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Study of Genetic Polymorphism of Xenobiotic Enzymes in Acute Leukemia

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This research is a trial to study the possible association between the main genetic polymorphisms of CYP2D6, GSTM1, GSTT1 and NQO1 and altered susceptibility to leukemia. Also to correlate these genetic polymorphisms with other clinical prognostic data of the patients, their response to therapy and the possibility of relapse. This study included thirty two leukemia patients, nineteen patients with AML and thirteen patients with ALL, it also included eleven normal subjects as control group. Basic investigations for the diagnosis of AML and ALL were performed including complete blood picture, bone marrow aspirate, with cytochemistry and immunophenotyping for the detection of ALL and AML subtypes. For the detection of CYP2D6, NQO1, GSTM1 and GSTT1 genetic polymorphisms, this work has applied a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. A follow up study was also carried out to investigate the association between the outcome of these patients and the different patterns of genetic polymorphism of these four genes. Our results have demonstrated a significant increase in the frequency of CYP2D6 wild type and GSTM1 null genotype in the acute leukemia group when compared with the control group. Studying the relationship between the genetic polymorphisms of these genes and the outcome of our cases revealed that the wild genotype of CYP2D6 significantly influenced the outcome of acute leukemia in particularly the AML cases, while the GSTM1null genotype was associated with bad prognosis among the ALL group. The GSTT1 null genotype had no impact on the outcome of acute leukemia cases. The study also revealed that patients with combined mutant CYP2D6/present GSTM1/present GSTT1 achieved the best prognosis, suggesting the synergistic impact of these genetic polymorphisms on the outcome of acute leukemia cases. This case-control study suggests a contribution of CYP2D6 and GSTM1 null variants in the development of acute leukemias. In addition GSTM1 and GSTT1 genotypes were apparently related with response, side effects and prognosis of patients with AML.

Key words: Xenobiotic enzymes, genetic polymorphisms, ALL, AML

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INTRODUCTION

The interaction between environmental exposure and genetic susceptibility has been postulated as a likely cause of many types of cancers. Functional polymorphisms in the genes encoding detoxification enzymes cause interindividual differences which contribute to leukemia susceptibility (Aydin-Sayitoglu *et al*., 2006).

Xenobiotic-metabolizng enzymes constitute one of the first lines of defense against environmental chemicals and are present in all higher organisms. A number of enzymatic systems, covering a vast range of substrates, have evolved as an adaptive response to environmental aggression and include the cytochrome P2D6, the glutathione S-transferases and the quinone oxido reductase (Davies *et al*., 2000).

Human cytochrome P450 2D6 (CYP2D6) (Debrisoquine hydroylase) plays a central role in drug metabolism, metabolising over 25% of the most commonly prescribed drugs. The CYP2D6 gene is highly polymorphic, leading to wide inter-individual and ethnic differences in CYP2D6-mediated drug metabolism.

The polymorphism of the enzyme results in poor, intermediate, efficient or ultra rapid metabolizers (Zanger *et al*., 2004).

NAD(P)H: quinone oxido reductase, NQO1 gene polymorphism results in reduction of NQO1 activity. In addition to its role in detoxifying quinone-containing carcinogens, it participates directly in the metabolism of quinone-containing chemotherapeutics NQO1 performs a dual action, participating in both activation and inactivation of cytotoxic agents (Yuille *et al*., 2002).

Glutathione S-transferases, GSTM1 and GSTT1 are polymorphic genes. Absence of enzymatic activity due to homozygous inherited deletion of these genes leads to reducing detoxification of carcinogens or harmful metabolites such as epoxides and alkylating agents, which in turn could cause injury to the genome leading ultimately to leukemogenesis (D'Alo *et al*., 2004).

The present research aims to study the possible association between the main genetic polymorphisms of CYP2D6, GSTM1, GSTT1 and NQO1 and altered susceptibility to leukemia. Also to correlate these genetic polymorphisms with other clinical prognostic data of the patients, their response to therapy and their therapy outcome.

MATERIALS AND METHODS

This study was conducted on 32 patients with newly diagnosed acute leukemia (19 AML and 13 ALL). They were attending the Nuclear Medicine Department, Kasr EL-Aini Hospital, Cairo University. They were followed up for six months after initiation of therapy. A control group of 11 subjects was also included.

