DISCUSSION
Chapter 4
Discussion

- Epidemiology
- Relationship between cyp1a1 (msp1), cyp1a1 (ile/val), cyp2e1, cyp2d6, gstm1 and gstt1 genotypes and lung cancer
- Relationship of lung cancer risk with combined genotypes of cyp1a1 (ile/val) and cyp1a1 (msp1) genes
- Relationship of lung cancer risk with combined genotypes of cyp1a1 and gstm1 genes
- Relationship of lung cancer risk with combined genotypes of cyp1a1 and gstt1 gene
- Relationship of lung cancer risk with combined genotypes of cyp1a1 and cyp2d6 genes
- Relationship of lung cancer risk with combined genotypes of gstm1, gstt1 and cyp2d6 genes
- Relationship of lung cancer risk with combined genotypes of gstm1 and gstt1 genes
- Relationship between cyp1a1 (msp1), cyp1a1 (ile/val), cyp2e1, gstm1 and gstt1 genotypes and lung cancer stratified according to smoking
- p53 gene mutations and lung cancer
• *p53* gene mutations and *cyp1a1* genotypes in relation to lung cancer
• *p53* gene mutations in combined genotypes of *cyp1a1* and *gstm1*
• *p53* gene mutations and *cyp2d6* genotype in relation to lung cancer
• *p53* Gene Mutations In Relation To *Gstm1* And *Gstt1* Genotypes
• Telomerase activity in bronchial washings of lung cancer patients
DISCUSSION

1. Epidemiology

This case control study pertains to 100 lung cancer patients and 76 controls. Of the various risk factors concerned with the development of lung cancer, smoking is the primary cause. About 90% of all lung cancer cases are due to smoking. In the present study, 86% were smokers. An important point in this study was that unlike Western countries, where the predominant mode of smoking is filtered low tar cigarettes (Doll, 1998), the Indian subjects were bidi smokers. Among the smokers, 83.7% of the patients were heavy smokers with a BI>400 and 16.3% light smokers (BI<400). The maximum number of cases was from the state of Himachal Pradesh followed by Haryana. Till date statistics are not clearly available regarding the incidence of lung cancer from different regions of India, but data here suggest that Himachal Pradesh and Haryana might have higher incidence of lung cancer as compared to other North Indian states. But in order to validate this point, a larger case/control study is warranted.

Among patients, 80% were less educated or illiterate and 20% educated. Since education leads to awareness regarding the use of tobacco and exposure to other pollutants, it seems that it might be playing a key role in the prevention of lung cancer. It was observed that patients were somewhat less educated than controls and more controls were non-smokers.

Among the lung cancer cases, 71% were with squamous cell carcinoma (SQCC), 24% with small cell lung carcinoma (SCLC), 4% with adenocarcinoma (ADCC) and 1% had large cell carcinoma (LCC). There is a less incidence of ADCCs and more prevalence of SQCCs, whereas in the West, the reverse position is seen. Earlier there was more incidence of SQCC as compared to ADCC, but there has been a shift in histology of lung cancers from SQCC to ADCC. This is attributed to the change in smoking habits in the Western countries, which have strict laws regarding the
manufacturing of cigarettes. In the past, cigarettes had high tar and nicotine content and were non-filtered. As a result, the incidence of peripherally arising tumours like SQCC was more because the smoker never used to inhale the tobacco smoke deeply into the lungs. As the new laws came into existence, cigarette manufactures were forced to make "good quality" cigarettes with less tar and nicotine content and are filtered. As a result, smokers who were initially smoking the old type of cigarettes are forced now to use the "good quality" stuff in order to satisfy the urge for nicotine thus, they started inhaling deeply into the lungs which lead to the development of centrally arising tumours like ADCC.

2. **Relationship between cyp1a1 (msp1), cyp1a1 (ile/val), cyp2e1, cyp2d6, gstm1 and gstt1 genotypes and lung cancer**

In the present study, investigations on the prevalence of three single nucleotide polymorphisms in the cyp1a1, cyp2d6 and cyp2e1 gene and also the polymorphism in the gstm1 and gstt1 genes have been carried out. Both among cases and controls, the frequency of val/val mutant allele is highly represented. But there is an over-representation of the val/val genotype in lung cancer cases as compared to controls (29% vs 19.7%). Similarly the frequency of the homozygous wild ile/ile genotype is under-represented both in cases and controls, with the cases having a less frequency than controls (4% and 10.5%). A high representation of the heterozygous (ile/val) and mutant (val/val) genotypes is reported for the first time. In fact, the frequency of val/val genotypes varies according to ethnicity. In Japanese, the allelic distribution of val/val genotype ranges from 5-10% (Nakachi et al., 1993; Kihara et al., 1995), in German population; the frequency of mutant genotype is 2% (Drakoulis et al., 1994), and same is the frequency in Brazilian population, (Hamada et al., 1995), 5% in Hawaiian population (Marchand et al., 1998) and 17% in Chilean population (Quinones et al., 2001). One particular study conducted on a Korean population, reports a high frequency of the heterozygous genotype (ile/val), but the representation of the mutant genotype is less (Hong et al., 1998). Another study on patients
with oral cancer in South India, has reported a high frequency of both the heterozygous and mutant genotypes (Sreelekha et al., 2001).

In this study, the individuals with the mutant genotype (val/val) were at a 2.5 fold elevated risk of lung cancer. There is a strong association between the val/val genotype and SCLC, but not for SQCC. A similar study carried out by Sugimura et al. (1998) also pointed out a risk for lung cancer with the val/val genotype. A study by Hamada et al. (1995) and another by Quinones et al. (2001) have shown an association of the ile/val polymorphism with lung cancer in Brazilian and Chilean populations. However, data from elsewhere in Japan, as well as Scandinavian countries, North America have not revealed a statistically significant association of this genotype with lung cancer susceptibility.

