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Molecular alterations in bladder cancer

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Abstract In recent years, significant information has been accumulated on the molecular alterations that take place during development of transitional cell carcinoma (TCC). A number of studies aimed at defining loss of heterozygosity have shown a general chromosomal instability in TCC with loss of parts of chromosome 9 at early stages of papillomas, and of chromosomes 11, 13, 3, 4, 8, 17 and 18 during further development of the tumor. Oncogenes are activated, exemplified by mutations in the ras gene family and overexpression of the c-*erb*B-2 gene, in a minor fraction of tumors. Alterations of tumor suppressors (involved in control of the cell cycle, DNA quality control and activation of apoptosis) seem to be frequently involved. Among these p53 has a key role, and one p53 allele is frequently lost in TCC followed by mutation of the remaining allele.These alterations are correlated with survival, disease progression, invasion and recurrence. Also frequently lost are the cell cycle control genes p16 and p15. The predictive value of this has not yet been determined. Studies of glycosylation genes have shown downregulation of the ABO gene, followed by loss of ABO blood group structures and accumulation of the Lewis cell adhesion molecules in high grade tumors. Functional proteome analysis has furthermore identified biomarkers that are correlated with grade and stage. Molecular models for TCC development can now be built, and clinical testing of these is urgently needed.

Keywords Bladder cancer · Molecular alterations · Tumorsuppressor genes · Glycosylation · Microsatellites

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Introduction

Multistage carcinogenesis is regarded as a consequence of the accumulation of somatic genetic alterations, which include activation of cellular proto-oncogenes, and inactivation of tumor suppressor genes. Tumor suppressor genes are a family of genes that encode proteins that have different cell biological functions, only sharing one condition in common, the suppression of tumor growth. As an example, some of these genes are involved in "quality control" procedures in the cell, as they control the quality of the DNA strands. If the DNA strands harbour incorrectly placed nucleic acids or other abnormalities, the cell is not allowed to divide, and may eventually be directed to programmed cell death. One way of inactivating a tumor suppressor gene is by deleting that part of the DNA strand that harbours the gene, or part of it.

Chromosomal deletions seem to be very common in cancer, and span from the deletion of small DNA fragments to loss of chromosomal arms or even entire chromosomes [7]. The deletions occurring in a given epithelial cancer seem to be non-random, and some seem even to be specific for a given cancer, whereas others are more generally present in cancers. In bladder transitional cell carcinomas (TCCs) chromosome 9 is subjected to deletions [22, 35], often very early in tumor development [22, 79], a finding also observed in other common cancers such as carcinoma of the lung [63], skin [80], ovary [88], and kidney [15]. A candidate tumor suppressor gene CDKN2;p16 was recently identified in the 9p21 region [13, 14, 24, 35, 46]. This gene encodes a protein involved in control of the cell cycle [45].

Various techniques have been used to identify non-random deletions. In the early 1980s cytogenetic analysis was used to identify monosomy, interstitial deletions, and isochromosomes with loss of genetic material at specific sites. In the 1990s molecular techniques have been developed (restriction fragment length polymorphism, polymerase chain reaction, fragment analysis)

that are able to identify very subtle deletions of DNA strands. These techniques are based on highly developed panels of informative markers that cover the complete human genome [25]. With the development of the polymerase chain reaction (PCR) it is now possible to use tandem repeat DNA sequences (e.g., CACACACA) known as microsatellites, as markers, as these are scattered throughout the genome, and highly polymorphic. The latter makes it easy to select microsatellites that can be expected to be heterozygous in the majority of examined individuals. To illustrate this, consider an individual who has a microsatellite of 60 CA repeats in the short arm (9p) of one of his chromosome 9 alleles, and one of 70 CA repeats on the other 9p allele. If this individual develops a bladder cancer that deletes part of, or the whole short arm of 9, there is only one microsatellite of 60 or 70 CA repeats left. In a gel system the 60 and 70 CA repeats will show up as two bands, the single 60 or 70 CA repeat as one band. In this simple way a deletion has been mapped to the area on 9p where we a priori know that the microsatellite is located. The knowledge on the physical microsatellite location in the genome is one of the benefits of large international research programs [25].

Several studies based on RFLP and microsatellite techniques have identified the most frequent aberration in bladder TCCs to be deletions of part of, or one entire, chromosome 9 [35, 79]. As evident from Fig. 1 some data are available on various parts of chromosome 9, making it possible to map those regions of chromosome 9 which are most prone to be deleted in TCC. These regions are marked by a black line in Fig. 1, and spans an area on 9p23 in which p16 is located, and an area on 9q31–34.

