Journal of the Egyptian Society of Haematology & Research, Vol. 11, No. 1, March: 1-7, 2015

Role of GSTP1Ile105Val SNP in the Risk of Developing AML and its Clinical Relevance

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ABSTRACT

Background: GSTP1, like other GST (s) plays an important role in the detoxification of several previously activated pro-carcinogens; GST (s) are phase 2 drug metabolizing enzymes responsible for detoxification of many environmental carcinogens. So it is anticipated that polymorphisms of GSTP1Ile105Val resulting in decreased or absent activity might be associated with increased risk of carcinogenesis.

Objectives: To determine the relation of GSTP1 SNPs (single nucleotide polymorphism) with the risk susceptibility to AML and evaluate its clinical relevance regarding response to induction chemotherapy and survival of AML patients.

Patients and Methodos: PCR-RFLP for GSTP1Ile-105Val was done for 60 AML cases and 100 age and sex matched healthy unrelated Egyptian control subjects.

Results: Our study showed that the GSTP1Ile105Val polymorphism distribution in AML cases (n=60; wild 46.7%, heterozygous 40% and homozygous 13.3%) was not significantly different from the control group (n=100; wild 53%, heterozygous 36% and homozygous 11%) (p-=0.730). GPST1 150 polymorphism was found to have no effect on response to induction chemotherapy. Although the median survival for patients with mutant GSTP1 was higher than that for patients with wild genotype (4 month versus 2.1 month respectively) but this was not statistically significant (p=0.136).

Conclusion: The presence of SNP in GSTP1 has no impact either on the risk of developing de novo AML or on the clinical outcome of patients with AML.

Key Words: *AML – SNP – GSTP1 – PCR-RFLP.*

INTRODUCTION

Acute Myeloid Leukemia (AML) is a clonal disorder characterized by the acquisition of somatic mutations in hematopoietic progenitors leading to disruption of differentiation. Exposition to DNA damaging agents may play an important role in the pathogenesis of AML. Detoxification and Deoxyribonucleic Acid (DNA) repair enzymes protect DNA from damage, due to both endogenous and exogenous sources. When detoxification or repair are ineffective, the DNA damage can cause chromosomal instability leading to severe failure of cell functions, and either apoptosis or oncogenesis. Genetic differences defined by polymorphisms altering the enzymatic activities in detoxification and DNA repair pathways are prime candidates for studies to explain variation in individual susceptibility to develop AML. Individuals with certain polymorphisms in genes metabolizing carcinogens have an increased risk of developing AML **[1]**.

The first line of defense to genotoxic agents is detoxification. This should occur before the agents are able to damage cellular molecules. Metabolism of endogenous and exogenous agents occurs by the same pathways and is divided into two phases. Phase I involves activation of substrates into electrophilic intermediates; these reactions are predominantly catalyzed by the cytochrome P450 (CYP) protein family, many of which harbor polymorphisms which affect their function. The products of the phase I reactions are highly reactive and liable to cause severe cellular damage and the phase II enzymes (conjugation) are required to inactivate the phase I products. Enzymes that participate in phase II include the glutathione S-transferases (GST) and NAD (P) H: Quinine oxidoreductase-1 (NQO1). These enzymes not only detoxify reactive phase I products but also

act on genotoxic agents that do not require phase I activation. The balance of phase I and II activity is critical and a consequence of high levels of phase I activity with low levels of phase II activity is the production of deleterious metabolites which will damage cell components, especially DNA. It follows that polymorphisms affecting the function of either phase I or II proteins, or indeed proteins from both phases, may upset the balance of detoxification activity and predisposes individuals to high levels of damaging agents **[2]**.

Differences in the activities of some GSTs are determined by genetic polymorphisms. Polymorphisms in GSTP1 were first reported by **[3]**. An A-G polymorphism at nucleotide 313 in exon 5 of GSTP1 gene leads to an amino acid substitution of Isoleucine (IE) by Valine (Val) at 105 amino acid position (Ile105Val). This substitution results in three GSTP1 genotypes: They are isoleucine/isoleucine (Ile/Ile) homozygous wild type, isoleucine/valine (Ile/Val) heterozygote and valine/valine (Val/Val) homozygous variant **[4]**.

