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SEX BIAS AND EFFECT OF GLUTATHIONE S-TRANSFERASE MU1 AND THETA 1 GENOTYPES IN DRUG RESISTANCE TUBERCULOSIS

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ABSTRACT

Purpose: Genotypes of human Glutathione S-Transferase Mu1 (GSTM1) and Theta 1 (GSTT1) have been reported to associate with drug resistance in cancer patients and also reported to influence the severity of malaria. This work aims to assess the effect of sex and human glutathione transferase null genotypes on TB drug resistance. The objectives were to determine the association between sex and TB drug resistance and to find the association of Glutathione null genotypes with TB Drug resistance.

Design/Methodology/ Approach: A total of 81 TB patients on treatment were purposively recruited in a hospital-based cross-sectional study. Among them 41% (n= 33) were known DR-TB patients (cases) while 59% (n=48) were non-resistance Tb patients (control). Data on genotypes of human GST Mu1 and Theta1 were generated using conventional polymerase chain reaction (PCR). Descriptive statistics were used to determine the frequency distribution of the genotypes. The association of sex of research participants and human GST Mu 1 and Theta 1 genotypes with drug resistance to DR-TB were analyzed using a binary logistic regression model at a confidence level of 95% and the significance of results were judged at p-value less than 0.05.

Findings: Sex of patients significantly associated with increased risk to DR-TB (OR 5.51, 95% CI: 1.88-16.17, P= 0.002) while GSTM1 and GSTT1 genotypes did not statistically associate with drug resistance in tuberculosis (P=0.418). Frequencies of GSTM1, GSTT1 and combined GSTM1/GST1 null genotypes were higher in DR-TB patients (39.4%, 42.4% and 21.21% respectively) against (31.3%, 39.6% and 12.5%, respectively) in controls. Sex of TB patients significantly associated with Drug resistance Tuberculosis while GSTT1 and M1 genotypes did not associate with DR-TB.

Research Limitation/Implication: This study has therefore explored the effect of human GSTs and the association of gender with drug-resistant tuberculosis.

Practical Implications: Sex is an important factor to consider in the management of MDR-TB patients.

Originality/ Value: Understanding the association of sex and human genetic factors associated with drug resistance to tuberculosis is also an important way of controlling the disease.

Keywords*: Drug- resistance. glutathione. GSTT1. S-transferase Mu1. tuberculosis*

INTRODUCTION

Tuberculosis (Tb) is one of the leading causes of death worldwide with an estimation of 1.3 million deaths and 6.3 million new cases (WHO, 2018). Tuberculosis is usually treated with a course of four standards, or first-line, anti-tuberculosis drugs i.e. isoniazid, rifampicin,

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ethambutol and pyrazinamide for two months (intensive phase), followed by a continuous phase of isoniazid and rifampicin for four months (MoHCDEC, 2017). However, treatment success is challenged by the emergence of multidrug résistance- tuberculosis worldwide. Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to both isoniazid and rifampicin. DR-TB is a continuing public health concern in developing countries where there are inadequate diagnostics and treatment resources. WHO estimated 600,000 new cases with resistance to rifampicin (RR- TB), the most effective first-line drug, of which 490,000 had multidrug-resistant TB in 2016 (WHO, 2018). It is a growing public health concern, with an estimation of 3.5% of new TB cases and 20.5% for those previously treated turning to MDR-TB. In Tanzania, about 1.3% (0.47–2.1) and 6.2% (5.1–7.4) of new and on retreatment TB cases respectively, are MDR-TB (WHO, 2018).

Statement of the problem

The Mycobacterium tuberculosis natural defence mechanisms against some drugs and genomic mutations that alter proteins that are the target of drugs have also been reported as the cause of drug-resistant tuberculosis (Hoza, 2015). However, for the drug to be effective requires several different enzymes such as glutathione s-transferases for its metabolic conversion/ biotransformation. Human genetic variability of the enzymes involved in drug metabolism has been explained to affect drug absorption and metabolism. The consequences of such variations can lead to therapeutic failure or adverse drug reactions (Lima Teixeira, Suffys, & Santos, 2014). Most studies have reported the association of Glutathione S-Transferases (GSTs) null genotype with the occurrence of anti-tuberculosis drug-induced hepatotoxicity. The association of genetic polymorphisms of GSTM1 and GSTT1 with drug resistance tuberculosis is not well known. This work aims to assess the effect of sex and human glutathione transferase null genotypes on TB drug resistance. The objectives were: To determine the association between sex and TB drug resistance and to find the association of Glutathione null genotypes with TB Drug resistance.

