AIM OF THE WORK

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This study aimed to investigate the prognostic value of two *XPD* polymorphisms (Asp312Asn (G→A) and Lys751Gln (A→C)) in response to remission induction chemotherapy for patients with newly diagnosed de novo cytogenetically normal acute myeloid leukemia. In addition, it aimed to evaluate the association between these *XPD* polymorphisms and chemotherapy-induced toxicities during remission induction for the same group of patients.

SUBJECTS AND METHODS

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1. Subjects

This study included 51 Egyptian adult patients (above 18 years old) with newly diagnosed de novo cytogenetically normal AML of both sexes presented to the Department of Hematology, Medical Research Institute, Alexandria University, during the period from October 2011 to October 2013. Those over 60 years old and those with comorbid conditions (liver, kidney or heart disease) and/or poor performance status were excluded. Patients with acute promyelocytic leukemia (AML M3) were not enrolled in this study, because of the different chemotherapy regimen adopted for them, and patients who died during the study were also excluded.

This study was carried out in accordance with the current recommendations as described by the ethical guidelines for investigation and were approved by the Ethical Committee of Medical Research Institute. Patients signed consent forms before being enrolled in the research.

All patients were subjected to the following investigations:

- Full medical history taking.
- Thorough clinical examination.
- Complete blood picture[163].
- Bone marrow aspiration[164].
- Liver function tests (Bilirubin, ALT, AST, Alkaline phosphatase)[165,166].
- Kidney function tests (Urea, Creatinine, Uric Acid)[167].
- Blood glucose level, serum calcium, potassium, sodium and magnesium[168,169].
- X-ray chest.
- Ultrasound Abdomen.

1.1. Patients' therapy and monitoring

Patients were treated by the standard remission induction protocol (3+7) comprising[9]:

- Daunorubicin 45 mg/m^2 on days 1-3 by intravenous push.
- Cytosine Arabinoside 100 mg/m^2 on days 1-7 by continuous intravenous infusion.

During remission induction, patients were monitored for possible toxic effects induced by chemotherapy [170]:

- Nephrotoxicity (increase of serum creatinine or presence of proteinuria)[167,171].
- Gastrointestinal toxicity (nausea, diarrhea, mucositis).
- Hepatotoxicity (increase of serum bilirubin, alkaline phosphatase, transaminases levels)[165,166].
- Lung Toxicity (dyspnea, cough, pulmonary edema, pneumonitis/infiltrates).
- Metabolic toxicity (hypoglycemia, hyperglycemia, hypocalcemia, hypokalemia, hyponatremia, hypomagnesemia)[168,169].
- Cardiac toxicity (conduction abnormalities, palpitations, prolonged QTc interval, arrhythmias, ischemia/infarction, hypertension, hypotension, left or right ventricular dysfunction, pericarditis, pulmonary hypertension, or cardiomyopathy).
- Monitoring also included clinical examination, electrocardiography, and imaging studies including echocardiography.

1.2. Evaluation of response

Response to chemotherapy was evaluated after regeneration from chemotherapy-induced bone marrow aplasia as regards clinical picture, complete blood picture and bone marrow aspiration[163,164]. Patients who failed to achieve complete remission were subjected to a second similar course of chemotherapy.

2. Methods

2.1. Sample collection

Three milliliters of venous blood were collected from each patient before receiving chemotherapy and after obtaining signed consent. These samples were collected into tubes containing 10mM ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. Immediately after collection, whole blood was stored in aliquots at -20^{o}C until used for genotyping of 2 *XPD* polymorphisms: Asp312Asn (G→A) and Lys751Gln (A→C) by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method[172].

2.2. DNA extraction

Total genomic DNA was extracted from the collected whole blood samples using GeneJET Genomic DNA purification kit (Feremntas, Germany) following manufacturer's protocols.

Principle

DNA extraction involves four main steps: lysis, binding, washing and elution. Lysis is achieved by digestion of whole blood samples with lysis solution which contains chaotrophic salts and proteinase K to denature and break down proteins. The lysate is then mixed with ethanol for the inactivation of the nucleases. The lysed samples are then loaded on the purification column (figure 9) in the binding step.

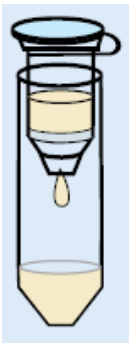

Figure 9: Spin Column (DNA Purification Column) inserted in a collection tube.

Nucleic acids will bind to silica particles with great affinity in the presence of chaotrophic salts and pH (<7.5). Washing step follows, effectively removing impurities by washing the column with the prepared wash buffers. Finally, elution of nucleic acid can then be achieved with low-salt elution buffer.

Reagents

- GeneJET Genomic DNA Purification Kit #K0721 (Feremntas, Germany) which contains the following reagents:
  - Proteinase K Solution (Tritirachium album serine proteinase).
  - Lysis Solution (Contains guanidinium hydrochloride).
  - Wash Buffer I (concentrated) (Contains guanidinium hydrochloride).
  - Wash Buffer II (concentrated).
    30 ml of ethanol (96-100%) was added to concentrated wash buffers I and II prior to first use.
  - Elution Buffer (10 mM Tris-Cl, pH 9.0, 0.5 mM EDTA).
- Ethanol (96-100%).

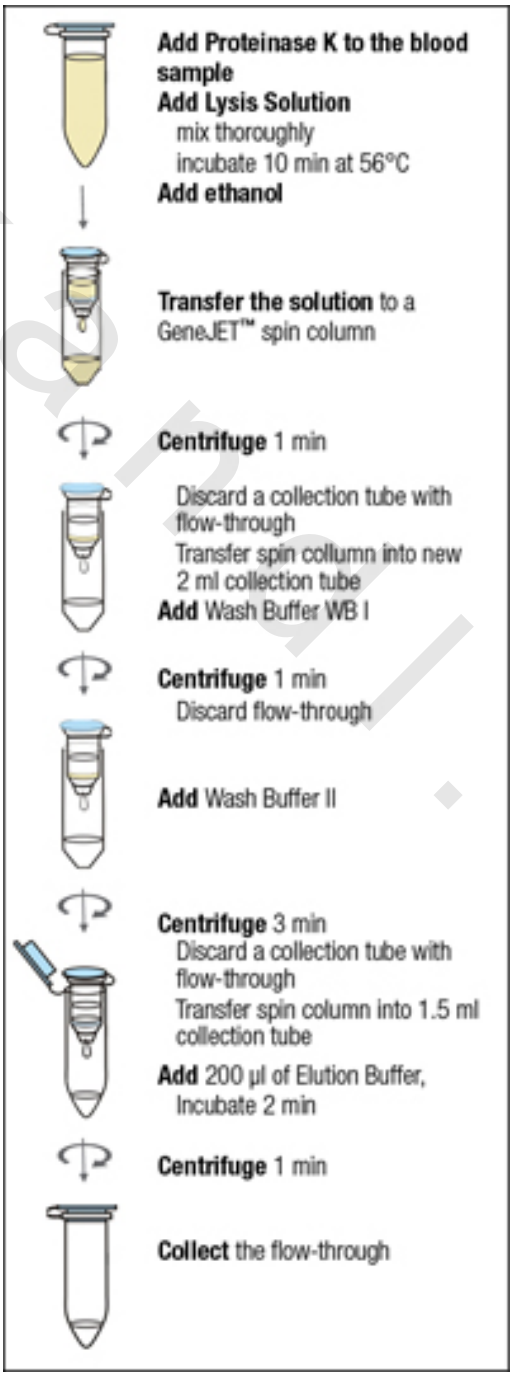

Figure 10: Genomic DNA purification procedure.

Genomic DNA purification procedure (figure 10)

1. **Lysate Preparation:** Gently vortex and briefly centrifuge reagents of the kit and whole blood samples after thawing. Add 400 µl of Lysis Solution and 20 µl of Proteinase K Solution to 200 µl of whole blood, mix thoroughly by vortexing to obtain a uniform suspension. Incubate the sample at 56°C while vortexing occasionally until the cells are completely lysed (10 min). Add 200 µl of ethanol (96-100%) and mix by vortexing.

2. **Bind DNA:** Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 xg. Discard the collection tube containing the flow-through solution. Place the column into a new 2 ml collection tube.

3. **Wash the column (Wash Buffer I):** Add 500 µl of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 xg. Discard the flow-through and place the purification column back into the collection tube.

4. **Wash the column (Wash Buffer II):** Add 500 µl of Wash Buffer II (with ethanol added) to the purification column. Centrifuge for 3 min at maximum speed (≥12000 xg). If residual solution is seen in the column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution.

5. **Elute DNA:** Transfer the purification column to a sterile 1.5 ml micro-centrifuge tube. Add 200 µl of Elution Buffer to the center of the purification column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 xg. For maximum DNA yield, repeat the elution step with additional 200 µl of Elution Buffer. Discard the purification column. Collect the flow-through solution containing the purified genomic DNA. Use the purified DNA immediately in downstream applications or store at -20°C.

2.3. Genotyping by PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism)

Principle

DNA sequence containing the polymorphism is amplified by PCR and then broken into pieces (digested) by restriction enzymes. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments.

2.3.1. Polymerase chain reaction (PCR)

Principle

PCR program consists of three steps, namely denaturation, annealing and extension, and is carried out using a thermo-cycler (figure 11). Complete denaturation of the DNA template is performed at the start of the PCR reaction at high temperature (about 95°C). Double stranded DNA can be disrupted by heat giving rise to single stranded DNA.

Polymerases require short regions of double stranded nucleic acid to initiate synthesis. This can be provided by synthetic oligonucleotides that are known as ‘primers’ and chosen according to the DNA region to be amplified. Two synthetic primers that flank the region of interest are used; one primer is complementary to the negative strand of DNA and second

primer to the positive strand. The annealing step involves attachment of these primers to the template DNA. As a general rule, the annealing temperature should be 5°C below the Tm (melting temperature of the DNA duplex).

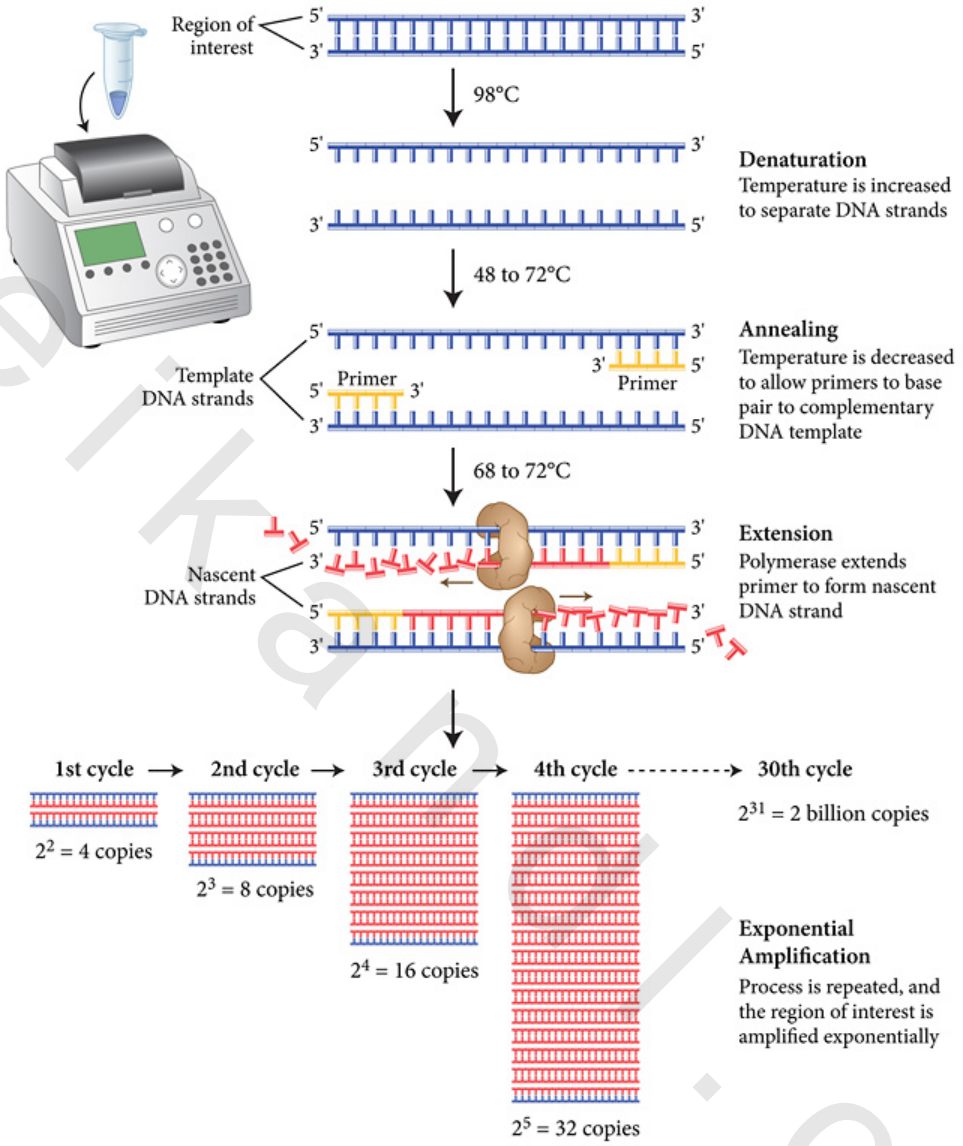

Figure 11: Steps of PCR.