All individuals included were subjected to the following:

- Full medical history and thorough clinical examination.
- Laboratory investigations:
  - Complete haemogram on Cell-Dyne 3700.
  - Bone marrow aspirate (for patients only).
  - Cytochemistry (for patients only).
  - Immunophenotyping by flow cytometry to detect AML subtypes (for patients only)
  - Genotyping of CYP2D6, GSTM1, GSTT1 and NQO1 genetic polymorphism.

Sample collection:

- Ten milliliter venous blood were obtained from each participant by a sterile vnepuncture and were divided as follows.
- Five milliliter blood in a sterile vacutainer containing EDTA for DNA extraction.
- Three milliliter blood in a sterile vacutainer containing EDTA for immunophenotyping.
- Two milliliter blood in a sterile vacutainer containing EDTA for complete haemogram.

Genotyping: DNA was isolated using Gentra DNA extraction kit (USA). A PCR-RFLP assay was Used for the detection of the G1934. A mutation responsible for the poor metabolizer allele CYP2D6*4. Amplification was carried out in a 50 μL reaction volume containing CYP2D6 (sense primer) 5'-GCTTCGCCAACCACTCCG-3' and antisense primer 5'-AAATCCTGCTCTTCCAGGC-3'. PCR cycle, 94°C for 5 min, 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 30 cycles. This yielded a 334 bp fragment, the PCR product was then digested by the restriction enzyme BstN1 (Biolabs Inc., Beverly, M.A.). Fragments were then visualized on 8% polyacrylamide gel stained ithethedium bromide. The wild-type allele produced 230 and 109 bp fragments. Alleles with the G1934A mutation do not have the restriction site for BstN1 and produced 334 bp undigested fragment.

For the detection of NQO1 mutation, amplification was carried out in a 50 μL reaction volume containing NQO1 (sense primer) 5'-AGTGGCATTCTGCATTTCTG TG-3' and antisense primer 5'-GATGGACTTGCCCAA GTGATG-3'. PCR cycle, 94°C for 8 min, 94°C for 30 sec, 56°C for 1 min, 72°C for 2 min for 35 cycles, followed by

10 min at 72°C. The PCR product was then digested by the restriction enzyme Hinf I (Biolabs Inc., Beverly. MA). Frament were then visualized on 4% agarose gel electrophoresis. The Pro/Pro genotype was identified by the presence of two bands at 188 and 85 bp. The Ser/Ser genotype was identified by the presence of two bands at 151 and 85 bp (loss of function polymorphism). The Pro/Ser genotype was identified by the presence of three bands at 181, 151 and 85 bp.

The polymorphic deletion of the GSTM1 gene was determined by amplification in a 50 μL reaction volume containing P15' CGCCATCTTGTGCTACATTGCCCG; P25' ATCTTCTCCTCTTCTGTCTC and P3 5' TTCTGGATTGTAGCAGATCA. PCR cycle, 94°C for 4 min, 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min for 35 cycles followed by 72°C for 7 min. P1 and P3 amplify a 230 bp product that is specific to GSTM1. The presence of one or both GSTM1 aside, identified by a 230 bp fragment, or its complete deletion (null genotype), was visualized on 1.5% agarose gel electrophoresis. The polymorphic deletion of GSTT1 gene was determined by amplification in a 50 μL reaction containing each of the following primers F46.5'GCCCTGGCTAGTTGCTGAAG and R137.5'GCATCTGATTTGGGGACCACA. PCR cycle, 94°C for 4 min, 94°C for 15 sec, 59°C for 30 sec, 72°C for 45 sec for 35 cycles, followed by 72°C for 7 min. The presence of one or two GSTT1 alleles identified by the presence of 112 bp fragment, or complete deletion (null genotype), was visualized by electrophoresis on a 1.5% agarose gel.

Statistical analysis was done using a commercially available statistics program, SPSS version 8.0 (SPSS Inc., Chicago, II, USA) applying Chi square and unpaired student's t-test.