LITERATURE REVIEW

Glutathione S-transferases (GSTs) belong to a major superfamily of ubiquitous conjugation enzymes, playing a crucial role in phase-II detoxification mechanisms catalyzing the conjugation of electrophilic compounds to glutathione. This conjugation usually renders hydrophobic compounds more water-soluble and predisposes them to excretion from the cell (Hayeset, Flanagan, & Jowsey,2005). The GST family consists of four superfamilies: cytosolic, mitochondrial, microsomal, and exclusive bacterial fosfomycin-resistance protein (Allocati, Federici, Masulli, & Ilio, 2009). Cytosolic GSTs consist of about fifteen classes namely: alpha, mu, pi, theta, sigma, omega, delta, epsilon and zeta. Others are lambda, phi, tau, chi, beta, and dehydroascorbate reductase. GST alpha, mu and pi are found in mammals, whilst GST phi, lambda, tau, and dehydroascorbate reductase in plants, the delta in insects, chi in bacteria and omega are found in both mammals and insects (Allocati et al., 2009). The human glutathione S-transferases (GSTs) possess both enzymatic and non-enzymatic functions. They are involved in many important cellular processes, such as phase II metabolism/ biotransformation of several drugs, stress response, cell proliferation, apoptosis, oncogenesis, tumour progression and drug

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resistance (Lo & Ali-osman, 2007). There are multiple variants in the genes encoding the GSTs families in the human body, which are classified into 4 major classes: GST-alpha (GSTA), GST-mu (GSTM), GST-theta (GSTT), and GST-pi (GSTP).

Most of these genes harbour polymorphisms that can influence their transcription which can affect the function of their encoded proteins and eventually can lead to drug resistance and/or toxicity (Lo & Ali-osman, 2007). The present study aimed at determining whether sex is associated with Drug resistance tuberculosis and the frequency distribution of human Glutathione S Transferase Mu 1 and Theta 1 genotypes in drug resistance TB patients.

Factors such as previous treatment for TB, traditional treatment, patients having diabetes mellitus, HIV-Tb co-infection, and suboptimal treatment adherence were found to be predictors for the occurrence of DR-TB (Tostmann, Boeree, Aarnoutse, De Lange, Van Der Ven, & Dekhuijzen, 2008; Mitnick, Rodriguez, Hatton, Brigden, Cobelens, Grobusch, & Van, 2016). Additionally, the sex of the infected host may also account for the susceptibility of individuals developing drug resistance tuberculosis as reported in South Africa (Pradipta, Boveneind-vrubleuskaya, Van, Akkerman, Alffenaar, & Hak, 2019).

RESEARCH METHODOLOGY

This study was conducted at Mawenzi Regional Referral hospital, Majengo health centre and Kibong'oto Infectious Disease Hospital (KIDH) Tb clinics in Kilimanjaro region North-Eastern part of Tanzania, from June to August 2018. Mawenzi Regional Referral Hospital and Majengo health centre are located in Moshi Municipality the capital of Kilimanjaro while KIDH is located at Sanyajuu in Siha district, 15 kilometres from Moshi - Arusha highway. KIDH is the centre of excellence for MDR-TB management in the country, receiving MDR-TB patients for care and treatment from almost all the country, particularly the Northern part of Tanzania. The KIDH also treats many patients with tuberculosis and HIV from the local area and the nearby mining district in Mirerani (Mpagama, Lekule, Mbuya, Kisonga,& Heysell, 2015). Being a touristic place for tourists visiting Mount Kilimanjaro and mining activities in the nearby district (Mirerani) might be one of the contributing factors to an increased risk of Tuberculosis in the region.

