Molecular genetics of urothelial cancer of the bladder

Ph.D. thesis

by

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INTRODUCTION

Epidemiology of bladder cancer

Tumours of the urinary bladder account approximately 3% of all types of cancers worldwide, being the 7th most common cancer. Bladder cancer is any of several types of malignancies arising from the epithelial lining of the urinary bladder. Transitional or urothelial cell cancer is the most common type of bladder cancer. Urothelial carcinoma (UC) makes up around 90% of all bladder cancer cases.

In 1973 the World Health Organization (WHO) classified urothelial papilloma and 3 grading levels such as well/moderately/poorly differentiated urothelial carcinomas. In 1998, the International Society of Urological Pathology (ISUP) proposed a new classification of non-invasive urothelial tumours. The majority of clinical trials published to date on bladder tumours have been performed using the 1973 WHO classification. Both classifications can be used according to different urological guidelines. The samples analysed in this thesis were diagnosed based on the 1973 WHO classification.

The Tumour, Node, Metastasis (TNM) classification is most widely used to classify the extent of cancer spread. In 2010, a seventh edition was published, but there are no significant modifications to this for bladder cancer compared with the previous (2002) edition.

Controversies of TNM and "clinical" classification

From a clinical point of view UCs are divided into non-muscle invasive (pTa and pT1) and muscle invasive (pT2-pT4) tumours. However, from the biological point of view the vast majority of pTa urothelial carcinomas may be considered as "benign" tumours, which rarely progress towards invasive diseases, whereas pT1 and pT2-4 urothelial carcinomas are simply correspond to different stages of tumour progression. Therefore, the difference between pT1 and pT2-4 urothelial carcinomas is only the difference in the time window in the clinical detection. The terms non-muscle-invasive and muscle-invasive cancer are clinical but not biological definitions.

Urologists classify bladder tumours into subgroups of non-muscle invasive and muscle invasive tumours regarding different treatment options. Because non-muscle invasive tumours (pTa and pT1) are restricted to the surface of urinary bladder, they can easily be removed endoscopically. However, these tumours tend to recur frequently and need a strict follow-up care. After several recurrencies pTa UCs may progress into invasive pT1,G3 tumours, whereas recurrent pT1,G3 UCs are almost all invasive tumours. Muscle-invasive tumours cannot be cured by endoscopical methods. These cancers – even in organ-confined cases – need radical surgery, radiation therapy or chemotherapy.

UC may be presented as solitary or multifocal tumour at the first observation. Asynchronous multiplex UCs, e.g. frequent recurrence are characteristic biological behaviour of papillary UCs and in the case of multiplicity or recurrence, the probability of future recurrences even increase up to 78%. Genetic studies showed that the majority of synchronous or asynchronous multicentric UCs are of monoclonal origin corresponding to intravesical homing of desquamated tumour cells. The rare occurrences of polyclonal UCs have been explained by the so-called "field effect" of environmental mutagenesis.

Genetics of UCs

In the past several studies have been carried out on UCs to find genetic changes of biological importance including tumour type specific alterations, prognostic markers or therapeutic targets. Loss of chromosome 9 is the most frequent genetic change in UCs. Monosomy or loss of heterozygosity (LOH) at all analyzed loci occurs in approximately 40% of the UCs removing one allele of all genes along the entire chromosome 9. The rest of genetic changes include partial deletions at distinct genomic sites of chromosome 9. Recurrent DNA alterations at small genomic

regions have already helped to identify the CDKN2A/B, PTCH, DBC1 and TSC1 genes. However, it is not yet known, whether losses at these genes at chromosome 9 occur at the same frequency in papillary pTa and solid growing invasive pT1-4 UCs or they mark subgroups of tumours of biological importance.

Previous cytogenetic, CGH and DNA studies revealed recurrent alterations of chromosome 8p at variable frequency in urothelial carcinomas. Nearly all studies suggested an association between alterations of chromosome 8p and tumour progression. Other studies found a strong association between LOH at chromosome 8p22, tumour grade and metastatic tumour growth. Putative suppressor gene loci were mapped to chromosome 8p21.1-pter and 8p21-q11.2. Muschek et al. have delineated a small region of common deletion at chromosome 8p23.3 region including the CSMD1 gene. Fluorescence in situ hybridization (FISH) and matrix CGH analysis indicated a high level amplification of DNA sequences including the FGFR1 locus at chromosome 8p12 in some of the UCs. Thus, multiple regions at chromosome 8p have been suggested to harbour genes involved in the development, maintenance and progression of UCs.

