Reflection of P53 Phenotype in Tumor Tissue by Genotypic Variants in Glutathione S-Transferases: An Association with DNA Damage in Lung Adenocarcinoma

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Abstract

Background: Genetic deficiency of glutathione S-transferase (GST) enzymes results in accumulating carcinogens from tobacco smoke. Consequently, the elevated risk of lung carcinoma (LC) is due to increased DNA damage and mutational frequency of the P53 caretaker gene.

Objective: To determine GST gene variants association with LUAD susceptibility and the relative risk of these variations developing the P53 mutation and DNA damage

Methods: We analyzed genetic polymorphisms in GST-M1 (+/-), T1 (+/-), and P1 (Ile¹⁰⁵ Val) genes using restriction fragment length PCR. The P53 phenotype was categorized as the mutant type (>50% and 2-3 + intensity in IHC) in M1 (+/-), T1 (+/-), and P1 (Ile¹⁰⁵Val) variants. Lymphocytic DNA damage was assessed using the comet assay.

Results: The M1 (-/-) and P1 Val/Val (GG) genotypes were significantly associated with the risk of the P53 mutant phenotype (RR: 1.81, p=0.03) and (RR: 2.23, p=0.05), respectively. In contrast, GSTT1 was not associated with the P53 phenotype. GSTM1 (-/-)/ GSTP1 Ile/Ile (AA) combined genotypes showed a significant synergistic effect on the P53 phenotype (RR: 8.50, p=0.01). DNA damage was significantly associated with GST-M1 (-/-), T1 (-/-), and P1 Val/Val (GG) genotypes and smoking (p=0.001) as compared to GST-T1 (+/+)/M1 (+/+)/P1 Ile/Ile (AA) genotypes. However, P53 expression was not associated with DNA damage.

Conclusion: The GSTM1 (-/-) and GSTT1 (-/-) null genotypes act as risk modifiers for LUAD and increase DNA damage. The GSTM1 (-/-)/ GSTP1 Ile/Ile (AA) combined genotype amplified 8-fold the risk of a P53 mutant phenotype.

Introduction

Lung cancer (LC) is characterized by various genetic alterations and somatic mutations that hamper normal cell biology and provide a selective advantage during tumorigenesis. Germline variation and somatic mutations have been studied extensively to explore various biological insights. However, the interactions between GST germline variations and somatic mutations influence an individual's susceptibility to lung adenocarcinoma (LUAD) and its clinicopathological characteristics are limited.

LUAD is the most prevalent histological subtype of LC. It represents about 40% of all LC and is strongly associated with smoking [1]. Tobacco smoke contains various carcinogens such as polycyclic aromatic hydrocarbons (PAHs), benzo (A) pyrenes (B) P), N-nitrosamines, and N-nitrosorosanicotin (NNN) are established as risk factors for LC [2]. PAHs and N-nitrosamines are potent genotoxic carcinogens that covalently bond with DNA and quickly form DNA adducts. It has been shown that P53 is a vulnerable target for critical DNA damage [3], and the accumulation of DNA adducts alters the frequency of expression and mutations in the tumor suppressor gene during tumor development [4;5]. Loss of P53 is attributed to uncontrolled cell growth, proliferation and tumorigenesis. The study also revealed that LUAD

is a highly heterogeneous disease influenced by inherent germline variations and somatic alterations in DNA [6].

The glutathione S-transferases, GST-P1, M1, and T1, are expressed in lung tissues and lymphocytes at higher levels [7] and can detoxify a variety of carcinogens such as PAHs, B(a)P, and NNK derived from tobacco smoke [8;9]. The loci of GSTM1 (1p13) and GSTT1 (22q 11.2) can be deleted, causing functional loss of the respective enzymes. In contrast, the A-G transition in codon 105 of GSTP1 resulted in a change from isoleucine (Ile) to valine (Val) in the hydrophobic binding site and altered catalytic efficiencies, thus increasing the susceptibility to the progression of LC [10]. Therefore, predisposing genetic factors to smoking-induced LC, such as xenobiotic detoxifying enzymes (XMEs) polymorphisms, may affect the tumor-suppressor gene and DNA mutational frequency damage.

