The Molecular Pathogenesis of Bladder Cancer

De moleculaire pathogenese van blaaskanker

Proefschrift

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"Wanneer je staat tegenover het numineuze dan
past alleen nederigheid. En voor kleine wezens zoals wij
is de onmetelijke uitgestrektheid van het heelal
alleen te verdragen wanneer je liefhebt."

Carl Sagan – Contact

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List of Abbreviations

CGH	Comparative Genomic Hybridization
CIS	Carcinoma *in situ*
DBCCR1	Deleted in Bladder Cancer Candidate Region 1
EST	Expressed Sequence Tag
FAI	Fractional Allelic Imbalance
FGFR3	Fibroblast Growth Factor Receptor 1
HDA	Heteroduplex Analysis
ISH	*in situ* Hybridization
LOH	Loss of heterozygosity
MIN	Microsatellite instability
PCR	Polymerase Chain Reaction
SCE	Sister Chromatid Exchange
SRO	Smallest Region of Overlap
SSCP	Single Strand Conformational Polymorphism
STS	Sequence Tagged Site
TCC	Transitional Cell Carcinoma
TGFBR1	Transforming Growth Factor Receptor 1
TUR	Transurethral resection
TSC1	Tuberous Sclerosis Complex gene 1
TSG	Tumor Suppressor Gene

Chapter 1. General Introduction

Bladder Cancer

Anatomy and histology of the normal bladder

The bladder is a hollow organ in the small pelvis. It stores urine that is produced when the kidneys filter the blood. Four different layers, the epithelium, lamina propria, muscularis, and connective tissue, define the bladder wall. The epithelium consists of 7 to 10 cell layers and rests on a basal lamina synthesized by epithelial and mesenchymal cells (Figure 1). The thickness of the epithelial cell layer and the lamina propria depends on the degree of distension of the bladder. The cells of the epithelium are referred to as transitional cells. They line the urinary tract starting at the kidney, down the ureter, into the bladder and most of the urethra. Their shape varies between cubical and flat. The latter cells form the barrier between urine and the epithelium. Because of their large flat morphology these superficial cells are sometimes named umbrella cells. The muscle coat of the bladder is also referred to as the detrusor muscle. It allows the bladder to get larger and smaller as urine is stored or emptied.

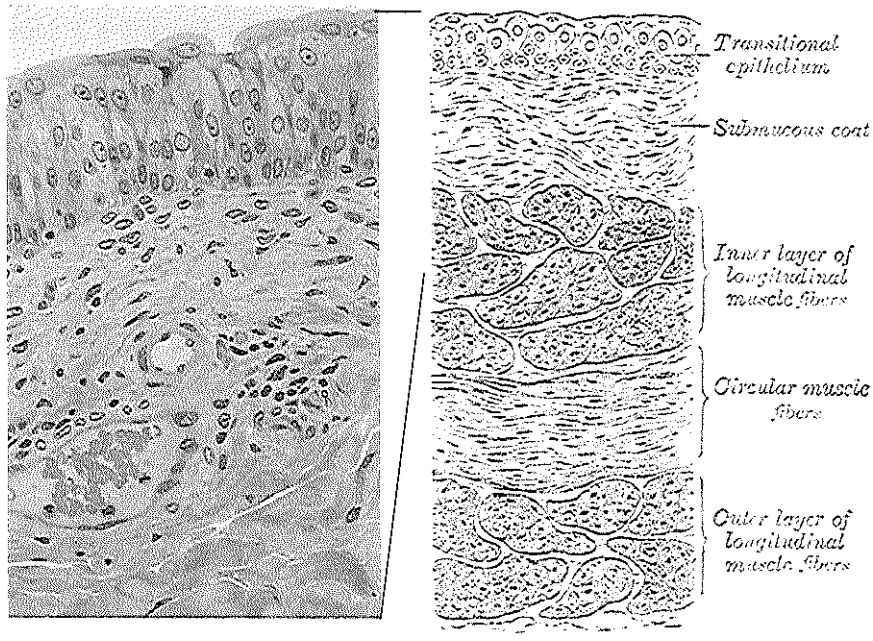

Figure 1. Histology of the bladder urothelium. Drawing from Gray's Anatomy, 1918.

Epidemiology

Bladder cancer is the eleventh most frequent cancer worldwide, but more common in Western Europe and the United States, while Japan has a lower incidence. In the Netherlands, approximately 2550 new cases present each year. Of these patients, 1200 will die from the disease (190, 191). The male-to-female sex ratio is at least 3:1. This can in part be explained by the gender difference in smoking behaviour and occupational exposure (see Risk Factors). The 5-year survival rate is approximately 50%, being high in local, superficial disease (92.8%) intermediate in locally invasive disease (48.3%) and low in metastasized disease (5.9%) (10, 130)

Histological subgroups

More than 90% of bladder tumors originate from the transitional cells lining the urinary tract. Many lower-grade tumors are papillary, and the cells covering its papillae are similar in appearance to those of the surrounding uninvolved urothelium. Other types of bladder cancer include squamous cell carcinoma and adenocarcinoma. These tumors constitute about 1% of all bladder tumors, and show a male predominance as well.

Carcinoma *in situ* is a flat, non-papillary, non-invasive, histopathologically anaplastic epithelium. Carcinoma *in situ* may present as a localized lesion adjacent to a superficial papillary or an invasive tumor, as a diffuse urothelial disease concomitant with macroscopic tumors, as a primary focal lesion in asymptomatic patients, or as symptomatic, diffuse, or multifocal lesions of the urothelium, not associated with macroscopic tumors at the time of diagnosis.

Table 1. Bladder cancer stages.

Tumor characteristics	Stage	Growth *a b c d e*
Carcinoma *in situ*	TIS	
Papillary tumor limited to mucosa	Ta	
Invasion lamina propria	T1	
Superficial muscle Invasion	T2	
Deep muscle and/or fat invasion	T3	
Invasion contiguous viscera	T4	

a, urothelium; b, lamina propria; c, muscularis propria; d, surrouding fat tissue; e, surrounding organs

Grading and staging

Three grades of urothelial carcinoma are recognized according to the WHO. Grade 1 represents well-differentiated papillary tumors with limited atypia and mitoses. At the other end, Grade 3 lesions show a marked disordered arrangement of the cell layers, cell size, and number of mitoses. Tumor grade appears to correlate significantly with prognosis. The higher the grade of the diagnosis, the higher the mortality within two years.

The staging of bladder cancer is based on the depth of invasion (Table 1). Tumors limited to the mucosa or lamina propria are often referred to as superficial, while tumors growing into the muscle and further are named invasive. Invasive bladder tumors tend to spread rapidly to the regional lymph nodes and then into adjacent structures.

Clinical Presentation

Patients with bladder cancer usually present with hematuria (blood in the urine) that is sometimes only visible under the microscope (microscopic hematuria). Other manifestations include bladder irritability and symptoms of urethral obstruction. Frequently the diagnosis of bladder cancer is delayed because bleeding is intermittent or attributed to other causes such as urinary tract infection, benign tumors, or bladder stones. Voided urine cytology is performed on a Papanicolaou stained smear of cells present in the urine. These can include inflammatory (blood) cells and cells previously lining the urinary tract that are exfoliated into the urine. Urinary cytology is highly specific, but has a low sensitivity. This means that if the urinary cytology is positive, transitional cell cancer of the urothelium is almost certainly present. But cytological examinations may be negative in up to half of patients with bladder cancer; thus, a negative smear does not rule out bladder cancer. Recently, several companies have started to offer dipstick tests of the urine to check for the presence of bladder cancer. Another possibility would be to look for DNA aberrations in cells collected from voided urine.

Superficial bladder cancer tends to recur frequently after their surgical removal; if this happens, it most often recurs as another superficial cancer. At presentation, approximately 80% of TCCs of the bladder are superficial papillary lesions (Ta/T1), the majority of which do not invade despite the common development of recurrences at the same or different sites in the bladder over a period of many years. For most of the 20% of tumors that are muscle invasive (T2) or metastatic (N+, M+) at the time of presentation, there is apparently no superficial papillary precursor lesion and these tumors may progress rapidly (130).

Treatment

Bladder cancer may be treated with surgery, biological therapy, radiation therapy, or chemotherapy. The choice of treatment depends on its stage and grade, particularly if, or

how deeply, the cancer has invaded the bladder wall. The urethra and bladder are inspected with a cystoscope to investigate the tumor size, number of tumors, and their location. Patients with low-grade superficial bladder cancer may be treated with a procedure called transurethral resection (TUR). A tool with a small wire loop on the end is used to remove the cancer (biopsy). Biopsies of normal-appearing bladder lining are performed to check for microscopic cancer that would otherwise be missed. Generally, this is followed by periodic cystoscopy, and cytologic evaluations.

Curative treatment of invasive bladder cancer comprises radical cystectomy, by which the entire bladder is removed, including nearby lymph nodes, and any surrounding organs that contain cancerous cells. For invasive cancers that appear to be limited to the bladder (stages T2-3), complete surgical removal of the bladder provides the best chance of a cure. When complete surgical removal of the bladder is performed, usually, a segment of small bowel is used to transfer urine directly from the kidneys and ureters through a stoma on the skin and into an external collection bag.

Patients with multiple tumors, high-grade tumors, carcinoma *in situ* or tumor penetration into the lamina propria are at high risk for tumor recurrence and progression. They are candidates for intravesical therapy with bacillus Calmette-Guerin (BCG), mitomycin, doxorubicin or thiotepa. Treatment with BCG involves placing a solution of BCG, a substance that stimulates the immune system, into the bladder for about 2 hours before the patient is allowed to empty the bladder by urinating.

In radiation therapy, high-energy rays are used to kill cancer cells. Like surgery, radiation therapy is local therapy. When bladder cancer has spread to other organs, radiation therapy may be used to relieve symptoms caused by the cancer. Radiation may come from outside the body (external radiation) or from a radiation implant, placed directly into the bladder (internal radiation).

Chemotherapy may be used alone or after TUR to treat superficial bladder cancer. Systemic chemotherapy may also be used to manage advanced bladder cancer, when cancer cells have deeply invaded the bladder and spread to lymph nodes or other organs. (130)

Risk Factors

Bladder cancer was one of the earliest cancers in which carcinogens were found to play a role in causing the disease. The most important known risk factor for bladder cancer is cigarette smoking. Cigarette smokers develop bladder cancer two to three times more often than non-smokers (28, 29, 119). Smoking does not only increase the risk for transitional cell carcinoma, but also for squamous cell carcinoma and adenocarcinoma of the bladder.

Smoking is estimated to be responsible for 25-60% of the bladder cancers in industrialized countries.

Occupational exposure to a certain class of organic chemicals called aromatic amines (beta-naphthylamines, xenylamine, 4-nirtobiphenyl, benzidine) is a well-established risk factor. Bladder cancer due to aromatic amine exposure has been documented in the textile, leather, rubber, dye, paint, hairdressing, and organic chemical industries. A period of 5 to 50 years may follow the exposure of carcinogenic agents and the diagnosis of bladder cancer.

Infection with Schistosoma haematobium, a parasite commonly encountered in Asia, Africa and South America, has been linked to bladder cancer. The eggs of the parasites are deposited in the bladder wall, and the morbidity of the disease is associated with chronic infection. Due to this infection, the proliferation rate of the urothelium is much higher. The tumors formed are mainly squamous cell carcinomas and adenocarcinomas.

Other factors that may contribute to the development of bladder cancer include bladder treatment with the anticancer drugs chlornaphazine or cyclophosphamide, long-term use of painkillers containing the drug phenacetin, recurrent urinary tract infections and stasis, dietary factors, tobacco products other than cigarettes (e.g., pipes and cigars), and genetic susceptibility.

Genetic susceptibility

In response to exposure to environmental agents that are toxic or mutagenic, organisms have evolved complex mechanisms by which they can protect themselves. This involves the expression of enzymes active in the metabolism and detoxification of the foreign chemicals. The best characterized of these enzyme systems are the cytochrome P450s, the glutathione-S-transferases (GSTs) and the N-acetylation enzymes (NATs). Genetic polymorphisms with well defined associated phenotypes have now been characterized in these genes (162). Indeed, many of these polymorphisms have been associated with a difference in the ability to detoxify substances that may otherwise act as carcinogens. This may render an individual more at risk for developing cancer. In the case of bladder cancer, both GST and NAT polymorphisms are implicated in an increased susceptibility.

Aromatic amines may be inhibited in their carcinogenic ability by acetylation. The acetylation speed in different individuals can, by nature, be slow or rapid. The slow N-acetylation genotype (NAT2) is a susceptibility factor in occupational and smoking-related bladder cancer (85, 142). The glutathione-S-transferase Mu1 enzyme (GSTM1) detoxifies arylepoxides which are formed after exposure to certain polycyclic aromatic hydrocarbons and possibly aromatic amines. According to the study of both Mungan *et al.* and Georgiou *et al.* (51, 121), individuals with the GSTM1 null genotype carry a substantially higher risk for bladder carcinogenesis. The GSTM1 null genotype is not associated with more aggressive

disease in terms of tumor grade, although there is a correlation between this genotype and stage of the disease.

Cancer genetics

Chromosomes and cancer

Chromosomal analysis of cancer cells has yielded a huge amount of information about the nature and incidence of chromosome abnormalities (74, 163). These abnormalities include numerical and structural changes. Numerical chromosomal changes lead to aneuploidy, which means any deviation of the normal set of 46 chromosomes. Polyploidy means the presence of whole sets of chromosomes in excess to the normal set of 46. Aneuploidy can be caused by non-disjunction, the failure of chromosomes to separate properly during the early stages of mitosis or meiosis. Aberrations in chromosome structure result from the breakage and reunion of chromosome segments and can involve one or more chromosomes. They can be divided in deletions, duplications, inversions, translocations, rings, and fragile sites. Chromosome breaks can occur from spontaneous errors in replication, crossing over, mutations in genes that normally repair breaks, and environmental agents such as ultraviolet light, radiation, chemicals, or viruses.

Oncogenes and tumor suppressor genes

Tumors arise from changes in the processes that control growth, division and mortality of cells. These changes are caused by mutations in oncogenes, tumor suppressor genes, or genes that are involved in DNA repair. Oncogenes were first discovered in transforming RNA viruses. They are derived from normal cellular proto-oncogenes with roles in normal growth and proliferation. When these genes are activated by a gain-of-function mutation, they may function as an oncogene and their products will for instance increase cell proliferation or inhibit cell death. Different types of oncogenes can be distinguished, based on their biological function, including growth factor receptors, transcription factors, or genes involved in protein phosphorylation or cell cycle regulation. Over 40 different oncogenes have been identified so far. Activation of a proto-oncogene can occur by a pointmutation, chromosomal translocation or by amplification of the gene.

Loss of growth suppression requires inactivation of both copies of a tumor suppressor gene for a phenotypic effect (Knudson's two-hit hypothesis, Figure 2) (91-93). Many different genetic incidents can eliminate both copies of a gene. Inactivation of one allele is often brought about by small sequence alterations like point mutations or small deletions. Inactivation of the other allele can be the result of loss of genetic material due to inaccurate chromosome segregation. In this case, the second copy of the gene, and usually flanking

regions of its chromosome as well, are either totally deleted or replaced by a copy of the corresponding region of the first defective chromosome.

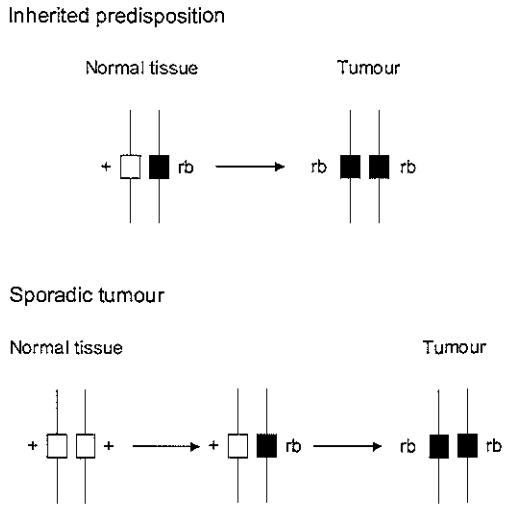

Figure 2. Knudson's two-hit hypothesis explained by the retinoblastoma model of tumorigenesis. + wild type allele, rb mutated allele. In patients with a germine mutation of the retinoblastoma gene only one mutation is necessary for the cell to become homozygous rb. In sporadic tumors, two mutations are required for the cell to become homozygous for the rb mutation. From: Hodgson & Maher, 1993 (69).

LOH in the cells of a tumor provides the basis for a method by which tumor suppressor genes can be identified and cloned. Tumor allelotyping (in analogy to karyotyping) is the analysis of all 23 pairs of human chromosomes for regions of LOH. The first allelotype was performed on colorectal carcinoma by Vogelstein *et al.* in 1989 (192). Allelotypes have been published for most of the major types of cancer. To date, despite the fine mapping of multiple common regions of deletion in sporadic tumors, only a small number of tumor suppressor genes mapped in this way have been authenticated. These include *DPC4* (65), and *PTEN* (102, 167), both of which have been found via a homozygous deletion. The usual heterozygous combination of maternal and paternal alleles of genes in that chromosomal region is lost (loss of heterozygosity or LOH, see Microsatellite instability and LOH). A tumor suppressor gene can also be inactivated by a small homozygous deletion within the gene, or extending only a short distance into DNA flanking the target gene. Another mechanism is de novo methylation of cytosine residues at CpG dinucleotides in the 5' CG-rich promoter region of genes. This leads to transcriptional silencing. These mechanisms are depicted in Figure 3. Several tumor suppressor genes have been identified to date that conform to the two-hit model. A list of these genes is given in Table 2.

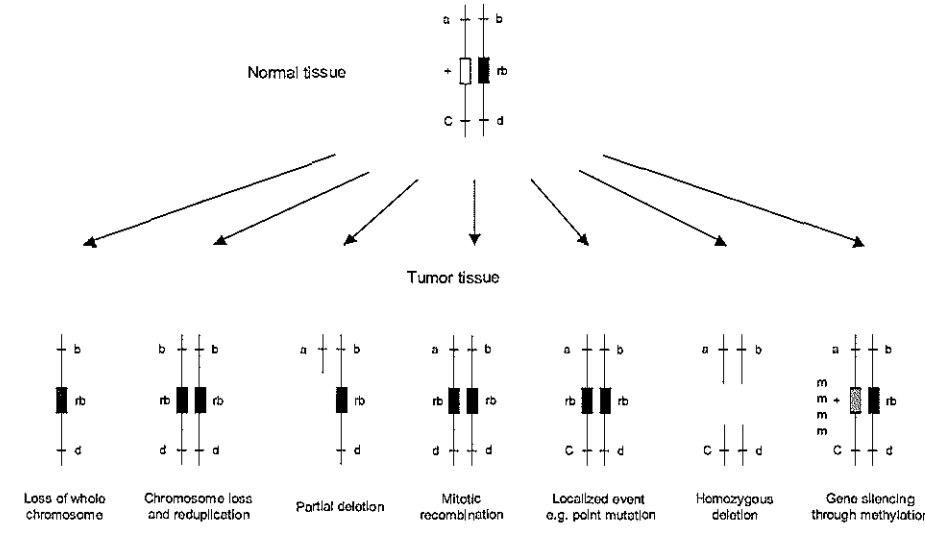

Figure 3. Model for the inactivation of tumor suppressor genes. +, wild type allele, rb, mutated allele, a/b and C/d are polymorphisms at two arbitrary marker loci either side of the tumor suppressor gene. The pair of chromosomes from the normal cell are heterozygous at both loci. The tumor either lost the tumor suppressor gene function due to a homozygous deletion or is homozygous for the rb mutation. In addition there is loss of heterozygosity at the marker loci in the tumor tissue unless it is a localized event. Note that the allele loss affects the chromosome with the wild type allele. Adapted from Hodgson & Maher, 1993 (69).

Chromosomal instability

Loss of a single tumor suppressor gene - even loss of both copies of the gene - is usually not sufficient by itself to cause cancer. In order for a tumor to develop, at least 3 to 6 mutations are needed in oncogenes or tumor suppressor genes (193). One way of reaching this number of mutations is by raising the mutation frequency. Cancer cells often display an enormous variability in the size and shape of their nuclei and in the number and structure of their chromosomes; in fact, abnormal nuclear morphology is one of the key features used by pathologists to diagnose cancer. Cancer cells grown in culture often have an unstable karyotype: genes become amplified or deleted and chromosomes become lost, duplicated, or translocated with a much higher frequency than in normal cells in culture. This chromosomal variability suggests that these cells have some defect in the control of chromosome replication, repair, recombination, or segregation, i.e. they have an unstable genome (101).

Table 2. Tumor suppressor genes and associated human cancers.
Adapted from Macleod, Curr Opin Genet Dev 10:81-93, 2000 (110).

Tumor suppressor gene	Human chromosomal location	Gene function	Human tumors associated with sporadic mutation	Associated cancer syndrome
RB1	13q14	Transcriptional regulator of cell cycle	Retinoblastoma, osteosarcoma	Familial retinoblastoma
WT1	11p13	Transcriptional regulator	Nephroblastoma	Wilms tumor
TP53	17q11	Transcriptional regulator/ growth arrest/apoptosis	Sarcomas, breast/brain tumors	Li-Fraumeni
NF1	17q11	Ras-GAP activity	Neurofibromas, sarcomas, gliomas	Von Recklinghausen neurofibromatosis
NF2	22q12	ERM protein/ cytoskeletal regulator	Schwannomas, meningiomas	Neurofibromatosis type 2
VHL	3p25	Regulates proteolysis	Hemangiomas, renal, pheochromocytomas	Von-Hippel Lindau
APC	5q21	Binds/regulates ß-catenin activity	Colon cancer	Familial adenomatous polyposis
INK4A	9p21	P16 cdki for cyclinD/ cdk4/6; p19 binds mdm2, stabilizes p53	Melanoma, pancreatic cancer	Familial melanoma
PTC	9q22.3	Receptor for sonic hedgehog	Basal cell carcinoma, medulloblastoma	Gorlin syndrome
BRCA1	17q21	Transcriptional regulator/DNA repair	Breast/ovarian tumors	Familial breast cancer
BRCA2	13q12	Transcriptional regulator/DNA repair	Breast/ovarian tumors	Familial breast cancer
DPC4	18q21.1	Transduces TGF-ß signals	Pancreatic, colon, hamartomas	Juvenile polyposis
FHIT	3p14.2	Nucleoside hydrolase	Lung, stomach, kidney, cervical carcinoma	Familial clear cell renal carcinoma
PTEN	10q23	Dual specificity phosphatase	Glioblastoma, prostate, breast	Cowden syndrome, BZS, LDD
TSC1	9q34	Cell-cylce regulator	Renal, hamartomas	Tuberous sclerosis
TSC2	16	Cell-cycle regulator	Renal, brain tumors	Tuberous sclerosis
NKX3.1	8p21	Homeobox protein	Prostate	Familial prostate carcinoma
LKB1	19p13	Serine/threonine kinase	Hamartomas, colorectal, breast	Peutz-Jeghers
ECAD	16q22.1	Cell adhesion regulator	Breast, colon, skin, lung carcinoma	Familial gastric cancer
MSH2	2p22	MutS homologue, mismatch repair	Colorectal cancer	HNPCC
MLH1	3p21	MutL homologue, mismatch repair	Colorectal cancer	HNPCC
PMS2	2q31	mismatch repair	Colorectal cancer	HNPCC
PMS2	7p22	mismatch repair	Colorectal cancer	HNPCC
MSH2	2p16	mismatch repair	Colorectal cancer	HNPCC

Currently, two different pathways to tumorigenesis are thought to exist (84). The first pathway involves gatekeeper genes. These genes are directly controlling cellular proliferation. Examples include the genes responsible for colon cancer, retinoblastoma and neurofibromatosis. The second pathway is represented by caretaker genes. These genes maintain the integrity of the genome. They sustain the intracellular machinery governing replication, recombination, and repair of DNA that is caused by, e.g., mutagens in the environment. People with the rare genetic disorder xeroderma pigmentosum, for example, have a defect in the system of enzymes required to repair the type of damage done to DNA by ultraviolet irradiation; as a result, the slightest exposure of the skin to sunlight is liable to provoke skin cancers (172, 200). A more general predisposition to cancer due to faults in DNA repair and replication occurs in the relatively common HNPCC syndrome, and in Bloom's syndrome, Fanconi's anemia, and ataxia-telangiectasia (189). Inactivation of a caretaker gene would not directly promote tumor initiation, but increase the mutation frequency, resulting in genetic instability and mutations in other genes, for instance gatekeeper genes.