Sampling Technique and Sample Size

The current study was a hospital-based and cross-sectional study. The purposive sampling method was used to select 81 participants aged 18 years and above, tuberculosis patients on treatment who were attending TB clinics (controls) and those admitted at KIDH (cases). Of these 33 were the cases known DR-TB patients diagnosed by GeneXpert and/or sputum culture-positive Mycobacterium tuberculosis, resistant to at least isoniazid and rifampicin, admitted at KIDH and 48 were unmatched drug-susceptible TB patients (controls)who turned to sputum smear and/or GeneXpert MTB/RIFnegative after 2nd, 5th, or 7th month of the course of treatment.

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Inclusion and exclusion criteria for research participants

The study participant(s) was/were considered eligible for participation if he or she met the following criteria: Cases were defined as being on second/third line anti- TB drugs and Confirmed DR-TB by GeneXpertMTB/RIF, sputum smears and culture-positive Tuberculosis at month five or later of the initial phase of treatment. Controls were defined as being on first-line anti-Tb drugs with a confirmation of sputum smear, GeneXpert or culture-negative after month two or later of the initiation of treatment. Individuals who were severely sick and unwilling to consent were excluded.

Data collection

Data were collected using designated case report forms (CRFs), from which information on the demographic data, treatment and diagnosis history were obtained. Individual-level variables were age (in years), sex, and marital status. The treatment variables were diagnosis and the treatment history.

Whole blood (50µl) from the study participants, was collected on the Whatman filter paper and left to air dry. The dried blood spots (DBS) were stored at room temperature in the plastic bag with silica gel pellets at the KCMUCo-Wet laboratory where all the analysis was performed.

Data analysis

The Chelex method was used for DNA extraction from dried blood spots collected on the filter paper. About 8mm of the hole-punched filter paper sample was placed in a 1.5ml microcentrifuge tube and 1ml 0.5% Saponin in PBS was added into it. After several inverts, the microcentrifuge tube was stored at 4 0C overnight to remove the haemoglobin while the nucleic acids and some other cell components remain on the filter paper. The solution was then discarded, followed by twice washing of the filter paper by adding 1ml PBS whereby the second wash was after storing the sample at 4 0C for 15 minutes. After discarding the liquid, 150µl 6% Chelex solution was added, and samples were heated at 1000C for 30 minutes in a heat block, with vortexing for a few seconds every 10 minutes, followed by centrifugation at maximum speed (14,000rpm) for 5 minutes to spin down Chelex. Then to avoid the Chelex resins from disrupting PCR, the supernatant that contained nucleic acids was placed into a new 1.5ml microcentrifuge tube and stored at -200C.

Polymerase chain reaction (PCR) for detection of GSTM1 genotype

Analysis of GSTM1 was performed as previously described by Brockmöller, Kerb, Drakoulis, Nitz, & Roots (1993). Briefly, PCR reactions were carried out in 50ml. Each reaction contained 25ml of TEMPase Hot Start 2x Master Mix A (containing 0.2 units/ ml TEMPase Hot Start DNA Polymerase, 3.0mM MgCl2, 0.2% Tween R 20, 0.4mM of each dNTPs, and Taq polymerase PCR buffer), 0.2mM of each primer and genomic DNA (100-150 ng).Primers: sense:5'- CTC CTG ATT ATG ACA GAA GCC- 3'antisense: 5'- CTG GAT TGT AGC AGA TCA TGC- 3'. Three allelic variants: GSTM1a, GSTM1b and GSTM1-null allele were investigated for presence

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or absence of the GST regardless of which of the two alleles 1a and 1b. Beta-globulin was used as an internal positive control, using the following primers: Forward: 5'-CAACTTCATCCACGTTCACC-3'Reverse: 5'-GAAGAGCCAAGGACAGGTAC-3'

Polymerase chain reaction for detection of GSTT1 genotype

GSTT1 were analyzed according to the methods that were prescribed by Pemble, Schroeder, Spencer, Meyer, Hallier, Bolt, ...& Taylor (1994). The GST-theta 1 PCR was carried out in 25m reaction mixture which contained:12.5ml of TEMPase. Hot Start 2x Master Mix A (containing 0.2 units/ml TEMPase Hot Start DNA Polymerase, 3.0mM MgCl2, 0.2% Tween R 20, 0.4mM of each dNTPs, TrisHCl pH 8.5 buffer, 0.2 TweenR20 stabilizer), genomic DNA template (100-150 ng),0.5mM of each primer and RNase free water. The reaction mixture was subjected to initial denaturation at 94°C for 15 min, followed by 35 cycles of 94°C for 60 seconds, 58°C for 30 seconds (annealing), and 72°C for 90 seconds elongation. The final extension was carried out at 72°C for 7 min. TEMpase Hot Start DNA Polymerase is a modified form of AmpliqonTaq DNA polymerase which is activated by heat treatment. The enzyme is inactive at room temperature and during the first ramp of thermal cycling. Primers were purchased from BiolegioInc, Nijmegen the Netherlands while enzymes and reagents were purchased from Ampliqon A/S, Stenhuggervej 22, DK-5230 Odense M, Denmark.

