6.1 Epidemiology

Urinary bladder cancer is the fourth most common neoplasm in men in the western world (Knowles, 1999). In the present investigations, it was tried to evaluate the role of epidemiological risk factors along with genetically susceptibility of North Indian population with regard to immuno- and DNA repair genes in developing bladder cancer. The patients in this study belonged to the states of Punjab, Haryana, Uttar Pradesh, Uttranchal, Himachal Pradesh as well as Union Territory of Chandigarh (India). Not much difference in the distribution of cases was seen. The state-wise demarcation had no effect on the incidence of this cancer, but according to absence or presence of risky area (like chemical plants or center with extensive agriculture activities), showed difference in the incidence of bladder cancer.

Uro-epithelial cancer, primarily a disease of people older than age 65 years, is rarely diagnosed before the age of 40. Additionally the diagnosis rate was higher in urban than rural areas (Devita et al., 2001). The maximum numbers of patients in this study were in the age-group of more than 60 years. The incidence in the urban areas was slightly more than in the rural areas (52.0 vs 48.0%). 90.3 of the patients were males and the rest were females indicating the predominance of this disease amongst males. It is agreed with the other findings in this regard (Landis et al., 1999; Qureshi et al., 1999; Lee et al., 2002). This might reflect a higher exposure of males to the carcinogenesis inducing factors, probably at their workplace. As many as 24.5 % of patients were educated (10.1% higher education and 14.5% matric) and 34.0 % had some education and rest (41.0%) were illiterate. The low socio-economic status and low education of patients lead to poor hygienic conditions of living as those might be playing some role in the initiation of this disease (Parashar, 1997)

Potential risky occupations included some jobs like those of workers in chemical industry, petrol pumps, gas stations, plastic and rubber industry, metal industry, printing, textile, painting, food processing industry, mechanics and drivers of truck, high voltage electricity technician and farmers applied or exposed to pesticide. Those lived in or so close to industrial area, factories, adjacent to petrol pumps or drainage channel or on area with extensive agriculture have a high risk of uro-epithelial cancer. In this study,
significant correlation between bladder cancer and risky occupation or residential place was found. Patients with risky occupation showed 1.83 increased risk of bladder cancer compared to controls. Many epidemiological studies have identified certain occupation leading to excessive risk of bladder cancer (Cole, 1973; Cartwright, 1982; Vinies and Simonoto, 1986; Anton-culvar et al., 1992; Mannetje et al., 1999). Approximately 10% of lung, 30% of bladder and 30% of leukemia have been thought to be occupation related (Higginson and Muir, 1979). Final conclusive statements can be made, if the studies are extended to a large population.

Hirayama et al. (1980) reported that incidence of urothelial cancer is closely related to tobacco smoking (Knapp et al., 2000). Evaluation of smoking as risk factor showed 2.45 increased risk of bladder cancer as compared to non-smokes and controls. Increased risk was observed in all the groups of smokers (active s, passive and ex-smokers). Those with pack-years more than 49.7 were found to be more prone to bladder cancer (2.80 times). The individuals with habit of chewing tobacco had 1.80 fold increased risk of developing bladder cancer. 67% women with bladder cancer were passive smokers particularly at home. These data support the findings of Pelucchi et al. (2002) who had seen smokers to be at 2-3 times higher risk of bladder cancer than non-smokers. Knapp et al. (2000) reported that smoking contributes to 50% or more to human bladder cancer in men and 33% or more in women. Polyaromatic hydrocarbons contained in cigarette smoke are known carcinogens and might be absorbed into the blood and transported to the bladder, where the cells are unable to withstand their carcinogenic effects (Gupta, 2001).

This study has shown that 55 % of patients had habit of consuming alcohol and significant but slight increase in the risk of bladder cancer (1.7 times). It was clarified that those consuming alcohol-units more than 26.8 per week were more prone (2 times) to develop bladder cancer as compared to those consuming lesser than 26.

A number of studies have investigated the possible role of alcohol in development of human bladder cancer (Howe et al., 1980; Thomas et al., 1983; Bravo et al., 1987; Slattery et al., 1988; Kunz et al., 1992). Several studies have indicated that there exists relationship between the consumption of alcohol and bladder cancer (Akdas et al., 1990;
Discussion...

Murta et al., 1996), but reports on inverse association are also available (Bruemmer et al., 1997; Chatenoud et al., 1998; Zeegers et al., 2001). The overall evidence does not support a direct role of alcohol in human bladder carcinogenesis. Most studies that have evaluated alcohol drinking as a risk factor for bladder cancer have not supported a positive association (Najem et al., 1982; Thomas et al., 1983; Cartwright et al., 1983; Kabat et al., 1986; Mills et al., 1991; Murata et al., 1996). Schifflers et al. (1987) demonstrated many people smoke cigarettes, while consuming alcohol; this may be a confounding factor as it can act as a promoter in the development of bladder cancer.

Several mechanisms have been postulated to explain ethanol-related carcinogenesis. Ethanol shows down protein synthesis. One consequence is that the cell repair mechanisms might be inhibited, which could lead to malignant changes. Furthermore, ethanol might improve permeability of membranes to carcinogens and enhance their activity (Freund, 1979; Garro and Lieber, 1990; Murta et al., 1996; Bagnardi and Vecchia, 2001). Other explanations include the effect of ethanol on cell proliferation, possibly caused by acetaldehyde (Garro and Lieber, 1990; Johansson and Cohen, 1997).

No positive association of food regime and development of bladder cancer was detected. It is because that Indian consuming less red-meat and have high application of vegetables in cooking. Steel utensils have been mostly used in cooking. Usage of high amount of oil (mostly unrefined mustered oil and Gee) in cooking is common. Steineck et al., (1989, 1990) reported that beef, pork or animal fat consumption may alter the incidence of urinary bladder cancer. Increased bladder cancer risk has also been associated with relatively high intake of cholesterol (Risch et al., 1988), with total fat (Silverman et al., 1989a) and saturated fat (Riboli et al., 1991), with fatty meals (Claude et al., 1986), fried foods (Chyoa et al., 1993; Bruemmer et al., 1996; Steineck et al., 1990), and with relatively high pork and beef consumption (Steineck et al., 1989). When meat is fired especially under high temperatures, heterocyclic amines are formed. Sugar and creatinine are utilized in a chemical reaction, known as Maillard, producing heterocyclic amines (Jagerstad et al., 1983). They are mutagenic, and all studied heterocyclic amines when given to rodents or monkeys, have been reported to be
carcinogenic. An excess risk of urinary bladder tumours has been found when the heterocyclic amine Trip-P-2 is fed to rats. This substance has some structural similarities to the strong bladder carcinogen 2-naphthylamine (Takahashi et al., 1993; Hashida et al., 1982). Un-recognized methodological problems may explain the inverse-U relation between meat intake (mainly beef and pork) and urinary bladder cancer risk. Messing (1992) has suggested that urinary pH may be of interest; a high meat intake may lower the urinary pH, and acid urine may inhibit certain growth factors that are important for tumour development. The intake of heterocyclic amines, in the doses typically ingested by the study populations, has not been found to be related to colorectal or kidney cancer risk (Augustsson et al., 1999). Available data are far from conclusive, but there is good evidence that consumers of such foods have an increased risk. The most consistent evidence supports a protective effect for vegetables and fruits. Relatively high vegetable and fruit consumption has been associated with low risk in most studies (La Vecchia et al., 1989; Mills et al., 1991; Chyou et al., 1993; Momaas et al., 1994; Bruemmer et al., 1996).

The correlation between amount of water drinking and also treatment process of drinking water with bladder cancer has been studied. It has been reported that diluted urine causes less damage to the urothelium than a concentrated one. Still, epidemiological evidence for a long time included the opposite. Early Danish (Jensen et al., 1986) and German (Kunze et al., 1992) studies for example, obtained increased relative risks of urinary bladder cancer in subjects with a high fluid intake. However, data from the physician health study had shown a decreased urinary bladder cancer risk in men with high fluid intake and had indicated that there is a need to drink 2 liter every day to obtain a preventive effect (Michaud et al., 1999). The complexity concerning the effects by fluid intake is further highlighted by the reports that chlorination of tap water may cause an increased risk of urinary bladder cancer (Cantor et al., 1998, 1987). In this study, slight increased (1.5 times) risk of bladder cancer was observed in those patients with average consumption of 2 liter or lesser water per day as compared to those use boiled or UV treated water (e.g. Aquagurd). Slight increased risk of bladder cancer was observed in patients who drank water directly from tap. Positive correlation (2.1 fold increased risk)
between drinking of natural water (wells or spring, particularly in rural area) and bladder cancer was detected. The continuous exposure to sunlight has been considered as indicator of perspiration and influences vitamin D synthesis and estrogen receptor with bladder cancer. 59.9% of patients in the present study were exposed to sunlight lesser than 3 hours per day. No positive association between 3 hours exposure to sunlight per day and bladder cancer was detected.

Three lines of evidence have suggested that the use of hair dyes may be associated with an increased bladder cancer risk. (i) Individuals with occupational exposure to these chemicals (hairdressers, barbers and beauticians) are at an elevated risk for bladder cancer (Clemmesen, 1981; Skov and Lynge, 1994; LaVecchia and Tavani, 1995). (ii) Findings from mutagenicity tests and animal experiments have indicated that some compounds in hair dyes are mutagens and possibly bladder carcinogens (Hartge et al., 1982). (iii) People who dye their hair appear to excrete dye compounds in urine (Hartge et al., 1982). In this research, no significant association between applying artificial hair-dye and uro-epithelial cancer was seen. Relative risk in patients using artificial hair-dye was found to be 1.5.

Evidence for familial predisposition to bladder cancer came mainly from clinical reports, but in a few case-control studies, elevated risks among persons with bladder cancers in close relatives was found (Cartwright et al., 1984; Piper et al., 1988; Kramer et al., 1991; Kunze et al., 1992; Kiemeney and Schoenberg, 1996). This indicates familial risks to be especially high among those with environmental exposures, such as heavy cigarette smoking. All these suggested genetic and environmental interactions. It has been shown that the risk of upper urinary tract, but not bladder TCC is increased more than 10-fold in families with hereditary nonpolyposis colon cancer (Watson and Lynch, 1993). Kiemeney et al. (1997) have found an increased risk in first-degree relatives, with an even greater risk for second- and third-degree relatives. In this study, the elevated risk of uro-epithelial cancer in the first-degree relatives was observed. There was with relative risk of 2.8 as compared to healthy controls with bladder cancer in close relatives.
6.2 Interaction of Single Nucleotide Polymorphisms in the risk of uro-
epithelial cancer

Identification of SNPs (single nucleotide polymorphisms) in human genome has
great implications in the study of disease susceptibility (Bid et al., 2004). A number of
studies have investigated the probable association between modulation of bladder cancer
risk and polymorphisms in metabolic, DNA repair and cell-cycle regulation genes
(Sanyal et al., 2004). The association between bladder cancer and various genetic
markers has helped to increase knowledge of the genetics of the immune response to and
pathogenesis of bladder cancer. The study included 227 histopathologically confirmed
cases and 232 population-based controls.