Microsatellite instability and LOH

Microsatellites or short tandem repeat polymorphisms (STRs) are short stretches of nucleotide sequences, usually repeated between 15 and 30 times. The repeated sequences are 2 to 5 nucleotides long. Microsatellites belong to a family of repetitive non-coding DNA sequences comprising satellites (5-100 bp), minisatellites (15-70 bp) and microsatellites. Between 35,000 and 100,000 different microsatellites are present in the human genome, a marker density of approximately one microsatellite every 100,000 bp. They are not uniformly spaced along chromosomes but tend to be underrepresented in subtelomeric regions. The most frequent occurring repeat motif is the $(CA)_n$ dinucleotide sequence.

No definite function can be ascribed to microsatellite sequences. The short size and high variability of microsatellite markers made them easy to type with the polymerase chain reaction (PCR). This has had a profound effect on genome analysis and the construction of a physical map of the human genome. An application of such a map is the linkage analysis, which tests whether a chromosomal region is correlated with transmission of a certain hereditary disease within a pedigree. A polymorphism is considered informative or heterozygous if the number of repeats differs between the two different chromosomes of an individual. The informativity of these polymorphisms depends on the number of repeats. The more variation in the stretch of repeats, the higher the chance that both chromosomes have a different repeat length. This makes them useful markers to study heterozygosity of genomes (95). Another application of these markers is to detect loss of heterozygosity in tumor DNA. This is explained in Figure 4.

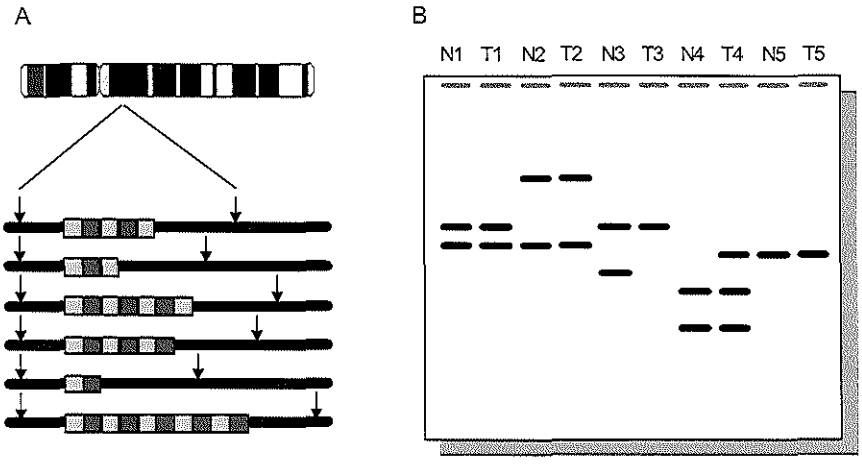

Figure 4. Detection of loss of heterozygosity or microsatellite instability in tumor DNA. A, a microsatellite marker is amplified via unique PCR-primer sequences on either site of the repeat. The length of the amplified band depends on the number of repeats (gray and black boxes). B, an example of the possible outcomes. N, normal DNA, T, tumor DNA. The first two individuals are heterozygous without loss, the third has loss of the lower allele in the tumor DNA, the fourth shows microsatellite instability represented by an extra band above the normal allele bands, the fifth is not informative (homozygous) because both alleles have the same length.

DNA polymerases are less able to process through repetitive sequences and therefore slippage is more likely to happen in these regions. The stable transmission of repeat size is no longer guaranteed when mutations are present in genes responsible for the detection and repair of replication errors, like the *hMSH2* or *hMLH1* genes (9). Tumors due to such mutations are called RER+, replication error positive. These tumors have a relatively normal, diploid karyotype and their cell cycle checkpoints are intact (11, 48). RER+ tumors show microsatellite instability (MIN) which means that the length of the microsatellites may become altered in cells of RER+ tumors (Figure 4).

Clonality versus field cancerization

Some cancer types, like bladder cancer, head and neck cancer and colon cancer, present themselves as multifocal. There has been considerable debate about whether tumors arise from a single mutated cell (clonal) as a multistep process, or whether there can be a process named field cancerization. Clonal development of multiple cancers is thought to occur through a series of recognizable stages, followed by spread and outgrowth of mutated progeny. Exposure of an entire field of tissue to repeated carcinogenic insults could give rise to multiple unrelated polyclonal tumors (50). It is not unlikely to think of a polyclonal process

in these multifocal cancer types, since environmental exposure could provide a constant mutation causing agent. To discriminate between the two possibilities, several groups have looked at X-chromosome inactivation patterns (46, 122) and the presence of specific mutations in for instance the *TP53* gene at chromosome 17p (198). When the same mutations or X-inactivation are observed in all multifocal tumors from one patient, the tumors are considered monoclonal. Another method that has been used to study clonality is MIN (112). Identical microsatellite alterations detected in multifocal tumors, recurrences, or metastases can serve as evidence for their clonal relation.

It is now widely accepted that most multifocal tumor types are monoclonal in origin, arising from a mutation or series of mutations in a single cell and its descendants. Possible exceptions are tumors of the aerodigestive tract, thought to be caused by chronic exposure to alcohol and tobacco (182, 198).

Genetic aberrations in bladder cancer

Cytogenetic studies and CGH in bladder cancer

Structural analyses of the genome changes in bladder cancer include cytogenetic studies, identification of deletions by LOH or homozygous deletion analysis, and comparative genomic hybridization (CGH) (79).

Cytogenetic studies of bladder cancer have given indications of the possible locations of relevant tumor suppressor genes (52, 135, 146). Monosomy 9 was described as the sole cytogenetic abnormality in some near diploid bladder tumors, sometimes accompanied by trisomy 7 and trisomy 10 (114). Other seemingly unique alterations on chromosome 9 include an interstitial del(9)(q11q21.2) (8). Another indication for the involvement of genes on chromosome 9 came from chromosome transfer studies. Following introduction of an intact chromosome 9 into bladder cancer cell lines, only clones with a rearranged chromosome 9 could be propagated *in vivo* (104, 205).

CGH analysis of bladder cancer showed losses on chromosome 2q, 3p, 4q, 5q, 11p, 11q, 8p, 9, 10q, 12q 17p, and 18q in more than 20% of the tumors (78, 159). Gains of DNA sequences were most often found at chromosomal regions distinct from the locations of currently known oncogenes. The bands involved in more than 10% of the tumors were 8q21, 13q21-q34, 1q31, 3p22-24, 3q24-q26, 1p22, 10p13-14, 12q13-15, 17q22-23, 18p11, and 22q11-13 (78, 196).

Oncogenes and tumor suppressor genes that play a role in bladder cancer

Several genes that are known to be altered in other tumor types have been studied for their involvement in bladder cancer. These include the oncogenes *HRAS, ERBB2,* and *MYC*, and

the tumor suppressor genes *TP53*, *CDKN2A, RB*, and *PTEN*. The characteristics of these genes are listed in Table 3.

HRAS is part of a family of genes whose products are involved in the transduction of mitogenic signals. *HRAS* mutations are reported infrequently in bladder tumors, with mutation frequencies varying between 6% (90) and 44% (47). *ERBB2* codes for an epidermal growth factor receptor-like protein and is amplified and overexpressed in 10-26% of bladder tumors (72, 126). *MYC* overexpression is sometimes found in bladder tumors, especially in tumors of higher grade (27, 148).

FGFR3 belongs to a family of four receptors that together contain the most frequent germline mutations in humans. More than 75 different mutations have been recorded, which account for more than seven skeletal syndromes, including achondroplasia, hypochondroplasia, thanatophoric dysplasia, and Muenke coronal craniosynostosis (80, 134, 180). All the mutant phenotypes cause a gain-of-function by receptor activation through three major mechanisms: receptor dimerization, kinase activation, and increased affinity for FGF. Paradoxically, the consequence of receptor activation is inhibition of chondrocyte cell growth through signaling pathways that are cell-type specific. The *FGFR3* gene is located on chromosome 4p16, and is activated by mutation in approximately 30% of bladder tumors (20). The role of the receptor in urothelial cells has yet to be established. Research in our lab so far suggests that screening for receptor mutations can identify patients with a lower recurrence rate of superficial tumors (183).

TP53 has been mapped to chromosome 17p13 and encodes a 53 kD protein, which is a transcription factor involved in cell-cycle regulation. The protein functions as a check point arresting cells in G1 when the genome is damaged. This allows cells to repair the DNA damage. The protein also induces apoptosis. Mutations in *TP53* will result in inactivation of the product and an increase in genetic alterations due to lack of DNA damage control. Genetic alterations of *TP53*, such as mutations, homozygous deletions and structural rearrangements are frequent events in bladder cancer (97, 203). With immunohistochemical staining, nuclear overexpression is often seen in tumors carrying *TP53* mutations. This can be explained by the fact that the presence of a mutation increases the half-life of the protein 4 to 20 times. Nuclear overexpression of the protein p53 can serve as a prognostic factor and is significantly related to tumor progression (41, 66, 137, 147, 177, 197).

The *CDKN2A* gene was identified as a candidate gene on chromosome 9p. Homozygous deletion mapping using quantitative duplex PCR showed that *CDKN2A* and/or the adjacent gene *CDKN2B* is the likely target of the 9p21 deletion in bladder cancer (204). Others have shown that point mutation and other small sequence changes in *CDKN2A* are rare in bladder cancer (13, 127, 204) The retained allele of the gene is in some bladder tumors silenced by methylation (54, 57). The identification of homozygous deletion as the

predominant mechanism of inactivation led to the hypothesis that inactivation of both *CDKN2A* and *CDKN2B* contributes to bladder cancer development and that a homozygous deletion provides an efficient means of inactivating more than one adjacent gene (128, 171).

The *RB* gene is located on chromosome 3 and codes for the RB protein, which functions as an important cell cycle checkpoint, the restriction point. Mutations that inactivate the *RB* gene were found in approximately 30% of bladder tumors, mostly of high stage and grade (15, 118).

Table 3. Oncogenes and tumor suppressor genes altered in bladder cancer.

Gene	Involved (%)	Clinical association	References
HRAS	6-44	Grade	47, 90
ERBB2	10-14	Grade, stage, recurrence	73, 115, 126, 150, 179, 194, 197
MYC		Grade, stage	148
FGFR3	30	Lower recurrence rate	20, 183
RB	30	High stage, progression, reduced survival	15, 31, 106, 118
TP53	10-70	Grade and stage, progression, reduced survival	63, 151, 156, 166, 174, 199, 203
CDKN2A	20-45	?	13, 54, 56, 127, 166, 204, 205
PTEN	10-30	?	4, 14

Adapted from Knowles, Br J Urol Int 84: 412-427, 1999 (86).

PTEN is located on chromosome 10q23 (102, 167). It is inactivated in several types of cancer including melanoma, prostate cancer and endometrial carcinoma. A search for mutations of *PTEN* in bladder tumors has yielded only a very small number of mutations and homozygous deletions (14), a frequency much lower than the frequency of LOH on 10q. It is possible that another gene in the same region is involved. However, the finding of intragenic homozygous deletions in bladder tumor cell lines (4, 118) indicates that *PTEN* plays a critical role in at least some cases.

Loss of heterozygosity in bladder cancer

To define the smallest region of deletion on a chromosome arm, a large number of tumor samples is used for microsatellite-based LOH and homozygous deletion analysis at low marker density. When the regions found in these tumors are combined, the resulting region of overlap is used to pinpoint the starting point of an attempt at cloning the gene involved. In 1994, an allelotype analysis was published based on a series of primary TCCs using 90 markers (88). About 60% of these tumors analyzed were superficial (pTa/T1). The most frequent losses were on chromosome 9 (9p, 51%; 9q, 57%). Other chromosome arms with frequent deletions were 11p (32%), 17p (32%), 8p (23%) 4p (22%) and 13q (15%). LOH on 17p and 13q is commonly associated with mutational inactivation of the retained copy of the *TP53* or *RB* genes, respectively (15, 203). LOH on all of these chromosome arms except

chromosome 9 is associated with high tumor grade and stage. Other studies have subsequently identified deletions on 3p (138), 10q (21, 77), 18q (12), and 14q (22) (Table 4).

Carcinoma *in situ* (CIS) is regarded as the most likely precursor lesion for invasive TCC. If so, the genetic aberrations identified in CIS are expected to be similar to those found in invasive TCC, but not superficial. A partial allelotype study of CIS using 29 markers on 13 chromosome arms (3p, 4p, 4q, 5q, 8p, 9p, 9q, 11p, 11q, 13q, 14q, 17p and 18q) has been reported (143). LOH of chromosome 9 was frequent (77%), as was LOH on 8p (65%), 17p (60%), 13q (56%), 11p (54%), 4q (52%) and 14q (70%). These frequencies, with the exception of chromosome 9 loss, are significantly higher than those found in superficial papillary TCC and resemble those found in invasive bladder cancer. This could indicate that CIS, although classified as a stage Ta lesion, represents a potentially aggressive entity.

A partial allelotype of squamous cell carcinomas (SCC) from patients with a history schistosomiasis revealed LOH on all chromosome arms studied (3p, 4p, 4q, 8p, 9p, 9q, 11p, 11q, 13q, 14q, 17p, 18q) (154). The most frequent regions of LOH were 9p (65%), 17p (58%), 3p (40%), 9q (39%) and 8p (37%). The most striking difference between this group of SCCs and TCCs was the high frequency of 9p LOH in the region of the *CDKN2A* gene (65%) and the relatively lower frequency of 9q LOH (39%). This suggests that a 9p gene, possibly *CDKN2A*, which is also implicated in TCC, may contribute to the development of the majority of schistosomiasis-associated bladder tumors and that a gene(s) on 9q plays a less important role.

A common region of deletion of about 750 kb was found close to the Huntington disease gene at 4p16.3 (39), together with a larger more centromeric region of deletion, a region at 4p15 and a region on 4q (136). The proximity of the 4p16.3 region of deletion to the HD gene provided many probes and allows mapping with a resolution of 30-40 kb. One candidate gene located within the region is the gene *SH3BP2*. Detailed mutation analysis failed to identify coding sequence mutations in bladder cell lines or primary tumors (6).

Allelotype analysis identified 8p LOH in approximately 23% of TCCs. Apart from a *TP53* mutation, LOH of 8p is the most frequent and specific alteration in muscle-invasive bladder tumors. Preliminary mapping showed that the deleted region was also deleted in several other tumor types, including prostate, which shows 70-80% 8p LOH (89). At least three regions are involved in the bladder, a large region at 8p21-22 encompassing two regions of deletion defined in prostate cancer, and two smaller, more proximal regions at 8p12-21, both contained within a third region defined in prostate cancer (111, 170). Most bladder tumors with 8p LOH, however, seem to have lost the entire chromosome arm (89, 170). Several candidate genes mapped within these 8p regions, including the *POLB* and *PPP2CB* genes, were tested with mutation analysis but revealed no mutations in bladder tumors or cell lines (42).

Table 4. Candidate regions for TSGs involved in TCC of the bladder.

Chrom	Regions (genes)	Association with tumor grade/stage	References
3	3p	High grade and stage	138
4	4p16.3, 4pcen-p14	All stages/grades	39
	4p15	High grade and stage	136
	4q33-34	High grade and stage	136
8	8p21.1-pter	High stage	170
	8p11.2-12	High stage	170
9	9p21 (*CDKN2A*)	All stages/grades	18, 36, 127, 204
	9q13-31	All stages/grades	13, 60
	9q32-33	All stages/grades	160
	9q34 (*TSC1*)	All stages/grades	60, 64, 160
10	10q23 (*PTEN*)	High stage	21, 77
11	11p15	High grade	44, 155, 176
	11q13-23.2	High grade	155
13	13q (*RB*)	High grade and stage	15
14	14q12	High stage	22
	14q32.1-32.2	High stage	22
17	17p13 (*TP53*)	High grade and stage	63, 125, 166, 176, 203
18	18q21.3-qter	High stage	12

Deletions of chromosome 11 can be detected in around 40% of TCCs (44, 176), and LOH of 11p is associated with higher tumor grade (125). Thirty-four percent of TCCs has LOH at one or more loci on chromosome 11p or 11q. A common region of deletion was defined at 11p15, which is coincident with a region deleted in breast (3, 109), testicular cancers (107), and NSCCL (201). The target of these deletions is not known at present. A candidate gene is *TSG101*, a suppressor gene identified by homozygous gene knockout through antisense RNA produced by a randomly introduced promotor (103), but this gene has not yet been shown to be mutated in human cancers.

The role of chromosome 9 in bladder cancer

LOH of markers on chromosome 9 is present in more than 50% of bladder tumors of all grades and stages and thereby is the most frequent genetic change identified (16, 17). These deletions are present at similar frequency in bladder tumors of all grades and stages and therefore represent a potential initiating event (176). In many tumors the region lost seems very large or involves regions on both chromosomal arms. This renders fine deletion mapping difficult. Several groups worldwide have studied large numbers of bladder tumors in order to identify small subchromosomal deletions that allow localization of the critical regions. Keen and Knowles (82) found a common region of deletion on 9p between D9S126 (9p21)

and the interferon-alpha cluster (IFNA) located also at 9p21. A single tumor showed a second site of deletion on 9p telomeric to IFNA, indicating the possible existence of 2 target genes on 9p. All deletions of 9q were large, with a common region of deletion between D9S15 (9q13-q21.1) and D9S60 (9q33-q34.1). The results suggested the simultaneous involvement of distinct suppressor loci on 9p and 9q in bladder carcinoma.

Refinement of the localization of loci on chromosome 9q has been described in a series of publications (16, 17, 36, 60, 64, 82, 186). This process is illustrated in Figure 5. First, the interpretation of the LOH data was based on the assumption that there was a single region of deletion on the chromosome or one on each chromosome arm (17, 82). LOH analysis of 9q based on larger numbers of markers suggested at least two regions at 9q13-22 and 9q34 (60, 160). At 9q22, Simoneau *et al.* (160), have examined the Gorlin syndrome gene (*PTCH*) and found no mutations. At 9q34, the *TSC1* gene was considered a candidate bladder tumor suppressor gene. Hamartomas developing in tuberous sclerosis patients with linkage to 9q34 show 9q34 LOH, indicating that *TSC1* may act as a tumor suppressor (184). SSCP analysis and sequencing have identified several mutations in bladder tumors with LOH in the region. This indicates that *TSC1* is a possible target of 9q34 deletions in a low percentage of bladder tumors (71, 127, 185).

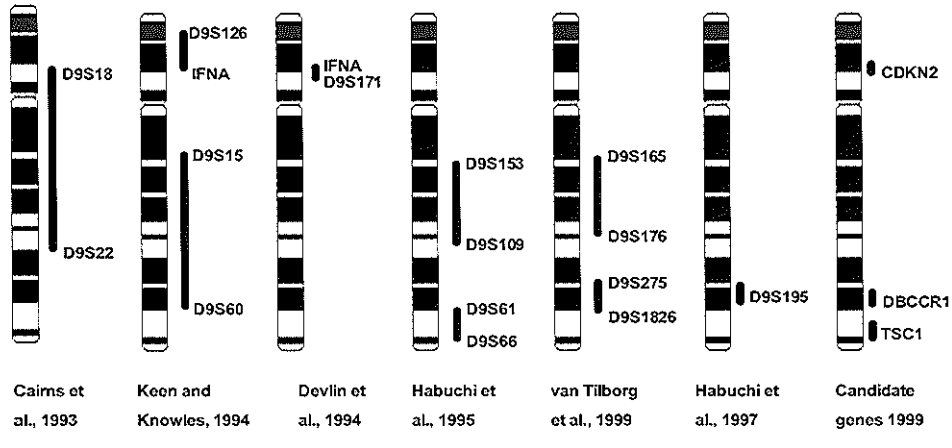

Figure 5. Sequential refinement of LOH regions on chromosome 9 in bladder cancer. The regions are indicated by black lines next to the chromosome, together with the markers determining the borders. Adapted from Knowles, 1999 (87).

A third possible tumor suppressor harbouring region was localized to 9q32-33, based on the finding of five tumors with small interstitial deletions in the region of D9S195 (127). A homozygous deletion was found in a region covered by a single YAC, whose estimated size was about 840 kilobase (64). This region was found to contain the cDNA sequence of the gene *DBCCR1* (for: deleted in bladder cancer chromosome region candidate 1) (61). The *DBCCR1* cDNA sequence contains an open reading frame with 8 exons encoding a protein of 761 amino acids with an estimated molecular weight of 89 kD. Mutation analysis of the coding region in the five critical tumors and a series of tumors with larger deletions of 9q by SSCP analysis and Southern blot analysis detected neither somatic mutations nor gross genetic alterations in primary TCCs of the bladder. *DBCCR1* is expressed in multiple normal human tissues including urothelium, but mRNA expression is absent in 5 of 10 bladder cancer cells lines. Methylation analysis of the CpG island at the 5' region of the gene and the induction of de novo expression by a demethylating agent indicated that this island might be subject to hypermethylation-based silencing.

Outline of this thesis

The identification of the genetic alterations in transitional cell carcinoma of the bladder could clarify the pathogenesis of this disease, provide insight into the possible presence of different genetic backgrounds, and eventually result in useful clinical tools. Genetic alterations on chromosome 9 are considered pivotal to bladder tumor development since deletions of this chromosome are present at high frequency in bladder tumors of all grades and stages. We first screened bladder tumors with several techniques that can detect loss of chromosome 9 in order to effectively select out those tumors that have lost an entire copy of this chromosome (Chapter 2). From this work we concluded that LOH analysis is to be preferred over ISH in order to reliably determine loss of chromosomes. We further searched for common regions of deletion with LOH analysis and homozygous deletion mapping in order to narrow down the candidate regions (Chapter 3). We subsequently selected several known genes in the regions of loss to screen for mutations and susceptibility polymorphisms (Chapter 4). When it became clear that there were several regions of LOH on chromosome 9 but very few mutations in any of the candidate genes, we started questioning the relevance of the regions. We therefore decided to search for common alterations in multiple superficial recurrent tumors in order to reconstruct the development of the genetic aberrations in time. This allowed us to order the tumors in a genetic tree, where tumors with few aberrations precede those with many. Based on the extent of the genetic damage and the accumulation of alterations during tumor development we hypothesize that LOH is presumably stimulated by an enhanced rate of mitotic recombination (Chapter 5). The genetic trees also allowed us

to interpret the LOH results on chromosome 9q and follow the development of as many as ten different events in tumors of individual patients. Since loss on chromosome 9q was almost never the characteristic first step in our patients and the regions lost varied between patients, we believe that this diminishes the probability of the presence of postulated gatekeeper genes on this chromosomal arm (Chapter 6).

Chapter 2. Loss of heterozygosity and loss of chromosome 9 copy number are separate events in the pathogenesis of transitional cell carcinoma of the bladder

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Summary

The most frequent genetic aberration found in transitional cell carcinoma (TCC) of the bladder involves chromosome 9. Loss of heterozygosity (LOH) analyses show deletions of both chromosome 9p and 9q, while *in situ* hybridization studies suggest a significant percentage of tumors with monosomy 9. To investigate the types of chromosome 9 losses that occur in bladder cancer, we studied forty tumors with different techniques such as *in situ* hybridization (ISH), flow cytometry and LOH analysis.