RESULTS

As regards to CYP2D6 genotyping (Table 1) among the acute leukemia group (AML and ALL), 17 cases (53.1%) were of the wild type and 15 cases (46.9%) cases were of the mutant type. Among the AML group, 13 cases (68.4%) were of the wild type and 6 cases (31.6%) were of the mutant type. Among the ALL group, 4 cases (30.8%) were of the wild type and 9 cases (69.2%) were of the mutant type. Among the control group, 5 subjects 945.5%) were of the wild type and 6 subjects (54.5%) were of the mutant type. Difference between cases of acute leukemia (AML%ALL) and the control group as regard the distribution of CYP2D6 was statistically significant ($p<0.05$) (Table 2).

NQO1 genotyping: It was found that all of the studied groups (AML, ALL and the controls expressed the Pro/Pro genotype.

GSTM1 genotyping: Among the acute leukemia group (AML and ALL), 13 cases (40.6%) expressed one band and 19 cases (59.4%) were of the null genotype. Among AML cases, 6 cases (31.6%) expressed one band and 13 cases (68.2%) were of the null genotype. Among ALL cases, 7 cases (53.8%) expressed one band and 6 cases (46.2%) were of the null genotype. Among the control group, all eleven subjects expressed one band (100%). Differences between cases of acute leukemia (AML and ALL) and the control group as regard the distribution of GSTM1 was statistically significant ($p<0.05$) (Table 3).

GSTT1 polymorphism distribution among the studied groups (Table 1). Among the acute leukemia group, 20 cases (62.5%) expressed one band and 12 cases (37.5%) of the null group. Among AML group, 10 cases (52.6%) expressed one band and 9 cases (47.4%) of the null genotype. Among ALL group, 10 cases (76.9%) expressed one band and 3 cases (23.1%) of the null genotype. Among the control group, 6 subjects (54.5%) expressed one band and 5 subjects (45.5%) of the null genotype. Difference between cases of acute leukemia (AML and ALL) and the control group as regard the distribution of GSTT1 was of no statistical significance (Table 4).

Study of the patients outcome as regard CYP2D6, GSTM1, GSTT1 polymorphism. The outcome of acute leukemia cases (AML and ALL) was classified into good outcome (complete remission) and bad outcome (others); as regard CYP2D6 genotype (Table 5), it was found that; the pattern of the outcome among the wild genotype was good in 5 cases (33.3%) and bad in 11 cases (73.3%). The pattern of the outcome among the mutant group was good in 10 cases (66.7%) and bad in 4 cases (26.7%). This was statistically significant ($p<0.05$) i.e., acute leukemia cases expressing the wild type achieved bad prognosis in comparison with those with mutant genotype who were achieving good prognosis. Regarding GSTM1 (Table 6) the pattern of the outcome among the group expressing the gene was good in 8 cases (50%) and bad in 3 cases (20%). The pattern of the outcome among the group of null genotype was good in 7 cases (46.7%) and bad in 12 cases (80%). As regard GSTT1 (Table 7) the pattern of the outcome among the group expressing one band of the gene was good in 11 cases (73.3%) and bad in 8 cases (60%). The pattern of the outcome among the group with null genotype was good in 4 cases (26.6%) and bad in

Table 1: CYP2D6, GSTMI polymorphisms distribution among the studied groups

		CYP2D6		GSTMI		GSTTI	
Cases		Wild	Mutant	Present	Null	Present	Null
Leukemia group	Number	17	15	13	19	20	12
	Percent	53.1	46.9	40.6	59.4	62.5	37.5
AML group	Number	13	6	6	13	10	9
	Percent	68.4	31.6	31.6	68.4	52.6	47.4
ALL group	Number	4	9	7	6	10	3
	Percent	30.8	69.2	53.8	46.2	76.9	23.1
Control group	Number	5	6	1	0.00	6	5
	Percent	45.5	54.5	100	0.00	54.5	45.5