6.2.1 SNP of repair genes and bladder cancer

The established risk factors for bladder cancer include cigarette smoking,
exposure to industrially relate aromatic amines and uptake of drugs like phenacetine and
cyclophosphamide (Steineck et al., 1995; Cohen et al., 2000). The exposure to such
environmental agents and byproducts of cellular metabolism results in damage to DNA,
which, if left un-repaired, can lead to carcinogenesis. The entire process results in DNA
damage and subsequent repair of the damage involves a host of enzymes (Friedberg,
2003). DNA damage itself is a consequence of a balance between activation and
detoxification of carcinogens that involves phase I and II metabolic enzymes, many of
which are polymorphic (Brockmoller et al., 2000). The repair of damaged DNA is
essential to protect cells against cancer (Friedberg, 2003). Different pathways of DNA
repair operate on specific types of damaged DNA, and each pathway involves numerous
molecules (Good et al., 2002). Till now, enzymes coded by more than 100 genes have
been found in human cells that are implicated in four major DNA repair pathways
including nucleotide excision repair (NER), base excision repair (BER), double strand
break repair (DBR), and mismatch repair (MMR). A deficiency in repair capacity due to
the defect in genes involved in DNA repair can lead to genomic instability and
carcinogenesis. Individuals with repair capacity below the population mean can be at an
increased risk of developing different kinds of cancer and DNA polymorphism can result
in subtle structural alterations of the repair enzymes and modulation of cancer susceptibility (Sanyal et al., 2004). It has been well known that DNA repair is a very important mechanism in protection against gene mutations and cancer initiation (Sobti et al., 2006).

In this study, the prevalence of nucleotide polymorphism in \textit{LIG-I}, \textit{APE-I} and \textit{NBS} and their associations with the risk of uro-epithelial cancer in north Indian population has been investigated.

Human \textit{DNA ligase I} (\textit{LIG-I}) is required for joining Okazaki fragments during DNA replication and for sealing single-strand breaks. It is involved in both nucleotide excision repair and long-patch base excision repair. Although \textit{LIG-I} has the same functions as some other DNA ligases, mutations in \textit{LIG-I} result in hypersensitivity to DNA-damaging agents. A single base change (A? C) in exon 6 of \textit{LIG-I} has been identified, however, it does not cause an amino acid change and its functional relevance remains to be determined (Shen et al., 2002). The \textit{LIG-I} exon 6(A? C) polymorphism does not cause an amino acid change and the biological relevance of this variant and whether it is in linkage disequilibrium with functional polymorphisms at other sites are unknown. It has been suggested that \textit{LIG-I} deficiency causes Bloom’s syndrome (Chan et al., 1987; Willis and Lindahl, 1987), in which increased cancer susceptibility is associated with increased chromosomal breakage and rearrangement, an elevated frequency of sister chromatid exchange, slow DNA-chain maturation, slow replication-fork progression, delayed conversion of replication intermediates to high-molecular weight DNA and slightly increased sensitivity to DNA damaging agents. Livak et al., (1998) have identified two \textit{LIG1} variants from HeLa cells as well as human tissues; one variant is a single-nucleotide polymorphism in which either A or C is found at a site in exon 6 and the other is a complex GT repeat at the 5’ end of intron 6, consisting of a 48-50 nucleotide polypurines. Because \textit{LIG-I} is involved in various DNA repair pathways (Nocentini, 1999; Levin et al., 2000), it is likely that common polymorphisms, rather than rare mutations of \textit{LIG-I} gene may be playing a role in cancer susceptibility in the general population. To investigate the role of this polymorphism in the etiology of uro-epithelial cancer, this case-control study was conducted to evaluate the association between the
LIG-I exon 6 polymorphism and risk of bladder cancer. To the best of our knowledge, this is the first study on the association between LIG-I polymorphism and uro-epithelial cancer risk. From this large case-control study, it was been concluded that A?C substitution in LIG-I exon 6 is very common in the general population. No evidence for an association between this polymorphism and risk of bladder cancer in transitional cell carcinoma (TCC) has been obtained. Therefore, the results suggest that this polymorphism may not be playing an important role in susceptibility of bladder cancer. A slight elevated risk of bladder cancer was observed when data were stratified by smoking status particularly in active smokers with pack-years of more than 50.1 and in those with high risky job or residential place having LIG-I (AA) genotype. Interestingly, the homozygote A allele of LIG-I showed an increased risk of bladder cancer in females, but, it can not be assured because of small sample size of females and consequently decrease of accuracy in statistic. Incidence of women with bladder cancer was very low in North Indian population. The lack of association was seen between LIG-I and risk of lung cancer even in smoker patients (Shen et al., 2002; Sobti et al., 2006).

The first step of DNA base excision repair (BER) typically involves the removal of a damaged or mismatched base by a DNA glycosylase, generating a baseless site. The major protein responsible for repairing a basic site in human DNA is APE-I. APE-I plays a central role in the BER pathway, which operates on small lesions such as oxidized or reduced bases, fragmented or non-bulky adducts, or those produced by methylating agents (Lu et al., 2001).

Hidemi et al. (2004) reported that APE-I Asp148Glu polymorphism might modify the risk of lung cancer attributable to cigarette smoke exposure (Heidemi et al., 2004). Polymorphisms in their encoding genes are associated with altered DNA repair capacity and, thus, may impact on cancer risk. Still there is no comprehensive study on the role of polymorphism in this gene and bladder cancer risk and a significant link of this DNA repair gene and susceptibility to bladder cancer is specified. Regarding biological significance, the Glu allele of this polymorphism appeared to be associated with hypersensitivity to ionizing radiation (Hu et al., 2001). (Hadi et al. (2000) had observed that the APE-I Asp148Glu polymorphism does not result in reduced endonuclease
activity, but their results suggested a reduced ability to communicate with other BER proteins giving rise to reduced BER efficacy. The polymorphism of APE1 Asp148Glu has so far only been looked at regarding lung cancer risk among male smokers and a lack of any link was reported for Caucasians (Misra et al., 2003). In this case-control study, no significant association has been detected in genotypes of APE-I and uro-epithelial cancer particularly in early stages of cancer. It was seen that APE-I (GG) or (Glu/Glu) genotype increase the risk of developing uro-epithelial cancer especially to more progressive stages (like T2 or T3). APE-I Asp148Glu polymorphisms appeared to play an important role in modifying the direction and magnitude of the association between cigarette smoking exposure (especially in active smokers with pack-years >50.1), chewing tobacco and drinker patients (particularly in those with alcohol-unit >26.8 per week) and risk of bladder cancer.

Defects in the detection and response to DNA double-strand breaks (DSBs) are the basis of hereditary chromosomal instability syndromes with high cancer risk, such as Nijmegen breakage syndrome (NBS) (Carney et al., 1998). NBS homozygotes are rare (Varon et al., 2002). The NBS1 protein is part of the Rad50 complex, which plays a role in DSB repair and meiotic recombination. This nuclear complex, composed of Rad50, Mre11, and NBS1/p95/Xrs2, becomes associated at sites of DSBs after treatment with ionizing radiation. In yeast, Xrs2, Rad50 and Mre11 are essential for processing the DSBs formed in meiosis and play a key role in DSB repair through homologous recombination in mitotic cells (Varon et al., 2002). The Rad50 complex is likely to play a similar role in DNA repair in mammalian cells. It is possible that inactivation of this complex contributes to chromosomal instability and participates in the mutational cascade leading to colon and other types of cancers (Vogelstein et al., 1998). The association of urinary bladder cancer with NBS1 exon-5 (Glu185Gln, G/C) was investigated. This is the first case-control study on Indian population which shows a statistically significant interaction between NBS1 homozygote for Gln allele and risk of bladder cancer particularly in males as compared to healthy controls. Somali et al. (2003) and Pedro et al. (2003) in case-control studies in the bladder cancer reported that the patients having homozygous for variant alleles for Glu185Gln NBS-I polymorphism
Discussion... showed statistically significant differences as compared to controls. Sanyal et al. (2004) in the study on $NBSI$ and bladder cancer risk found marginally significant differences in Sweden population. The present study almost supported their observation. Increased risk of bladder cancer was observed in both the age-groups as well as all stages of tumours in patients with $NBSI$ (Gln/Gln) genotype; hence it was shown that age had no significant effect on interaction of $NBSI$ genotypes on risk of bladder cancer risk. The same trend was seen about tumour stage that $NBSI$ (Gln/Gln) had a resemble elevation of risk of bladder cancer in all stages. It was shown that $NBSI$ (Gln185Gln) mostly has a significant effect on developing bladder cancer in low histopathological grades (GI=non-invasiveness and GII=moderate). The Gln allele genotypes for the SNPs in $NBSI$ showed a marginal association with bladder cancer in smokers in especially in active smokers and particularly with pack-years >50.1 and those who have the habit of chewing tobacco. The same trend was also observed in alcohol-drinker patients particularly with alcohol-units more then 26.8 per week. The link of $NBSI$ (Gln/Gln) with increased risk of bladder cancer in those with exposure to variant known risk factors (in job or residential place) was observed. It was deduced that uro-epithelial cancer in individuals with homozygote Gln allele of $NBSI$ and drinking water lesser than 2 lit/day, particularly in those using natural water without any curing, and also in the group with exposure-time of sunlight more than 3hr/day and consequently, high precipitation is more prone. Notable interaction of variant alleles of $NBSI$ with usage of artificial hair-dye, food regime and inheritance in developing bladder cancer was not seen.

### 6.2.2 Relationship between SNP of FAS and uro-epithelial cancer

Apoptosis is a physiological process that regulates normal homeostasis. Alterations of apoptosis-related genes are likely to contribute to the pathogenesis of malignant tumours. Among various death receptors, Fas/CD95, a transmembrane receptor, is known as a member of necrosis factor (TNF) receptors superfamily (Ueda et al., 2005). Down-regulation of FAS with resultant resistance to death signals has been reported in many cancers (Butler et al., 1998; Lee et al., 1999; Shimonishi et al., 2000). A previous LOH study has suggested that loss of one or more tumour suppressor genes at
chromosome 10q24.1–24.3 may be involved in the development of bladder TCC. One of the candidate genes in this region is FAS, which is located at chromosome 10q24.1 (Lee et al., 1999).

Since single nucleotide polymorphism at -670 in the enhancer region (A/G) situates at a binding element of gamma interferon activation signal (GAS), it may affect the level of transcription of the FAS protein. Previous work has suggested that the substitution of G to A in the position –670 (TTCCAG G/A AA) would change the gamma interferon activation site (GAS) (TTCnnnGAA) (Decker et al., 1997; Chatterjee-Kishore et al., 2000; Bauvois et al., 2000). This site is involved in IFN-? and IFN-a signaling (Shuai, 1994). GAS elements are known to bind to homodimers of a phosphorylated form of the 91 kDa transcription factor, STAT1. IFN-? could cause tyrosine phosphorylation of STAT1 by the IFN-/? receptor-associated Janus kinases 1 and 2. Subsequently, phosphorylated STAT1 forms homodimers and translocated into the nucleus where it induces transcription of GAS containing genes (Gao et al., 1997).

The present study has tried to demonstrate the relationship of A/G SNP at -670 of FAS gene promoter with human bladder TCC. This is the first report in this regard particularly in North Indian population. Significant association between FAS (GG) and increased risk of bladder cancer especially among men has been detected, conversely, the genotype FAS (AA) showed a protective effect against bladder cancer. Lai et al. (2003) have stated that A allele and AA genotype in cervical cancer, conferring an intact GAS element and more efficient FAS expression could be one of the mechanisms that cells use to avoid of carcinogenesis. Hunag et al. (1999) and Kanemitsu et al. (2002) have also reported that G allele results in an abolishment of the GAS element and a significant decrease in FAS gene expression in response to IFN-? stimuli in rheumatoid arthritis and systemic lupus erythematosus patients. Interestingly, significant difference has been found between patients and controls carrying homozygote G allele in early onset (age=61.9). FAS (GG) genotype makes patients more prone to risk of bladder cancer and have a significant role in developing bladder cancer in early tumour stages (Ta and T1) and in low and moderate histopathological grades (GI, GII). Also, FAS (AA) also increases the tolerance against of bladder cancer in lower stages (Ta and T1) and low and
moderate hisopathological grades. It can be explained because of the role of this allele to avoid carcinogenesis through IFN-γ pathway. No association between smoking and FAS genotypes for developing of bladder cancer was found. Decreasing the risk of bladder cancer by \textit{FAS (AA)} was observed in smokers as well as non-smokers.