Correlation of chromosomal deletions with histopathological parameters

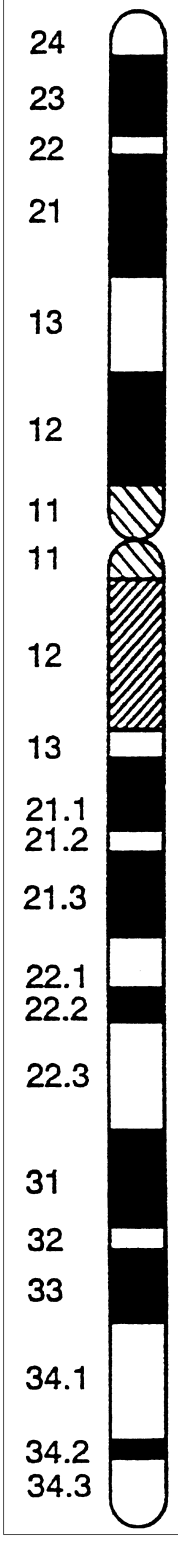
It is generally believed that chromosome 9 very frequently shows deletions in bladder cancer, however, when it occurs during the development of bladder cancer is not known. Chromosome 9 deletions have been shown to be independent of tumor grade and stage in several papers [46, 53, 65], and to occur much more frequently in low-grade, low-stage tumors in other papers [22, 36]. These last authors used their finding to advocate a very early role of chromosome 9 deletions in bladder tumorigenesis. Unfortunately, the number of tumors examined in all the cited papers is small (2–5 tumors in each group) and the conclusions reached should be regarded as preliminary. One explanation for the differences could be that the loci studied on chromosome 9 are deleted at different grades and stages, as shown in [71] in which some loci like D9S17, ASS on chromosome 9q and IFNA on chromosome 9p are lost in low-grade, low-stage tumors, and loci like D9S28 and D9S54/D9S13 on chromosome 9q lost in high-grade, high-stage tumors. This finding is still based on a small number of tumors; however, it indicates that it is important to realize what small part of chromosome 9 that is under investigation.

A confounding parameter is the number of normal cells present in the tumor tissue examined, as these may mask deletions in the tumor if they are present in a significant number. Furthermore, the cut-off level, as well as the techniques used, varies much from paper to paper. In some papers, the complete deletion of chromosome 9 – monosomy 9 – is taken out of the data when deletions are shown, and those papers only report on partial deletions of chromosome 9. This is important as the number of tumors showing monosomy 9 is reported to be as frequent as 32% [46], 41% [85], 54% [12], and 59% [53]. In conclusion, it seems that chromosome 9 alterations occur early in bladder carcinogenesis, and are partly independent of stage and grade. Approximately half of bladder tumors have lost one chromosome 9, and the chromosome 9 remaining is suffering from deletions in both the p and q arms, in more than two of three tumors. The deleted area on 9p seems to be between D9S126 and D9S109 reflecting 9p21–23, and on 9 q the area between D9S167 and D9S176, reflecting 9q21.1–22.3. Some more telomeric placed regions on 9q are also very frequently lost: they are placed around D9S154-D9S103, reflecting 9q32–33, and D9S60–66, reflecting 9q34.

Chromosomal changes, apart from chromosome 9, which occur early in bladder tumorigenesis seem to be difficult to define. Some papers describe findings in Ta gr I/II tumors of 11p[22, 32, 36] and 13q [22], deletions, but most deletions occur at a frequency of less than 20% in these superficial tumors. In high-grade, high-stage bladder tumors several deletions seem to occur systematically compared with low-stage, low-grade tumors: 3p21–25 ($P < 0.004$ [79]); 4p /D4S1608–404 ($P < 0.005$ [78]); 4 q 33–34/D4S408-D4S426 ($P < 0.002$ [78]); 8p21.3 ($P < 0.05$ [47]); 18q21.3 ($P < 0.02$ [9]) and DCC ($P < 0.05$ [9]). Interestingly, deletions of chromosome 17p11–13, which is the location of tumor inhibitor p53, are also frequent in high-grade ($P < 0.06$ [70, 79]), high-stage ($P < 0.0015$ [79]) tumors, and may be related to vascular invasion [79].

