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Chromosomal deletions in bladder cancer: shutting down pathways

Reimar Abraham [1], Francesco Pagano [1], Leonard G. Gomella [2] and Raffaele Baffa [2]

[1] Venetian Institute of Molecular Medicine, Padova, Italy, [2] Department of Urology, Thomas Jefferson University, Philadelphia, U.S.A.

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1. ABSTRACT

Bladder cancer is one of the most common cancers in the world, leading to approximately 145,000 deaths annually. Bladder cancer is typically managed by surgical removal of the tumor; however, the recurrence rate is disappointingly very high, often requiring systemic chemotherapy. Improvement in the diagnosis and prognosis of bladder cancer will only come from a comprehensive understanding of the genetic factors that lead to its development. In this review, we focus on the chromosomal deletions that contribute to the downregulation of tumor suppressor pathways in bladder cancer. Chromosomal deletions are not a random event, since bladder cancer progression has been associated with specific chromosomal deletions and this progression correlates with specific stages of tumor development. The most commonly found chromosomal deletion in all stages of bladder cancer involves deletions in chromosome 9, resulting in the loss of three genes encoding proteins that activate the Rb and p53 tumor suppressors. Additionally, chromosome 9 harbors the *TSC1* tumor suppressor which downregulates the well-known anti-apoptotic Akt/mTOR pathway. Hence, deletions on one chromosome may have a crucial influence on the initial steps in tumor development. Other deletions targeting the tumor suppressors *Rb, p53, FHIT* and *LZTS1* occur at later stages of tumor development. Considering the central importance of these tumor suppressor pathways in the formation and evolution of tumors, the time has come to evaluate available drugs in bladder cancer that target the positive regulators of these pathways.

2. INTRODUCTION

Cancer is a genetic disease that is formed by the erroneous activation of proto-oncogenes and inactivation of tumor suppressor genes. While proto-oncogenes are activated by mutation or overexpression, tumor suppressor genes are lost from the genome of cancer cells by mechanisms described below. These events transform normal cells so that they acquire the phenotypes that are inherent to any cancer cell: self sufficiency in growth signals, insensitivity to anti-growth signals, the capacity to invade other tissues and to metastasize, limitless replicative potential, the capacity of sustained angiogenesis and the evasion of apoptosis (1).

We will focus our review on the most important chromosomal deletions for the inactivation of tumor suppressor genes in bladder cancer. We will review the consequences of these deletions for tumor development and discuss the cellular signaling pathways that are subsequently influenced.

2.1. Bladder cancer epidemiology

Bladder cancer is one of the most frequently occurring cancers; it is the 5th most common cancer in developed countries and the 9th most common cancer worldwide. It is estimated that annually there are 357,000 new cases and 145,000 deaths worldwide (2, 3).

The disease is thought to be caused exclusively by somatic genetic changes induced by environmental carcinogens. Smoking therefore constitutes the main risk factor for developing this cancer. Bladder cancer is more widespread among men than women, with around three times more men than women being diagnosed with the disease. Various factors have been attributed to this difference including smoking habits among men and women (4). No contributions of bladder cancer specific germline mutations have been described, i.e. no families with a history of increased bladder cancer risk have been found. This does not exclude higher susceptibility to bladder cancer due to polymorphisms in certain genes (5).

2.2. Bladder cancer progression and treatment

In contrast to most epithelial tumors, which are thought to develop in a single pathway from benign lesions via primary tumor to metastatic cancer, bladder cancer can arise through two diverse pathways that both may culminate in the formation of muscle invasive bladder cancer (6). It is the muscle invasive tumor that constitutes the biggest threat in bladder cancer because these high grade tumors give rise to metastatic cancer. The two pathways start as superficial bladder cancer either with a low grade benign papillary tumor or with a non-papillary form - carcinoma in situ (CIS). Around 80% of patients present with papillary tumors at first diagnosis, and those tumors can be surgically removed by trans-urethral resection followed by intravesical immuno- or chemo-therapy. These tumors have a high chance of recurrence but only a 15% chance to develop into invasive high grade tumors. In contrast, the tumors which evolve via the CIS-pathway (i.e. 20% of the patients) are more aggressive and invariably form invasive tumors. Once a muscle invasive tumor has been found, the tumor is resected by partial or complete removal of the bladder, a treatment that can be combined with adjuvant chemotherapy. While this eliminates the primary cancer, in most cases metastases will form within 2-3 years upon which point systemic chemotherapy is the only available treatment option. However, overall median survival time after systemic chemotherapy is only 14 months, explaining the high death toll that this cancer affords (7).