Deletion mapping and CGH analysis of UCs revealed loss of different chromosome 11p regions and loss or amplification of the 11q13 region. The region of interest was determined by allelotyping between loci D11S902 and D11S569 on the short arm and between the FGF3 gene and locus D11S490 on the long arm. A correlation between LOH at loci D11S490 and D11S928 and frequent recurrences of the UCs has been suggested. Although gain or amplification of DNA sequences has been shown at the chromosome 11q12-13 region in some UCs, no any correlation between amplification/expression of CCND1, FGF3, FGF4 and EMS1 and staging or grading of UC has been detected.

Alterations at several other chromosomal regions have been implicated in the genetics of UCs, most of them were correlated with tumour progression. Whether the secondary DNA alterations are associated with gene alterations, e.g. mutation, methylation or haploinsufficiency of the gene(s) at these regions or they simply reflect the genetic instability of the tumour genome, is not yet known.

Proposed molecular pathways of UC progression

Based on the histological pattern and genetic data several pathways from dysplastic urothel towards frankly malignant tumours involving distinct chromosomal regions/genes have been proposed. One of the simplest models suggested that the development of papillary UCs is associated with LOH at chromosome 9, followed by mutation of the p53 gene in invasive tumours, whereas flat tumours, e.g. carcinoma *in situ* (CIS) are initiated by mutation of the p53 gene. Recently, similar pathways have been proposed, one from hyperplasia towards papillary UC showing FGFR3 mutation in over 60% of the cases and another pathway involving dysplasia, CIS, pT1+CIS, pT2 muscle invasive carcinoma with deletion and/or mutation of the TP53 gene in 70% of the tumours. Although several studies have reported a LOH at chromosome 9 not only in papillary non-invasive but also in solid invasive UCs, it remained unclear whether alteration of chromosome 9 including the CDKN2A/B, PTCH, DBC1 and TSC1 genes is "the primary" genetic change in the development of all types of UCs or characteristic only for papillary UCs.

The origin of multiplex UCs: monoclonal vs. field effect theory

Approximately 30% of non-muscle invasive (pTa, pT1 and carcinoma *in situ*) UCs appear as multifocal tumour at the time of first detection (synchronous multiplex UCs). More than 70% of pTa or pT1 solitary UCs will recur after the first treatment (metachronous multiplex UCs) and several of them will progress to muscle invasive disease within 5 years of follow up. These cases pose a serious oncological problem of regular control and treatment. Therefore, "superficial" or non-muscle invasive UCs represent a chronic cancerous disease with uncertain outcome.

The origin of multiplex synchronous or asynchronous UCs was controversial for long time. The "field effect" theory has postulated, that diffuse carcinogen effect lead to the development

of several genetically different tumours in the bladder, others proposed a clonal origin of multiplex tumours. Genetic analysis of multiplex UCs by applying microsatellites, which clearly differentiate between parental alleles, revealed the loss of the same parental alleles at distinct chromosomes in multiplex tumours. These studies confirmed the monoclonal origin of the vast majority of synchronous or asynchronous multiplex tumours of the bladder. Thus, in these cases a local intravesical metastasis led to multiplex tumour nodules on the surface of the urothelium. However, in few cases development of genetically distinct UCs, e.g. polyclonal origin has been demonstrated confirming the "field-effect" theory, at least in these exceptional cases.

It is suggested that synchronous or asynchronous appearance, especially the latter is a characteristic biological behaviour of non-invasive papillary UCs. The recurrent growth of tumours during the years of observation in around 70% of the cases supports this hypothesis. We cannot exclude a similar biological behaviour of invasive, high-grade tumours (pT2-4). It is difficult to answer this question, because these tumours are removed by radical cystectomy. However, it would be important to differentiate between tumours having the capacity of intravesical spreading and homing and those, which remain solitary tumours without local metastasis. Why multiplex tumours arising synchronously or asynchronously in several cases and why not in others is not yet known.