Potential diagnostic and predictive use of microsatellite alterations

In normal biological conditions, DNA is very well conserved, and any error in the base sequence is immediately corrected by either the mismatch repair system [66] or the excision repair system [86]. However, in malignant transformed cells a general feature seems to be chromosomal instability with incorporation of mismatched bases giving rise to either missense, or nonsense mutations. The former results in the substitution of one amino acid for another which often leads to a defect in protein function, the latter results in either a truncated protein, as the translation may stop before the total



Microsatellite		N	Loss in TCC (%) Mean	Min/Max	References
D9S	143	1	67		[24]
D9S	129	1	57		[24]
D9S	54	2	46	36/38	[24,53]
D9S	47	1	33		[12]
D9S	199	4	80	43/100	[14,24,46,35]
D9S	144	2	100	100/100	[14,12]
D9S	168	1	82		[24]
D9S	156	1	60		[85]
D9S	157	3	82	60/100	[14,24,85]
D9S	162	2	84	75/92	[14,24]
IFNB1		3	29	0/66	[12,53]
IFNA		7	65	33/100	[12,53]
D9S	736	1	100		[24]
D9S	171	2	69	57/80	[14,24]
D9S	126	5	52	38/69	[24,46,65,12,53]
D9S	104	3	52	13/88	[24,46,53]
D9S	161	3	47	0/50	[14,85,53]
D9S	3	3	0		[65,12]
D9S	50	2	42	8/75	[46,12]
D9S	165	1	33		[85]
D9S	19	2	38	25/50	[53]
D9S	200	4	37	17/75	[14,24,46,35]
D9S	55	1	55		[53]
D9S	145				
D9S	146	1	0		[85]
D9S	15	6	61	33/100	[24,46,65,85,35,53]
D9S	153	1	40		[35]
D9S	201	1	50		[35]
D9S	283	1	66		[35]
D9S	119	1	100		[35]
D9S	12	3	51	33/100	[46,85,35]
D9S	176	2	50	20/75	[85,35]
D9S	109	3	54	25/86	[46,85,35]
D9S	127	2	52	33/71	[46,35]
D9S	53	2	65	60/69	[46,53]
D9S	105	3	72	50/100	[46,85,35]
D9S	58	1	69		[53]
D9S	59	2	73	66/80	[46,35]
HXB		3	73	50/100	[46,35,53]
D9S	103	2	84	67/100	[46,35]
GSN		4	68	33/100	[46,85,35,53]
D9S	60	2	86	71/100	[46,35]
ASS		3	42	25/100	[71,53]
ABL		5	73	20/100	[71,24,46,65,12,35]
D9S	66	3	68	66/75	[46,35,53]
D9S	67		55	33/75	[46,35,53]
D9S	7	3	40	21/50	[71,65,53]
D9S	11	3	72	40/100	[65,12,53]

Fig. 1 Deletions of chromosome 9 in transitional cell carcinoma (TCC). *N* number of studies

protein has been transcripted, or a loss of part of the protein if an exon is skipped completely.

The chromosomal instability is not completely understood. One protein involved in "quality control" of the DNA is p53, and it is well documented [33] that changes affecting p53 lead to accumulation of mutations in other genes. However, p53 changes (loss of intact alleles in heterozygotes, non- or missense mutations) seem to occur late in most cancers and cannot be responsible for the important early steps in the development of cancer [33].

Chromosomal instability

Genes in which mutations are known to lead to a greatly increased susceptibility to the development of cancer are the mismatch repair genes MSH2 [82], 3 [59, 83], 6 [59]; MLH1 [77]; PMS1, 2 [23, 69]; and others [66 and references therein]. Inherited heterozygosity in these genes greatly increases the risk of colorectal, endometrial and to some extent urothelial cancer, as well as cancer at other sites [23, 56]. Urothelial cancer in families with mismatch repair gene heterozygosity occurs relatively late in life compared with colorectal cancer (median 42 years [2]). These patients were initially identified by clinical criteria (Amsterdam criteria [56]), and later by both clinical and molecular criteria [23]. The molecular finding was microsatellite instability leading to massive alterations in several microsatellites at the same time. This phenomenon was called the replication error (RER) phenotype [1]. The many alterations which have been published in bladder cancer resemble – in some ways – this RER phenotype even in non-invasive cancers. Another frequent alteration in bladder cancer is LOH. Thus, by examining only five loci Habuchi et al. [36] found 19 of 30 non-invasive tumors showed LOH, and several of these in more than two loci. In invasive tumors 16 of 19 showed LOH and of these 14 of 16 lost more than two loci [36].