Lastly the extension step involves the formation of nascent DNA sequence by DNA polymerase. The single stranded DNA serves as a template for synthesis of a complementary strand by replicating enzymes, DNA polymerases. In order to imitate the accelerated form of DNA replication for a gene region, a special form of DNA polymerase resistant to thermal denaturation is used, Taq polymerase, an enzyme isolated from the bacterium *Thermus aquaticus*.

Because only one amplification is not enough, PCR is a cyclic process. The sample is heated again to separate the strands, and then cooled to allow another round of DNA duplication. After 20 cycles of amplification, a million copies of DNA can be generated from a single copy. After several rounds of amplification (about 40 times), the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain.

Reagents

- Forward and reverse primers (Fermentas, Germany)
  To each primer, a volume of nuclease free water was added for reconstitution as indicated by the manufacturer (Table 4) to obtain a stock solution of 100 pmoles/µl. Each solution was vortexed gently to ensure homogeneity. From this stock solution, 10 µl was moved to a 1.5 ml micro-centrifuge tube and diluted to 200 µl with nuclease free water to obtain a solution of 5 pmoles/µl.
- DreamTaq Green PCR Master Mix #K1082 (ThermoScientific, USA) which contains Taq DNA polymerase. It also contains dATP, dCTP, dGTP and dTTP, 0.4 mM each, 4 mM $MgCl_2$, a density reagent (Glycerol) and two dyes for monitoring electrophoresis progress: the blue dye migrates with 3-5 kb DNA fragments in a 1% agarose gel and the yellow dye migrates faster than 10 bp DNA fragments in 1% agarose gel.
- Nuclease Free Water.

Table 4: Volume of nuclease free water added to each primer for reconstitution.

Primer	Volume added
XPD 312 F	186.3 µl
XPD 312 R	214.0 µl
XPD 751 F	114.5 µl
XPD 751 R	125.7 µl

Primers

Two sets of primers were used, one for each polymorphism[172]:

1. *XPD* Asp312Asn

a. Forward: 5′CTGTTGGTGGGTGCCCGTATCTGTTGGTCT3′
b. Reverse: 5′TAATATCGGGGCTCACCCTGCAGCACTTCCT3′

2. *XPD* Lys751Gln

a. Forward: 5′GCCCGCTCTGGATTATACG3′
b. Reverse: 5′CTATCATCTCCTGGCCCCC3′

PCR amplification procedure

1. Gently vortex and briefly centrifuge PCR Master Mix, primer solutions and purified DNA after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 25 µl reaction: 12.5 µl DreamTaq Green PCR Master Mix, 1 µl Forward primer, 1 µl Reverse primer and 10.5 µl Template DNA.
3. Gently vortex the reaction mixture and transfer the tubes to the thermo-cycler.
4. Perform PCR using the thermal cycling conditions specific for each polymorphism[172]. *XPD* Asp312Asn (G→A) PCR starts with an initial denaturation at 94°C for 3 min., followed by 30 cycles of denaturation at 94°C for 45 sec., annealing at 65°C for 45 sec. and extension at 72°C for 60 sec. and ends with a final extension step at 72°C for 7 min. While PCR reaction of *XPD* Lys751Gln (A→C) starts with an initial denaturation at 94°C for 3 min. followed by 38 cycles of denaturation at 94°C for 45 sec., annealing at 50°C for 45 sec., extension at 72°C for 60 sec. and ends with a final extension step at 72°C for 7 min.
5. For detection of PCR product, run 10 µl of the PCR product on gel electrophoresis using 2% agarose gel for 20 min. at 100 V. Compare results with DNA ladder (GeneRuler 50 bp, ThermoScientific) to ensure the amplification of the desired DNA sequences. *XPD*

Asp312Asn (G→A) PCR reaction gives a 751 bp DNA sequence, while *XPD* Lys751Gln (A→C) PCR reaction gives a 436 bp DNA sequence.

6. Apply restriction digestion on the PCR product immediately or store at -20°C.

2.3.2. Restriction fragment length polymorphism (RFLP)

Principle

Restriction endonucleases are enzymes that recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to these sequences to produce fragments, called restriction fragments. Because of restriction enzymes' specificity for certain nucleotide sequences, it is possible for them to discriminate polymorphisms in a DNA sequence which can change an already existing recognition site or create a previously non-existing one.

2.3.2.1. *XPD* Asp312Asn polymorphism (rs1799793): is a **G ⇒ A** variation at position 11587 (figure 12), codon 312 (**G**AC ⇒ **A**AC) in exon 10 with a residue change in protein product: **D[Asp] ⇒ N[Asn]**.

```
CTG CCC GAC GAA GTG          CTG CCC AAC GAA GTG
 L   P   D   E   V     ⇒      L   P   N   E   V
GAC GGG CTG CTT CAC          GAC GGG TTG CTT CAC
 Wild variant                 Mutant variant
```

XPD Asp312Asn (G→A) PCR product is digested by FastDigest StyI (Eco130I) restriction enzyme (#FD0414) (Thermo Scientific).

Recognition site for StyI (Eco130I): `5'...C↓C W W G G...3'` (W can be A or T)
`3'...G G W W C↑C...5'`

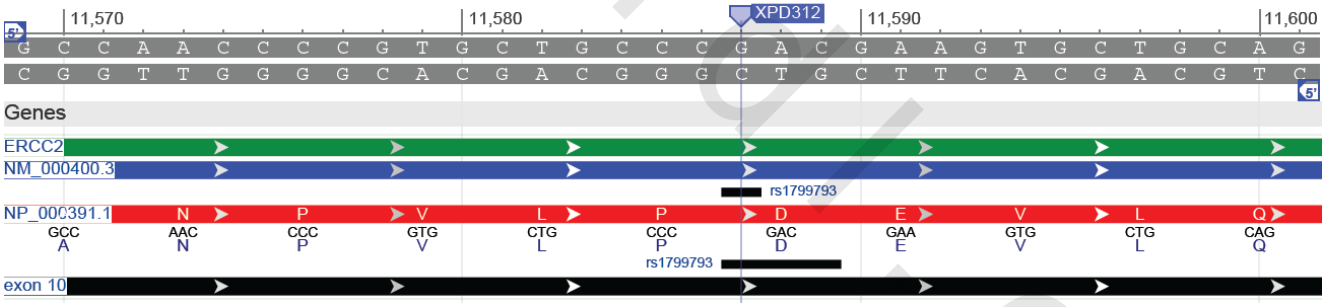

Figure 12: Small sequence of *XPD* gene showing the wild variant of the *XPD* Asp312Asn (G→A) polymorphism and marking its position on the gene[(173)].

XPD Asp312Asn (G→A) 751 bp PCR product with wild polymorphic variant (*XPD* 312 Asp (G)) has one StyI recognition site resulting in 507 bp and 244 bp fragments upon digestion. While the mutant variant (*XPD* 312 Asn (A)) creates another recognition site for the enzyme, producing three fragments, 474 bp, 244 bp and 33 bp. Therefore, there will be three possible outcomes for the StyI (Eco130I) digestion of *XPD* Asp312Asn (G→A) PCR product, according to the genotype of the two alleles (table 5, figure 13).

Table 5: Restriction fragments produced by the digestion of *XPD* Asp312Asn (G→A) PCR product by StyI (Eco130I).

Genotype	Digestion Fragments
GG (Asp/Asp)	507 bp, 244 bp
GA (Asp/Asn)	507 bp, 474 bp, 244 bp, 33 bp
AA (Asn/Asn)	474 bp, 244 bp, 33 bp

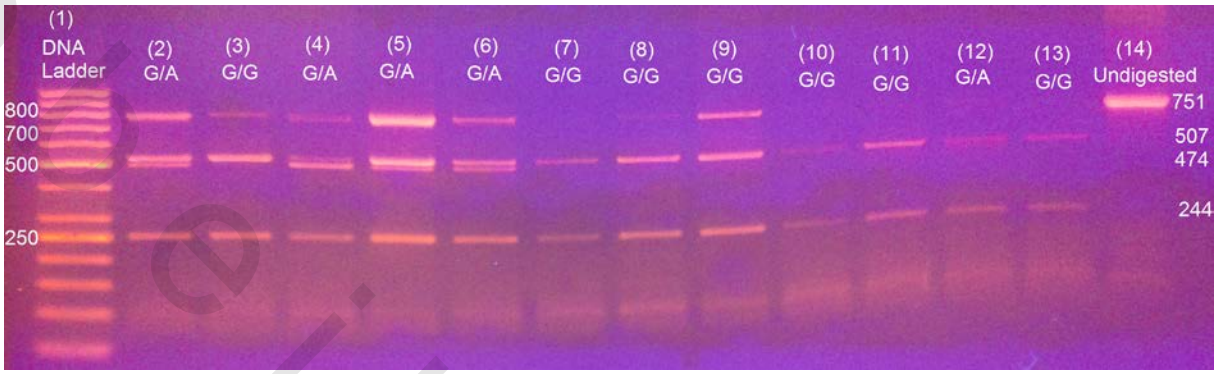

Figure 13: Gel electrophoresis for *XPD* Asp312Asn (G→A) polymorphism showing DNA ladder (50-1000 bp) at the first lane on the left, undigested PCR product at the last lane on the right, GA genotype at lanes 2, 4, 5, 6 and 12, and GG genotypes at lanes 3, 7, 8, 9, 10, 11 and 13.

Reagents

- FastDigest StyI (Eco130I) restriction enzyme kit #FD0414 (Feremntas, Germany) which contains:
  - 200 µl FastDigest StyI (Eco130I) restriction enzyme, Source: *Escherichia coli* RFL130. It is an isoschizomer of StyI (source: *Salmonella typhi*) having the same recognition and cleavage specificity.
  - 1 ml of 10X FastDigest Green Buffer which includes a density reagent (glycerol) along with blue and yellow tracking dyes that allow for direct loading of the reaction mixtures on a gel. The blue dye of the FastDigest Green Buffer migrates with 3-5 kb DNA fragments in a 1% agarose gel, while the yellow dye of the FastDigest Green Buffer migrates faster than 10 bp DNA fragments in a 1% agarose gel.
- Nuclease free water.