The same trend was seen for drinking, chewing tobacco or having risky job or living in risky zone. FAS (GG) marginally increased risk of bladder cancer in both drinkers and non-drinkers; chewers and non-chewers of tobacco; and those classified in the risky as well as non-risky groups. A allele also showed its protective effect against cancer in both smokers and non-smokers, drinkers and non-drinkers, and in those with risky job or residential place and non-risky group. Almost this trend was observed in other studied risk factors like food regimes, continues exposure-time to sunlight, the kind and amount of drinking water and usage of hair-dye. It can be concluded that effect of FAS genotype as a pro-apoptosis gene is independent of risk factors. It was found that inheritance does not play significant role with respect to \textit{FAS} genotypes and risk of bladder cancer.

\subsection{6.2.3 Relationship between SNP of cytokine and uro-epithelial cancer}

Cytokines mediate many immune and inflammatory responses contributing to tumourigenesis (Ahirwar \textit{et al.}, 2008). The relationship between cytokines and the severity of bladder cancer is worth to investigate; further studies could lead to immunotherapy of bladder cancer (Tsai \textit{et al.}, 2005). The present study evaluated the impact of polymorphisms of \textit{IL-1β}, \textit{IL1Ra}, \textit{IL4}, \textit{IFN-γ} and \textit{IL18} genes on the risk of development of transitional cell carcinoma of bladder.

The interleukin-1 (\textit{IL-1}) gene cluster on chromosome 2q and contains 3 related genes within a 430-kilobase (kb) region, \textit{IL1A}, \textit{IL1B} and \textit{IL-1RN}, which encode the pro-inflammatory cytokines \textit{IL-1α} and \textit{IL1β} as well as their endogenous receptor antagonist \textit{IL-1Ra} respectively (Tamandani \textit{et al.}, 2008). All three molecules bind to IL-1 receptors (Sehouli \textit{et al.}, 2003). The polymorphism of \textit{IL1a}, \textit{IL1β}, and \textit{IL-1Ra} produce alterations of their expression, and it may have crucial effects on oncogenic processes (Sehouli \textit{et al.}, 2003). Mutations in one of these genes up-regulate the expression, \textit{e.g. IL-1Ra}
polymorphism is associated with enhanced IL-1β production in vitro (Santtila et al., 1998). Also, IL-1Ra genotype plays a major role as a modulator in IL-1β release (Vamvakopoulos et al., 2002). El-Omar et al. (2000) and Machado et al. (2001) have reported an association of the IL-1 gene cluster polymorphism with enhanced production of IL-1β and gastric cancer.

IL-1β is a potent immunomodulator, which mediates a wide range of immune and inflammatory responses including the activation of B and T cells (Boulay and Paul, 1992). IL-1β actively participates in the regulation of vascular cell functions, including the stimulation of leukocyte adhesion to the endothelial cells, permeability of vessels, matrix metalloprotease production, suppression of vascular contractility, regulation of the pathways of coagulation within the cell and the production of pro-coagulator (Omar, 1999). Interleukin-1β is a potent pro-inflammatory agent which is central for immunoregulation, fever, inflammation and cancer formation (Tsai et al., 2005). In the present study, the impact of polymorphism of IL-1β (C-31T) as the risk of bladder cancer was studied. Slight increased risk of bladder cancer (1.40 fold) was observed in individuals carrying IL-1β (TT). Increasing risk of bladder cancer in higher stage T3 has been observed in the carriers of IL-1β (TT) genotype. No significant correlation of genotypes of IL-1β and smoking, drinking, artificial dye and even with risky job with bladder cancer was found. Increasing risk of bladder cancer was seen in the patients drinking less water and it might be due to the hidden stress caused by low water. Slightly increased risk of bladder cancer was observed in those exposed for more than 3 hours continuously in sunlight. Patients with risky job or living in risky zone on the grounds of hazards and carrying IL-1β (TT) showed 2.13 fold more susceptibility to bladder cancer.

The IL-1Ra gene is also polymorphic due to a variable number (2–6) of tandem repeats of 86-bp (VNTR) within its second intron (Tarlow et al., 1993). The common allele (allele 1) generated a 410-bp band (including four copies of an 86-bp repeat). The uncommon alleles generated a 240-bp band (two copies of the same repeat; allele 2) a 500-bp band (five copies of the same repeat; allele 3) and a 325-bp band (allele 4). The allele 2 of IL-1Ra seemed to be the critical point in the molecular pathway of different diseases. The two-repeat allele was associated with different benign diseases: vestibulites,
Discussion...

ulcerative colitis, alopecia areata, psoriasis, autoimmune conditions and idiopathic recurrent miscarriage (Arend et al., 1998; Jeremias et al., 2000). The polymorphic gene that encodes IL-1Ra seems to play an important role in the development of various diseases (Witkin et al., 2002), seems to be involved in the induction of different solid tumours (Sehouli et al., 2002). IL-1Ra may have a function in the host immune responses in the local and general environments of gynaecological cancers. IL-1Ra decreased tumour growth and angiogenesis (Sehouli et al., 2003). The plasma level of IL-1Ra was influenced by the number of repeats in the IL-Ra VNTR (Arend, 1990). The objective of this study was to provide the relationship between polymorphism of IL-1Ra (86-bp repeat VNTR in intron 2) gene and susceptibility to bladder cancer in North Indian population. Attempts were made to reveal the correlation between this genotype and studied risk factors since there is no report regarding the relationship of risk factors and this polymorphism particularly in North Indian population. It was seen that those carrying allele C (510bp) were significantly more susceptible (2.30 times) to risk of bladder cancer. Carriers of allele 2 of IL-1Ra homozygote had an increased risk of developing gastric cancer with odds ratios of 2.7 (95% CI 1.5–4.9) and 3.1 (95% CI 1.5–6.5), respectively (Sehouli et al., 2003). Bid et al. (2006) suggested that IL-1Ra intron-2 polymorphism plays a prominent role in bladder cancer in North India population.

In the present study, it was demonstrated that the allele C polymorphism of the IL-1Ra gene is significantly associated with bladder cancer. Patients carrying at least one C allele in individuals of both the age-groups more and less than 60 years, showed almost same chance of having bladder cancer and this genotype was not much age-dependent. The susceptibility towards higher stages (T2 and T3) of bladder cancer increased in patients with at least one copy of C or D alleles. Smoking (active smoker only) also increased the risk of bladder cancer by 1.93 fold, in the carriers of IL1-Ra (CC) genotype as its expression decreased. Drinking also had the same trend especially in those drinking more than 26 alcohol-units per week. Carriers of at least one C allele of IL1-Ra and having risky job or living in risky zone showed 3.1 times more chance to get bladder cancer. The correlation with the amount of water, the time of continuous exposure to sunlight, usage of artificial hair-dye, and heritage with risk of bladder cancer in carriers of
Discussion...

IL-IRa genotype in bladder cancer was not found. It was found that patients homozygotes for CC or carriers of D allele and using directly natural water have more chance to have bladder cancer. It might be due to the presence of bacteria or other parasites in this kind of water.

IL-4 gene has been mapped in q23-31 of chromosome 5 and is in a cluster of cytokine genes (IL-3, -5, -9, -13, -15), granulocyte colony-stimulating factor, and interferon regulatory factor (Rosenwasser et al., 1995). IL-4 protein of 129 amino acids is synthesized as a precursor containing a hydrophobic secretory signal sequence. IL-4 is glycosylated at two arginine residues (positions 38 and 105) and contains six cysteine residues involved in disulfide bond formation. The disulfide bonds are essential for biological activity. Some glycosylation variants of IL-4 that differ in their biological activities have been described. IL-4 enhances expression of class II MHC antigens on B-cells. It can promote their capacity to respond to other B-cell stimuli and to present antigens for T-cells (Boulay and Paul, 1992). IL-4 is a key cytokine that induces the activation and differentiation of B cells, and the development of the Th2 subset of lymphocytes. Th2 cytokines such as IL-4, -6, and -10 primarily support antibody production and many studies have confirmed that patients with cancer have high levels of such cytokines in their serum. IL4 also inhibits macrophage activation and might be involved in cancer formation (Tsai et al., 2005). The relationship between uro-epithelial cancer and IL-4 gene polymorphism has been less studied; only one study has been carried out on Taiwan population. This present study has tried to screen the polymorphism in IL-4 (70-bp repeat VNTR in intron-3) and its role in bladder cancer in Indian population. It was observed that Rp1 severely decreased tolerance against bladder cancer and enhances the cancer formation in all the age-groups and both genders. An increased risk of bladder cancer in those carriers of Rp1 was observed in almost all the stages, but more prominent in T2 and T3. The Rp1/Rp2 polymorphism (183 bp / 253 bp) in intron-3 might be enhancing cancer formation either through an IgE pathway, or its transcription activity, although the function of this polymorphism is unknown; possibly distinct numbers of VNTR copies might be affecting the transcriptional activity of the interleukin-4 gene (Tsai et al., 2005). No correlation of smoking, tobacco chewing,
drinking, risky factors, amount and kind of water, usage of artificial hair dye, heritage and diet with genotypes of \textit{IL-4} and bladder cancer risk was found. But an increased risk was observed in all groups carrying Rp1 allele.

Interferon-\(\gamma\) (IFN-\(\gamma\)) was first recognized for its antiviral activity, but a great deal of data have since been accumulated which establish that this multifunctional cytokine plays an important role in modulating almost all phases of the immune response (Awad et al., 1999). The multifunctional cytokine IFN-\(\gamma\) demonstrates an antiproliferative and antifibrotic capacity among various other immunological features (Xie et al., 2001). One critical aspect of IFN-\(\gamma\) activity is its role in inflammatory responses. IFN-\(\gamma\) increases collagen synthesis and fibroblast collagen matrix deposition as well as enhancing the transcription of other matrix genes, including fibronectin (Awad et al., 1999). IFN-\(\gamma\) acts as a regulator of antigen presentation and of proliferation and differentiation of lymphocyte populations. High-level production of IFN-\(\gamma\) during this phase of host defense is now classically seen as a hallmark of a type I reactions, characterized by activation of macrophages and by strong cellular inflammatory reaction (Billiau et al., 1998).

The IFN-\(\gamma\) gene, normally present as a single copy, has been assigned chromosome position 12q24.12. There are two well-known single-nucleotide polymorphisms in the first intron of \textit{IFN-\(\gamma\)} gene: CA repeat microsatellite sequence in the noncoding region; and a single nucleotide T/A polymorphism at the 5' end of the CA repeat region (+874 A/T polymorphism) (Khani-Hanjani et al., 2000). Five different alleles of the CA repeat microsatellites in the first intron of the gene have been demonstrated in the control population (Awad et al., 1999). Sequence analysis has shown that allele #1 corresponds to 11 CA repeats, allele #2 to 12 repeats, and alleles #3–#5 have 13–15 repeats, respectively (Pravica et al., 2000). It has been tried in the present study to find the correlation of \textit{IFN-\(\gamma\)} +874A/T SNP with uro-epithelial cancer. It was found that patients homozygotes for AA were more susceptible to bladder cancer, particularly those with age lesser than 60 years. Smokers featuring \textit{IFN-\(\gamma\)} (AA) (especially in active smoker with pack-years more than 50) showed increased risk of bladder cancer. The same trend was observed in those chewing tobacco and drinking alcohol (especially
with alcohol-units more than 26 per week). The interaction of smoking with IFN-γ (AA) was found to be stronger than drinking of alcohol. IFN-γ (AA) genotype along with risky jobs or living in risky zone and consuming less water (especially natural water) or having exposure to sunlight for more than 3 hours per day, showed increased susceptibility to bladder cancer. Enhancement in cancer formation may be due to decrease in immune response and stress in body due to availability or less water.