LOH for one or more markers was found in 43% of the tumors. This percentage does not differ from previous reports. With ISH, complete monosomy for chromosome 9 was observed in only 1 of the 40 tumors. Four other tumors had monosomic subpopulations, representing 23-40% of the cells. In 18 cases an underrepresentation of the chromosome 9 centromere relative to chromosome 6 or to the ploidy of the tumor was observed, these include the cases with monosomy. In 5 of these 18 cases the relative loss could not be corroborated by LOH. In addition, when LOH and a relative underrepresentation were observed in the same tumor, the extent of LOH as measured by the intensity of allele loss was often not related to the extent of underrepresentation. We therefore conclude that complete monosomy of chromosome 9 is rare in TCCs of the bladder and that a relative loss of centromere signal may not be related to loss that is meant to inactivate a tumor suppressor gene. LOH was found in TCCs of all stages and grades. This suggests that loss of tumor suppressor genes on chromosome 9 is an early event in the pathogenesis of bladder cancer.

Introduction

Transitional cell carcinoma (TCC), the most common form of urinary bladder cancer, is presented in two ways: superficial papillary tumors (Ta/T1) and invasive tumors (T2-T4). Other types of bladder tumors include carcinoma *in situ* (CIS), squamous cell carcinomas and adenocarcinomas. Aberrations concerning chromosome 9 are found in TCCs of all grades and stages, suggesting that the inactivation of a tumor suppressor gene (TSG) on this chromosome is an early event in the development of bladder cancer. Loss of heterozygosity studies show loss of heterozygosity (LOH) on both chromosome arms in more than 50% of the tumors (60, 88). Fluorescent *in situ* hybridization (FISH) analyses show that the number of chromosome 9 centromeres is often lower when compared to other chromosomes (149, 202). Thus, chromosome 9 is relatively under-represented. In addition, subpopulations of tumor nuclei are found in which only one spot for the centromere 9 probe is observed, which suggests monosomy for chromosome 9 in at least a part of the tumor

cells. Finally, with comparative genomic hybridization (CGH) loss of chromosome 9 is observed in about 25% of the TCCs (78, 196). A combination of LOH, FISH and CGH results led to the interpretation that monosomy of chromosome 9 may occur in over 50% of the TCCs of the bladder.

Besides possible loss of entire copies of chromosome 9, interstitial deletions have been observed on both chromosome arms (82). This suggests that at least two and perhaps even three TSGs on this chromosome may contribute towards the development of bladder cancer. The TSG on chromosome 9p is generally believed to be the *CDKN2A (MTS1, p16)* gene and in many cases even homozygous deletions within this gene have been found (13, 127). On the q arm, two shortest regions of loss have been determined (60, 186). These two regions are still too large to start cloning of the putative TSGs by positional cloning. In order to narrow down these regions we wanted to screen TCCs for LOH and, in addition, to search for homozygous deletions. For determining a shortest region of loss by LOH analyses tumors with monosomy for chromosome 9 are not useful. On the other hand, these tumors can be used to search for homozygous deletions. We therefore decided to screen our tumors first by *in situ* hybridization and use the results to determine whether the sample would be used for LOH or homozygous deletion analysis. To our surprise, monosomy for chromosome 9 was observed in only 1 of the 40 tumors.

To further evaluate the types of chromosome 9 losses that could inactivate tumor suppressor genes in TCCs of the bladder, we also determined the DNA-index and searched for LOH of chromosome 9 in tumor DNA. From the data obtained it appeared that a relative underrepresentation of chromosome 9 and LOH are often present in the same tumor. However, a quantitative interpretation of these data suggests that a relative underrepresentation and LOH are not causally related. Therefore, we conclude that complete monosomy of chromosome 9 is rare in these tumors and that a relative loss of centromere signal may not be related to loss that is meant to inactivate a tumor suppressor gene.

Materials & Methods

Flow cytometry and *in situ* hybridization

Forty paraffin-embedded archival bladder tumors were examined microscopically by a pathologist (T. v/d K.), and the parts which represented tumor tissue were punched out of the original paraffin blocks and newly embedded. Necrotic and/or inflammatory parts were avoided. An H&E stained section was made before and after every tissue handling, to ensure the comparability of the samples. These sections were again valuated by a pathologist. The percentage of tumor cells in the new blocks was estimated to be at least 90%. For flow cytometry, cell suspensions were made according to the method of Hedley (68). After deparaffinization, cells were resuspended in Hank's BBS with ethidium bromide (50 µg/ml), treated with RNase and filtered (40 µm). Samples were counted on a FACScan (Becton Dickinson Co, Sunnyvale, CA). A total of 10,000 nuclei were analyzed for each sample. Peaks were only considered significant when they comprised more than 20% of gated events. The DNA-index was determined as the ratio of the aneuploid mean channel number divided by the diploid mean channel number.

For *in situ* hybridization, interphase nuclei were isolated from 20 µm sections of the new blocks. Sections were deparaffinized and digested with 0.1% subtilisin (Sigma protease XXIV) at 37°C in 0.1 M Tris, 0.07 M NaCl, pH 7.2, for 25-40 min. Suspensions were filtered (40 µm). The nuclei were spinned onto slides. Centromere associated probes for chromosome 9 (pHUR98) (120), and, as a control, chromosome 6 (p308) (75), were used. Probes were labeled by nick-translation with biotinylated dNTPs. DNA probes were stored at -20°C. The ISH procedure was performed as described by Van Dekken *et al.* (181). Briefly, the slides were subjected to a microwave treatment of 10 min at 85°C in 2xSSC, followed by a 0.1% pepsin digestion. Nuclear DNA was denatured in 70% formamide/2xSSC, pH=7.0 at 70°C for 2 min, followed by dehydration in ethanol. The hybridization mixture was denatured for 5 min at 70°C. Hybridization was performed overnight at 37°C in a humidified chamber. Visualization was done with diaminobenzidin (DAB). The CARD technique (CAtalyzed Reporter Deposition) (83), was used to amplify the signal for chromosome 9. The distribution of centromeric signals was determined by counting 200 nuclei per tumor per probe. All intact nuclei were included in the analysis. A tumor was considered disomic if no other sub-population had >20% of tumor cells. A tumor was considered to have a monosomic sub-population of cells when this sub-population comprised more than 20% of the cells. When the monosomic population was the largest population of cells in a tumor, representing over 70% of the cells, this was interpreted as a complete monosomy for chromosome 9. Underrepresentation of chromosome 9 was considered when the average number of spots for chromosome 9 differed more than 0.3 from the average number of spots for chromosome

6 and/or the tumor had a considerable sub-population of tetra-/aneuploid cells as determined with FCM while the number of spots for chromosome 9 was lower than the ploidy would predict.

DNA preparation and LOH analysis

Matched pairs of bladder tumors and normal control tissue of the same patient was isolated by proteinase K (2 mg/ml) digestion of deparaffinized 5 µm sections, followed by phenol/chloroform extraction and ethanol precipitation. For LOH analysis, microsatellite primer sequences were obtained from the Genome Data Base (http://gdbwww.gdb.org/gdb). The following primers where included: D9S178, D9S168, D9S156, D9S171, D9S165, all located on chromosome 9p, and D9S166, D9S153, D9S264, D9S283, D9S197, D9S196, D9S280, D9S180, D9S272, D9S173, D9S154, D9S275, D9S195, D9S179, D9S164, D9S158, all located on chromosome 9q. Primers are listed in linkage order. Template DNA (±50 ng) was amplified in a total volume of 15 µl reaction mixture containing 2.5 µM dNTPs, 10 pmol of the appropriate primer combination, and 0.25 units of Taq polymerase (Promega). Products were labeled with a-^{32}P-dATP. Thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of each 55°C for 45 sec, 72°C for 45 sec, and 94°C for 45 sec. The final elongation step was 72°C for 10 min. PCR-products were separated on 6% denaturing polyacrylamide gels. Detection was done by autoradiography and, when necessary, followed by quantification using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). An allele was considered to be lost when the intensity of the remaining signal was less than 50% compared to the signal of the same allele in the matching control DNA of the same patient. However, the intensity of the lost allele in most tumors was 30% or less.

Statistical analysis

The Chi-square test was used to determine the correlation between tumor stage and grade, loss of chromosome 9 as detected with polymorphic markers or *in situ* hybridization, and DNA-index. A p-value of <0.05 was considered significant.

Results

DNA analyses

In order to obtain a better insight in the loss of chromosome 9 sequences in TCCs of the bladder and the possible mechanisms by which these losses occur, we investigated forty TCCs with different techniques. The combined results are depicted in Table 1. The grade and stage of the tumors is included in the table. Loss of heterozygosity (LOH) was determined by PCR with primers for several microsatellite markers for both the p and q arm of chromosome 9. In Table 1, LOH is indicated by Y(es) or N(o), and the number of microsatellites that were examined for a given tumor is indicated between brackets. Flow cytometry was used to determine the DNA-ploidy of the tumor.

The results are shown in Table 1 as the DNA-index, i.e. the ratio between the distance of a possible aneuploid peak and the peak representing the diploid nuclei. In addition, the percentage of nuclei in the diploid (peak 1) and aneuploid (peak 2) peaks is given to reflect the relative number of tumor cells within each subpopulation. *In situ* hybridization (ISH) was performed with probes for centromeres of chromosomes 6 and 9. In Table 1 the results obtained are represented by the average number of spots for each probe per nucleus. In order to facilitate the interpretation, Table 1 is divided into tumors with an underrepresentation of chromosome 9 centromeres relative to either the number of spots for chromosome 6 and/or the DNA-ploidy (Table 1A) and those with LOH but without any indication for numerical aberrations of chromosome 9 (Table 1B) and finally, Table 1C represents those cases in which no indication for numerical or structural aberrations were obtained. The complete *in situ* hybridization data for the tumors from Table 1A and B are shown separately in Figure 1.

In 5 tumors (tcc5, 8, 20, 36, and 57), monosomy for chromosome 9 was observed in more than 20% of cells. The ISH countings of these tumors are shown in Figure 1. Tumor 57 is the only example with monosomy in the largest population of cells: over 70% of the nuclei have one spot for the centromere 9 probe. Figure 2 shows representative areas of the slides with interphase nuclei of this tumor hybridized with a probe for chromosome 6 (Figure 2A) and chromosome 9 (Figure 2B). This result is corroborated by the LOH analysis in which a clear loss of all but one microsatellite marker was observed.

Table 1. Histopathological stage and grade, chromosome 9 loss and DNA-index in transitional cell carcinoma of the bladder

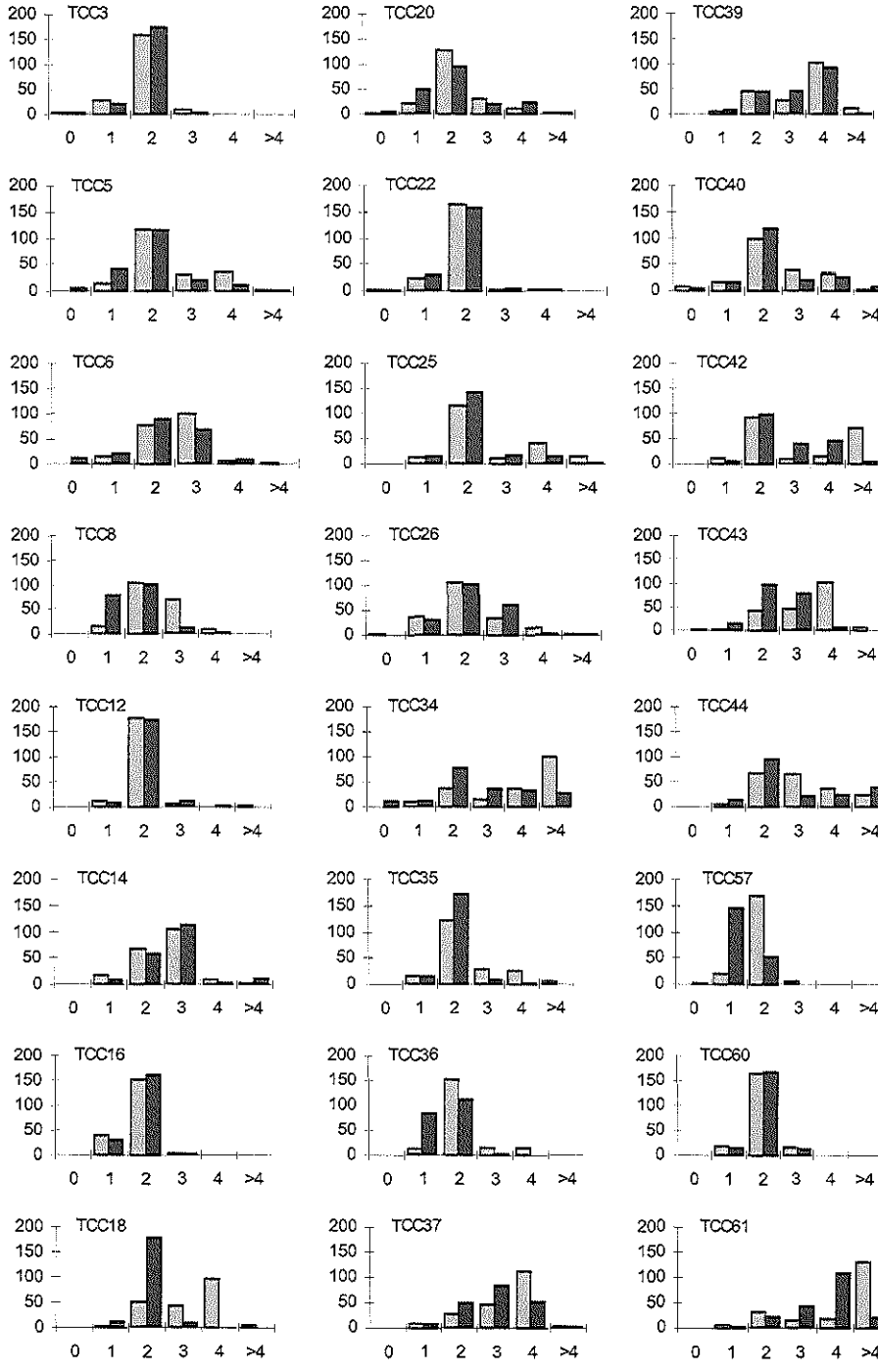
Pathology			LOH		ISH		FLOW CYTOMETRY		
					average # of spots		peak1	peak2	DI
TCC	T	G	9p	9q	chrom. 6	chrom. 9	%	%	
A) tumors with an underrepresentation of chromosome 9 centromeres									
5	3	3	Y(1)	Y(4)	2.46	1.99	59	6	1.00
6	4	3	N(1)	Y(1)	2.50	2.21	64	11	1.00
8	1	3	nd	nd	2.38	1.70	64	3	1.00
14	3	3	N(1)	N(3)/Y(1)	2.55	2.74	16	49	2.00
16	CIS?	3	nd	N(1)/Y(2)	1.80	1.88	29	23	1.98
18	2	3	Y(2)	Y(4)/N(2)	3.25	2.00	46	21	1.87
20	1	3	nd	N(2)	2.21	2.07	31	20	1.98
25	2	3	N(1)	N(6)	2.64	2.23	64	6	1.00
26	2	3	nd	N(1)	2.20	2.22	39	24	2.00
34	2	3	nd	nd	3.89	2.76	20	28	2.34
35	2	2	Y(1)	Y(3)	2.43	2.01	37	32	1.90
36	1	2	N(1)	Y(3)/N(14)	2.20	1.59	65	7	1.00
37	2	3	nd	N(3)	3.35	2.93	19	35	1.70
40	a?	?	Y(1)	Y(5)	2.42	2.37	40	34	2.10
42	2	3	Y(1)	Y(3)/N(2)	3.19	2.74	47	9	1.00
43	1	3	nd	N(4)	3.33	2.36	49	9	1.00
44	2	3	Y(2)/N(1)	Y(6)/N(1)	3.02	2.89	26	9	1.00
57	1	2	Y(1)	Y(10)/N(1)	1.92	1.26	53	8	1.00
61	2	3	nd	nd	4.19	3.61	23	31	2.90
B) tumors with LOH and no underrepresentation of chromosome 9									
3	a	1	nd	Y(1)	1.87	1.88	nd	nd	nd
12	1	3	Y(1)	Y(4)	2.02	2.06	nd	nd	nd
22	a	2	N(5)	Y(7)	1.89	1.91	59	8	1.00
39	a	2	N(3)	N(2)/Y(7)	3.36	3.16	47	20	2.00
60	a	2	Y(1)	Y(6)/N(2)	2.00	1.99	57	7	1.00
C) tumors without indication for loss of chromosome 9									
2	?	2	N(1)	N(3)	1.95	1.93	65	4	1.00
4	2	3	N(2)	nd	2.76	2.75	57	10	1.00
7	a	1	N(1)	N(4)	2.11	2.04	44	8	1.00
10	1	3	N(1)	N(1)	3.31	3.19	nd	nd	nd
29	1	2	N(2)	N(4)	1.93	1.88	22	21	1.10
33	2	3	nd	N(6)	2.00	1.97	62	9	1.00
38	2	3	N(2)	N(10)	2.28	2.30	22	35	1.60
46	?	3	N(1)	N(4)	3.44	3.52	19	35	1.80
49	1	2	N(1)	N(7)	3.60	3.53	57	9	1.00
50	2	3	nd	N(2)	3.43	3.38	24	26	2.00
53	?	?	N(1)	N(9)	2.01	1.96	69	1	1.00
54	a	2	N(1)	N(9)	1.96	1.99	58	7	1.00
56	2	3	nd	N(4)	1.99	1.99	42	7	1.00
58	a	1	N(1)	N(8)	2.01	1.91	68	4	1.00
59	a	1	nd	N(9)	1.88	1.84	46	8	1.00
62	2	3	nd	N(1)	2.17	2.16	49	6	1.00

Tcc8 and 36 apparently are heterogeneous and have a subpopulation of monosomic tumors cells, reflected by the 40% nuclei with one spot for chromosome 9. Tcc36 is a special case in which the 40% monosomic sub-population is accurately reflected by a lower signal for 7 LOH markers (about 60-70% signal remaining). This is not reported as LOH in the table because the percentage signal that is left, is higher than the definition we use for LOH. In addition, this tumor harbours an interstitial deletion on one chromosome 9, which results in 3 markers with about 30% remaining signal (the markers with LOH in the table). Unfortunately no DNA was available for LOH analysis of tcc8. In tcc5 and tcc20 only a small fraction of the cells appeared to have lost one copy of centromere 9. However, for tcc5 the LOH on both p and q arms as judged from the relative intensity of the autoradiographic signals is much higher than can be explained by the percentage of cells with monosomy (results not shown). In tcc20 no LOH was observed. A discrepancy between loss of chromosome 9 centromere spots relative to chromosome 6 and/or the ploidy on the one hand and the extent of loss in the LOH analyses on the other was observed for tcc5, 6, 14, 16, 18, 20, 25, 26, 35, 37, 42, and 43. Taken together, this means that a concordance between the LOH results and the ISH/flow cytometry was obvious in 3/18 cases (tcc36, 40 and 57), and no concordance was observed in 12/18 cases. In 3/18 cases no LOH data were obtained due to insufficient tumor material.

Statistical correlations between parameters

No correlation could be found between chromosome 9 loss as detected with LOH and tumor stage or grade. The chromosome 6 probe correlated well with the DNA-index ($p=0.024$). Although an association was found between chromosome 9 underrepresentation as detected with *in situ* hybridization and LOH ($p=0.0063$), this is misleading, since the extent of loss in the LOH analyses is much higher than can be explained by the extent of underrepresentation of chromosome 9.

Figure 1. (next page) Distribution of centromere 6 and 9 signals as determined by in situ hybridization of the tumors with LOH or underrepresentation of chromosome 9. Shown are the number of spots for chromosome 6 and 9 per nucleus in 200 nuclei per tumor. On the X-axis are the number of spots, varying between no spots (0) and 5 or more spots (>5). On the Y-axis are the number of nuclei. The results for chromosome 6 are represented by light bars, the results of chromosome 9 by dark bars. Only tcc5, 8, 20, 36 and 57 have a significant population of cells with monosomy 9.


TCC3
TCC20
TCC39
TCC5
TCC22
TCC40
TCC6
TCC25
TCC42
TCC8
TCC26
TCC43
TCC12
TCC34
TCC44
TCC14
TCC35
TCC57
TCC16
TCC36
TCC60
TCC18
TCC37
TCC61
200
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100
50
0
0 1 2 3 4 >4

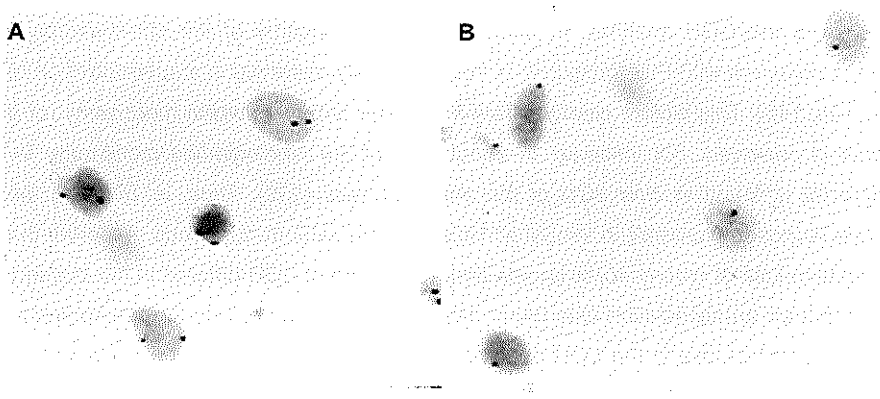

Figure 2. DNA-ISH on isolated interphase nuclei of bladder tumor 57. Biotinylated probes were visualized with DAB. (A) With probe p308, specific for the centromeric region of chromosome 6, two spots are visible per nucleus. (B) Monosomy 9 is detected after hybridization with the centromere 9 associated probe pHuR98.

Discussion

In our series of TCCs, 18 tumors displayed a relative loss of the chromosome 9 centromere when compared with chromosome 6 or the ploidy. In 5 of these, no loss was observed in the LOH analysis. This suggests that in these cases the relative under-representations had nothing to do with monosomy 9 as a way of inactivating a tumor suppressor gene. In the cases where LOH was detected, the extent of loss did not correlate with the relative numerical loss observed with ISH and flow cytometry in 12/18 cases. Moreover, in 7/10 samples with an underrepresentation of chromosome 9 and LOH the loss was regional and did not cover the entire chromosome. Again this suggests that relative loss and LOH are two different entities and that the relative loss is not instrumental in bringing about LOH.

Aberrations concerning chromosome 9 have been observed by several investigators using techniques such as LOH analysis, FISH and CGH. However, there are some important differences between the techniques and this affects the significance of the obtained results with respect to the interpretation of tumor pathogenesis. When LOH is scored, in general this will imply that the intensity of the lost allele is visually much lower than that of the control. Thus, the loss is relatively clear-cut and it can safely be concluded that the region in which the marker(s) is located has been deleted in most of the tumor cells. The remaining signal can convincingly be explained by the presence of a percentage of non-tumorous endothelial or stromal cells in the tissue block. This is much less the case when loss is studied using FISH. Using this technique, most authors conclude that loss of chromosome 9 has occurred when a relative underrepresentation of chromosome 9 is observed when compared with the

number of spots for other chromosomes. A relative underrepresentation will also lead to an observed loss in the CGH analysis. In addition, when monosomy of the chromosome 9 centromere is observed with FISH, this loss is frequently only observed in a subpopulation of nuclei (70, 135, 149). This picture clearly differs from other tumors in which monosomy for a chromosome is observed. For instance, in about 60% of the meningiomas monosomy for chromosome 22 is found in over 80% of the analyzed cells (100). Thus, in TCCs of the bladder, pure monosomy present in almost all cells is rather rare. In our series of tumors the degree of relative loss of chromosome copies and the degree of LOH differed in the majority of tumors. Although an association was found between chromosome 9 underrepresentation and LOH, the extent of loss in the LOH analyses is much higher than can be explained by the extent of underrepresentation. We therefore conclude that there is no causal relationship between underrepresentation and LOH and that the observed correlation is spurious.