We hypothesized that smoking behavior in GST-M1, T1 (-/-), and P1 Val/Val (GG) genotypes and DNA damage at baseline would help predict an individual's risk consistent with exposure to multiple carcinogens. If DNA adducts are bypassed incorrectly by DNA polymerase during replication, mutations may arise. Further, it has appeared that germline variants and somatic occurrences can be intricately interlinked, and germline variation was shown to influence gene expression in tumors [11;12]. The present study aimed to evaluate the emerging link between GSTs (M1, T1 and P1) gene family germline variants with the P53 phenotype (expression) and association with the extent of DNA damage in LUAD cases.

Subjects And Methods

A total of 163 patients with histologically documented LUAD based on GSTM1 (-/+), GSTT1 (-/+), and GSTP1 I105V genotypic variants were enrolled. A subset of untreated 80 stratified cases (from 163 patients) containing adequate formalin-fixed paraffin-embedded (FFPE) tissue samples were selected to assess the relative risk of P53 phenotypic (expression/mutation) status and DNA damage in LUAD cases. This protocol was approved by the Human Ethics Committee (IEC No.-11/16) of Dr. Ram Manohar Lohia Institute of Medical Sciences (Dr.RMLIMS), Lucknow, India. This protocol complied with the 1996 Declaration of Helsinki. Informed consent was obtained from all participants.

Samples collection

A 4 mL blood sample was collected from all study subjects (with a self-administered questionnaire containing demographic information, smoking, and medical history) visiting the outpatient department (OPD) facility, Respiratory Medicine Department, King George's Medical University (KGMU), Lucknow, India. Blood samples were collected in EDTA (01 mL) and heparin (03 mL) vials from the participants. EDTA samples were immediately stored at -80 °C for genotyping, and 03 mL of blood from newly diagnosed untreated cases (n=48) was used for lymphocytic DNA damage. For each participant, FFPE tumor tissue was retrieved for P53 expression (Immunohistochemistry).

DNA extraction:

According to the manufacturer's recommendations, genomic DNA was extracted from whole blood using a Genomic DNA mini extraction kit (Invitrogen, USA). The extracted DNA was quantified and checked for purity by using a NanoDrop spectrophotometer (DS-11-spectrophotometer, Bio-rad, USA).

Genotypes analysis:

Detection of GSTT1 (+/-) GSTM1 (+/-) null and GSTP1 (Ile¹⁰⁵ Val) genotype:

Polymerase chain reaction determined GSTT1 genotype. A 20 µL PCR reactions have 100 ng of DNA, 0.8 µM of primers for GSTT1 (F5'-TTCCTTACTGCTCATC-3' R5'- TCACCGGATCGCCAGCA-3') and 0.5µM primers (F5'-GCCCTGCTAACACCTAC-3' R5'GCCCTAAAAAATCGCCAATC-3') for albumin gene and 12 µL EmeraldAmp GT PCR Master Mix (2X Premix) (Takara Bio, Japan). The amplification conditions were denaturation at 98°C for 10 s, followed by 35 cycles of annealing at 64°C for 45 s, and extension at 72°C for 1 min (Thermocycler Bio-Rad, USA). PCR products were resolved on a 1.2 % agarose gel stained with ethidium bromide (EtBr). After electrophoresis, GSTT1 (+/+) Fuc. genotype showed two bands of 480 bp and 375 bp, but only 375 bp showed GSTT1 (-/-) null genotype (SupplementaryFig.1a). The albumin gene (375 bp) acts as an internal control forthe reaction.