Restriction digestion procedure:

1. Combine the following reaction components at room temperature in the order indicated to obtain a 30 µl final reaction volume: 17 µl nuclease-free water, 2 µl of 10X FastDigest Green Buffer, 10 µl *XPD* Asp312Asn (G→A) PCR product and 1 µl FastDigest StyI enzyme. Mix gently and spin down
2. Incubate at 37°C for 20 min.
3. Inactivate the enzyme by heating for 5 min at 65°C.
4. To detect restriction fragments, run 10 µl of the restriction digest on gel electrophoresis using 2% agarose gel for 30 min. at 100 V. Compare results with DNA ladder (GeneRuler 50 bp, ThermoScientific) to measure the length of the restriction fragments and determine the genotype of each sample.

2.3.2.2. *XPD* Lys751Gln (rs13181) polymorphism is a **A ⇒ C** variation at position 23927 (figure 14), codon 751 (**A**AG ⇒ **C**AG) in exon 23 with a residue change in protein product: **K[Lys] ⇒ Q[Gln]**.

```
ACG CTG AAG AGG ATA          ACG CTG CAG AGG ATA
 T   L   K   R   I     ⇒      T   L   Q   R   I
TGC GAC TTC TCC TAT          TGC GAC GTC TCC TAT
 Wild variant                 Mutant variant
```

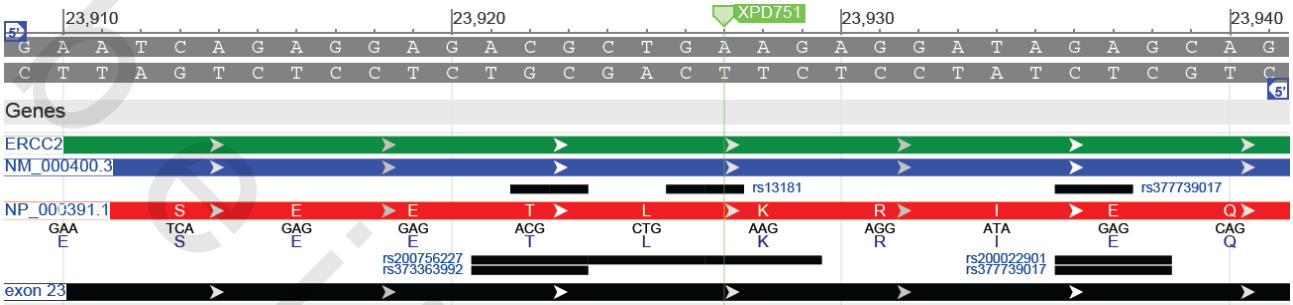

Figure 14: Small sequence of *XPD* gene showing the wild variant of the *XPD* Lys751Gln (A→C) polymorphism and marking its position[173].

XPD Lys751Gln (A→C) PCR product is digested by PstI restriction enzyme (#ER0611) (Thermo Scientific)

Recognition site for PstI:

```
5'...C T G C A↓G...3'
3'...G↑A C G T C...5'
```

XPD Lys751Gln (A→C) 436 bp PCR product with wild polymorphic variant (*XPD* 751 Lys (A)) has one PstI recognition site resulting in 290 bp and 146 bp fragments upon digestion. The mutant variant (*XPD* 751 Gln (C)) creates a new recognition site for the enzyme, producing three fragments, 227 bp, 146 bp and 63 bp. Therefore, there will be three possible outcomes for the PstI digestion of *XPD* Lys751Gln (A→C) PCR product, according to the genotypes of the two alleles (table 6, figure 15).

Table 6: Restriction fragments produced by the digestion of *XPD* Lys751Gln (A→C) PCR product by PstI.

Genotype	Digestion Fragments
AA (Lys/Lys)	290 bp, 146 bp
AC (Lys/Gln)	290 bp, 227 bp, 146 bp, 63 bp
CC (Gln/Gln)	227 bp, 146 bp, 63 bp

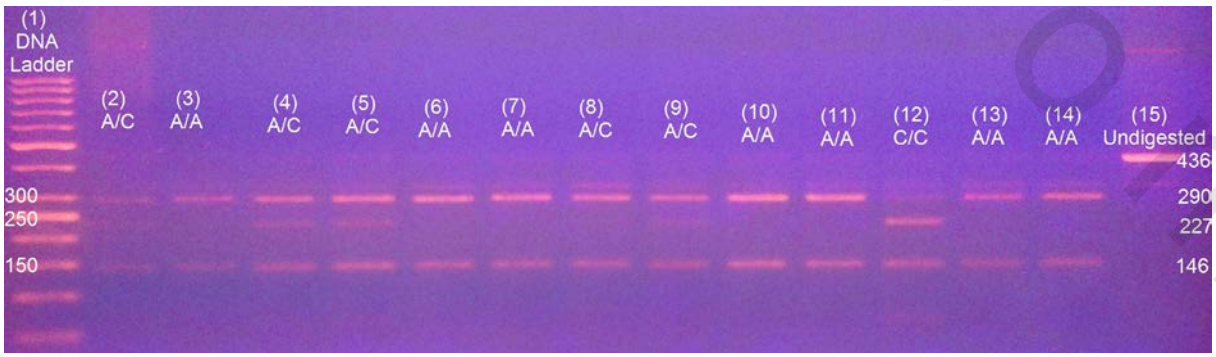

Figure 15: Gel electrophoresis for *XPD* Lys751Gln (A→C) polymorphism showing DNA ladder (50-1000 bp) at the first lane on the left, undigested PCR product at the last lane on the right, AC genotype at lanes 2, 4, 5, 8 and 9, AA genotype at lanes 3, 6, 7, 10, 11, 13 and 14, and CC genotype at lane 12.

Reagents

- PstI restriction enzyme kit #ER0611 (ThermoScientific, USA) which contains:
  - 300 µl PstI restriction enzyme, source: *Providencia stuartii*.
  - 2 ml of 10X Buffer O which consists of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA.
- Nuclease free water.

Restriction digestion procedure:

1. Combine the following reaction components at room temperature in the order indicated to obtain a 30 µl final reaction volume: 17 µl nuclease-free water, 2 µl of 10X Buffer O, 10 µl *XPD* Lys751Gln (A→C) PCR product and 1 µl PstI enzyme. Mix gently and spin down
2. Incubate at 37°C for 16 hours.
3. Stop the digestion reaction by adding 1 µl of 0.5 M EDTA, pH 8.0, and mix thoroughly.
4. To detect restriction fragments, mix 10 µl of the restriction digest with 2 µl 6X loading dye and run 10 µl of this mixture on gel electrophoresis using 2% agarose for 30 min. at 100 V. Compare results with DNA ladder (GeneRuler 50 bp, ThermoScientific) to measure the length of the restriction fragments and determine the genotype of each sample.

2.3.3. Agarose gel electrophoresis

Principle

In gel electrophoresis, samples to be separated are applied to a porous gel medium, made of a material such as agarose, in an electrophoresis chamber with an electrode at each side. The gel is then covered with an ion containing buffer, which controls the pH of the system and conducts electricity. The chamber is connected to a power supply and an electrical current is applied. Once the electric field is established, charged molecules in the samples migrate through the pores of the gel toward their pole of attraction.

Since DNA is negatively charged, it will move towards the anode. The mobility of DNA molecules during gel electrophoresis depends on its molecular size. Small molecules maneuver more easily through the pores than larger molecules and therefore travel relatively quickly. Large molecules encounter more resistance as they make their way through the tiny pores and therefore travel at a slower rate.

Visualization of DNA fragments is accomplished by complexation of DNA with a fluorescent dye (ethidium bromide) during electrophoresis.

Reagents

- Electrophoresis buffer, 10X Tris-Borate-EDTA (TBE) pH 8.3 # B52 (ThermoScientific, USA), which is diluted to a concentration of 1X before use. For each electrophoresis fresh 1X buffer should be used (composition of 1X buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA).

- Ethidium bromide 1% w/v. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows visual detection of DNA fragments in gels when exposed to UV light.

- Agarose powder.

- DNA ladder, GeneRuler 50 bp, ready-to-use #SM0373 (ThermoScientific, USA), (figure 16).

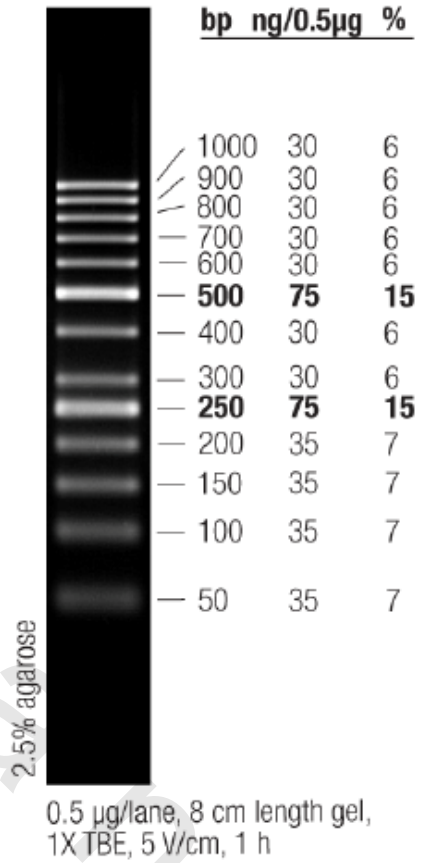

Figure 16: DNA ladder, GeneRuler 50 bp, on 2.5% agarose.

This ladder is composed of thirteen chromatography-purified individual DNA fragments (in base pairs): 1000, 900, 800, 700, 600, **500**, 400, 300, **250**, 200, 150, 100, 50. It contains two reference bands (500 and 250 bp) for easy orientation. The ladder is ready to use, it is premixed with 6X DNA Loading Dye for direct loading on gel. Storage and Loading Buffer: 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.005% bromophenol blue, 0.005% xylene cyanol FF and 10% glycerol.

- 6X Loading Dye Solution #R0611 (Fermentas, Germany), which is used for loading DNA markers and samples on agarose or polyacrylamide gels. It contains dense agent (e.g. glycerol) to allow the sample to "fall" into the sample wells, and two dyes, bromophenol blue and xylene cyanol FF, for easy visual tracking of DNA migration during electrophoresis. Composition: 10mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60mM EDTA. In 1% agarose gels bromophenol blue co-migrates with ~300 bp fragment and xylene cyanol FF – with ~4000 bp fragment.

Agarose gel electrophoresis procedure

A. Preparation of the gel bed

1. Close off the open ends of a clean and dry gel bed (casting tray) and place a comb at the desired notch to allow formation of the wells (figure 17).

2. Use a conical flask to prepare 2% agarose gel solution using 1X electrophoresis buffer. Heat the mixture to boiling on a hot plate with occasional swirling. Boil until all the agarose is completely dissolved and solution appear clear without any undissolved

particles. Cool the molten agarose to about 55–60°C. Add an appropriate volume of ethidium bromide 1% to reach a final concentration of 0.5 µg/ml with careful swirling to promote even distribution of the dye.

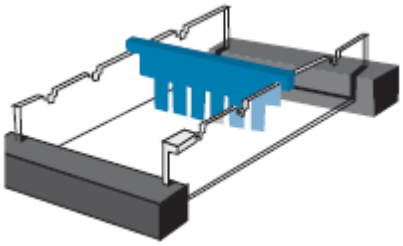

Figure 17: Casting tray (gel bed).

3. Make sure the casting tray is on a level surface and pour the cooled agarose solution into the casting tray (figure 18). Allow the gel to completely solidify.

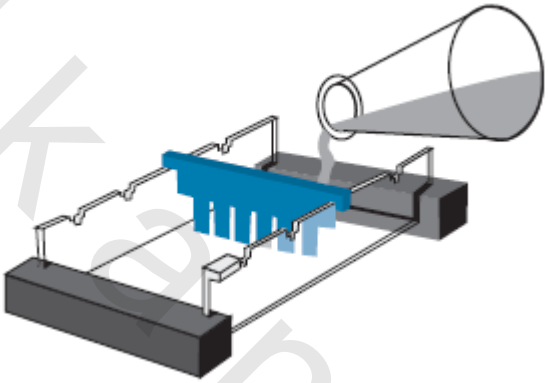

Figure 18: Pouring the agarose solution into the casting tray.