Superficial bladder cancer usually manifests with multiple papillary lesions in the urothelium. Two theories have been put forward to explain this observation. In the first hypothesis, bladder cancer is of polyclonal origin. Here, the exposure to carcinogens leads to a field effect on the entire urothelial epithelium that gives rise to multiple, genetically distinct tumors. In the second hypothesis, bladder cancer arises from a single clone and migrates across the epithelium to form papillary tumors. Commonly found genetic differences between these tumors can be explained by the genetic instability of cancer cells that results in different genetic lesions in these sub-clones. Evidence exists for both theories (For a recent review see Ref (8)).

2.3. Chromosomal deletions and tumor suppressor genes

According to the classical view of tumor suppression, tumor suppressor genes are dominant, such that both copies of a tumor suppressor gene must be inactivated in order for cancer to develop. Therefore, gross deletions of chromosomes are usually a prerequisite for the inactivation of tumor suppressors. Consequently, chromosomal aberrations can be observed in every cancer cell and are the most predictive diagnostic factor for distinguishing tumor from normal cells. This process has been termed loss of heterozygosity (LOH). The second allele is generally inactivated by subtle mutations or by transcriptional silencing, for instance through promoter methylation or histone deacetylation.

Since LOH is such an important mechanism for inactivation of the tumor suppressor, chromosomal aberrations today are mainly investigated with the aim of finding tumor suppressor genes whose inactivation contributes to cancer formation. The region harboring the tumor suppressor gene(s) can be found by mapping the smallest chromosomal region that is commonly lost in a given cancer. Various methodologies have been developed for this purpose and are described below. Once a minimal region of chromosome loss has been defined, the tumor suppressor in that region is usually positionally cloned and further characterized. Until recently, a requirement for the definition of a new tumor suppressor was the finding of LOH of that gene in cancer. For a putative tumor suppressor gene this means that, apart from the loss of one allele by deletion, the other allele should be inactivated too, either by mutation or transcriptional silencing.

In recent years, it has become increasingly clear that the classical view necessitating the inactivation of both

alleles is not true under all circumstances. There have been numerous reports that attribute cancer formation to the inactivation of only one tumor suppressor allele. This leads to a reduction in gene dosage which can promote tumor formation at a rate in between that of wild type cells and cells with both alleles inactivated. When this occurs, the tumor suppressor is said to be haploinsufficient. Clear evidence for haploinsufficiency of many well known tumor suppressors have been realized through mouse models. In humans, the contribution of haploinsufficiency to tumor formation is much more difficult to prove since haploinsufficiency most probably cooperates with other unknown genetic lesions. Thus, the already weaker effect of haploinsufficient tumor suppressors can be considerably diluted by the individual genetic variations in cancer patients. However, these mechanisms make it experimentally harder to rule out contributions of a gene to tumor suppression in the absence of LOH.

3. EXPERIMENTAL TECHNIQUES FOR DISCOVERING CHROMOSOMAL DELETIONS

The discovery and understanding of chromosomal deletions has come only by the introduction of high throughput, molecular techniques, some of which will be described below.

3.1. Polymerase Chain Reaction (PCR)-based techniques

PCR has become widely used in clinical diagnostics to identify chromosomal regions lost in cancer. A PCR is run on microsatellite DNA markers of normal and tumor tissue and the reaction products are quantified. LOH is scored when the amount of PCR product amplified from the tumor sample is substantially lower than that from the sample with normal cells.

3.2. Fluorescence-in-situ hybridization (FISH)

FISH provides researchers with a way to visualize and map specific genes or portions of genes. This is important for understanding a variety of chromosomal abnormalities and other genetic mutations. A DNA probe specific for a sequence of a known chromosomal location is fluorescently labeled and hybridized to metaphase or interphase chromosomes. The number of signals (two for a normal diploid cell) indicates gain or loss of the investigated chromosomal region. The technique is versatile because a sample can be collected by spinning the precipitates from a patient's urine.

3.3. Comparative Genomic hybridization (CGH)

CGH is a molecular-cytogenetic method for the analysis of copy number changes (gains /losses) in the DNA content of tumor cells. The method is based on the hybridization of fluorescently labeled tumor (fluorescein) and normal DNA (texas red) to normal human metaphase preparations. Using epiflourescence microscopy and quantitative image analysis, regional differences in the fluorescence ratio of tumor vs. control DNA can be detected and used for identifying abnormal regions in the tumor cell genome. CGH will detect only unbalanced chromosomes changes. Structural chromosome aberrations such as balanced reciprocal translocations or inversions cannot be detected.