Aims of the study

This study was focused on the molecular characterisation of urothelial carcinomas with the aim:

➢ to identify small DNA alterations at chromosomes 8p, 9, 11p and 17p (including mutation of the p53 gene), which are associated with the development or progression of urothelial carcinomas,

➢ to identify specific molecular alterations in solitary versus synchronous and metachronous multiplex urothelial carcinomas and

➢ to establish the role of genetic changes in the development and progression of UCs.

MATERIALS AND METHODS

Tissue samples and clinical data

Fresh tumour tissues were obtained by transurethral resection or radical cystectomy of consecutively operated urothelial carcinomas at the Departments of Urology, Philipps-University of Marburg and University Medical Center Mannheim, Ruprecht-Karls-University of Heidelberg and Department of Urology, Medical School, University of Pécs, Hungary.

A small part of the tumour tissue was immediately snap-frozen in liquid nitrogen and stored at –80°C, the larger part was processed for routine histological diagnosis at the corresponding pathological departments. Haematoxylin and eosin stained histological slides were re-evaluated by two pathologists (Professors Gyula Kovacs and Antonio Lopez-Beltran). Solitary tumour was diagnosed in cases without preoperative clinical history and at least three years of tumour free postoperative course. All tumours having a histological report on UC of the bladder during the pre- or postoperative course in the clinical report were designed as asynchronous multiplex tumours. Synchronous multiplex tumours were diagnosed in the cases having at least two UCs of the bladder at the time of first observation. Both solid and papillary growing UCs representing all histological grades and histological-clinical stages were included in this study.

Methods

The isolation of nearly pure tumour cell suspension was carried out by a pathologists experienced in this technique (Professor Gyula Kovács). Extraction of DNA and RNA was carried our by standard methods used in the laboratory. The tumor DNA was characterized by microsatellite allelotyping, high resolution oligo array-CGH, sequencing and methylation specific PCR. The gene

expression was estimated by RT-PCR. The technical details are beyond the scope of this summary. A detailed description of methods is available in the Thesis.

RESULTS

Genetic analysis of chromosome 8p in UCs

The frequency of allelic changes for 34 microsatellite loci at chromosome 8p and for 3 loci at the long arm of chromosome 8 were determined by analyzing paired normal and tumour DNA from 122 UCs of the bladder. Allelic changes, i.e. decrease or loss of signal intensity of one allele at one or more loci were seen in 58 (48%) of the 122 tumours. There was a breakpoint cluster at the centromere of chromosome 8 leading to the loss of the entire chromosome 8p in 23 (19%) cases. Partial deletions involving different parts of the chromosome 8p were detected in 35 (29%) tumours. Although LOH at chromosome 8q was seen in some cases, most alteration at chromosome 8q were designed as allelic imbalance suggesting a loss of the chromosome 8p and duplications of chromosome 8q sequences.

Identifying small chromosomal regions of allelic changes

The saturated microsatellite allelotyping detected five small non-syntenic regions of allelic changes indicating putative tumour genes at chromosome 8p. One of them was localised to the locus D8S504 at chromosome 8p23 harbouring the ARHGEF10 gene. A small region of allelic changes exclusively at the ARHGEF10 gene was seen in three cases but taking into account the large deletions involving this locus as well, one allele of the ARHGEF10 gene was deleted in 48 (39%) of the 122 UCs. The genomic sequences of 2 Mb at the CSMD1 gene were covered with 8 microsatellites. Allelic changes at the entire genomic sequences of the CSMD1 gene or intragenic deletion affecting exclusively the CSMD1 gene occurred in 6 (5%) UCs. Altogether, one allele of the CSMD1 was deleted in 57 (47%) of the 122 UCs. Another putative tumour gene locus was located to a 140 kb genomic region at chromosome 8p12 between D8S1477 and D8S1758 including D8S375 and D8S278, both within introns of the NRG1 gene. One of the UCs showed LOH exclusively at loci D8S375 and D8S278 disrupting the integrity of one allele of NRG1. Another region of approx. 2.3 Mb was mapped between loci D8S1821 and D8S1104. A candidate gene for Wolf-Hirschhorn syndrome, a putative protein LOC441345, the leucine-zipper-EF-hand containing transmembrane protein 2 (LETM2) and FGFR1 genes are mapped to this region. The last region of approx. 500 kb between loci D8S1023 and D8S268 harbours the SFRP1 and a hypothetical protein FLJ13842.