B. Preparing the gel for electrophoresis

1. After the gel is completely solidified, carefully open the ends of the casting tray and place it in the electrophoresis chamber (figure 19).

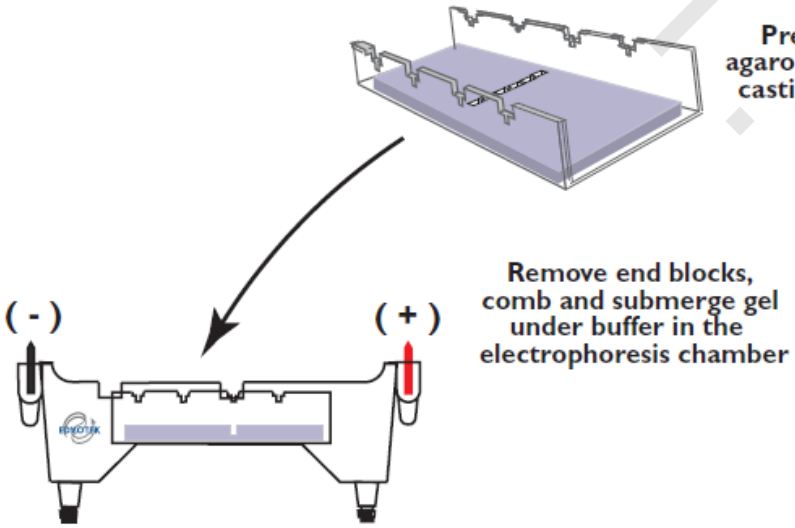

Figure 19: Placing the casting tray in the electrophoresis chamber.

2. Fill the electrophoresis chamber with 1X electrophoresis buffer solution making sure the gel is fully submerged in the buffer.

3. Remove the comb by slowly pulling straight up. Do this carefully and evenly to prevent tearing the sample wells.

C. Sample Delivery (Gel Loading)

1. Load 10 µl of the ready-to-use DNA ladder in the first well and then load the same volume for each sample into the other wells using a micropipette (figure 20).

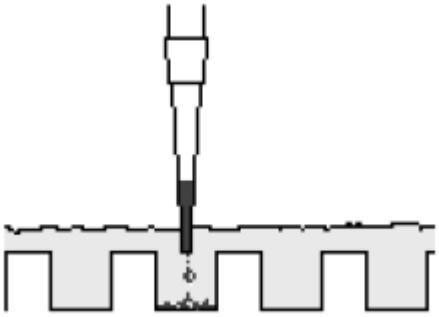

Figure 20: Loading the sample into the well.

D. Running the gel

1. After the samples are loaded, carefully snap the cover down onto the electrode terminals. Insert the plug of the black wire into the black input of the power source (negative input) and the plug of the red wire into the red input of the power source (positive input) (figure 21).

2. Set the power source at the required voltage (about 100 V) and conduct electrophoresis. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.

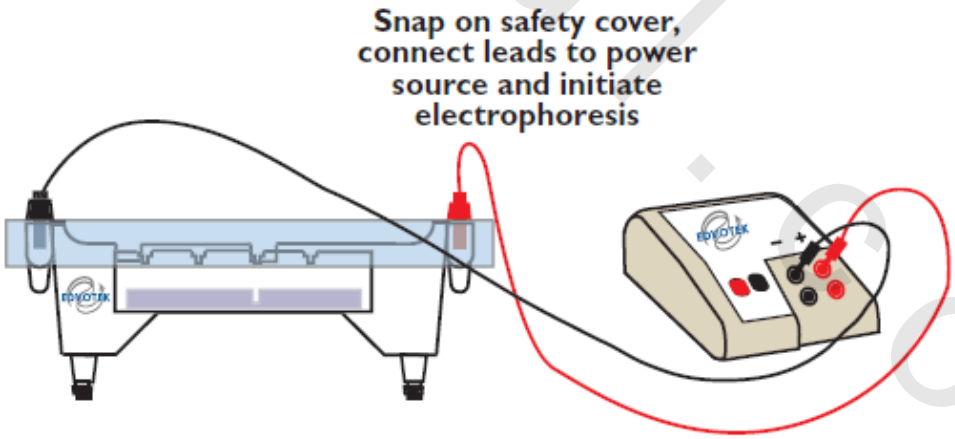

Figure 21: connect the electrophoresis chamber to the power supply and start electrophoresis.

3. Track the progress of electrophoresis using the colored tracking dyes. After the electrophoresis is completed, turn off the power, unplug the power source, disconnect the electrodes and remove the cover.

E. Visualizing results

1. Carefully transfer the casting tray containing the gel to the ultraviolet box, close the safety cover, turn on the UV lamp and visualize the DNA bands stained with ethidium bromide.

Compare bands of the DNA fragments of the samples with those of the DNA ladder to determine the genotypes. Take a photo of the gel to document the results.

2.4. Statistical analyses of the data

Data were fed to the computer using IBM SPSS Statistics software package, version 20.0 (New York, USA).

Quantitative data were described using median, minimum and maximum as well as mean and standard deviation. The distribution of continuous variables was tested for normality using Shapiro-Wilk test, because of small sample size (≤ 2000).

Differences in continuous variables between groups were compared using parametric tests (ANOVA) for normally distributed data and non-parametric tests (Mann-Whitney U test for 2 samples and Kruskal-Wallis test for k samples) for abnormally distributed data.

Qualitative data were described using number and percent. Differences between groups regarding categorical variables were tested using Pearson Chi-square (χ^2) test on cross tabulated data. When more than 20% of the cells had expected count of less than 5, correction for Chi-square (χ^2) was conducted using Fisher's Exact test. Monte Carlo approximation was used instead of Fisher's Exact test with large samples (N>100 for 2 x 2 contingency tables and N>30 for r x c contingency tables).

Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 95% confidence level.

RESULTS

RESULTS

Clinical and laboratory parameters of AML patients

Fifty-one newly diagnosed de novo cytogenetically normal AML patients were enrolled in our study. Their ages ranged from 19 – 57 years with a median of 32 years and a mean of 34.3 ± 10.3. Age was tested for normality using Shapiro-Wilk test and was found to be significantly deviated from normal distribution (p=0.036). 28 Patients were males comprising 54.9%, while 23 were females (45.1%). (Table 7 and Figure 22).

Regarding AML FAB subtypes, the most frequent was M2 with 12 patients (23.5%), followed by M1 and M4 each represented by 11 patients (21.6%), while M5, M6 and M0 were found in 8 (15.7%), 4 (7.8%) and 3 (5.9%) patients, respectively. The least frequent was M7 with only 2 patients, representing 3.9% of the subjects (Table 7 and Figure 23).

Flow cytometry data showed that 9 patients (17.6%) tested positive for CD34 and 11 (21.6%) showed lymphoid marker co-expression. Additionally 6 patients (11.8%) suffered from CNS-infiltration (Table 7 and Figure 24).

As for chemotherapy-induced toxicities, hepatotoxicity was the most frequent, appearing in 14 patients (27.5%), followed by metabolic toxicity 12 (23.5%). While nephrotoxicity and cardiac toxicity, each, affected 7 patients (13.7%) (Table 7 and Figure 25).

Out of the 51 AML patients, 21 (41.2%) failed to respond to induction chemotherapy while 30 patients (58.8%) achieved complete remission (Table 7 and Figure 26).

Among the 30 responders, 19 (63.3%) needed one course of chemotherapy to achieve complete remission, while 11 (36.7%) needed two courses (Table 7 and Figure 27).

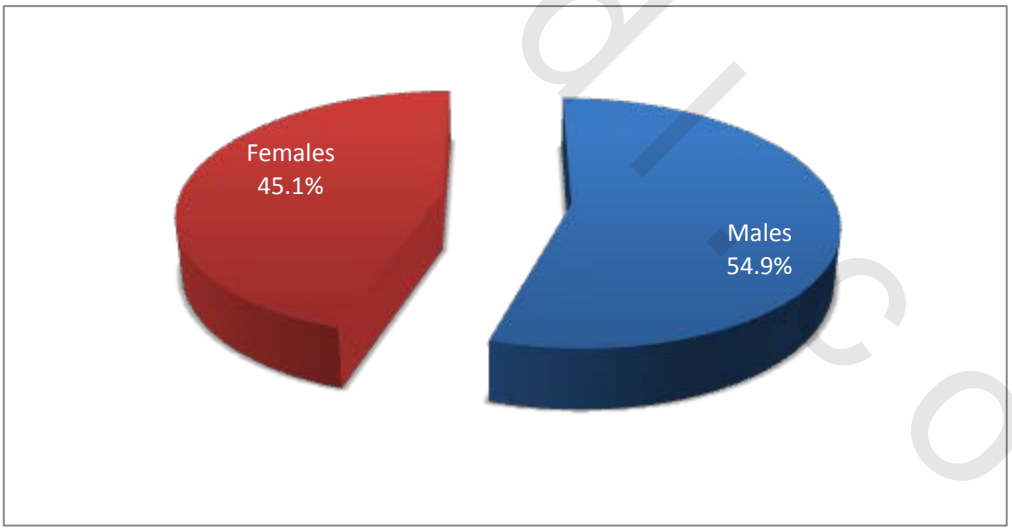

Figure 22: Distribution of AML patients according to gender.

Table 7: Clinical and laboratory parameters of AML patients.

Parameter		AML patients (n=51)	
Age (years)	Min – Max	19.0 – 57.0	
	Mean ± SD.	34.3 ± 10.3	
	Median	32.0	
		no.	**%**
Gender	Male	28	54.9
	Female	23	45.1
AML FAB subtypes	M0	3	5.9
	M1	11	21.6
	M2	12	23.5
	M4	11	21.6
	M5	8	15.7
	M6	4	7.8
	M7	2	3.9
CD34 expression	Positive	9	17.6
	Negative	42	82.4
Lymphoid marker co-expression	Positive	11	21.6
	Negative	40	78.4
CNS-infiltration	Yes	6	11.8
	No	45	88.2
Hepatic toxicity	Yes	14	27.5
	No	37	72.5
Renal toxicity	Yes	7	13.7
	No	44	86.3
Metabolic toxicity	Yes	12	23.5
	No	39	76.5
Cardiac toxicity	Yes	7	13.7
	No	44	86.3
Response	Complete response	30	58.8
	Resistance	21	41.2
Number of induction courses to CR (n=30)	One	19	63.3
	Two	11	36.7

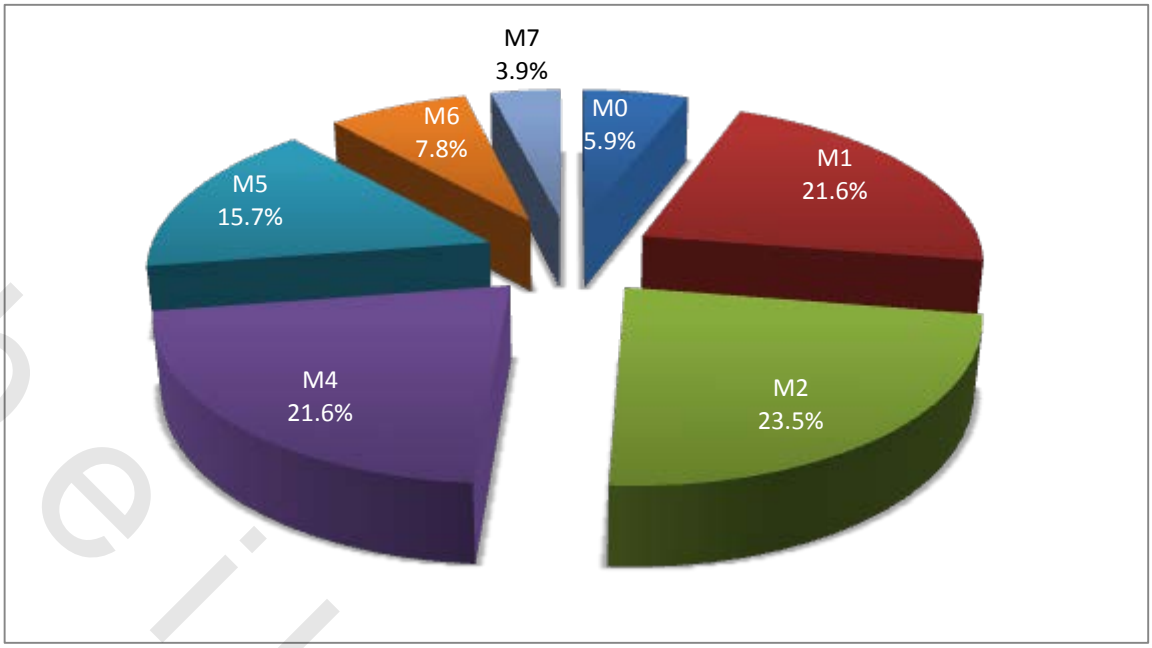

Figure 23: Distribution of the studied cases according to FAB subtypes.

