2.1. ANATOMY OF BLADDER

The urinary bladder is a hollow, muscular and distensible organ that sits on the pelvic floor (superior to the prostate in males). On its anterior border lies the pubic symphysis and, on its posterior border, the vagina (in females) and rectum (in males). The urinary bladder is normally capable of storing 1.1-1.3 liters of urine, but because it is made up of transitional epithelium, it is able to stretch to volumes of even several liters (Spiritus-Temporis, 2005; McKinley, 2008).

![Fig. 1: Anatomy of bladder (McKinley, 2008)](image)

2.2. CANCER

Cancer is generally defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells. It is a major public health burden in the United States and in other developed countries. Currently, one out of four deaths in the United States is due to cancer (Jemal et al., 2007).
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2.3. BLADDER CANCER

According to a World Health Organization report, during the year 2005, cancer killed approximately 826,000 people in India, of which, nearly 519,000 were under the age of 70 years. The study also indicated that in 2005, amongst the 10 leading causes of cancer deaths in India, bladder cancer ranked 8th and the subsequent death rate per 100,000 people was 4 (World Health Organization, 2005) (Table 2.1).

Table 2.1: Incidence of bladder cancer in the world (WHO, 2005)

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence Rate of Bladder Cancer (per 100,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>35</td>
</tr>
<tr>
<td>Denmark</td>
<td>31</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>30</td>
</tr>
<tr>
<td>Finland</td>
<td>22</td>
</tr>
<tr>
<td>Italy</td>
<td>21</td>
</tr>
<tr>
<td>Israel</td>
<td>20</td>
</tr>
<tr>
<td>England</td>
<td>18</td>
</tr>
<tr>
<td>Australia</td>
<td>16</td>
</tr>
<tr>
<td>Russia</td>
<td>14</td>
</tr>
<tr>
<td>Sweden</td>
<td>13</td>
</tr>
<tr>
<td>Japan</td>
<td>10</td>
</tr>
<tr>
<td>India</td>
<td>6</td>
</tr>
<tr>
<td>China</td>
<td>4</td>
</tr>
<tr>
<td>Iran</td>
<td>4</td>
</tr>
</tbody>
</table>

According to the US National Cancer Institute, bladder cancer affected approximately 68810 people in America in 2008 and estimated 14100 people probably had died from this disease. Bladder cancer is three times more common in men than in women and is the
fourth most common cancers and second most common urologic cancer found amongst men in the United States.

In India, amongst the genitourinary cancers, carcinoma of the urinary bladder is one of the leading causes of death (Vaish et al., 2005).

The incidence of bladder cancer is different between genders and races and the underlying reasons are currently not well understood (American Cancer Society, 2006).

During the recent decades, the overall incidence of bladder cancer has appeared to be rising and this may be due to the latent effects of tobacco abuse and industrial carcinogens, as well as the overall ageing of our population (Ries et al., 2003).

In the same report of the American Cancer Society (2006), bladder cancer is a disease of older individuals, with greater than 90% of diagnoses in patients over 55 years of age; although uncommon, bladder cancer can occur in young adults and even in children. Most bladder cancers present as a "superficial" disease, confined to the bladder mucosa or submucosal layer, without muscle invasion. Superficial tumours consist of papillary tumours that are mucosally confined (Ta), papillary or sessile tumours extending into the lamina propria (T1), and carcinoma in situ, which occurs as "flat" mucosal dysplasia, which can be focal, diffuse, or associated with a papillary or sessile tumour (Amling, 2001).

2.3.1. ETIOLOGY

The etiology of bladder cancer appears to be multifactorial with exogenous environmental factors, as well as endogenous molecular factors, playing possible roles. First postulated by Rehn (1895), the link between bladder cancer and environmental carcinogens has long been observed (Smith et al., 1999). A large body of epidemiologic evidence linking bladder cancer to certain chemical agents, occupations, and industries has been generated since this time. As
the bladder functions as a reservoir of urine, it is, therefore, possible that it is susceptible to the effects of a variety of potential environmental carcinogens in the process of waste elimination. Rising rates of bladder cancer in recent decades, the increased incidences observed in industrial countries, and the relatively long latency periods observed between exposure and cancer development suggest a potentially cumulative effect of carcinogens on malignant transformation of the urothelial lining of bladder (Liu et al., 2005; Samanic et al., 2006).

2.3.2. RISK FACTORS RELATED TO THE BLADDER CANCER

Several chemical and environmental exposures have been linked to the development of bladder cancer. However, in most cases, the long latency period between the exposure and development of clinical disease has limited the ability to establish causality (Matthew and Galsky, 2007).

2.3.2.1. Industry-related carcinogens

A link between chemical carcinogens and the development of bladder cancer was first made in the early 1800. Since then, multiple compounds have been implicated. The majority of these carcinogens are aromatic amines, including 2-naphthamine, 4-aminobiphenyl and benzidine, or derivative of aromatic amines. The time from exposure to death from bladder cancer has been estimated to be over 20 years (Golka et al., 2006).

Occupations linked to the development of bladder cancer include workers in the aluminum, dye, paint, petroleum, rubber and textile industries. Occupations involving exposure to combustion gases including chimney sweepers, truck drivers and garage or gas station workers have also been associated with an increased risk (Goebell et al., 2004).

Several case-control studies have suggested an increased risk
in hairdressers and barbers, thought to be related to exposure to hair dyes (Gago-Dominguez et al., 2001). However, no significant association has been shown with personal hair dye use (Andrew et al., 2004).

2.3.2.2. Tobacco Smoking

Cigarette smoking is the most important risk factor for bladder cancer and is thought to contribute to 1 to 2 of every three diagnosed cases of bladder cancer (Zeegers et al., 2002).

The risk of developing bladder cancer increases with the extent of exposure to cigarette smoking; heavy smokers have a six-to ten fold increase in risk as compared with non-smokers. Smoking cessation leads to a decreased risk. In a meta-analysis of case-control studies, the risk decreased by 60% after 25 years (Brennan et al., 2000).

So far, the precise carcinogens in tobacco linked to the development of bladder cancer are not clear. However, the aromatic amines, 2-naphthylamine and 4-aminobiphenyl and heterocyclic amine Trp-p-2 are likely candidates (Riedel et al., 2006).

2.3.2.3. Diet

Several dietary factors have been implicated in the development of bladder cancer. A case-control study suggested an increased risk with the consumption of fried or fatty foods and a decreased risk with fruit and vegetable intake (Radosavljević et al., 2005).

The quality of drinking water, through the by-products of water chlorination and concentration of arsenic, has also been associated with an increased risk (Janković and Radosavljević, 2007).

However, the consumption of coffee and dietary sweeteners has not convincingly been associated with the development of bladder cancer (Matthew and Galsky, 2007).
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2.3.2.4. Drugs

Heavy use of the analgesic phenacetin has been associated with chronic renal disease and development of cancer of the ureter and renal pelvis (Kaye et al., 2001).

Exposure to certain drugs, like cyclophosphamide used in chemotherapy and heavy consumption of phenacetin-containing analgesics, has been shown to cause bladder cancer and in patients with a history of superficial bladder cancer, a single dose of cyclophosphamide poses a significantly increased risk of tumour recurrence in the lower as well as upper urinary tract (Volkmer et al., 2005; Janković and Radosavljević, 2007).

2.3.2.5. Others

Pelvic irradiation for prostate, cervical, or ovarian cancer increases the risk of a secondary bladder cancer (Maroñas et al., 2005).

Schistosomiasis, caused by the trematode Schistosoma haematobium, is an endemic to the Middle East, Southeast Asia, and Africa. Bilharzial bladder disease, secondary to infection with S. haematobium, has been linked to the development of bladder cancer (Ross et al., 2002).

In endemic regions, bladder cancer is the most common solid tumour. The majority of cases are squamous cell carcinomas. Paraplegic patients (often with bladder stones or chronic indwelling Foley catheters) are also thought to be at an increased risk for squamous cell carcinoma of the bladder (Matthew and Galsky, 2007).

2.3.3. Molecular Mechanisms for Urothelial Carcinogenesis

Abnormal metabolic pathways and molecular instabilities may likewise play a role in bladder cancer development and progression. These include: (1) altered metabolism/detoxification of carcinogens,
and (2) inherent or acquired genetic abnormalities that may promote tumour development (oncogenes), inhibit tumour cell proliferation (tumour suppressor genes), or impair DNA repair (DNA repair enzymes). Pathways involved in altered chemical metabolism of exogenous carcinogens have included aberrant cytochrome P450 metabolism (associated genetic defects), glutathione-s-transferase abnormalities, and N-acetyltransferase genetic and metabolic derangements (Marcus et al., 2000; Engel et al., 2002; Hazra et al., 2006).

In addition, DNA abnormalities may be inherent or acquired secondarily to carcinogenic exposure. Genetic instability may result in abnormal activity of oncogenes (e.g., ras and myc families) resulting in aberrant protein expression (e.g., GDP/GTP binding proteins), cellular proliferation, and resistance to apoptosis (Theodorescu, 2004; Hazra et al., 2006).

Abnormalities in tumour suppressor genes that may be mutated or inactivated, and such defects associated with bladder cancer have also been well studied. These may thereby predispose to cell cycle dysregulation and tumour cell development and progression (Malats et al., 2005).

Similarly, alterations in DNA repair (e.g., ner, ber and dsb repair genes) (have also been associated with polymorphisms) may result in bladder urothelial carcinogenesis (Sanyal et al., 2004; Matullo et al., 2005).

Furthermore, other potential inherent and acquired pathways including telomere dysfunction, apoptosis and cellular inflammation have also been identified and may also be involved (Wu et al., 2003; Leibovici et al., 2005).

2.3.4. MAJOR PATHOLOGIC SUBTYPES

More than 90% of tumours are conventional urothelial carcinoma (Transitional cell carcinoma) and the rest consisting of
urothelial carcinoma with aberrant differentiation (squamous/glandular differentiation, small cell carcinoma, sarcomatoid carcinoma and micropapillary carcinoma) may have worse prognosis and response to therapeutic than pure urothelial carcinoma or non-urothelial carcinoma (squamous cell carcinoma and adenocarcinoma) (Black et al., 2008).

Amongst these, the adenocarcinomas account for approximately 2.5% of all urothelial neoplasms (Bitar et al., 2007), while the squamous cell carcinoma accounts for upto 65% of bladder cancers in certain regions of the world in which schistosomiasis (also known as Bilharziasis) infection is endemic (Mungadi and Malami, 2007).