The human IL-18 gene is located on chromosome 11q22.2-q22.3, and is composed of six exons and five introns (Kruse et al., 2003). Interleukin-18 (IL-18) is a pro-inflammatory cytokine initially isolated from liver cells. It shares structural features with interleukin-1β (IL-1β). Both these cytokines are produced as inactive precursor molecules, which are then processed caspase-1 thereby biologically active molecules are released. IL-18 binds to the IL-18 receptor formerly known as the IL-1 receptor (Giedraitis et al., 2001). Being involved in the pro-inflammatory cytokine network, IL-18 is mainly produced by activated macrophages and, like interleukin-12 (IL-12), is able to induce IFN-γ and TNF-a, as well as enhancing the cytotoxicity of NK cells and FasL expression (Hoshino et al., 1999; Kretowski et al., 2002; Takada et al., 2002), and decreases the production of interleukin-10 (Ushio et al., 1996). IL-18 was first described as an IFN-γ inducing factor, and has multiple functions including induction of the synthesis of IFN-γ by T and NK cells, promotion of Th1-type immune responses, augmentation of proliferative response and cytokine production of activated T cells. Meanwhile, IL-18 leads to activities against pathogens, it activates effector cells involved in the cellular interactions that occur during inflammation, and are part of the acute and chronic stages of viral hepatitis, induce target-cells apoptosis (Zhang et al., 2005). Giedraitis et al. (2001) have described that there are three SNPs at position -656G/T, -607C/A, and -137G/C in the promoter of IL-18 gene. Two SNPs of the promoter of IL-18 gene at position -607 and -137 have been suggested to cause the differences in transcription factor binding site and have an impact on IL-18 gene activity and potentially also to IFN-γ (Zhang et al., 2005). A change from C to A at position -607 disrupts a potential cAMP-responsive element-binding protein binding site and a change at position -137 from G to C changes the H4TF-1 nuclear factor binding site (Giedraitis et al., 2001).
In the present project, it was observed that carriers of IL-18 (CC) at -137 position have significantly increased tolerance against bladder cancer and age or genderness. Zhang et al. (2005) have reported that potentially G/C polymorphisms at position -137 could play a main role in the expression of IL-18. Individuals with CC genotype at position -137 have higher levels of IL-18 mRNA compared to other genotypes that have a clear correlation between IL-18 and IFN-γ mRNA expression. No significant interaction of smoking, drinking, diet, the amount and kind of water, usage of artificial hair-dyes, continuous time of exposure to sunlight, heritage factor in carriers of IL-18 (CC) with bladder cancer was found. But there was decrease of its protective effect of IL-18 (CC) in smokers, drinkers, and risky jobs. There may be a possible link between increased production of IL-18 in the carriers of G/C polymorphism at position -137 of the promoter of IL-18 gene. The presence of allele C at position -137 of each of these polymorphisms has been related with high production of IL-18, which may augment the production of IFN-gamma, modulate activity of NK and CTL cells, and trigger the complex immunological processes to eliminate HBV and its complex. Ridele et al. (2004) mentioned that IL-18 enhances the immune defense against tumour cells by activating and inducing the production of IFN-γ. The increased IL-18 plasma levels were found in patients with malignant diseases such as acute lymphoblastic leukemia or chronic myelotic leukemia, breast carcinoma, or gastric cancer (Ridele et al., 2004).

6.3 Combination of genotypes of studied genes and the risk of uro-epithelial cancer

The individuals carrying LIG (AA) and NBS (Gln/Gln) showed 1.9 fold increased risk of bladder cancer. In the carriers of combined genotype of LIG (AA) and APE (Glu/Glu), there was 1.61 times increased risk of bladder cancer. In the combination LIG and FAS, it was found that Fas alleles have controlling role. For example, combination of A allele of LIG-I and A allele FAS, promoted tolerance against bladder cancer (like LIG (AA) + FAS (AA)), whereas combination of LIG (AA) with FAS (AG) and FAS (GG) caused about 1.4 fold increased risk of bladder cancer, hence it indicated the controlling
effect of A or G alleles of FAS on A allele of LIG-I in inducing the promotion of susceptibility or tolerance against bladder cancer.

The combination of A allele of LIG and Rp1 alleles promoted the development of bladder cancer. LIG (AC) IL4 (Rp2/Rp2) genotype increased the tolerance against bladder cancer but LIG (AA) and IL4 (Rp1/Rp1) increased 1.90 fold the risk. Combination of C allele of LIG-I with C of IL-18 induces resistance against bladder cancer (OR=0.56). 2.0 times increased risk for bladder cancer was observed in individuals carrying A allele of LIG-I and A alleles IFN-γ. The presence of C allele of IL-1β and C of LIG-I induced slightly tolerant effect against the development of bladder cancer. Combination of A allele of LIG-I with C of IL-1Ra caused 2.0 folds susceptibility towards bladder cancer. Combination of NBS (Gln/Gln) and APE (Glu/Glu) conferred 1.8 increased risk of uro-epithelial cancer. An elevation of risk of bladder cancer by 1.4 folds was seen in the carriers of NBS (Gln/Gln) and FAS (GG) genotype. The combination of NBS (Glu/Glu) and FAS (AA) enhanced tolerance against uro-epithelial cancer (OR=0.43).

The presence of at least one Rp1 allele in IL-4 genotype with NBS (Gln/Gln) showed 2.0 fold increased risk of bladder cancer. The presence of C allele of IL-18 showed the moderating effect of Gln allele of NBS, in such a way that combination of NBS (Glu/Glu) and IL18 (CC) severely decreased the development and susceptibility to uro-epithelial cancer (OR=0.31). NBS (Gln/Gln) and IFN (AA) combined genotype significantly increased (1.80 times) the risk of bladder cancer. 1.95 times increased susceptibility to the development of bladder cancer was observed in those having combined NBS (Gln/Gln) and IL-1β (TT) genotype. The presence of C or D alleles in IL-1Ra genotype and NBS (Gln/Gln) showed an increased risk of uro-epithelial cancer by 2.2 times.

The interaction of APE (Glu/Glu) with FAS (GG) was seen to be associated with the increased risk of bladder cancer (1.6 fold). The combination of FAS (AA) with APE (Asp/Asp) decreased the risk of the studied cancer. Combination of APE (Glu/Glu) and IL-4 with at least one Rp1 allele showed 1.8 -2.4 fold increased in the risk of bladder cancer. 2.4 times increased risk of bladder cancer was observed in individuals carrying
Discussion...

APE (Glu/Glu) and C or D alleles of IL-1Ra. Decrease in the risk of bladder cancer was seen in individuals carrying at least one Asp allele in APE and C in IL-18. It was seen that those with IFN-γ (AA) with APE (Asp/Glu) or APE (Glu/Glu) had 1.9-2.1 times increased the risk of bladder cancer. The significant association between combined genotypes of APE and IL-1B and risk of uro-epithelial cancer was observed.

The presence of at least one G allele in FAS genotype (GA+GG) and one Rp1 allele in IL-4 was seen to be associated with the risk of bladder cancer (1.6 to 2.1 folds). The effect of Rp1 allele in IL-4 clearly decreased the effect of A allele of FAS. The combination of FAS (AA) with C allele of IL-18 showed strongly increasing tolerance against bladder cancer (OR=0.25). About 1.4 fold risk of uro-epithelial cancer was observed in individuals carrying combined genotype of FAS (GG) and IFN-γ (AA). Combination of A allele of FAS with T allele IFN-γ showed decrease the risk of bladder cancer. The combination of FAS (GG) and (TT) revealed slight increase in the risk of bladder cancer (OR=1.6). Slightly decrease in the risk of bladder cancer was also observed in interaction of A allele of FAS with IL-1B alleles. The combination of FAS (GG) with allele C or D of IL-1Ra, statistically increased the risk (2.0 fold) bladder cancer.

It was found that Rp2 allele of IL-4 and C of IL-18 and T of IFN-γ decreased the chance of development of bladder cancer. A 1.9 fold increased risk of uro-epithelial cancer was found in individuals carrying IL-4 (Rp1/Rp1) and IL-1B (TT). The combination of IL-4 (Rp1/Rp1) and IFN-γ (AA) caused 1.8 times risk of uro-epithelial cancer. Combination of IL-4(Rp1/Rp1) and IL-1Ra (CC) genotype showed 2.0 times increased risk of bladder cancer and for combination of IL-4(Rp1/Rp1) and IL-1Ra (AD), the risk was 1.8 times.

Combination of IFN-γ (AA) and IL18 (GG) elevated 1.9 times the susceptibility to bladder cancer, whereas individuals having at least on copy of variant allele T in genotype of IFN-γ and IL18 (CC) slightly increased tolerance against uro-epithelial cancer. It was observed that combination of IFN-γ (AA) and IL-1B (TT) slightly increased the development of bladder cancer (OR=1.56). Individuals having at least one copy of A allele of IFN-γ and that of C or D allele in IL-1Ra have 1.7–2.7 fold increased
risk towards bladder cancer. No significant association with the interaction of IL-IRA (VNTR at intron 2) with IL-1β (C-31 T) and uro-epithelial cancer was seen. The same trend was seen in the interaction of IL-IRA (VNTR at intron 2) with IL-18 (G-137C).

The results of this study revealed that presence of allele (A) in exon-6 of LIG-I, allele Gln in position -185 of NBS-I, allele Glu in position +148 of APE-I, (G) allele at -670 of FAS, T allele in -31 of IL-1β, (A) allele in position of +874 of IFN-γ, Rpl (183 bp) allele in minisatellite located in intron 3 of IL-4, and (C) allele (510bp) in minisatellite located in intron 2 of IL-IRA in combination with other genes, increased the risk of the bladder cancer. Conversely, A allele at position -670 of FAS and C allele of -137 of IL-18 decreased the susceptibility or moderated the risk against uro-epithelial cancer.

6.4 Study of expression profiles of interleukins IL-13, IL-18 and IFN-γ in uro-epithelial cancer

Malignant tumours often impair host immune responses. T helper lymphocytes based on their cytokine production profile are classified into two subsets. Development of Th1 cells is driven by IL-12 produced by macrophages and denderitc cells via the transcription factor STAT4 signaling. On the other hand, commitment to the Th2 lineage is induced by IL-4 via the STAT6 signaling pathway. Th1 immune response is activated primarily by IL-2, IL-12, IFN-γ and TNF-β, which are secreted during cell-mediated immune response like killing activity by either cytotoxic T cells (CTLs) or NK cells (Mosmann et al., 1991).

Th1 response is prominent in autoimmune disorders, graft rejection, and chronic inflammatory disorders. Otherwise, Th2 immune response generally plays a role in humoral immunity such as antibody-mediated immune response related to B cells (produce IgE) and contributes to the eradication of extracellular parasites. It also induces atopic reaction and is generally activated by IL-4, IL-5, IL-6, IL-10, and IL13 (Coyle et al., 1995). These Th responses regulate each other antagonistically.