Similarly for the cyp1a1 (msp1) genotype, there is a less representation of the mutant msp1 genotype (m1/m1); 6% in cases and 6.6% in controls. Studies carried out in Japanese and Chinese show a high frequency of the m1/m1 mutant genotype (21% and 10.5%) (Nakachi et al., 1991; Lin et al., 2000). Studies carried out in Caucasians have reported a less representation of the mutant genotype. The frequency of the m1/m1 genotype is 1.7% in Finnish (Hirvonen et al., 1992), 1.3% in Swedes (Alexandrie et al., 1994), 0.7% in Germans (Drakoulis et al., 1994), 2.3% in Belgians (Jacquet et al., 1996), 2% in French (Bouchardy et al., 1997) and 1% in Norwegian and American (Tefre et al., 1991; Garcia-Closas et al., 1997) populations. In this study the frequency of the mutant alleles is intermediate between those of South-East Asians and Caucasians. It is considered that the m1/m1 genotype may not be a susceptibility risk factor for lung cancer in the present population from India because of its less frequency. But there is a more representation of the heterozygous genotype (wt1/m1) (45%). When both the genotypes i.e. wt1/m1 and m1/m1 are combined as a single genotype (wt1/m1/m1/m1), they have no association with lung cancer in the present population. But there is a slight increased risk in case of SQCC. The risk for SQCC is 5-fold higher in Japanese
(Kawajiri et al., 1990) for the mutant m1/m1 genotype than for wt1/wt1 genotype. This ethnic difference might be due to the higher frequency of the m1/m1 allele in South East Asian populations. The association of the cyp1a1 (msp1) genotype with risk towards SQCC has also been observed in Hawaiian and some Caucasian populations (Marchand et al., 1998). The interindividual difference in genetically determined susceptibility to SQCC of the lung has been investigated in relation to smoking dose (Nakachi et al., 1991). The total amount of cigarettes consumed over one’s lifetime has been compared among the three genotypes of msp1 polymorphism. The patients with the mutant m1/m1 genotype contracted lung cancer after a fewer cigarettes than those with the other genotypes.

For the cyp2e1 gene, no single variant allele both in cases and controls has been observed and the wild homozygous genotype (c1/c1) is predominant. It is hypothesized that the pst1 polymorphism located in the 5’ flanking region of the cyp2e1 gene might not be an important risk factor in the present population. There is a possibility that other polymorphic regions located in the cyp2e1 gene such as dra1; rsa1 and taq1 might be associated with lung carcinogenesis. The associations between cyp2e1 pst1 or rsa1 genotypes and lung cancer susceptibility have been evaluated in Finnish, American and Swedish populations, but results are inconsistent and remain controversial (Hirvonen et al., 1993a; Person et al., 1993). The dra1 cyp2e1 RFLP has been found to be associated with lung cancer in Japanese case-control study (Uematsu et al., 1991b). However, subsequent studies have failed to implicate the dra1 RFLP as a cancer risk factor in American whites and African-Americans (Kato et al., 1994).

Conflicting reports concerning the pst1 or rsa1 mutation are available in the literature. The rare pst1/ rsa1 allele has been associated with decreased risk for cancer (Marchand et al., 1998; Wu et al., 1998). Wu et al. (1998) have reported that the individuals who lack the rare allele (c2) might be at higher risk for developing lung cancer in Mexican-Americans, but not in African-Americans. Using the combined genotypes c1/c2 and c2/c2 as the
reference, the OR for c1/c1 genotype is 14.0 (95%CI=1.9-101.15) among Mexican-Americans. Given the low gene frequency of the *cyp2e1* in the European and African-American population studies to date and the relatively modest ORs associated with this polymorphism, the demonstration of a *cyp2e1* polymorphism and a relationship with lung cancer susceptibility would require case and control groups of greater than 1000 subjects each (Kiyohara et al., 2002).

For the *cyp2d6* gene, no poor metabolizer (PM) was observed. Studies carried out on Caucasians have shown a 7% frequency of the PM genotype; in Swedish population, the frequency of the PM genotype is 9.5% (Rannug et al., 1995), 7.1% in Italian (Shaw et al., 1998), 5.4% in French (Laforest et al., 2000), 3.7% in German (Roots et al., 1992) and 3.6% in British (Wolf et al., 1992) populations. In Japanese and Chinese populations, the frequency of the PM is less than 1% (Gao et al., 1999). It is thought that the PM may not be a susceptibility risk factor for lung cancer in this present population as it is absent. The heterozygous alleles (HEM) are more represented in this population and they are at a moderate risk for lung cancer (OR=1.48), with increased risk for SQCC (OR=1.62) than for SCLC.

Both for the *gstm1* and *gstt1* null genotypes, there is a lack of association for risk of lung cancer. Subjects with the null genotype for *gstm1* and *gstt1* have ORs of 1.2 for both genotypes and have no statistical significant relation for lung cancer risk. But when stratified for tumour histology, there is a negligible risk towards SQCC development with null *gstm1* genotype (ORs=1.35), but the risk is higher in the case of *gstt1* genotype and SQCC (OR= 1.72 than for *gstt1* genotype. For the SCLC, the association is weak with both the *gstt1* and *gstm1* null genotypes. The role of null *gstt1* gene as a susceptibility factor for lung cancer is not well established (Rebbeck et al., 1997; D’Errico et al., 1999). Initial reports have pointed out a non-significant risk for lung cancer (Kiyohara et al., 2000; London et al., 2000), while a statistically significant OR of 1.41 for the null *gstt1* genotype has been reported for it (Spitz et al., 2000).
It is generally postulated that the individuals with null \textit{gstt1} gene would be at a higher risk for cancer because they have reduced ability to conjugate carcinogens to extractable hydrophilic metabolites (Rebeck et al., 1997). Epidemiological studies conducted on the role of null \textit{gstt1} genotype on lung carcinogenesis have not demonstrated a consistent increase of lung cancer risk in smokers (Figueras et al., 1997). Some \textit{gstt1} metabolites could act as tissue specific mutagens (Rebeck et al., 1997). Regarding other tobacco related neoplasms, studies have found a statistically significant increased risk for bladder cancer among non-smokers with \textit{gstt1} null genotype.