2.3.5. PRESENTATION AND DIAGNOSIS

Hematuria, occurring in the majority of patients with bladder cancer, is continuous or intermittent and either visible (gross) or microscopic. From microscopic hematuria screening studies, it has been estimated that approximately 1.3% of patients have an underlying diagnosis of bladder cancer (range 0.4 to 6.5%), although this is more likely in patients with gross hematuria (Golin and Howard, 1980; Caremel and Pfister, 2007).

As such, the 2007 AUA Best Practice Policy on Asymptomatic Microscopic Hematuria recommended that all patients with hematuria, particularly those without evidence of infections, stones or other causative factors, should undergo cystoscopy and upper tract imaging.

Irritative voiding symptoms including frequency, urgency and dysuria are particularly associated with carcinoma in situ. Indeed, the diagnosis of bladder cancer is a consideration in patients with irritative voiding symptoms in the absence of infection. The physical examination of patients with bladder cancer is often unremarkable especially in the case of nonmuscle invasive disease. A bimanual
examination of the patient at the time of transurethral resection of the bladder tumour (TURBT) may help with clinical staging, especially for patients with muscle invasive disease. Transurethral resection of bladder tumour provides essential histopathologic information for bladder tumour diagnosis as well as staging and grading of the cancer. The diagnosis of bladder cancer depends on cystoscopy and histologic evaluation of the resected tissue. A complete and correct transurethral resection (TUR) is essential for the prognosis of the patient. When the initial resection is incomplete or when a high-grade or T1 tumour is detected, a second transurethral resection within 2-6 week needs to be performed (American Urological Association, 2007; Babjuk et al., 2008).

Common imaging techniques include intravenous urogram, retrograde pyelography, computerized tomography, and magnetic resonance imaging and fluorescence cystoscopy also used for diagnosis of bladder cancer (Zaak et al., 2005).

2.3.6. URINE-BASED MARKERS

Whereas the diagnosis and surveillance of patients with non-muscle invasive urothelial cancers rely on cystoscopy, cytology and biopsy when necessary, in recent years there has been an intense search for noninvasive adjunctive urine-based markers that could improve or perhaps replace cytology and cystoscopy. These may aid both in the diagnosis and the surveillance of patients with nonmuscle invasive urothelial cancers. Currently available Food and Drug Administration (FDA)-approved tests include the bladder tumour antigen STAT test (Bard Diagnostics, Redmond, WA, USA), the BTA TRAK test (Poly Med Co, Cortlandt Manor, NY, USA), the nuclear matrix protein (NMP) 22, and NMP22 BladderChek assays (Matritech, Newton, MA, USA), ImmunoCyt test (Diagnocure Inc, Quebec City, Quebec, Canada), and fluorescence in situ hybridization (FISH) analysis (Urovysion Systems Vysis, Abbott Laboratories, Abbott Park,
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IL, USA). Other recently investigated tests and identified markers include Quanticyt (Gentian Scientific, Niawer, The Netherlands), BLCA-4, hyaluronic acid, telomerase, LewisX blood group antigens, microsatellite polymorphism analysis, cytokeratins, and surviving (Gaston and Pruthi, 2004; Konety, 2006).

2.3.7. TUMOUR CHARACTERISTICS

2.3.7.1. Staging

The bladder cancer is divided into clinical and pathological stages. Clinical stage reflects the histologic findings at TURBT, the clinician’s physical exam (including bimanual exam under anesthesia), and findings on radiologic imaging. Pathological staging (also known as surgical staging) is based on the extent of disease following surgical resection of the bladder (partial versus radical cystectomy) and of the adjacent pelvic lymph nodes. In the past, the panel avoided using the term "superficial" in their report when categorizing the three nonmuscle invasive stages of bladder cancer, Ta, T1, and Tis. The panel agrees with the International Society of Urological Pathology’s recommendation that use of such terms should be discouraged as Ta, T1, and Tis tumours behave differently from one another particularly with regard to tumour recurrence and progression (Epstein et al., 1998).

Currently, the staging system of the American Joint Committee on cancer, also known as the Tumour-Node-Metastases (TNM) classification, is the most commonly used and universally accepted staging system for bladder cancer (Greene et al., 2002). Under this system, nonmuscle invasive tumours include: (1) papillary tumours confined to the epithelial mucosa (stage Ta), (2) tumours invading the subepithelial tissue (i.e., lamina propria; T1), and (3) Tis (Table 2.2).
Table 2.2: Staging of primary tumours (T) in bladder cancer
(http://health.ucsd.edu/cancer/patcare/urologic/bladder/staging.htm; Greene et al., 2002)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>Ta</td>
<td>Noninvasive papillary carcinoma</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour invades lamina propria</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour invades muscularis propria</td>
</tr>
<tr>
<td>T2a</td>
<td>Invades superficial muscularis propria (inner half)</td>
</tr>
<tr>
<td>T2b</td>
<td>Invades deep muscularis propria (outer half)</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour invades perivesical tissue/fat</td>
</tr>
<tr>
<td>T3a</td>
<td>Invades perivesical tissue/fat microscopically</td>
</tr>
<tr>
<td>T3b</td>
<td>Invades perivesical tissue fat macroscopically (extravesical mass)</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour invades prostate, uterus, vagina, pelvic wall, or abdominal wall</td>
</tr>
<tr>
<td>T4a</td>
<td>Invades adjacent organs (uterus, ovaries, prostate stoma)</td>
</tr>
<tr>
<td>T4b</td>
<td>Invades pelvic wall and/or abdominal wall</td>
</tr>
</tbody>
</table>

Data gathered during the past few decades has demonstrated that approximately 70 to 75% of bladder cancers present as nonmuscle invasive tumours. The term noninvasive bladder cancer should be used and qualified with the appropriate American Joint Committee on Cancer Stage (i.e., Ta, T1, Tis). Seventy percent of noninvasive cancers occur as stage Ta, 20% as T1, and 10% as Tis. The invasion refers to muscularis propria involvement and not that of lamina propria. Therefore, stage T1 cancer invades lamina propria, but is classified as noninvasive (Jones, 2006;
On the other hand, the majority of Tis cases occur in association with high-grade nodular tumours, only 3 to 5% occur as isolated Tis disease (Lopez-Beltran and Cheng, 2006).
malignant potential was created to describe lesions with an increased number of urothelial layers when compared with papilloma but without cytologic features of malignancy.

The authors also categorized nonmuscle invasive papillary carcinomas as either low or high grade. This classification attempts to avoid the classification of intermediate grade (1973 WHO Grade 2), the grade that often represents the default grade diagnosis (Eble et al., 2004; Pavone-Macaluso et al., 2006). Indeed some reports have shown that Grade 2 tumours represent upto 65% of urothelial carcinoma diagnosed (Sylvester et al., 2005). Under the new system, some Grade 2 lesions are classified as low grade and others as high-grade tumours. This new system is to potentially allow for enhanced prognostic significance, but certainly is to be greatly dependent on the pathologist for making these distinctions.

2.3.8. OTHER PROGNOSTIC INDICATORS

Although stage and grade represent perhaps the most important features of nonmuscle invasive urothelial neoplasm with regard to prognosis, other important clinical, histologic and molecular features may also help predict prognosis and therapeutic response (Millan-Rodriquez European meta-analysis). Tumour multiplicity and tumour size represent two often used clinical features with prognostic significance with regard to disease recurrence (Sylvester et al., 2006). Some authors have evaluated the potential prognostic value of histologically sub staging T1 tumours to portend disease recurrence, response to therapy, and ultimately progression (Bernardini et al., 2001; Orsola et al., 2005).

A variety of other molecular markers in bladder cancer have been studied with regard to their ability to predict disease recurrence, response to therapy, and progression. These include flow cytometry, blood group antigens (e.g., Lewis X), tumour suppressor genes (e.g., p53 and Rb), proliferative indices (Ki-67), urinary growth factors (e.g.,
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epidermal growth factor, basic fibroblast growth factor, and CD44), matrix metalloproteinases (e.g., MMP-9), and urinary plasminogen activator (uPA) (Lopez-Beltran et al., 2004; Habuchi et al., 2005). Such indicators may prove to be valuable adjuncts to stage and grade in the management of nonmuscle invasive urothelial cancers in future.

2.4. SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

Inherited differences in DNA sequence contribute to phenotypic variation, influencing an individual's anthropometric characteristics, risk of disease and response to the environment. A central goal of genetics is to pinpoint the DNA variants that contribute most significantly to population variation in each trait (Sachidanandam et al., 2001).

Single nucleotide polymorphisms (SNPs) serve as effective markers for localizing disease susceptibility genes (Wenlei et al., 2006). By a strict definition, SNP is a site where two nucleotides have been found in a specific population with the minor allele present in greater than 1% (Lewin, 2006).

An analogous disease-causing mutation typically has an allele frequency of less than 1% with highly penetrating phenotype. The sequencing of the human genome was completed in April 2003.

The humans are almost identical to each other in their genomic DNA sequences. On an average, our genomes differ only by 0.1% from each other and we are approximately 98.8% identical to chimpanzees at the nucleotide level (Clark et al., 2003).

Various groups studying polymorphisms and human diseases however identified various DNA variations. The mapping of the genome has not only accelerated the cloning of disease associated genes but also increased our understanding of how disease-causing variations differ from normal polymorphisms (http://www.sanger.ac.uk/Info/Press/2003/030414.shtml 2003).
The majority of SNPs have a minor allele frequency of less than 10%. These are relatively new variations found only in specific populations. Individual genes also differ in their nucleotide diversity (Sachidanandam et al., 2001). A critical step in complex disease gene mapping is to identify the most informative markers to use in linkage and association analysis. Single nucleotide polymorphisms (SNPs) are preferred to other genetic markers because of their high abundance and ease for genotyping. It has been estimated that a polymorphism (an SNP with minor allele frequency > 1%) occurs about once every 290 base pairs, which allows us to infer the existence of 11 million SNPs among the 3.2 billion base pair human genome (Kruglyak and Nickerson, 2001). These new alleles are introduced to gene loci by spontaneous endogenous processes or induced by various exogenous agents, such as UV radiation or tobacco smoke. Although these processes are rare, they constantly create new variations in the human population (Matthew and Galsky, 2007). However, the fate of the new mutation is dependent on its effect on the phenotype.