Allelic changes at chromosome 8p is associated with tumour grading

A strong correlation was observed between loss of heterozygosity at the chromosome 8p regions and grading of UCs irrespectively of tumour staging. Two of the G1 tumours revealed allelic imbalance along the chromosome 8p, one another displayed an allelic imbalance at the CSMD1 gene and one UC showed an LOH at the ARHGEF10 gene . Thus, only 4 (12%) out of the 34 UCs of G1 (all belong to histological stage pTa) displayed allelic changes at chromosome 8p whereas 41% and 71% of the G2 and G3 tumours showed LOH-allelic imbalance at chromosome 8p, respectively .

Allelic changes at chromosome 8p is associated with tumour staging

Increased frequencies of allelic changes have been found along with the stage of UCs. Of interest, a clear cut difference was detected between non-invasive and invasive tumours irrespectively of the deepness of the invasion. Only 21% of the non-invasive papillary UCs showed allelic changes at chromosome 8p, whereas non-muscle invasive pT1 as well as muscle-invasive pT2-4 tumours displayed an allelic alteration at chromosome 8p in 71% and 73% of the cases, respectively. These data corresponds to the high frequency of G2 and G3 cases among invasive UCs.

NRG1 and SFRP1 are down-regulated in UCs

Because NRG1, FGFR1 and SFRP1 are the target of small interstitial deletions, their expression was analysed in a panel of 8 normal urothel samples and 28 UCs including tumours of stages Ta, T1 and T2-4 and one dysplasia by quantitative RT-PCR technique. A variable expression of FGFR1 was detected in normal urothelial cells and also in tumour cells. There was no correlation between expression profile of FGFR1 and staging/grading of UCs. Therefore, the role of FGFR1 in the progression of UCs, at least by the mechanisms of LOH and haploinsufficiency can be excluded. High expression of NRG1 was seen in normal urothel samples but the gene expression was significantly reduced or absent in all UCs and in the dysplastic lesion. Similarly, the SFRP1 gene was also expressed in all normal urothelial samples and was down-regulated in all but one UCs.

SFRP1 is frequently methylated in UCs with or without LOH

The methylation status of CpG islands in the 5' UTR region and exon 1 of the SFRP1 was analysed in a set of UCs. At least a weak methylation of this region was detected in all UCs and a higher level of methylation in 11 of 19 UCs. No unmethylated DNA sequences were detected in 5 UCs. Unmethylated sequences were detected in all DNA samples obtained from normal cells and also in nearly all DNAs isolated from UCs. LOH data were available for 12 tumours subjected to analysis of methylation. LOH or AI was detected in 4 of the 7 cases showing methylation of the promoter region whereas LOH occurred in 3 of 5 cases without methylation of this region. Methylation occurred in Ta, G1 UCs as well as in T4, G3 tumours. The case with severe dysplasia without LOH at the SFRP1 locus also showed a strong methylation of the CpG island of the gene.

Genetic analysis of chromosome 9

Allelic changes at chromosome 9

A high saturation microsatellite analysis at 39 loci with an average distance of 3.6 Mb was carried out on a panel of 129 UCs of all stages and grades. LOH at all informative loci along the entire chromosome 9, indicating a monosomy 9 occurred in 49 (38%) tumours. In addition to LOH and allelic imbalance a homozygous loss of DNA sequences was seen at loci of four genes, which are suggested to be involved in the genetics of UCs. The most frequent homozygous loss occurred, as expected, at the CDKN2A/B region on the short arm of chromosome 9. Further homozygous losses were detected at the DAPK1, PTCH and DBC1 loci on the long arm of chromosome 9.