GSTs serve two distinct roles in the development of drug resistance via direct detoxification as well as acting as an inhibitor of the MAP kinase pathway. Hence, it is not surprising that high levels of GSTs have been reported in a large number of tumor types. A survey of the NCI cancer drug screening panel of cell lines showed a correlation between GST expression and sensitivity toward alkylating agents **[5]**. Some of these agents are substrates of GSTs and can be directly inactivated through catalytic conjugation to GSH through thioether bond formation. Many cancer drugs that decompose to produce electrophilic species can be detoxified via glutathione metabolism **[6]**.

Previous studies showed that GSTP1105Val genotype has been associated with favorable prognosis following chemotherapy with drugs known to be GSTP1 substrates in a variety of malignancies such as pediatric acute lymphoblastic leukemia, breast and colon cancers **[7-9]**.

However, the results of GSTs concerning risk and prognosis of acute myeloid leukemia are conflicting in studies conducted in different ethnicities **[4,10,11]**.

PATIENTS AND METHODS

Patients: The study was performed on 60 de Novo AML patients presented to the Medical Oncology Department, NCI, Cairo University in the period from June 2012 to January 2014. Twenty eight patients were (46.7%) males and 32 (53.3%) were females with an age range of 18 to 78 with a median of 32.years. One hundred age and sex matched apparently healthy unrelated individuals selected from blood donors served as a control group; they included 58 (58%) males and 42 (42%) females with an age range of 17 to 59 and a median of 32.5 years. The study was approved by the IRB of the NCI, Cairo University and an informed consent was obtained from each subject before enrollment.

Methods: All patients were subjected to complete history taking and clinical examination as well as baseline chest X-ray and abdominal US and other radiological investigations as indicated. The diagnosis of AML was done according to standard methods (WHO, 2008) and classification was made using the French-American-British (FAB) criteria **[12]**. The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University. All patients signed informed consent before treatment.

Laboratory investigations included:

- Complete and differential blood picture.
- Bone marrow aspiration and examination of Romanowsky stained smears, supplemented with cytochemical stains such as Peroxidase (MPO) or Sudan Black Stain (S.B.B), Estrases, Acid Phosphatase and PAS when indicated.
- Immunophenotyping using monoclonal antibodies and flow cytometric analysis.
- Conventional karyotyping was performed for all cases.
- FISH as a complementary tool to conventional cytogenetics when indicated.

GSTP1Ile105Val genotyping:

Blood or bone marrow samples were obtained into EDTA tubes. DNA was extracted from WBCs by salting out method (REF) followed by Polymerase Chain Reaction (PCR) as described by Hohaus et al., **[13]**. Amplification of the extracted DNA was performed in 25µl reaction mix containing 200ng DNA, 200ng

each primer, 1.5mmol/L $MgCl_2$, and 1 unit Taq DNA polymerase in a total volume of 25µL. Following initial denaturation at 95ºC for 7 minutes, 40 PCR cycles were done. Amplification conditions included initial denaturation at 95ºC for 5 minutes followed by 30 cycles of 94ºC for 1 minute, 61ºC for 1minute, and 72ºC for 1 minute with a final elongation step at 72ºC for 7 minutes. The primer sequences were: P105F (5´-ACC CCA GGG CTC TAT GGG AA-3´) and P105R (5´-TGA GGG CAC AAG AAG CCC CT-3´). Enzymatic digestion of the PCR products was performed using one unit Bsm A1 restriction enzyme. Digestion was performed at 37º for 30 minutes in 20µl reaction mix containing 10ul fast digest restriction enzyme mixture (7ul H2O + 2ul buffer + 1u enzyme) + 10ul PCR product. The PCR and the digestion products were visualized with ethidium bromide after electrophoresis on 2% agarose gel at 100 volts for 30min.