Contradictory reports are there in the literature regarding the null \textit{gstm1} genotype with the risk of lung cancer, especially in relation to histology. Sun et al. (1997) have reported an increased risk for lung cancer especially for SCLC, but not for SQCC. Similar results have been obtained from Chinese lung cancer patients (Persson et al., 1999). Studies in a Finnish population have pointed out an elevated risk for SQCC and null \textit{gstm1} genotype (Hirvonen et al., 1993b). These findings suggest that in this particular study \textit{gstm1} and \textit{gstt1} null genotypes are associated with risk towards SQCC of the lung, but not SCLC. It is generally assumed that, since SQCC (being epithelial in origin) is related to smoking, higher exposure to tobacco carcinogens present in smoke might increase the risk to develop lung cancer. Thus individuals lacking the \textit{gstm1} and \textit{gstt1} gene have decreased capacity to detoxify carcinogens present in cigarette smoke and are thus more susceptible to develop SQCC, but not SCLC in relation to the null \textit{gst} genes.

It is hypothesized that individuals with both null \textit{gst} genotypes would be at the highest risk to develop lung cancer, since the ability to completely detoxify decreases substantially (Nelson et al., 1995). Nevertheless, results in this study do not support such a contention. In agreement with these findings, other study groups did not find an increased lung cancer risk among smokers with these genotypes (Figuereas et al., 1997, Malats et al.,
On the other hand, the $gstm1$ (+) and null $gstt1$ genotype has an excess risk for lung cancer, although these differences do not reach statistical significance (OR=1.60, 95%CI: 0.54-4.78). The greatest risk of this genotype is for SQCC, where there was a 3-fold increased risk as compared to both $gst$ genes being present. Although the polymorphic expression of $gstm1$ and $gstt1$ genes appears to be linked to individual sensitivity to chemical carcinogens, this, however, cannot explain the entire observed individual variations for the different combinations of $gst$ genes.

3. **Relationship of lung cancer risk with combined genotypes of $cyp1a1$ (ile/val) and $cyp1a1$ (msp1) genes**

There is a 3-fold elevated risk for overall lung cancer (OR= 2.85, 95%CI: 1.01-8.05) among the individuals with mutant val/val allele and the variant $msp1$ genotype (wt1/m1/m1/m1). Thus, it is evident that the two genotypes play an important role in lung carcinogenesis. When evaluated as single genotype, except for the val/val genotype, the associated risk is not that significant, but the two polymorphisms in tandem coordination with each other increase the risk for lung cancer. When stratified for tumour histology, both the SCLC and SQCC have 3 and 2-fold increased risk respectively towards lung cancer. In the Japanese, the association has been stronger for SQCC (Nakachi et al., 1991; Nakachi et al., 1995). In contrast, studies on $cyp1a1$ gene and lung cancer conducted in Caucasians have been mostly inconsistent with some early studies finding no association (Tefre et al., 1991; Hirvonen et al., 1992; Shields et al., 1993) and later ones reporting an increased risk with the variant alleles (Drakoulis et al., 1994; Xu et al., 1996). Further the prevalence of $msp1$ genotype is increased in lung cancer as a consequence of linkage with ile/val (Cascorbi et al., 1996).

An association between $cyp1a1$ hyper-inducibility and lung cancer has been earlier reported (Jacquet et al., 1996; Stucker et al., 2000). However, most of the studies performed in Caucasians have not been large enough to compensate for the low frequency of the ile/val alleles as compared to the Asians. Another reason for these discrepancies might be
related to differences in linkage or genetic associations between alleles in different populations as shown in Africans that display no linkage between \( msp1 \) and \( ile/val \) polymorphisms in contrast to Asians or Caucasians (Garte, 1998). In this study among the lung cancer group, 21 cases of \( val/val \) genotype have shown genetic linkage with the \( msp1 \) variant genotype. Overall 55% of the lung cancer cases have shown genetic linkage with both the \( cyp1a1 \) genotypes. Interestingly, in lung cancer patients, singular A to G transition (\( ile \rightarrow val \)) has been found in 8 of the 21 cases without showing linkage to T to C transition (\( msp1 \)). This overrepresentation of the single \( val/val \) genotype without linkage to the \( msp1 \) may be a special risk constellation.

Zhang et al. (1996b) have reported that the isoleucine \( \rightarrow \) valine substitution alone cannot increase the enzymatic activity, but have suggested that the \( ile/val \) genotype in linkage with \( msp1 \) genotype might be resulting in increased inducibility and has been linked to the increased lung cancer risk. The functional effects of the \( cyp1a1 \) polymorphisms have been investigated with some contradictory results. When expressed in yeast, both the \( cyp1a1 msp1 \)and \( ile/val \) variants have exhibited only small differences in enzymatic properties (Persson et al., 1997). However, Kiyohara et al. (1996) have shown an increased, non-induced AHH activity in mitogen treated lymphocytes from Japanese subjects homozygous for the \( val/val \) allele and increased AHH inducibility in those homozygous for \( m1/m1 \) allele. This implies that these polymorphisms might be causing higher enzyme concentrations in vivo, due to enhanced inducibility and increased stability. Thus it might be possible that in the present study the presence of single variant allele (\( wt1/m1m1/m1 \)) in combination with a homozygous mutant allele (\( val/val \)) might have increased the metabolic activation of procarcinogens to ultimate carcinogen, thus leading to an increase in risk towards lung cancer. Hence \( cyp1a1 \) polymorphisms might not only be playing an important role for lung cancer risk in South East Asian populations, but there is a greater impact on Indian population too.
4. Relationship of lung cancer risk with combined genotypes of cyp1a1 and gstm1 genes