According to the cytokine profiles, T helper (CD4+) cells, and cytokine network act together to cause a compartmentalized inflammatory process. Inflammation is mainly driven by Th1 cytokines such as IL-1, IL-2, IL-6, IFN-γ, or TNFα which have pro-
inflammatory effects (Hauber et al., 2003). Th2 subset predominance in peripheral blood has been observed in the patients with malignant tumours (Kawabata et al., 2001). It has been suggested that an immunosuppressed state contributes to the development of an environment favourable for further growth of tumour cells (Gastl et al., 1993). As far as our knowledge goes, there is no report regarding the expression profiles of IL-13, IL-18 and IFN-γ in patients with uro-epithelial cancers, in particular, in Indian population.

A large number of occupations and occupational chemicals have been found to have an excess incidence of urinary bladder cancer. There is also no doubt that exposure to combustion gases, probably including engine exhausts, increase the risk to contract the disease. Combustion gases contain a plentitude of chemicals, for example, small amounts of aromatic amines, nitrosamines and several different polycyclic aromatic hydrocarbons (Droller, 2001) and it has the role in the stimulating immune response. The urban atmospheric pollution is one of the potential culprits. Exhaust from diesel-powered engines is now the main source of particles in urban air pollution (Fahy et al., 2002). In particular, polyaromatic hydrocarbons (PAH) adsorbed on the carbon core of diesel exhaust particles (DEP) are believed to exacerbate the allergic inflammatory reaction. For example, DEP-PAHs induce Ig switch towards IgE and act as adjuvants by potentiating both total and specific IgE production by committed B cells (Takenaka et al., 1995; Fujieda et al., 1998). DEP-PAH can also act directly on many effector cells, such as macrophages, neutrophils, mast cells, and eosinophils, by inducing their recruitment and by triggering the release of pro-inflammatory mediators (Saneyoshi et al., 1997; Terada et al., 1997; Kanemitsu et al., 1998). Interestingly, combined exposure to diesel extracts and allergen strongly potentiates the effect of each stimulus, further enhancing IgE production, and skewing the immune response toward a type-2 cytokine profile (Diaz-Sanchez et al., 1998).

6.4.1 Expression of IL-13 in uro-epithelial cancer

Anti-inflammatory cytokines have not been well characterized. Since patients often have only mild symptoms, counteracting mechanisms such as anti-inflammatory cytokines have been suggested for immunotherapy. Interleukin IL-13 plays a major role
in various inflammatory diseases including cancer, asthma and allergy. It mediates a
variety of different effects on various cell types including B cells, monocytes, natural
killer cells, endothelial cells and fibroblasts (Joshi et al., 2006). IL-13 is a potent
immunosuppressive cytokine and Th2 cytokine that regulates the effector functions and
alters the phenotype and function of normal macrophages switching to alternatively
activated or type II polarized macrophages. The type II polarized macrophages differ
from normal macrophages greatly in terms of receptor expression, NO and other cytokine
production. It produces chemokines that preferentially attract Th2 cells, which increase
the local concentration of Th2 cytokines including IL-13 (Deepak et al., 2008). IL-13
suppresses the production of nitric oxide (NO) and expression of inducible nitric oxide
synthase (iNOS), and pro-inflammatory cytokines. The expression of iNOS and pro-
inflammatory cytokines is dependent largely upon the activation of nuclear factor-kappa
B (NF-?B). Activation of NF-kappaB involves the degradation of cytoplasmic inhibitor I-
kappaBalpha, allowing the nuclear translocation of NF-kappaB and thereby transcription
of the iNOS gene (Deepak et al., 2007). Nuclear factor-kappa B (NF-?B) is a
transcription factor that resides in the cytoplasm of every cell and translocates to the
nucleus when activated. Its activation is induced by a wide variety of agents including
stress, cigarette smoke, viruses, bacteria, inflammatory stimuli, cytokines, free radicals,
carcinogens, tumour promoters, and endotoxins. On activation, NF-kappaB regulates the
expression of almost 400 different genes, which include enzymes (e.g., COX-2, 5-LOX,
and iNOS), cytokines (such as TNF, IL-1, IL-6, IL-8, and chemokines), adhesion
molecules, cell cycle regulatory molecules, viral proteins, and angiogenic factors. The
constitutive activation of NF-?B has been linked with a wide variety of human diseases,
including asthma, atherosclerosis, AIDS, rheumatoid arthritis, diabetes, osteoporosis,
Alzheimer's disease, and cancer. Several agents are known to suppress NF-kappaB
activation, including Th2 cytokines (IL-4, IL-13, and IL-10), interferons, endocrine
hormones (LH, HCG, MSH, and GH), phytochemicals, corticosteroids, and
immunosuppressive agents. Because of the strong link of NF-kappaB with different stress
signals, it has been called a "smoke-sensor" of the body (Ahn et al., 2005).
Interleukin-13-producing natural killer T cells that signal Gr-1(+) cells to produce transforming growth factor-beta(1) (TGF-β (1)), a cytokine that suppresses the activity of tumour-inhibiting cytolytic CD8(+) T cells (Fichtner-Feigl et al., 2008). Sasaki et al. (2007) have reported that IL-13 expression is dramatically increased in canine TNF-α-generated mature dendritic cells.

Ashpord et al. (2007) have demonstrated that similar to Hodgkin’s cells, breast cancer cells express pSTAT6, suggesting that IL-13 actually delivers signals to cancer cells and the role of IL-13 in the indirect suppression of tumour surveillance is linked with suppression of cytotoxic T cell function.

IL-13 binds to two primary receptor chains IL-13Ra1 and IL-13Ra2. The IL-13Ra2 but not IL-13Ra1 chain binds IL-13 with high affinity and is over-expressed in a variety of human cancer cells derived from glioma, squamous cell carcinoma of head and neck, and AIDS-associated Kaposi’s sarcoma. IL-13Ra2-expressing cells are involved in various pulmonary pathological conditions. In contrast, normal tissues such as brain, lung, endothelial cells, and head and neck tissues express IL-13Ra1 chain, but show only marginal expression of IL-13Ra2 chain. Thus, IL-13Ra2 chain may serves as a novel biomarker for diseased cells such as cancer or fibrosis and a target for receptor-directed therapeutic agents (Joshi et al., 2006).

IL-13 shares many of its biologic activities with the Th2 cytokine IL-4, including induction of a class switch to IgE and anti-inflammatory properties (van der Pouw et al., 1996). IL-13 gene is located on chromosome 5 band q31 in close proximity to the IL-4 gene, and in the same cluster of genes encoding for IL-5, IL-3, and granulocyte-macrophage CSF (McKenzie et al., 1993). In humans, IL-13 and IL-4 has been shown to have many of their activities in common. Both cytokines are able to enhance expression of CD23 on monocytes and B cells and also induce IgE production. Production of many LPS induced monokines, such as IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-7, macrophage inflammatory protein-1α (MIP), granulocyte-macrophage CSF, and granulocyte CSF, is inhibited by IL-13, whereas IL-1 receptor antagonist is up-regulated. These properties are shared with IL-4 and IL-10. Therefore, IL-13 is a pleiotropic cytokine that can be considered as anti-inflammatory and immuno-regulatory activities.
that down-modulate macrophage activity, reducing the production of pro-inflammatory cytokines (van der Pouw et al., 1996). IL-13 mRNA is produced by PBMC and not expressed in heart, brain, placenta, lung, liver, and skeletal muscle tissues (Minty et al., 1993). Other cell types such as transformed B cell lines has been shown to produce IL-13 mRNA, and activated murine mast cells also produce bioactive IL-13 (Fior et al., 1994; Burd et al., 1995).

There are several reasons to investigate IL-13: (i) it is mainly produced by CD4+ T cells (ii) IL-13 has been shown to inhibit TNF-α production in blood monocytes. A study has found that IL-13 could suppress TNF-α secretion by human blood monocytes in healthy subjects (Cosentino et al., 1995), therefore, it might have some anti-inflammatory effects or influence the Th1 immune response (Hauber et al., 2003).

The mRNA expression in peripheral blood mononuclear cells (PBMC) in patients with bladder cancer (n=48) and in healthy controls (n=15). IL-13 levels were measured in sera of patients and controls. Only 9 out of 15 controls showed IL-13 expression of mRNA and protein and only 4 of them revealed high expression level. The expression of IL-13 was observed in all the patients. In this study IL-13 mRNA (2.3 fold) and protein (2.5 fold) expression was seen to be significantly increased both in PBMC and sera of patients with carcinoma of bladder as compared to controls. It led to conclusion that blood cells are one of the major sources of expression of IL-13 in response to carcinoma of bladder. Hauber et al. (2003) have reported that alveolar macrophages might be an important source of IL-13 in sarcoidosis.

It was found that age, smoking and drinking have no significant effect on the expression of IL-13 in patients with uro-epithelial cancer as compared to controls. The patients with lower stages of cancer (Ta and T1) showed increased level of expression of IL-13 mRNA in PBMC in comparison to those at higher stages.

It was observed that percentage of up-expression of IL-13 was more in lower stages. It might be because of disturbance of Th1/Th2 balance in higher stages towards more activation of Th1 immune response and pro-inflammatory cytokines in these patients. Lower stages of tumour are usually limited to epithelium layer of bladder wall, whereas stages T2 and T3 may be associated with a more progressive course. These
findings suggested that IL-13 may be involved in early inflammation of bladder cancer and may inhibit pro-inflammatory reactions, thus supporting limitation of disease. IL-13 may not be playing an important role in more advanced carcinoma when muscle layer of bladder layer is involved in cancer.

It has been shown that IL-13 stimulates TGF-β1 production in transgenic mice, indicating that the fibrogenic effects of IL-13 are mediated to a great extent by a TGF-β1 pathway (Lee et al., 2001). It is tempting to speculate that IL-13 might also activate TGF-β1 leading to regression of malignant cells. IL-13 has been shown to be a promising modality in the treatment of uveitis (Roberge et al., 1998), and intratracheal instillation of IL-13 has been found to have anti-inflammatory activity in the airways of TNF-α- or antigen-challenged guinea pigs (Watson et al., 1999). Over-expression of IL-13 in an animal model resulted in subepithelial fibrosis (Zhu et al., 1999). IL-13 may not be a suitable therapeutic agent for treating bladder cancer, but it may help to restore the disturbed Th1/Th2 balance in these patients. Another possible option might be to selectively induce anti-inflammatory activity via the IL-13 receptor. Joshi et al. (2005) demonstrated that a variety of solid human tumour cell lines express a large number of receptors for interleukin-13 (IL-13). These receptors could be targeted with a chimeric fusion protein consisting of human IL-13 and a truncated form of Pseudomonas exotoxin (PE). In conclusion, it was found that IL-13 plays a role as an anti-inflammatory mediator in carcinoma of bladder. Further studies are needed to determine whether or not IL-13 influences other mediators of inflammation in bladder cancer like TNF-a.

One hypothesis has been raised that some mutation in study sequence of IL-13 might be influencing the amount of its expression. Hence, The RT-PCR product was sequenced. The used method of sequencing DNA-the dideoxy or chain termination method-was developed by Fred Sanger (1977). The key to the method is the use of modified bases called dideoxy bases i.e. ddNTPs along with dNTPs. When a piece of DNA is replicated, a dideoxy base gets incorporated into the new chain stopping the replication reaction as there is no 3’-OH for the next nucleotide to be attached. Samples of both the high and less expression production from patients as well as controls was selected and exposed to sequencing in order to compare with each other and with
BLAST. No mutation was detected in study sequence of mRNA which was amplified in RT-PCR.