As implicated above, the best evidence for involvement of chromosome 9 associated tumor suppressor genes in pathogenesis of transitional cell carcinoma of the bladder is obtained by LOH analyses. We observed LOH in 16/37 tumors, this means that in at least 16/37 tumors (43%) of the TCCs putative tumor suppressor genes on chromosome 9 may play a role in pathogenesis. One tumor suppressor gene identified on 9p is the *CDKN2A (p16, MTS1)* gene. Cairns (13), showed that in 71% of primary bladder tumors homozygous deletions targeting this gene were detected. Besides the *CDKN2A* gene on 9p there is evidence for at least two other loci on chromosome 9q (60, 186).

In Table 1, 5 tumors (tcc3, 5, 12, 35, and 40) have LOH for all tested markers. These tumors have 2 centromere 9 signals per nucleus. A possible mechanism by which this situation has occurred, is that first one copy of chromosome 9 was lost, for instance by non-disjunction, later followed by reduplication of the preserved copy in the case of the diploid tumors or by tetraploidization in tcc35 and tcc40. Alternatively, the LOH is due to interstitial deletions on both arms with retention of the centromere. In tcc16, 18, 22, 39, 57 and 60 not all markers show LOH. Tumor 57 is monosomic with respect to centromere 9 and tumor 39 has an interstitial deletion on 9q (186). For the other 4 cases the regional losses may have occurred by interstitial deletions or by translocations. These tumors have 2 signals for centromere 9. The best explanation therefore is that the two centromere signals represent the two different copies of chromosome 9 and that the LOH is due to structural rather than numerical aberrations. A reduplication of the unaltered chromosome would be evident from the relative intensities of the microsatellite signals in the regions without LOH. These would differ from the allele intensities found in normal control DNA of the same patient and this was not found (results not shown).

To check whether the tumors discussed in this article are a representative selection of transitional cell carcinomas of the bladder, we also determined their pathological

parameters. From the 40 TCCs 18 were Ta/T1 and 17 T2-T4, 4 were grade 1, 10 grade 2 and 24 grade 3. These numbers do not differ significantly from those found by other investigators. In addition, it appeared that the chromosome 6 copy number is related to the ploidy of the tumors, suggesting that the chromosome 6 centromere probe can reliably be used to reflect the ploidy of the tumors. No significant relations were observed between LOH persé, LOH for 9p or 9q, and tumor grade, stage or DNA-index. Also this finding is in agreement with other investigators (16). This suggests that the putative tumor suppressor genes on chromosome 9 that are the target of the LOH events most probably are involved in the early stages of the pathogenesis of these tumors.

Acknowledgements

The authors like to thank Dr. M. Giphart-Gassler for helpful discussions.

Chapter 3. Evidence for two candidate tumor suppressor loci on chromosome 9q in transitional cell carcinoma (TCC) of the bladder but no homozygous deletions in bladder tumor cell lines

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Summary

The most frequent genetic alterations in transitional cell carcinoma (TCC) of the bladder involve loss of heterozygosity (LOH) on chromosome 9p and 9q. The LOH on chromosome 9p most likely targets the *CDKN2* locus, which is inactivated in about 50% of TCCs. Candidate genes that are the target for LOH on chromosome 9q have yet to be identified. To narrow the localisation of one or more putative tumor suppressor genes on this chromosome that play a role in TCC of the bladder, we examined 59 tumors with a panel of microsatellite markers along the chromosome. LOH was observed in 26 (44%) tumors. We present evidence for two different loci on the long arm of chromosome 9 where potential tumor suppressor genes are expected. These loci are delineated by interstitial deletions in two bladder tumors. Our results confirm the results of others and contribute to a further reduction of the size of these regions, which we called TCC1 and TCC2. These regions were examined for homozygous deletions with EST and STS markers. No homozygous deletions were observed in 17 different bladder tumor cell lines.

Introduction

Bladder cancer is the fifth most common cancer in males. Over 95% of all bladder cancers in industrialised countries are transitional cell carcinomas (TCCs). TCCs are presented in two ways: superficial papillary tumors, confined to the mucosa and lamina propria, and invasive tumors spreading beyond the lamina propria into detrusor muscle. The remaining 5% of tumors include squamous cell carcinomas, adenocarcinomas, and carcinoma *in situ* (CIS).

Frequent somatic allelic loss is regarded as a hallmark of tumor suppressor gene (TSG) inactivation. In TCCs, cytogenetic studies and loss of heterozygosity (LOH) analyses have revealed a number of chromosomal aberrations, including deletion of chromosome 9p and/or 9q (16, 36, 82, 105, 129), and deletions of chromosome 11p (157), 18q (12), chromosome 8 (89, 170), 4p (39, 136), and 14q (22). Loss of heterozygosity of markers on chromosome 9 is found in TCCs of all grades and stages, suggesting that the inactivation of a putative tumor suppressor gene (TSG) on this chromosome is an early event in the development of bladder cancer. Several groups (60, 144, 160), reported evidence for the presence of more than one TSG that can contribute to the development of bladder cancer on chromosome 9. The *CDKN2A* (*p16, MTS1*) and *CDKN2B* (*p15*) genes are localised on the short arm of chromosome 9. Recent studies showed the inactivation of these genes in as much as 40-50% of bladder tumors (2, 13). More detailed deletion mapping on the long arm revealed two regions of loss (60, 160). Small interstitial deletions covering the location of the marker D9S195 were recently reported in 5 TCCs. The shortest region of overlap of these

deletions is estimated to amount to about 840 kb. The putative TSG in this Deleted in Bladder Cancer region (DBC1) was called DBCCR1 (61). This DBC1 region does not overlap with the two other regions described by Habuchi and Simoneau *et al.* Thus, the combined data provides evidence for 3 TSGs on chromosome 9q that may play a role in the pathogenesis of bladder cancer.

In the present study we used a PCR-based microsatellite assay to further delineate the extent of the deletions at chromosome 9q. A combination of our data with those of others, supports the view that apart from the DBC1 region two other putative TSG loci may exist on chromosome 9q. These two regions were called TCC1 and TCC2. We screened 17 bladder tumor cell lines for homozygous deletions in these areas. No evidence for homozygous deletions was obtained.

Materials and Methods

DNA preparation

Matched pairs of 59 paraffin-embedded bladder tumors and normal control tissue of the same patient were selected. Paraffin sections were examined microscopically by a pathologist (Th. v/d K). Parts that represented tumor tissue were punched out of the original paraffin blocks and newly embedded. DNA was isolated by proteinase K (2 mg/ml) digestion of deparaffinised 5 μm sections, followed by phenol/chloroform extraction and ethanol precipitation. A haematoxylin/eosin staining of sections flanking the sections used for DNA isolation was again controlled by the pathologist. In general the percentage tumor tissue in the material dissected by this procedure was estimated to be over 90%.

The following bladder tumor cell lines were used: 253J, 575A, 647V, 1207, 5637, J82, Jon, RT4, RT112, SCaBER, SD, SW780, SW800, SW1710, T24, VMCubI, and VMCubII. Dr. D. Chopin, Paris, kindly provided the cell lines 1207 and 647V. Genomic DNA was prepared according to standard procedures (145).

LOH analysis

For LOH analysis, microsatellite primer sequences were obtained from the Genome DataBase (http://gdbwww.gdb.org/gdb). Thirty primer pairs were used. On the short arm, the markers D9S178, D9S171, D9S168, D9S165, and D9S156 were included. On the long arm, the markers D9S153, D9S154, D9S158, D9S164, D9S166, D9S173, D9S176, D9S177, D9S179, D9S180, D9S195, D9S196, D9S197, D9S257, D9S264, D9S272, D9S275, D9S278, D9S280, D9S283, D9S287, D9S1783, D9S1818, D9S1826, D9S1838 were used. Template DNA (50 ng) was amplified in a total volume of 15 μl reaction mixture containing

2.5 mM dNTPs, 10 pmol of the appropriate primer combination, and 0.25 units of Taq polymerase (Supertaq). Products were labelled with a-^{32}P-dATP.

Thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 32 cycles of each 55°C for 45 sec, 72°C for 40 sec, and 94°C for 40 sec. The final elongation step was 72°C for 10 min. PCR-products were separated on 6% denaturing polyacrylamide gels. Detection was done by autoradiography and, when necessary, followed by quantification using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). An allele was considered to be lost when the intensity of the remaining signal was less than 50% compared to the signal of the same allele in the matching control DNA of the same patient.

HD screening

For the homozygous deletion mapping, 98 primer sequences were obtained from the Whitehead Institute (http://www-genome.wi.mit.edu/), the Sanger Centre (http://www.sanger.ac.uk), and The Institute of Genome Research (TIGR) (http://www.tigr.org). All primer sequences were from sequence tagged sites (STS) or expressed sequence tags (EST) mapped between our TCC1 and TCC2 border markers (http://www.ncbi.nlm.nih.gov/Science96). Amplification was done as described for the LOH analysis, with the exception of the presence of a second control primer set in the reaction mixture. As a control, primers were used for the NF2 (exon 5 and 11, (76)) or MN1 genes on chromosome 22 (bp 5304-5421, forward: MN1-16, 5'- AGG TTG GTA CCT GCT TAG TG, reverse: MN1-13, 5'- GGG TTA ACA CTG GTA ACA TAC), since there are no data suggesting the involvement of either of these genes or the chromosome in bladder cancer. Since the presence of a homozygous deletion in the *CDKN2A* gene was known in 8 of the 17 cell lines used, primers were included for a 167 bp product spanning an intron-exon boundary of the *CDKN2A* gene (123). The detection of these deletions was used as a positive control.

Results

LOH analysis

Fifty-nine bladder tumors were screened for LOH of markers on chromosome 9. Twenty-six tumors (44%) showed LOH for one or more markers. No microsatellite instability was seen. Of these, 2 tumors had a deletion confined to the p arm, in 10 the loss was confined to the q arm and in 12 cases both p and q arms were affected. Losses on the short arm overlap the region containing the *CDKN2* locus, which is located telomeric to marker D9S171. Two individual tumors were found to obtain different interstitial deletions on chromosome 9q, suggesting two different TSG loci on this chromosome arm. These are discussed in detail in

the following sections. Other regions of loss that were observed on 9q could target both putative TSG loci on 9q and/or the *CDKN2* locus and did not contribute to a further delineation of these loci.

An interstitial deletion between D9S165 and D9S176

In tcc39 an interstitial deletion was observed between the flanking markers D9S165 and D9S176. No loss was observed for 3 microsatellite markers on 9p. Examples of the LOH analysis of 9q are shown in Figure 1A. Between D9S165 and D9S176, a clear LOH was observed for 7 microsatellites. The autoradiogram for one of these, D9S283 is shown in Figure 1A. The extent of loss was also calculated with the Phosphorimager. The results obtained are depicted in Table 1 in the lane marked Tcc39. For D9S283 the signal of the lost allele was measured to be 13% of that of the control allele from normal tissue. For some of the other markers slightly higher values were observed. This is probably due to the fact that when two alleles are relatively close together and comprise several stutter bands, they contribute to each others background. In some cases, i.e. for D9S272 and D9S1783, this makes the quantitative analysis impossible, although with the eye a clear LOH is evident. The region deleted in tcc39 is approximately 48 cM in size. Tcc39 was classified by the pathologist as Ta/grade II.

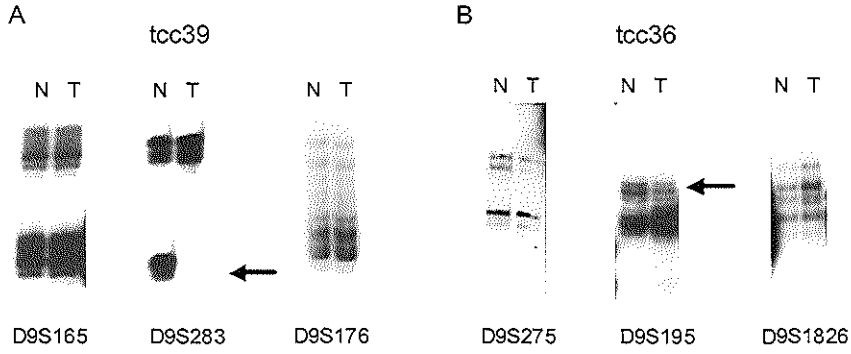

Figure 1. Autoradiographs illustrating the LOH analyses for tcc36 and tcc39. N: matched control DNA; T: tumor DNA. Arrows indicate deleted alleles. (A) tcc39: markers D9S165 and D9S176 show retention, while marker D9S283 shows loss of the lower allele. (B) tcc36: markers D9S275 and D9S1826 show retention, while marker D9S195 shows a lower intensity of the upper allele.

An interstitial deletion between D9S275 and D9S1826

Tcc36 is a T1/grade II bladder tumor in which 40% of the cells are monosomic for chromosome 9 as determined by *in situ* hybridisation using the chromosome 9 heterochromatin region probe pHUR98 (187). Thus tcc36 is heterogeneous with respect to its genomic constitution. This partial loss of one copy of chromosome 9 is also observed in the LOH analyses and is reflected by the measured intensities of allele signals of around 60% as shown in Table 1. Three microsatellites show a remaining signal of approximately 30-40% when compared to the control DNA. This most likely reflects an interstitial deletion of 31 cM flanked by markers D9S275 and D9S1826. Figure 1B shows representative autoradiograms of the LOH analyses of tcc36. Based on the signal intensities as measured by the Phosphorimager, the most plausible model to explain these findings would be that one allele of a putative TSG which is located within the interstitially deleted area was first inactivated and that two individual second hits targeting the other allele occurred in separate cells: a) loss of an entire copy of chromosome 9 as reflected by the subpopulation of 40% of the tumor cells that are monosomic for chromosome 9 and b) an interstitial deletion of the same copy of chromosome 9 present in approximately 25% of the tumor cells.

Figure 2. The borders of the TCC1 region. Markers are shown in linkage and physical mapping order according to the Whitehead Institute contig data and CEPH/Généthon data. White circles: retention; black circles: loss of heterozygosity; grey circles: not informative. On the right side, a vertical bar indicates the potential smallest region of overlap, based on our results with tcc39 and the results of Habuchi et al. (60).

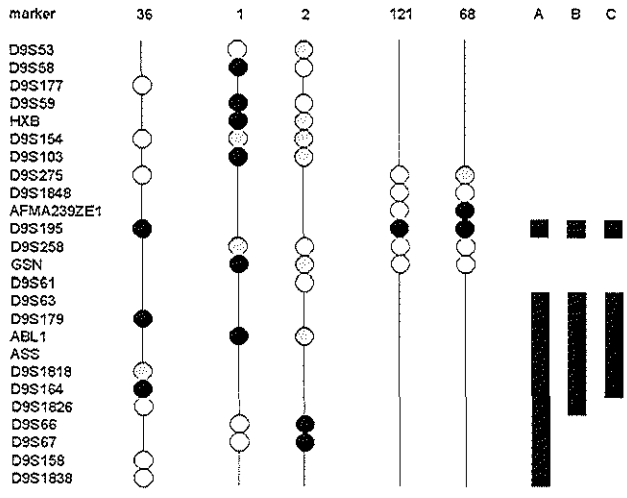

Figure 3. The borders of the DBC1 and TCC2 regions. Markers are shown in linkage and physical mapping order according to the Whitehead Institute contig data and CEPH/Généthon data. White circles: retention; black circles: loss of heterozygosity; grey circles: not informative. On the right side, vertical bars indicate the possible smallest regions of overlap, based on our results with tcc36 and the results of Habuchi et al. (60, 64), and Simoneau et al. (160). For an explanation of the 3 possible SROs for TCC2 (A, B, and C), see text.

Table 1. Percentages of allele signal left for tcc36 and tcc39 as determined with the Phosphorimager.[a]

Marker	Tcc36	Tcc39
D9S165		0.94
D9S166		0.34
D9S264		0.15
D9S283	0.87	0.13
D9S197	0.51	
D9S280		0.19
D9S287	0.57	
D9S180	0.59	0.22
D9S272		N.M.[c]
D9S1783		N.M.
D9S176		0.77
D9S173		0.89
D9S177		1.14
D9S154	0.64	
D9S275	0.73	
D9S195	0.37	
D9S179	0.38	0.76
D9S164	0.44	
D9S1818	N.I.[b]	
D9S1826	0.60	
D9S158	0.86	0.60
D9S1838	0.65	

[a]*The percentages were determined by comparing the intensity of the lost allele in the tumor with the intensity of the same allele in normal control DNA, in relation to the intensity of the other retained allele.* [b]*N.I., Not Informative;* [c]*N.M., Not Measured.*

Homozygous deletion analysis

We next screened DNA from 17 bladder tumor cell lines for homozygous deletions in these areas. For the more centromeric region, 83 ESTs and STSs were selected between the markers D9S153 and D9S176, a region of 27 cM. The borders of this region are indicated in Figure 2, they were deduced based on our results with tcc39 and the results of Habuchi *et al.* (60). The markers for the homozygous deletion analysis are listed in Table 2. When the markers are randomly distributed, this results in a density of one marker per 300 kb. Special attention was paid to the prevention of contaminating the PCR-reaction to avoid false positives, by strictly separating the equipment used to handle amplified DNA from other equipment. Separate work areas were used for pre- and post-amplification steps. Random negative controls were included. No homozygous deletions were found. In addition, the region with an interstitial deletion as defined by tcc36 was screened with 15 sets of PCR primers, representing a density of 1 marker per 500 kb. However, no evidence for homozygous deletions was obtained. As a control we also screened the cell lines for deletions of the *CDKN2* locus. Deletions were observed in 8 of the 17 cell lines tested. This confirms data obtained by others (164, 171, 204).

Table 2. Overview of the ESTs and STSs used for HD mappng, ordered from centromere to telomere.

Located in TCC1					Located in TCC2
1 stSG8675	22 A006N11	43 A002D08	64 WI-7285	84 U18543	
2 WI-30336	23 A006U15	44 A008R29	65 WI-11414	85 IB3089	
3 A002Y36	24 NIB973	45 IB543	66 A001T44	86 WI-13592	
4 WI-11585	25 stSG1471	46 WI-2958	67 WI-8684	87 WI-11542	
5 CKS2	26 stSG2118	47 WI-6937	68 TGFBR1	88 WI-6257	
6 WI-11909	27 NIB722	48 IR10	69 A008N47	89 WI-12734	
7 WI-16825	28 stSG2205	49 WI-13546	70 A005N10	90 ROP	
8 WI-12646	29 stSG3724	50 PTCH	71 WI-7447	91 WI-11957	
9 stSG2370	30 WI-7541	51 WI-14826	72 WI-5249	92 FB23F1	
10 stSG8105	31 WI-13139	52 WI-1941	73 WI-14669	93 WI-15097	
11 WI-2414	32 A007K29	53 WI-2013	74 WI-7344	94 WI-13608	
12 stSG9248	33 WI-6338	54 stSG9221	75 WI-3790	95 NIB1929	
13 WI-4860	34 WI-6428	55 WI-9350	76 NIB1437	96 WI-14271	
14 A004T01	35 WI-4577	56 A006I15	77 WI-15742	97 WI-11577	
15 IB2336	36 WI-532	57 WI-6378	78 WI-688	98 WI-12991	
16 IB3559	37 WI-8025	58 stSG8121	79 SGC31311		
17 WI-9447	38 stSG1737	59 WI-9840	80 WI-11370		
18 WI-2331	39 stSG2403	60 WI-9914	81 WI-2008		
19 WI-6758	40 WI-15517	61 WI-9212	82 A008T08		
20 WI-17567	41 A001U11	62 A003P31	83 WI-4017		
21 WI-9905	42 WI-2820	63 WI-7974			

Discussion

The purpose of this study was to further define chromosome 9q deletions in TCCs. Previous LOH analyses predicted that 57% of tumors had deletions on chromosome 9p and 9q (60). The putative presence of two or more TSGs on the same chromosome complicates the interpretation of the LOH analysis. Most estimations for the losses of the whole chromosome are based on allelotyping studies in which a limited number of 9p and 9q markers were used. This causes high percentages of apparent complete loss of chromosome 9. It is our experience that when more markers are used most of these apparent cases of monosomy are in fact large terminal or interstitial deletions. This emphasises the importance of testing as many informative polymorphic markers as possible. The *CDKN2A (p16)/CDKN2B (p15)* tumor suppressor genes are located on the short arm of chromosome 9. Loss of one or even both copies of these genes was shown to occur in at least 40-50% of TCCs (2, 13). In some cases the region of LOH spreads from the *CDKN2* region beyond the centromere into the q arm of chromosome 9. Such a deletion could target the *CDKN2* region, a locus on the q arm or even both. In addition, losses that are confined to the q arm of chromosome 9 suggest the existence of more than one candidate bladder gene on this arm. Also here it is often not possible to define to which region the observed LOH contributes.

Our LOH results confirm the hypothesis that there are at least two different putative tumor suppressor gene loci on the q arm. The first, more centromeric region is called TCC1. The borders of this new region, as depicted in Figure 2, are defined by the interstitial deletion in tcc39 as reported in this work and an interstitial deletion in a bladder tumor number 1 as published by Habuchi *et al.* (60). For the definition of the region we have excluded tumors in which LOH was observed based on only one tested marker. Our results place the lower border of the TCC1 region at marker D9S176, instead of D9S109. This reduces the size of the region with 6 cM from 33 to 27 cM. In both our case tcc39 and case 1, the signal intensities of the remaining alleles are very low, suggesting that the gene targeted by these deletions is inactivated in most if not all tumor cells. Thus, inactivation of this gene may represent an early event in the pathogenesis of these tumors.

For the definition of a second region, several possibilities exist. These are shown in Figure 3. In this figure the interstitial deletion in tcc36 (this paper) is shown next to deletions as published by the group of Knowles (60, 64). The DBC1 region was deducted from short interstitial deletions in 5 separate tumors that have a shortest region of overlap of 840 kb in which marker D9S195 is located (64). The first conclusion from these combined data is that it is impossible to attribute all deletions to one region. For instance, cases 36 and 1 can both target the DBC1 region, but case 2 clearly falls outside this region. The 3 different possible SRO regions based on these data are also shown in Figure 3. In A, it is assumed that both

tcc36 and tcc1 target the DBC1 region. This would result in a TCC2 region defined by case 2. In option B, tcc36 targets DBC1 and tcc1 theTCC2 region and reversely in option C, the case 2 deletion targets DBC1 and tcc36 TCC2. As a result, the size of the TCC2 region can vary from 30 to 40 cM.

Approximately 40% of the cells in tcc36 are monosomic for chromosome 9 and in an additional 25% an interstitial deletion of the same copy of chromosome 9 occurred. This suggests that both the interstitial deletion and the loss of an entire chromosome may target the same tumor suppressor gene and that for this gene these two events represent separate second hits. These findings suggest that the inactivation of the proposed TSG at this location may not have been one of the first hits in the pathogenesis of tcc36.

Losses of chromosome 9q have also been observed in basal cell carcinoma (153), squamous cell carcinoma of the head and neck (1), oesophagus carcinoma (117), ovarian cancer (152), renal cell carcinoma (19), and small cell lung cancer (116). Since the SROs for bladder cancer are still very large, with the exception of the DBC1 region, it cannot be excluded that the losses seen in other tumors target the same TSGs. Recently, the gene responsible for sporadic basal cell carcinoma of the skin and the hereditary disorder NBCCS was identified. This *PTCH* gene is located within the TCC1 region. However, no mutations in this gene in bladder tumors were observed (160, our unpublished results). For oesophagus carcinoma, the region containing a putative tumor suppressor gene has been narrowed down to about 200 kb, between the markers D9S155 and D9S177. These microsatellites are positioned distal of the TCC1 region and proximal to the DBC1 region. In ovarian cancer, LOH is found around the gelsolin gene (*GSN*), where the DBC1 gene is expected.