Polymerase chain reaction determined the GSTM1 genotype. In a 20 µL PCR reaction mixture comprising 100 ng of DNA and 0.8 µM of primers for GSTM1(F5'-GAACTCCTGAAAGCTAAGC-3';R5'-GAAGCCAAGGACGTAC-3') and 0.5µM primers ('F5'-GCCCTCTGCTAACAAGTCCTAC-3';R5'GCCCTAAAAAAGAAATC-3) for the albumin gene and 12 µL EmeraldAmp GT PCR Master Mix (2X Premix)(Takara Bio, Japan). The amplification conditions were denaturation at 98°C for 10 s followed by 35 cycles of annealing at 64°C for 45 s, and extension at 72°C for 1 min (Thermocycler Bio-Rad, USA). Amplified PCR products were resolved on a 1.5 % agarose gel stained with ethidium bromide (EtBr). After electrophoresis, GSTM1 (+/+) Fuc. genotype was characterized by two bands of 215 bp and 375 bp, while only 375 bp showed GSTM1 (-/-) null genotype (Supplementary Fig.1b). The Albumin gene (375 bp) acts as an internal control for the reactions.

The GSTP1 gene in exon-5 contains the Ile105Val substitution genotypic variants by RFLP. The PCR reaction mixture in 20 µL contained 0.5 µM of each primer (F5'- ACCCCAGGGCTCTATGGGAA-3'R5' TGAGGGCACAAGAAGCCCCT -3'), and 12 µL EmeraldAmp GT PCR Master Mix (2X Premix)(Takara Bio, Japan) and 100 ng DNA. The amplification conditions were denaturation at 98°C for 10 s followed by 35 cycles of annealing at 60°C for 1 min and extension at 72°C for 1 min (Thermocycler Bio-Rad, USA). Amplified PCR products were digested with 6 units of the Alw261 (BsmA1) restriction enzyme (Thermo Fisher Scientific, USA) for 10 h at 37°C. The digested PCR products were analyzed using 3.5% agarose stained with ethidium bromide (EtBr). The banding pattern was as follows: wild genotype Ile/Ile (AA): 176 bp; heterozygous genotype Ile/Val(AG): 176 bp, 91 bp, and 85 bp; and homozygous mutant Val/Val (GG): 91 bp and 85 bp) (Supplementary Fig.1c).

All genotyping of 10% random samples was performed repeatedly to confirm the reproducibility of the findings and was 98%.

Expression of P53 by immunohistochemistry

Immunohistochemistry (IHC) protocol for P53 expression was performed using the BenchMark XT platform (Roche, Switzerland). Briefly, formalin-fixed- paraffin-embedded (FFPE) tissues were sectioned into 3-4µm sections using a microtome (Leica, Germany). The slides were subjected to heat treatment in Ventana CC1 retrieval solution for 30 min and then incubated with the primary antibody (Dako clone DO-7, Denmark) for 30 min. The immune complexes were detected using ultraview 3,3-diaminobenzidine (DAB) as the chromogen (Ventana). Positive and negative controls were used in each batch (Fig.2). The IHC slides were evaluated for the percentage (%) of stained cells and staining intensity. Cells expressing P53 immunostaining were assessed by nuclear staining at 20X magnification. A tumor was recorded as positive if >50% of tumor cells with 2+ and 3+ intensity were considered as mutant type P53 expression and staining of <50% cells with 1+ and 2+ intensity as wild type P53 expression [13].

DNA damage analysis by comet assay/ single-cell gel electrophoresis (SCGE)

Lymphocytes were separated using HISTOPAQUE-1077 from 03 ml of peripheral heparinized blood from untreated cases. Lymphocytes were washed and re-suspended in PBS and adjusted to 1000000 cells/mL

The comet assay was employed for DNA damage analysis as previously described Singh et al.[14] with slight modifications. Briefly, 30 µL PBS suspended cells (40000 cells) were stretched onto an 80 µL high melting point agarose (HMPA) pre-coated microscope slide. An 80 µL low melting point agarose (LMPA) was poured onto slides and left at 4°C to solidify. Slides were placed in a lysis buffer for one h then placed in an alkaline solution for 20 min. Electrophoresis was applied for 30 min at 4°C, after which the slides were neutralized in 5% acetic acid and stained with ethidium bromide (EtBr). Cells were scored using an image analysis system (Komet-5.0; Kinetic Imaging, Liverpool UK) connected to a fluorescent microscope (DMLB, Leica, Germany). Approximately 100 cells/slide were analyzed. The DNA lesions in the cells were quantified as a percentage (%) of tail DNA (100% head DNA) (Fig.3a, b, c and d).