### 6.4.2 Expression of IL-18 in uro-epithelial cancer

Interleukin-18, formerly called interferon-γ inducing factor (IGIF), produced by activated macrophages, keratinocytes, kupffer cells, intestinal epithelial cells and osteoblasts as a biologically inactive form requires cleavage by CASPase-1 to get activated (Okamura et al., 1995).

IL-18 is a member of IL-1 family and its receptor also belongs to IL-1 receptor (IL-1R) family. IL-18 receptor (IL-18R) consists of a ligand binding domain α-chain and a signal-transducing domain β-chain (Born et al., 1998). When IL-18 binds to IL-18Ra, IL-18Rβ transduces its signal to stimulate the MAPK pathway involved in producing IFN-γ (Tsujii-Takayama et al., 1997). Interestingly, IL-18 is expressed in a biologically inactive form, pro-IL-18, with 24 KDa molecular weight, and it confers functional activity by proteolytic cleavage of IL-18 converting enzyme (ICE) resulting in an 18.3 KDa final molecule, which can then act as a biological signal transducer (Gu et al., 1997).

IL-18 is a pleiotropic cytokine that is produced by many cancer cells. IL-18 induces T helper type-1 (Th1) immune response (Okamura et al., 1995). IL-18 along with interleukin-12 (IL-12), synergistically stimulates activated T cells and natural killer (NK) cells to produce IFN-γ (Hu et al., 2004). IL-18 enhanced the immune defense against tumour cells by activating and inducing the production of IFN-γ (Fukumoto et al., 1997). In addition to its IFN-γ-enhancing capacity, IL-18 also augments the cytotoxic activity of natural killer and T-cells and enhances their production of other proinflammatory mediators such as TNF-α, IL-1 and IL-8 (Park et al., 2001; Gunel et al., 2002). Activated NK or T cells by IL-18 eliminate spontaneous cancer or pathogen infected cells. Contrary to the anti-cancer effect of IL-18, its pro-cancerous effect has been recently suggested (Park et al., 2007). Bioactive IL-18 induces interferon (IFN)-gamma production, Fas Ligand (FasL) expression, and inhibits angiogenesis, raising the issue of anti-tumour effects of a tumour-derived cytokine (Carbone et al., 2005).
IL-18 prevents NK cell death initiated by different and distinct stress mechanisms. IL-18 reduces NK cell self-destruction during NK-targeted cell killing, and reduces CASPase-3 activity. The critical regulatory step in this process is downstream of the mitochondrion and involves reduced cleavage and activation of CASPase-9 and CASPase-3. The ability of IL-18 to regulate cell survival is not limited to a CASPase death pathway in that IL-18 augments tumour necrosis factor (TNF) signaling, resulting in an increased and prolonged mRNA expression of c-apoptosis inhibitor-2 (cIAP2), a pro-survival factor and CASPase-3 inhibitor, and TNF receptor-associated factor 1 (TRAF1), a pro-survival protein. The cumulative effects of IL-18 define a novel role for this cytokine as a molecular survival switch that functions to both decrease the cell death through inhibition of the mitochondrial apoptotic pathway and enhance TNF-a induction of pro-survival factor (Hodge et al., 2006).

The present study is the first on mRNA and protein expression of IL-18 in patients with bladder cancer. Attempt has been made to address this question whether or not serum IL-18 is increased in patients with bladder cancer. Although elevated serum IL-18 levels have been reported in patients with advanced tuberculosis and acute-graft-versus-host disease (Yamada et al., 2000; Fujimori et al., 2000), the clinical impact of IL-18 remains unclear in patients with solid malignancies.

The level of IL-18 in the sera of the patients had a mean level of 232.5 pg/ml, while the mean IL-18 serum leveling the healthy controls was found to be 199.8 pg/ml. IL-18 serum levels agreed with the findings reported by other workers. Taniguchi et al. (1997) found the increases of IL-18 plasma levels in patients with malignant disease such as acute lymphoblastic leukemia or chronic myelocytic leukemia. The increased of IL-18 expression in plasma have also been found in haemophagocytic lymphohistiocytosis (Takada et al., 2001), breast carcinoma (Gunel et al., 2002), gastric cancer (Kawabata et al., 2001) and head and neck squamous cell carcinoma (HNSCC) (Riedel et al., 2004). Hu et al. (2004) found that IL-18 secreted by nasopharyngeal carcinoma (NPC) tumour cells plays a role in initiating the leukocyte infiltration process. Increased IL-18 serum levels were found in patients with cutaneous T-cell lymphoma and natural-killer-cell lymphoma (Amo et al., 2001). Merendino et al. (2001) showed that patients with
metastatic breast cancer have significantly higher IL-18 serum levels as compared to those without metastasis or healthy controls. Gunel et al. (2000) demonstrated increased IL-18 serum concentrations in breast carcinoma with significant higher levels in metastatic patients as compared to non-metastasis and healthy subjects. Higher IL-18 serum levels were seen in metastatic cancer patients as compared to non-metastatic patients and healthy individuals (Lissoni et al., 2000). Kabawata et al. (2001) showed that the survival rate of patients with gastric cancer who had high levels of IL-18 in their serum were significantly low. IL-18 levels and interestingly, IL-18 serum levels for all patients who underwent surgerical resection was decreased as compared to the levels before surgery. IL-18 was detected at elevated levels in the bone marrow of patients with some haematological malignancies, and might be involved in the proliferation of certain leukemic cells \textit{in vivo} through an autocrine mechanism (Zhang et al., 2003).

IL-18 plays an important role in the interactions among T cells, NK cells and macrophages. IL-18 stimulates T and NK cells to produce IFN-\(\gamma\), which consequently activate macrophages and other immune cells to secrete chemokines to start a leukocyte recruitment cascade. Okamoto et al. (2004) indicated that IL-18 inhibits the growth of Dunn osteosarcoma cells \textit{in vivo} by enhancing the cytotoxic activity of CD8\(^{+}\) T lymphocytes through the FasL-Fas system. Eissa et al. (2005) and Merendino et al. (2001) reported that IL-18 is also an important marker of breast cancer progression because higher IL-18 levels have been detected in sera from breast cancer patients with metastasis as compared to healthy volunteers and breast cancer patients without metastasis.

In the present investigations, IL-18 serum level were found to be significantly enhanced in patients with bladder cancer as compared to control group. The majority of patients with bladder cancer showed high concentrations of serum IL-18. These results suggested that IL-18 participates in the immune responses during the progression of uroepithelial cancer. The mRNA expression of IL-18 in PBMC was found to be similar in the patients with bladder cancer and healthy controls; hence it came to light that the cellular source of circulating IL-18 in serum of patients could not be PBMCs. As it was found that IL-18 serum level is increased in higher stages of bladder cancer and
Discussion...

metastatic. Consequently it can be concluded that IL-18 might be secreted by tumour infiltrating inflammatory cells and gains access to the circulation. It was been observed that mRNA expression in PBMC is increased in higher stages of bladder cancer, but no significant difference was observed between primitive (Ta and T1) and progressive (T2 and T3) stages. Kabawata et al. (2001) have stated that IL-18 production may be induced in response to tumour cells or other tumour growth factors. Though, macrophages are the major source to produce IL-18, it is still not clear which cell populations synthesize IL-18 in malignancy condition (Reidel et al., 2004).

Zheng et al. (2007) found that positive expressions of IL-12 and IL-18 can play an important role in progression and metastasis of gastric cancer. According to Ye et al. (2007) IL-18 was highly expressed in the tumour region in comparison with non-tumour region and related with distant metastasis. Majima et al. (2006) indicated that gastric cancers exploit IL-18 to grow/invoke and evade immunosurveillance in the hosts. Kim et al. (2006) demonstrated that IL-18 plays a key role in regulating the immune escape of melanoma and gastric cancer cells. Hacker et al. (2008) reached to this conclusion that increased expression of IL-18 can be considered as a marker of UVR-induced melanoma, both in animal models and humans. Findings of Dzierzanowska-Fangrat et al. (2008) suggested that IL-18 and macrophages may have an important function in gastric inflammatory response to H. pylori infection in children. IL-18, and possibly CD14 receptor signaling pathway, may be involved in macrophage activation and subsequent IL-8 and IL-18 release.

Initially, IL-18 has also multiple biologic functions such as promoting the production of granulocyte-macrophage-colony-stimulating factor (GM-CSF) and IL-2 and activating NK cells and macrophages (Golab, 2000). Hue et al., (2005) found that IL-18 plays a critical role as a regulatory factor for stem cell factor (SCF) expression via ROI and p38 MAPK.

Kim et al. (2007) identified the pro-metastatic function of VEGF that stimulates cell migration of gastric cancer cells through IL-18 production. Moreover, VEGF stimulates IL-18 mRNA and protein levels, which then affect the migration ability of gastric cancer cells. Neutralization of IL-18 by using IL-18 binding protein (IL-18BP)
has also been shown to decrease hepatic metastasis of melanoma cells (Carrascal et al., 2003). Jiang et al. (2003) also demonstrated that IL-18 is an important stimulator of lung cancer metastasis. IL-18 stimulates transmigration of human myeloid leukemia cell line, HL-60 through MMP-9 (Zhang et al., 2004). Pro-angiogenic function of IL-18 in cancer was verified. IL-18 stimulates thrombospondin-1 (TSP-1) production which is a pro-angiogenic factor in gastric cancer (2004). IL-18 is increased by VEGF via the regulation of reactive oxygen species (ROI) and the ERK1/2 pathway. This means that pro-angiogenic factors can also induce the maturation of IL-18, suggesting a positive relationship between IL-18 and VEGF production in cancer. IL-18 stimulates the binding of AP-1 to VEGF promoter, resulting in VEGF production (Park et al., 2007). Amin et al. (2007) reported that IL-18 increases the production of various angiogenic factors such as stromal cell-derived factor 1α (SDF-1α)/CXCL12, MCP-1/CCL2, and VEGF in rheumatoid arthritis (RA) synovial tissue (ST) through the phosphorylation of JNK-1/2, PI3K, p38MAPK, PKC and NF-κB.

While evaluating the effect of age, smoking and drinking on the IL18 mRNA and protein expression on patients with uro-epithelial cancer, no significant difference has been observed in patients with different age-groups, smokers and non-smokers, and drinkers and non-drinkers.

It was clearly seen that mRNA and protein expression in individuals carrying IL-18 (CC) were higher than those carrying IL-18 (GG) genotype. Generally, this trend has been observed between those carrying at least one C allele (GC+CC) and homozygous for G allele. No significant difference was found for mRNA expression between patients and controls with one genotype. Protein expression between patients and controls having at least one C allele (GC+CC) was significantly different.

In summary, this is the first report on the elevated serum concentration of IL-18 in bladder cancer patients. Irrespective of IL-18 biological activity, it is suggested to as a biomarker of developing bladder cancer. Further studies regarding mechanism of IL-18 expression and its pathway, determination of the expression of IL-18 in tumour tissue of bladder cancer, correlation between the IL-18 serum level and recurrent cancer and follow-up of cancer therapy as clinical application need to be persuaded.
6.4.3 Expression profile and study of microsatellite in first intron of IFN-γ in uro-epithelial cancer

In the present investigation, IFN-γ level in the sera was significantly enhanced in patients with bladder cancer (208.9 pg/ml) as compared to healthy controls (186.3 pg/ml), but mRNA expression in PBMC of patients was slightly increased, but the difference was not significant as compared to healthy controls.