Based on the data presented above, a hypothesis for the steps involved in general development of bladder cancer can be formed: (i) loss of a mechanism that protects against LOH, or acquisition of a mechanism which enables LOH, (ii) loss of a *tumor inhibitor* on chromosome 9, (iii) loss of genes responsible for *chromosomal stability*, such as mismatch repair genes.

These three changes leads to tumor growth and accumulation of further mutations. We have tested step iii), the possible LOH of mismatch repair genes, by placing microsatellites around the MSH2 locus on chromosome 2, and the MLH1 locus on chromosome 3. In 14 relatively young bladder cancer patients we found LOH of the MSH2 locus in 12 of 13 informative cases, and LOH of the MLH1 locus in 11 out of 13 informative cases. In both cases the LOH was independent of grade and stage. When a locus on chromosome 17 was examined only one of four examined showed LOH[18]. A summary of the functional aspects of chromosomal losses in TCC is shown in Table 1.

Predictive use of microsatellite instability

Several data on small clinical materials are available; however, before clinical use of microsatellite instability can be implemented, we need much more profound data on carefully controlled prospective clinical materials. As described above we know that loss of some chromosomal areas is associated with a high grade and a high stage; however, we do not know whether the LOH will occur in low-grade, low-stage tumors before the tumor acquires invasive properties. If this is the case, the LOH of areas like 4p15.1, 8p21.3,18q21.33 and 17p4.13 could be used to predict a later invasive disease course, and serve as a signal to initiate a more radical therapy. As LOH seems to be the event associated with high grade and stage it is ideal for predictive purposes. For diagnostic purposes the combination of alterations of the length of the individual band (mobility shift) with LOH

Table 1 Functional aspects of chromosomal losses during progression of transitional cell carcinoma

Tumor development	Chromosomal event	Depicted function
Low grade & stage	Monosomy of chr. 9	Loss of tumor inhibitor on chromosome 9
	Loss of heterozygosity: 9p21–22.3 (p15/p16) 9q22–23 + 33–34	Loss of control with cell cycle (p16)
	2q	Loss of DNA repair efficiency (MSH2), leading to microsatellite instability
	11p 13q	*Ras* oncogene activation
High grade & stage	Loss of heterozygosity: 3p 21–25 4p15.1 4q33/34 8p21.3 17p11–13 (p53) 18q21.3 (DCC)	Loss of DNA quality control and loss of apoptosis mechanism (p53) as well as other unknown effects

seems most promising, as many band alterations occur early in tumor development. These band alterations may be both the addition of extra repeats leading to longer repeat sequences, or the removal of repeats leading to shortening. This change in repeat length has been attributed to malfunctioning of mismatch repair protein complexes involving MSH2 and MSH3 (lengthening of repeats) and MSH2 and MSH6 (shortening of repeats)[3,83]. In a recent letter [57] microsatellite analysis was used to diagnose bladder cancer in urine DNA. The hypothesis was that tumor cells shed into the urine due to the reduced cohesion and increased proliferation in tumors could be identified by their altered microsatellites. Nineteen of 20 tumors were identified by microsatellite alterations in urine, independent of stage and grade. Three of the 19 tumors were identified by band shift alone, and these were grade I and II Ta and T1 tumors, and one grade III T1 tumor. LOH alone identified seven tumors of which four were grade III. We have found the same tendency in our own material (to be published), LOH identifies high-grade, high-stage tumors, whereas band mobility shifts identify lower-grade, lower-stage tumors. If the most vulnerable microsatellite loci are used for diagnosis, as few as five loci may be sufficient to identify bladder cancer by testing on urine sediments (to be published). Future prospective studies will show the sensitivity and specificity of this test compared with cytology and cystoscopy. In a preliminary study analysis of microsatellite instability in urine was more sensitive than conventional cytology [57].

P16 and p15 gene deletions

The cell has to pass through well-defined steps during the cell cycle, and this process is controlled by protein kinases. The active molecule is a cyclin which is regulated by cyclin-dependent protein kinases (Cdk). To pass through the cell cycle some proteins need to be phosphorylated, among these the retinoblastoma protein. The cell cycle passage can be stopped by a new group of inhibitory molecules known as Cdk – inhibitory molecules [89]. Among these we find the p15 and p16 proteins also known as p15(INK4B/MTS2) and p16 (INK4A/MTS1/CDKN2) [37, 45, 89]. Both p15 and p16 bind to Cdk4 and Cdk6 and inhibit phosphorylation of the retinoblastoma protein, thereby inhibiting the cell cycle progression. These genes are regarded as candidate tumor suppressor genes, as the chromosomal regions they map to are frequently altered in cancer. This region is small, as p16 and p15 are located within 80 kilobases of each other on chromosome 9p21.