3.4. Array CGH

This is a recent extension of the normal CGH technique that allows a higher resolution mapping of DNA copy number changes. Instead of metaphase chromosomes, DNA fragments immobilized on a solid support are used for hybridizing the two differentially labeled probes. This allows mapping of chromosome changes down to a resolution of 500 base pairs (9, 10).

3.5. Single-Nucleotide Polymorphism (SNP) arrays

Like Array CGH, this array-based methodology allows the high-resolution mapping of chromosomal changes by comparing the abundance of specific SNPs in normal and tumor DNA. Oligonucleotides whose sequences cover known SNPs are deposited on a solid support in the same way as for DNA microarrays. An increase or decrease in the signal by a multiple of two indicates the gain or loss of chromosome fragments harboring the SNP. The resolution achieved by this kind of mapping depends on the number of SNPs covered. Using 1000 to 100,000 SNPs, results obtained with the more traditional and laborious PCR based methods could be reproduced and extended in bladder cancer, lung cancer and breast cancer (11-13). The latest SNP array offered by Affymetrix covers 500,000 SNPs allowing an even greater resolution.

4. IDENTIFIED CHROMOSOMAL DELETIONS

Numerous gross but not random chromosomal deletions have been detected in bladder cancer. Except for deletions in chromosome 9, these deletions are found in high grade, high stage bladder cancer. An overview of the regions commonly lost in bladder cancer is presented in Table 1.

4.1. Deletions on Chromosome 3

Deletions on chromosome 3p have been investigated early on in bladder cancer because studies in other types of cancer suggested that this chromosomal region harbors tumor suppressor genes. Indeed, deletions on chromosome 3p can be found in approximately 25% of the studied cases and this genomic loss is associated with invasive tumors (14-18). Two genomic regions have been identified that are most frequently deleted, 3p12-14 and 3p21-23 (17), but only the former location has been investigated further in bladder cancer.

4.1.1. 3p14.2

This region is the site of a familial reciprocal chromosomal translocation from 3p14.2 to chromosome 8q24 that segregates with the early onset of renal cell carcinoma. Therefore, as early as 1979, this translocation was suggested to affect a tumor suppressor gene (19). Coincidentally, 3p14.2 is also a fragile site in the human genome, which means that exposure to DNA replication stress, presumably due to structural constraints, constitutively leads to a gap or chromosome break at that

Table 1. Overview of the best characterized chromosomal deletions in bladder cancer and associated putative tumor suppressor genes.

Chromosome	Frequency of deletion	Clinical correlations	Tumor suppressor/alteration in the remaining allele	Reference
3p	25%	High stage	*FHIT*/promoter methylation (60%)	14-17
8p	25-50%	High grade and stage	*LZTS1*/transcriptional silencing	23, 32-36, 42
9p	60-80%	Deletions found throughout all tumor stages	*P16*/sub-chromosomal deletions and methylation *ARF*/sub-chromosomal deletions and methylation *P15*/sub-chromosomal deletions *MTAP*/no mutational analysis	13, 63, 65, 72, 74-76, 121
9q	55-75%	Deletions found throughout all tumor stages	*PTCH*/low frequency of mutation (4%) *DBCCR1*/promoter methylation *TSC1*/low frequency of mutation (8%)	13, 61, 69, 97, 107-109, 122
10q	30%	High grade and stage	*PTEN*/low frequency of mutation (14%)	115-117, 123
13q	30%	High grade and stage	*Rb*/loss of expression and overexpression can lead to inactivation	52-57
17p	60%	High grade and stage	*P53*/high frequency of mutation (60%)	58-60, 62, 112, 124

Note: More chromosomal deletions have been described but not fine-mapped for identification of tumor suppressors. Aside from that, using high-throughput technologies, many more chromosomal deletions have been detected on almost every chromosome (13, 112). These newly detected lesions also await further characterization.