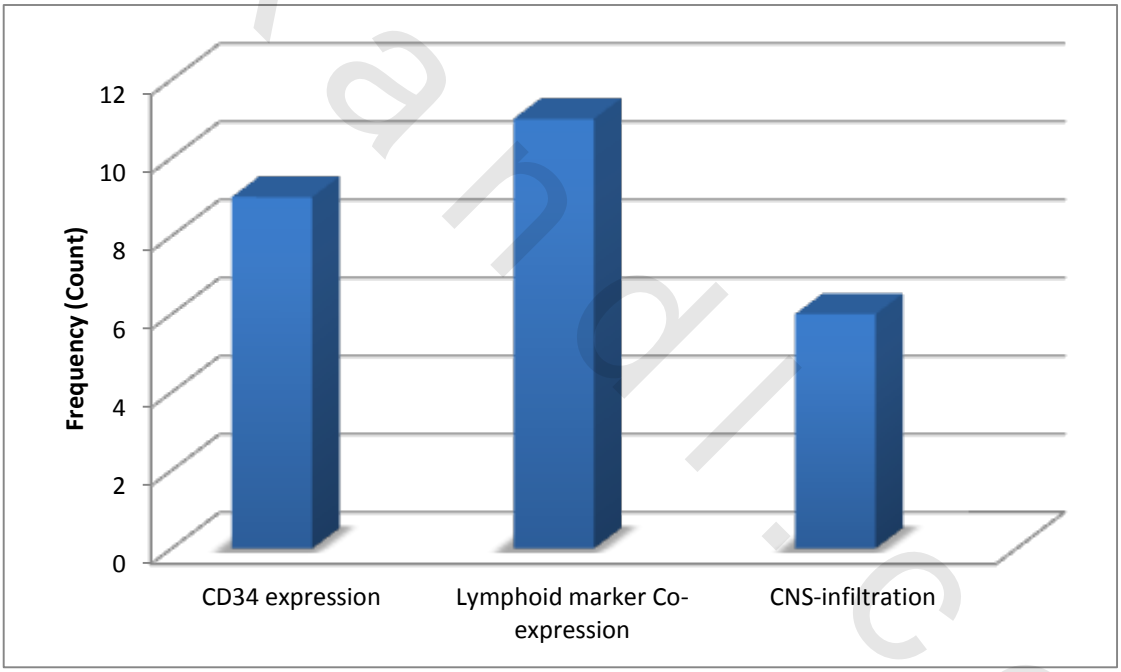

Figure 24: Frequencies of flow cytometry markers and CNS-infiltration in AML patients.

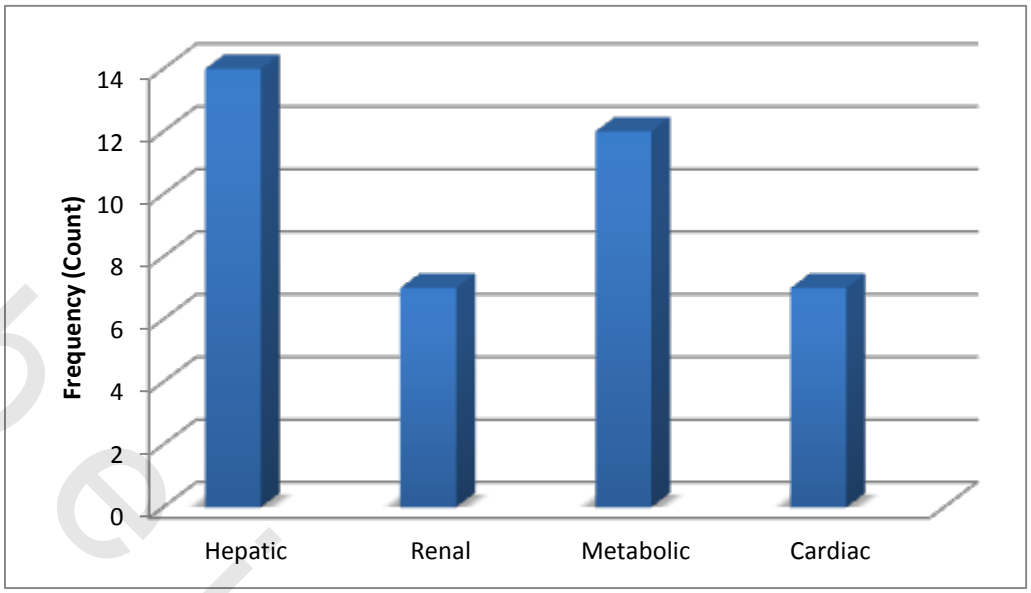

Figure 25: Frequencies of chemotherapy-induced toxicities in AML patients.

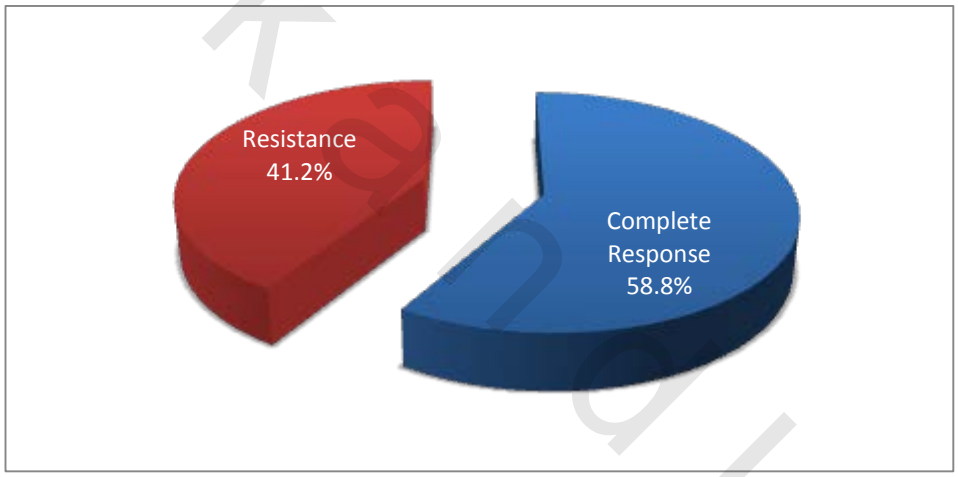

Figure 26: Response of AML patients to induction chemotherapy.

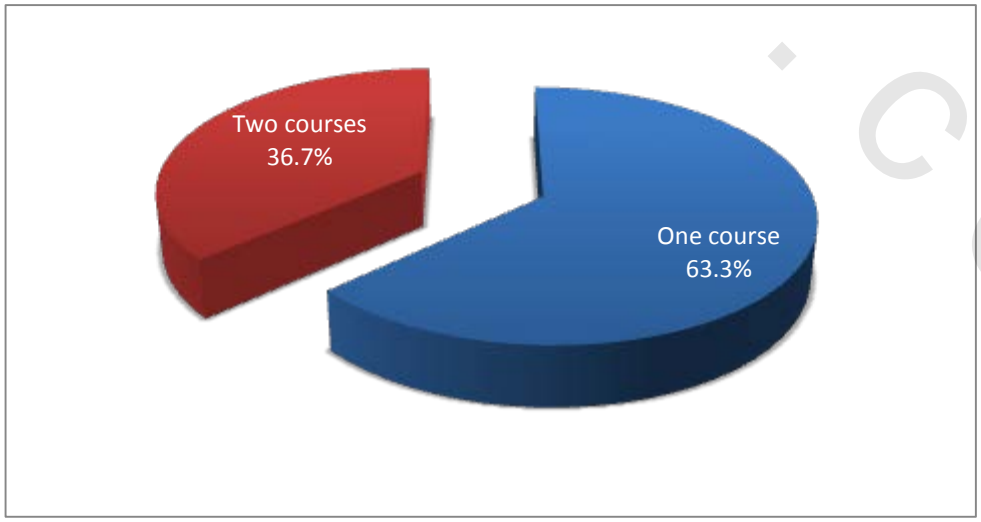

Figure 27: Number of courses needed to achieve complete remission.

Distribution of genotype and allele frequency of *XPD* Asp312Asn (G→A) and *XPD* Lys751Gln (A→C) polymorphisms in AML patients.

Our data regarding *XPD* Asp312Asn polymorphism showed that *XPD* Asp312Asn GG genotype was the most frequent with 29 patients (56.9%), followed by the GA variant in 19 patients (37.2%), while the AA variant was the least frequent, found in only 3 patients (5.9%). *XPD* 312 G (Asp) allele had a frequency of 75.5% and *XPD* 312 A (Asn) represented 24.5% (Table 8, Figures 28 and 29).

As for *XPD* Lys751Gln polymorphism, the AA genotype was the most frequent, in 25 patients (49.0%), followed by the AC and CC variants in 20 (39.2%) and 6 (11.8%) patients, respectively. Allele frequency for *XPD* 751 A (Lys) was 68.8% and for *XPD* 751 C (Gln) was 31.4% (Table 8, Figures 30 and 31).

Genotype frequencies for both polymorphisms were consistent with the Hardy-Weinberg equilibrium ($p>0.05$). Yet, this result is not accurate for the *XPD*312 polymorphism because one genotype (AA) is possessed by 3 (<5) individuals, a consequence of sample size restrictions.

Table 8: Distribution of genotype and allele frequency of *XPD* Asp312Asn (G→A) and *XPD* Lys751Gln (A→C) polymorphisms in AML patients.

Polymorphism			n=51	%	Hardy-Weinberg equilibrium		
					Expected n	χ^2	p
***XPD* Asp312Asn (G→A)**	**Genotypes**	Asp/Asp (GG)	29	56.9	29.1	0.002326	0.912
		Asp/Asn (GA)	19	37.2	18.9		
		Asn/Asn (AA)	3	5.9	3.1		
	Alleles	(G) Allele	----	75.5	----	----	----
		(A) Allele	----	24.5			
***XPD* Lys751Gln (A→C)**	**Genotypes**	Lys/Lys (AA)	25	49.0	24.0	0.406569	0.524
		Lys/Gln (AC)	20	39.2	22.0		
		Gln/Gln (CC)	6	11.8	5.0		
	Alleles	(A) Allele	----	68.6	----	----	----
		(C) Allele	----	31.4			

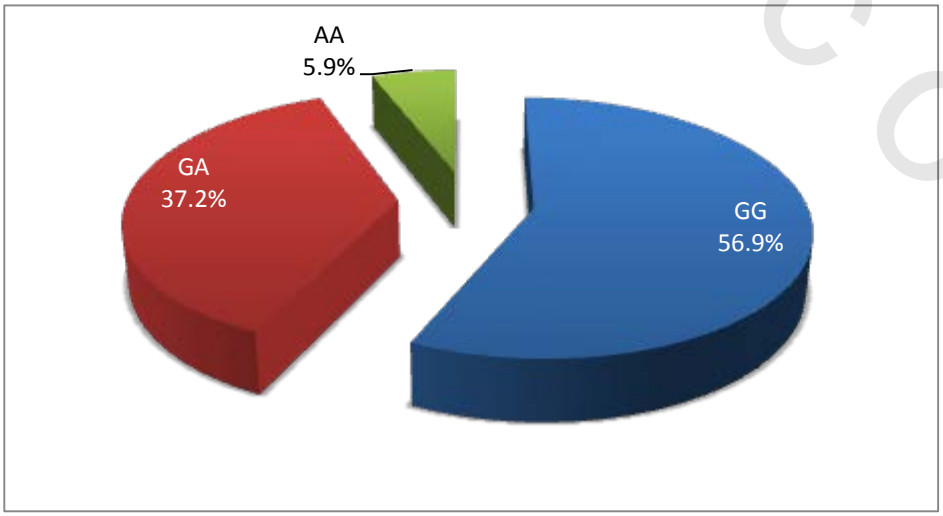

Figure 28: Distribution of the *XPD* Asp312Asn (G→A) genotypes.