Statistical Analysis

The frequency distributions of gender, smoking status, genotypes, age and pack-years in subgroup groups; Chi-square (χ^2) and t-test were used to compare variables. To assess the risk of LUAD risk ratio (RRs) along with 95% confidence intervals (95% CIs) were calculated to determine the association with specific genotypes, and P53 immunoexpression using Graph Pad Instat Version 3.05 and SPSS(IBM, USA). DNA damage was expressed as mean $\pm$ standard deviation (SD). All p values were two-tailed, and $p < 0.05$ were considered statistically significant.

Results

Demographic and clinicopathological characteristics of the study group

Table (Supplementary) summarizes the cases; the mean $\pm$ SD age was 56.45 ± 11.20 . Among the cases with a mean pack-year of 11.76 ± 16.08 . Smoking beedi was more prevalent (45.40 % and 42.63%) than cigarettes. TNM was available in 82.2% of cases (stage II: 1.22%, III: 17.79% and IV: 63.19%) The GSTP1 variant genotype frequency of 113(69.33%) was more than double the wild genotype frequency of 50(30.67%). Similarly GSTT1 and GSTM1 showed 42(25.77%) and 37(22.70%) variants/deletion genotypes (Supplementary Fig. 1d)

Association between the germline genetic variation in GSTs (M1, T1, and P1) and P53 phenotype in cases of LUAD

The characteristics of stratified cases based on GSTT1, GSTM1 (+/-), and GSTP1 variant genotypes are shown in Table 1.

The mutant type P53 expression was demonstrated in 29.03% of cases, while 26.54% had a wild type P53 expression. There was no significant difference between the P53 mutant and P53 wild type expression groups in the distribution of males and females ($p = 0.80$). A higher incidence of P53 mutant type expression was observed in heavy smokers (68.75%) (> 40 pack-years). The number of pack-years was significantly higher in the P53 mutant type expression group($p = 0.03$). TNM stage data were available for 75 patients (93.75%) and P53 expression was not associated with TNM ($p = 0.65, 0.67$ and 0.10). However, the early disease stage (stage II) had a higher frequency (11.11%) in mutant type P53 expression than the wild type p53 expression group (6.25%).

The GSTs genotype distribution in the subgroup of cases carrying the mutant type and wild type P53 expression is illustrated in Table 2. The patients carrying GSTP1 Val/Val (GG) genotype significantly increased the relative risk of mutant type P53 expression (RR: 2.23, $p = 0.05$). Likewise, GSTM1 (-/-) was significantly increased but had a slightly lower relative risk to the mutant P53 expression (RR: 1.81, $p = 0.03$). Nevertheless, for the GSTT1 genotypes, no association was observed with p53 expression (RR: 1.01, $p = 0.96$). The combinatorial effect of GST genotypes demonstrated that double GSTM1 (-/-)/GSTP1 Ile/Ile (GG) genotypes were significantly and synergistically increased the risk of the mutant P53 expression (RR: 8.5, $p = 0.01$) and GSTT1 (-/-)/GSTP1 Ile/Ile (GG) elevated the risk of mutant P53 expression also, but could not reach a significant level (RR: 4.28, $p = 0.13$). However, a low number of cases in combination must be considered.

DNA damage among the GSTM1 (+/-), GSTT1 (+/-) and GSTP1 (Ile¹⁰⁵ Val) genotypic variants and the P53 wt/mut phenotype in untreated LUAD patients

DNA damage was significantly associated with null genotypes of GSTM1 (-/-) and GSTT1 (-/-) and GSTP1 Val/Val (GG) mutant genotypes in LUAD cases ($p = 0.001$). In the combination of GSTT1 (-/-)/GSTM1 (-/-)/GSTP1 (AG + GG) genotypes were significantly associated with DNA damage as compared to GSTT1 (+/+)/GSTM1 (+/+)/GSTP1 Ile/Ile (AA) genotypes ($p = 0.001$). Likewise, a significant tail DNA level was observed in the smoker group (mean $\pm$ SD = 9.45 ± 4.69 $p = < 0.001$). However, P53 expression was not associated with DNA damage in lymphocytes (Table 3).