PAHs require metabolic activation by phase I enzymes to their ultimate carcinogenic forms and the intermediates, thus, formed are then further subjected to detoxification by phase II enzymes (Kiyohara et al., 2002). Thus, genetically determined susceptibility to lung cancer might be depending on the metabolic balance between phase 1 and phase 2 enzymes (Kiyohara et al., 2002). It is, therefore, important to genetically identify individuals who are at high risk of lung cancer in terms of polymorphisms of genes for encoding phase I and phase II enzymes. In this study, neither the cyp1a1 variant allele nor the null gstm1 genotype alone, are associated with significant increase in lung cancer risk. However, the presence of a single copy of the variant msp1 allele (wt1/m1/m1/m1) and null gstm1 genotype does show a slight elevated risk towards lung cancer with an OR of 1.5. The evidence for such an association is stronger for SQCC (OR=2.02, 95%CI: 0.73-5.59). In Japanese, it has been reported that individuals with the homozygous mutant msp1 genotype and null gstm1 gene are at significantly high risk with an OR of 16.00 for SQCC (Nakachi et al., 1993). In Caucasian populations, neither the cyp1a1 msp1 heterozygous genotype alone, nor the null gstm1 gene are associated with a significant increase in lung cancer risk, although having both genetic traits is associated with a 2-fold increase in lung cancer risk (Garcia-Closas et al., 1997). Similarly Marchand et al. (1998) have reported that the presence of at least one copy of the cyp1a1 msp1 variant allele and null gstm1 gene is associated with a 3.1 fold increase in the risk of SQCC. In the French population, the effect of gstm1 null genotype on lung cancer is not modified by the cyp1a1 genotype (Jourenkova et al., 1997).

Additional evidence regarding the possible interaction of cyp1a1 (msp1) and gstm1 genetic polymorphism has been provided by the Scandinavian and Japanese populations. Alexandrie et al. (1994) while comparing the combined distribution of gstm1 and cyp1a1 (msp1)
genotypes have reported that these are significantly over-represented in patients with SQCC. Similarly Antilla et al. (1994) have studied the distribution of the \textit{gstm1} genotype on the basis of \textit{cyp1a1} inducibility by immunohistochemistry on frozen sections of lung tissue and have found that the \textit{gstm1} null genotype is over-represented among individuals with \textit{cyp1a1} inducible phenotypes. The combination of \textit{gstm1} (+) and \textit{cyp1a1} (\textit{wt1/m1/m1/m1}) has been associated with the lowest risk of developing SQCC and SCLC. Similar observations have been made by Kihara et al. (1995), perhaps individuals with this genotype combination are able to eliminate absorbed PAHs most efficiently and rapidly and, thus, are able to tolerate exposures to tobacco smoke. The observations by Antilla et al. (1994) appear to support the above line of reasoning. They have reported that bronchial tumours (normally SQCC and SCLC) are much less common in \textit{cyp1a1} inducible patients having the \textit{gstm1} gene than those lacking it. This suggests that the expression of \textit{gstm1} gene in patients with inducible \textit{cyp1a1} phenotype has a protective role against bronchial lung carcinomas.

Among the \textit{gstm1} and \textit{ile/val} genotypes, the combined genotypes of \textit{val/val} and null \textit{gstm1} has been found to be associated with a slight risk towards lung cancer with an OR of 1.6. When stratified according to the histological cell types, there is a 3.5-fold increased risk towards SCLC, but weak association for the SQCC with an OR of 1.15. Similar results have also been reported in Japanese population having a 2 to 5 fold risk towards lung cancer with the \textit{val/val} and \textit{gstm1} null genotype (Nakachi et al., 1993). Although it is expected that the combination of \textit{val/val} and \textit{gstm1} null genotype might be strongly linked to lung cancer, the present results also show that the combined genotypes of \textit{val/val} and \textit{gstm1} (+) genes are associated with lung cancer (OR= 1.75). This discrepancy might be due to the small number of cases examined that might have limited the power to detect combined effects of gene.

5. Relationship of lung cancer risk with combined genotypes of \textit{cyp1a1} and \textit{gstt1} gene
There is a 3-fold increased risk for lung cancer for individuals carrying a single copy of the variant \textit{msp1} allele (wt1/m1/m1/m1) and the null \textit{gstt1} genotype (OR=2.91, 95%CI; 0.74-11.46). Thus the presence of a single copy of the variant \textit{cypl1} allele and null \textit{gstt1} genotype plays an important role in lung carcinogenesis. When stratified according to histology, the relative risk is increased 4-fold in the case of SQCC (OR=3.70, 95%CI; 0.88-15.61), thus individuals with such type of genotype are at a 4 times higher risk to develop SQCC as compared to those with the homozygous wild \textit{cypl1} (\textit{msp1}) and \textit{gstt1} (+) genotype. In the case of SCLC, such genotype combinations do not play a significant role and are not associated with the development of lung cancer. Similarly the \textit{val/val} and null \textit{gstt1} genotype has a marginal risk towards overall lung cancer with an OR of 1.64 (95%CI=0.39-6.90). The combination of the \textit{val/val} and both the null and (+) \textit{gstt1} genotypes has been found to have a 3 and 4-fold increased risk towards SCLC, but on the other hand the evidence for such an association has not been seen for SQCC. Till date few studies have concentrated on the combined genotypes of \textit{cypl1} and \textit{gstt1} genotypes. Lear \textit{et al.} (1997) have studied the effect of \textit{cypl1} and \textit{gstt1} on basal cutaneous carcinoma (BCC). Their data suggest that individuals deficient in their ability to repair oxidative stress induced DNA damage are more likely to develop BCC. Thus in this study, it has been considered that the null \textit{gstt1} genotype and \textit{cypl1} gene play an important role in lung carcinogenesis. It is suggested that individuals with the variant or mutant \textit{cypl1} gene and null \textit{gstt1} genotype have reduced capability to metabolize the carcinogens present in tobacco smoke and thus are unable to tolerate the reactive carcinogenic intermediates, which then might build up and increase the risk towards cancer.