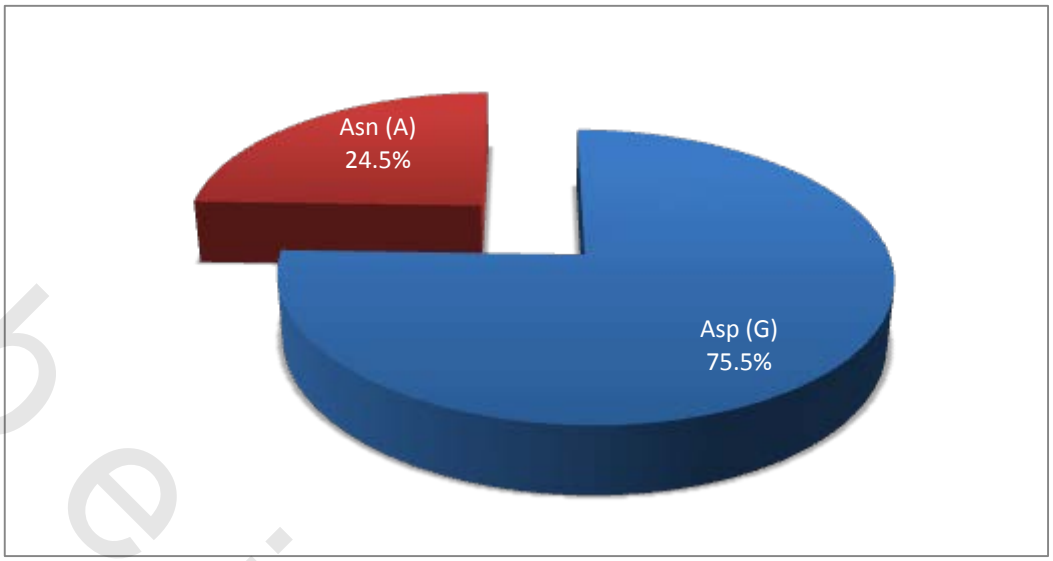

Figure 29: Frequencies of *XPD* Asp312Asn (G→A) alleles.

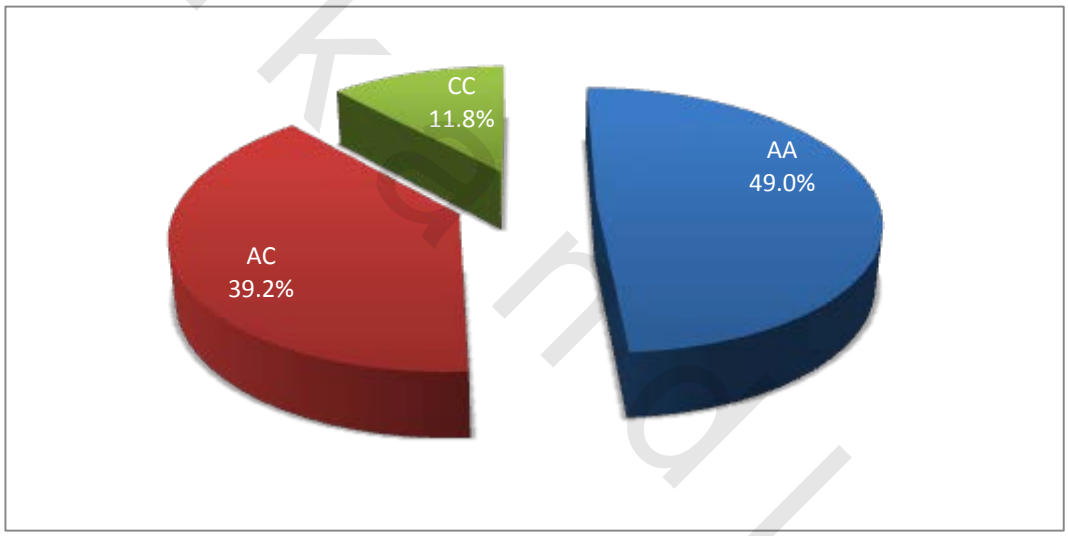

Figure 30: Distribution of *XPD* Lys751Gln (A→C) genotypes.

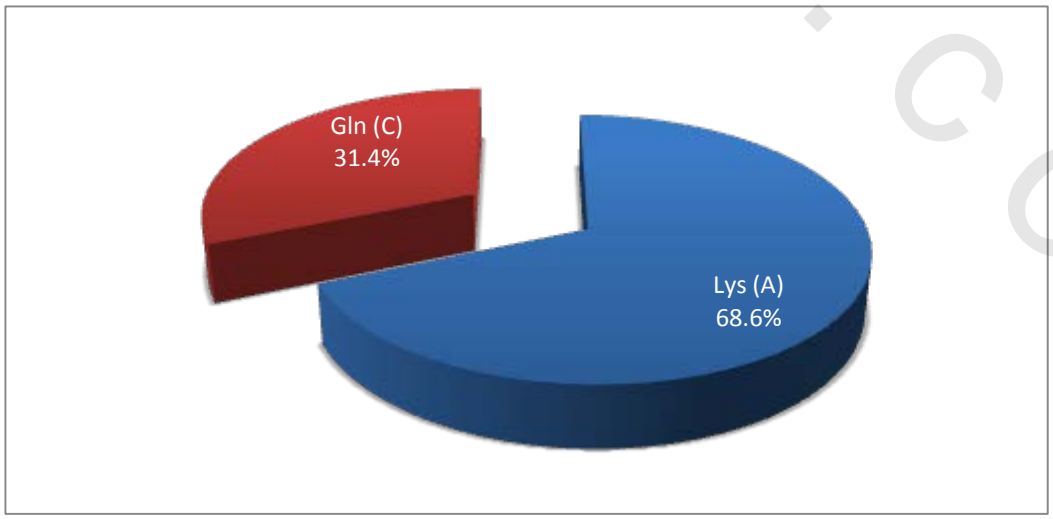

Figure 31: Frequencies of *XPD* Lys751Gln (A→C) Alleles.

Distribution of combined genotypes for both *XPD* Asp312Asn (G→A) and *XPD* Lys751Gln (A→C) polymorphisms in AML patients.

Combined genotypes for the two *XPD* polymorphisms were reported as *XPD*312/*XPD*751. The combined genotype found most frequently was GG/AA 15 (29.4%) followed by GG/AC 12 (23.5%), then GA/CC 9 (17.6%) and GA/AC 8 (15.7%). Each of the combined genotypes, AA/CC, GA/CC and GG/CC, were found in 2 patients (3.9%). The least frequent was AA/AA, which was present in only one patient (2%). Out of the 51 patients, none had AA/AC genotype combination (Table 9 and Figure 32).

Table 9: Distribution of combined genotypes for both *XPD* Asp312Asn (G→A) and *XPD* Lys751Gln (A→C) polymorphisms in AML patients.

Combined genotypes (*XPD*312/*XPD*751)	**n=51**	**%**
Homozygous Wild both: Asp312Asp (GG) & Lys751Lys (AA)	15	29.4
Homozygous Mutant both: Asn312Asn (AA) & Gln751Gln (CC)	2	3.9
Heterozygous both: Asp312Asn (GA) & Lys751Gln (AC)	8	15.7
Homozygous Wild / Heterozygous: Asp312Asp (GG) & Lys751Gln (AC)	12	23.5
Homozygous Mutant / Heterozygous: Asn312Asn (AA) & Lys751Gln (AC)	0	0.0
Heterozygous / Homozygous Wild: Asp312Asn (GA) & Lys751Lys (AA)	9	17.6
Heterozygous / Homozygous Mutant: Asp312Asn (GA) & Gln751Gln (CC)	2	3.9
Homozygous Wild / Homozygous Mutant: Asp312Asp (GG) & Gln751Gln (CC)	2	3.9
Homozygous Mutant / Homozygous Wild: Asn312Asn (AA) & Lys751Lys (AA)	1	2.0

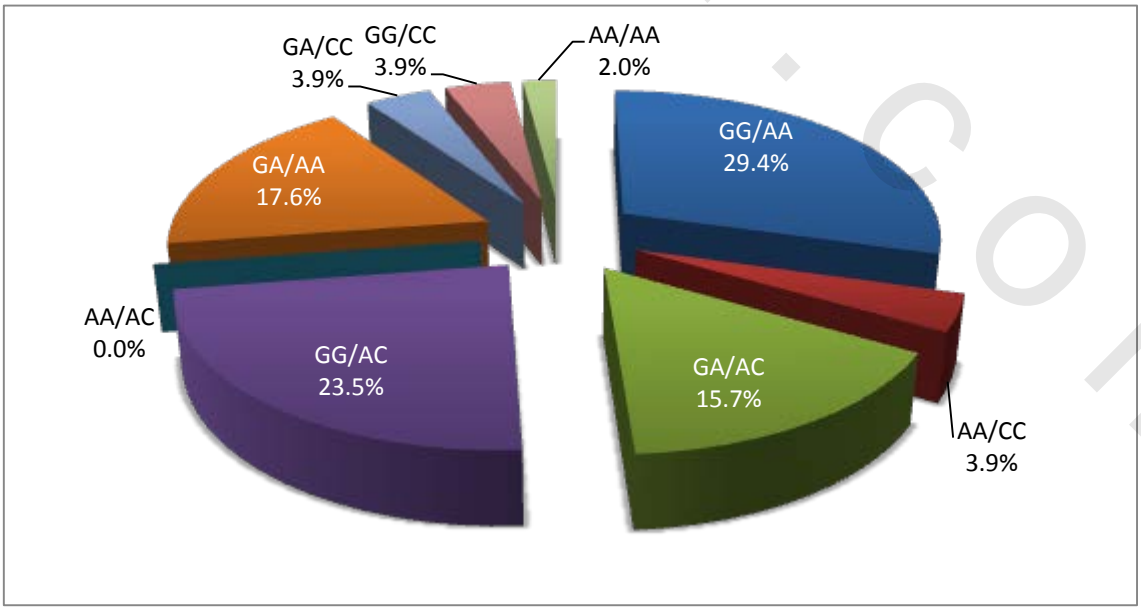

Figure 32: Distribution of *XPD*312/*XPD*751 combined genotypes.

XPD Asp312Asn (G→A) polymorphism in relation to clinical and laboratory parameters of AML patients.

There was no association between *XPD* Asp312Asn (G→A) genotypes and age, gender, AML FAB subtypes, CD34, lymphoid marker co-expression or CNS-infiltration (Table 10).

Furthermore, *XPD* Asp312Asn (G→A) variants were not associated with chemotherapy-induced toxicities, namely, hepatotoxicity, nephrotoxicity, metabolic toxicity and cardiac toxicity (Table 10, Figure 33).