Table 2: CYP2D6 polymorphism distribution among the studied groups

			CYP2D6		
Groups			Mutant	Wild	100
GP General studied population)	AML	Count	6	13	19
		% Within GP	31.6%	68.4%	100.0%
	ALL	Count	9	4	13
		% Within GP	69.2%	30.8%	100.0%
	Control	Count	6	5	11
		% Within GP	54.5%	45.5%	100.0%
Total		Count	20	23	43
		% Within GP	40.5%	53.5%	100.0%

p-value <0.05

Table 3: GSTMI polymorphism distribution among the studied groups

			GSTMI		
Groups			Present	Null	Total
GP	AML	Count	6	13	19
		% Within GP	31.6%	68.4%	100.0%
	ALL	Count	7	6	13
		% Within GP	53.8%	46.2%	100.0%
	Control	Count	11	0.00	11
		% Within GP	100%	0.00%	100.0%
Total		Count	23	20	43
		% Within GP	53.5%	46.5%	100.0%

p-value <0.05

Table 4: GSTTI polymorphism distribution among the studied groups

			GSTTI		
Groups			Null	Present	Total
GP	AML	Count	9	10	19
		% Within GP	47.4%	52.6%	100.0%
	ALL	Count	3	10	13
		% Within GP	23.1%	76.9%	100.0%
	Control	Count	5	6	11
		% Within GP	45.5%	54.5%	100.0%
Total		Count	16	27	43
		% Within GP	37.2%	62.8%	100.0%

Table 5: Outcome of the leukemia group good (remission) or bad (others) in relation to genetic polymorphism of CYP2D6 gene

			CYP2D6		
Condition			Mutant	Wild	Total
Outcome	Good	Count	10	5	15
		% Within outcome	66.7%	33.3%	100.0%
	Bad	Count	4	11	15
		% Within outcome	26.7%	73.3%	100.0%
Total		Count	14	16	30
		% Within outcome	46.7%	53.3%	100.0%

p<0.05

Table 6: Outcome of the studied group good (remission) or bad (others) in relation to genetic polymorphism of GSMI gene

			GSTMI		
Condition			Present	Null	Total
Outcome	Good	Count	8	7	15
		% Within outcome	53.3%	46.7%	100.0%
	Bad	Count	3	12	15
		% Within outcome	20%	80%	100.0%
Total		Count	11	19	30
		% Within outcome	36.7%	63.3%	100.0%

Table 7: Outcome of the studied group good (remission) or bad (others) in relation to genetic polymorphism of GSTTI gene

			GSTTI		
Condition			Null	Present	Total
Outcome	Good	Count	4	11	15
		% Within outcome	26.6%	73.4%	100.0%
	Bad	Count	6	9	15
		% Within outcome	40%	60%	100.0%
Total		Count	10	20	30
		% Within outcome	33.3%	66.7%	100.0%

6 cases (40%). Combined patterns of CYP2D6, GSTM1 and GSTT1 genotypes in relation to the outcome of the leukemia cases were also demonstrated in Table 8. It was found that, the 4 cases expressing combined mutation of CYP2D6, presence of GSM1 and presence of GSTT1 achieved good outcome.

The risk of acute leukemia (cancer susceptibility) in relation to CYP2D6, GSTM1 and GSTT1 polymorphisms; regarding CYP2D6, comparison between the wild and the mutant genotype distribution among the acute leukemia group and the control group in relation to susceptibility of acute leukemia demonstrated an odd ratio equals 1.03 (statistically not significant). As regard GSTM1, comparison between the presence and absence of GSTM1in relation to susceptibility of acute leukemia demonstrated that the odds ratio equals 6.06 (statistically significant) i.e., the risk of acute leukemia among those with null genotype is 6 times more than those expressing GSTM1. Regarding GSTT1; comparison between the presence and absence of GSTT1 among the acute leukemia cases and the control group in relation to the susceptibility of acute leukemia demonstrated an odd ratio equals 1.03 (statistically not significant).

Table 8: Combined CYP2D6, GSTMI and GSTTI polymorphism in relation patients outcome

Condition			CYP2D6/GSTMI/GSTTI: Mutant CYP2D6/ Present GSTMI Null GSTTI	Mutant CYP2D6/ Present GSTMI Present GSTTI	Mutant CYP2D6/ Null GSTMI Null GSTTI	Mutant CYP2D6 Null GSTMI/ Present GSTTI	Wild CYP2D6 Present GSTMI/ Present GSTTI	Wild CYP2D6 Null GSTMI/ Null GSTTI	Wild CYP2D6/ Null GSTMI/ Present GSTTI	Total
Outcome	Good	Count	1	4	2	2	2	2	2	15
		% Within outcome	6.8%	26.7%	13.3%	13.3%	13.3%	13.3%	13.3%	100%
	Bad	Count	2		1	2	2	2	6	15
		% Within outcome	13.3%		6.8%	13.3%	13.3%	13.3%	40.0%	100%
Total		Count	3	4	3	4	4	4	8	30
		% Within outcome	10.0%	13.3%	10.0%	13.3%	13.3%	13.3%	26.8%	100%