Gel electrophoresis of GSTM1 and GSTT1 PCR products

The PCR products were then electrophoresed in a 2% agarose gel (AGTCBioproducts Ltd, Hessle Hull-UK) stained with ethidium bromide (Et-Br) and visualized under the UV light illuminator (VWR SCIENTIFIC Tran illuminator-USA). DNA from samples positive for GSTM1 and GSTT1 yielded 650bp and 480bp bands respectively and the absence of any fragment was considered as null genotype. Each set of reactions included the external negative control (RNAse free water) and internal positive control beta globulin which yielded 100bp bands as described above in GSTM1 analysis.

Data were entered into Microsoft Excel 2007. STATA 20 software was used for data analysis. The age of participants was categorized into two groups of ≤35 and >35. The Chi-square test was used to test the difference in proportions and determine the relationship between categorical variables. Binary logistic regression was used to identify factors that independently associate with DR-TB. A p-value of 0.05 or less was considered statistically significant.

Ethical consideration

Approval for this study was obtained from KCMUCo Research and Ethics Review Committee (certificate no. 2031). Permission to conduct the study at Mawenzi regional referral hospital and Majengo health centre was authorized by the Regional and District Medical Officers of Kilimanjaro Region and Moshi Municipality Council, respectively; and from the Director-General of Kibong'oto Infectious Disease Hospital. All participants were informed of the objectives, procedures and benefits of the study and assured of anonymity and confidentiality. Written informed consent was obtained from all participants who voluntarily agreed to

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participate, and was free to refuse and refusal did not affect them in any way. Confidentiality and privacy were maintained throughout the study conduct and reporting.

RESULTS AND DISCUSSION

Socio-demographic characteristics of Research participants

A total of 81 participants were included in the analysis, of which 32(39.5%) were females. Thirty-three were DR-TB (cases) and 48 were Non-DR-TB (controls) patients. In terms of age categories, all participants were above 18 years old and were categorized into two levels: ≤ 35 and >35, of which 28 (34.6%) were between the age of 18-35 years; and 53 (65.4%) wereabove35years. Of all the participants, 52 (64.2%) were new cases Tb and 29 (35.8%) were previously treated, patients. Concerning HIV status: 30 (37%) were seropositive. Among the seropositive; 14 (46.7%) were the cases and 16(53.3%) were controlled.

Distribution of GSTT1 and GSTM1 genotypes

Overall, 33(41%) of the patients possessed both GSTM1 and GSTT1 genotypes, while 13 (16%) were having a deletion of both genes (null genotypes). Twenty eight (34.57%) were GSTM1-null genotype while 33(40.7%) were GSTT1-null genotype. Concerning groups, the GSTM1null genotype was 39.4% in cases as compared to 31.2% in controls but the difference was not statistically significant (P=0.301). The frequency of GSTT1null genotype among the cases and controls was almost similar (42.4% and 39.9%, respectively) (Table 1).

Furthermore, when the GSTM1 and T1 polymorphisms were compared to sex the frequency of GSTM1 null genotype was 36.7 %(18) in males while in females was 31.3% (10). However, the prevalence of GSTT1 null genotype was slightly higher in females 56.2% (18) than in males 30.6% (15) and the difference was statistically significant (P= 0.02).