6. Relationship of lung cancer risk with combined genotypes of \textit{cypl1} and \textit{cyp2d6} genes

A 1.8-fold increased risk for lung cancer for individuals carrying a single copy of the variant \textit{msp1} allele (wt1/m1/m1/m1) and the HEM genotype of \textit{cyp2d6} has been seen. This becomes 2-fold in SQCC.
(OR=2.17, 95%CI; 0.72-6.53), as compared to homozygous wild cyp1a1 (msp1) and EM genotype of cyp2d6 gene. In the case of SCLC, such genotypic combinations do not play a significant role and have not been associated with lung cancer development. The val/val and HEM genotypes have also been found to have a 2-fold risk towards overall lung cancer with an OR of 2.10 (95%CI= 0.62-7.13).

This risk increased to 4-fold when only SCLC was taken into account (OR=3.92 95%CI; 0.75-20.5).

7. Relationship of lung cancer risk with combined genotypes of gstm1, gštť1 and cyp2d6 genes

The presence of one copy of the variant cyp2d6 (HEM) allele and null gstm1 genotype have slightly been associated with risk towards lung cancer (OR= 1.80, 95% CI; 0.60-5.43). But when SQCC are taken alone, the risk is 2-fold (OR= 2.29, 95%CI= 0.70-7.49). This increase is 2.5-fold for SCLC as compared to those with the gštť1 (+) and cyp2d6 EM genotype. On the whole, the presence of at least one copy of the cyp2d6 genotype (HEM) and gštť1 (+) has a slight more risk than with HEM null gštť1 gene. There is a 2-fold risk for SQCC with the HEM and gštť1 (+) genotype. Data on genotypic interactions between cyp2d6 and gštť1 and gštţ1 is sparse. Roots et al. (1992) using phenotyping assays have shown that cyp2d6 metabolism of debrisoquine is significantly lower in lung cancer patients with low gštţ1 activity as compared to those with high activity. Studies carried out on cervical neoplasia, astrocytoma and cutaneous basal cell carcinoma have not shown any type of genotypic interaction between cyp2d6 and gšt genotypes (Ramachandran et al., 1999).

8. Relationship of lung cancer risk with combined genotypes of gštţ1 and gštţ1 genes

Activated forms of benzo(a)pyrene, like benzo(a)pyrene 7,8-diol, epoxides, PAHs, halogenated organic compounds are conjugated both by gštţ1 and gštţ1 of the phase II to water soluble forms which then can easily
be excreted (Bouchardy et al., 2001; Murata et al., 2001). It is generally hypothesized that individuals with null \textit{gstm1} and \textit{gstt1} genotypes would be at highest risk to develop lung cancer since the ability to completely detoxify toxic metabolites substantially decreases (Nelson et al., 1995). In this study, both null \textit{gst} genotypes have not been found to increase the risk towards lung cancer (OR=1.08, 95% CI; 0.28-4.1). The association is marginal with both null genes in SQCC and SCLC with ORs of 1.22 and 1.37. Among all other \textit{gst} genotypes, the null \textit{gstt1} and \textit{gstm1 (+)} genes are associated with a slightly increased risk towards lung cancer (OR=1.60, 95% CI; 0.54-4.78). On the other hand, there is a 3-fold increased risk of SQCC with the \textit{gstt1} null and \textit{gstm1 (+)} genotypes (OR=3.04; 95% CI; 0.90-10.30) when both the genes are present. Such an association has not been observed in the case of SCLC. Other study groups have also not found an increased lung cancer risk among smokers with the null \textit{gstt1} and \textit{gstm1} genotypes (Figuereas et al., 1997; Malats et al., 2000). A significant association has, however, been observed for the concurrent lack of both \textit{gstm1} and \textit{gstt1} genes and SQCC when compared with other genotypic combinations (Saarikoski et al., 1998).

Kelsey et al. (1997) and Kiyohara et al. (1996) have shown increased ORs for the association of lung cancer with both the polymorphisms. It is reported here for the first time that the null \textit{gstt1} and \textit{gstm1 (+)} genotypes are associated with risk to lung cancer especially SQCC in our population group. It is assumed that \textit{gstt1} genotypes carriers have a high uptake, high metabolic clearance, rapid post exposure decline and low post exposure exhalation, while non-conjugators i.e. lacking the \textit{gstt1} gene impart opposite characteristics. Individuals lacking \textit{gstt1} enzyme activity seem to entirely lack metabolic capacity to detoxify certain carcinogens like methyl chloride, methyl bromide, methyl iodide and ethylene oxide (Hallier et al., 1993).

In the present study most of the cases were bidi smokers and till date it is not known what type of carcinogens are present in the smoke from bidis. It is possible that there might be certain degree of carcinogens whose substrates are specifically metabolized by \textit{gstt1} enzymes only. Furthermore,
the presence of dietary constituents might also be playing an important protective role against lung cancer. Isothiocyanates (ITCs) are non-nutrient compounds in cruciferous vegetables having anti-carcinogenic properties (Spitz et al., 2000). One proposed mechanism for the protective role of ITC is through the direct inhibition of their catalytic activities, with induction of phase II enzymes (Spitz et al., 2000). ITCs are potent inhibitors of NNK metabolism (Morse et al., 1989). The protective effects of ITCs are primarily seen among individuals with a deletion of gstm1 gene (Spitz et al., 2000). Thus the cases in this study with both null genotypes that are considered to have the highest risk towards lung cancer might be at a better protection, if they are having a more intake of ITCs as compared to those in with fewer intakes. The effect of ITCs is more pronounced in case of gstm1 genotypes as compared to the gstt1 genotypes. Individuals with the null gstt1 genotype seem to excrete ITCs more slowly as compared to those with other genotypes and non-null gstt1 genotypes (Lin et al., 2002). Thus, it is possible that interaction of dietary habits and null gstt1 genotype play an important role in lung cancer progression in North Indian subjects. However, before coming to any plausible conclusion, it might be important to know that possible population bias, case-control selection criteria, presence of confounding factors, prevalence of other environmental factors and ethnicity might have led to bias and possible discrepancy.