location. In 1996 the gene Fragile Histidine Triad (*FHIT)* was cloned that is affected by the aforementioned translocation and lies at the fragile site (20). LOH was subsequently shown for the *FHIT* locus in a wide variety of investigated tumor types including bladder cancer. The remaining *FHIT* allele was found to be inactivated by intragenic deletions or downregulation of the expression (21-24). In bladder cancer, reduced *FHIT* expression has recently been correlated with promoter hypermethylation (25). Restoration of *FHIT* expression in cancer cell lines endogenously lacking the gene lead to reduced tumorigenicity *in vivo* and mice heterozygous for *FHIT,* show elevated levels of stomach cancer development after carcinogen exposure (26, 27). Intriguingly, the frequency of tumor formation was similar in mice heterozygous and nullizygous for *FHIT* (28). In chemically induced bladder cancer, the heterozygous mice showed even an elevated frequency of tumor formation with respect to the nullizygous mice (29). These data strongly support a role for *FHIT* as a haploinsufficient tumor suppressor. How precisely *FHIT* influences tumor formation is not entirely clear. Fhit has dinucleoside 5',5'''-P1,P3-triphosphate hydrolase activity generating dinucleoside monophosphates from dinucleoside polyphosphates (30). However, this enzymatic activity is not required for its tumor suppressing function (26). Ectopic expression of *FHIT* in tumor cells lacking this gene leads to inhibition of the cell cycle and apoptosis but no further insights have been garnered so far regarding the exact signaling mechanisms involved in this action of *FHIT*. In conclusion, there is overwhelming evidence for a tumor suppressor activity of *FHIT*. Moreover, since *FHIT* is inactivated in 60% of the tumors it is the most commonly altered gene in human cancer.

4.2. Deletions on chromosome 8

The focus of research on chromosome 8 in bladder cancer has been deletions of the short arm of this chromosome. Deletions on chromosome 8p occur at a frequency of 25-50% in bladder cancer (31-34). In contrast to chromosome 9 deletions, these aberrations are also significantly correlated with cancer progression, namely tumor grade, stage and invasiveness (34, 35). In an effort to narrow down the common region of deletion on chromosome 8p, deletion mapping using PCR based techniques was undertaken which led to the assignment of the locus at 8p21-22 as the most frequently deleted (31, 35). There are four known putative tumor suppressor genes in this region.

4.2.1. 8p22

The best characterized of these tumor suppressors is *FEZ1/LZTS1* (leucine zipper putative tumor suppressor 1) which has been shown to be inactivated by LOH plus mutation or transcriptional silencing in several cancers including esophageal, breast, prostate, gastric, oral and lung cancer (36-39). In bladder cancer, mutation seems not to be the primary means by which the remaining *LZTS1* allele is deactivated after LOH because only one non-conserved mutation was found among 54 primary bladder tumors and 34 bladder cancer derived cell lines. (40). Rather, it appears that transcriptional silencing plays the main role in switching off *LZTS1*. *LZTS1* expression has been shown to be reduced by immunohistochemistry and at the mRNA level in around 25% of cases, a frequency that is compatible with the frequency of LOH of 8p22 (23, 40). In gastric cancer cell lines, hypermethylation of the *LZTS1* promoter could explain the reduced Lzts1 expression (37), but in bladder cancer, the methylation status of the *LZTS1* promoter has not been examined yet. Re-expression of *LZTS1* in breast and bladder cancer cells that do not express this gene, results in growth inhibition and reduction of tumorigenicity through cell cycle inhibition at the G2/M phase (41, 42). These data therefore provide compelling evidence for a tumor suppressor function of *LZTS1* in bladder cancer as well as in other cancer types.

Another tumor suppressor in this chromosomal region, *DBC2* (deleted in breast cancer 2), was identified as a gene that is homozygously deleted in breast cancer and whose re-expression in *DBC2*-defective breast cancer cells resulted in growth inhibition (43). *DBC2* was suggested to influence multiple cellular functions, including cell cycle and apoptosis. However, data supporting this suggestion comes only from the observation of transcriptional changes when overexpressing or knocking down the gene in one cell line (Hela) (44). No functional assays were undertaken in order to underscore the conclusions and therefore these results await further experimental proof. In bladder cancer,

similar to *LZTS1*, *DBC2* has a very low mutation rate. At least in bladder cancer cell lines, *DBC2* shows also a reduced expression compared to normal urothelial cells (40). Thus, no conclusions can be drawn yet with respect to a possible tumor suppressor function of *DBC2* in bladder cancer.

Two other putative tumor suppressors, deleted in liver cancer 1 (*DLC1*) and fibrinogen-like 1 (*FGL1*) have been first found to be deleted in liver cancer (45, 46). Solid evidence has been accumulated regarding the tumor suppressor activity of DLC1 in liver, lung and breast cancer (47-51). *FGL1* has only recently been described for the first time as a tumor suppressor and this currently remains the sole publication regarding the tumor suppressor status of this gene (46). However, none of these two genes has been further investigated in bladder cancer.