Taking into account all alterations, allelic changes at one or more microsatellite loci was found in 114 (88%) of the 129 UCs. Allelic changes occurred at nearly the same frequency at both chromosomal arms. Hetero- or homozygous losses at 9p has been found in 93 (72%) and at chromosome 9q in 98 (76%) of the 129 tumours. However, some tumours displayed allelic changes at either the long arm or the short arm of chromosome 9. Allelic changes at chromosome 9p but not at 9q have been detected in 11 (9%) UCs, whereas 17 (13%) UCs showed LOH exclusively at the 9q region.

The evaluation of all type of allelic changes, e.g. hemizygous or homozygous losses and allelic imbalances in general at the chromosome 9 in UCs of distinct grades and stages did not reveal any association between genetic alterations and tumour stages and grades. Although the percentage of allelic imbalance was higher in G2 and G3 tumours, it can be explained by the observation that several of the G2 and G3 tumours were obtained from cystectomy specimens with T2-T4 UCs. Therefore, the tumour DNA was in some cases contaminated with infiltrating lymphocytes leading to "allelic imbalance" call.

Detection of two new regions of LOH at chromosome 9p

Because 9p21 region has earlier been showed to be the main target of LOH at chromosome 9p, 5 microsatellite markers (D9S1749 to D9S1870) covering the 300 kb genomic sequence harbouring the MTAP and CDKN2A/B genes were analysed in our series of UCs. LOH at

these genes occurred in 85 (66%) of the 129 UCs . Homozygous deletion involving either CDKN2A or CDKN2B or all the three genes was found in 40 (31%) UCs. In three tumours the only LOH at the short arm of chromosome 9 occurred at the D9S1749 locus, which is located within the genomic sequences of the MTAP gene.

The high resolution microsatellite study delineated two other small regions of allelic changes, which have not been described earlier in UCs. Overlapping allelic changes were seen at loci D9S286, D9S921 and D9S269, which are mapped to chromosome 9p24.1 region. The D9S921 is localized within an intron of the PTPRD gene, D9S286 is mapped 250 kb distal and D9S269 450 kb proximal to the PTPRD genomic sequences. Allelic changes exclusively at these loci occurred in 9 (7%) UCs. Together with larger deletions involving several other genes or the entire chromosome 9p or 9, 77 (60%) tumours showed LOH at the PTPRD gene. Another small region of allelic losses was seen at loci D9S156 and D9S157 at 9p22.3 bracketing the BNC2 gene. The D9S156 is located 150kb distal and the D9S157 700kb proximal to the BNC2 gene. Again, a loss exclusively at this region of less than 1 Mb was detected in 7 (5%) UCs. Together with large deletions, 78 (60%) tumours showed LOH at the BNC2 gene.

Detection of one new region of LOH at chromosome 9q

The long arm of chromosome 9 was saturated with 18 microsatellites including those mapped to or flanking the three known genes, the PTCH (9q22.32), DBC1 (9q33.1) and TSC1 (9q34.2) genes. An LOH affecting exclusively the PTCH gene (D9S1809) was found in 3 (2%) tumours, but the PTCH genomic region together with large chromosome 9q regions was deleted in 80 (62%) of 129 UCs. In 3 (2%) tumours a homozygous loss of genomic sequences the PTCH locus was detected. LOH at D9S1872 and D9S195 (DBC1) occurred in 86 (67%) of UCs including 18 (14%) tumours with LOH only at this region. In two cases a homozygous deletion at the DBC1 occurred. LOH involving only the TSC1 locus (D9S1830) was found in 12 (9%) UCs, but the TSC1 region was deleted together with large chromosome 9q deletions in 79 (61%) of the 129 UCs. No homozygous loss at this gene locus was seen.

The microsatellite alleotyping identified a small deletion at chromosome 9q21.33, which has not been described in UCs yet. LOH at the locus D9S257 with retention of heterozygosity of the flanking loci has been found in 12 (9%) UCs. The D9S257 is located within intronic sequences of the DAPK1 gene. In six UCs a homozygous deletion of the DAPK1 genomic sequences were detected. Taking into account large chromosome 9q deletions including the MAPK1 gene locus as well, 80 (62%) of the 129 UCs showed LOH at the DAPK1 gene.

