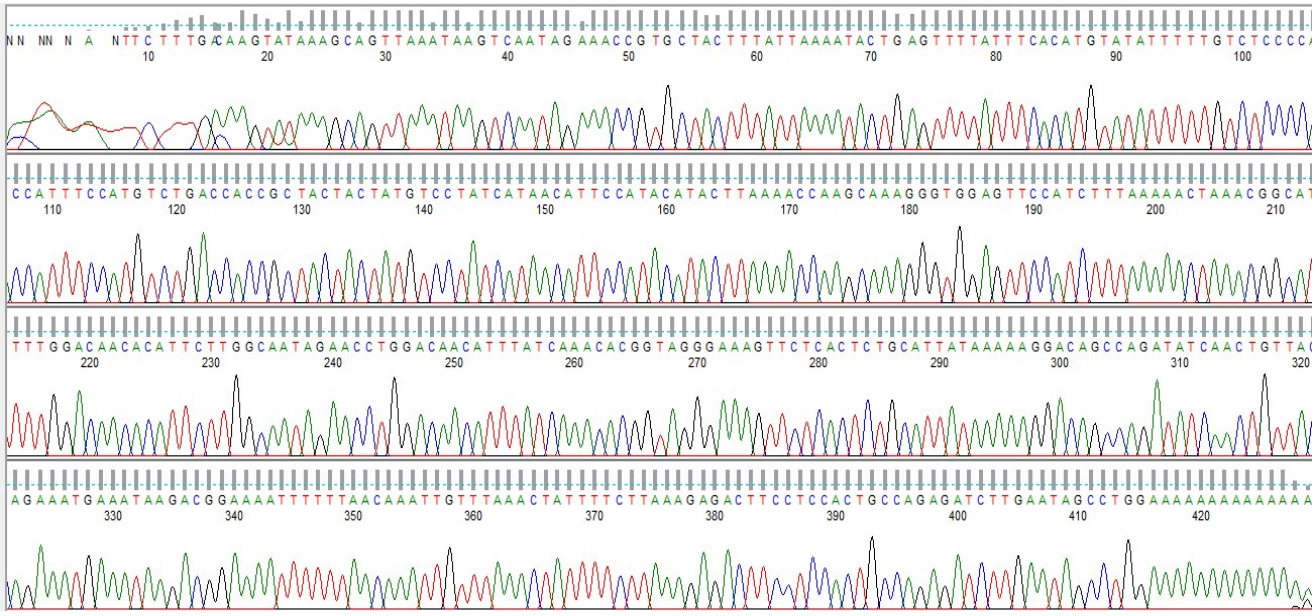
Appendix I

Ensembl GRCh 37 showing the reference sequence containing the maximum base pair with ENST number.



Appendix II

Sanger sequence result of *NPM1* gene



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