2.4.1. CYTOKINES

Cytokines are soluble mediators secreted by many cell types in response to several stimuli and they reach the peak level very early. For example, TNF-α in 1h, IL-1β in 2h and IL-6 in 3h. They significantly regulate and determine the nature of the immune response by promoting survival, proliferation, differentiation and activation of cells or cell death. Cytokines are not usually stored as preformed molecules and their synthesis is usually initiated by new gene transcription as a result of cellular activation. Such transcriptional activation is transient and the mRNAs encoding most cytokines are unstable, so cytokine synthesis is also transient. The production of some cytokines may be additionally controlled by RNA processing and by posttranscriptional mechanisms, such as...
proteolytic release of an active product from an inactive precursor. Once synthesized, cytokines are rapidly secreted, resulting in a burst of release as needed (Janis and Kuby, 2007).

Like hormones, cytokines initiate action by binding to specific receptors on the cell surface of target cells. However, unlike hormones, cytokines are usually made by a variety of cells, rather than in a gland, and act locally (autocrine or paracrine) instead of distally (endocrine). Cytokines may be pleiotropic (one cytokine, multiple effects), redundant (multiple cytokines, one effect) and antagonist (one cytokine inhibits another cytokine). They act at very low concentrations (μg/ml and pg/ml) and to achieve a maximal biological effect, only a fraction of their receptors are required to be occupied. The interaction of cytokines can be synergistic, inhibitory or modulating. Many cytokines have been cloned, sequenced and characterized, which has further promoted research on them. Cytokine functions are regulated in multiple ways: at the levels of both the producer (by means of transcriptional, translational and post-translational regulation) and the target (the accessibility of the specific receptor or its signal transduction can be modulated) cells. The binding capacity of a cytokine can be modified in many ways: by decreasing the number or affinity of its receptors, receptor antagonists, non-receptor binding proteins, anti-cytokines autoantibodies and soluble cytokine receptors (Janis and Kuby, 2007).

Cytokines play an important role in the pathogenesis of many solid cancers. Several single nucleotide polymorphisms (SNPs) identified in cytokine genes are thought to influence the expression or function of these proteins and many have been evaluated for their role in inflammatory disease and cancer predisposition (Balasubramanian et al., 2006).

Originally, the cytokines were named according to their function (e.g., T cell growth factor, now called IL-2), but with the pleiotropy of
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cytokines observed, this made functional specific names confusing. Main classes of cytokines include:

- **Interleukins** - regulate interactions between leukocytes
- **Interferons** - response to viral infections (α, β and γ)
- **Chemokines** - chemotaxis and inflammation (e.g., GM-CSF)
- **Tumour necrosis factor** - derived from macrophages, antitumour activity

Interleukins are cytokines that are secreted from leukocytes. Cytokines that are secreted from lymphocytes are also termed lymphokines, while those secreted by monocytes or macrophages are termed as monokines. The term interleukin derives from the fact that these cytokines are secreted from leukocytes, but also affect the cellular responses of leukocytes. More specifically, interleukins are growth factors targeted to cells of haematopoietic origin (Abbas and Andrew, 2005).

As the inflammatory process leads to the activation of the cytokine network, in the early phase of this process, proinflammatory cytokines (TNF-α, IL1-β, IFN-γ and IL-12) are released. The activity of proinflammatory cytokines is counteracted by the production of anti-inflammatory cytokines (IL-4, IL-10, IL-13 and TGF-β) and soluble inhibitors of proinflammatory cytokines (soluble TNF-α receptor, soluble IL-1 receptor, and IL-1 receptor antagonist) (Cavaillon and Duff, 1999; van der Poll and van Deventer, 1999; Golab, 2000). Thus, the key point in the influence of cytokines on infection is the balance between the proinflammatory and anti-inflammatory cytokines, for example in the bacterial infection, the balance between IL-10 and TNF-α (Lenz et al., 2007). Current data support the notion that inflammation triggered by tumour-infiltrating host leukocytes does not always exert normal immunoprotective mechanisms which could lead to eradication of the evolving cancer (antitumour immunity). Instead, excessively and chronically produced
proinflammatory mediators are thought to contribute to tumour promotion and progression (Balkwil et al., 2005; Ben-Baruch, 2006) as, in the tumour microenvironment, a delicate balance occurs between antitumour immunity and tumour-originated proinflammatory activity (Ben-Baruch, 2006; Kim et al., 2006). These activities depend on different mediators that are released by host inflammatory, cancer, and other types of tumour associated host (such as fibroblasts and endothelial cells) cells. When host mediated antitumour activity is weaker than tumour mediated immunosuppressive activity, tumour cells undergo immune escape and grow rapidly. By contrast, when the host mediated antitumour immunity is stronger than the tumour mediated immunosuppressive activity, tumour cells are eliminated (Hadden, 2003). The net outcome of a persistent inflammatory microenvironment is enhanced tumour promotion, accelerated tumour progression, invasion of the surrounding tissues, angiogenesis and often metastasis (Lin and Karin, 2007).

Several proinflammatory cytokines are known to promote tumour growth, such as tumour necrosis factor (TNF-\(\alpha\)), interleukin 1 (IL-1), interleukin 6 (IL-6) or interleukin 8 (IL-8) (Pikarsky et al., 2004; Karin and Greten, 2005).

In addition, a link between activating mutations in oncogenes and inflammation has recently been reported, as activation of Ras proto-oncogenes in cancer results in up-regulation of the inflammatory cytokine IL-8, which, in turn, acts as a chemokine that promotes tumour associated inflammation, angiogenesis and eventually tumour growth (Karin, 2005).

The precise molecular events leading to malignant transformation in an environment of chronic inflammation have not been fully elucidated. Cancer cells, in order to facilitate a survival advantage, may usurp some physiologic functions of the immune system. For instance, tumour associated macrophages frequently
infiltrate neoplastic tissues and provide the tumour with angiogenic and lymphangiogenic growth factors, cytokines, and proteases — all of which enhance cancer progression (Schoppmann et al., 2002).

In addition, proinflammatory signals that target the elimination of infection in the acute phase of inflammation subsequently switch their function from the killing of the intruder to tissue healing, thereby providing further growth opportunities for incipient tumours (Nathan, 2002). Studies have suggested an association between chronic inflammation, modulating the tissue microenvironment, and tumour biology. Tumour environment consists of tumour, stromal and endothelial cells, and infiltrating macrophages, T lymphocytes, and dendritic cells, producing an array of cytokines, chemokines and growth factors, accounting for a complex cell interaction and regulation of differentiation, activation, function and survival of tumour and surrounding cells, responsible for tumour progression and spreading or induction of antitumour immune responses and rejection (Lin and Karin, 2007). Indeed, it has been shown that cancer is preceded by chronic inflammation in many cases (Pelekanou et al., 2008). Examples include inflammatory bowel disease and colorectal cancer (Freeman, 2008), chronic bronchitis and lung cancer (Kampa and Castanas, 2008), papillomavirus infection and cervical cancer (Datta et al., 2008), Barrett’s metaplasia and oesophageal cancer (Hawk and Viner, 2008), H. pylori–induced gastritis and gastric cancer (Franco et al., 2008), and persistent inflammation of the bladder, as with schistosomiasis or chronic indwelling catheters and bladder cancer (Kim et al., 2008).

Cytokines, expressed by various immune cells, bind to specific receptors and activate distinct signal pathways to transcriptionally activate a plethora of downstream factors (Kidd, 2003).

The role of cytokines in cancer immunity and carcinogenesis in general has been well established (Smyth et al., 2004). Single nucleotide polymorphisms in specific candidate genes are thought to
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...influence expression and/or activity of the encoding proteins thereby predisposing to solid cancers especially bladder cancer (Nonomura et al., 2006).

Many cytokine polymorphisms have been studied for associations with susceptibility to gastric (Lu et al., 2005; Zambon et al., 2005), liver (Nieters et al., 2005), lung (Campa et al., 2005), prostate (Burmester et al., 2004) and ovarian (Hefler et al., 2002) cancers but with mixed results.

Cytokines secreted by tumour and inflammatory/immune cells can either promote tumour development and tumour cell survival or exert antitumour effects. Chronic inflammation develops through the action of various inflammatory mediators, including TNF-α, IL-6, and IL-17, leading to eradication of antitumour immunity and accelerated tumour progression. However, TNF-related apoptosis-inducing ligand (TRAIL), through direct induction of tumour cell apoptosis, IL-10, through antiinflammatory effects, and IL-12, through activation of CTLs and NK cells and expression of cytotoxic mediators, can lead to tumour suppression. The multiple actions of TGF-β (cytotoxic in cancer cells, and having both positive and negative effects on the tumour microenvironment) and IL-23 (Fig. 2.3) explain their dual roles in tumour development.
Fig. 2.3: Two outcomes of interactions between tumour cells and infiltrating inflammatory and/or immune cells in the tumour microenvironment (Lin and Karin, 2007).

2.4.1.1. IL-6

2.4.1.1.1. IL-6 as a proinflammatory cytokine

IL-6, a phosphorylated glycoprotein containing 185 amino acids, which is a pleiotropic cytokine with a wide range of biological activities in immune regulation, haematopoiesis, inflammation, and oncogenesis, is produced by lymphoid and nonlymphoid cells, such as T and B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several kinds of tumour cells and involved in regulation of the immune response, acute phase reaction, and cell
Interleukin-6 (IL-6) is an immunomodulatory cytokine and also plays a role in growth stimulation, metastasis, and angiogenesis in secondary tumours in a variety of malignancies. IL-6 can be released from tumour infiltrating leukocytes, but is produced to a large extent by tumour cells themselves (Brozek W., 2008).

Furthermore, IL-6 activates target genes involved in differentiation, survival, apoptosis and proliferation (Heinrich et al., 2003).

The pleiotropy and redundancy of IL-6 functions have been identified by using a unique receptor system comprising two functional proteins: an IL-6 receptor (IL-6R) and gp130, the common signal transducer of cytokines related to IL-6. Signal transduction through gp130 is mediated by two pathways: the JAK-STAT (Janus family tyrosine kinase-signal transducer and activator of transcription) pathway and the Ras mitogen-activated protein kinase pathway. The negative regulators of IL-6 signaling have also been identified, although the physiological roles of the molecules are not yet fully understood. The pathological roles of IL-6 have also been clarified in various conditions, such as inflammatory, autoimmune, and malignant (Naka et al., 2002) diseases.
It has been suggested that IL-6 may play a significant role in the pathophysiology of cancer. Several clinical reports have highlighted the prognostic importance of IL-6 in a variety of human solid tumours, including prostate (Wegiel et al., 2008), breast (Sansone et al., 2007), bladder (Sanchez-Carbayo et al., 2001) and ovarian (Hefler et al., 2003) cancers, suggesting that circulating IL-6 level is correlated with the extent of disease and disease recurrence, and is associated with worse survival. In some of these tumours, higher levels of IL-6 were found in patients unresponsive to hormone therapy, radiotherapy or chemotherapy (Sanchez-Carbayo et al., 2001; Wegiel et al., 2008).