LOH at the seven regions occurs at similar frequency in each stage and grade of UCs

The genes localized at the seven small non-syntenic chromosome 9 regions have different biological function and are involved in different pathways. Therefore, it was temptating to look for the possible role of these genes in the cell growth (nuclear grade) as well as in the cell motion-invasion (pathological stage). To evaluate the possible role of alterations at the already known and new tumour gene loci, the results of allelotyping for each region was analysed in relation to the grade and stage of UCs. LOH at the loci of the PTPRD, BNC2, CDKN2A/B, DAPK1, PTCH, DBC1 and TSC1 genes occurred at nearly the same frequency in different stages and grades of UCs

Evaluation of the LOH at the seven regions in solitary UCs and in synchronous or asynchronous multiplex tumours yielded the same results for all but one gene loci. Again, alterations at the PTPRD, BNC2, CDKN2A/B, PTCH, DBC1 and TSC1 regions occurred nearly at the same frequency in solitary vs. multiplex tumours. However, LOH at the DAPK1 region occurred in 59 (73%) of the 81 UCs with multifocal growth or recurrence but only in 12 (29%) of the 41 solitary UCs without recurrence. This suggests a role of DAPK1 in the multifocal and recurrent growth of UCs.

Allelic changes at chromosome 11

The entire chromosome 11 was first analysed for allelic changes at 18 microsatellite loci covering the 135 Mb genomic sequences at an average distance of 7.5 Mb. After detecting frequent alterations at 11p15.5, 11p11.2 and 11q12.1 chromosomal bands, these regions were saturated with additional microsatellites to reach an average distance of approximately 600 kb between the loci analysed. LOH at all informative loci corresponding to monosomy of chromosome 11 was detected in only 2 of the 121 UCs. Loss of the short or long arm of chromosome 11 or small non-synthenic deletions occurred in 85 (70%) cases including 5 (4%) LOH at a single marker.

Delineation of two regions of allelic changes at 11p

The most frequent interstitial deletions occurred at chromosome 11p15.5 region, which was saturated in the second round of analysis with microsatellites at an average distance of 600 kb. An overlapping LOH between D11S922 and D11S1318 harbours approximately 600 kb genomic sequences known as an imprinted region. This region contains the IGF2 (insulin-like growth factor 2) gene, which expresses only from the paternal allele and the H19, which expresses a non-coding RNA only from the maternally inherited chromosome 11. Allelic changes at these two genes were observed in 49 (40%) of the 121 UCs. The second overlapping LOH also occurred within the imprinted gene domain of the chromosome 11p15.5 region between loci D11S2345 and D11S988. This region of approximately 700 kb harbours the RHOG, STIM1, RRM1 and TRIM21 genes. Allelic alterations at this region were detected in 58 (48%) of the 121 tumours. The third region at chromosome 11p11.2, which was saturated with 10 microsatellites at an average distance of 600 kb displayed several non-syntenic losses, which probably reflect the instability of this region.

Frequent allelic changes at the 11q12.1 region

The region of approximately 4.8 Mb between loci D11S1920 and D11S4191 at chromosome 11q12.1 shows frequently losses of non-syntenic small region involving only one or two of the 5 microsatellite loci analysed. This region harbours the members of olfactory receptor family genes. The genes SSRP1, PRG3, FAM11A and CTNND1 are mapped to a small region between loci D11S1809 and D11S4191. The latter genomic segment was involved in the allelic alterations in 41 (34%) of the 121 UCs.

Correlation between pathological and genetic data

Allelic changes occurred in 18 (53%) of the 34 G1 tumours, 13 (65%) of the 20 G2 tumours and in 56 (84%) of the 67 G3 UCs. Although the frequency of allelic changes increased with the grading score, it was high even in G1 tumours. Similarly, 31 (58%) of the 53 pTa papillary UCs showed LOH, 17 (68%) of the 25 pT1 and 38 (86%) of the 44 pT2-4 tumours displayed LOH at chromosome 11. Taking into account only the allelic changes at the imprinted region of H19 and IGF2, 44%, 50% and 50% of the G1, G2 and G3 tumours showed allelic changes at this region, respectively. In pTa UCs, 28% of the cases displayed LOH at the imprinted region whereas pT1 and pT2-4 UCs showed LOH in 52% and 47% of the cases, respectively. Thus, no clear association between genetic alterations at chromosome 11 or at one of the specific region can be established.

