SUMMARY AND CONCLUSIONS

India is a high burden country with respect to tuberculosis with 25% of the world’s TB patients. Drug resistance is hampering the effectiveness of the RNTCP despite various efforts and money spent. Drug resistance in general is associated with irregular adherence to therapy or opting out of therapy due to side-effects or secondary complications. But with an effective programme implementation like DOTS the drug resistance problem should be minimized. The focus of the present work is rational use of drug in a more personalized way to check the resistance problem and a more consistent positive outcome of the therapy. The findings of the present study indicate that individuals with rapid and intermediate acetylator genotypes appear to be at higher risk of developing of INH resistance even with successful completion of multidrug therapy (DOTS). Such associations were reported earlier in pre-MDT era and are found to be still relevant. After development of adequate infrastructure and clinical research the patients could be screened for their genetic predisposition factors before initiating therapy optimizing the therapeutic regimen. A genotyping test costing around 200-250 INR which could be conducted in many settings even today could stratify the patients receiving therapy enabling implementation of a patient specific therapy regimen.

To achieve this goal the study was focused on Isoniazid which is one of the first line drugs for the treatment of TB. Isoniazid metabolic differences in the population are known since 1980s but pharmacokinetic and pharmacogenetic aspects are not adequately explored and used to develop tailor-made regimens to yield optimum results. Since the Human genome was sequenced, genetic markers and polymorphisms are more worked out and it should be possible to classify patients according to their genetic predispositions. The personalized therapy regimen could also be applied in the administration of INH in TB suspect and co-infection cases and optimizes its chemo-prophylactic as well as its chemotherapeutic potential.

Objectives of the study are:
1. To work out relationship, if any, between long term therapeutic response with NAT2 allelic frequencies & distribution.

2. Establishment & standardization of primary hepatocyte culture from mice for transcriptomics analyses.
3. Genome wide high throughput screening for other drug metabolizing enzymes, transporters, binding proteins interacting with drug or affecting the drug responses.


**NAT2 Genotyping**

**Methodology**

In the present study NAT2 genotyping was undertaken on 204 pulmonary tuberculosis patients with varied therapeutic response to DOTS. One hundred of these patients were infected with *M.tb* strains mono-resistant to INH, while other 104 were infected with *M.tb* strains sensitive to all first line TB drugs. Out of these 204 patients, 62 patients could be followed up for one year after completion of DOTS.

**Association of NAT2 alleles with INH mono-resistance**

We have tried to assess impact of NAT2 genotypes on occurrence of INH mono-resistance. When the wild type NAT2 haplotype is considered dominant a significant association with INH-mono-resistance [p-Value < 0.05 (0.047933)] which assumes that both homozygous & heterozygous are at same risk to develop mono-resistance. If the multiplicative effect is considered i.e. subjects with one copy of the wild type NAT2 haplotype are at an intermediate risk the association is highly significant [p-Value < 0.01 (0.009691)]. Thus a significant association of rapid inactivation of drug with occurrence of INH mono-resistance becomes more significant as the effect is not diminished by other possible coexistent factors such as primary resistance, highest resistant mutation frequency and rapid elimination in hyper-micturation conditions such as diabetes. Therefore the relationship between fast and intermediate acetylator status and INH-resistance is highly probable. The implications of the findings in this study might be clinically useful that it might be worthwhile to consider administering higher dosages of INH to TB patients who are either fast or intermediate acetylators of INH.

**Association of NAT2 alleles with long term therapy response**

The response of patient to anti-TB therapy (ATT) and correlation with NAT2 genotype has not been conclusive due to sample size but shows some insight into its possible effect on
outcome of therapy response particularly in relation to INH monoresistance. These results help us to calculate the sample size of future prospective studies.

**Microarray Analysis of Mice Hepatocyte Genes post INH challenge**

**Methodology**

Mice primary Hepatocytes were cultured in artificial medium and exposed to INH. The genome wide expression change induced by INH was analyzed with the aid of microarray technology. Various statistical tests were performed which showed that the experimental conditions were comparable among control and INH induced groups. The differentially expressing genes were studied by pathway analysis.

**Leads Obtained**

In the present study microarray has proved to be a useful tool to identify various genes differentially expressed in cultured mice primary Hepatocytes. Our data is suggestive that a number of high-level GO terms (functional annotations) were influenced by the corresponding mapped expression data. Our observations indicate that INH may influence many cancer and mutation associated pathways such as base excision repair, basal cell carcinoma, cell cycle and p53 signalling pathway. These changes observed could be due to hydrazine toxic intermediate generated after hydrolysis of INH. Most important is the xenobiotic transformation pathway which is mainly responsible for drugs inactivation and neutralization. Other Also INH is known to react with Vitamin B6 (pyridoxal) and thereby inactivating the later. Many genes involved in Vitamin B6 metabolism as well as genes depended on pyridoxal for their function are differentially expressed. Overall, the differentially expressed genes could be considered as important leads for future investigations and human orthologous counterparts need to be subjected to Real time PCR analysis in human primary Hepatocytes. As the starting sample for Human primary Hepatocytes is always small a Real time PCR analysis targeting the genes found to be differentially expressed in mice will generate a better picture of therapeutic relevance in humans.
Development of spot test to detect INH acetylation status

Methodology

After trying various reagents and reaction profiles a method yielding reproducible results was standardized. Hydroxynepthaldehyde Test is a strip based test which can detect INH in biological fluids (serum/urine). Urine samples were tested as the sampling is non invasive and simpler.