4.3. Deletions on chromosome 13 – Rb

Chromosome 13 harbors the Retinoblastoma tumor suppressor at 13q14. Frequent LOH in around 30% of the cases at this locus was described by several groups and correlated to tumor progression (52-55). Since the tumor suppressor activity of Rb has been known before, many studies investigated the expression of Rb directly. Loss of Rb expression but also elevated Rb expression was shown to correlate with higher rates of recurrence and shorter survival time. Importantly, alteration of p53 and Rb is often found in the same tumors and leads, in a cooperative way, to enhanced tumor recurrence and reduced survival (56). Reduced as well as elevated Rb expression leads probably to Rb inactivation since elevated expression of Rb leads to its hyperphosphorylation retaining Rb in the cytoplasm. This prevents the inhibition of the E2F family of transcription factors by Rb in the nucleus, an event that normally culminates in stop of the cell cycle (57).

4.4. Deletions on chromosome 17 – p53

Deletions in chromosome 17 occur in approximately 60% of the cases and deletions of the 17p arm are strongly correlated with tumor progression (18, 58-61). Moreover, deletions of 17p have been identified in almost every cancer studied. Several putative tumor suppressors are known to reside in this chromosomal region, the most prominent one being *p53*. Together with Rb, p53 is arguably the best characterized tumor suppressor and plays a role in a host of cellular processes ranging from DNA damage, apoptosis and cell cycle to redox regulation and ageing.

Numerous studies have shown that mutations in *p53* are common in bladder cancer (60% of the cases) and are accompanied by LOH at the 17p13.1 locus, the site harboring the p53 gene. Alterations in *p53* are more prevalent in advanced cancer than in low grade superficial bladder cancer and these alterations have been correlated with a worse clinical prognosis for the affected patients (59, 60, 62). Despite the strong association with tumor progression and prognosis, the influence of *p53* mutations on chemotherapy is still controversial. While some studies have found a worse response of tumors with altered *p53*, other studies have found the opposite. Currently, clinical trials are ongoing that assess the influence of *p53* mutation on treatment outcome.

There are several other putative tumor suppressors on 17p but none of these has been investigated in bladder cancer.

4.5. Deletions on chromosome 9

Deletions in chromosome 9 have long been considered the most frequent chromosomal aberration in bladder cancer. Various laboratories have reported loss of microsatellite markers across the entire length of chromosome 9 in 30 to 70% of the studied cases [63-68]. Loss of chromosome 9 has been the only chromosome loss at the early tumor stages T0 and T1 while at later stages loss of other chromosomes was detected concomitantly with loss of chromosome 9. Thus, it was concluded that loss of the entire chromosome 9 represents an initial event in bladder tumor formation (63-66). Only 10 to 12% of deletions involving chromosome 9 are partial deletions that allow finer mapping of sites with putative tumor suppressor genes. Using those cases, as many as 5 sites for putative tumor suppressor genes have been mapped. These sites include 9p21, 9q22.3, 9q31, 9q33 and 9q34 (67-71).

4.5.1. Chromosome 9p

The best documented subchromosomal deletions are the ones covering 9p21 which harbors three tandem loci that code for 4 assumed tumor suppressors. The *INK4A* locus codes for two proteins, p16 and *alternate reading frame* (ARF), which are generated from this same locus through alternative promoters and using different exons. However, no amino acid sequence homology exists between p16 and ARF. Intriguingly, both proteins act by inhibiting the cell cycle through either Rb or p53, two of the most important tumor suppressors (Figure 1). p16 inhibits the cell cycle by binding to and inhibiting the cyclin dependent kinases CDK4 and CDK6, which during cell cycle progression phosphorylate Rb, thus inactivating its cell cycle inhibiting function. ARF, on the other hand, stabilizes p53 by binding and inhibiting Mdm2, a protein that mediates the degradation of p53. In bladder cancer, mutations of the remaining *INK4A* or *ARF* allele are very rare (around 2% among the investigated cases) (72). Instead, the remaining second allele is inactivated by subchromosomal deletions leading to homozygous loss of the locus or by methylation (72-76). In line with these observations, ectopic expression of *INK4A* in *INK4A* negative bladder cancer cell lines induces growth arrest and there is a correlation between tumor recurrence as well as cancer progression and decreased expression of *INK4A* (77-80).