Discussion

This study has investigate possible associations between germline genetic variations in GSTs xenobiotic metabolic enzymes(XMEs) and the relative risk of P53 expression (phenotype) in cases of LAUD and whether cases carrying GSTs variant genotypes have a high frequency of DNA damage?Futher association of GSTs genotype, P53 and DNA damage was correlated with smoking habits.

The GSTM1 and GSTT1 genes both exhibit homozygous deletions also called null genotypes, resulting in a lack of enzyme activity [15]. Similarly, the GSTP1 gene (Ile¹⁰⁵Val) residue lies in the enzyme substrate-binding site of the enzyme and the amino acid substitution has been shown to affect enzyme activity. A decrease in enzyme activity of GSTs may lead to poor detoxification of different carcinogens, leading to genetic damage and increased cancer risk [16].

Furthermore, it has been established that a decrease in GSTs enzyme activity could lead to a higher frequency and a specific pattern of expression for the tumor suppressor gene (P53) [16; 17]. Our results of P53 phenotype interpretation based on a tumor to be P53 mutated or P53 wild expression by immunohistochemical (IHC) method because P53 has a very short half life, which is not be detected in healthy lung tissue. In contrast, the mutated P53 gene has greater stability and can be seen by IHC. The oveexpression of P53 is generally regarded as a indicative of missense mutation [18].

Studies have demonstrated that the 33% of adenocarcinoma of lung showed mutant type P53 expression in the tumor and a link between the high rates of P53-positive tumor cells and tumor progression (patients with regional lymph node metastasis) for both adenocarcinomaand squamous cell carcinoma histological subtype of NSCLC[19, 20] Our result parallels the overhead line of 38.75 % LUAD cases with P53 mutant type expression which was significantly associated with heavy smoking (> 40 pack-years, p = 0.03) and higher frequency of T3N2M1 i.e; progressive disease.

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healthy lung tissue. In contrast, the mutated P53 gene has greater stability and can be seen by IHC. The overexpression of P53 is generally regarded as indicative of a missense mutation[21].

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We found a significant association with the P53 mutant type expression in patients carrying the GSTM1 (-/-) genotype as compared with the GSTM1 (+/+) genotype (RR: 1.81, $p = 0.03$). The GSTP1 Val/Val (GG) genotype exhibited a significant 2.23 fold increased risk of mutant type P53 expression in the LUAD cases as compared to P53 wild type expression with GSTP1 (AA) genotypes. Similarly, the combinatorial effect of GSTM1 (-/-)/GSTP1 Val/Val genotypes synergistically (RR: 8.50) enhanced the risk of P53 mutation in patients with LUAD. These findings support the hypothesis that polymorphic variants of GSTM1 or GSTP1 are moderately strong susceptibility factors for LUAD, but may become a dominant factor in the presence of a particular gene-gene interaction in a specific group, as has been demonstrated for cases carrying GSTM1null/ GSTP1 Val/Val genotype with P53 mutant expression. Consistent with our findings, Ryk et al. [17] showed that the GSTP1 variant genotype was associated with cancer risk and was overrepresented in P53 mutated cases. P53 is frequently mutated in GSTM1 null genotypes. Similarly, Gudmundsdottir et al. [16] observed a higher risk of developing breast cancer in the GSTP1 Val/Val (GG) genotype and P53 mutated phenotype of the tumors. Another study revealed that the GSTP1 gene contains a functional canonical P53 binding motif and the capacity of P53 to activate the human GSTP1 gene transcriptionally [21]. These results indicate that deletion of the GSTM1 and GSTP1 amino acid substitution Ile-Val, possibly leading to ineffective detoxification of certain mutagens or carcinogens, could increase DNA damage and frequency of P53 mutations. Another possible explanation could be that GSTs (GSTp and GSTm) play a regulatory role in cellular signalling by forming protein: protein interactions with critical kinases involved in controlling stress response, apoptosis, and proliferation via GSTp-JNK-P53, and GSTm-ATF2-P38 [22]. DNA damage mediated by decreased Phase II function (GST-P1 Val/Val (GG), T1, and M1 deletion) altered tumor suppressor gene P53 may lead to carcinogenesis. Studies have also demonstrated that GSTP1 and P53 are both independently involved in regulating cell growth, cell death, and survival and cellular protection against genotoxins [23;24].