DISCUSSION

Acute leukemia is a heterogeneous disease with distinct biological and prognostic groups (Cui *et al*., 2004). The etiology of acute myeloid leukemia is largely unknown. Biological and epidemiological data implicate exogenous toxins, including cytotoxic drugs, benzene, radiation and cigarette smoking (Bowen *et al*., 2003). On the other hand many environmental factors (e.g., exposure to ionizing radiation and electromagnetic fields, parental use of alcohol and tobacco) have been investigated as potential risk factors of acute lymphocytic leukemia (Pui *et al*., 2001).

The present study revealed a significant increase in the frequency of CYPD6 wild type and GSTM1 null genotype in the acute leukemia group when compared with the control group. While the frequency of GSTT1 and NQO1 were not. Human Cytochrome P450 2D6 (CYP2D6) (Debrisoquine hydroxylase) plays a central role in drug metabolism (phase I), metabolizing over 30% of the most commonly prescribed drugs. The polymorphism of the enzyme results in poor, intermediate, efficient or ultra rapid metabolizers (Zanger *et al*., 2004).

In this study the analysis of CYP2D6 polymorphism revealed two genotypes a wild type (two bands; 230, 104 bp) Extensive Metabolizers (EM) and a mutant type (one band; 334 bp) Poor Metabolizers (PM). Present study demonstrated significant increase in the frequency of CYP2D6 wild type in the acute leukemia group when compared to the control group. Among the AML group, 68.4% were of CYP2D6 wild genotype and among the ALL group, 30.8% were of CYP2D6 wild genotype compared to 54.5% among the control group.

In agreement with our results Joseph *et al*. (2004) reported a higher frequency of CYP2D6 wild genotype among children with ALL compared with the control group. Aydin- Saitoglu *et al*. (2006) in a case-control study suggested a contribution of CYP2D6, null variants to the development of acute leukemia This study also reported a worse prognosis among the group of acute leukemia cases with wild genotype in comparison with better prognosis among those with the mutant genotype, where 78.6% of cases with wild genotype had unfavorable outcomes while 68.8% of acute leukemia cases with mutant genotype achieved remission. This significant difference was also noticed when applying it on the AML group separately where only 16.7% among the wild genotype group achieved a good prognosis compared with 60% among the mutant genotype. In contrast the outcome of ALL cases was good in the majority of cases whether wild or mutant genotype (75 and 77.8%, respectively).

Glutathione S-transferases (GSTs) are enzymes involved in the detoxification of several environmental mutagens, carcinogens and anticancer drugs (phase II). GSTs polymorphisms resulting in decreased enzymatic activity have been associated with several types of solid tumors (Dirksen *et al*., 2004).

Recently, some studies have addressed the role of GST polymorphisms in the development of hematological malignancies. Few data were reported so far for adults with Acute Myeloid Leukemia (AML) and in particular, to our knowledge, there are only few reports on the relationship between GST genotypes, clinical outcome and established indicators of prognosis in adult AML (Voso *et al*., 2002).

The analysis of GSTM1 polymorphism revealed an increased frequency of the null genotype among the acute leukemia group when compared to with the control group, where frequency of the null genotype among the AML and ALL groups was 68.4 and 46.2%, respectively compared with 9.1% among the control group.

Aydin-Sayitoglu *et al*. (2006) found that patients with ALL and AML had a higher prevalence of the GSTM1 deletions compared to controls but only the difference among adult AML patients was statistically significant. Nevertheless, a significant relationship was found between the null GSTM1 genotype and the outcome of patients with ALL, where all cases expressing the gene (100%) achieved a good outcome and only 50% of the null

group achieved a good outcome. No significant relationship was found between the null GSTM1 genotype and the outcome of patients with AML.