However, whether IL-6 is directly involved in the development of treatment resistance is not clear as yet.

2.4.1.1.2. IL-6 gene and polymorphic sites

The human IL-6 gene is located at chromosome 7p21 (Sehgal et al., 1986) and consists of 5 exons and 4 introns (Zilberstein et al., 1986) (Fig. 2.5). Within the gene, the positions of exon/intron boundaries, exon lengths, and location of cysteine residues within exons are conserved across species. The amino acid sequence identity is 96% between human and macaque and 39% between human and mouse IL-6 (Simpson et al., 1997).

![Fig 2.5: Human IL-6 gene. Closed and open boxes represent the coding and non-coding regions respectively, of the transcripts (Zilberstein et al., 1986).](image)

Polymorphisms in the promoter region of the IL-6 gene may result in inter-individual variations in transcription and expression. Genetic variants could, therefore, influence an individual's
susceptibility to a diverse range of diseases. There are precedents for this; for example, the $\text{TNFa-308TNF2}$ allele is associated with elevated risk of cerebral malaria (McGuire et al., 1999). Fishman and Olomolaiye (1998) submitted $-174$ C/G polymorphism and $A_nT_n$ polymorphism to GenBank. They investigated that a polymorphism in the 5' flanking region of the $\text{IL-6}$ gene at position $-174$ (G→C) appears to affect its transcription, and the presence of the C allele may be associated with systemic onset juvenile chronic arthritis.

Understanding the influence of genetic variation on the control of IL-6 expression may provide insight into inter-individual variations in disease risk and underlying pathogenesis.

Polymorphisms do not exist in isolation, and it may be the combination of base changes at several sites, i.e. the haplotype, that influence function. The 5' region of the $\text{IL-6}$ promoter at position $-572$ (G→C), and position $-597$ (G→A) and the effect of the $-373$ AT run polymorphism and the $-174$ (G→C) points substitution relative to transcription factor binding sites (Fishman et al., 1998). The $-550$ to $+61$ bp of $\text{IL-6}$ constructs transfected into HeLa cells have been compared in reporter gene studies (Fishman et al., 1998). Furthermore, the region between $-225$ and $-164$ has previously been reported to represent a negative regulatory domain (Ray et al., 1990).

In the promoter region at position $-373$, there is also a polymorphic $A_nT_n$ tract with six alleles (Fishman et al., 1998) and previous data have demonstrated that the $-174C$ allele is usually associated with the $A_8T_{12}$ pattern.

In the study by Fishman et al. (1998), 19 subjects out of 20 analysed had this combination and correspondingly, in the study by Terry et al. (2000), 39 subjects out of 40 analysed had it (Fishman et al., 1998; Terry et al., 2000). In addition, the genotypes of the $-597G$→A polymorphism are in strong allelic association with $-174$ genotypes (Brull et al., 2001).
Terry et al. (2000) studied the functional effects of four polymorphisms (−597G→A, −572G→C, −373AnTn, −174G→C) on the IL-6 promoter and found that these different haplotypes may have an important role in determining the transcription levels of the IL-6 gene.

2.4.1.1.3. Association studies on the IL-6 −174 polymorphism

A common G/C polymorphism of the IL-6 promoter on position −174 has been investigated in a wide variety of diseases, including acute myocardial infarction (Licastro et al., 2007), breast (Hefler et al., 2005) and ovarian (Hefler et al., 2003) cancers.

In the first report of 102 healthy subjects, the IL-6 −174CC homozygotes had the lowest IL-6 plasma levels and the reporter vector analyses in the same study supported this in in vivo finding (Fishman et al., 1998). However, the relationship between the IL-6 genotypes and IL-6 plasma levels appears to be more complex. Results from subsequent large scale studies showed no significant association between IL-6 levels and the −174 polymorphism among healthy subjects (Rauramaa et al., 2000; Kilpinen et al., 2001; Veres et al., 2002). In a group of patients undergoing elective coronary artery bypass graft surgery (CABG), preoperative IL-6 levels did not differ between genotypes (Brull et al., 2001). Secondly, the C allele and the CC genotype were related to higher IL-6 levels in subjects with chronic inflammation (Jones et al., 2001), or in subjects after acute severe injury (Brull et al., 2001). On the other hand, the GG genotype was associated with elevated plasma levels in primary Sjogren's syndrome patients and in HIV-infected males with Kaposi sarcoma – both disease conditions with chronic inflammation.

Subsequent studies of this polymorphism showed that the −174 C allele decreases susceptibility to systemic juvenile idiopathic arthritis (Ogilvie et al., 2003) and increases the risk of coronary artery disease presumably through inflammatory mechanisms (Georges et al., 2001; Humphries et al., 2001). It was also shown to increase the
risk of bladder (Leibovici et al., 2005), colorectal (Landi et al., 2003) cancers and Kaposi's sarcoma in HIV infected men (Foster et al., 2000). On the other hand, Vishnoi et al. (2007) could not find any significant role of -174G/C IL-6 in the susceptibility to gall bladder cancer in Indian population.

Studies by Hefler et al. (2003) also showed that the mutant -174 C allele of IL-6 influences the biological phenotype of ovarian cancer because it is associated with early stage and improved disease-free and overall survival and the presence of at least one mutant allele to be associated with early tumour stage as well as an expanded length of disease-free (DFS) and overall survival with a dose-dependent effect regarding the carriage of 0, 1 and 2 alleles.

2.4.1.2. IL-10

IL-10 polymorphisms are of particular interest in relation to cancer because IL-10 has both immunosuppressive (potentially cancer-promoting) and antiangiogenic (potentially cancer inhibiting) properties. A possible role for IL-10 polymorphisms in modulating cancer susceptibility and tumour development was, therefore, considered in more detail (Howell et al., 2007).

The IL-10 gene is comprised of 5 exons that span approximately 5.2 Kb and is located on chromosome 1, at 1q31-1q32 (Eskdale et al., 1997).

To date, at least 49 IL-10 associated polymorphisms have been reported, and an even larger number of polymorphisms are recorded in SNP databases (e.g., Ensembl Genome Browser) (European Bioinformatics Institute, 2006). Of these, 49 polymorphisms, 46 are SNPs, 2 microsatellite polymorphisms, and 1 a small (3 bp) deletion. Twenty-eight polymorphisms occur in the promoter region of the gene, 20 are noncoding intronic or synonymous substitutions, and only 1 results in a change in amino acid sequence (Howell, 2006). A simplified configuration of the IL-10 gene shows key SNP and
microsatellite promoter polymorphisms and also examples of intronic (+19) and 3' untranslated region (+117) SNPs to illustrate that polymorphism occurs across the full length of the *IL-10* gene (Fig. 2.6).

**Fig. 2.6: The *IL-10* gene showing key SNPs and microsatellite polymorphisms. Black boxes: exon sequences (Howell et al., 2007).**

Promoter polymorphisms were subject to the most scrutiny, particularly with regard to possible influences on gene transcription and protein production. For example, the *IL-10* -1082 SNP and -1082, -819, and -592 haplotypes were reported to be associated with differential IL-10 expression *in vitro*, with the -1082 A, -819 T, and -592 A haplotypes associated with decreased IL-10 expression compared with the -1082 G, -819 C, and -592 C haplotypes (Turner et al., 1997; Crawley et al., 1999; Hoffmann et al., 2001). This was thought to reflect, at least in part, differential transcription factor binding associated with -1082 SNP (Reuss et al., 2002). In addition, *IL-10*R and G microsatellite haplotypes were also been shown to be associated with differential levels of IL-10 expression *in vitro* (Reuss et al., 2002).

Some workers suggested that as much as 75% of inter-individual variation in IL-10 expression might be a result of genetic variation (Eskdale et al., 1998), although others believed that the contribution of individual SNPs, such as the best-described -1082 SNP, might be much less than this (Westendorp et al., 1997). However, the precise role of *IL-10* promoter polymorphisms, both individually and as part of defined haplotypes and mosaics, in *IL-10* transcription and expression levels is still a subject under active investigation.
Howell et al. (2005) reviewed 22 studies in 13 different malignancies with respect to associations between IL-10 polymorphisms and cancer susceptibility/prognosis. In 17 of these studies, positive associations between IL-10 genotype or haplotype and disease susceptibility and/or progression were reported. In some of these cancers, genotypes associated with low IL-10 expression were a risk factor for disease or disease progression, while in others genotypes associated with high IL-10 expression were a risk factor. Published findings in breast cancer are as yet conflicting. Most, but not all of the studies reviewed were based on small sample sizes and hence, a limited number of IL-10 polymorphisms. However, the preliminary data obtained thus far indicate that larger studies are required in a number of cancers, in order to confirm initial results; studies need to be extended on more detailed genotype/haplotype analysis and combined genotype and gene expression studies in the same subjects. Such studies will significantly contribute to our understanding of the biological role of IL-10 in cancer development.

Although a large number of investigations of possible associations between IL-10 genotypes and immune-mediated disease and were performed, the literature with regard to IL10 polymorphisms and cancer is as yet small, but is growing. Following Table 2.3 are some of the published studies including 13 different malignancies.