We observed that the mean tail length in LUAD cases was significantly higher in patients carrying the GSTM1 (-/-), GSTT1 (-/-), and GSTP1 Val/Val (GG) genotypes compared to the GSTM1 (+/+), GSTT1 (+/+) and GSTP1 Ile/Ile (AA) genotype. A possible explanation is that deletion GSTM1, GSTT1 gene (null genotype), and GSTP1 Val/Val (GG) genotype; results in no functional enzymatic activity, thereby failing to detoxify several xenobiotics, including tobacco smoke constituents and other environmental carcinogen and finally leading to increased DNA adduct formation occurs, if not repaired results in accumulated DNA damage. Some studies revealed the association between the level of DNA damage in

PBLs and cancer risk [25;26;27]. The effect of environmental genotoxic factors is supposed to cause an increased level of DNA damage in PBLs and malignant cells and substances released in cancer patients during metabolic processes [28]. The increased DNA damage could be due to either carcinogen-DNA adducts formation or the disease progresses. Measuring DNA damage using the comet assay could be of potential diagnostic value to assess cancer's progression, histopathological features and genetic instability [26]. The present study revealed that DNA damage is significantly associated with smoking and GSTT1/M1 deletion and GSTP1 variant genotypes. P53 mutation is also significantly associated with smoking and GSTs variant genotypes. Identification of a link between DNA damage and P53 accumulation in tumor cells will strengthen understanding of the extent to which tobacco smoking is responsible for tumorigenesis in humans. This idea is based on the knowledge that the P53 phenotype may strongly influence DNA damage induced by tobacco carcinogens. However, P53 was not associated with lymphocytic DNA damage.

The study has some limitations that need to be addressed in future studies. First, this was a pilot study of the P53 expression and DNA damage with stratified GSTs genotypes. Secondly, samples were available at one time for DNA damage assay during the disease.

The GSTs gene family is also detoxifying the chemotherapeutic drugs such as cisplatin and carboplatin [29], homozygous deletion of GST may dismiss the detoxification of drugs, resultant increase the toxicity, poor treatment response, and survival; thus, DNA damage in follow-up samples can help to monitor the treatment. Finally, the detailed mechanistic approach can be analyzed through the GSTp-Jnk-P53 and GSTm-ASK1-P38 cascading pathway of cell survival and cell death.

Conclusion

The altered detoxification due to the null genotype and amino acid substitution may influence P53 mutations in LUAD. This influence may result from decreased detoxification of tobacco smoke carcinogens and possibly the formation of carcinogen-DNA adduct (increased DNA damage evidenced in circulating lymphocytes). The relative risk increases 2.0 fold more in the P53 mutated cases and carrying GSTM (-/-) deletion and GSTP1 Val/Val (GG) genotype, respectively. The P53 mutant patients had the combination of GSTM1 (-/-) and GSTP1 Val/Val (GG) genotype, the relative risk increases synergistically. DNA damage may be diagnostic markers in stratified patients based on smoking, tumor progression, and genetic variation of carcinogens detoxifying the glutathione S- transferases gene.

Abbreviations

LUAD: Lung adenocarcinoma; **GSTs:** Glutathione S transferases; **AA:** Ile/Ile; **GA:** Val/Ile; **GG:** Val/Val; **min:** minute; **s:** Second; **h:** hour; **Mutant phenotype:** immunopositive; **Wild phenotype:** immunonegative; **MAPKs:** Mitogen-activated protein kinases;

Declarations

Conflicts of interest-

The authors state that there are no conflicts of interest to disclose.