Allelic changes at chromosome 17p and mutation of the p53 gene

Allelic changes at chromosome 17p13.1 and mutation of the p53 gene was analysed in 120 UC including all stages and grades . The vast majority of the 55 pTa papillary UCs displays G1 and only 3 of them was diagnosed as G3. None of the 65 pT1 and pT2-4 tumours showed G1 and only 4 of them were diagnosed as G2, whereas the vast majority displayed G3. Loss of heterozygosity at the p53 locus was detected in 44 (37%) of the 120 UCs analysed for allelic changes at chromosome 17p13.1 region. The frequency of LOH has also been increased along with the higher grade of UCs, with a clear-cut difference between grade 1-2 (16%, 17%) and grade 3 (55%) tumours . The frequency of allelic loss at 17p13.1 was similar in so-called non-muscle invasive

(superficial) pTa and pT1 tumours (20% and 18%, respectively) but increased in pT2 and pT3-4 tumours (55% and 69% respectively) .

Mutations in exons 5-8 of the p53 were found in 25 (21%) of 120 UCs. Sequence changes were detected 3 times in exons 5, 6 times in exon 6, 7 times in exon 7, and 9 times in exon 8. Most tumours revealed a missense point mutation. All but one (a pT1,G3) of the 25 UCs with p53 mutation revealed also an LOH at the chromosome 17p13.1 locus. None of the 55 pTa UCs showed p53 mutation, whereas mutation was seen in 23% of the minimal invasive pT1 UCs, and 36% and 37% of the muscle invasive pT2 and pT3-4 UCs, respectively. None of the G1 or G2 tumours showed alterations of exons 5-8 of the p53 gene. Summarising the results of LOH and p53 mutation analysis, LOH at chromosome 17p13.1 occurred in all stages and grades albeit at different frequencies, but mutation of the p53 gene was seen exclusively in high grade (G3) UCs including a CIS. The occurrence of p53 mutation in invasive UCs (pT1-pT4) reflects the observation that the overwhelming majority of G3 tumours were diagnosed in these stages.

Allelic changes at chromosome 9 and mutation of the p53 gene

Because it was suggested that deletional/mutational inactivation of the p53 gene and LOH at chromosome 9 marks distinct pathways of UC development (papillary vs. solid tumours), we have evaluated the LOH data obtained from 39 loci in 21 UCs showing p53 mutations. LOH at chromosome 9 occurred in 19 (90%) of the 21 UCs displaying p53 mutation. Most tumours showed LOH along the entire chromosome 9. Three UCs displayed LOH only at chromosome 9p, one of them showing a homozygous deletion of the CDKN2A/B region as the only change. Three tumours displayed LOH only at chromosome 9q including two cases with LOH only at 9q22 (PTCH) and 9q33 (DBC1) regions. A homozygous deletion of the CDKN2A/B region was seen in 9 cases whereas a homozygous deletion at the DAPK1 and PTCH genes each in one UC.

Allelic changes at the UC genome

Previously, an allelotyping of UCs at chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, 18 and 20 (3-5 loci on each chromosome) have been carried out in the Laboratory of Molecular Oncology by others. Chromosome 2q and 8p regions were also analysed for several additional loci. By exploiting the mechanism of probabilistic reasoning in Bayesian Networks and reconstructing the possible flow of progression of allelic changes, Bulashevska et al. suggested primary and secondary events in UC pathogenesis. LOH at chromosome 9 was found to be the primary event whereas LOH at chromosome 8, 11 and 17 as secondary genetic changes. The results of high resolution microsatellite analysis of chromosomes 8, 9 and 11 was evaluated together with the results of previous studies mentioned above. Summarising previous and recent results indicates that LOH (frequently a homozygous loss) at chromosome 9 occurs at similar frequency in G1, G2 and G3 UCs and in UCs of different stages . Alterations at chromosomes 2q, 5, 6, 8p 11p, 17p and 18q are seen more frequently in G2 and G3 tumours, e.g. associated with the grading of UCs .