Interferons belong to a protein family involved in viral replication prevention, cell growth inhibition and cell differentiation modulation (Borden and Balkwill, 1999). The IFNs are divided into three main classes: alpha, beta, and gamma, and are defined by their differences in amino acid sequences, physicochemical properties, and induction by different agents from different cell types. The inducing agents include viruses, bacteria, bacterial products, polymers, low molecular weight compounds, and antigens or mitogens. Studies on the mechanisms of action of IFNs have mainly been focused on their antiviral actions. IFN gamma employed alone and in combination with IFN alpha may dramatically increase IFNs activity (Stanton et al., 1987).

IFN-γ has been described as a 17 KDa peptide that is secreted by antigen activated lymphocytes and natural killer cells. It has also been reported that IFN-γ has antiviral activity, inhibits cell growth and modulates cell differentiation (Gutterman, 1994). IFN-γ acts throughout a specific membrane receptor, which is composed of two different subunits: IFNγ-Rα and IFNγ-Rβ (Soh et al., 1994). IFNγ-Rα is sufficient for ligand binding, but the presence of IFNγ-Rβ is necessary to begin the IFNγ signal transduction (Linehan et al., 1990). IFN-γ receptor complex has been described in a number of different tissues as endothelial cells, fibroblasts, neuronal cells, melanocytes and prostate cells (Aguet et al., 1988; Royuela et al., 2000). In addition to its role in immune cells, IFN-γ inhibits the growth of a number of nonhematopoietic cell types, including several tumour types. In fact, it has been considered as an antitumour agent (Havat et al., 1996; Muller et al., 1996; Fujishima et al., 1998).

The pro-inflammatory cytokine IFN-γ plays an important role in diverse cellular processes, including activating innate and adaptive immune responses against pathogens and tumours, inhibiting cell proliferation, and inducing apoptosis (Boehm et al., 1997;
Stark et al., 1998). Activation of adaptive immune responses by IFN-γ is in part due to transcriptional induction of genes encoding MHC class I and class II antigens, invariant chain, HLA-DM/H2-DM, transporters associated with antigens processing (TAPs) and the immunoproteasome subunits LMP-2, LMP-7, and LMP-10 (Boehm et al., 1997).

The regulation of cellular responses to IFN-γ is very complex and is mediated by the equilibrium between the activities of the JAKs and STAT-1, and a number of negative regulatory molecules, which include SOCS-1 (by interacting with JAK-2 and either inhibiting kinase activity), protein inhibitor of activated STAT (PIAS) (by PIAS1 directly blocks IFN-γ-inducible transcription by inhibiting STAT-1 DNA-binding activity), and PTPs (by suppressing the initiation of IFN-γ signaling in human choriocarcinoma cells) (Shuai et al., 2003).

Induction of apoptosis and cell-cycle arrest occurs through activation of CASPase and p21 gene expression, respectively (Boehm et al., 1997; Stark et al., 1998). It has been shown that IFN-γ causes this antitumoural effect by up-regulating the expression of p21 and resulting cell cycle arrest in breast cancer cell lines (Gooch et al., 2000). In prostate cancer cells, it has been reported that IFN-γ apoptotic effects are promoted by p21 stimulation, which inhibits G1 and S phase of cell cycle (Hobeika et al., 1998). In breast cancer cell lines, IFN-γ treatment produces an increase in p21 (Gooch et al., 2000).

p21 has been detected in the cytoplasm of epithelial cells in situ carcinomas, and p21 cytoplasmic product does not reach to enter the nucleus, and therefore, it would not be functional and would not contribute to the cell cycle arrest. Furthermore, this p21 location has been related to breast cancer resistance to the TNF-α apoptotic effect, and it could be explained by the IFN-γ effects mediated by TNF-RI. IFN-γ effects mediated by TNF-RI would be inhibited by the presence of p21 in the cell cytoplasm, the p21 location in situ carcinomas would be capable to inhibit the apoptotic pathway of TNF-RI at ASK-1 level, and thus, to prevent the IFN-γ function as cell cycle inhibitor. Briefly, IFN-γ could be non-functional and unable to activate p21 to stop the cell cycle (García-Tuñón et al., 2007).

IFN-gamma regulates the immunogenicity of target cells by increasing the expression of HLA class I molecules (Malmberg et al., 2002). Fas-mediated apoptosis is
one of the mechanisms for IFN-gamma to exercise its anti-tumour effect (Zhang et al., 2002). Langaas et al. (2001) demonstrated that IFN-γ sensitizes human colon carcinoma cells to TRAIL-mediated apoptosis, partly by elevated CASPase-8 expression.

It has been indicated that IFN-gamma sensitizes human myeloid leukemic cells (Varela et al., 2001) and breast tumour cells (Ruiz-Ruiz et al., 2000) to a death receptor-induced, mitochondria-mediated pathway of apoptosis through the elevation of CASPase-8 levels. IFN-γ-induced up-regulation of HLA-DR results in a potent enhancement of the in vivo antimaleroma activity (Carlo-Stella et al., 2007). Inaba et al. (2004) reported that IFN-γ sensitizes osteosarcoma cells to Fas-induced apoptosis through up-regulation of Fas receptor and CASPase-8.

As far as our knowledge goes, no studies of IFN-γ mRNA and protein expression are available in the literature, in malignant and carcinomatous human uro-epithelial tissue. The aim of this study was to elucidate the expression patterns of IFN-γ in human bladder cancer, and to relate stages of tumour tissue.

It was observed that majority of patients with bladder cancer had high concentrations of serum IFN-γ. These results have suggested that IFN-γ participates in the immune responses during the progression of uro-epithelial cancer. The mRNA expression of IFN-γ in PBMC has been found to be slightly increased in patients with bladder cancer (the mean average of IFN-γ: β-actin ratio was 0.224) and healthy controls (IFN-γ:β-actin ratio = 0.204), it has therefore been come to light that the cellular source of circulating IFN-γ in serum of patients could not be totally from PBMCs and it is possible that IFN-γ secreted by tumour-infiltrating inflammatory cells gains access to circulation.

As it was found that IFN-γ serum levels are increased in higher stages of bladder cancer, this finding can be considered as hypothesis that the cells around the tumour could also be one of IFN-γ secretion sources. Also, it has also been observed that mRNA expression in PBMC is increased in higher stages of bladder cancer. The difference has not significantly been between primitive (Ta and T1) and progressive (T2 and T3) stages.

Champelovier et al. (2004) demonstrated that in the low-grade bladder cancer cell lines, the effect of IFN-γ is dose dependent (i) high doses (>5 ng/ml) induce apoptosis, whereas low doses (<5 ng/ml) induce a resistance to the cytotoxic effect of tumour
necrosis factor alpha and increase the metastatic potential. Csiszár et al. (2001) have demonstrated that changes in IFN-γ and TNF-α mRNA in TIL and tumour cells could be related to tumour progress or causation of metastases, respectively. In breast cancer patients with skin metastasis, local injection of IFN-γ results in the total or partial regression of the skin lesions (Habi et al., 1995). It has been shown that IFN-γ increases the growth inhibitory effect of tamoxifen in breast metastatic carcinomas (Macheleidt et al., 1991; Seymour et al., 1993). Gao et al. (2008) demonstrated that IFN-γ can decrease the adhesiveness and invasiveness of the prostate cancer cell line by down-regulating the expression of annexin-2. According to Le Poole et al. (2001) IFN-γ may enhance inflammatory responses, yet hamper effective recognition of melanoma cells.

In the study on the effect of age, smoking and drinking on the IFN-γ mRNA and protein expression in patients with uro-epithelial cancer, the amount of IFN-γ expression was slightly decreased in drinkers and smokers, but no significant difference of mRNA and even protein expression was observed between smoker and non-smoker; drinker and non-drinker patients, and it has led to the conclusion that in spite of confirmed role of risk factors like smoking and drinking in tumourigenesis, the present study failed to find significant correlation on the expression of cytokines and consequently, the immune response of host against bladder tumours.

The ability of IFN-γ to inhibit the growth of several tumour cell lines, including breast cancer cells, has been demonstrated in different studies (Wadler et al., 1990; Kirchhoff et al., 1999; Ruiz-Ruiz et al., 2000). This effect requires the signal transduction through the IFN-γ receptor, so the tumour proliferation rate has been found to be higher in animal cells that expressed lower number of functional receptors (Dighe et al., 1994; Doherty et al., 1996).

Yamashita et al. (2001) reported the up-regulation of IFN-γ inducible genes and oxidative stress-inducible genes in both the chronic hepatitis C (CH-C) and hepatocellular carcinoma (HCC). Aberle et al. (2000) indicated that reduced IFN-gamma expression may be an important factor in the pathogenesis of severe respiratory syncytial virus (RSV) disease in infancy. Cui et al. (2007) have reported that Th1 cytokines (IFN-γ, TNF-α, IL-12A and IL-18) are increased in local tissues of colorectal carcinoma.
(CRA) and decreased in colorectal adenoma (CRC). Csizsár et al. (2004) detected that more IFN-γ transcripts in PBMC samples from patients with colorectal cancer than those from normal controls.

As far as our knowledge goes, functional significance of the relationship between two polymorphisms A+874T and microsatellite CA repeat in first intron and expression of IFN-γ and susceptibility against of bladder cancer was investigated for the first time. The mRNA and protein expression in individuals carrying IFN-γ (TT) were elevated as compared to those having IFN-γ (AA) genotype. This trend was observed between those carrying at least one T allele (TA+TT) and homozygous for A allele. However, no significant difference was found for mRNA expression between patients and controls with same genotype. Protein expression between patients and controls having at least one T allele (TA+AA) was found to be significantly different.

Pravica et al. (2000) reported that T-allele to be associated with high IFN-γ production in healthy control population. Lo’pez-Maderuelo et al. (2003) found that IFN-γ production to be closely linked to the +874(T/A) alleles that allele A homozygous patients with progression of pulmonary tuberculosis produced significantly lower levels of IFN-γ as compared with those carrying one or two copies of allele T.

Microsatellites can be used as polymorphic dinucleotide repeats that occur within genes for several human cytokines, including IFN-γ. Five alleles of the human IFN-γ gene, which differ by number of CA repeats in the first intron have been described. Sequence analysis has shown that allele #1 corresponds to 11 CA repeats, allele #2 corresponds to 12 repeats and alleles #3 to #5 have 13-15 repeats (Awad et al., 1999).

Regarding the microsatellite CA repeats in first intron of IFN-γ, it was seen that those carrying IFN-γ (+874 T/T) were also homozygous for 12 CA repeat (allele #2) and surprisingly, those with IFN-γ (+874 T/A) were heterozygous for allele #2 and those with genotype of IFN-γ (+874 A/A) lacking any allele #2. Elevated levels of expression of mRNA and protein expression was observed in the carriers of allele #2 (12CA repeat), but significant difference between patients and controls was found only in protein expression in those carrying at least one copy of allele #2 (or T allele). mRNA and protein expression level were increased in higher stages in patients carrying...
Patients homozygous for allele #2, was not observed in higher stages. It might be indicated the role of allele #2 (12 CA repeat) in immune response. This finding was in agreement with those of other workers (Pravica et al., 1999 and 2000; Khani-Hanjani et al., 2000).

Pravica et al. (1999) stated that allele #2 is associated with the production of higher concentrations of IFN-γ in vitro. Pravica et al. (2000) reported that individuals homozygous for allele #2 produced significantly more IFN-γ than those having other allelic combinations. Khani-Hanjani et al. (2000) described CA repeat microsatellite sequence to be associated with rheumatoid arthritis susceptibility and severity in Canadians. Pravica et al. (1999) demonstrated that CA repeat polymorphism to be strongly associated with the post-transplant outcome of lung graft survival it may serve as a useful prognostic indicator of chronic transplant rejection resulting from graft fibrosis and may, therefore, help in tailoring immunosuppressive therapy.