Both the TCC1 and TCC2 regions were screened for the presence of homozygous deletion in 17 bladder tumor cell lines with in total 100 microsatellite markers, with an average spacing of 300-500 kb. Deletions of the *CDKN2* region are often between 50-500 kb or more in size (204). Other homozygous deletions vary between 130 kb in B-cell chronic lymphocytic leukaemia (30), and 3 Mb in NSCLC (94). This suggests that the homozygous deletion targeting the TCC1 and TCC2 loci are either much smaller in size than those observed for other loci or that the putative TSGs are not readily inactivated by homozygous deletions.

Several interesting genes in the TCC2 region are known: the Transforming Growth Factor β receptor type I gene (*TGFBR1*), the Death Associated Protein Kinase 1 gene (*DAPK1*) and theTuberous Sclerosis 1 gene (*TSC1*). Further studies to identify the genes involved in bladder cancer, should include these genes as candidates.

Chapter 4. The chromosome 9q genes TGFBR1, TSC1 and ZNF189 are rarely mutated in bladder cancer

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Abstract

We assessed a series of bladder tumours and bladder tumour cell lines for sequence variation in the Krüppel-like zinc finger gene *ZNF189*, the Tuberous Sclerosis Complex gene 1 (*TSC1*) and the TGF beta receptor type I (*TGFBR1*). All three genes have been mapped to 9q regions commonly deleted in transitional cell carcinoma of the bladder. Mutation analysis of the coding sequence of these genes revealed several variant bands that were shown to represent polymorphisms. Mutation analysis of the *ZNF189* gene in bladder cancer cell lines identified one amino acid substitution (lysine → isoleucine) at position 323 in exon 4. For the *TSC1* gene, two mutations were identified in two out of 27 independent cell lines. Both mutations result in a truncated protein. Furthermore, one out of 36 bladder tumours had a frameshift mutation in exon 7 of the *TSC1* gene. No tumour-specific mutations were found in the *TGFBR1* gene. We also investigated the length of the polyalanine tract present in exon 1 of the *TGFBR1* gene. It has been suggested that the allele with 6 alanines (6A) is more frequent in patients with bladder and other cancers. We therefore compared bladder cancer patients with normal controls. In both groups the percentage of heterozygotes was 17%. Thus, our data do not support a role for the 6A allele in bladder cancer susceptibility.

Introduction

Tumors of the transitional epithelium of the bladder often present as superficial, non- or minimally invasive, papillary structures. The most prominent genetic aberration in these tumors is loss of (part of) chromosome 9 (141). So far, several groups have tried to identify putative tumor suppressor genes on chromosome 9q by testing for loss of heterozygosity (LOH) with microsatellite markers. This approach has led to the establishment of different regions of loss on both the short arm and the long arm (60, 64, 82, 160, 161, 186). A further narrowing down of these regions has proven to be difficult because of the complicated loss-patterns present in many tumors. Another approach therefore, would be to look for the involvement of candidate genes known to be located in the regions of interest. Presently, only a few chromosome 9 genes have been considered, including *CDKN2A*, *GAS1*, Gelsolin, *PTCH*, and *DBCCR1* (61, 160, 173). Although the involvement of the 9p gene *CDKN2A* has been shown in several studies, no significant percentage of tumors has been correlated with a specific gene on chromosome 9q (61, 173).

In this study, we describe the analysis of three different candidate genes located on chromosome 9q that could be involved in bladder cancer. The *ZNF189* gene encodes a Krüppel-like zinc finger protein with a predicted KRAB A domain at the N terminus, a spacer region, and 16 zinc fingers of the Cys_2His_2 type (124). These domains are frequently found in

proteins involved in transcription repression. Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the growth of multiple benign tumors (hamartomas) in many tissues and organs. The *TSC1* gene was identified on chromosome 9q34 (184). The LOH observed in TSC associated hamartomas suggests that *TSC1* acts as a tumor suppressor gene. Transforming growth factor ß regulates cell cycle progression via binding to the type II receptor and activation of the type I receptor. Both receptors are transmembrane serine/threonine kinases. Various types of human tumor cells are insensitive to TGF-ß mediated cell cycle arrest, suggesting inactivation of the TGF-ß signalling pathway, either by mutation in the type I or type II receptor. *TGFBR2* mutations have been found in different tumor types, mostly in combination with the MIN+ phenotype (113). The knowledge of the tumorigenic role of the *TGFBR1* gene has been limited to the identification of a single mutation in breast cancer metastases (23). A polyalanine stretch in exon 1 of this gene shows variation in length, the most frequent alleles being TßR-I with nine alanine residues and TßR-I(6A) with six alanine residues (133). Recent studies suggest a difference in functionality of both alleles, where the TßR-I(6A) variant could function as a susceptibility allele for bladder and several other types of cancer (24, 132, 133).

Materials and Methods

Bladder cancer cell lines and tumors

The following bladder cancer cell lines were screened for mutations in the coding sequence of the candidate genes: 253J, 575A, 647V, 1207, 1266, 5637, EJ, HcV29, HT1197, HT1376, J82, Jon, KK47, RT4, RT112, SCaBER, SD, SW780, SW800, SW1710, T24, TCCSUP, UMUC3, VMCubI, VMCubII, VMCubIII, VT (available from ATCC or DNA kindly provided by Dr. F. Radvanyi). Genomic DNA was prepared according to standard procedures (145). Thirty-six archival, paraffin-embedded tumours (10xTa, 9xT1, 14xT2, 3xT3) were tested for mutations in the TSC1 gene. DNA was isolated as described previously (186).

Microsatellite analysis

Microsatellite analysis of the bladder cancer cell lines was done as previously described (186). At least six different informative polymorphic markers for chromosome 9 were tested per cell line.

Mutation Analysis and sequencing

Primers for the *ZNF189* gene were as follows: exon 1: 5'-CCCAATTCCTGCCCCTA TTC-3', 5'-AAAGCAGTGCGGCCTA-3', exon 2: 5'-GGTTCGCGAACAAACTGC-3', 5'-CTTGGGGTCCAGGCACTGAG-3', exon 3: 5'-CCTCTGTCACTTTTAGTGG-3', 5'-

CAGTGAGACCAGGTTTCC-3', exon 4A: 5'-GAACAGAGATAAGGATGAGG-3', 5'-TTTATGGGGTCTTTCCCCAG-3', exon 4B: 5'-CCAACTCAGAGAGAAATGC-3', 5'-CTGCTGTATAACAAGACTGC-3', exon 4C: 5'-CCAGGGAGAAGACTTATCC-3', 5'-GGTACTAGTCTCAAATCAAATTG-3'. For *TSC1*, primer sequences for amplification of the 21 coding exons (exon 3-23) were obtained from http://expmed.bwh.harvard.edu/projects/tsc/. For exon 15, 21, 22, and 23, new primers were designed (exon 15: 5'-GTAAAGGCTCAGGGTTCACG-3', 5'-CGTGAACCCTGA GCCTTTAC-3', 5'-AGGCTGCCCGCTTCCAAAG-3' 5'- CTTTGGAAGCGGGCAGCCT-3', exon 21: 5'-CACAAAAGCCTTTCCTGATG-3', exon 22: 5'-CATCAGGAAAGGC TTTTGTG-3', exon 23: 5'-TTGAACTGGGAAGTGAGCCC-3', 5'-GGGCTCACTTCC CAGTTCAA-3') to be able to amplify DNA isolated from the paraffin-embedded tumors. The primers for the *TGFBR1* gene were adapted from Vellucci and Reiss (188). PCR conditions were standardised at 35 cycles of 95°C for 30', 55°C for 30', and 72°C for 30', with the addition of α-^{32}P dATP. Amplification products were analysed for heteroduplex formation using weakly denaturing polyacrylamide gels (29:1 acryl:bisacryl) (32), or via single strand conformational polymorphism (SSCP) at room temperature with 6% polyacrylamide gels (49:1 acryl:bisacryl). Since in the case of cell lines, no normal control DNA of the same patient could be used and most of the cell lines are hemizygous for regions on chromosome 9q, we mixed the amplified DNA of two different samples prior to the formation of heteroduplexes. Aberrant bands were isolated from the acrylamide gels by incubating the slice in water for three hours at 37°C, followed by reamplification and sequencing using the Sequenase sequencing kit (USB, Cleveland, Ohio). For the polyalanine stretch in exon 1, primers were used according to Pasche *et al.* (133), Because of the high CG-content of this part of the gene, we used the Clontech GC-melt kit (Clontech, Palo Alto, CA) in combination with a touchdown PCR in the presence of α-^{32}P dCTP.

Statistical analysis

Frequency distribution data were analysed by the Chi-square test. A p-value of <0.05 was considered significant.

Results

Mutation analysis for the three candidate genes was done by SSCP and Heteroduplex Analysis (HDA) in 27 bladder tumor cell lines and, for *TSC1*, in 36 bladder tumors. Since many of the cell lines used will have LOH for the studied regions of chromosome 9, the hetero- or homozygosity of chromosomes 9 regions in 20 different bladder cancer cell lines was determined with a series of highly informative microsatellite markers. This is depicted in Figure 1. LOH of a region was deduced when three or more closely spaced markers revealed only one allele.

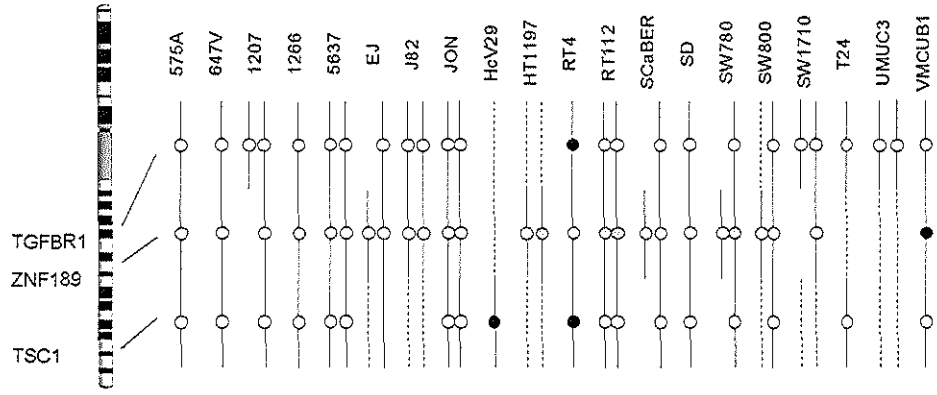

Figure 1. Determination of the chromosome 9 status of 20 bladder cancer cell lines with microsatellite markers and sequence determination. Cell lines 253J, HT1376, KK47, TCCSUP, VMCubII, VMCubIII and VT were also included in the mutation screening. At least six different informative polymorphic markers for chromosome 9 were tested per cell line. The chromosomes are represented by lines. The genes ZNF189, TSC1 and TGFBR1 are represented by circles. Informativity is indicated by a circle on both chromosomes. A dotted line is given when the hetero- or hemizygous state is unknown. Loss is represented by a gap in the chromosome. Mutations are indicated with a black circle. Polymorphisms are given in grey circles. For a description of the polymorphisms or mutations, see text.

For HDA, cell line DNA but also mixtures of DNA samples from two different cell lines were used to circumvent the possibility that mutations would go undetected because of LOH of the region of interest. Several aberrant bands were found in the coding sequence of the *ZNF189* gene (results not shown). Figure 2A and Figure 2B show two polymorphisms, Figure 2C shows the only mutation found in this gene. The effect of this mutation on the function of the protein is unknown. Table 1 summarizes the mutations and polymorphisms found.

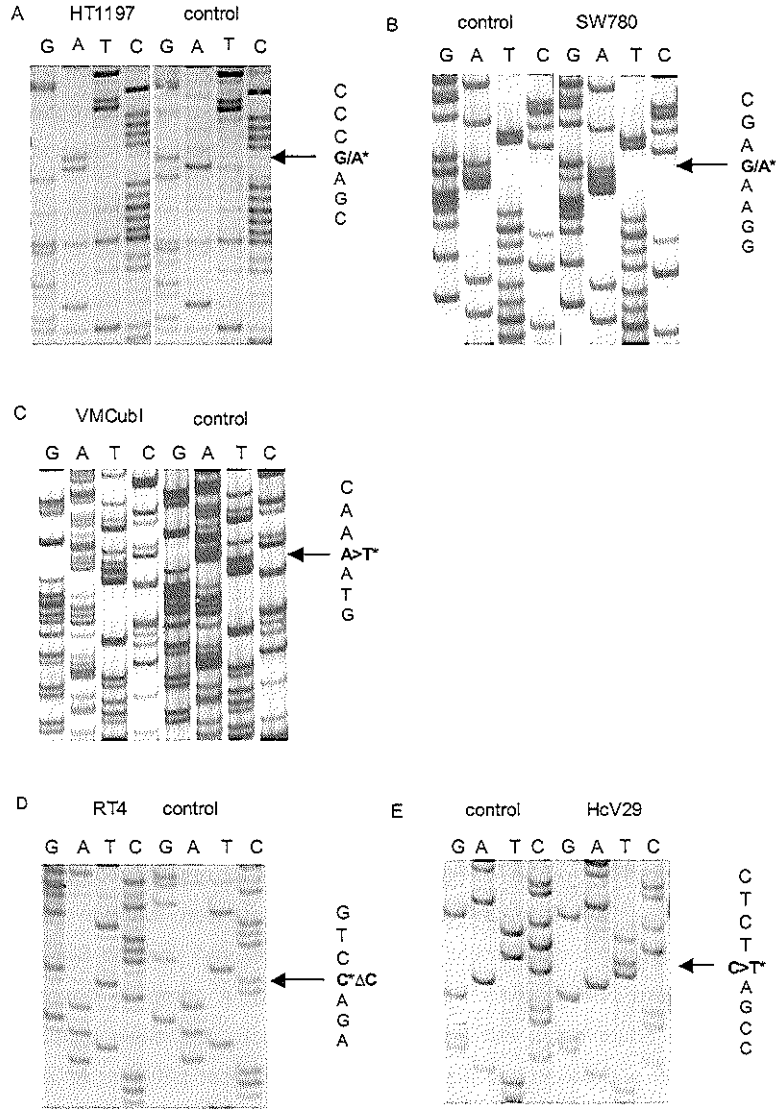

Figure 2. Sequence analysis of mutations found in bladder tumor cell lines. A; A G/A polymorphism is present in exon 1 bp 12 of the ZNF189 *gene in cell line HT1197. B; in exon 4 of the* ZNF189 *gene, a G/A polymorphism was found at bp 1602. Cell line SW780 is heterozygous at this position. C; an aminoacid substitution was found in cell line VMCubI at position 323 replacing a lysine with isoleucine (bp 969, AAA → ATA). D; A 1 bp deletion causes a frameshift mutation in exon 15 of the* TSC1 *gene in bladder tumor cell line RT4 and a stopcodon at amino acid 701. E; A C→T transition in exon 4 of* TSC1 *causes a stopcodon at aa 54 in bladder cell line HcV29.*

Table 1 also summarizes the results of the *TSC1* mutation screening. We found a one-basepair deletion in exon 15 (1890ΔC) in cell line RT4 (Figure 2D). This mutation has been reported before in bladder cancer (71). A second mutation was found in exon 4 in cell line HcV29 (Figure 2E). Furthermore, 36 tumors were included in the analysis. One patient had a mutation in exon 7 (data not show), resulting in a frameshift. In total, three mutations (2 out of 27 cell lines, 1 out of 36 tumors) were found in the *TSC1* gene.

Table 1. Alterations in ZNF189 *and* TSC1 *found in bladder cancer cell lines and bladder cancer patients.*

Gene	Cell line/tumor	Mutation	Consequence
ZNF189	HT1197, VMCubII, VMCubIII	Exon 1, 12G>A	Polymorphism
	24 of 27 cell lines	Exon 4, 1602G>A	Polymorphism
	VMCubI	Exon 4, 969A>T	K>I substitution
TSC1	RT4	Exon 15, 1890delC	Frameshift
	HcV29	Exon 4, 384C>T	stopcodon
	1207, SD, VMCubIII, tcc20, tcc56	Exon 22, 3050C>T	Polymorphism
	tcc42	Exon 7, 747insT	frameshift

Exons 2-8 were used for HDA of the *TGFBR1* gene. Primers for amplification were adapted from Vellucci and Reiss (188). No mutations or polymorphisms were found in the 27 tested cell lines. The analysis of the polyalanine tract present in exon 1 showed two of the four different alleles as were published by Pasche *et al.* (133). A complete absence of the polyalanine tract was observed in cell line RT4. Furthermore, the allele distribution was determined in a control population and a group of bladder cancer patients. An overview of the allele distribution is given in Table 2. In both our populations of random individuals and patients with TCC, the frequency of the TβR-I(6A) allele is significantly higher than previously reported, 17.5 and 17.1%, respectively. Our findings, in contrast to those of Pasche *et al.*, suggest that the TβR-I(6A) allele does not predispose towards the development of bladder cancer.

Table 2. Frequency of the polyalanine tract polymorphisms in normal blood donors, bladder cancer cell lines, and peripheral blood lymphocytes of bladder cancer patients.

Samples	9A/9A	9A/6A	6A/6A
Normal blood donors	148	32 (17.5%)	3
PBL of TCC patients	121	25 (17.1%)	0

Discussion

In this study we describe the mutation analysis of 3 9q genes in bladder cancers and bladder cancer cell lines. LOH of 9q is a very frequent finding in these tumors, suggesting that a gatekeeper gene for bladder cancer is located on this chromosome arm. Indeed, our analysis of the bladder tumor cell lines shows that 13 out of 20 cell lines display chromosome 9q aberrations. Unfortunately however, so far no gatekeeper role could not be assigned with certainty to any of the genes from this chromosome that have been investigated. Likewise, our study excludes an important mutational contribution from the *ZNF189*, *TSC1* and *TGFBR1* genes. The sensitivity of the methods used (SSCP and HDA) is around 80%, meaning that we could have missed some mutations. Even when this is taken into account, the number of mutations in the genes tested here will be too low to support a significant role in bladder pathogenesis. However, when considering the ZNF189 gene we cannot completely rule out the possibility that mutations causing bladder cancer are preferentially located in another part of the gene, especially since it has been suggested that the gene has more exons than the 4 that were published initially (124). Our percentage of TSC1 mutations in bladder cancer is lower than the 10% found by Hornigold *et al.* (71). A possible explanation for this could be that we did not select for tumours with LOH at the 9q34 region.

The *TGFBR1* gene comprises a polymorphic alanine stretch in its first exon. Alleles with 5, 6, 9 and 10 alanines have been observed in the population, with the 9A allele being the most frequent. When Pasche *et al.* compared the frequency of the TβR-I(6A) allele in a control population with a group of patients with different types of cancer, they found a significantly higher frequency of this allele in the cancer patients (133). From this work, they conclude that the 6A allele is a candidate tumor susceptibility allele. In addition, their experiments suggest that the protein with 6 alanines is less active in signal transduction, providing a logical explanation for the association of the 6A allele with cancer predisposition. These authors observed 13% and 10.6% 6A/9A heterozygotes in bladder cancer patients and the control population, respectively. However, in contrast, in the study described here we observe percentages of 17% for both groups. The 17% in the control population is much

higher than the 10.6% heterozygotes in Pasche's control group and even higher than the average percentage of 14.6 that they observe in all cancer patients together. It is not clear how the discrepancy between their and our findings can be explained. Analogous to their study, our control group is approximately 20 years younger than the bladder cancer cases and consists of people that donated blood to screen for recessive hereditary non-cancer diseases. Because of privacy reasons we have no further information about these people, however, we have no reason to think that this group harbours a high percentage of future cancer patients or that the ethnic make up of both groups differs significantly. Therefore, we must conclude that our data do not support a role for the *TGFBR1/6A* allele in a predisposition towards bladder cancer.

Chapter 5. Molecular evolution of multiple recurrent cancers of the bladder

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Abstract

We describe the reconstruction of bladder tumor development in individual patients spanning periods of up to 17 years. Genomic alterations detected in the tumors were used for hierarchical cluster analysis of tumor subclones. The cluster analysis highlights the clonal relationship between tumors from each patient. Based on the cluster data we were able to reconstruct the evolution of tumors in a genetic tree, where tumors with few aberrations precede those with many genetic insults. The sequential order of the tumors in these pedigrees differs from the chronological order in which the tumors appear. Thus, a tumor with few alterations can be occult for years following removal of a more deranged derivative. Extensive genetic damage is seen to accumulate during the evolution of the tumors. To explain the type and extent of genetic damage in combination with the low stage and grade of these tumors, we hypothesize that in bladder cancer pathogenesis an increased rate of mitotic recombination is acquired early in the tumorigenic process.

Introduction

Tumorigenesis is a process that is largely occult. It is generally accepted that most cancers will develop through an accumulation of mutations in oncogenes and tumor suppressor genes, a process, which precedes clinical detection of the tumor. The tumor that is finally clinically detected is more often than not a heterogeneous mixture of cancer cell subclones, which makes it difficult to establish the order of the genetic insults. The postulated steps in tumorigenesis are almost invariably based on a retrospective comparison of genomic alterations in tumors from different patients of different stage and grade. Early steps are then defined as genetic alterations that are present in all grades and stages, whereas later steps are detected solely in the higher stages and grades. The prototype for genetic evolution in cancer is presented by the colon cancer model that describes the distinctive stages from benign adenoma through carcinoma by successive alterations in *APC*, *KRAS*, *TP53* and a gene on chromosome 18q, respectively (45). The only example so far, in which neoplastic development was monitored in time in one and the same patient by repeated biopsies, is Barrett's esophagus, a premalignant condition that predisposes to esophageal adenocarcinoma. In these patients regions of metaplasia, low- and high-grade dysplasia and adenocarcinoma can be distinguished in the same area. These histologically distinct stages are clonally related and presumably derived from a single precursor and a model for the genetic evolution of these different stages has been designed (5, 49).

Bladder cancer is a disease that presents as superficial in ~75% of patients. Although these papillary tumors that extend into the lumen of the bladder are easily removed by

transurethral resection (TUR), as many as 60-80% of patients will eventually develop one or more recurrences (96). New tumors arise most of the time at a different location and are not regrowths of an incompletely removed tumor (67). The multiple recurrences are most probably clonally related as appears from X chromosome inactivation studies and genetic and cytogenetic analyses (43, 158). Therefore, these tumors are the result of dissemination and re-implantation of tumor cells in the bladder wall and/or of spreading of tumor cells via expansion within the urothelium. Due to this rather unique property, bladder cancer provides the opportunity to study the genetic relation and evolution of the different tumor subclones over long periods of time in one and the same patient because of their separate locations. Previous genetic studies of bladder cancer established that the most frequent alterations represented by loss of heterozygosity (LOH) are on chromosomes 4p, 8p, 9p, 9q, 11p and 17p (7, 88, 157, 170). Furthermore, it has been found that recurrent tumors may have both concordant and discordant genetic alterations, suggesting that genetic evolution is an ongoing process in tumor development (169). However, a thorough description of the tumor evolution process is still lacking. In this study, we explored the unique possibilities of bladder cancer as a model for cancer evolution in general. To this end, we systematically mapped the individual tumor genotypes of 11 patients with 104 recurrent bladder cancers.