Wild type GSTP1Ile/Ile retained the 176bp product of the amplification step, the homozygous GSTP1Val/Val showed 91 and 85bp fragments of complete digestion and the heterozygous GSTP1Ile/Val showed the three bands Fig. (1).

Treatment plan:

Patients received standard induction chemotherapy using cytosine arabinoside and anthracycline as 7 and 3 protocol. Patients who achieved CR were consolidated by the same regimen then HLA typing was done for those below 40 years of age with good general condition, –ve inv (16) & *t* (8,21). Those with identical donor were referred for allogeneic BMT. Those with no HLA identical donor, those with favorable risk (+ve inv 16 or *t* (8,21)) and those 40 years were given 4 cycles of HAM consolidation. Intra-thecal prophylaxis was given only for cases with AML M5 (high risk of CNS disease) after achieving CR by induction chemotherapy. Triple intrathecal prophylaxis was given every 8 weeks for a total of 6 injection using methotrexate 15mg, Ara-C 40mg and dexamethasone 4mg.

Patients with AML M3 were treated with all trans-retinoic acid (ATRA) 45mg/m^2 oral daily in 2 divided doses until CR or for maximum of 90 days in combination with adriamycin 30 mg/m^2 for 3 days every month for 3 months as induction treatment. Patients who achieve CR received maintenance treatment with ATRA (45 mg/m^2 oral daily for 2 weeks every 3 month), 6 mercaptopurine (60mg/m^2 daily) and methotrexate (20mg/m^2 IV once weekly) for 2 years.

Response to induction chemotherapy:

Complete remission was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements, no circulating blast cells, no evidence of extramedullary leukemia and recovery of granulocytes to 1500/µl and platelets to 100,000/µl. Unfavorable outcome included refractory cases (didn't achieve CR) and early death (death within 30 days of diagnosis and before evaluation of the response).

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either Student *t*-test or Mann-Whitney test (non-parametric *t*-test) as apprporiate. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds Ratio (OR) with it 95% Confidence Interval (CI) were used for risk estimation. All tests were two-tailed. A *p*-value <0.05 was considered significant.

RESULTS

This study included 60 de novo AML patients, as well as 100 age and sex matched healthy controls.

Patient's characteristics:

A total of 60 de novo AML patients included in this study, 28 males (46.7%) and 32 females (53.3%) with an age range of 18-78 years with a median age of 32 years.

The presenting total leucocytic count in the study cases ranged from 2.2 to 183X10^9/L with mean of 60.7±56.5 and a median of 43.5X10^9/L. The platelet count ranged from 6 to 297X10^9/L with a mean of 59.4±54.7 and a median of 40

X10^9/L. Hemoglobin ranged from 4.2 to 12 gm/dl, with a mean of 7.69±1.8 and a median of 7.3gm/dl. Blasts in peripheral blood ranged from 3 to 90% with a mean and SD of 40.4% ±28 and a median of 32%. The mean percentage of blasts in marrow was 70.1%±21.4, the median was 77% and the range was 22-97%. The FAB classification of the studied cohort is presented in (Table 1). The most frequent was M2 followed by M4 while the least was M5 and M7; no M6 cases were encountered in our cohort. Cytogentics and molecular genetics findings are presented in (Table 2); the majority of our patients (70%) showed normal karyotype.

GSTP1 105 genotypes in AML vs. control:

The wild type GSTP1105 Ile/Ile (1578AA) was encountered in 28 (46.7%) AML patients compared to 53 (53%) controls, the heterozygous genotype GSTP1 105 Ile/Val (1578AG) in 24 (40%) AML patients compared to 36 (36%) controls and the homozygous genotype GSTP1 105Val/Val (1578GG) in 8 (13.3%) AML patients compared to 11 (11%) controls. The difference was found to be statistically insignificant (*p*.value=0.730). The frequency of heterozygous and homozygous genotypes (mutant types) showed insignificant difference between AML and control groups (*p*.value=0.438).