The mechanisms of altered gene expression associated with polymorphisms are still poorly understood. There is an evidence that sequence changing in non-coding regions of cytokine genes may influence production of the corresponding peptide due to linkage with another markers directly affecting gene expression (Lo'pez-Maderuelo et al., 2003). Among numerous intronic polymorphisms in the IFN-γ gene, +874(A/T) polymorphism is related to the translation start site located at the 5' end of the CA repeat region that correlates with the presence or absence of the high-producing allele #2. It has been speculated that this polymorphic site may be affecting IFN-γ gene expression as it coincides with a putative NF-κB binding site (Pravica et al., 2000). This T to A polymorphism coincides with the middle of a putative NF-κB binding site (Heinemeyer et al., 1998), which could have functional consequences for the transcription of the human IFN-γ gene. The consensus NF-κB motif is GGANTYYCC (Sica et al., 1992), with 'N'; representing any nucleotide and ‘Y’ representing C or T. The -874*T allele creates the DNA sequence AATCTC.

Considering the role of IFN-γ in inhibition of cell proliferation and cell-inducing apoptosis, administration of IFN-γ is one of prominent options in immunotherapy of cancer and IFN treatment combined with chemotherapy may also give enhanced anti-
tumour activity. Inaba et al. (2004) combined immunotherapy with IFN-gamma and either anti-Fas monoclonal antibody or cytotoxic T cells that bear Fas ligand might be a useful adjunctive therapy for patients with osteosarcoma. Blanck et al. (2002) stated this hypothesis that IFN-gamma signaling pathway is anti-tumourigenic. Few drugs have stimulated as much research interest or clinical promise as the IFNs. Clinical trials in patients have shown most promise in coryza, herpes virus infections, papilloma virus tumours, hairy cell leukemia, multiple myeloma, and renal cell carcinoma (Stanton et al., 1987). Detjen et al. (2001) found that IFN-gamma treatment profoundly inhibits anchorage dependent and independent growth of pancreatic cancer cells. Interferon-gamma could provide a promising anti-tumour therapeutic approach as it has been described to enhance cellular susceptibility to apoptosis in a variety of tumour cells (Ruiz de Almodóvar et al., 2004). IFN-$\gamma$ treatment of short-term ovarian carcinoma cell lines (OVACs) had resulted in resistance of tumour cells to lysis by peptide- and allospecific CD8(+) T cells (Malmberg et al., 2002). Ramani et al. (1987) found that pre-treatment with rMuIFN-$\gamma$ (recombinant murine IFN-gamma) render colon cell line resistant to \textit{in vivo} NK-cell lysis via a mechanism that does not involve changes in MHC expression.

In summery, this is the first study that has demonstrated elevated serum concentration of IFN-$\gamma$ in bladder cancer patients. IFN-$\gamma$ serum level may be as a biomarker of developing bladder cancer. There are several reports regarding the role of BCG vaccine in treatment of bladder cancer through increased IFN-$\gamma$ level (O'Donnell et al., 1999; Ikeda et al., 2002; Nadler et al., 2003). The correlation between the IFN-$\gamma$ serum level and recurrent cancer and its role in immunotherapy of bladder cancer has been suggested for further study.

### 6.5 Methylation pattern of \textit{CASP-8} and \textit{Rb1} in uro-epithelial cancer and its effect on their expression

This present study was conducted to evaluate the promoter methylation of \textit{CASP-8} (a key component of the death-inducing signaling complex (DISC) and \textit{Rb1} gene (as key gene in cell cycle regulator) in tumour tissue samples of patients with bladder cancer. The difference of RNA expression between methylated and unmethylated states of these two
genes has been analyzed to find whether or not methylation of the promoter has any effect on the RNA transcription? The effect of etiological risk factors like smoking and drinking and clinicopathology (like age, stage and grade on methylation in bladder cancer) was evaluated.

Methylation of various genes including RARβ, DAPK, E-cadherin, p15, p16, MGMT, and GSTP1 was reported in transitional carcinoma cell (TCC) of bladder in different stages and grades (Chan et al., 2002). As far as our knowledge goes, this is the first study to identify CASP-8 and Rbl methylation in bladder cancer.

There has not been significant trend towards increasing risk of early onset disease with methylation of CASP-8 gene in tumour tissue, this condition is completely reverse among patients having age more than 60 years. Methylation of CASP-8 takes place in patients in the age-group of more than 60 years, whereas it was observed that methylation of Rbl occur in both the age-groups of patients and it enabled us to conclude that methylation of Rbl is one of the factors for the early onset of bladder cancer. It seems increasing age has a main role in methylation of CASP-8 which is one of the main executioner gene in apoptotic pathway induced by TNF-α and FAS ligands in cell death program. Age does not have any impact on the methylation of Rbl. However, the methylation after 60 years of old seems more prone to increased risk of bladder cancer.

Bladder cancer is more common in men than in women, with a worldwide male: female ratio of 10:3 (Globocan-2002: http://www-dep.iarc.fr/globocan/database.htm.). In the study of gender effect and methylation of two genes in bladder cancer, the above mentioned pattern was again repeated in male.

In the present population, it was found that methylation of CASP-8 and Rbl in tumour tissue plays an important role in developing bladder cancer. Bladder tumours are graded as low (GI), intermediate (GII), or high (GIII). Grading is more important for non-invasive tumours because almost all invasive neoplasms (T1 or more) are high grade. For invasive tumours, stage (Not grade) is the most important independent prognostic variable for progression and overall survival (Devito et al., 1999). It was observed that methylation of CASP-8 and Rbl did not take place in low grade (GI) tumour. Methylation of CASP-8 and Rbl was mostly starts in intermediate grade (G2). Methylation of CASP-8
was significantly correlated with bladder cancer of high grade (G3), whereas correlation between methylation of \textit{RBI} and bladder cancer was found in grade 2 and more. Interestingly, this pattern was observed to be related with the progression of disease. Significant correlation between methylation of \textit{Rbl} and bladder cancer was found in stage pT2 and greater, whereas this situation for \textit{CASP-8} was in stage pT3 only. It is noted that invasion to the superficial muscle takes place in stage T2. Therefore, it can be concluded that methylation of \textit{Rbl} plays a significant role in early onset of muscle-invasive of tumor, but methylation of \textit{CASP-8} is significant in higher stage when tumor already penetrated to deep muscles and near to metastasis.

The effect of smoking and drinking as two main etiological risk factors on the methylation of these two key genes was studied. It was found that methylation has mostly been occurred among drinker and particularly smoker patients. It was observed that smoking might be having effect on methylation of these two genes in bladder cancer. Interestingly, methylation of \textit{CASP-8} and \textit{RBI} was mostly observed in patients who smoked pack-years more than 44.7.

While considering the result of study on the effect of age on methylation, it was observed that the exposure of people to carcinogens including tobacco smoking or tobacco chewing with the increasing age increased the risk of methylation of \textit{CASP-8} and \textit{Rbl} and consequently, the probability of developing bladder cancer to higher stages and grades, because of significant occurrence of \textit{Rbl} methylation (as a regulator cell cycle) in higher stages of bladder cancer.

The impact of drinking of alcohol on methylation pattern was also studied. Although the above pattern was again repeated in this case also, the role of alcohol in methylation and consequently increasing risk for developing bladder cancer was not so much like tobacco smoking. Methylation of \textit{CASP-8} and \textit{Rbl} was mostly appeared among drinker patients particularly in those consuming high doses of alcohol. Therefore, it was concluded that consumption alcohol almost more than 30 units per week correlates with methylation of \textit{CASP-8} and increased risk of bladder cancer. It could be concluded that age, smoking and drinking will increase the probability of methylation of these genes.
in people and consequently increased risk of developing of bladder cancer because mostly methylation happened in higher stage and histological grade.

Aberrant hypermethylation of CpG islands in the promoter region of genes is associated with transcriptional silencing and is a frequent event in human cancers (Jones and Baylin, 2002). Epigenetic events, a key driving force in the development of cancer, are alterations in gene expression without changes in the DNA coding sequence that are heritable through cell division. Such changes occur throughout all stages of tumourigenesis, including the early phases, and are increasingly recognized as major mechanisms involved in silencing of genes (Costello and Plass, 2001). Hypermethylation results in loss of expression of a variety of genes critical in the development of breast cancer. These include steroid receptor genes, cell adhesion genes, and inhibitors of matrix metalloproteinases genes (Yang et al., 2001). Transcriptional silencing by the hypermethylation of CpG islands in the promoter regions has been recognized as a common mechanism for the inactivation of cancer-related genes (Murao et al., 2006). The essential promoter region of \( Rbl \) lies 185–206 bp upstream of the initiation codon and contains putative binding sites for the transcription factors RBF-1, Sp1, ATF, and E2F (Ohtani-Fujita et al., 1993). Cote et al. (1998) found that in invasive transitional carcinomas of the bladder, loss of \( Rbl \) expression, together with p53 protein accumulation, is associated with significantly shorter survival. The hypermethylation is associated with transcriptional silencing of the gene and, therefore, acts as an alternative mechanism of inactivation of a tumour suppressor gene allele (Baylin et al., 1998; McCabe et al., 2005). Genes identified as hypermethylated in cancer cells include tumour suppressor genes of clear biological significance already known to be involved in bladder tumourigenesis, such as \( Rbl, p16^{INK4a}, \text{ and } p14^{ARF} \) (Greger et al., 1989; Sakai et al., 1991; Jones and Laird, 1999). One mechanism by which methylation can be increased is by up-regulation of expression of DNA methyltransferase-1 (DNMT1), which occurs in cells with loss of Rb function (Merloet et al., 1995).

Decreasing the RNA transcription by methylation of promoter of CASP-8 and Rbl was proved, but the complete silencing of these genes has not been seen in patients with bladder cancer. Interestingly, significant reduction of \( CASP-8 \) expression because of
methylation has been observed only in smoker and drinker patients (specially patients with pack-years >44.7 and alcohol-unit >30 per week) with age more than 60 years old, whereas methylation in promoter led to significant decrease of \( Rbl \) expression in both the age-groups of patients, smokers and drinkers as well as non-smokers and non-drinker patients with bladder cancer. It can thus be concluded that methylation by itself may be has a main effect on reducing the expression of \( Rbl \), but not in \( CASP-8 \).

Meanwhile, It was observed that significant decrease in the expression of \( Rbl \) started from stage T2 (starting invasion to the superficial muscle) with intermediate histological grade (G2), whereas this significant reduction of expression of \( CASP-8 \) was seen in stage T3a or greater and high grade (G3). Consequently, it can be deduced that methylation in promoter of \( RB1 \) plays a main role in the progression and development of bladder cancer.

Briefly, it could be concluded that methylation of \( CASP-8 \) and \( Rbl \), may have a main role in developing bladder cancer, and this role is strengthened the presence of certain ethological risk factors like smoking and drinking. Methylation of \( Rbl \) in tissues in intermediate phase may have a role in progression of bladder cancer in higher stage and grade. It seems that methylation of \( Rbl \) in patients with bladder cancer can be considered as one of prognosis indicators for development and progression of tumours. However, further study on methylation of \( Rbl \) in the other populations whether or not it can be considered as biomarker of development of bladder tumours and study of the role demethylating agents to inhibit the progression of bladder tumours or at least its acceleration is needed.