Table 2.3: IL-10 polymorphisms and cancer

<table>
<thead>
<tr>
<th>Disease</th>
<th>IL-10 polymorphism</th>
<th>Cases</th>
<th>Controls</th>
<th>Association</th>
<th>Genotype, allele or haplotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutaneous malignant melanoma</td>
<td>-1082, -819, -592</td>
<td>153</td>
<td>158</td>
<td>Susceptibility, advanced stage of disease, greater tumour thickness</td>
<td>-1082 AA</td>
<td>Howell et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Greater tumour thickness</td>
<td>ACC/ACC, ACC/ATA, ATA/ATA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Noninvasive growth phase</td>
<td>-1082 GG</td>
<td></td>
</tr>
</tbody>
</table>
## Review of literature

<table>
<thead>
<tr>
<th>Disease</th>
<th>IL-10 polymorphism</th>
<th>Cases</th>
<th>Controls</th>
<th>Association</th>
<th>Genotype, allele or haplotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninvasive growth phase</td>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Survival (shorter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ACC/ATA</td>
<td>Martinez-Escribano et al. (2002)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>-1082</td>
<td>247</td>
<td>263</td>
<td>Susceptibility</td>
<td>-1082 AA</td>
<td>McCarron et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>-1082, -819, -592</td>
<td>1320</td>
<td>1255</td>
<td>No</td>
<td>—</td>
<td>Michaud et al. (2006)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>-1082</td>
<td>144</td>
<td>263</td>
<td>No</td>
<td>—</td>
<td>Smith et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>-1082</td>
<td>125</td>
<td>100</td>
<td>Susceptibility</td>
<td>-1082 AA</td>
<td>Giordani et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>-592</td>
<td>500</td>
<td>500</td>
<td>Protection</td>
<td>-592 AA</td>
<td>Langsenlehner et al. (2005)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>-1082</td>
<td>77</td>
<td>69</td>
<td>Susceptibility</td>
<td>-1082 AG</td>
<td>Stanczuk et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>-1082, -819, -592</td>
<td>144</td>
<td>179</td>
<td>No</td>
<td>—</td>
<td>Roh et al. (2002)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>-1082, -819, -592</td>
<td>220</td>
<td>230</td>
<td>Susceptibility, advanced stage</td>
<td>GCC (1 or 2 copies)</td>
<td>Wu et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>-1082</td>
<td>150</td>
<td>220</td>
<td>Association with EBV - negative gastric carcinoma</td>
<td>-1082 G allele</td>
<td>Wu et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>-1082, -819, -592</td>
<td>188</td>
<td>212</td>
<td>Susceptibility (noncardia gastric cancer)</td>
<td>ATA haplotype</td>
<td>El-Omar et al. (2003)</td>
</tr>
<tr>
<td>Squamous cell carcinoma of skin (post renal transplant)</td>
<td>-1082, -819, -592</td>
<td>70</td>
<td>70</td>
<td>Susceptibility</td>
<td>GCC haplotype</td>
<td>Alamartine et al. (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protection</td>
<td>ATA haplotype</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma (post HBV infection)</td>
<td>-1082, -819, -592, +117</td>
<td>230</td>
<td>792</td>
<td>Susceptibility, accelerated age of onset</td>
<td>ACCT haplotype</td>
<td>Shin et al. (2003)</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>-1082</td>
<td>166</td>
<td>161</td>
<td>Susceptibility</td>
<td>-1082 AA</td>
<td>Havranek et al. (2005)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>IL-10G, IL-10R microsatellites</td>
<td>73</td>
<td>109</td>
<td>Susceptibility</td>
<td>IL-10G 136/136, IL-10R 112/114, IL-10R 114/116</td>
<td>Zheng et al. (2001)</td>
</tr>
<tr>
<td>Myelodysplasia</td>
<td>-1082, -819, -592</td>
<td>150</td>
<td>Up to 1000</td>
<td>No</td>
<td>—</td>
<td>Gowans et al. (2002)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Disease</th>
<th>IL-10 polymorphism</th>
<th>Cases Controls</th>
<th>Association</th>
<th>Genotype, allele or haplotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hodgkins lymphoma</td>
<td>−1082, −819, −592</td>
<td>126 302</td>
<td>Susceptibility to aggressive disease</td>
<td>−1082 AA, ATA, ACC haplotypes</td>
<td>Cunningham et al. (2003)</td>
</tr>
<tr>
<td>Non-Hodgkins lymphoma (in AIDS patients)</td>
<td>−592</td>
<td>139 1011</td>
<td>Susceptibility</td>
<td>−592 CC</td>
<td>Breen et al. (2003)</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>−1082, −592</td>
<td>149 111</td>
<td>No</td>
<td>—</td>
<td>Munro et al. (2003)</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>−1082</td>
<td>135 —</td>
<td>Protection from poor response to prednisone treatment</td>
<td>−1082 GG</td>
<td>Lauten et al. (2002)</td>
</tr>
</tbody>
</table>

These published studies included both solid tumours and haematological malignancies and common and less-common diseases. Most of these studies comprise small, single-center case-control investigations, which might be prone to sampling bias and type 1 errors.

It is striking that in 17 of the 23 studies summarized in Table 2.3, positive associations between IL-10 genotype or haplotype and disease susceptibility and/or progression were detected. In some of these cancers (e.g., cutaneous malignant melanoma, noncardia gastric cancer, and renal cell carcinoma), genotypes associated with low IL-10 expression were a risk factor for disease development or disease progression, whereas in others (e.g., cervical cancer, cardia gastric cancer, hepatocellular carcinoma after hepatitis B virus infection, posttransplant squamous cell carcinoma of the skin, and multiple myeloma), genotypes or haplotypes believed by the authors to be associated with high IL-10 expression were a risk factor.

Results with respect to breast and prostate cancer and NHL were conflicting. In breast cancer, 4 studies were performed. One small study reported that the −1082 AA low-expression genotype was
associated with susceptibility to the disease. On the other hand, a larger case-control study (500 cases, 500 controls) demonstrated an association between the -592 AA genotype and reduced breast cancer risk. In the same study, no associations were found with respect to IFN-10 -592 genotype and tumour size, histological grading, estrogen or progesterone receptor status, or age at diagnosis. In this study, the -592 SNP was studied as a marker of the -3575, -2763, -1082, -819, and -592 haplotypes with the -592 A allele marking the TCATA low-expression haplotype. Accordingly, these results were interpreted to suggest that genetically programmed low IL-10 expression is protective in susceptibility to breast cancer, but not in further development of the disease (Langsenlehner et al., 2005). However, 2 independent studies failed to confirm these findings, most notably in a larger series of over 2000 breast cancer cases and 2000 controls (Bulpitt et al., 2004).

McCarron et al. (2002) reported associations between the IFN10 -1082 AA “low expression” genotype and susceptibility to prostate cancer. However, a second study failed to confirm this association (Michaud et al., 2006) although patients were recruited to this study as part of a screening trial and so might have had less advanced disease.

One study of NHL reported that genotypes and haplotypes associated with low IL-10 expression to be a risk factor for aggressive disease, whereas a second study suggested that genotypes associated with increased IL-10 expression to be a risk factor for the development of the disease in patients with AIDS. Another study on diffuse large B-cell lymphoma (the most frequent form of NHL in North America and Western Europe) suggested that although the IFN-10 -1082 G (high expression) allele might be a risk factor for disease susceptibility (at a marginal level of significance), this same allele is protective in patients with NHL.
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Taken together, these apparently conflicting data might reflect the dual biological function of IL-10 as an antiinflammatory (potentially cancer-promoting) and antiangiogenic (potentially cancer-inhibiting) cytokine in relation to the biological growth patterns of the cancer in question. Alternatively, association between IL-10 genotype and IL-10 expression might be differing between tissues and disease states and antigenic and nonantigenic stimuli. In any event, at this stage, all of the above findings should be regarded as highly preliminary because of the small sample sizes of most of the studies, limited numbers of IL-10 polymorphisms examined, and differing ethnicity of the study groups. In addition, only a few studies examined levels of IL-10 production in vivo in the subjects genotyped.

The preliminary data obtained thus far indicate that much larger studies are required on a number of common cancers to confirm initial results, extend studies to include more detailed genotype and haplotype analyses, and combine genotype and gene expression studies in the same subjects. In this way, our understanding of the biological role of IL-10 in tumour development will be greatly increased, with implications for cytokine immunotherapy in cancer. Besides, although results from studies of IL-10 polymorphism and breast cancer are at best conflicting, further studies of other cytokine and related growth factor polymorphisms in this disease are certainly required. A number of studies investigated SNPs in TNF-α (Smith et al., 2004) and other cytokines (Ito et al., 2002; DeMichele et al., 2003) in breast cancer also, but with conflicting findings. A number of studies investigated transforming growth factor-β SNPs and reported predisposing effects (Ito et al., 2002; DeMichele et al., 2003; Saha et al., 2004; Lee et al., 2005), including a large case-control study (Dunning et al., 2003).

Alamartine et al. (2003) investigated a possible association between IL-10 gene promoter polymorphisms and the occurrence of skin carcinomas after renal transplantation. Seventy kidney
transplant recipients who developed a squamous cell carcinoma or a basal cell carcinoma were examined for polymorphisms in the IL-10 gene promoter using PCR-based methods. Single base pair mutations were studied at positions −1082, −819, and −592. These patients were compared to 70 healthy controls and to 70 matched renal transplant recipients without cancer. The IL-10 secretion capability was tested in a subgroup of 40 of these patients by *in vitro* stimulation of peripheral mononuclear cells. IL-10 genotypes and haplotypes were differently distributed in kidney transplant recipients who developed a skin carcinoma, especially a squamous cell carcinoma, with an increased frequency of the GCC haplotype and a decreased frequency of the ATA haplotype. The author found a shift in the predicted phenotypes from the low production phenotype to the high production phenotype. Secretion of IL-10 was strongly correlated to the production predicted phenotype, and tended to be higher in patients who developed a squamous cell carcinoma than in the others. These results indicated that IL-10 gene polymorphisms and IL-10 production capability may contribute to the development of skin squamous cell carcinomas after renal transplantation.

*IL-10* is thought to play a key role in psoriasis. Its highly polymorphic promoter contains 2 informative microsatellites, *IL-10.G* and *IL-10.R*. Asadullah *et al.* (2001) analyzed *IL-10* promoter polymorphisms in 78 patients and 80 healthy controls. The distribution of *IL-10.G* and *IL-10.R* microsatellite alleles did not vary between patients and controls. In addition, when the psoriasis patients were stratified according to age of onset (younger than 40, or 40 and older), no difference in allele distribution was observed; however, a clear differential distribution was revealed at the *IL-10.G* locus when patients were stratified according to whether they had a positive family history of psoriasis (*p* = 0.04). This difference was due to an overrepresentation of the *IL-10.G13* allele in those patients with familial disease (40.4% vs 19.6%, *p* = 0.007). The positive association of allele *IL-10.G13* with familial psoriasis was especially
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strong when patients with early onset were compared with those with early onset against a non-familial background (39.6% vs 14.5%, p = 0.003). Patients with age of onset of less than 40 were 4-fold more likely to have a psoriatic family background if they carried the IL-10.G13 allele. These data suggested that the IL-10 locus contributes to the heritability of psoriasis susceptibility.