Association of GSTP1 105 genotypes with various clinical and hematological parameters:

There was no association between GSTP1 genotypes on one side and age (*p*=0.629), Gender (*p*=0.744), Hb (*p*=0.358), TLC (*p*=0.901), platelets (*p*=0.493), PB blasts (*p*=0.883), BM blasts (*p*=0.625) or cytogenetics (*p*=0.136) on the other side. As regards the FAB subtypes, we have only 2 cases of M5 and M7 so association could not be statistically analyzed.

Impact of GSTP1 105 genotypes on response to induction chemotherapy:

Complete remission was achieved in 24/60 patients (40%). Twenty two out of 60 patients achieved CR after one course of induction chemotherapy, while 2 patients achieved CR after a second induction. Out of 32 patients with mutant GSTP1, 14 patients (43.7%) achieved CR compared to 10/28 (35.7%) with wild disease (*p*= 0.526). The CR rate was 50% (4/8) for patients with homozygous GSTP1 105 Val/Val genotype and 41.7% (10/28) for heterozygous lle/Val polymorphism.

Impact of GSTP1 105 genotypes on overall survival:

The median follow-up period for the entire group of 60 AML patients was 2 month (range 0.233-24 month), the median OS for the whole group was 3 month (95% CI 1.463-4.537) and cumulative survival of 47.1% at 3 month Fig. (2). There was no statistically significant difference in the OS of the patients with regards to the GSTP1 105 polymorphism. The cumulative survival at 3 months for patients with GSTP 105 Ile/Ile genotype was 41.2% with a median of 2.1 month (95% CI of0.00-8.355) while the cumulative survival at 3 months for patients with the mutant variant was 85% with a median survival of 4 month (95%CI 0.599-3.734) (*p*= 0.136) Fig. (3).

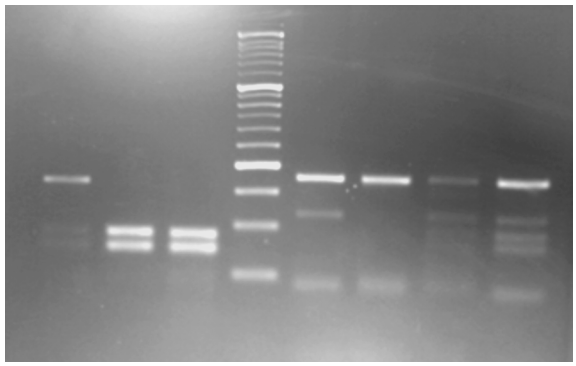

Fig. (1): PCR products for GSTP1Ile105Val after digestion with Bsm A1 restriction enzyme.

Lanes 1, 7, 8 : Heterozygous (Ile/Val), 85, 91, 176bp.
Lanes 2, 3 : Homozygous (Val/Val), 85, 91bp.
Lane 4 : 50bp ladder
Lanes 5, 6 : Wild type (Ile/Ile), 176bp.

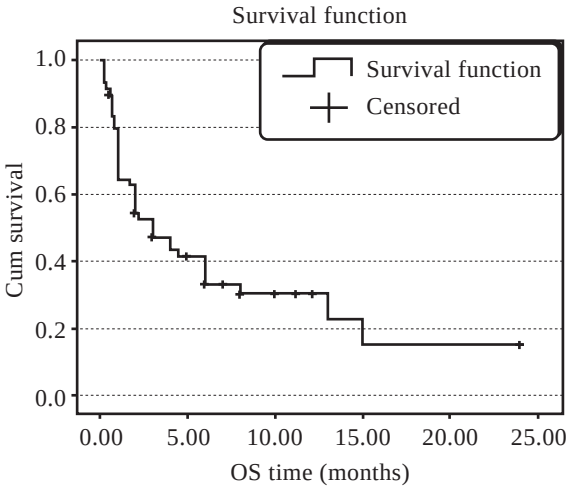

Fig. (2): Cumulative overall survival of 60 adult acute myeloid leukemia patients.