Table1. Distribution of GSTM1 and GSTT1 genotypes among Tb patients (n=81)

GST Polymorphisms	Cases n (%)	Controls n (%)	FE*	P value
GSTM1Positive	20(60.6)	33(68.8)	ref	
GSTM1Null	13(39.4)	15 (31.2)	0.483	0.301
GSTT1Positive	19(57.6)	29(58.3)	ref	
GSTT1Null	14(42.4)	19(39.9)	0.822	0.489
BothGSTM1/T1 positive	13(39.39)	20(41.67)	ref	
Either GST positive	13(39.39)	22(45.83)		
GST M1/T1 Null	7(21.21)	6(12.5)	2.76	0.418

*Fisher's Exact test

Figure1. Gel electrophoresis for analysis of GSTM1/T1 PCR products. 1(a) GSTM1 which yielded a 650 bp bands, (b) GSTT1 DNA with 480 bp bands. Markers (M) are the 1 kb ladder (ThermoFisher Scientific). Positive internal control Beta globulin gave a positive yield of 100 bp for all 81 DNA preparations

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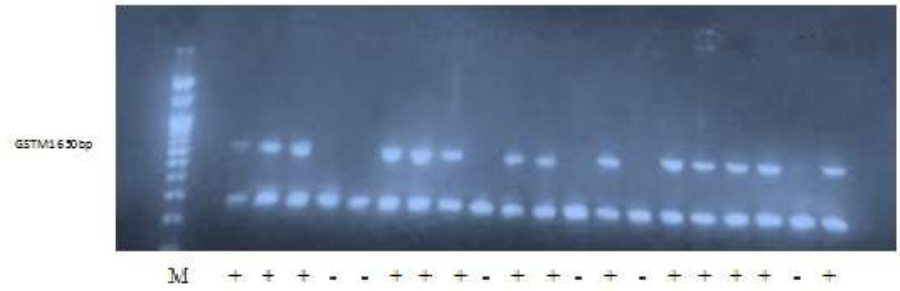

Figure 1(a) Gel electrophoresis for analysis

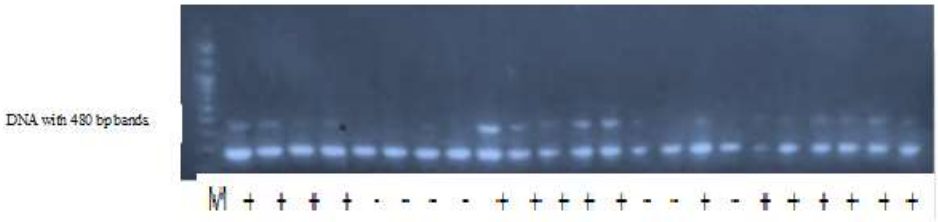

Figure 1(b) Gel electrophoresis for analysis

Association of GSTs genotypes with drug-resistant tuberculosis

A binary logistic regression model was used to determine the association of human GST Mu1, Theta 1 genotypes and other variables with drug resistance tuberculosis (Table3). Possession of both GSTMu1 and Theta 1 was used as a reference group in the analysis of the genetic factor. Adjusted OR (95% CI) for GSTM1 null was 0.51(0.14, 1.88) p=0.309, compared to those with GSTT1 null genotype which was 1.27(0.32, 5.04), p=0.73, whereas the OR (95% CI) among those with combined GSTM1/T1 null genotype was 1.37(0.31, 5.92), p= 0.678.Unadjusted OR (95% CI) of females Tb patients was 4.62 (1.77, 12.01) P=0.002 as compared to males. After adjusting for other variables OR (95% CI) of the female group was 5.51 (1.88, 16.17), p=0.002.

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Table 2: Logistic regression analysis of the association of DR-TB and GSTs with other factors

Variable	Crude OR (95%CI)	P-value	Adjusted *OR (95%CI)	P-value
Age group				
<35 years	Ref			
≥35 years	0.88 [0.35, 2.22]	0.778	0.64 [0.21, 1.9]	0.419
Sex				
Male	Ref			
Female	4.62 [1.77, 12.01]	0.002	5.51 [1.88, 16.17]	0.002
Treatment history				
Previously treated	Ref			
New cases	0.49 [0.2, 1.25]	0.136	0.4 [0.13, 1.25]	0.115
Genetic factors				
GSTM1&T1	Ref			
GSM1 null	0.83[0.26,2.63]	0.749	0.51[0.14,1.88]	0.309
GSTT1 null	1.03 [0.29,3.57]	0.968	1.27[0.32, 5.04]	0.73
[1]None	1.79 [0.49,6.55]	0.376	1.37[0.31,5.92]	0.678

*Adjusted for all other variables in the table. [1]Null genotype of the combined GSTM1/GSTT1

Generally, the findings of the present study show that sex is significantly associated with MR-TB resistance while the human Glutathione S Transferase Mu1 and Theta1genotypes do not influence the TB drug resistance.