Regarding the risk of acute leukemia and GSTM1 polymorphism, the present work reported that the risk of acute leukemia was significantly higher in subjects with null genotype than normal individuals (Odds ratio = 6.06) i.e., the risk of acute leukemia among GSTM1 null genotype was 6 times higher than those expressing the gene. Rocha *et al.* (2005) found that, the genotype/outcome relationships maintained prognostic significance in a therapeutic setting in which race itself was unimportant. Moreover, the same genotypes were predictive for hematological relapse within the major subgroups of whites and blacks. Dosing based on pharmacogenetics holds the promise for delivery of color-blind therapy: genotyping allows for individualized dosing according to genetic rather than racial characteristics.

Norppa (2004) agreed that the lack of GSTM1 enzyme activity (null genotype) appears to be associated with increased sensitivity to genotoxicity of different carcinogens and consequently different types of malignancies including leukemia. Also, Barnette *et al.* (2004) agreed that the lack of GSTM1 enzyme activity (null genotype) is not only associated with increased cancer risk but also with altered response to and toxicity from chemotherapy and therefore the outcome of the disease.

The analysis of GSTT1 genotype revealed no significant difference in the frequency of GSTT1 between acute leukemia patients and the control group. In agreement with the present study, Krajinvic *et al.* (2002) reported no association between GSTT1 genotyping and outcome of ALL. Contradictory to our results Barnette *et al.* (2004) reported that, the lack of GSTT1 enzyme activity (null genotype) is associated with increased cancer risk. Dirksen *et al.* (2004) found that the GSTT1 null genotype may modulate the metabolism of exogenous pollutants or toxic intermediates. The absence of the GSTT1 enzyme, leading to genetic susceptibility toward certain pollutants, might determine the individual risk for development of acquired aplastic anemia in children.

NAD (P) H: quinone oxidoreductase 1 (NQO1) is a cytosolic enzyme that catalyzes the two-electron reduction of quinoid compounds into hydroquinones, their less toxic form. A sequence variant at position 609 (C T) in the NQO1 gene encodes an enzyme with reduced quinone reductase activity *in vitro* and thus was hypothesized to affect cancer susceptibility (Chao *et al.*, 2006).

In this research all the studied groups (AML, ALL and the control groups) expressed the Pro/Pro genotype and therefore no linkage to leukemia susceptibility or prognostic outcome could be made. Complete lack of NQO1 activity (Ser/Ser genotype) is rare and marked differences in the ethnic distribution of the NQO1 polymorphism have been described by Zhang *et al.* (2004). They reported that the null-allele is approximately twice as common in Chinese as in Caucasians.

Bowen *et al.* (2003) reported that NQO1 polymorphic variant, which confers reduced phase II metabolism, is associated with a predisposition to therapy related AML and selected subgroups of de novo AML. Krajinvic *et al.* (2002) also found that individuals with the NQO1 variant associated with reduced enzyme activity had worse therapeutic outcome of childhood ALL.

The present research also studied the impact of combined genetic polymorphism on the frequency of AML and ALL in order to assess the influence of any synergistic effect of these genotypes. Our study demonstrated higher frequency of combined wild CYP2D6/null GSTM1 and null GSTT1 among AML group (100%) compared to 0% among the control group, while the frequency of mutant CYP2D6/presence of GSTM1 and GSTT1 was 0% among AML and 27.3% among the control group.

Also the work studied the impact of combined genetic polymorphisms on the outcome of the leukemia groups and elicited that the combined mutant CYP2D6/one band GSTM1/one band GSTT1 was associated with the best outcome (100% achieved good outcome).

Similarly Davies *et al.* (2002) studied the impact of combined null GSTM1 and GSTT1 on both the frequency and the outcome of ALL and reported higher frequency of double null genotype in black children with ALL compared to normal controls but they could not relate this combination to their outcome.

CONCLUSIONS

The present study revealed that the wild type of CYP2D6 and the null GSTM1 genotype could be associated with increased risk of acute leukemia. Also GSTM1 and GSTT1 genotypes were apparently related with response, drug side effects and prognosis of patients with AML. GSTM1 and GSTT1 genotype might be useful in selecting appropriate chemotherapy regimens for patients with acute leukemia.

CYP2D6 and GSTM1 being members of phase I and phase II drug metabolizing enzymes respectively, the genetic analysis of xenobiotic metabolizing enzyme is

clinically important for the appropriate dosage of certain drugs during the therapeutic course of acute leukemia; thus it prevents therapeutic failures, adverse effects and toxicity.

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