The *INK4B* locus adjacent to the *INK4A* locus encodes for the cell cycle inhibitor p15 that inhibits CDK4 and 6 in a similar manner as p16 (Figure 1). An investigation of *p15* and p16 expression in superficial and invasive bladder cancer revealed a significantly lower expression of *p15* in superficial but not in muscle invasive bladder cancer suggesting that loss of *p15* contributes to establishment of the tumor but not to progression (81). A

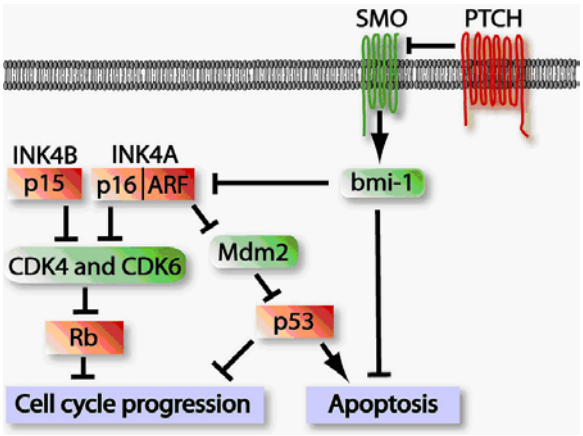

Figure 1. Rb and p53 pathways. *Red boxes*- tumor suppressors that are deleted in bladder cancer; *Arrows* depict activation, *blunt-ended lines* depict inhibition. See the text for details.

fourth gene, adjacent to the two before described loci codes for the methylthioadenosine phosphorylase (MTAP) and is frequently homozygously co-deleted with the *INK4A* and *INK4B* loci in bladder cancer as well as in various other cancer types (82-88). MTAP is a regulator of polyamine metabolism and S-adenosylmethionine dependent methylation reactions. It has been shown to suppress tumorigenicity of the MCF-7 cancer cell line (89).

All together, these findings show the unique contribution that the 9p21 locus has on tumor formation and explain the high frequency of deletion of this locus in bladder cancer and many other tumor types.

4.5.2. Chromosome 9q

The four discovered deleted loci on the long arm of chromosome 9 harbor 3 putative tumor suppressor genes that will be described in detail below.

4.5.2.1. 9q22.3

This region has been described to be deleted by two groups [22, 41] and it harbors the homolog of Drosophila Patched (*PTCH*). *PTCH* is the gene that is mutated in the Gorlin syndrome which is characterized by multiple basal cell carcinomas at an early age (90-92). The protein product of *PTCH* functions as a plasma membrane receptor for the soluble sonic hedgehog (SHH). Binding of SHH to PTCH prevents normal inhibition of Smoothened (SMO) by PTCH (93). SMO is a seven transmembrane G-protein coupled receptor that activates the transcription factor Bim1. Bim1 activates proliferation and inhibits differentiation by repressing *INK4A/ARF* and is an oncogene that inhibits apoptosis (94-96). Hence, deactivation of PTCH does not only lead to repression of another important tumor suppressor but also leads to activation of an oncogene (Figure 1). It seems therefore feasible that deactivation of PTCH can substitute for deletions at the *INK4/ARF* locus. One study so far investigated mutation of *PTCH* in bladder cancer. McGarvey et al. found a low number of mutations (3.8%), all of which were accompanied by LOH of the *PTCH* region (97).

4.5.2.2. 9q31

While deletions encompassing this locus have been described in two independent studies, (71, 98) no putative tumor suppressor has been described that resides in this region.

4.5.2.3. 9q33

The putative tumor suppressor in this region was localized to an 840 kb stretch of DNA and this region was named deleted in bladder cancer 1 (*DBC1*) (70). Subsequently, a gene was cloned that maps to this region: deleted in bladder cancer chromosome region candidate 1 (*DBCCR1*). While no mutations were found in the remaining allele after LOH at the 9q33 locus, the *DBCCR1* gene was not expressed in 50% of the investigated bladder cancer samples. This repression in the expression of *DBCCR1* was due to hypermethylation of the promoter (99). Additionally, DBCCR1 was shown to inhibit cell proliferation when re-introduced in mouse fibroblasts (NIH3T3) and to inhibit the formation of stable cell lines when re-introduced in DBCCR1 negative bladder cancer cell lines (100). However, further studies with the stable bladder cancer cell transfectants could not reproduce the results obtained with the NIH3T3 cells. Another group reported that DBCCR1 expression in DBCCR1 negative bladder cancer cell lines results in non-apoptotic cell death in these cells (101). Thus, while DBCCR1 is a good candidate tumor suppressor, further studies are warranted to verify the influence of DBCCR1 on tumor formation.