Wei et al. (2007) investigated that interindividual variations in IL-10 production were due to the polymorphisms within IL-10 promoter region. Their study was to determine whether single nucleotide polymorphisms (SNPs) at positions −1082 (A/G), −819 (T/C) and −592 (A/C) in the IL-10 gene promoter were involved in predisposing an individual to nasopharyngeal carcinoma. The genotypes of one hundred and ninety-eight patients with NPC and 210 age- and sex-matched controls were determined using polymerase chain reaction-restriction fragment length polymorphism. Significant differences in the genotype and allele distribution of -1082 A/G polymorphism of the IL-10 gene among cases and controls were observed. The −1082 AG and GG genotypes were associated with a significantly increased risk of nasopharyngeal carcinoma as compared with the −1082 AA genotypes. Haplotype analysis showed that the homozygosity of the GCC haplotype (defined by SNPs at positions −1082, −819 and −592) of IL-10 gene conveys the highest risk for NPC compared with the homozygosity for the ATA haplotype. This study showed for the first time an association between IL-10 gene promoter −1082 A/G polymorphism and its haplotype that might be contributing to genetic susceptibility to NPC in a Chinese population. Silva et al. (2007) observed tIL-10 1082 A allele to be more frequent in non responders to BCG treatment in patients with superficial bladder cancer, so this result suggested that host genetics of immune regulatory molecules may be playing a role in predicting the antitumoural response after BCG treatment of bladder cancer.
2.4.1.3. IL-12

IL-12 (formerly NKSF, for natural killer cell stimulatory factor, or CLMF, for cytotoxic lymphocyte maturation factor) is a novel cytokine cloned from B-cell lines. It has a broad array of potent biologic activities, acting at picomolar and subpicomolar levels on both T and NK cells.

IL-12 is a heterodimeric cytokine of 70 kDa composed of two chains, a heavy chain of 40 kDa (p40) and a light chain of 35 kDa (p35). The p40 subunit is thought to be primarily involved in receptor binding, while p35 is critical for signal transduction. IL-12 p40 is a very useful reporter gene (D’Aiuto et al., 2008).

The p35 subunit is a member of the four-helix bundle cytokines, IL-6, and granulocyte-colony-stimulating factor, whereas p40 resembles the soluble extracellular domains of the IL-6, growth hormone, and ciliary neurotrophic factor receptors (Merberg, 1992; Dwyer, 1996).

By Southern blot and PCR analysis of DNA from rodent/human cell hybrids containing translocation chromosomes, Sieburth et al. (1992) localized IL-12A, the gene encoding the p35 subunit, to 3p12-q13.2. The gene for the p40 subunit (IL-12B) maps to 5q31-q33 (Schweitzer et al., 1996).

Basile et al. (2008) found that IL-12 appears to have significant clinical potential as a hematological adjuvant cancer therapy.

Interleukin-12 (IL-12) is well-known for its essential role in bridging the innate and adaptive arms of immunity, in regulating inflammatory responses, innate resistance to infection, and adaptive immunity. Moreover, based on its potent antitumour responses in mice, IL-12 was clinically evaluated as a single agent immunotherapy that possibly could provide clinically relevant antitumour responses in humans (Basile et al., 2008).
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Besides forming heterodimers with p35, the p40 subunit may also form homodimers. These p40 homodimers are capable of competing with the p35-p40 heterodimers and so may modulate IL-12 function (Mattner et al., 1993), while many cell types express the p35 chain, the p40 subunit is expressed principally by activated macrophages and B cells, and its expression may be upregulated by interferon gamma. Given the importance of the IL-12 p40 subunit in the immune system, the p70 heterodimer, occurs mainly in dendritic cells, macrophages and monocytes after one of several stimuli (Ebner et al., 2001).

The genomic sequence of the IL-12 p40 subunit was determined Huang et al. (2000). IL-12B gene contains 8 exons, the first and last of which are noncoding. Polymorphisms in IL-12B have been reported at promoter (Morahan et al., 2002), intron 2 (Davoodi-Semiromi et al., 2002), intron 4 (Huang et al., 2000), exon 5 (Noguchi et al., 2001), and 3’ untranslated region (UTR) (Hall et al., 2000; Huang et al., 2000). The allelic variants at 3’ UTR and promoter have different effects on IL-12B mRNA (Morahan et al., 2001) and IL-12p70 protein expression levels (Morahan et al., 2002; Seegers et al., 2002). Whereas no polymorphisms were found in the coding region of this gene, indicating a high level of conservation in humans, several intronic polymorphisms and a TaqI polymorphism in the 3’ UTR at position 1188 were identified (Davoodi-Semiromi et al., 2002; Tsunemi et al., 2002). The latter may have functional relevance, since the 3’UTR region is able to influence the amount of translated protein by several mechanisms, including effects on mRNA stability as well as on transcriptional activity (Grzybowska et al., 2001).

To elucidate the biological significance of the 3’ UTR polymorphism in the etiology or course of a disease, it is mandatory to study the relation between genotype and phenotype. For this reason, Seegels et al. (2002) studied in vitro IL-12 secretion by stimulated human monocytes in relation to this polymorphism.
Fig. 2.7: The polymorphic loci in the *IL-12 p40* gene. The regions are shown by a horizontal line below gene with start and end positions according to the first nucleotide of the initiation codon as +1 (Vasilescu *et al*., 2004).

They found the *TaqI* polymorphism to be associated with increased secretion of IL-12 p70 by stimulated human monocytes. Individuals homozygous for the polymorphism were high producers of IL-12 p70 *in vitro*, non-carriers were low producers, and individuals that were heterozygous were intermediate producers. In contrast, they did not find an association between genotype and the ability of monocytes to produce IL-12 p40.

Interleukin-12 (IL-12) is well characterized to induce cellular antitumoural immunity by activation of NK-cells and T-lymphocytes. However, systemic administration of recombinant human IL-12 resulted in severe toxicity without perceptible therapeutic benefit. Even though intratumoural expression of IL-12 leads to tumour regression and long-term survival in a variety of animal models, clinical trials have not yet shown a significant therapeutic benefit. One major obstacle in the treatment with IL-12 is to overcome the relatively low expression of the therapeutic gene without compromising the safety of such an approach (Wulff *et al*., 2007).

It has been reported in several animal tumour models that administration of IL-12 not only prevented the taking of grafted tumours, but also inhibited the development of established primary or metastatic tumours and induced anti-tumour immunity (Chen *et al*., 1997). In humans, either after intratumour injection of fibroblasts engineered to release IL-12 or systemic administration of rhIL-12 (Atkins *et al*., 1997), some clinical responses have been observed.
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It has been claimed that the anti-tumour efficacy of IL-12 is dependent on its ability to inhibit angiogenesis (Voest et al., 1995).

Induction of a cellular antitumoural immune response is directed not only against the primary lesion, but also against distant organ metastases opening therapeutic options for those patients considered incurable at the present time. Tumour regression at the treatment site as well as in distant organ metastases was illustrated in 1997 utilizing a gene gun technique to incorporate plasmid DNA into intradermal tumours in mice (Rakhmilevich et al., 1997). In this particular tumour model, IL-12 resulted in a superior antitumoural immune response and lasting systemic antitumoural immunity as compared with a variety of other cytokines (Interleukin-2, -4, -6, IFN-γ, TNF-α or GM-CSF).

Despite incomplete understanding of the human immune system, the rapid progress in tumour immunology provides a framework for more effective and safe interventions in the near future. Early approaches in patients with cancer that have focused on the nonspecific and broad stimulation of the immune system by interferons and IL-12 will be replaced by the highly specific stimulation of immune reactions targeting precisely defined tumour antigens. IL-12 has several biologic properties that seem useful in immune therapy for bladder cancer. The striking antitumour responses with IL-12 in preclinical animal models of bladder cancer provide optimism that future clinical trials involving this agent may impact on the risk and mortality associated with this disease (Clinton et al., 2000).

2.4.2. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

Major histocompatibility complex (MHC) class I and II molecules are vital parts of the cellular immune system presenting self and/or foreign peptides to CD8 positive (cytotoxic) and CD4 positive (helper) T cells. Both classes of genes reside in a 4 Mb gene
dense region on human chromosome 6 shared with many other immune genes (Shiina et al., 2004). These four million base pairs of DNA contain 128 genes as well as 96 pseudogenes (non-functional gene remnants). Many, but by no means all of the genes in this complex play important roles in the immune system (Twyman, 2003).

The major histocompatibility complex (MHC) class I is a large family of vertebrate genes that was discovered as the molecular basis for the rejection of grafted tissue (Boulanger, 2004). MHC class I genes play an essential role in the immune defence against intracellular pathogens by binding peptides mainly derived from viral and bacterial proteins and cancer infected cells. They are expressed on the surface of all nucleated somatic cells. Stabilizing these antigens at the cell surface enables interactions with antigen-specific cytotoxic T cells, which subsequently kill infected cells. One of the central features of the adaptive immune response is the ability to quickly and accurately identify and dispatch cellular sources of foreign proteins (Boulanger, 2004).

In contrast, MHC class II genes are predominantly involved in monitoring the extracellular environment by presenting peptides mainly derived from parasites to the T-cells (e.g. bacteria, nematodes, cestodes) (Klein and Horejsi, 1997; Dengjel et al., 2005). They are primarily expressed on antigen-presenting cells of the immune system, such as B cells and macrophages. Within class II genes, most research in mammals focused on the second exon of DRB genes because these loci code for parts of the functionally important antigen binding sites (ABS) (Ohta, 1998). Alternatively, the β-chain in general is used if loci assignment is not possible due to missing information e.g. in teleosts (Reusch et al., 2001; Wegner et al., 2003). The class II region genes are closely linked in humans and all other mammals examined. The variants at these genes are generally in strong linkage equilibrium (Marsh, 2000). Thus, the pattern observed for DRB loci should be a good indicator of the
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genetic variation in other class II genes and even some other less closely linked genes in the MHC (Gutierrez-Espeleta et al., 2001; Stenzel et al., 2004; Kelley et al., 2005).

The MHC has long been believed to be the most important region in the human genome with respect to infection, inflammation, autoimmunity and transplant medicine (Lechler and Warrens, 2000).

This was recently confirmed by the largest genome-wide association study carried for seven common diseases, including two autoimmune diseases (type 1 diabetes and rheumatoid arthritis) and one inflammatory disease (Crohn’s disease). The highest associations were found between the MHC and these two autoimmune diseases (The Wellcome Trust Case Control Consortium, 2007).

The complex etiology of MHC-associated disease coupled with high density, polymorphism, linkage disequilibrium (LD) and frequent non-Mendelian inheritance of gene loci made it challenging to identify variations that cause or contribute to disease phenotypes. The other limiting factors include incomplete knowledge of the allelic variation of genes and regions flanking the nine classical human leukocyte antigen (HLA) loci and the lack of a single haplotype reference sequence, the original reference sequence being a composite of multiple MHC haplotypes (The MHC Sequencing Consortium, 1999; Mungall et al., 2003).