The loss of p16, most often accompanied by loss of p15, is one of the most frequent findings in TCC [34, 107, 111]. In a large study of 140 tumors, Knowles and coworkers [107] were able to estimate the frequency of homozygous loss of p16 to approximately 38% in primary tumors. An area of approxiomately 40 Kb, frequently including p15, was lost [107]. In cases with monosomy of chromosome 9, 58% of tumors had lost p16 on the remaining chromosome [107]. Similar [76] or lower figures have been reported from other groups, but in these cases the number of examined tumors was much smaller. An inactivating mechanism in which one p16/p15 allele is lost and the other inactivated by a mutation, has been described as common in cancer of the esophagus [68] and in melanomas [34, 45]. However, it seems that mutations of p16 and p15 are rare in bladder tumors [107], allele instability being the predominant course of p16/p15 inactivation in this tissue.

Rb gene

The retinoblastoma susceptibility gene *Rb* was the first tumor suppressor gene isolated [93]. It is a nuclear phosphoprotein that maps to chromosome 13q14. The phosphorylation of *Rb* inhibits the entry of the cell into the S-phase, by blocking transcription factors necessary for transcription of DNA.

The importance of the *Rb* gene was demonstrated in vitro by stable transfection of an *Rb* gene into a bladder carcinoma cell line devoid of *Rb* expression. The transfected cell line was shown by W- and N-blots to express the *Rb* protein for several months. During this time the cell line showed lack of ability to form colonies, a decreased growth rate, and reduced tumorigenicity in nude mice [97].

Several studies on clinical materials have shown a reduced expression of the *Rb* gene to be associated with invasive properties, and a high tumor grade [44,109]. Data on survival revealed a significantly reduced survival during 4 years of follow-up in those patients with reduced *Rb* expression [109].

In a study of 30 bladder tumors, eight were shown to have *Rb* mutations by a RNA-SSCP examination of the entire coding region [64]. Furthermore, the same study showed LOH of the *Rb* locus in one of three bladder tumors [64]. LOH and mutations were not related to stage or grade in these tumors.

Ras gene

The genes Ha-, Ki-, and N-*ras*, constituting the *ras* gene family, have been demonstrated to be involved in initiation, promotion, and progression of carcinogenesis in many tissues [7, 8, 27, 30, 91, 98, 106, 112]. The *ras* oncogene is a transforming gene, encoding a 21-kDa protein involved in the regulation of cell growth and differentiation [43].

The *ras* genes harbour some "hot" spots that are susceptible to point mutations. Frequent mutations are glycine to valine in codon 12, glycine to cysteine at codon 13, and glutamine to arginine/lysine/leucine at codon 61 [51]. Mutations of H-*ras* are often seen in TCC, but in a frequency probably less than one mutation per five tumors examined.

In a SSCP (single-stranded conformation polymorphism) study of 111 bladder tumors, 26 tumors showed the codon 12 mutation, and seven mutations were distributed on codons 13 and 61 [51]. In another large SSCP study of 152 bladder tumors, only nine tumors contained mutations, four located to codon 12, two to codon 13, and three to codon 61 [48]. The *ras* mutations are not correlated to tumor grade and/or stage in some papers [11, 48, 51], or correlated to a high grade and aneuploidy in another paper [21]. Attempts have been made to use identification of *ras* mutations in urine sediments to complement cytology in the detection of bladder tumors. The combined use of H-*ras* mutation detection and cytology increased the overall detection of tumors from 33% to 60% [28].

P53

The p53 protein is a component in biochemical pathways central to human carcinogenesis. P53 is located on chromosome 17p13.1 and encodes a 53-kDa nuclear phosphoprotein with DNA binding properties.

The p53 protein is involved in several different mechanisms such as gene transcription, DNA synthesis, repair and quality control, and programmed cell death [38–40, 52, 67, 103]. It seems that loss of p53 function leads to malignant transformation of the cell by two mechanisms simultaneously, loss of tumor suppressor function, and gain of oncogenic activity.