9. **Relationship between cyp1a1 (msp1), cyp1a1 (ile/val), cyp2e1, gstm1 and gstt1 genotypes and lung cancer stratified according to smoking**

Unlike cigarettes, bidi (type of cigarette) is the most prevalent form of smoking. Manufactured in India, bidi consists of tobacco wrapped in a tendu or temburni leaf. Due to the cruder form of tobacco in bidi, it has a higher concentration of tar and nicotine and, thus, is more carcinogenic than the cigarette. The present data clearly indicates that individuals having val/val genotype and who are heavy smokers (Bl>400) have a 5-fold risk over light smokers (Bl<400). The present data are consistent with the study of Song
et al. (2001) who have found a strong tendency of increased risk of lung cancer for heavy smokers for the val/val genotype. Other reports have demonstrated a stronger tendency for light smokers (Nakachi et al., 1993; Sugimura et al., 1998).

For the cyp1a1 msp1 genotype, not much attributable difference has been seen among the light and heavy smoker group with susceptible genotype (wt1/m1/m1m1) and lung cancer. But when compared with non-smokers, both groups have a 3-fold increase risk towards lung cancer risk.

In earlier studies on Japanese populations it has been shown that susceptibility to lung cancer for individuals with a cyp1a1 polymorphism is remarkably high at a low level of tobacco exposure and that the difference in susceptibility of high-risk groups is decreased at high dose levels (Nakachi et al., 1991; 1993). In the present study an apparent interaction between bidi smoking and the variant cyp1a1 genotypes among North Indians has been observed. The heaviest smokers who have the variant cyp1a1 genotypes are at the highest risk of lung cancer.

When the gstm1 null gene is evaluated with lifetime smoking exposure, a slight elevation in risk of lung cancer for heavier smokers (B\(>400\)) (OR=2.01, 95%CI; 0.73-3.77) is apparent. Several epidemiological studies have evaluated the interaction between gstm1 genotype and cumulative smoking, but contradictory results have been obtained. In four studies, stronger associations have been found for heavier smokers (Seidegard et al., 1986; Hirvonen et al., 1993b; Stewart et al., 1993; Kihara et al., 1995). Two studies have found a strong association with a low smoking exposure (Nakachi et al., 1993; London et al., 1995) and one saw no evidence for the differences in both the categories of smoking and lung cancer (Brockmoller et al., 1993).

With respect to life time tobacco exposure and lung cancer risk in relation to null gstt1 gene, an increased risk (OR=4.34, 95%CI; 0.70-27.03) for heavy smoking dose compared to light smoker has been observed. Even
in non-smokers, the null genotype has been seen to be at high risk for lung cancer (OR=3.87, 95%CI: 0.56-26.82). Previous epidemiological studies on the role of null *gstt1* genotype have not demonstrated a consistent increase in lung cancer in smokers (Figueras *et al.*, 1997). Jourenkova *et al.* (1997) reported high risk towards lung cancer at a low level of tobacco exposure and null *gstt1* gene.

The non-smokers with null *gstt1* have been found to be at a high risk to develop SQCC as compared to both heavy and light smokers. A high risk for laryngeal cancers in relation to null genotype at a low level of tobacco exposure has also been reported (Journekova *et al.*, 1998). In contrast, there is an increased risk towards SQCC at high smoking (Bl>400) exposure in relation to *gstm1* genotype as compared to light and non-smokers. London *et al.* (1995) have seen a higher risk (4-fold) of SQCC for smokers with less than 40 pack years.

For the *cyplal msp1* genotype not much attributable difference is seen among the light and heavy smoker groups with genotype (*wt1/m1/m1m1*) as risk for lung cancer. But when compared with non-smokers, both groups have a 2-fold increased risk.

Heavy smokers (Bl>400) with the *val/val* genotype are at a very high risk to develop SCLC with an OR of 29.30 (95%CI=2.42-355.34, p=0.008). This risk was reduced to less than half in non-smokers. Similarly the *wt1/m1/m1/m1* genotype has a 7-fold increased risk towards SCLC as compared to non-smokers and light smokers.

In the case of null *gstm1* genotype, non-smokers and light smokers together have a more attributable risk for developing SCLC. These results are in accordance with those of London *et al.* (1995) who have reported an increased risk for SCLC at a low smoking exposure.

10. *p53* gene mutations and lung cancer

In the present study, the status of *p53* gene has been investigated by PCR-SSCP analysis. Mutational analysis of the *p53* gene is restricted to
exons 5-8, where 90% of mutations have been found (Hollstein et al., 1991). The overall mutations detected in lung cancer patients by PCR-SSCP is 80% if we include all the exons, but when the different exons are taken individually the total number of samples of all the exons corresponds to 224 samples i.e. exons 5,6,7 and 8 each in 56 samples. In all these samples (224), 22.32% of mutations have been found in p53 gene. Of these 35.7% (20/56) in exon 5, 1.78% (1/56) in exon 6, 28.5% (16/56) in exon 7 and 23.2% (13/56) in exon 8. This is in coherence with other reports of 20-60% prevalence of p53 mutations in lung cancer (Mitsudomi et al., 1993, Fong et al., 1995, Top et al., 1995). In the present study, the maximum numbers of mutations have been found in exon 5 followed by exons 7 and 8 and just one mutation in exon 6. This is in coherence with the studies of Vega et al. (1997) and Skaug et al. (2000) who too have reported a high number of mutations in exons 5 and 7. Maximum mutations have been seen in exon 5 followed by exon 7 in patients with SQCC. Mutations in multiple exons have been seen in 6 cases and these mainly pertain to exons 5 and 7. This suggests that both these exons are mutational hot spots.