Materials and Methods

LOH analysis

LOH was assessed with the following polymorphic markers or gene markers: D2S423, D2S405, D2S1326, D2S1397, D4S186, D4S230, D4S243, FGA, FGFR3, D5S492, ACTBP2, D8S258, D8S298, D9S171, D9S153, D9S152, D9S252, D9S278, D9S283, D9S1816, D9S280, D9S1851, D9S180, D9S176, D9S747, D9S275, D9S195, D9S242, D9S752, D9S1826, D10S168, D10S575, D10S676, D10S169, D11S1776, D11S4200, D13S802, D14S288, D14S267, D17S695, D17S960, D17S786, D18S51, D22S686, D22S685, D22S684, D22S683, D22S445, D22S444, or nrs 1-49, respectively, as used in Figure 1. Primers were chosen in regions with relative frequent losses in bladder cancer. Markers on chromosome 2 and 22, which, so far, did not show many changes in bladder cancer, served as controls. Primer sequences were obtained from the Genome Database (http://gdb.www.gdb.org) or the Cooperative Human Linkage Consortium (http://lpg.nci.nih.gov/CHLC) and were chosen for their high degree of informativity and for a clear visualization of the alleles (i.e. as few stutter bands as possible). In most cases ratios of upper and lower alleles were quantified using the Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). All LOHs were performed in duplicate. Phosphor Imager graphs without clear peaks, due to low signal intensities were dismissed and the marker was considered not

evaluable. LOH was defined when the ratio between the upper and lower alleles in tumor DNA was <0.6 or >1.67 when compared with control DNA: (T1/T2) / (N1/N2) = ratio. Note that this distinguishes between losses of upper versus the lower allele. This representation was deemed necessary because of the observed alternate allele loss in some patients. A calculation of all losses shows that lower and upper alleles are lost with similar frequency. This indicates that our approach is valid because there is no preference for loss of, for instance, the upper, sometimes naturally weaker, allele. Approximately 40% of the LOH ratios were <0.3 or >3.33. Changing the cut-off values to 0.3/3.33 did not significantly alter the results. Detailed information is available online as supplementary data.

Human tumor tissues

We selected 104 paraffin-embedded bladder tumor specimens from 11 different patients with five or more recurrences. Sections were examined microscopically by a pathologist (Th. v/d K). Parts that represented tumor tissue were punched out of the original paraffin blocks. In general the percentage tumor tissue in the material dissected by this procedure was estimated to be >90%. Normal bladder epithelium of the same patient served as a constitutive control for each patient. A group of unrelated blood DNA samples was analyzed for all markers in order to correct for variation in ratio between allele-specific combinations and to serve as alternative control in those instances where the normal epithelium DNA was not reliable or unavailable. DNA isolation was done as described previously (186).

Mutation analysis

Patients were screened for the recently described mutations in the *FGFR3* gene (20) (exons 7, 10 and 15) with SSCP-analysis at room temperature on 6% polyacrylamide gels (49:1 acryl:bisacryl) or amplification products were analyzed for heteroduplex formation using weakly denaturing polyacrylamide gels (29:1 acryl:bisacryl) (32). The nature of the mutation was confirmed by subsequent sequence analysis. Dr. F. Radvanyi kindly provided primers for the *FGFR3* exons 7, 10 and 15.

Cluster analysis

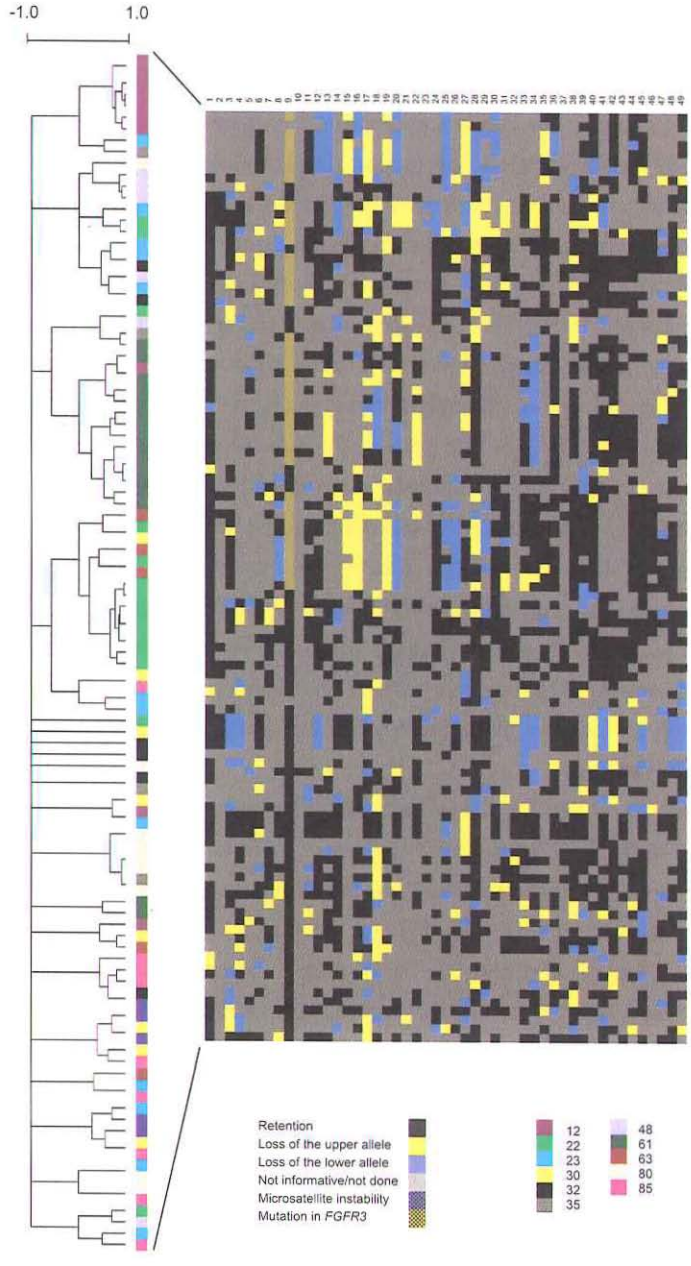
We used the cluster analysis program available at http://rana.stanford.edu/software to apply a hierarchical clustering algorithm to the tumors. The starting data table consisted of the following options: 0; retention, -1000; loss of the upper allele, 1000; loss of the lower allele, 500; MIN, –500; point mutation in the *FGFR3* gene. The result of this process is a dendrogram in which short branches connect similar genotypes and longer branches reflect diminishing similarity. To avoid confusion with micro-array results, we chose to change the colors to yellow and blue for loss of the upper and lower allele, respectively.

Results

A total of 48 microsatellite markers were used to determine a genotype for each tumor based on the number and nature of markers with LOH. In addition, the *FGFR3* gene was screened for specific point mutations. The LOH data were re-interpreted to be used for a one-dimensional hierarchical cluster analysis as described by Eisen *et al.* (38). Figure 1 shows the results of such an analysis when all 104 tumors are used for clustering based on the LOH and mutation analysis results. Patients in the figure are identified by colors next to the dendrogram. From this figure it is evident that the tumors from one patient tend to cluster together. For instance, 13 of the 15 tumors from patient 61 cluster in one sub-branch of the cluster dendrogram. Therefore these results suggest that tumors from one patient are more related to each other than tumors between patients, providing further support for a monoclonal process of tumorigenesis.

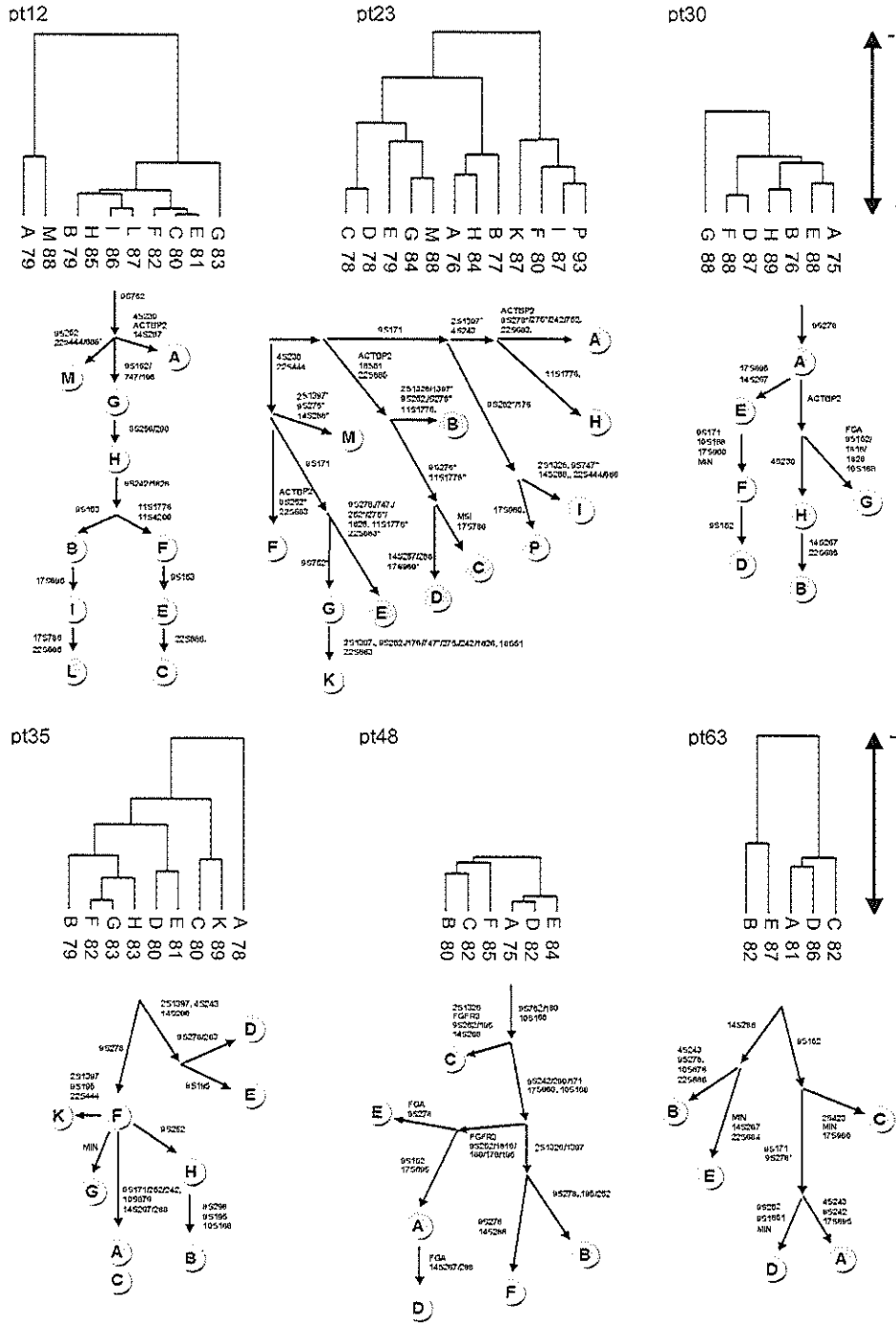
Subsequently, we assessed the relationships between the different tumors of single patients. Figure 2A shows the number, stage and grade of the 15 bladder tumors from patient 22. These tumors were removed between 1977 and 1990. Identification number, stage and grade of the tumors are indicated. Tumors are ordered in a chronological order, i.e. tumor A was removed before tumor B and so on. Twenty-nine microsatellite markers were informative for this patient and 17 showed LOH in one or more tumors, ranging from no loss to a loss of 11 markers. The extent of loss is indicated by the ratio between upper and lower alleles as calculated by the Phosphor Imager.

Figure 1. (next page) Cluster analysis of 104 tumors from 11 patients shows that the different tumors from one patient are clonally related. The color bar underneath the dendrogram depicts the different patients. These are represented by different colors (patient number and color are indicated on the lower right). The length of the branches represents the relation between individual tumors, i.e. short branches descending from a node indicate highly related samples. The scale on top is a quantification of these relations, with -1 indicating no relation and +1 the maximal relation. In the array table the different genetic aberrations used for the calculation are depicted as indicated underneath the table. The genetic markers are shown on top of the table and for reasons of clarity they are numbered from 1 to 49. Their identity can be found in the materials and methods section. Note that the relatively large proportion of gray cells in the table is due to the fact that in this analysis all markers had to be used for the cluster analysis, including the markers that were not informative for a given patient.


-1.0
1.0
Retention
Loss of the upper allele
Loss of the lower allele
Not informative/not done
Microsatellite instability
Mutation in FGFR3
12
22
23
30
32
35
48
61
63
80
85

An identical point mutation in the *FGFR3* gene was detected in 10/15 tumors. When the genetic aberrations seen in the individual tumors of this patient are compared, it is clear that these cannot be explained by a linear model based on the chronology of appearance, simply because consecutive tumors have genotypes of different complexity. For example, no genetic alteration was seen in tumor I, removed in 1985, while previously resected tumors A-H all displayed loss of one or more markers and/or had a mutation in the *FGFR3* gene. Note also that these losses do not appear to be random and unrelated, since for most markers LOH in different tumors concerns the same allele. We then reordered the tumors with respect to genetic events. A representation of the data based on a one-dimensional cluster analysis is given in Figure 2B. The scale next to the dendrogram indicates the correlation coefficient calculated by the program. From this calculation it appears that all tumors except I are considered to be highly related. Because the cluster analysis does not provide a direction to the tumor evolution process, we then reordered the tumors based on the cluster data but with the assumption that a tumor with no or little genetic damage will have evolved before a tumor with extensive damage. In addition, this handmade reconstruction allows the introduction of hypothetical steps in the evolution process. The resulting evolutionary tree of the tumors in patient 22 is depicted in Figure 2C. Tumor I is considered to be the primary tumor and, for instance, tumor B, which was removed 7 years before I, as a descendant from tumor I. As can be seen in Figure 2B and C, B is several genetic steps removed from I. Based on this analysis, we propose that the genetic tree reflects the development of, and relationships between, the different tumors from this patient better than the linear chronological order in which the tumors were removed.

Patient 61 also developed 15 tumors between 1976 and 1990. Twenty-four markers were informative and of these 16 showed LOH in one or more tumors, with a maximum of 8 markers with LOH in a single tumor (Figure 2D). Again, the genotypes of the tumors suggest a different order in genetic events than their chronological appearance. The strikingly consistent loss of the lower allele of D10S169 in all tumors indicates that loss of this marker is the first or a very early event and that a clonal relationship between recurrences is very likely. An identical *FGFR3* mutation was observed in 14/15 tumors. In Figure 2E the clustered analysis is shown. In this patient, the correlation coefficient between tumors ranges from 0.05 to ~1, again suggesting an intimate relationship between these tumors. As for the previous case, we then reconstructed the tumor clustering assuming a direction in the genetic build-up and by the introduction of hypothetical genetic steps. The adjusted tumor tree representing the genetic pathways along which the recurrences have developed in patient 61 is shown in Figure 2F. All tumors derive from tumor L, since in this tumor only one genetic insult was detected. Tumor A from patient 61 was removed in 1976 and tumor L in 1987. Tumor A has many additional genetic aberrations that are lacking in L.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M 88
B 79
H 85
I 86
L 87
F 82
C 80
E 81
G 83
pt23
C 78
D 78
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M 88
A 76
H 84
B 77
K 87
F 80
I 87
P 93
pt30
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1
G 88
F 88
D 87
H 89
B 76
E 88
A 75
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B 79
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C 80
K 89
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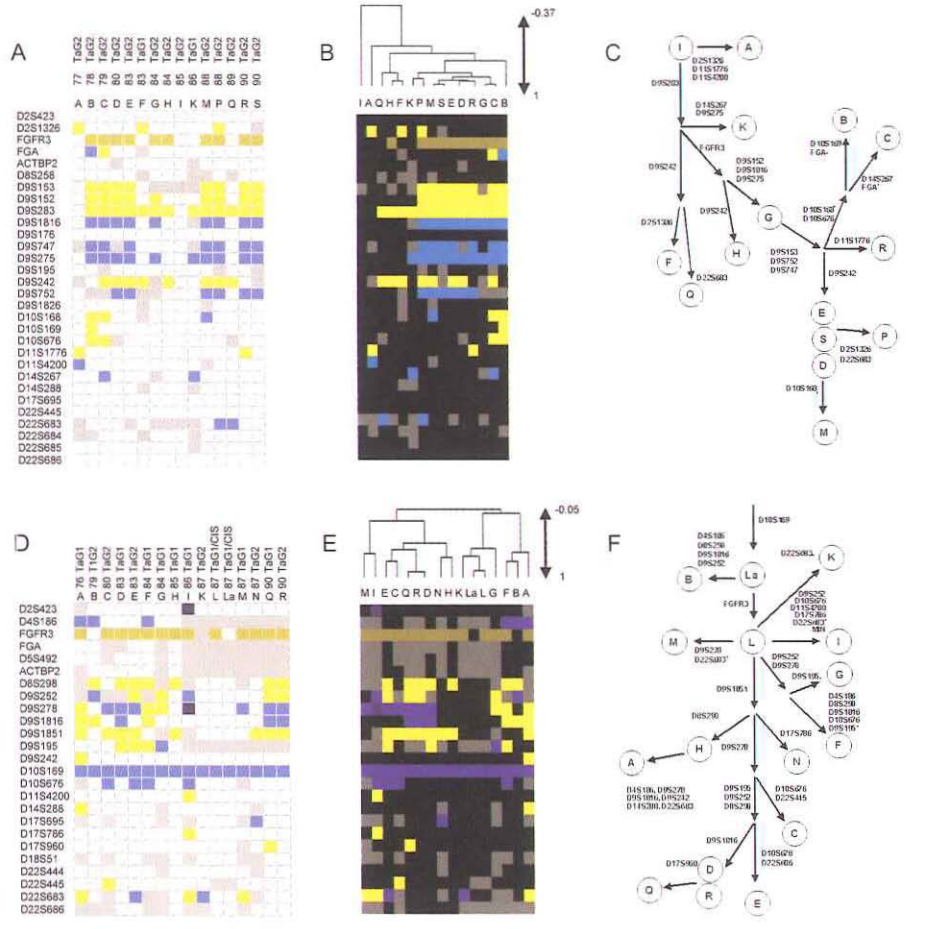

Figure 2. Genotypes, hierarchical tree clustering and deduced evolutionary trees for the multiple tumor recurrences from patient 22 and 61. For an explanation of colors, see Figure 1, with the exception that in Figure 2A and D retention is indicated by white cells. A, LOH analysis and genotypes of the 15 tumors in patient 22. Tumors are ordered chronologically in columns, microsatellite data and FGFR3 mutation analysis in rows. Markers are ordered per chromosome from pter to qter, non-informative markers were excluded. B, cluster analysis of the tumors. One-dimensional hierarchical clustering was performed using the genetic data from A. C, evolutionary genetic tree depicting the relationship between the recurrences of patient 22. Each circle represents a tumor. Arrows indicate the different genetic steps and the markers involved are listed next to each arrow. Because of the alternating losses observed for some markers, an asterisk indicates whether the upper or lower allele is lost, when relevant. D, genotypes and LOH analysis of the 15 tumors in patient 61. E, clustered correlations between recurrences. F, evolutionary genetic tree of patient 61.

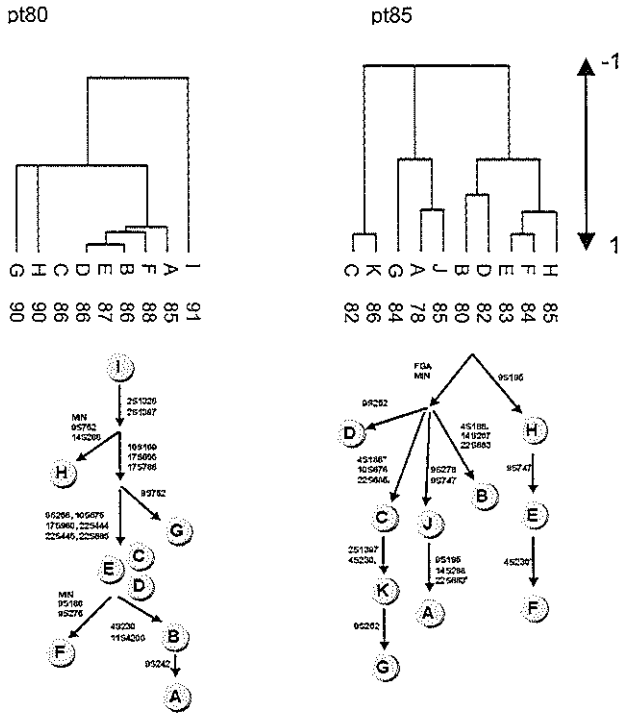

Figure 3. (previous page and above) Cluster dendrograms and reconstructed genetic trees of the remaining eight patients. For reasons of clarity, markers are depicted without the initial letter D. Because of the alternating losses observed for some markers, asterisks indicate whether the upper or lower allele is lost. The scale on the right can be used to estimate the degree of genetic relation. A complete description of the genetic analyses can be found online as supplementary information..

Thus, in the genetic tree, L precedes A. It can be seen in Figure 2D that tumor L is a mixture of CIS and Ta. Considering the extent of LOH, loss of D10S169 is most probably present in all cells of this tumor. The intensity of the SSCP signal, however, suggested that the *FGFR3* mutation was restricted to a fraction of the tumor (results not shown). We therefore divided tumor L in the fraction with mutation (L) and the fraction without receptor mutation (La). In the model, La is the founding tumor moiety, giving rise to B, the only tumor without the *FGFR3* mutation and L, from which the remaining tumors derive (Figure 2F).

We were able to establish such a representation of sequential events, linking the tumors to one or more common precursor clones, for all patients, except one. In the six tumors from this latter patient (no. 32) in total only seven genetic hits were scored and this number is too low for a reliable cluster analysis. A representation of the cluster data and the deduced genetic trees for the other eight patients is given in Figure 3. In two of these

patients, a first tumor was detected from which all other tumors developed. For the others, a hypothetical first tumor or tumor cell has been assumed. It also appears that for only one patient (patient 30) the first clinically presenting tumor (tumor A) is also the founding tumor in the genetic pedigree (Figure 3). When the positions of the tumors in all trees are compared to their clinical manifestation, it is apparent that the chronology of tumor presentation does not parallel the genetic evolution of the tumors at all. Thus, this appears the leading principle rather than an exception.

Although most losses in the tumors from a certain patient concern the same allele, alternate allele loss was found for 19% of the LOHs in total (indicated by superscript and subscript asterisks in Figure 3). Especially in patient 23, the alternate allele loss is very pronounced and concerns 10 of the 28 markers with LOH. This results in a more extensive branching of the tree than for the other patients.

Discussion

An accumulation of mutations in essential genes can transform a normal cell into a cancer cell. This transformed cell may then grow out to form a tumor with additional mutations occurring during this process. The tumor that is finally clinically detected is more often than not a heterogeneous mixture of cancer cell subclones, which makes it difficult to establish the order of the genetic insults. Bladder cancer, however, provides the unique property that different tumor subclones grow at separate sites and thus can be studied independently. The presence of identical alterations in different bladder tumors from one patient and the increase in the number of genetic alterations allowed us to order the multiple tumors in each patient in the form of evolutionary genetic trees or pedigrees. In such a model, an original transformed cell grows out and sheds cells into the lumen of the bladder. Some of these cells will have acquired additional genetic damage. They attach to the bladder wall, grow out and can themselves lead to secondary disseminations and so on, thus creating the different branches of the tree. This model resembles the evolution of cell lineages in Barrett esophagus (5). In their model esophageal adenocarcinoma evolves from premalignant conditions such as metaplasia and dysplasia. Their results also indicate that this clonal evolution is more complex than predicted by a linear model. Here we show that bladder cancer cell lineages evolve, like in Barrett's model, over a period of many years, giving rise to clonal expansion and outgrowth due to newly acquired aberrations, and continue to do so after the emergence of recurrent tumors.