P53 protein alterations due to missense mutations, and loss of p53 due to frameshift or nonsense mutations, provide a selective growth advantage for the p53-insufficient cell. This may be one of the mechanisms behind the frequent presence of p53-mutated cells in high-grade invasive cancers. Expression of high levels of wild-type p53 arrests the cell cycle at late GI phase. This function is induced rapidly in response to DNA damage, which may allow time for repair of DNA before continuation of the cell cycle.This is obviously an advantage in a situation with increased DNA instability, as seen in low-grade TCC.

Exons 5–8 of the p53 gene are conserved during evolution, and this 600 base pair region houses the majority of the mutations seen in sporadic tumors. In TCCs as in several other cancers, the most common mutation is a G:C–A:T transition. This base shift occurs due to methylation of guanine by alkylating agents, leading to mispairing, as the methylguanine is misread by the DNA polymerase. The polymerase pairs methylguanine with thymidine and not cytosine, which would have been correct [42].

The importance of p53 in bladder tumorigenesis was suggested by the high frequency of LOH of chromosome 17p in TCCs. [70]. Other reports have demonstated that p53 follows the classic tumor suppressor theory, the loss of heterozygosity of one allele accompanied by a mutation of the remaining allele [4].

The frequency of p53 mutations in TCCs is reported to be between 20% in Japan [94] and up to 50% among arylamine-exposed workers in New Jersey [99]. The frequency is dependent on the distribution of examined tumors on histological grades, as the mutations seem to be rare in low-grade tumors and frequent in high-grade tumors [26, 102]. The methods used to detect p53 mutations can be divided into two, those that use molecular methods (SSCP and sequencing), and those that use immunohistochemistry. The latter technique is based on the fact that the p53 wild-type protein has a half-life in the nucleus of 6 to 30 min, whereas the mutated protein binds to heatshock protein hsc70, and thereby increases the half-life to many hours [26]. By immunostaining with an antibody to p53, positive nuclei will indicate a mutated p53. Several papers have shown a good overall concordance (accuracy 90.3% [19]) between the two techniques; however, the immunotechnique seems to be positive in approximately one of four tumors that have undetectable mutations, and mutations are found in more than 10% of tumors that do not stain [26,58]. The latter could be due to loss of the nuclear localization signal, as speculated in [58]. The former is unambigiously explained by the fact that biopsies usually contain a mixture of normal and malignant cells, and the powerful amplification in the PCR step could lead to loss of detection of the mutation, if the tumor cells only account for a minor fraction.

A property unique to urothelial tumors is the large fraction (28%) of tumors carrying multiple mutations in p53 [99]. This is rarely ($< 5\%$) seen in other tumors, and could indicate a higher genomic instability in TCC than in most other tumors. This is supported by the finding of marked heterogeneous chromosome 17 copy numbers in carcinoma in situ, two out of six of these showing 1–7 chromosome 17 signals per cell [104].

Most clinical studies have been performed by the use of p53 immunostaining. These studies have generally shown correlation with survival [58, 102], disease progression [90], invasion and stage [26, 29, 60], and histological grade [29, 60]. One study in addition found a positive correlation with disease recurrence [90].

Epidermal growth factor receptor and C-erbB-2

Epidermal growth factor receptor (EGF-R) is an Mr 170000 transmembrane glycoprotein, which is activated upon binding of the ligand's EGF or transforming growth factor-α. Activation results in phosphorylation of tyrosine residues on proteins, and leads to cell division. The c-*erb*B-2 gene is a human proto-oncogene with significant sequence homology to EGF-R. It lacks the extracellular part, as well as part of the C-terminus, and is constitutively active [87].

Several papers have examined the possible amplification and overexpression of these genes [50, 55, 100, 101, 108]. In general, it seems that an overexpression, and maybe an amplification of the EGF-R takes place in

grade II and III tumors, and that this is correlated to a high T-stage and a poor outcome [55, 101]. However, correction for stage shows that this information is stage-dependent and does not give additional information [101]. The c-*erb*B-2 gene is amplified in a small fraction of tumors [108], and overexpressed in around one-third of these [108]. It does not show a correlation to disease recurrence and disease progression that can be used for clinical purposes [50, 100, 101].

E-cadherin and catenins

E-cadherin is a calcium dependent intercellular adhesion molecule, which forms an epithelial junction complex that involves other proteins, and triggers signal transduction. Loss of E-cadherin is an important step in the progression of many carcinomas, and is believed to lead to a decreased cell adhesion in tumors [31]. The loss of E-cadherin function can also be due to defects in its linkage to the cytoskeleton, which is mediated through catenins [92].