Analysis of the mutations in p53 gene in relation to smoking has also been made. It has been found out that both NSCLC and SCLC have 8 fold-probability of having mutated p53 in smokers as compared to non-smokers. When the smokers are further stratified according to smoking dose, the heavy smokers (BI>800) have an OR of 12.5 (95%CI=1.76-88.74) as compared to the light smokers (BI<800) (OR=7.5; 95%CI=1.02-55). Similarly in SQCC, smokers showed a higher frequency of mutations in p53 gene than in non-smokers with an OR of 14.5 (95%CI=1.69-124.24). When the smokers were further classified accordingly to smoking dose, the frequency of mutation in p53 gene in patients with SQCC was more in heavy smoking group (BI>800) with an OR of 17 (95%CI=1.3-223.15). These results indicate a relationship between heavy tobacco exposure and mutations in p53 gene in SQCC. The G: C→T: A transversion found in p53 gene are positively correlated with lifetime cigarette smoking and exposure.
(Suzuki et al., 1992). Cigarette smoke and some of its components have been shown to damage DNA and cause defects in DNA repair, which may contribute to mutations in p53 gene (Grafstorm et al., 1983). It is generally assumed that, since SQCC (being epithelial in origin) is related to smoking and higher exposure to carcinogens present in tobacco, smoke might be increasing the probability of binding to DNA and, thereby, resulting in mutations in p53 gene and hence leading to lung cancer. This is further substantiated by the fact that the maximum number of mutations have been seen in exons 5, 7 and 8 in relation to heavy smoking and cumulative tobacco exposure (BI>800). On the contrary, results here do not point out such a relationship for SCLC.

The likelihood that PCR-SSCP analysis might have missed mutations within the screened area is low. The sensitivity and specificity of this technique to detect mutations even if only present in low amounts, is more than 90%.

11. p53 gene mutations and cyp1a1 genotypes in relation to lung cancer

It has been observed that a single copy of the variant cyp1a1 (msp1) allele i.e. wt1/m1/m1/m1 has a 15-fold higher probability of harboring a mutation in the p53 gene as compared to the homozygous wild cyp1a1 genotype (wt1/wt1). The association is greater in the case of SQCC (OR=9.5, 95%CI: 0.99-92.2, p= 0.075) than in SCLC (OR= 3.43). Kawajiri et al. (1996) have reported similar findings in a Japanese population in which patients carrying the mutant msp1 genotype have 5-fold increased risk of having a mutated p53 gene. Similarly val/val genotype has a 6-fold higher risk of having mutations in the p53 gene than ile/val genotype, (OR of 5.90 95%CI= 0.69- 50.14, p= 0.15). An increased probability of a mutation has been observed in SCLC (OR=5.00, 95%CI; 0.44-56.8). In the case of SQCC, the risk of having a mutation in p53 is less SCLC (OR= 2.77). It has
also been shown that the *p53* gene is more frequently mutated in patients with the *cyp1a1 val/val* genotype (Kawajiri *et al.*, 1996).

The genotype *wt1/m1/m1* and *ile/val* have a 2-fold high probability of having a mutated *p53* (OR=13.5, 95%CI; 1.64-126, p=0.01) than *wt1/m1/m1/val/val* genotype (OR=7.6, 95%CI; 0.73-62.8, p=0.15). Similarly both for SQCC and SCLC, the frequency of mutations was high in *wt1/m1/m1/ile/val* genotype (OR=6.5, 95%CI; 0.71-69.49, p=0.17) as compared to *wt1/m1/m1/val/val* (OR=4, 95%CI; 0.33-36.86, p=0.588). Such an association has not been observed for the SCLC.

The present results clearly indicate that the variant *cyp1a1* genotypes play an important role in lung carcinogenesis. An important point is that the both the *cyp1a1* genotypes have different risks for developing SQCC and SCLC. The *msp1* variant genotypes associated with SQCC and *val/val* genotype with SCLC. The apparent reasons for this are unknown. It may be possible that the difference in enzyme inducibility and expression controlled by transcriptional control elements is different in the two histological subtypes.

12. *p53* gene mutations in combined genotypes of *cyp1a1* and *gstm1*

The presence of a single copy of the variant *msp1* allele and the null *gstm1* gene [*wt1/m1/m1/m1* and null *gstm1* (--)] has a 13-fold high risk of having mutations in *p53* gene (OR=13.5, 95%CI; 1.47-123.7, p=0.021). The frequency of mutations in *p53* gene in SQCC was estimated to be very high (OR=16.25, 95%CI; 1.44-183.1, p=0.036). Even for with *wt1/m1/m1/m1* and *gstm1* (+) genotypes there is a risk of having a mutation in the *p53* gene (OR=10, 95%CI; 0.85-117.02, p=0.133). The risk is more in case of *wt1/m1/m1/m1* and null *gstm1* genotype. Such a strong association has not seen in SCLC.

The highest risk of having a mutated *p53* gene has been in the combined genotypes of *val/val* and null *gstm1* (OR=6.00, 95%CI; 0.65-
In SQCC this genotype shows 4-fold increased probability of having a mutated \( p53 \) gene (OR=4.1, 95%CI; 0.40-41.66, \( p=0.439 \)) as compared to the \( il6/val \) and \( gstm1 (+) \) genotype. Such an association has also been seen in the case of SCLC, where there four fold probability of having a mutated \( p53 \) gene (OR= 4.00, 95%CI; 0.3-53.47, \( p=0.62 \)). These were confirmed by the observations made on Japanese population. The latter have reported an 9-fold increased risk in the case of null \( gstm1 \) and mutant \( msp1 \) genotype (Kawajiri \textit{et al.}, 1996).

It is apparent that a loss of metabolic balance, including the activation of carcinogens by \( cyp1a1 \) and detoxification by \( gstm1 \) acts synergistically to enhance lung cancer risk. This hypothesis might be supported by a report that there is a correlation between increased DNA adduct levels of polycyclic aromatic hydrocarbons (measured by ELISA) and combined susceptible genotypes of the \( cyp1a1 \) and \( gstm1 \) gene (Dickey \textit{et al.}, 1995).