The MHC shows a high degree of polymorphism (100 times higher than the genome average i.e., a 10 per cent difference between any two unrelated individuals). Many of these polymorphisms appear to be associated with either increased or decreased susceptibility to a range of infectious diseases including malaria, tuberculosis, leprosy, typhoid fever, hepatitis and HIV/AIDS. Defects in certain MHC genes lead to autoimmune disorders in which the body fails to recognize self-antigens. Examples of such diseases include multiple sclerosis, some forms of arthritis and diabetes, and
inflammatory bowel disease (Twyman, 2003).

On the other hand, the variability of the MHC-molecules is correlated with the diversity of the T-lymphocyte receptors which in turn determine the disease and parasite resistance of an organism and thus may influence the long-term survival probability of populations (Paterson et al., 1998; Hedrick and Kim, 2001; Hedrick et al., 2001; Langeors et al., 2001). The antigen binding sites show high levels of variation not only in the number of alleles, but also in the extent of sequence variation between alleles (Hughes and Yeager, 1998).

2.4.2.1. TAP (Transporter Antigen Peptide)

Two genes encode components of the proteasome complex, which degrades cytosolic proteins and may generate antigenic peptides which are transported into the endoplasmic reticulum by the product of another set of MHC class II genes, TAP1 and TAP2.

The genes, TAP1 and TAP2, encode subunits of a transporter that presumably translocates peptides into an exocytic compartment where they associate with class I molecules. The location of these genes in the MHC in close linkage to the class I and class II gene families suggests that they coevolved to optimize functional interactions. If this is so, the question is raised whether the proteasome subunits and the transporter genes, like most class I and class II genes, are polymorphic. Allelic variation in the peptide transporter might influence the selection of peptide epitopes presented by class I molecules. Colonna et al. (1992) investigated variability of TAP1 and TAP2 by SSCP analysis and DNA sequencing. They identified several variants and examined possible involvement of the TAP1 and TAP2 genes in susceptibility to MHC-associated diseases such as ankylosing spondylitis, insulin-dependent diabetes mellitus, and coeliac disease. Two allelic variants were identified in the TAP1 and 3 in the TAP2 genes.
RING4 (TAP1), a gene within the human MHC class II region, was found to have sequence homology with members of the ABC (ATP-binding cassette) transporter superfamily (OMIM). TAP2 gene is located 7 kb telomeric to gene family member ABCB2 (ATP-binding cassette) (GenID: 6890-entrez gene).

![Diagram of MHC class II and MHC class I genes and TAP1 and TAP2 genes](image)

**Fig. 2.8:** The location of the TAP genes in the human MHC class II complex (HLA) in a schematic representation (Lankat-Buttgereit and Tampe, 2002).

Neefjes et al. (1993) defined the conditions by which the TAP1 and TAP2 heterodimer is involved in assembly of class I molecules and presentation of endogenous peptides derived from nuclear and cytosolic proteins to CD8(+) T cells.

TAP1 and TAP2 polypeptides possess a nucleotide-binding domain (NBD). Karttunen et al. (2001) presented biochemical and functional evidence that the NBDs of TAP1 and TAP2 are nonequivalent. Photolabeling experiments with 8-azido-ATP demonstrated a cooperative interaction between the 2 NBDs that can be stimulated by peptide. The substitution of key lysine residues in the Walker A motifs of TAP1 and TAP2 suggested that TAP1-mediated ATP hydrolysis is not essential for peptide translocation, but that TAP2-mediated ATP hydrolysis is critical, not only for translocation, but for peptide binding.
By comparative sequence analysis, Ritz et al. (2003) predicted that glu263 or phe265 of TAP1 are critical for TAP transporter function. They found that deletion or mutation of phe265 of TAP1 had little effect on TAP function, whereas deletion or alteration of glu264 had a major effect.

The antigen processing machinery plays an important role in the cellular immune response of vertebrates for the identification of infected or malignantly transformed cells (Trombetta and Parodi, 2003).

Presentation of "non-self" peptides at the cell surface to cytotoxic T-cells triggers the elimination of infected or transformed cells. Lack of TAP expression e.g., in tumour tissue is associated with a dramatic loss of surface major histocompatibility complex class I and consequently promotes strongly the evasion of malignant cells from a proper cellular immune response (Ritz and Seliger, 2001).

Several immune escape mechanisms that allow the metastatic tumour to go undetected have been observed; however, most tumours down-regulate a cassette of genes involved in antigen processing and presentation (Gabathuler et al., 1994; Seliger et al., 1997; Seliger et al., 2000). These genes include those for beta-2 microglobulin, the transporters associated with antigen processing 1 and 2 (TAP-1 and -2), tapasin, the low-molecular-weight proteins LMP-2 and -7, and the proteasome activator PA28 (Gabathuler et al., 1994; Seliger et al., 2000; Lankat-Buttgereit and Tampe, 2002).

Tumours that are deficient in components of the MHC-I antigen presentation pathway, such as TAP and MHC-I, are often observed to escape T cell responses. This is due to failure in the presentation of tumour antigens on the cell surface when TAP and MHC-I are absent (Zhang et al., 2008).

TAP-1 down-regulation has specifically been attributed to tumour growth and metastatic ability and is used as a predictor of
rapid tumour progression and poor survival rates in humans (Alimonti et al., 2000; Seliger et al., 2000; Lankat-Buttgereit and Tampe, 2002).

Vermeulen et al. (2007) have shown that defective TAP expression in cervical carcinoma is often associated with LOH in the TAP region, but not with mutations in the TAP1 gene. A TAP-1 polymorphism in exon 10 region may, however, be a contributing factor for MHC class I down-regulation in colon cancer (Yang et al., 2005).

Loss of TAP function leads to a loss of cell surface expression of MHC class I molecules. This may be a strategy for tumours and virus-infected cells to escape immune surveillance (Lankat-Buttgereit and Tampe, 2002).

TAP2 appears to be involved in some human diseases. Mutation of TAP2 may cause defective processing of HLA I, leading to primary immunodeficiency (Teisserenc et al., 1997). TAP2 mutation has been associated with familial bronchiectasis and with susceptibility to Sjögren’s syndrome (Donato et al., 1995; Kumagai et al., 1997). TAP2 may increase the risk for nickel allergy (Silvennoinen-Kassinen et al., 1997) TAP2 polymorphism may also be involved in inflammatory bowel disease (Heresbach et al., 1997).

Dutta and his colleagues (2006) in India suggested that the establishment of EBV latent infection in gastric tissues allows malignant cells to avoid the immune surveillance of both cytotoxic T-lymphocytes and natural killer cells by regulating the differential expression of HLA class I molecules. Understanding the regulation of TAP2 is essential to elucidate its role in the pathogenesis of these as well as EBV-associated diseases.

The lack of association between TAP and HLA class I antigen expression is compatible with the possibility that multiple mechanisms underlie HLA class I antigen down-regulation in primary ovarian carcinoma lesions. The potential role of immunologic events in the clinical course of ovarian carcinoma suggests that the
association between HLA class I antigen down-regulation and disease progression may reflect the escape of tumour cells from immune recognition and destruction (Vitale et al., 2005).

2.5. EPIGENETICS

Cancer is a polygenetic and polyepigenetic disease (Wong, 2001).

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence. DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target (Wong, 2001).

Epigenetics has evolved as a rapidly developing area of research. Moreover, recent studies have shown that epigenetics plays an important role in cancer biology (Singal and Ginder, 1999; Jones and Baylin, 2002) viral infections (Baylin, 1997), activity of mobile elements (Costello and Plass, 2001), somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health and X-inactivation (Amir et al., 1999; Laird, 2003).

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylation occurs in the sequence context 5'CG3' (also called the CpG dinucleotide), some involve CpA and CpT dinucleotides (Ramsahoye et al., 2000).

In contrast to the rest of the genome, smaller regions of DNA, called CpG islands, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60 to 70%), have a ratio of CpG to GpC of at least 0.6, and thus do not show any suppression of the frequency of the dinucleotide CpG (Antequera and Bird, 1993; Cross and Bird, 1995). Approximately half of all the genes in humans have CpG islands (Antequera and Bird, 1993) and these are present on both
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housekeeping genes and genes with tissue specific patterns of expression (Singal and Ginder, 1999).

The promoter region of a gene is located upstream, at the 5' end, from the transcription start site. Promoter regions of genes are richer in cytosine-guanine dinucleotides than would be expected by chance alone, relative to the rest of the genome (Santini et al., 2001). Many mammalian genes have CpG islands in their promoter regions and are not methylated, if the gene is to be transcribed within the cell. Methylation of cytosine residues in CpG islands, by DNA methyl transferase enzymes, is associated with transcriptional silencing.

Increased methylation in the promoter region of a gene leads to reduced expression, whereas methylation in the transcribed region has a variable effect on gene expression (Jones, 1999; Singal et al., 2002).

Moreover, DNA methylation can also affect histone modifications and chromatin structure, which, in turn, can alter gene expression. The underlying patterns of methylated cytosines are important in guiding histone deacetylation to certain residues (Bender et al., 1998).

Earlier it was suggested that histone modification was secondary to DNA methylation, but some studies on fungus revealed that histone modification can on its own commence the process of DNA methylation (Tamaru and Selker, 2001).

The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation (Fahrner et al., 2002).
2.5.1. HYPERMETHYLATION IN CANCER

Hypermethylation of the CpG islands of gene promoter is one of the earliest and most frequent alterations leading to cancer (Baylin and Herman, 2000; Jones and Baylin, 2002) as well as in development (Partha et al., 2004). It is an important epigenetic mechanism for gene silencing, which may confer tumour cells of growth advantage (Jones and Laird, 1999; Baylin and Herman, 2000). Many cellular pathways are inactivated by this epigenetic event, including DNA repair, cell cycle, apoptosis, cell adherence, and detoxification (Esteller, 2002). Evidence showed that CpG island hypermethylation is widespread in human genome. But it is not randomly distributed in carcinogenesis, and is gene-specific and cancer type specific (Costello et al., 2000; Patel et al., 2003). The specific patterns of CpG island hypermethylation between tumour types may provide a useful signature for tumour diagnosis and prognosis (Esteller, 2003; Patel et al., 2003). Moreover, genes that are frequently aberrantly methylated in specific tumours have been used as molecular targets for the detection of neoplastic cells in body fluids such as urine and plasma which provide additional targets for noninvasive early diagnosis and monitoring for cancers (Cottrell, 2003; Laird, 2003).

DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns (Baylin and Herman, 2000). Hypomethylation usually involves repeated DNA sequences, such as long interspersed nuclear elements, whereas hypermethylation involves CpG islands (Ehrlich, 2002).