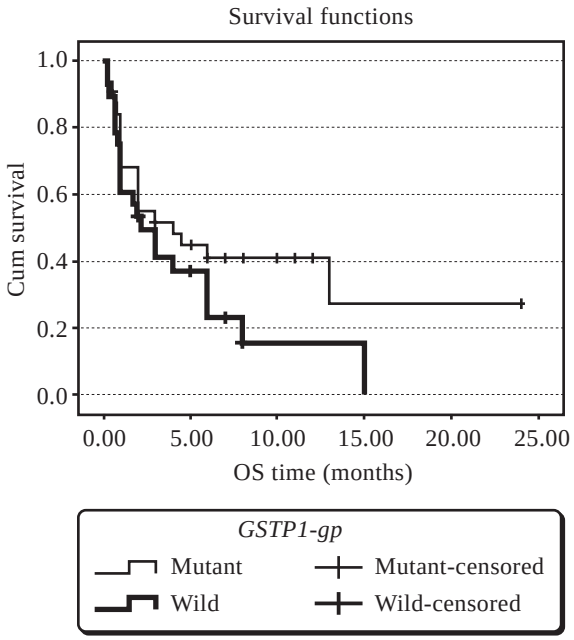

Fig. (3): Effect of GSTP1105 polymorphism on overall survival of 60 adult acute myeloid leukemia patients.

Table (1): French-American-British classification (FAB) of 60 adult acute myeloid leukemia patients.

FAB subtype	No	%
M1	12	20
M2	25	41.7
M3	6	10
M4	13	21.7
M5	2	3.3
M7	2	3.3

Table (2): Karyotyping of 60 adult acute myeloid leukemia patients.

Karyotype	No	%
Normal karyotype	42	70
Abnormal:	18	30
t (8;21)	6	10
t (15;17)	6	10
inv (16)	4	6.7
8 trisomy	1	1.7
Hyperdiploidy	1	1.7

Table (3): Comparison of the frequencies of GSTP1 polymorphisms among healthy Egyptians and other Ethnic groups.

Country (Ethnic)	Exon 5 (codon Ile105Val)				Reference	*p*-value
	No	A/A%	A/G%	G/G%		
Egypt	100	53	36	11	Current study	
Australia	292	39	46*	15**	20	0.123* 0.312**
Finland	293	53	38	9	15	0.881
Germany	64	50	34	16	7	0.586
Italy, Caucasian	70	56	34	10	14	0.911
Japan	50	52	44	4*	16	0.04*
Poland	170	49	44	7	18	0.397
Spain, Caucasian	200	50	44	6	17	0.308
UK, Caucasian	946	49	37	14	21	0.767
USA, PA, NY, Caucasian	163	53	39	8		0.743
African-American	83	58	29	13	19	0.564

p-value: Vs. Egyptians.

DISCUSSION

GSTP1, like other GST (s) plays an important role in the detoxification of several previously activated pro-carcinogens; GST (s) are phase 2 drug metabolizing enzymes responsible for detoxification of many environmental carcinogens. So it is anticipated that polymorphisms of GSTP1 Ile105Val resulting in decreased or absent activity might be associated with increased risk of carcinogenesis on one side and might affect response to therapy, by substrate drugs, on the other side.

In the present study, we tried to elucidate the role of GSTP1 Ile105Val single nucleotide polymorphisms in AML. Their significance was investigated in relation to risk susceptibility, various clinical, laboratory and standard prognostic factors, as well as to treatment response and clinical outcome of patients.

The population frequency of GSTP1 polymorphism among different ethnic groups varies. However, the frequencies of GSTP1 Ile105Val in our study are in concordance with Italian Caucasian reported by Viezzer et al., **[14]** and

also similar to Finland population **[15]**. The Japanese population shows slight decrease in GSTP1 Ile105Val homozygous genotype **[16]** which is statistically different from our population (*p*-value=0.04). Also Spanish and Polish populations show mild increase in GSTP1 Ile105Val heterozygous polymorphism **[17,18]**. However, the USA Caucasian population **[19]**, Australians **[20]** show higher heterozygous type while UK Caucasians show slightly higher homozygous type **[14]** as shown in (Table 3).