13. \( p53 \) gene mutations and \( cyp2d6 \) genotype in relation to lung cancer

A single copy of the variant \( cyp2d6 \) allele [HEM] has a 6-fold high risk of having mutations in \( p53 \) gene. The association is greater in the case of SQCC (OR=3.16, 95%CI; 0.33-30.43, \( p= 0.075 \)) than in SCLC (OR= 1.34). The role of \( Cyp2d6 \) in modulating lung cancer susceptibility is still controversial. In this study on North Indian population no poor metabolizers PM allele was observed. The predominant genotypes were EM and HEM. The data here suggest that individuals with EM genotype of \( cyp2d6 \) gene and carrying variant allele for \( cyp1a1 \) (Msp1) are at a high risk towards lung cancer especially SQCC. Such an association has not been seen for \( ile/val \) genotype. On the other hand an associative risk for lung cancer has been seen in individuals with null \( gstm1 \) gene and HEM genotype of \( cyp2d6 \) gene.

14. \( p53 \) Gene Mutations In Relation To \( Gstim1 \) And \( Gstt1 \) Genotypes

A null \( gstim1 \) genotype has a 5.4 fold higher risk of mutations in \( p53 \) gene than \( gstim1 (+) \) genotype (OR= 5.4, 95%CI; 1.00-26.82, \( p= 0.07 \)). A B-
fold increased risk of having a mutated $p53$ gene has been observed in SQCC patients carrying the null $gstm1$ gene (OR= 8.3, 95% CI: 0.19-16.45, $p=0.104$). The association is weak and non-significant for SCLC. As many as 11.1% (6/54) of the lung cancer patients with mutated $p53$ gene had the null $gstt1$ gene (OR= 1.80, 95% CI: 0.19-16.45, $p=0.998$). The association is weak in SQCC (OR=1.15, 95% CI: 0.11-11.8, $p=0.65$). In the case of SCLC, of the 11 (64.75%) cases with mutated $p53$ gene, there is not a single subject with the null $gstt1$ genotype. Initial reports have suggested that $gstm1$ null heavy smokers are at a higher risk of mutations in the $p53$ gene (Ryberg et al., 1994), other investigators have not been able to confirm this observation (Kawajiri et al., 1996). In the Silesian case, the spectrum of $p53$ mutations has been similar among smokers both with $gstm1$ wild type and null genotypes (Rusin et al., 1999). The patients with SQCC having null $gstm1$ genotype in this study have a higher incidence of mutations in $p53$ gene than SCLC. It is because of the fact that since there is a more frequency of null $gstm1$ genotypes among individuals with SQCC and as reported within this study and the heavy smokers have a 17-fold higher risk for having a mutation in the $p53$ gene. It is therefore hypothesized that patients with null $gstm1$ genotype have reduced capacity to metabolize carcinogens present in tobacco smoke, and thus more probability of having a mutated $p53$ with null $gstm1$ gene.

The present results on $p53$ gene mutations spectra differ for same of the earlier studies probably because of possible differences in sample sizes, and variations in the distribution of risk factors between countries like Japan, Europe and India. The $p53$ mutation spectra have, however, been found to be different between different populations, which could indicate that risk factors are not the same in all the countries. This could affect the importance of $gst$ and $cyp1a1$ gene products in detoxifying carcinogens and thus could have an effect on study results.
15. Telomerase activity in bronchial washings of lung cancer patients

Telomerase activity has been seen in most malignant tumours (85%), but absent in normal somatic tissues (Shay et al., 1997). A lot of studies till date have focused on assessing telomerase activity in lung cancer tissues but those on bronchial washings are looked. Most lung cancer tissues, obtained during surgical resection of lung tumours have telomerase activity when measured by TRAP assay (Kim et al., 1994). Transbronchial lung biopsies or brushings are often used to obtain lung tissues, but these techniques are often used for more advanced stages and are not effective in the early detection of lung cancer (Yahata et al., 1998). Considering these issues, it was reasoned to detect telomerase activity in bronchial washings of lung cancer patients. In the present study, telomerase activity was detected in 66.66% (30/45) of lung cancer cases. It was found in more than 73.35 of SCLC cases as compared to 63.3% of SQCC cases. Yahata et al. (1998) have found telomerase activity in 82% of bronchial washings obtained from lung cancer patients. These differences in results may be due to the fact that Yahata et al. (1998) used two methods to detect telomerase activity, one by in situ TRAP assay and the other by extract-based TRAP assay. Both these techniques are highly sensitive as compared to the conventional TRAP assay and that’s why there are differences in results. The present cases telomerase was found to be low. The possible reason may be that the samples were kept for a long time (2 years) at -80°C and these too might have influenced the results telomerase is a highly sensitive enzyme and is affected by long-term storage. Another reason may be that the telomerase activity was assessed in total of 5ml of bronchial lavage, it is possible that the number of cancer cells having telomerase activity in this much amount of bronchial lavage may not be sufficient. On the other hand Yahata et al. (1998) used 20ml of bronchial washings from lung cancer patients to assess the telomerase activity. More important the use of sensitive methods to detect telomerase activity play a crucial role; here
silver-staining method was used to detect the telomerase activity whereas most of the workers have used autoradiography for detecting it. In spite of the drawbacks as reported, telomerase activity has been seen in 66% of bronchial washings, thus it is highly encouraging that this molecule can be used as diagnostic biomarker and can replace conventional cytology. Assessing telomerase activity in cells from bronchial washings is usually easy and less invasive than obtaining tissue biopsy specimens (Yahata et al., 1998). The fact that telomerase activity appears to be expressed in virtually advanced malignancies should lead to new diagnostic and therapeutic applications. Assays for telomerase could help clinicians to determine the status of suspect tumours, while a drug that can block telomerase might have significant anti-cancerous effects (Aragona et al., 2001).