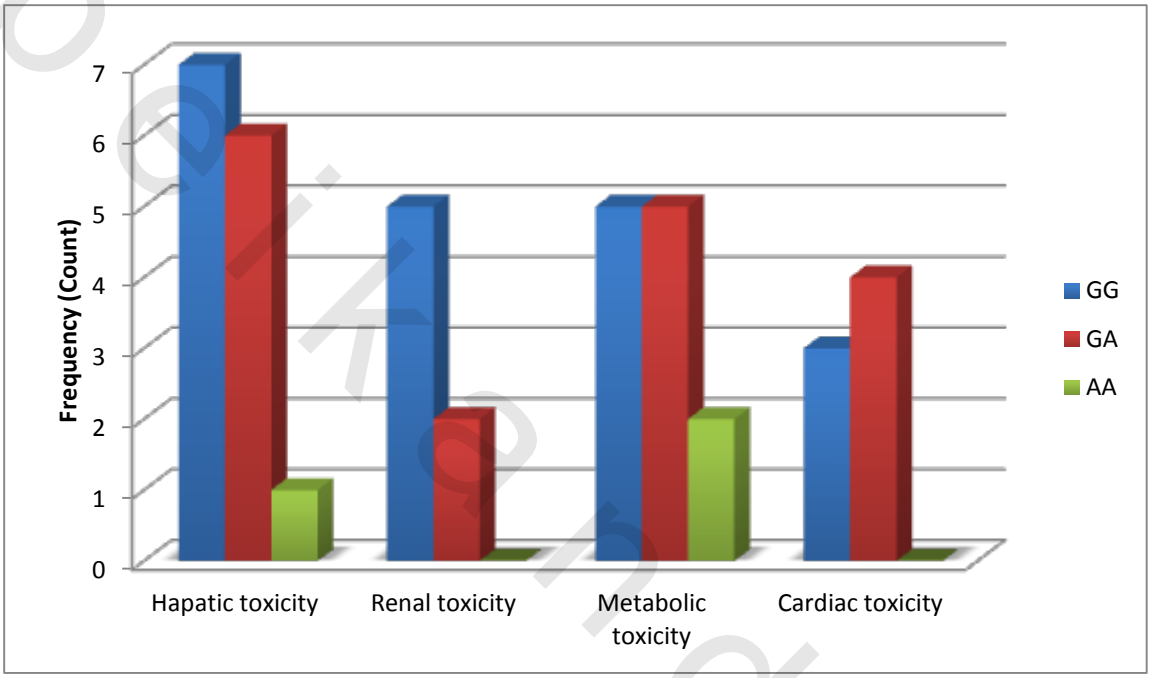

Figure 33: *XPD* Asp312Asn (G→A) polymorphism in relation to studied toxicities.

Table 10: *XPD* Asp312Asn (G→A) polymorphism in relation to clinical and laboratory parameters of AML patients (n=51).

Parameter		Asp/Asp (GG) (n=29)		Asp/Asn (GA) (n=19)		Asn/Asn (AA) (n=3)		χ^2	p
Age (years)	Min - Max	19.0 – 56.0		21.0 – 57.0		25.0 – 50.0			
	Mean ± SD	32.4 ± 9.7		36.1 ± 10.4		40.7 ± 13.7		2.395	0.302^{KW}
	Median	31.0		36.0		47.0			
		no.	**%**	**no.**	**%**	**no.**	**%**	χ^2	**p**
Gender	Male (n=28)	12	41.4	14	73.7	2	66.7	5.017	0.083^{MC}
	Female (n=23)	17	58.6	5	26.3	1	33.3		
FAB	M0 (n=3)	1	3.4	2	10.5	0	0.0	14.139	0.295^{MC}
	M1 (n=11)	9	31.0	2	10.5	0	0.0		
	M2 (n=12)	7	24.1	5	26.3	0	0.0		
	M4 (n=11)	7	24.1	2	10.5	2	66.7		
	M5 (n=8)	3	10.3	4	21.1	1	33.3		
	M6 (n=4)	2	6.9	2	10.5	0	0.0		
	M7 (n=2)	0	0.0	2	10.5	0	0.0		
CD34 positive	No (n=42)	22	75.9	17	89.5	3	100.0	2.146	0.323^{MC}
	Yes (n=9)	7	24.1	2	10.5	0	0.0		
Lymphoid marker co-expression	No (n=40)	23	79.3	14	73.7	3	100.0	1.091	0.560^{MC}
	Yes (n=11)	6	20.7	5	26.3	0	0.0		
CNS-disease	No (n=45)	26	89.7	17	89.5	2	66.7	1.429	0.585^{MC}
	Yes (n=6)	3	10.3	2	10.5	1	33.3		
Hepatic toxicity	No (n=37)	22	75.9	13	68.4	2	66.7	0.375	0.882^{MC}
	Yes (n=14)	7	24.1	6	31.6	1	33.3		
Renal toxicity	No (n=44)	24	82.8	17	89.5	3	100.0	0.944	0.805^{MC}
	Yes (n=7)	5	17.2	2	10.5	0	0.0		
Metabolic toxicity	No (n=39)	24	82.8	14	73.7	1	33.3	3.822	0.128^{MC}
	Yes (n=12)	5	17.2	5	26.3	2	66.7		
Cardiac toxicity	No (n=44)	26	89.7	15	78.9	3	100.0	1.619	0.432^{MC}
	Yes (n=7)	3	10.3	4	21.1	0	0.0		

χ^2: Chi-square value
^{KW}: Kruskal-Wallis test *p* value
^{MC}: Monte Carlo test *p* value

XPD Lys751Gln (A→C) polymorphism in relation to clinical and laboratory parameters of AML patients.

No association was found between *XPD* Lys751Gln (A→C) genotypes and age, gender, AML FAB subtypes, CD34, lymphoid marker co expression or CNS-infiltration (Table 11).

Additionally, no statistically significant association was found between any of the *XPD* Lys751Gln (A→C) variants and hepatotoxicity, nephrotoxicity or metabolic toxicity. However, there was a significant association with chemotherapy-induced cardiotoxicity, which was more frequent among patients having the CC polymorphic variant (Table 11, Figure 34).

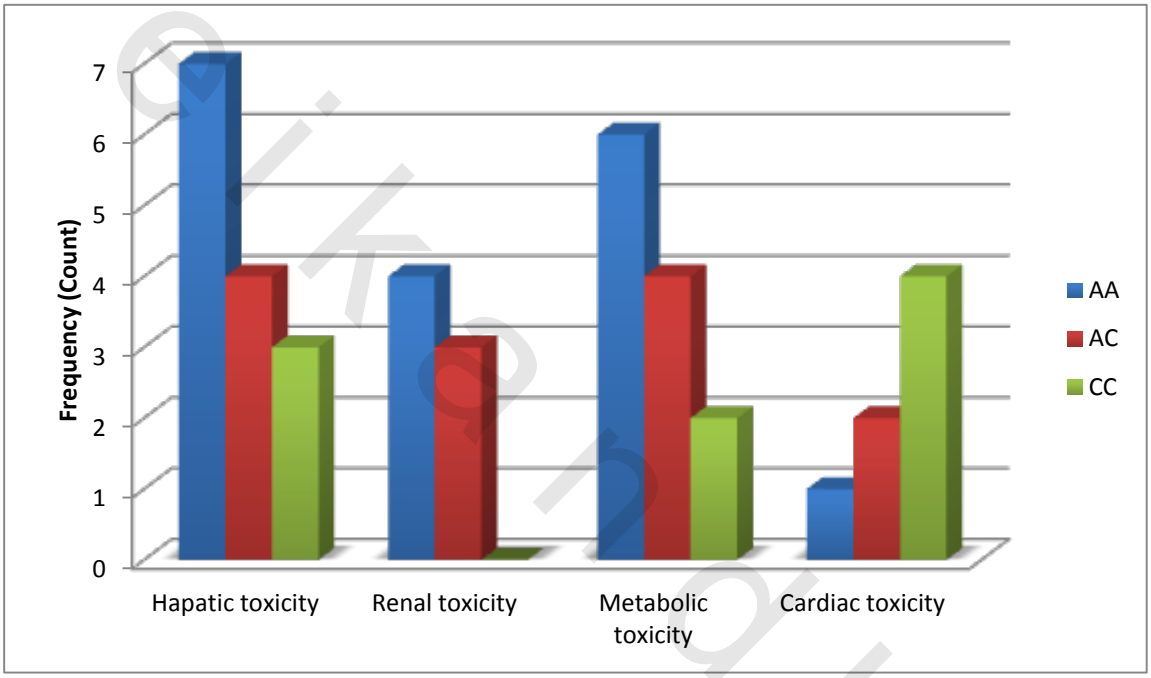

Figure 34: *XPD* Lys751Gln (A→C) polymorphism in relation to studied toxicities.

Table 11: *XPD* Lys751Gln (A→C) polymorphisms in relation to Clinical and laboratory parameters of AML patients (n=51).

Parameter		Lys/Lys (AA) (n = 25)		Lys/Gln (AC) (n = 20)		Gln/Gln (CC) (n = 6)		χ^2	p
Age (years)	Min - Max	19.0 - 57.0		21.0 - 53.0		21.0 - 50.0		0.948	0.623^{KW}
	Mean ± SD	34.6 ± 11.6		32.9 ± 8.3		37.8 ± 10.8			
	Median	35.0		31.0		39.5			
		no.	**%**	**no.**	**%**	**no.**	**%**	χ^2	**p**
Gender	Male (n=28)	11	44.0	12	60.0	5	83.3	3.369	0.212^{MC}
	Female (n=23)	14	56.0	8	40.0	1	16.7		
FAB	M0 (n=3)	2	8.0	1	5.0	0	0.0	17.933	0.111^{MC}
	M1 (n=11)	5	20.0	4	20.0	2	33.3		
	M2 (n=12)	3	12.0	9	45.0	0	0.0		
	M4 (n=11)	7	28.0	2	10.0	2	33.3		
	M5 (n=8)	6	24.0	1	5.0	1	16.7		
	M6 (n=4)	1	4.0	3	15.0	0	0.0		
	M7 (n=2)	1	4.0	0	0.0	1	16.7		
CD34 positive	No (n=42)	21	84.0	15	75.0	6	100.0	2.076	0.368^{MC}
	Yes (n=9)	4	16.0	5	25.0	0	0.0		
Lymphoid marker co-expression	No (n=40)	19	76.0	16	80.0	5	83.3	0.202	1.000^{MC}
	Yes (n=11)	6	24.0	4	20.0	1	16.7		
CNS-disease	No (n=45)	22	88.0	18	90.0	5	83.3	0.200	1.000^{MC}
	Yes (n=6)	3	12.0	2	10.0	1	16.7		
Hepatic toxicity	No (n=37)	18	72.0	16	80.0	3	50.0	2.093	0.409^{MC}
	Yes (n=14)	7	28.0	4	20.0	3	50.0		
Renal toxicity	No (n=44)	21	84.0	17	85.0	6	100.0	1.091	0.745^{MC}
	Yes (n=7)	4	16.0	3	15.0	0	0.0		
Metabolic toxicity	No (n=39)	19	76.0	16	80.0	4	66.6	0.462	0.897^{MC}
	Yes (n=12)	6	24.0	4	20.0	2	33.3		
Cardiac toxicity	No (n=44)	24	96.0	18	90.0	2	33.3	16.433	$0.001^{MC,*}$
	Yes (n=7)	1	4.0	2	10.0	4	66.7		

χ^2: Chi-square value
^{KW}: Kruskal-Wallis test *p* value
^{MC}: Monte Carlo test *p* value
*: Statistically significant at $p \leq 0.05$

Response to induction chemotherapy in relation to clinical and laboratory parameters of AML patients.

There was no statistically significant association between response to chemotherapy and age, gender, AML FAB subtypes, CD34 expression or lymphoid marker co expression. On the other hand, there was an association with CNS infiltration. No patient with CNS infiltration achieved complete response (Table 12).

No statistically significant association was found between response to induction chemotherapy and chemotherapy-induced toxicities, namely, hepatotoxicity, nephrotoxicity, metabolic toxicity and cardiac toxicity (Table 12 and Figure 35).