To the best of our knowledge, reports on hypermethylation far outnumber the reports of hypomethylation in cancer. However, global hypomethylation has also been recognized as a cause of oncogenesis (Partha et al., 2004). Several protective mechanisms prevent the hypermethylation of the CpG islands. These include...
active transcription, active demethylation, replication timing and local chromatin structure preventing access to the DNA methyl transferase (Clark and Melki, 2002).

However, DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. As methylation occurs early and can be detected in body fluids, it may be of potential use in early detection of tumours and for determining the prognosis. Because DNA methylation is reversible, drugs like 5-azacytidine, decitabine, and histone deacetylase inhibitors are being used to treat a variety of tumours. Novel demethylating agents such as antisense DNA methyl transferase and small interference RNA are being developed, making the field of DNA methylation wider and more exciting (Partha et al., 2004).

A vast amount of knowledge has been gained in the last 8 years about altered methylation patterns in human cancers. The genes that are susceptible are involved in cell cycle regulation (p16INK4a, p15INK4a, Rb, p14ARF), those associated with DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance, detoxification, differentiation, angiogenesis and metastasis (Partha et al., 2004).

The potential clinical application of this information is in cancer diagnosis, prognosis, and therapeutics.

Although certain genes such as RASSF1A (Kwong et al., 2002) and p16 (Sanchez-Cespedes et al., 2000) are commonly methylated in a variety of cancers, other genes are methylated in specific cancers.

2.5.2. METHYLATION STUDY TECHNIQUES

Recent advances in the techniques for detection of methylation include powerful tools such as sodium bisulfite conversion, cDNA microarray, restriction landmark genomic scanning, and CpG island microarrays (Fraga and Esteller, 2002). The sodium bisulfite method is ideal for mapping the normal and aberrant patterns of methylation.
Bisulfite converts unmethylated cytosines to uracil, leaving methylated cytosines unchanged.

Within these techniques, methylation-specific polymerase chain reaction (Herman et al., 1996) and methylation-sensitive single nucleotide primer extension (Gonzalgo and Jones, 1997) are among the most widely used for identification and characterization of novel methylation changes associated with bladder carcinogenesis. This method has been used to detect methylation differences between the genomes of normal, bladder cancer tissues and cell lines (Liang et al., 1998).

With methylation-specific PCR (MSP) technique, it is possible to identify up to 1 methylated allele in 1000 unmethylated alleles (Herman et al., 1996), appropriate for the detection of neoplastic cells in a background of normal cells. MSP also allows rapid analysis of multiple gene loci, does not require prior knowledge of epigenetic alteration, and can provide a "yes or no" answer (Herman et al., 1996; Baylin et al., 2000).

In one issue of the Journal of Clinical Oncology, Catto et al. (2005) reported their studies on hypermethylation at 11 CpG islands in a large cohort of upper urinary tract transitional cell carcinomas (UTTs) and lower tract (bladder) urothelial carcinomas (UCs). They provided some interesting insights into differential epigenetic features of the two types of malignancies. Despite morphologic similarities between the two, more extensive promoter hypermethylation was found in UTTs (96%) than in bladder UCs (76%). The presence of methylation was seen to be significantly associated with advanced stage, higher tumour progression rates, and higher mortality rates. On combining the data from numerous publications, the role of methylation in many genes relevant in bladder cancer, it is becoming obvious that not only these marker genes, but also that the process of aberrant methylation itself, may provide a therapeutic target in bladder cancer.
2.5.3. TUMOUR SUPPRESSOR GENES

Tumour suppressor genes protect cells from undergoing malignant transformation. Tumour suppressor genes function by one of the following mechanisms: protect the genome from mutagenic events, impede dysregulated progression through the cell cycle, induce apoptosis in cells that escape normal cell cycle controls, and inhibit cellular migration and metastasis. Classically, tumour suppressor genes have been described to acquire loss of function mutations or deletions leading to their inability to delay malignant transformation. Alternatively, epigenetic events, such as methylation, represent a distinct mechanism of tumour suppressor gene inactivation. Of all the epigenetic modifications, hypermethylation, which represses transcription of the promoter regions of tumour suppressor genes leading to gene silencing, has most extensively been studied (Partha et al., 2004). Aberrant gene promoter methylation of these genes is associated with gene silencing and is functionally equivalent to a deleted gene. Gene silencing by DNA methylation has been considered to be permanent in non-embryonic cells, only reversible pharmacologically during cell division (Hayslip and Montero, 2006).

Significant changes in the global levels and regional patterns of DNA methylation are among the earliest and most frequent events known to occur in human cancers. CpG islands located in tumour suppressor gene promoters are normally unmethylated. Abnormal methylation of these regions may lead to progressive reduction in gene expression, thus transcriptionally silencing suppressor genes by a variety of mechanisms, including remodeling of local chromatin structure or inhibition of transcription factor binding, ultimately altering normal cellular growth properties. Application of novel molecular biologic techniques has increased our appreciation of the widespread changes in methylation patterns that occur during urinary bladder carcinogenesis. Epigenetic events, such as methylation,
occur throughout all stages of tumourigenesis, including the early phases, and are increasingly recognized as major mechanisms involved in silencing tumour suppressor genes (Richard et al., 2005).

A large number of genes have been reported to be hypermethylated in bladder cancer. Some examples include the p16 (at CDKN2A\textsuperscript{INK4a} locus on chromosome 9p21), E-cadherin (CDH1, encoding a transmembrane glycoprotein that modulates calcium-dependent intercellular adhesion), and RASSF1A (ras association domain family gene 1, isoform A) genes (Chan et al., 2003; Chang et al., 2003; Horikawa et al., 2003). Diminished CDH1 expression may lead to an increase in beta-catenin activity and proliferation in urothelial carcinomas (Thievessen et al., 2003).

\textit{INK4A-ARF} is an important locus on chromosome 9p21 that controls both \textit{P53} and \textit{RB}. The transcript of the \textit{INK4A-ARF} gene is alternatively spliced to give two mRNAs that code for proteins with no sequence relationship. P16\textsuperscript{INK4a} is upstream of RB. The second protein is called P19\textsuperscript{ARF} in mouse and P14\textsuperscript{ARF} in human. The loss of \textit{INK4A-ARF} activity either through deletions, mutation or promoter hypermethylation of the locus are common in human cancers (almost as common as mutations in \textit{p53}) and common step in tumour development and progression. There is a highly significant effect, because they eliminate both \textit{p16\textsuperscript{INK4a}} and \textit{p14\textsuperscript{ARF}} and therefore lead to loss of both the \textit{RB} and \textit{p53} tumour suppressor pathways (Lewin et al., 2004).

![Fig. 2.9 : Structure of the \textit{INK4a}/\textit{ARF} and \textit{INK4b} loci (Serrano, 2000)](image)
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2.5.3.1. P16

P16 is the most commonly altered gene in human malignancies. It is also known by the following names: INK4, INK4A, CDK4I, MTS1, and CDKN2 (Hirama and Koeffler, 1995). P16 is a 148 amino acid protein containing 4 ankyrin repeats. It inhibits the phosphorylation of retinoblastoma protein and, thereby, impedes mitosis at G1-S transition of the cell cycle pathway (Serrano et al., 1993). P16 functions as an important tumour suppressor, by blocking unregulated cellular proliferation. p16\textsuperscript{INK4A} inhibits the cdk4/6 kinase. It, therefore, prevents the kinase from phosphorylating RB. In the absence of this phosphorylation, progress through the cell cycle (and therefore growth) is inhibited. Aside from deletion and mutation, the p16 gene has been shown to be inactivated by promoter methylation (Sanchez-Cespedes et al., 2000). Mutation or deletion of one p16 allele and hypermethylation of the remaining allele may be sufficient for loss of functional activity. Progressive inhibition of p16 expression by DNA methylation may result in loss of adequate growth regulatory function. Methylation of p16 has been observed in 27 to 60% of primary urothelial carcinomas (Chan et al., 2002; Chang et al., 2003).

Arvind et al. (2001) found a 42% incidence of methylation for P16 in cervical cancers, and in 24% of high-grade dysplasia. Methylation was rare (3%) in non dysplasia/low-grade CIN specimens.

![Fig. 2.10: Tumour suppressor pathways controlled by the products of the INK4a/ARF locus. These pathways are activated by the presence of oncogenic stresses. Multiple crosstalks are known to exist between the p16\textsuperscript{INK4a}/Rb and p19\textsuperscript{ARF}/p53 pathways (Serrano, 2000).](image)
In another study, p16 promoter methylation was detected in 42% of tumours and was seen to be more frequent in Dukes’ stage C and D than in stages A and B (Yi et al., 2001).

2.5.3.2. P14ARF

The p14 protein, also known as p19ARF or CDKN2A, is also a cyclin-dependent kinase inhibitor known to induce cell cycle arrest at G1 and G2. P14 is encoded by three exons designated 1-β, 2, and 3. Exons 2 and 3 are shared with p16 although exon 1 is unique for each of the proteins (Quelle et al., 1995). P14ARF has been ascribed a role in modulating the cellular amounts of p53 through direct interaction with MDM-2 and P14ARF inhibits MDM-2, tags p53 for degradation through the proteosome pathway (Kamijo et al., 1997). Homozygous deletion of the P14ARF locus has been reported in a variety of cancers (Xing et al., 1999) and gene knockout of P14ARF to be correlated with tumourigenesis. Esteller et al. (2001) demonstrated that P14ARF hypermethylation occurs frequently in sporadic colorectal cancer.

Many studies have shown several methylated genes to be closely related to the prognosis. In colorectal cancers, the promoter region of the P14 gene was found to be hypermethylated in 61.1% of tumour samples, and this was correlated with the traditional prognostic indicators, such as tumour grading and Dukes’ staging (Maeda et al., 2003).

Both p16INKa and p14ARF hypermethylation may be involved in bladder carcinogenesis (Dominguez et al., 2003). The p14ARF promoter hypermethylation in plasma DNA may also be an indicator of disease recurrence in bladder cancer patients (Dominguez et al., 2002).

Essel et al. (2004) also observed hypermethylation of P14 in 16 of 45 (35%) bladder tumours by methylation specific-PCR. Masayoshi et al. (2003) suggested significant correlation between loss of P14
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expression as detected by immunohistochemistry and homozygous deletion/promoter methylation \((p = 0.02)\). They have found a significantly higher frequency of \(P14\) gene alteration than that in \(P16\) gene \((p = 0.02)\).

Though lot of work has been carried out on the detection of susceptibility genotype for Bladder Cancer, but no clear cut marker are available. Moreover the work on Indian population is very scanty. It is because of this the present project was undertaken.