The GSTP1 105Val allele is present in 30% of caucasians and is associated with a decreased activity of the enzyme, when compared to the Ile allele, as measured by the conjugation of the substrate 1-chloro-2, 4-dinitrobenzene and thiotepa **[6]**.

A meta-analysis of case control studies published between 1998 and 2009 was performed to investigate the association of GSTP1 polymorphism with AML risk. Pooled Odds Ratio (ORs) were assessed and heterogeneity between studies was calculated. Overall, OR for GSTP1 Val105 allele was 1.03 with 95% CI (0.80-1.33) and *P*-value=0.80. Significant heterogeneity was found between studies relating to GSTP1 (p=0.162). From the limited studies on the association of GSTP1 with risk of AML, the role of the gene cannot be fully ascertained **[22]**.

Also, our results are in concordance with Zhou et al., **[11]** who found that GSTP1 Val/Val carriers had a non-significant risk of AML, with OR of 1.64 and 95 CI of 1.03-2.63 (p>0.05).

However in contrary to the results of the current study, some studies found that GSTP1 Val/Val genotype is associated with the risk of development of acute leukemia **[4,23]**.

Concerning standard prognostic factors, Dunna et al., **[4]** reported that the proband GSTP1 Val/Val genotype frequency was increased in female AML patients as compared to male patients, whereas sex association was not observed in our patients. Val/Val genotype was also associated with early onset of AML (<20 years) but in our study, all patients are adults (>18 years old). The mean white blood cells count (WBCs) in Dunna study was substantially higher in AML with Val/Val genotype ($97.35X10^9$/L) compared to $46.06X10^9$/L in association with Ile/Ile genotype (p-value <0.05). Also Voso et al., **[10]** reported lower WBC counts (median $10.8X10^9$/L) in AML patients with GSTP1 105Val allele than in patients with GSTP1 105Ile allele (median 18.6X 10^9/L) with *p*-value of 0.02. However, no association was encountered in our patients.

GSTP1105Val genotype has been associated with favorable prognosis following chemotherapy with drugs known to be GSTP1 substrates in a variety of malignancies such as pediatric acute lymphoblastic leukemia **[7]**.

There is a better survival for patients with multiple myeloma and the GSTP1105Val allele treated with conventional chemotherapy but not in those treated with high-dose therapy **[24]**.

Another study performed on 166 Chinese patients with metastatic colorectal carcinoma treated with first-line FOLFOX-4 showed that patients with Val105 allele variants had a higher response (56.1% vs 37.6%, p=0.04), and a longer progression-free (p<0.01) as well as overall (p<0.01) survival **[25]**.

Voso et al., **[10]** reported that at a median follow-up of 46 months (range 0-221 months), the GSTP1 105 genotype was significantly associated with Relapse Free Survival (RFS) (p=0.03), whereas the OS was not significantly influenced (p=0.15). Using a competing risk analysis to distinguish between failures due to relapse and failures due to toxicity, they found that the cumulative incidence of relapse was significantly lower for carriers of the variant GSTP1105Val allele (p=0.05), whereas the cumulative incidence of toxic death did not differ according to the GSTP1 105Val allele (p=0.86).

In contrast **[4]** reported no association between GSTP1 polymorphism and the rate of complete remission failure.

The results of GSTs and risk of acute leukemia are conflicting in studies conducted in different ethnicities. The reason might be inducted by ethnic difference, case selection and variation of clinical characteristics. Further studies are needed to verify the association between GSTP1 polymorphism on AML risk and validate its impact on survival.

In conclusion our data suggested that GSTP1 105 polymorphism has no effect on the risk of development of de novo AML. Also in the

current study, GSTP1 polymorphism failed to show a predictive or prognostic value among the AML patients.

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