4.5.2.4. 9q34

Deletions in this region have been firmly established by several laboratories (69, 71). This region harbors the prominent tumor suppressor Tuberous Sclerosis Complex 1 (*TSC1*) whose germline deletion leads to tuberous sclerosis complex, a condition that is characterized by the development of hamartomas in multiple organs. *TSC1* acts in concert with *TSC2* on chromosome 16p13. Lately, a great body of evidence has been accumulated showing that dimerized TSC1 and TSC2 act as negative regulators in the signaling cascade leading to activation of the serine/threonine kinase mTor (Figure 2). The dimer composed of TSC1 and TSC2 acts as a GTPase activating protein for RHEB, a small GTPase that activates mTor in its GTP bound state. Activated mTor stimulates protein synthesis by enhancing the translation rate via several effectors of the translational machinery. mTor itself is activated by nutrients and by the well known anti-apoptotic protein kinase Akt/PKB (102). Recently, it has been shown that Akt inhibits apoptosis and contributes to tumorigenesis through its activation of mTor *in vitro* and in mouse models of tumorigenesis *in vivo (103-106)*. Inactivation of TSC therefore leads to increased signaling through mTor and thus increased resistance to apoptosis.

A search for mutations in the remaining *TSC1* allele after LOH by deletion of 9q34 yielded a low percentage of mutations of 5 to 12% (107-109). Therefore, *TSC1* in bladder cancer cannot behave like a classical

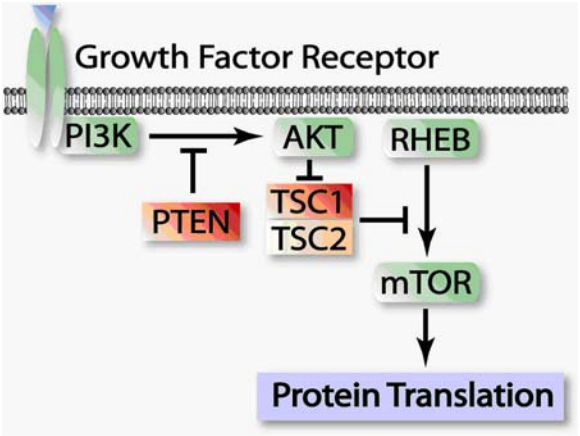

Figure 2. The AKT/mTOR signaling pathway. *Red boxes*-tumor suppressors that are deleted in bladder cancer. *Arrows* depict activation, *blunt-ended lines* depict inhibition. Increased protein translation leads to inhibition of apoptosis but which proteins are translated to achieve this action is currently unknown. While the chromosomal region where TSC2 resides (16p13.3.) has been found to be deleted in bladder cancer (13, 120) the TSC2 mutation spectrum has not been examined in this cancer. See the text for further details.

tumor suppressor that requires two inactivating mutations/deletions to shut off its tumor suppressing function. An expression analysis on *TSC1* in bladder cancer which could indicate a downregulation by transcriptional repression like methylation has not been done. However, the recent advent of DNA microarray technology makes it possible to search databases containing expression data of thousands of genes. We searched the Oncomine DNA microarray gene expression database (110) for genes that are statistically significantly downregulated in bladder cancer compared to normal bladder. Remarkably, using the data generated by Dyrskjot et al. (111), we find that *TSC1* is one of only 88 genes downregulated in bladder cancer (unpublished data). In light of the significant functional evidence for TSC1 as a negative regulator of the Akt-mTor-pathway, it appears also very likely that a lower dosage of TSC1 may lead to fewer complexes with TSC2 and consequently to increased signaling through mTor; i.e. *TSC1* might be haploinsufficient. Haploinsufficiency was also suggested by Knowles et al. based on observations that, in some bladder tumors, *TSC1* was mutated but retained heterozygosity (109).

4.6. Other chromosomal deletions

With the advent of high throughput methodologies such as SNP arrays that allow the mapping of chromosomal alterations at an ever increasing speed, chromosomal lesions in bladder cancer have been discovered that comprise every chromosome. Importantly, in these studies, the frequency of known chromosomal lesions was verified by the SNP arrays and extended to more chromosomal sites (13, 112). Hence, many more chromosomal lesions than previously thought occur in bladder cancer and the putative tumor suppressors inactivated in bladder cancer very likely abound. For this reason, delineation which tumor suppressors are targets of the newly discovered deleted regions in bladder cancer will be an important area of future investigations.