Funding acquisition-

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Tissue samples-Saumya Sukla

Experimental Investigation- Anumesh K Pathak

Supervision- Nuzhat Husain

Funding acquisition- Nuzhat Husain

Writing – Anumesh K Pathak

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Ethics approval-

This protocol was approved by the Human Ethics Committee (IEC No.-11/16) of Dr. Ram Manohar Lohia Institute of Medical Sciences (Dr.RMLIMS), Lucknow, India

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Tables

Table 1.

Frequency of p53 expression and clinicopathological characteristics of patients in LUAD cases

Variables		*P53 wt (%) (N=49)	P53 mut (%) (N=31)	p-Value
Age(years)	20-40	06 (12.24)	5 (16.12)	0.91
	41-60	28 (57.14)	18 (58.07)	
	≤60	15 (30.62)	08 (25.81)	
	Mean ±SD	54.69±10.62	54.96±11.89	
Gender	Male	36 (73.46)	22 (70.97)	0.80
	Female	13 (26.54)	09 (29.03)	
Smoking Status	Non-Smoker	19 (38.77)	15 (48.39)	0.39
	Smoker	30 (61.23)	16 (51.61)	
Pack-Year	<20	09 (30.00)	02 (12.5)	0.03*
	20-40	07 (23.33)	03 (18.75)	
	>40	14 (46.67)	011(68.75)	
	Mean ±SD	8.92±10.11	15.23±13.99	
#Pathological Staging	II	03 (6.25)	03 (11.11)	0.21
	III	12 (25.00)	11 (40.74)	
	IV	33 (68.75)	13 (48.15)	
#Tumor Status(T)	T1	02 (4.17)	01 (3.70)	0.65
	T2	05 (10.42)	04 (14.81)	
	T3	12 (25.00)	09 (33.34)	
	T4	29 (60.41)	13 (48.15)	
#Nodal Status(N)	N0	01 (4.17)	02 (7.41)	0.69
	N1	03 (6.25)	01 (3.70)	
	N2	13 (27.08)	09 (33.34)	
	N3	31 (64.58)	15 (55.55)	
#Metastasis(M)	M0	14 (29.17)	13 (48.15)	0.10
	M1	34 (30.83)	14 (51.85)	

Abbreviations:*p53 expression was determined by immunohistochemistry (IHC). A tumor was considered to be a positive (Mutant) phenotype if > 50% of tumor cells were stained.>50% of stained tumor cells were considered as negative (wt) phenotype. *p < 0.05 was considered statistically significant. # only 75(93.75%) cases TNM was available.

Table 2.

Frequency of expression in the p53 gene in stratified GSTs genotypes in LUAD cases.

Cases of LUAD				
Genotypes	P53 ^{mut} (n=31)	P53 ^{wt} (n=49)	RR (95% CI)	p- value
GSTM1 (rs.366631)				
Fuc. (+/+)	17 (54.84%)	38 (55.56%)	Ref.1	
Del. (-/-)	14 (45.16%)	11 (22.45%)	1.81 (1.07-3.06)	0.03*
GSTT1 (rs.2266637)				
Fun. (+/+)	22 (70.97%)	35 (71.43%)	Ref.1	
Del. (-/-)	09 (29.03%)	14 (28.57%)	1.01 (0.55-1.85)	0.96
GSTP1(rs.1695),(Ile;A/Val;G)				
AA (Wild)	07 (22.58%)	18 (36.73%)	Ref.1	
AG (Heterozygous)	14 (45.17%)	25 (51.02%)	1.28 (0.66-2.73)	0.51
GG (Mutant)	10 (32.25%)	06 (12.25%)	2.23 (1.07-4.65)	0.05*
Genotype distribution of described GSTs in p53 mut and p53 wt cases of LUAND				
GSTM1(+)/P1(AA)	04	17	Ref.1	
GSTM1(-)/P1(GG)	06	03	8.50 (1.45-49.5)	0.01*
GSTT1(+)/P1(AA)	07	12	Ref.1	
GSTT1(-)/P1(GG)	02	05	4.28(0.64-28.20)	0.13

Abbreviations: Fuc.(+): present; Del(-): gene deletion; RR: risk ratio; 95% CI: confidence interval; p-value :Pearson,s χ^2 test (Fisher's Exact), *p=>0.05 was considered as significant. Ref.; reference category, p53 wt phenotype and p53 and Fuc./AA as the reference category for calculating RR (95% CI). rs: Reference sequence of a gene.