Interestingly, it appeared that the chronology of tumor appearance does not run in parallel with the genetic evolution. This also implies that the earliest genetic events must be deduced from the genetic tree rather than from the first appearing tumor. Thus, the

evolutionary trees could theoretically lead to the identification of a common first or early genetic step for these superficial bladder tumors. However, it appears that in the early steps of the trees from the 11 patients represented here, no evidence for a common first LOH event can be identified. We rather suggest that the extensive LOH found is due to random genomic instability, appearing already very early in the development of superficial bladder cancer. In some of the pedigrees theoretical early tumors/tumor cells have been introduced. A second question that can be raised is whether there is a certain identifiable genetic step that can lead to the clinical appearance of a tumor, i.e. a step that, for instance, induces rapid growth. Again the pedigrees do not reveal such a common denominator.

The standard treatment for low-stage, low-grade bladder tumors involves TUR, although there appears to be a general agreement that TUR alone does not prevent the development of new tumors. TUR is therefore often followed by intravesical treatment with bacillus Calmette-Guerin (BCG) in order to provoke an immune response that is thought to lead to rejection of urothelium and remaining tumor cells. Besides possible differences in growth rate, this might, at least to some extent, explain why an apparent precursor with few alterations appears so much later than a descendant subclone. Any tumor that reaches the detection threshold at a certain point in time will be removed and, subsequently, all other existing, but not yet visible, subclones will be affected or even wiped out by the adjuvant treatment.

A surprising finding is the sheer number of alterations in some tumors. There is a great variability in the number of LOH events that was observed per tumor, ranging from none or a few alterations in the early steps of the genetic trees to LOH of 65% of the informative markers. When these numbers are extrapolated it appears that a large part of the genome may be affected. A related extrapolation has recently been presented by Stoler *et al.* (168). In their paper the authors show that colonic polyps, representing early steps in the tumor progression pathway, have a mean number of 11,000 genomic events per cell. Our findings also illustrate that the number of genomic alterations even in early tumor stages is already astoundingly complex. These findings can best be explained by assuming that genomic instability is already present early in tumorigenesis.

There are two levels of genetic instability: at the nucleotide level (microsatellite instability or MIN) and at the chromosome level (chromosome instability or CIN), the latter being much more frequent in cancer (101). We found microsatellite instability in 19 tumors, but only few of these showed instability for several markers. In general, MIN is not considered to play a major role in bladder cancer (55), although it is reported to be more frequent in young patients with bladder cancer (26). What type of instability mechanism could best explain the findings presented here? It appears that the LOH events that we observe on the best studied chromosome, chromosome 9q, reveal what can best be described as a

patchwork pattern of losses and retentions, rather than loss of an entire chromosome or chromosome arm. For instance, in patient 22 (Figure 2C) loss D9S283 is followed by loss of D9S275 and D9S752; moreover, all 3 areas of loss increase in size as is apparent from subsequent losses of adjacent markers. Likewise, in patient 61, LOH of the marker D9S1851 is followed by losses of the adjacent markers D9S1816 and D9S278. However, in the case of these latter markers loss of alternate alleles occurs in different tumors. To explain these findings, we suggest a model in which the losses of heterozygosity are caused by an increased rate of mitotic recombination. Recombination between two homologous chromatids during mitosis could result in multiple crossovers (35). As a consequence, the crossover region in the recipient chromosome becomes identical in sequence to the donor chromosome. When recombinations occur frequently this leads to an expansion of the region of loss of heterozygosity. A model to explain this mechanism is given in Figure 4.

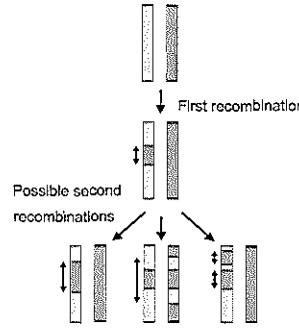

Figure 4. Enhanced rates of mitotic recombination may create multiple regions of LOH and expand existing LOH. The recombination takes place between homologous chromatids. For reasons of clarity, sister chromatids are not included. The arrows next to the chromosomes indicate the extent of LOH.

The consequence of mitotic recombination is that no actual loss of chromosome regions occurs; only the sequence of part of one chromosome is now an exact duplicate of the other. Thus, the tumor genome in later stages of the genetic tree becomes more and more homozygous. Such a mechanism would be compatible with the low-stage, low-grade phenotype of the papillary bladder tumors. Although some of the LOH events could perhaps be explained by tetraploidization followed by loss of a chromosome, we believe that this is not the major explanation for our findings for the following reasons. Firstly, this would not explain the patchwork nature of the losses; secondly, in >40% of the cases, the LOH is far too profound; and thirdly, flow cytometry of bladder cancers has shown that especially the low-stage, low-grade papillary tumors, like the ones in this study, are mostly diploid (175).

An enhanced rate of mitotic recombination is seen in hereditary syndromes like Bloom's syndrome, Fanconi anemia and Werner's syndrome (37, 98, 207). The pattern of chromosome instability in especially Bloom's syndrome is characterized by sister-chromatid exchanges and homologous chromatid interchanges reflected in a gain of homozygosity for polymorphic loci (40, 59). Patients with these diseases have an increased risk of developing several cancers. The genes responsible for these syndromes have, in part, been cloned and the protein products of both the *BLM* and *WRN* genes are DNA helicases (58, 81). Therefore, we reason that it is not unlikely that a gene that functions in these diseases or a gene with similar characteristics may play a role in bladder cancer pathogenesis. Because of the increase in LOH with each step in the genetic trees, we favor a model in which such a type of genomic instability, caused by an enhanced rate of mitotic recombination generating functional homozygosity, occurs early in tumor evolution and may even be the elusive first step.

Acknowledgements

The authors thank Dr. F. Radvanyi for providing primer sequences and SSCP-conditions for the *FGFR3* mutation analysis, Dr. P. Hogeweg for advice about the cluster analysis and Dr. J.H. Hoeijmakers for helpful discussions.

Chapter 6. Variable losses of chromosome 9q regions in multiple recurrent bladder tumors prohibit the localization of a postulated tumor suppressor gene

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Submitted for publication

Abstract

Allelic loss on chromosome 9q is a very frequent event in bladder carcinogenesis. In recent years, efforts have been directed towards identifying the responsible genes on this chromosome arm by deletion mapping and mutation analysis of candidate genes. Here we describe the genesis and development of chromosome 9q alterations in multiple recurrent superficial bladder cancers of 10 patients. We show that loss of heterozygosity on this chromosome is almost never the characteristic first step. The regions of loss are multiple and variable in different tumors of the same patient and expand in subsequent tumors. Moreover, the regions of loss vary form patient to patient. We conclude that, even when 9q harbors a bladder cancer gatekeeper gene, it is unlikely that the gene will be identified through LOH analysis alone.

Introduction

Loss of one copy of a tumor suppressor gene can create a hereditary predisposition to cancer. The second copy of the gene can be inactivated in many ways, such as loss of a (part of a) chromosome or chromosome arm. The key insight in this process came from a rare type of human cancer, retinoblastoma, which arises from cells in the body that are transformed by an unusually small number of mutations. Polymorphic markers have been very useful in linkage analysis in hereditary cancer syndromes, leading to the identification of what are often called gatekeeper genes like *APC* or *RB*. Tumor suppressor genes detected in hereditary cancer syndromes frequently play a role in sporadic tumors as well. These tumors can have loss of heterozygosity (LOH) in the area where the gene is located. This is relatively easily detected by screening tumor DNA with polymorphic markers. Many investigators therefore searched for loss of heterozygosity in their tumor type of interest in order to pinpoint the location of putative tumor suppressor genes.

Bladder cancer usually presents itself as a superficial, papillary tumor of the transitional cells of the urothelium. The tumors are low stage, low grade and can be removed by transurethral resection. However, about 70% of patients will develop one or more recurrences, a process that can continue for many years and eventually may lead to invasive disease in 15-25% of patients. It has been suggested that chromosome 9 harbors several genes that play a role in the early steps of bladder cancer pathogenesis. This theory stems from the observation of chromosome 9 underrepresentation by *in situ* hybridization (ISH) (135, 140, 149), comparative genomic hybridization (CGH) (78, 196) and LOH. This underrepresentation is sometimes seen as the sole abnormality, and can be found in tumors of low stages and grades.

On the short arm of chromosome 9, the *CDKN2A* gene was thoroughly investigated as the target of LOH (127, 204). Although the gene does not appear to be frequently mutated, it is often homozygously deleted or transcriptionally silenced in bladder cancer. On the long arm, LOH analyses so far show a multiplicity of events suggesting to some authors that chromosome 9q might harbor 2, 3 or even 4 genes that may have a role in bladder cancer (17, 34, 36, 60, 64, 82, 108, 186). Tumor suppressor genes like *PTCH* and *TSC1*, both found by a combination of linkage analysis and LOH in hereditary syndromes, are, although located in candidate regions, not frequently mutated in bladder tumors[3] (71, 160). The only candidate tumor suppressor gene on this arm found in bladder tumors at the moment is the *DBCCR1* gene (61). This gene was found in a homozygously deleted region and although it appears to be transcriptionally silenced in bladder cancer, no inactivating mutations have been found. One of the explanations for the difficulty of finding a single smallest region of overlap could be that there are different subgroups of bladder tumors with different loss patterns, one being the continuously low grade papillary tumors, the other being the more invasive tumors, including carcinoma *in situ*. Therefore, investigators have been dividing tumors in separate groups with different stages and some suggest that the role of chromosome 9 is more important in the (non-invasive) superficial group (141, 159, 165). Only a few studies have looked at losses in tumor recurrences, mainly because for a long time it was suggested that bladder cancer is a field cancerization process, even after X-chromosome inactivation studies showed otherwise (67, 158).

We previously showed that recurrent tumors in a patient are clonal and can be arranged in a genetic tree based on their genetic relationships. In these trees, tumors with few aberrations precede those with many (Chapter 5). The trees reflect the history of tumor development and allow the identification of the earliest genetic events that must have occurred in tumor evolution. We have used this background to study the development of loss of heterozygosity on chromosome 9q in 11 patients. We show that loss on this chromosome arm is almost never the characteristic first alteration in recurrent bladder cancer. The regions of loss are multiple and variable in different tumors of the same patient and their size expands in subsequent tumors. Moreover, the regions of loss vary form patient to patient. We conclude that, even when 9q harbors a bladder cancer gatekeeper gene, it is unlikely that the gene will be identified through LOH analysis alone.

Materials and Methods

Tumor samples

We selected paraffin-embedded bladder tumor specimens from 11 different patients with five or more recurrences. Sections were examined microscopically by a pathologist. Parts that

represented tumor tissue were punched out of the original paraffin blocks. In general the percentage tumor tissue in the material dissected by this procedure was estimated to be over 90%. Normal bladder epithelium served as a constitutive control for each patient. A group of unrelated blood DNA samples was analyzed for all markers in order to characterize the alleles and to serve as alternative control in those instances where the normal epithelium DNA was not reliable or unavailable. DNA isolation was done as described previously (186).

Microsatellite analysis

LOH was assessed with the following polymorphic markers: D2S405, D2S423, D2S1326, D2S1397, D4S186, D4S230, D4S243, FGA, D5S492, ACTBP2, D8S258, D8S298, D9S152, D9S153, D9S171, D9S176, D9S180, D9S195, D9S242, D9S252, D9S275, D9S278, D9S283, D9S747, D9S752, D9S1816, D9S1826, D9S1851, D10S168, D10S169, D10S575, D10S676, D11S1776, D11S4200, D14S267, D14S288, D17S695, D17S768, D17S960, D18S51, D22S444, D22S445, D22S683, D22S684, D22S685, D22S686. Primer sequences were obtained from the Genome Database (http://gdb.www.gdb.org/) or the Cooperative Human Linkage Consortium (http://lpg.nci.nih.gov/CHLC). In most cases ratios of upper and lower alleles were quantified using the Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). LOH was scored when the ratio between the upper and lower alleles in tumor DNA was <0.6 or >1.67 when compared with control DNA; (T1/T2)/(N1/N2) = ratio. Note that this distinguishes between losses of upper vs. lower allele. This representation was deemed necessary because of the observed alternate allele loss in some patients. A calculation of all losses shows that lower and upper alleles are lost with similar frequency. This indicates that our approach is valid because there is no preference for loss of, for instance, the upper, sometimes naturally weaker, allele.

Results

In a previous paper we describe the construction of genetic trees for the recurrent tumors of 11 patients (Chapter 5). To this end we analyzed LOH of 46 microsatellite markers and performed mutation analysis of the *FGFR3* gene. Hierarchical cluster dendograms were calculated based on these data. The cluster analysis demonstrated the clonal relation between the different tumors of a patient. The dendograms provided the starting point for the construction of genetic trees assuming that tumors with few aberrations precede those with many genetic lesions. We established that in the trees the order of the tumors differs from the chronological order in which they appeared. Since, these trees reflect the actual history of genetic tumor development they can be used to search for the first common genetic aberrations. In this study we focus on the development of LOH on chromosome 9q during

tumor evolution in order to gain more insight in the localization of possible tumor suppressor genes on this chromosome arm. Figure 1A shows the result of the partial genomic allelotyping for the 46 microsatellite markers. The exact number of tumors with loss for a certain marker is shown as the fraction of all informative tumors. Of the 17 chromosome 9 markers shown, only D9S171 is located on 9p, all others are on 9q. On chromosome 9q, the loss is distributed across two regions, with a decrease in LOH around marker D9S176 (16%). The mean fractional allelic imbalance (FAI) on chromosome 9 is 47%, and this is about twice as high as the FAI on all other chromosomes combined (21%)(Figure 1B). This corresponds with results of others and appears to point in the direction of bladder cancer-specific alterations on this chromosome. However, a high frequency of loss was also found on chromosomes 10 (40% for D10S169) and 22 (44% for D22S683). For chromosome 2, there seems to be a difference between loss on the short arm (1% and 6% for D2S423 and D2S405) and the long arm (28% and 39% for D2S1326 and D2S1397, respectively).

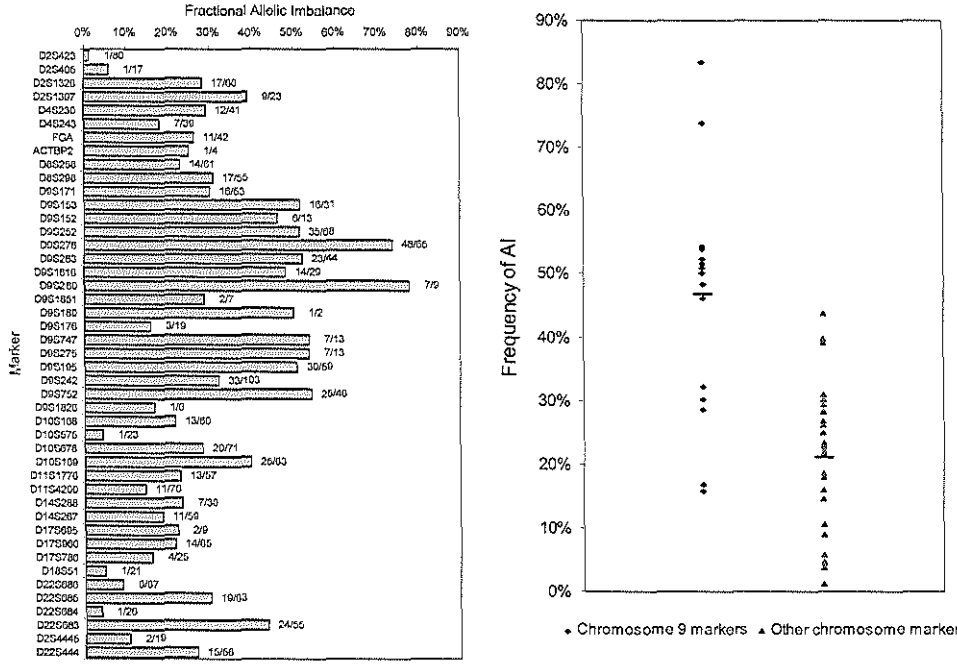
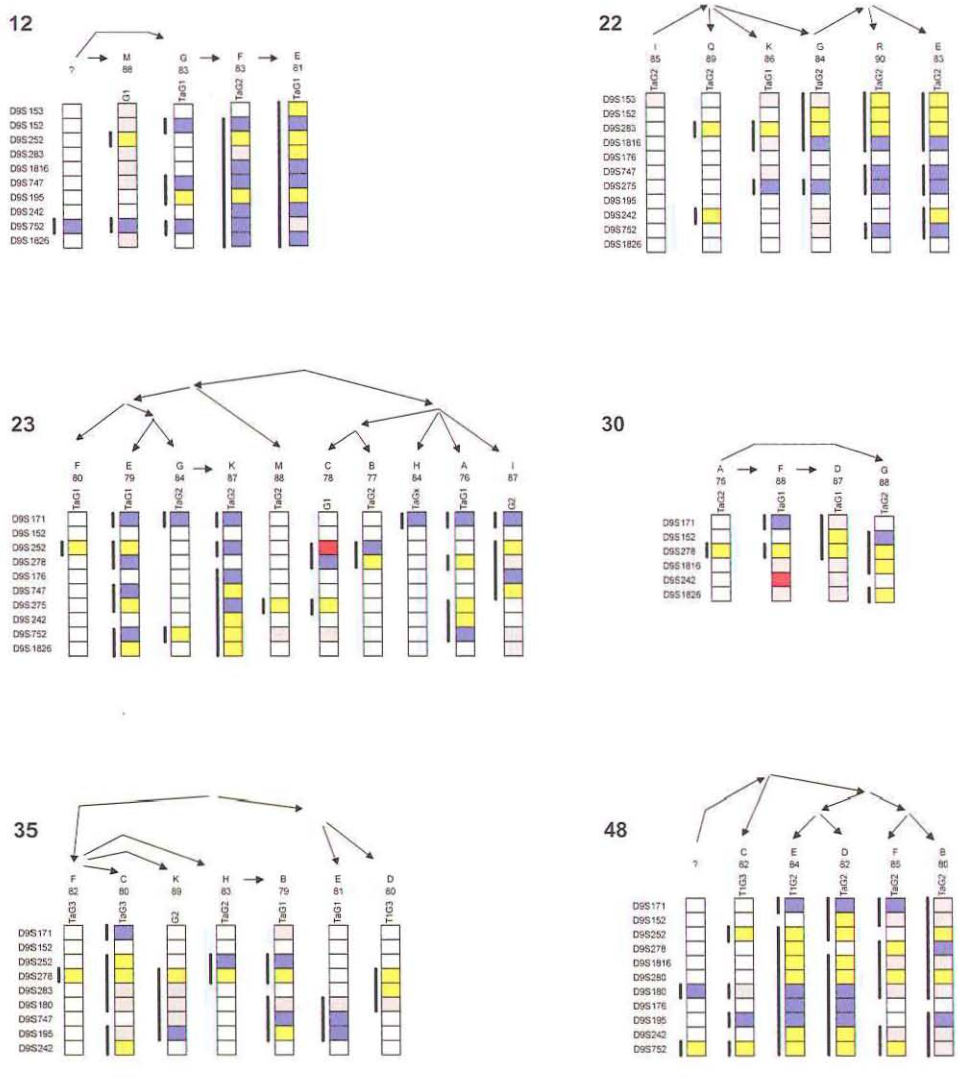
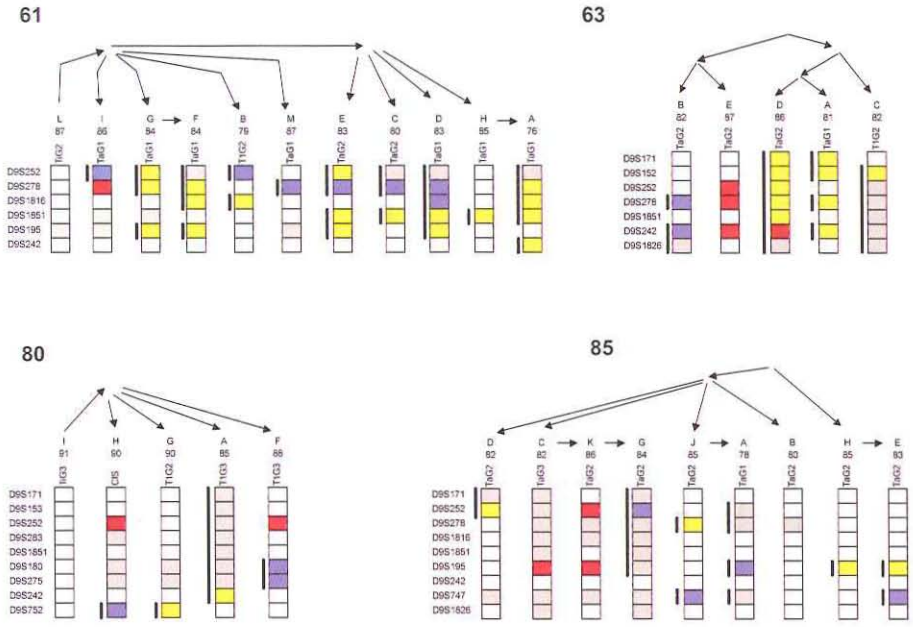


Figure 1. Allelic imbalance in a partial allelotype of 104 tumors. A, imbalance depicted per marker. Next to the bars, the number of tumors with loss is given as a fraction of the number of informative tumors. For chromosome 9, all markers are located on 9q, with the exception of marker D9S171, which is located on 9p. The markers are ordered from pter (top)-qter (bottom) for each chromosome, where relevant. B, Mean loss on chromosome 9 compared to mean loss on other chromosomes. Every datapoint represents a marker. A horizontal bar indicates the mean loss.

Figure 2. (below and next page) Overview of the development of homozygosity on chromosome 9 in evolutionary trees based on whole genome typing. Every tumor is represented by an image of the status of chromosome 9. The arrows above the chromosomes indicate the branches of the trees that were deduced from accumulating genomic alterations. Only those tumors from each patient that have alterations on chromosome 9 are shown and only one example is shown in those cases where two or more tumors had identical changes. We included a hypothetical tumor of patients 12 and 48 to visualize a developmental step for reasons of clarity. On the left side of the images, the marker names are listed. Tumor names, year of removal and stage and grade are listed above the images. White blocks; retention of a marker, yellow; loss of the upper allele, blue; loss of the lower allele, gray; no signal/not done, red; microsatellite instability. Black bars on the left of the chromosome image emphasize the areas of homozygosity.





From a gatekeeper tumor suppressor gene one would expect that it is inactivated in a substantial percentage of tumors from different patients and that its inactivation can be detected in the first step in tumor development. We therefore focussed on the build-up of chromosome 9q events in the genetic trees of 10 of the 11 the patients. One case, patient 32, was omitted because in the six tumors that were investigated too few genetic aberrations were detectable to build a genetic tree. The constructed genetic trees provide the framework for Figure 2, in which the individual events that took place on chromosome 9 are illustrated. For reasons of clarity, only those tumors are depicted in which an additional 9q alteration occurred.

Tumors with an LOH pattern that is identical to another tumor are omitted. For every tumor, the status of the informative markers along chromosome 9q is given. The arrows above each set of tumors indicate the evolutionary tree as deduced previously. The stage, grade and year of removal of the tumors are listed. For patient 30, for instance, we examined 7 recurrences. All 7 tumors have lost the upper allele of D9S278. From tumor A, two different branches of the tree develop. In tumor G the LOH area around D9S278 expands in size and an additional loss was observed for D9S1826. In the other branch, reflected by F and D, subsequent losses include D9S171 and D9S152. Thus, for patient 30, a putative chromosome 9q suppressor gene could be located in the region covered by marker D9S278. In contrast, however, all 10 tumors from patient 12 show LOH for the lower allele of D9S752

and all tumors in the genetic tree derive from a hypothetical tumor that has lost this marker as its only genetic aberration. This hypothetical tumor clone is indicated in Figure 2 with a question mark. As can be seen in the Figure, two developmental lines lead to different additional areas of loss, in tumor M around marker D9S252 and in tumor G around marker D9S152 and a second region around D9S747 and D9S195. Although not shown here, tumor G cannot have developed from M, based on the alterations found on other chromosomes. The two regions that are lost in tumor G are enlarged in the subsequent tumors F and E. Thus, for patient 12, a causative chromosome 9q gene could be located in the area marked by D9S752. D9S752 is also lost in all 6 tumors from patient 48, but all tumors also show loss for D9S180. These 3 patients, 30, 12 and 48, represent the only 3 of the 11 patients in which loss of a chromosome 9q marker was found in all tumors and/or was deduced to be the first or one of the first concomitant events in tumor formation. Loss of 9q markers was found for all other patients, but not in all tumors of a patient. Six patients (patients 22, 32, 61, 63, 80 and 85) have one or more tumors without apparent loss on this chromosome arm. In total 13 tumors were found without detectable LOH on 9q. These tumors are all situated in the early steps of the genetic trees. In addition, the genetic trees reveal that in the latter patients and in cases 23 and 35 loss of markers on other chromosomes precede a 9q event. A clear example in this respect is patient 61. All 15 tumors of this patient have lost the lower allele of D10S169. Losses on 9q apparently occur later and quite chaotically with alternate allele losses for several markers (Figure 2). From these results we conclude that LOH on chromosome 9q, although frequent, may not be a prerequisite for bladder cancer tumor formation.