Leads Obtained

The test developed to check the INH acetylation status (retention time) in urine specimen and follow-up during the course of INH therapy is simple with application to field settings. The spot test strip eliminates the time bound degradation of INH in the samples and can be done in field settings. This test may potentially prove to be simple aid for personalizing the medicine of the patients in the future. Also the same strips can be utilized to monitor compliance with the therapy among the patients.

Important findings from our study are summarized below:

Effect of genetic polymorphisms in Nat2 gene responsible for acetylation and deactivation of INH on the long term therapy outcome in TB patients.

Highlights:-

1. This study shows significant pharmacogenetic variability that may influence treatment even in MDT scenario.

2. Rapid acetylators i.e. homozygous wild type allele as well as Intermediate acetylators i.e. heterozygous with one wild type and one any of the slow allele have been observed to be at a significant risk of developing INH mono resistance.

3. The results obtained with genotype association are compelling evidence to initiate a dose modulation study to improve therapy outcome i.e. decrease in resistance.

4. Prospective studies with initial genotyping and a follow-up under therapy with effective sample sizes needs to be carried out to address effect of these polymorphisms in the long term.
India is a country with many population genetic niches specific to geographical regions. Thus a countrywide survey of polymorphisms may aid us to consider and design regional based changes in the therapy under our national programme.

**High Throughput (Whole genome microarray) screening of genes in INH induced cultured murine primary hepatic cell lines.**

**Highlights:-**

1. In the present study many relevant pathways were found to be significantly affected by INH induction. But there were numerous differentially regulated genes that were not classified in any pathway or were hypotheticals leaving a scope of further investigations.
2. Most of the affected pathways (Phagosome, RNA degradation, lysine degradation and DNA repair) could be attributed to drug toxicity of INH or its metabolites on Hepatocytes.
3. Many cancer related pathways were affected which could be due to hydrazine or oxidative stress.
4. NAT8 and Cyp1a1 were found to be over expressed which are associated with INH acetylation and oxidation respectively.
5. Many other Phase I enzymes of xenobiotic transformation were differentially expressed. This was expected as INH modulates metabolism of numerous other drugs.
6. Ces1 was differentially expressed and could be involved in INH hydrolysis.
7. Present study shows that INH or its metabolites can affect the expression of several relevant genes related to glucose export and then may act as glucose export inhibitors by repressing vesicular transport and reducing glucose export transporters.
8. Oxidative stress pathway of the liver causing regeneration is also upregulated which explains the under lying mechanism of INH mediated hepatitis.
9. Abcc12 a less studied ABC transporter was found to be over-expressed. This is of particular interest as its polymorphisms could be another contributing factor for INH toxicity.
10. Psat1 was found upregulated, suggesting that higher dose of Vitamin B6 may need to be administered to pregnant women receiving INH.
Development of a simple spot test to detect INH acetylation status of the patients.

Highlights:-

1. Time bound degradation of INH hampers sampling from remote field settings.
2. We have devised a strip based test for detecting INH. To check the acetylation status (retention time) follow-up testing with patient urine samples is required post INH consumption at different time ranges.
3. The same strips can also be utilized to monitor compliance with the therapy among the patients.

Conclusions, Rationale and Future implications
To conclude, our study provides the following significant useful findings:

1. New information unraveled in pulmonary TB patients from Uttar Pradesh regarding Nat2 polymorphisms association with INH monoresistance. This information can be used to develop a case specific pre-screening methodology for TB patients as one of the approaches, followed by dose modulation according to patient genotype.

2. Gene expression studies of murine primary hepatocytes induced with INH have shown differential expression of genes of various pathways relevant for xenobiotic transformation, Insulin pathway, Oxidative damage and various toxicity associated pathways etc. These aspects need to be validated in humans. There appears to be a possible danger of drug interactions of INH with drugs being administered for other coexistent conditions such as HIV, pregnancy, diabetes and substance abuse etc.

3. Psat1 was found to be affected by INH which is vitamin B6 dependent enzyme and essential for fetus development. Re-examining of current practices; for example preventive and or therapeutic options to supplement vitamin B6 in all pregnant females, especially those conceiving during the anti-TB therapy may be considered. Relevance of our findings thus needs to be validated further in pregnant TB patients receiving DOTS who could be monitored on regular intervals to ensure adequate vitamin B6 supplementation mitigating the toxicity.
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