Table 3.

DNA damage among the GSTT1, GSTM1, GSTP1 genotypic variants and p53 wt /mut. the phenotype in LUAD cases.

Variables	No. of Cases (%)	Tail length (µm) Mean±SD	p-Value
GSTM1			
Fuc. (+/+)	28 (58.33)	5.50±1.62	
Del.(-/-)	20 (41.67)	8.74±3.46	0.001*
GSTT1			
Fuc. (+/+)	29 (60.41)	5.60±2.09	
Del.(-/-)	19 (39.58)	8.76±3.20	0.0018
GSTP1			
AA	13 (27.08)	5.15±2.26	
AG	24 (50.00)	6.80±3.84	0.16
GG	11(22.92)	7.24±2.81	0.05*
T1 (Fuc.) /M1 (Fuc.)/P1 AA	06 (12.5)	5.6±1.33	
T1 (Del.)/M1 (Del.)/P1 AG+GG	06 (12.5)	11.72±2.98	0.001*
Smoking Status			
Non- smokers	23 (47.92)	03.76±1.07	
Smokers	25 (52.08)	9.45±4.69	<0.001*
P53 Phenotype			
Wt	28 (58.33)	7.98±3.19	
Mut	20 (41.67)	6.66±2.76	0.62

Abbreviations: Peripheral lymphocytes from untreated LUAD patients were subjected to comet assay, and the mean comet length for each subject was scored in 100 cells and calculated as tail length (µm) = maximum total length-head diameter. Values are shown in Mean ± SD. *p<0.05 compared to various genotype combinations and p53 phenotype groups of the LUAD cases was considered significant.

Figures

Figure 1 is not available with this version of the manuscript.

Figure 1

1a: Genotyping of GSTT1 PCR: PCR product; 480 bp & 375 bp show GSTT1 (+/+) wild genotype (lane 2&4-7). Only 375 bp GSTT1(-/-) null genotype (lane 3); Lane 1: 100 bp ladder, albumin 375 bp as internal control. Fig.1: b Genotyping of GSTM1 PCR: PCR product; 215 bp & 375 bp show GSTM1 (+/+) wild genotype (lane 2-4&6-7). Only 375 bp GSTM1(-/-) null genotype (lane 5); Lane 1: 100 bp ladder, a 375 bp as internal control. Fig.1c: Electrophoresis of the digested with Alw26I; PCR products showing individuals for the GSTP1 polymorphism (lane 3&6; GG- homozygous- mutant), heterozygous for the polymorphism (lane 3; AG- heterozygous) and lane 2 & 7 Homozygous wild (AA). Lane-1 DNA Ladder 20bp. Fig.1d: WT/WT: Wild genotypes, Variants: Heterozygous+Mutant for GSTP1 and (+/+): present; (-/-): gene deletion for GSTT1 and GSTM1.

Fig.2

Figure 2

Photomicrograph showing immunostaining for p53. A) Positive control – p53 in gliosarcoma (mutated) (DAB, 20X), B) Negative control- p53 in diffuse glioma (wild type) (DAB, 20X), C) p53 mutated in lung adenocarcinoma (DAB, 20X) and D) p53 wild type in lung adenocarcinoma (DAB, 20X).

Figure 3

Fluorescence Micro Images of comet assay showed the formation of tail DNA (200X). Lymphocytes cell of LUAD patient a) Case carrying with GSTM1(+/+), GSTT1(+/+) b) Case with GSTP1 (AA) wild genotype c) Case with GSTP1 (GG) mutant genotype. d) Case carrying with GSTM1(-/-), GSTT1(-/-)

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