Discussion

The majority of bladder cancers display losses of chromosome 9 sequences, irrespective of stage and grade. These investigations resulted in an accumulation of LOH data, finally leading to the suggestion that as much as 7 separate chromosome 9 tumor suppressor genes (3 on 9p and 4 on 9q) may contribute towards bladder cancer pathogenesis (33, 87, 161, 186). Our data again show that alterations on chromosome 9q are very frequent and occur more often than losses on other chromosomes. This genomic distribution of LOH is in accordance with other studies on superficial pTa/pT1 bladder tumors, like the ones studied here. When the 9q LOH data for all 104 tumors studied in this work are added as was done in Figure 1A, one could, in analogy with the previous investigations, deduce that two regions of loss are observed on this chromosome arm. However, we have shown here that, taking into account the development of the losses, a completely different picture emerges. No common region of loss could be established in the early steps of the genetic trees in our

patients. Six out of 11 patients have one or more tumors without detectable LOH on chromosome 9q. This shows that LOH of 9q is not a prerequisite for bladder cancer initiation. In only 3 patients we have reason to believe that loss of a marker on chromosome 9q may be an early event. In two cases this concerned marker D9S752, which could indicate inactivation of a gene in that region, but in one of those two patients LOH is present at another region (D9S180) at the same time, which makes it difficult to determine the relevance of those regions. Moreover, the third patient has loss at marker D9S278, which again is at a completely different position on the long arm of chromosome 9. Therefore, the postulated early, or even initiating, loss of function of one or more tumor suppressor genes on chromosome 9q in bladder cancer pathogenesis is not supported by our data. The nature and apparent randomness of the mechanism that inflicts the regions of loss on chromosome 9 is best illustrated by the finding that chromosome 9q can be the target of as much as 10 independent LOH events in one patient (23 and 61) and by patients in which some tumors show loss of alternate alleles.

Studying bladder cancer gives us the possibility to monitor the LOH in multiple recurrences over time. This is an important advantage in understanding the role of LOH in tumorigenesis. Loss of heterozygosity analyses have been performed since the discovery of the first tumor suppressor gene and a comprehensive list of deleted chromosome regions has been made for most tumor types. Despite the apparent straightforwardness of this method, the number of actual genes isolated in this way is disquietingly scarce. The regions where *PTEN* (102) and *DPC4* (65) are located, do display LOH in tumor types like glioblastomas, prostate and breast cancer, and pancreatic and colorectal cancer, however, the genes were cloned only because of the identification of homozygous deletions. Bladder cancer is not the only tumor type where confusing LOH results have led to the assumption that multiple closely spaced tumor suppressor genes exist on one chromosome (53, 99, 131), and chromosome 9 is not even the only chromosome in bladder cancer where this phenomenon has been observed (195). Considering the many tumor types with a similar patchwork LOH pattern, we feel that it is rather unlikely that multiple tumor suppressor genes for a certain type of cancer all reside on one chromosome (arm). Comparative mapping of the mouse genome shows that human chromosome 9q is spread out over 3 different mouse chromosomes. Moreover, it is difficult to envisage an evolutionary advantage for man to cluster tumor genes on one chromosome in order to induce cancer more easily. In our view, most of the LOH events will be due to random genomic instability. Thus, even if some of these losses target growth-inhibiting genes, the localization of the relevant gene is severely hampered by the enormous background of random genetic events. We think it therefore unlikely that a bladder cancer gatekeeper gene on chromosome 9q, if present, will be identified through LOH analysis alone. Perhaps the higher frequency of LOH events on

chromosome 9 when compared to the other chromosomes even has a mechanistic rather than a tumor promoting reason. In this respect it is relevant to mention that chromosome 9, together with 1 and 16, is one of the 3 chromosomes with a large heterochromatin region. It has been suggested that heterochromatin is involved in specific forms of chromosomal instability (178, 206). Although this is speculative, it is therefore not impossible that the frequent alterations on chromosome 9 are due to such a chromosomal instability mechanism for which chromosome 9 is extremely sensitive and that the elusive gatekeeper gene may not reside on this chromosome after all.

Chapter 7. General Discussion

Aim of this thesis

Bladder cancer is a frequently occurring disease with many different histopathological forms. These different phenotypes are thought to reflect a heterogeneous collection of genotypes, and, perhaps, pathways along which bladder cancer might develop. The identification of the genetic alterations in carcinoma of the bladder is therefore considered pivotal in our understanding of the pathogenesis of the disease and will be helpful in designing improved strategies for prevention, diagnosis, prognosis, and therapy. This thesis focusses on identification of the alterations (especially on chromosome 9q) in transitional cell carcinomas. The results will be discussed in a wider context, and also suggestions for future research will be made.

No correlation between underrepresentation of chromosome 9 and LOH

Aberrations concerning chromosome 9 are found in TCCs of all grades and stages, and sometimes it is the sole abnormality seen. We therefore started the work described here with the classical hypothesis that this chromosome harbors a gatekeeper gene for bladder cancer. Consequently, it should be possible to localize this gene by mapping of the deletions on this chromosome in tumors. A combination of previous LOH, FISH and CGH results led to the interpretation that monosomy of chromosome 9 may occur in over 50% of the TCCs of the bladder. Tumors that have lost an entire copy of chromosome 9 cannot be used to map a TSG. We therefore decided to analyse all tumors first with *in situ* hybridization (ISH) and use only those with 2 copies of chromosome 9 for LOH analysis (Chapter 2). However, with ISH, complete monosomy for chromosome 9 was observed in only 1 of 40 tumors. Four other tumors had subpopulations of cells with only one chromosome 9. A rescreen of the literature revealed that a tumor is already labeled as having monosomy for chromosome 9 when as low as 20% of the cells have lost this chromosome. Moreover, we found that relative loss of chromosome 9 (relative to chromosome 6) does not always coincide with LOH for chromosome 9. This can be explained by assuming that relative loss of 9 in a, for instance, tetraploid tumor may reduce the chromosome 9 copy number from 4 to 3 or 2. However, both paternal and maternal copies can still be present and no actual loss of genomic material exists. Although an association was found between chromosome 9 underrepresentation and LOH, the extent of loss in the LOH analyses was much more pronounced than can be explained by the extent of underrepresentation. This can be explained by partial or interstitial deletions without the loss of the centromere of chromosome 9. We therefore conclude that underrepresentation of chromosome 9 does not necessarily lead to the inactivation of the postulated TSG. The LOH analyses further revealed that several tumors had more than one region of loss on 9 and confirmed that only 1 tumor had lost an entire copy of this chromosome. We then decided to map the LOH regions in further detail.

Many candidate regions but no mutant genes

To narrow the localisation of one or more putative tumor suppressor genes on chromosome 9 that play a role in TCC of the bladder, we examined tumors with a panel of microsatellite markers along the chromosome (Chapter 3). We found evidence for two different loci on the long arm of chromosome 9 where potential tumor suppressor genes are expected. These loci are delineated by interstitial deletions in two bladder tumors. Both regions were examined for homozygous deletions with EST and STS markers, but no homozygous deletions were observed in 17 different bladder tumor cell lines.

Because of the difficulty in narrowing down the smallest region of overlap (SRO), we decided to look for possible candidate genes in the regions known to be lost at that moment. In Chapter 4, the mutation analysis of the Krüppel-like zinc finger gene *ZNF189*, the Tuberous Sclerosis Complex gene 1 (*TSC1*) and the TGF beta receptor type I (*TGFBR1*) in a series of bladder tumors and bladder tumor cell lines is described. All three genes have been mapped to 9q regions commonly deleted in transitional cell carcinoma of the bladder. Our study excludes an important contribution from the *ZNF189*, *TSC1* and *TGFBR1* genes. We investigated the frequency of the 6A allele of the polyalanine tract present in exon 1 of the *TGFBR1* gene since it was suggested that the protein with a shorter alanine tract (6A) is less active in signal transduction than the most frequent 9A allele. This would provide a logical explanation for the association of the 6A allele with cancer predisposition. We found no evidence to support a role for the 6A allele in bladder cancer susceptibility. In contrast, we found higher percentages for both bladder cancer (17 vs. 13%) and control (17 vs. 10.6%) groups. It is not clear how the discrepancy between their and our findings can be explained.

At this moment, combined LOH analyses in bladder cancer show a multiplicity of events suggesting to some authors that chromosome 9q might harbor 2, 3 or even 4 genes that may have a role in bladder cancer. Several candidate genes have been included in mutation analysis, besides the ones tested here. So far, none of these genes shows a significant number of alterations in bladder tumors.

Mitotic recombination as an explanation for multiple LOH events

The finding of multiple regions of LOH on chromosome 9 raises the question whether all these regions are indeed harboring relevant TSGs. We therefore decided to investigate multiple bladder tumors from a limited number of patients in order to investigate the development of the genetic aberrations in time. If there is a gatekeeper gene on chromosome 9, alterations around the location of this gene would be expected early in the disease and stay present throughout. In Chapter 5, we describe the reconstruction of bladder tumor development in individual patients spanning periods of up to 17 years. Genomic alterations detected in the tumors were used for hierarchical cluster analysis of tumor

subclones. The cluster analysis highlights the clonal relationship between tumors from each patient. Based on the cluster data we were able to reconstruct the evolution of tumors in a genetic tree, where tumors with few aberrations precede those with many genetic insults. The sequential order of the tumors in these pedigrees differs from the chronological order in which the tumors appear. Thus, a tumor with few alterations can be occult for years following removal of a more deranged derivative. Extensive genetic damage is seen to accumulate during the evolution of the tumors. We also observed a very variable loss pattern in tumors of the same patient, with many small and expanding regions of loss of heterozygosity. To explain the extent of genetic damage in combination with the low stage and grade of these tumors, we hypothesize that in bladder cancer pathogenesis an increased rate of mitotic recombination is acquired early in the tumorigenic process. This type of damage to the genome is without actual loss of genetic material, more consistent with the mostly diploid tumors.

A second look at chromosome 9q LOH: multiple recurrent bladder cancers

The first allelotyping studies involved two or three markers on every chromosome arm. Loss of these markers swiftly led to the conclusion that a whole arm was deleted, or that a tumor showed monosomy for an entire chromosome. When, in subsequent studies, more markers were included, more (interstitial) deletions were detected and used to define the SRO. This is a valid method, as long as tumors are compared with an identical genetic background (caused by inactivation of the same gatekeeper gene). In bladder cancer, this is difficult since it has been suggested that there is a genetic difference between superficial recurrent cancer and more invasive cancer, although the histopathological distinction is not always clear and both presumed types show LOH on the same chromosome. Thus, in pooling these different tumor types there is a risk that a specific alteration will be obscured. Furthermore, some tumors appear to have more than one region of deletion on the same arm and therefore they cannot and should not be used to delineate a possible gene locus, since the relevance of the different regions to one another cannot be determined. The interpretation of LOH results is at best very complicated when four genes are suspected on the same chromosome arm, and seven on the chromosome in total. In our study, the ongoing process of multiple alterations on chromosome 9 is elucidated by deletion mapping in multiple recurrent bladder cancer. In Chapter 6, we describe the genesis and development of chromosome 9 alterations within 11 patients with multiple superficial bladder cancer. We show that loss of heterozygosity on this chromosome is almost never the characteristic first step. The regions of loss are multiple and variable in different tumors of the same patient and expand over time. This, we believe, makes it unlikely that a bladder cancer gatekeeper gene on chromosome 9q, if present, will be identified through LOH analysis alone.

Future directions

The patchwork LOH pattern and the increase in LOH during tumor development in mostly diploid superficial papillary bladder tumors might be due to an increased frequency of mitotic recombination. Future research should focus on trying to find proof for this hypothesis. Patients with Bloom's syndrome, an autosomal recessive disorder characterized by growth deficiency; sun-sensitive skin, predisposition to malignancy, and chromosomal instability, exhibit an increased recombination between homologous chromosomes and between sister chromatids. The high sister chromatid exchange (SCE) is a diagnostic feature of the disease. It will therefore be interesting to determine the rate of SCE in bladder tumors as well. In order to do so, it would be favorable to propagate these small sized tumors *in vitro*, because the tumor cell lines in use today are mainly derived from invasive, aggressive tumors and these may not reflect an accurate genetic representation of superficial tumors. Another possibility is to search for mutations in the gene mutated in Bloom's syndrome, *BLM*. This gene encodes a DNA helicase involved in unwinding DNA to make it available for replication and repair. Furthermore, the number of alterations on chromosome 9 is clearly higher than on other chromosomes. Maybe this represents a chromosome breakage defect, like in Bloom's syndrome or Fanconi's anemia. These syndromes are known as nonspecific chromosomal breakage syndromes. Conversely, the ICF syndrome (Immunodeficiency - Centromeric instability - Facial anomalies) only shows instability of chromosomes 1, 9 and 16. These chromosomes differ in this respect that they have a large pericentromeric heterochromatin area. Mutations in the *DNMT3B* gene, encoding DNA methyltransferase 3B cause undermethylation of classical satellites II and III, which are present in this heterochromatin. This makes the heterochromatin more susceptible to breakage. It is possible that a similar phenomenon is operating in bladder cancer. Therefore, next to mutation analysis of the *DNMT3B* gene, it will be worthwhile to test this hypothesis by determining the methylation profile of the 9q heterochromatin region in bladder tumors.

Samenvatting

Blaaskanker is in Nederland de zesde meest voorkomende vorm van kanker. Jaarlijks krijgen zo'n 2550 mensen blaaskanker en zullen er ongeveer 1200 aan overlijden. De meeste mensen krijgen een relatief onschuldige, oppervlakkige tumor die met behulp van een transurethrale resectie kan worden verwijderd. Kenmerkend is dat na verwijdering van deze oppervlakkige tumoren, meer dan 70% van de patiënten één of meerdere recidieven ontwikkelt, die meestal ook oppervlakkig zijn. Blaaskanker is, door de veelvuldige recidivering, uitermate geschikt om als model te dienen voor het bestuderen van de tumorevolutie binnen een patiënt. Het ontstaan en de progressie van tumoren naar meer invasief gedrag wordt toegeschreven aan een opeenstapeling van genetische afwijkingen in tumor suppressor genen (TSGs) en oncogenen. Moleculair genetisch onderzoek van kanker heeft als doel deze genen op te sporen en door de analyse van hun biologische functie inzicht te verkrijgen in de regulatie mechanismen die in de kankercel zijn veranderd. Verlies van een specifiek chromosomaal gebied, aantoonbaar via LOH (loss of heterozygosity) analyse, wordt vaak gebruikt om de plaats van TSGs te bepalen. In blaaskanker wordt de meeste LOH gevonden op chromosoom 9, waardoor men veronderstelt dat op dit chromosoom een TSG moet liggen dat blaaskanker veroorzaakt.

Het doel van het in dit proefschrift beschreven onderzoek was om te bepalen wat het belang is van LOH in het algemeen en chromosoom 9 in het bijzonder in de pathogenese van blaaskanker. Hiertoe werd in eerste instantie in 40 blaastumoren het aantal chromosomen 9 bepaald met verschillende technieken, zoals *in situ* hybridisatie en LOH analyse. Dit is beschreven in hoofdstuk 2. Alhoewel 18 blaastumoren verhoudingsgewijs weinig chromosomen 9 bleken te hebben, was er maar één tumor die compleet verlies van één van beide kopieën had. In 5 van deze 18 tumoren kon de onderrepresentatie van dit chromosoom niet worden bevestigd door LOH analyse. Hieruit kan worden geconcludeerd dat een verlies van chromosoom 9 zoals aangetoond met *in situ* hybridizatie niet gerelateerd hoeft te zijn aan het verlies dat wordt aangetoond door LOH analyse. Uit de LOH analyse bleek verder dat meerdere tumoren meer dan één gebied verloren hadden op chromosoom 9. Daarom werd besloten deze gebieden verder in kaart te brengen.

In hoofdstuk 3 wordt beschreven hoe met LOH analyse is geprobeerd te bepalen welk specifiek deel van chromosoom 9q verloren is gegaan. Onze resultaten onderschreven de hypothese dat er twee verschillende gebieden van verlies zijn, wat zou kunnen betekenen dat er twee verschillende TSGs op de lange arm liggen. De gebieden, genaamd TCC1 en TCC2, zijn getest in 17 blaastumor cellijnen op de aanwezigheid van homozygote deleties, maar deze werden niet gevonden. Dit kan betekenen dat deleties in deze gebieden zo klein

zijn dat ze moeilijk op te sporen zijn, of dat de genen in deze gebieden niet snel worden uitgeschakeld door een homozygote deletie. Overigens zijn door anderen nog meer gebieden van verlies op chromosoom 9 gevonden, zodat het totale aantal wordt geschat op vier gebieden op de lange arm en drie gebieden op de korte arm. Het is echter de vraag of het waarschijnlijk is dat al deze gebieden een voor blaaskanker relevant TSG bevatten.

Aangezien de TCC1 en TCC2 gebieden niet verder verkleind konden worden, hebben we gekozen voor mutatie analyse van de kandidaat TSGs *ZNF189*, het tubereuze sclerose complex gen 1 (*TSC1*) en de TGF beta receptor type I (*TGFBR1*). Deze liggen alle drie op chromosoom 9q in die gebieden die vaak verloren gaan in blaastumoren. Analyse van deze genen toonde verschillende polymorfismen en mutaties aan in *ZNF189* en *TSC1*, maar niet in het *TGFBR1* gen (hoofdstuk 4). In exon 1 van het *TGFBR1* gen is een polyalanine stretch aanwezig met een variabele lengte. De meest voorkomende lengtes zijn 6 of 9 alanines. Er zijn aanwijzingen dat het allel met 6 alanines (6A) vaker voorkomt bij patiënten met bepaalde vormen van kanker. Om dit te onderzoeken, hebben we de lengte van de stretch bepaald in een groep patiënten met blaaskanker en een controle groep. In beide groepen was het percentage heterozygoten (6A/9A) 17%. Het lijkt er dus op dat het 6A allel in blaaskanker geen rol van betekenis speelt. Behalve de drie door ons onderzochte genen, zijn nog veel meer genen op chromosoom 9 getest op de aanwezigheid van mutaties. In veel van die genen wordt wel eens een mutatie gevonden, maar tot nu toe is er geen enkel gen gevonden met mutaties in een significant aantal blaastumoren.

De veelvoud aan LOH gebieden en het gebrek aan mutaties in kandidaatgenen, geeft aanleiding te denken over andere mechanismen waarmee LOH tot stand kan komen. Dit bracht ons op het idee om de ontwikkeling van deze gebieden te volgen in meerdere opeenvolgende tumoren van een klein aantal patiënten. De blaastumoren zijn verzameld van een groep patiënten die tot 15 recidieven hadden gekregen in de loop van 6 tot 17 jaar tijd. Met LOH analyse zijn de genetische afwijkingen van deze tumoren bepaald. Hieruit bleek dat de verschillende recidieven van éénzelfde patiënt clonaal aan elkaar gerelateerd waren en dus uit elkaar, of uit gemeenschappelijke voorlopertumoren moeten zijn ontstaan. De moleculaire evolutie van de aandoening kon met behulp van clusteranalyse worden gereconstrueerd in de vorm van een genetische stamboom, waarbij elke vertakking een nieuwe afwijking representeert. Aan de hand van deze stamboom kon worden geconcludeerd dat de volgorde waarin de tumoren zich ontwikkelen binnen een patiënt sterk kan afwijken van de volgorde waarin ze in de kliniek worden ontdekt. De tumorevolutie staat beschreven in hoofdstuk 5. Opmerkelijk was verder de grote toename in het aantal afwijkingen dat werd gevonden binnen een stamboom, wat suggereert dat deze blaastumoren genetisch instabiel zijn. Uit eerdere onderzoeken is echter gebleken, dat oppervlakkige blaastumoren meestal een redelijk normale hoeveelheid DNA bevatten (ze zijn

diploïd). Een mogelijke verklaring voor deze bevindingen is dat de genetische afwijkingen worden veroorzaakt door een verhoogde mitotische recombinatie frequentie aangezien dit een gebeurtenis is waarbij geen genetisch materiaal verloren gaat, maar wel LOH optreedt.

Met de informatie die deze studie heeft opgeleverd is ook duidelijk geworden dat de rol van TSGs op chromosoom 9 in het ontstaan van blaaskanker wordt overschat. In hoofdstuk 6 wordt beschreven hoe de afwijkingen aan chromosoom 9q zich binnen patiënten ontwikkelen. De afwijkingen treden in de meeste patiënten pas in een later stadium op en zijn dus niet de karakteristieke eerste gebeurtenis in het ontstaan van de tumor. Daarnaast zijn de gebieden van verlies variabel binnen de tumoren van een patiënt en worden ze in de loop van de tijd groter. Het aantal afzonderlijke gebeurtenissen kan in sommige patiënten zelfs oplopen tot tien. Tenslotte variëert de plaats van de verloren gebieden ook nog tussen de patiënten. Hierdoor wordt de hypothese dat dit chromosoom een, voor blaaskanker, belangrijk gen bevat minder waarschijnlijk.

Als de vele gebieden van LOH in blaaskanker inderdaad worden veroorzaakt door een verhoogde mitotische recombinatie frequentie, is het interessant om dit in een vervolgstudie te onderzoeken. Dit is onder meer mogelijk door het niveau van recombinatie tussen zusterchromatiden vast te stellen (sister chromatid exchange, SCE). Dit is een maat voor de totale recombinatie frequentie en wordt als zodanig ook toegepast bij patiënten met het syndroom van Bloom. Deze patiënten hebben een verhoogd risico op het krijgen van kanker door een gebrekkig herstel van fouten in hun DNA. Daarnaast is blaaskanker niet het enige tumor type waarbij meerdere gebieden van verlies op één chromosoom arm worden gevonden. Het is interessant om te onderzoeken of aan dit verschijnsel bij andere typen tumoren ook een verhoogde mitotische recombinatie frequentie ten grondslag ligt. Dit zou ook een verklaring kunnen bieden voor het feit dat LOH analyse alleen nog niet succesvol is geweest in het cloneren van TSGs. Tot slot kan het mogelijk zijn dat de vele afwijkingen op chromosoom 9 in blaaskanker duiden op een chromosoom specifieke instabiliteit. Dit is bijvoorbeeld het geval in het ICF syndroom. Hierbij zijn alleen chromosoom 1, 9 en 16 aangedaan. Deze chromosomen verschillen van de andere doordat ze onder het centromeer een groot heterochromatine gebied hebben. ICF wordt veroorzaakt door mutaties in het methyltransferase gen *DNMT3B*, waarna het heterochromatine te weinig gemethyleerd wordt. Hierdoor wordt het gevoeliger voor breuken. Misschien is er in blaaskanker iets vergelijkbaars aan de hand.

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(Zucht....)™

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