Several immunohistochemical studies have shown a decreased E-cadherin expression, associated with high grade and high stage [10, 41, 84, 92, 105].The staining is heterogeneous, which has lead to the use of image-analyzing equipment for standardized evaluation [84].

The decreased E-cadherin expression is associated with a short recurrence-free survival of Ta-T1 tumors, in those where less than 50% of cells stain for E-cadherin [54]. A reduced E-cadherin expression has also been reported to be a predictor of reduced survival [10, 92, 95]. The predictive value has, however, been questioned by other researchers, who did not find independent prognostic value of reduced E-cadherin expression, when using multivariate survival analysis [41,54]. The predictive value of reduced E-cadherin expression may be better when combined with increased expression of the autocrine motility factor [75]. A single immunohistochemical study has shown that loss of beta-catenin and alpha-catenin expression has a prognostic value similar to E-cadherin [92].

Alterations of blood group gene and antigen expression

Glycosylation of cell surface glycoproteins and lipids is altered remarkably in cancer cells compared with normal cells. The blood group ABO, Lewis and T/Tn antigens are such glycostructures that change during the malignant progression of bladder tumors, as the ABH antigens are lost from the cell surface and replaced by Lewis and T antigens in invasive cancers, reviewed in [72]. The Lewis antigens are cell adhesion molecules that bind to E-selectin on activated endothelial cells, and the expression of these could be involved in haematogenous metastases [96]. A number of glycosyltransferases are involved in the biosynthesis of glycoconjugates and transferase measurements of normal and cancer tissues have identified relative up-and down regulation of enzymatic activity as tumor-associated events. In human TCC the most significant event is the loss of activity of the blood group ABO transferase, and consequently loss of AB antigen expression [62,74]. As the ABO locus is located at chromosome 9q34, a region often showing LOH in bladder tumors, it was speculated that loss of ABO activity was due to LOH of this locus. However, examination of tumor cells, separated from normal cells by flow cytometry, from informative heterozygous individuals did not show any LOH [61]. Recent research has shown that the loss of AB transferase activity is due to a reduced transcription of the ABO blood group gene in TCC, a reduction which could be reproduced by growth stimulation of urothelial cell lines [73]. The latter indicates that the mechanism of reduced transcription could be an increased cell proliferation in cancer cells.

Another blood group-related structure is detected by the monoclonal antibody M344. The structure is a sialylated, O-linked structure related to the T antigen [5,6]. The structure is absent from normal and inflamed urothelium, occurs in three of four low-stage tumors, and may be used to identify premalignant lesions [20, 49].

Functional genome analysis in bladder cancer

Studies of protein analysis have lagged behind studies of genome alterations mainly due to the complexity of the technology required to separate, analyze and identify the thousands of polypeptides that constitute the proteome of a given cell type. Proteins are frequently the functional molecules and are likely to reflect qualitative (expression of new proteins, changes in post-translational modification) and quantitative (coregulated proteins in pathways) differences associated with various stages of cancer development. Recently, using a database approach and state of the art proteome analysis techniques (mass spectrometry, microsequencing) some aspects of proteome changes in bladder tumors have been identified [16,17]. In these papers, it was shown that the protein psoriasin is a biomarker of squamous cell metaplasia in TCC and that psoriasin is externalized to the urine in these patients [17]. Examination of 63 TCCs with two-dimensional gel electrophoresis, microsequencing and mass spectrometry, fingerprinted and partially characterized the protein expression profile of TCCs of various grades and T-stages [16]. Four abundant biomarkers, A-FABP, GST-μ, PGDH, and keratin 13 were identified. They all showed a gradual disappearence from normal to grade IV tumors. A-FABP (adipocyte-type fatty acid binding protein) was absent from two of three grade III tumors, and in all grade IV tumors ($P < 0.0006$), and correlated to T stage ($P < 0.0269$). Other data implies that A-FABP is an inhibitor of cell proliferation [110], thus, A-FABP could be involved in the lack of control of cell proliferation in high grade TCCs. Further knock out studies are underway to identify the exact function of these proteins