In the present study, almost half of the participants (59%) were GSTT1 positive while 65% were GSTM1 positive. Deletion of both genes was found in 16% of all of the Tb patients enrolled in the study and their absence was not found to be statistically associated with drug-resistant tuberculosis.

When GSTs genotypes were analyzed concerning sex, the proportion of GSTT1 null genotype was statistically higher in females than males (P=0.02). The overall proportion of GSTM1 and GSTT1null genotypes observed in this study was 34.6% and 40.7%, respectively. This finding agrees with previous studies which reported similar findings on the proportion of null genotypes of GSTM1 and GSTT1in different African populations (Mo, Gao, Cao, Gao, & Jian, 2009; Kavishe, Bousema, Shekalaghe, Sauerwein, Mosha, van Der Ven, & Koenderink, 2009; Saguti, Balthazary, Manjurano, Max, & Tenu, 2013) which may most likely give the clue on the proportion of these genes. However, the deletion of genotypes may differ among the different ethnic groups as previously reported on healthy individuals (Roy, Majumder,& Roy, 2008).In this study, it was observed that the proportion of both GSTM1 and GSTT1 null genotypes was slightly higher in cases as compared to the controls, though the difference was not found to be statistically significant the findings point to a biological variation that exists between controls and cases.

In binary logistic regression analysis of GSTs and other factors, we observed that null genotypes of either GSTs or both were not statistically associated with DR-TB. However, other studies

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have reported the association of GSTM1/T1 null genotypes with other disease treatment outcomes (Ramappa& Aithal,2013; Lima, Teixeira, Suffys, & Santos, 2014). The study conducted in Morocco observed a higher risk of developing chronic myeloid leukaemia (CML) in males with GSTT1 null genotype than their counterpart females (Kassogue, Dehbi, Quachouh, Quessar, Benchekroun, & Nadifi, 2015). Although most of these epidemiological studies have established the importance of GSTs as determinants of therapeutic response in different types of cancers, probably the same phenomenon might be applicable in Tb treatment, whereas the absence of these GST enzymes could have an indirect influence on an increased risk of drug-resistant tuberculosis.

We observed in this study also that the female sex had a higher risk of developing drug resistance tuberculosis than males (P= 0.002). Similar findings were observed in the study done in South Africa where a greater proportion of women had a higher risk to DR-TB as compared to men (Andrews, Shah, Weissman, Moll, Friedland, & Neel,2010; WHO, Global Report, 2010). Pradipta and others reported that males had a slightly higher risk of DR-TB (54%) than females (Pradipta, Boveneind-vrubleuskaya, Van, Akkerman, Alffenaar, & Hak, 2019). These results deviate from other studies conducted in Tanzania that neither of the sex was reported to be associated with DR-TB (Hoza, 2015: Lema et al., 2016; Nagu et al., 2015). The reason for this association is unclear, whether is genetic, environmental or contact rate to an infectious agent.

CONCLUSION

As an infectious disease with a high burden, understanding the association of sex and human genetic factors associated with drug resistance to tuberculosis is also an important way of controlling the disease. For the drug to be effective it depends on its metabolism which is controlled by human genetic factors responsible for enzymatic activity and other factors such as gender (Belle and Singh, 2008). Poor drug metabolism can lead to the emergence of resistant Mycobacteria tuberculosis which is more difficult and expensive to treat. This study has therefore explored the effect of human GSTs and the association of gender with drug resistance tuberculosis and the following conclusions may be drawn:

- GSTT1 and M1 genotypes were not found to be significantly associated with drug-resistant tuberculosis.
- Also, show the association of the female gender with an increased risk of drug-resistant tuberculosis.
- Moreover, the differences in proportions of GSTM1/T1 null genotypes observed in this study were slightly higher in drug-resistant patients as compared to controls.

Recommendation: Sex is an important factor to consider in the management of MDR-TB patients. Further studies need to be conducted to explore more the effect of human Glutathione S Transferase Mu1 and Theta1genotypes on drug-resistant tuberculosis.

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