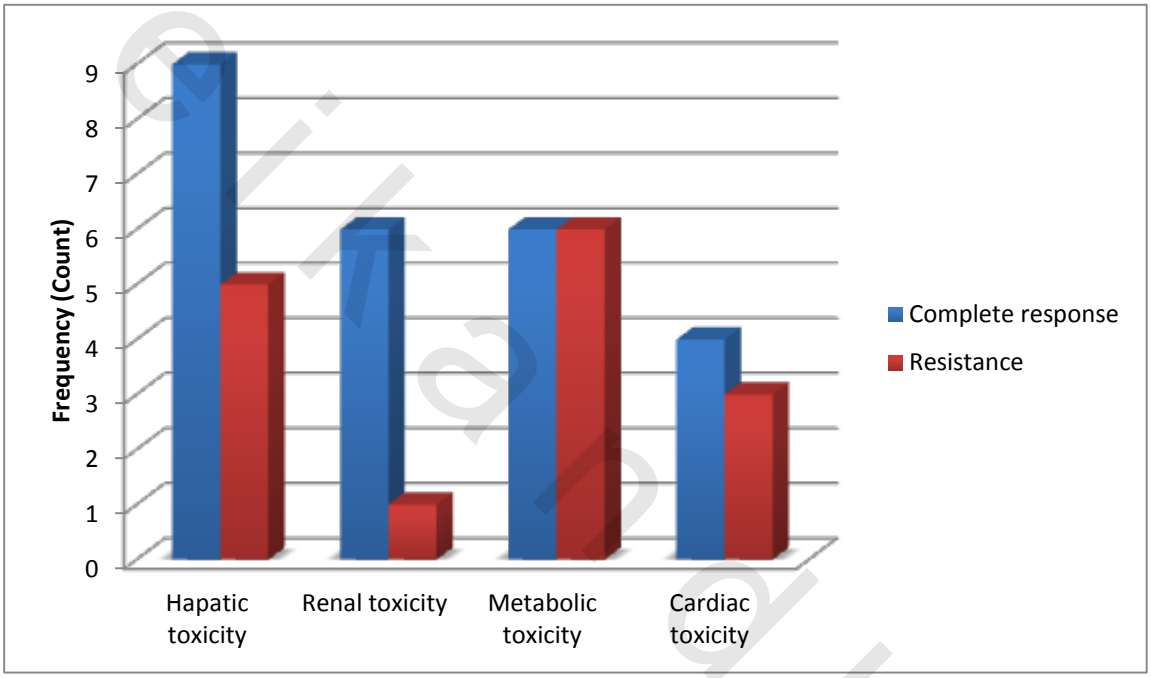

Figure 35: Response to induction chemotherapy in relation to studied toxicities.

Table 12: Response to induction chemotherapy in relation to clinical and laboratory parameters of AML patients.

Parameter		Complete response (n = 30)		Resistance (n = 21)		χ^2	p
Age (years)	Min - Max	19.0 – 57.0		21.0 – 54.0		<0.001	0.985^{MW}
	Mean ± SD	34.3 ± 10.6		32.3 ± 10.0			
	Median	33.0		32.0			
		no.	**%**	**no.**	**%**	χ^2	**p**
Gender	Male (n=28)	16	53.3	12	57.1	0.072	0.788^{AS}
	Female (n=23)	14	46.7	9	42.9		
FAB	M0 (n=3)	2	6.7	1	4.8	8.620	0.196^{MC}
	M1 (n=11)	9	30.0	2	9.5		
	M2 (n=12)	4	13.3	8	38.1		
	M4 (n=11)	7	23.3	4	19.0		
	M5 (n=8)	6	20.0	2	9.5		
	M6 (n=4)	1	3.3	3	14.3		
	M7 (n=2)	1	3.3	1	4.8		
CD34 expression	No (n=42)	23	76.7	19	90.5	1.621	0.277^{FE}
	Yes (n=9)	7	23.3	2	9.5		
Lymphoid marker co-expression	No (n=40)	23	76.7	17	81.0	0.134	0.746^{FE}
	Yes (n=11)	7	23.3	4	19.0		
CNS-disease	No (n=45)	30	100.0	15	71.4	9.714	$0.003^{FE,*}$
	Yes (n=6)	0	0.0	6	28.6		
Hepatic toxicity	No (n=37)	21	70.0	16	76.2	0.238	0.626^{AS}
	Yes (n=14)	9	30.0	5	23.8		
Renal toxicity	No (n=44)	24	80.0	20	95.2	2.422	0.217^{FE}
	Yes (n=7)	6	20.0	1	4.8		
Metabolic toxicity	No (n=39)	24	80.0	15	71.4	0.504	0.518^{FE}
	Yes (n=12)	6	20.0	6	28.6		
Cardiac toxicity	No (n=44)	26	86.7	18	86.3	0.009	1.000^{FE}
	Yes (n=7)	4	13.3	3	13.7		

χ^2: Chi-square value
^{MW}: Mann-Whitney U test *p* value
^{AS}: Asymptomatic test *p* value
^{MC}: Monte Carlo test *p* value
^{FE}: Fisher Exact test *p* value
*: Statistically significant at $p \leq 0.05$

Response to induction chemotherapy in relation to *XPD* Asp312Asn (G→A) and *XPD* Lys751Gln (A→C) polymorphisms in AML patients.

There was no statistically significant association between the response to induction chemotherapy and any of the *XPD* Asp312Asn genotypes, however, it was significantly associated with *XPD* Lys751Gln genotypes. Patients having the AA genotype were more likely to achieved complete remission. Furthermore, patients with the C allele (*XPD*751Gln) had lower chance of achieving complete response (Table 13, Figures 36 and 37).

Table 13: Response to induction chemotherapy in relation to XPD Asp312Asn (G→A) and XPD Lys751Gln (A→C) polymorphisms in AML patients.

Polymorphism		Complete response (n = 30)		Resistance (n = 21)		χ^2	p
		no.	%	no.	%		
XPD Asp312Asn (G→A)	Asp/Asp (GG) (n=29)	19	63.3	10	47.6	4.829	0.113^{MC}
	Asp/Asn (GA) (n=19)	11	36.7	8	38.1		
	Asn/Asn (AA) (n=3)	0	0.0	3	14.3		
XPD Lys751Gln (A→C)	Lys/Lys (AA) (n=25)	19	63.3	6	28.6	6.164	$0.048^{MC,*}$
	Lys/Gln (AC) (n=20)	8	26.7	12	57.1		
	Gln/Gln (CC) (n=6)	3	10.0	3	14.3		
	Lys/Gln & Gln/Gln (AC&CC) (n=26)	11	36.7	15	71.4	5.973	$0.015^{AS,*}$
	Lys/Lys (AA) (n=25)	19	63.3	6	28.6		

χ^2: Chi-square value
^{AS}: Asymptomatic test *p* value
^{MC}: Monte Carlo test *p* value
*: Statistically significant at $p \leq 0.05$

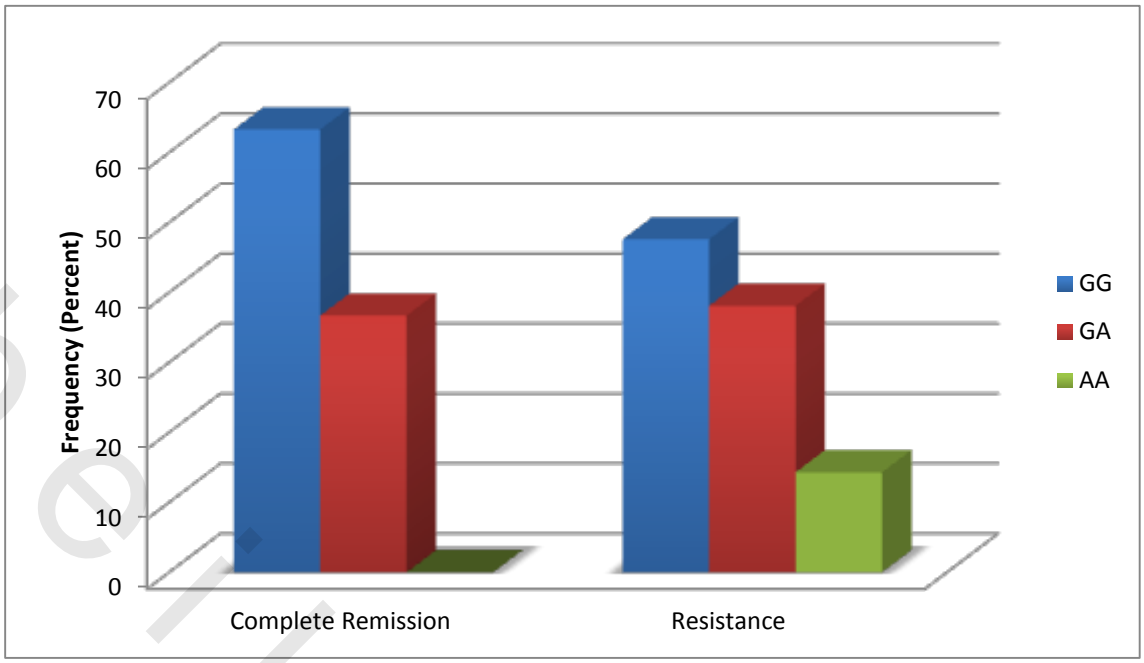

Figure 36: Response to chemotherapy in relation with *XPD* Asp312Asn (G→A).

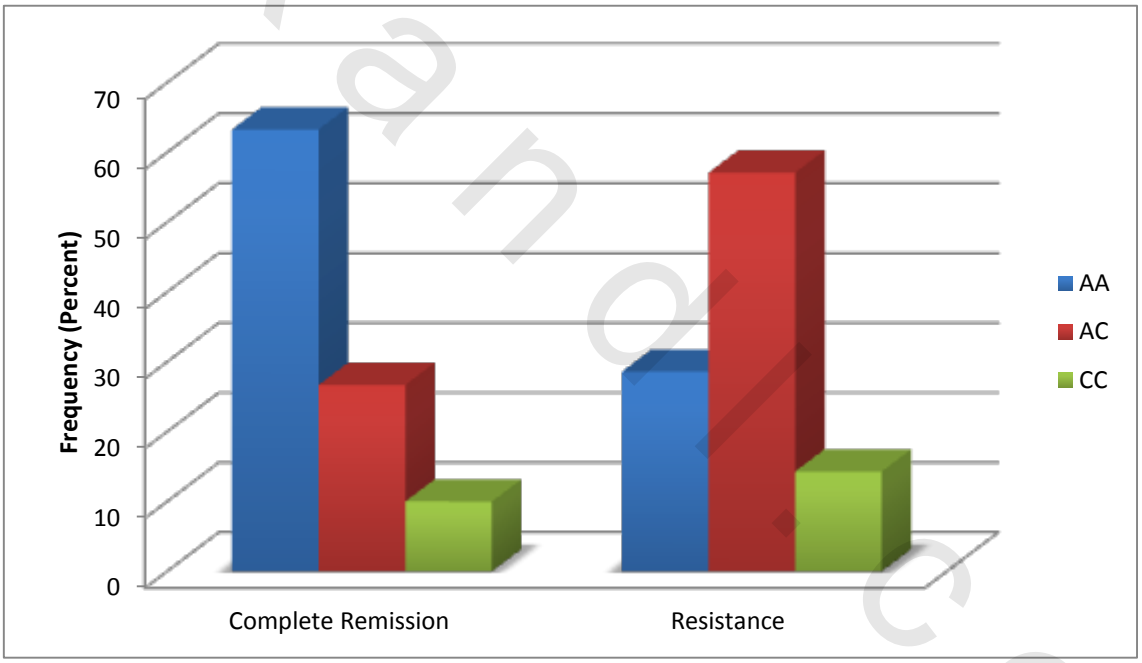

Figure 37: Response to chemotherapy in relation with *XPD* Lys751Gln (A→C).