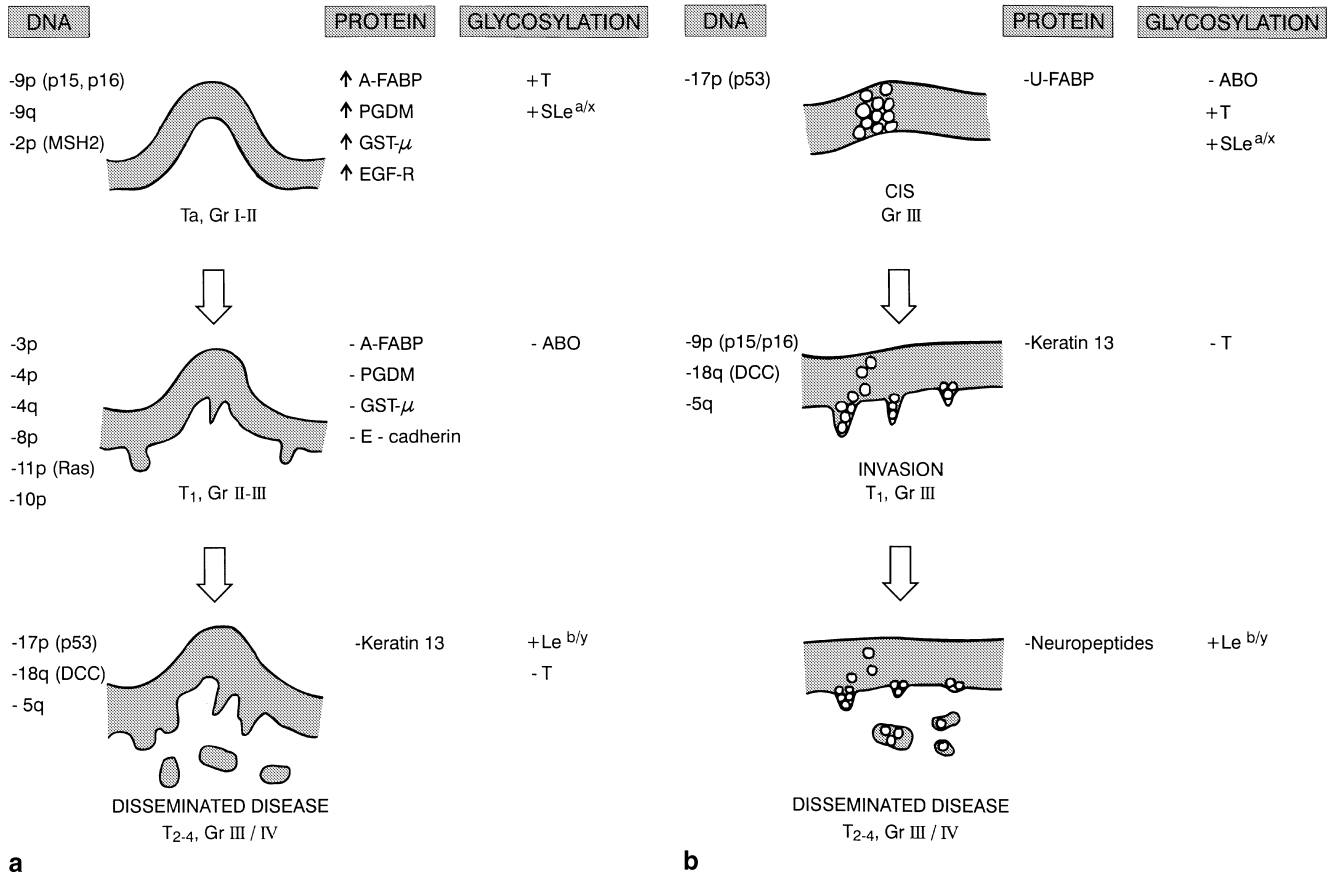


Fig. 2 A model for the different pathways leading to invasive bladder cancer. **a** Papillary bladder carcinomas, **b** non-papillary bladder carcinomas

in tumor development (J.E. Celis, personal communication).

Computer technology offers a new way of dealing with the many thousand data obtained from protein blots. As an example, a comprehensive database has been developed of proteins from the urine of TCC patients [81]. The aim of this database is to identify tumor markers in the urine that might serve as prognostic factors for the development of superficial bladder cancer.

A model for the development of bladder cancer

Based on the data presented above, a model for the different pathways leading to invasive bladder cancer is shown in Fig. 2. It is evident that flat lesions represent one pathway that is distinct from the pathway initiated by papilloma formation. The model tries to unify data from both genomic, proteomic, and glycobiology analyses of TCCs. A substantial amount of knowledge is already available on molecular alterations in TCC, and large clinical follow-up studies are now urgently needed to test the clinical relevance of the many available markers. Hopefully this will lead to better ways to prevent, diagnose, treat and follow bladder cancer.

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