5. PATHWAY REGULATION IN BLADDER CANCER

In recent years, it has become clear that groups of genes mediating a cellular function are regulated rather than single genes belonging to the group. For instance, mutations of single genes coding for components of the oncogenic PI3K/Akt signaling in colon cancer occur at a frequency of not more than 4%. However, the observed mutations in different pathway members are found mutually exclusively, i.e. in any given cancer sample only one pathway member is mutated. Therefore, in contrast to the single gene level, mutations in any one of the pathway members are found in 40% of the patients (113). Likewise, in DNA microarray expression experiments investigating tumor and normal tissue, there is little overlap in the differentially expressed genes found in different patient cohorts. Rather, the differentially expressed genes belong to the same pathways that are similarly altered in the different groups of patients (114).

Also in bladder cancer, multiple inactivating deletions and mutations can be attributed to common pathways. Pathway deregulation in bladder cancer is particularly prominent in case of the Rb and p53 pathways. As explained above (see also Fig.1), the Rb pathway is either deactivated indirectly by deletions on 9p and 9q22.3 or directly by deletion of Rb on chromosome 13. Likewise, deletion of 9p leads to inactivation of the p53 pathway indirectly while deletion of p53 is achieved through chromosome 17 deletions. It would be interesting to know whether different lesions targeting components of the Rb or p53 pathways occur together in the same bladder tumor sample or not. Again, if they occurred in different samples, it would parallel the mutually exclusive mutations in Akt pathway members in colon cancer.

Also in bladder cancer, components of the Akt pathway are deregulated. As mentioned before, the tumor suppressor *TSC1* is often inactivated in bladder cancer. The same observation has been made about the other prominent tumor suppressor in the Akt pathway, phosphatase and tensin homolog (*PTEN*). PTEN is a lipid phosphatase that dephosphorylates phosphatidylinositol-3,4,5-trisphosphate, a lipid that is generated by the action of phosphatidylinositol-3-kinase (PI3K) and triggers the activation of Akt. Like for *TSC1*, a rather low mutation rate between 6 and 25% has been described for *PTEN* (115-117). In analogy to the above described pathway regulation, this low mutation rate may well have a higher significance when the mutations in *PTEN* and *TSC1* are mutually exclusive in the tumor, raising the incidence of pathway inactivation considerably.

So far, no study has stressed the question of pathway inactivation in bladder cancer further, leaving promising avenues for future research.

6. PERSPECTIVE

The increasing penetrance of high content methodologies like SNP-arrays, array-CGH and DNA microarrays in modern biology are transforming the way bladder cancer research is conducted. Only recently has it been feasible to simultaneously investigate a great number of genetic changes in bladder cancer tissue. We foresee an ever increasing application of these new methodologies in bladder cancer research allowing the identification of new genetic lesions but also combinations of these lesions that associate with tumor development. We believe that combinations of chromosomal lesions and not single chromosomal lesions will be most informative regarding the behavior of any given tumor much in the same way combinations of expression tumor markers can classify tumor sub-groups where expression profiles of single genes cannot.

If we consider pathways as the basic regulatory and regulated unit in cancer, it has important implications for the field of tumor suppressor research. Traditionally, when LOH for a putative tumor suppressor gene is found and the remaining allele is mutated only at low frequency, it is assumed that the putative tumor suppressor either acts in a haploinsufficient way or that it plays only a minor role in bladder cancer. Generally this gene is then viewed as scientifically less interesting. However, it may simply be that the pathway, to which the tumor suppressor belongs, is deactivated by LOH and mutation in this tumor suppressor in a certain subset of patients. In the remaining patients, the pathway may be deactivated in other pathway members. As a consequence, in deciding whether a certain gene acts as a tumor suppressor, more emphasis must be based on multiple evidences coming from LOH studies as well as from other experiments investigating the contribution of the gene to *in vitro* and *in vivo* tumor models. If evidence exists that a gene product can act as a tumor suppressor (for instance in the case of PTEN), even a low rate of alteration in that tumor suppressor strongly suggests that the respective pathway (here, the Akt/PI3K pathway) plays an important role in cancer. Investigation of the other pathway members (if known) therefore would be valuable to pursue.

Recently, several cancer therapeutics like Herceptin, Glivec and Avastin have proven that a better understanding of cancer biology can lead to improved disease management (118, 119). Understanding the consequences of chromosomal deletions will undoubtedly continue to reveal the fundamental mechanisms behind cancer development, ultimately leading to alleviation of the suffering for cancer patients.

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Send correspondence to: Reimar Abraham, Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padova, Italy, Tel.: 39-049-7923-234, Fax: 39-049-7923-250, E-mail: reimar.abraham@unipd.it

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