Similarly, LOH at chromosomes 2q, 5, 6, 8p, 11p, 12q, 14q, 17p and 18q occurred more frequently in T1-T4 UCs. Most UCs showed LOH at several chromosomal regions. Only two cases (one Ta,G1 and one Ta,G2) were without any changes at the chromosomal loci analysed in this study.

Clinical application of microsatellite allelotyping: analysis of urine samples for detection of recurrent UCs of the bladder.

Several different chromosomal loci might be involved in development of an individual UCs. Therefore, taking into account, that 1, the DNA extracted from urine sediment is highly contaminated with normal DNA and 2, we do not know, which chromosomal regions are involved in the genetic changes of each UCs (if any), a screening with microsatellite allelotyping is not possible.

However, if the genetic changes in a primary tumour are known, it is possible to search for these specific alterations in the urine sediment by regular controls. It was established earlier and

in this study, that both synchronous and asynchronous multiple bladder cancer tumours develop by intravesical spreading (i.e. intravesical metastases). This means that that vast majority of multiplex and recurrent bladder cancers have a monoclonal origin . If one know the genetic alterations occurring in the primary UC, can search for the same alterations in urine sediments as well. Unfortunately, papillary pTa,G1 tumours display only few and sometimes uncommon alterations, therefore the detection of genetic changes in such tumours and detecting the alterations in the urine sediments is difficult. Altogether 36 cases with matched blood, UC and urine samples were subjected to microsatellite analysis. Four cases were excluded because the UCs did not showed allelic changes at any of the 13 loci. Finally, 32 urine samples were analysed for microsatellite loci, which showed LOH in the corresponding UC tissue. At least one of the microsatellite markers identified allelic changes in the urine specimens in 25 of the 32 cases, whereas in seven cases the allelotyping did not detected any changes at the loci analysed. Summarising the results, in a pilot experiment simulating a control urine analysis with microsatellite markers positive in the primary tumours, an UC was diagnosed in 78% of the cases.

Array-CGH analysis of multiplex versus solitary UCs

The high resolution array-CGH analysis has several advantages against microsatellite allelotyping. The array used in this study give an insight in the genetic alterations at 60,000 loci along the entire tumour genome. Moreover, array-CGH detects not only copy number loss of DNA sequences, but also duplications and amplifications. However, this technique does not differentiate between parental alleles and therefore, it does not give information on the clonal growth of multiplex tumours. The clonal development of multiplex UCs analysed in this study was confirmed by microsatellite allelotyping previously by Dr. Sigrun Langbein in the Laboratory of Molecular Oncology, University of Heidelberg.

Microsatellites differentiate between parental alleles and therefore allow determining the origin of multiplex tumours. All multiplex UCs used in the study of array-CGH (see below) were analysed for specific chromosomal regions by microsatellites. Each tumour obtained from the same patient displayed the loss of the same parental alleles confirming the monoclonal origin of tumours.

Evaluating genetic changes occurring in multiplex tumours allow the establishment of clonal evolution during tumour progression. Based on the primary genetic alterations occurring in all tumour cells and in each tumour and secondary alterations occurring only in subclones of one or more of the metastatic tumours a step-by-step genetic developmental tree can be established

Differential gross DNA alterations in multiplex versus solitary UCs

Our aim was to find genomic changes occurring preferentially in multiplex or solitary UCs. Array-CGH analysis indicates that chromosomal losses at 2q, 8p and 18p occur preferentially in solitary UCs, whereas multiplex UCs show the tendency to copy number gain at these regions. Gain at chromosome 3p, 5p was seen preferentially in multiplex tumours with tendency towards loss of the same region in solitary UCs. One of the most important finding of this study is a detection of frequent loss at chromosome 9q, 10q, 11q, 18q and 21q in multiplex UCs. Of interest, a slight tendency to increased copy number at these regions was detected in solitary UCs. In addition, copy number gain at the X chromosome was found in solitary UCs. In several cases of multiplex UCs only the long arm of chromosome 9 was deleted whereas the short arm remained without copy number changes.

Copy number losses at small regions occurs preferentially in multiplex UCs

Altogether, 80 small homozygous losses were detected at chromosome 9p 10q, 11q, 18q and 21q in the 56 UCs. None of the solitary UCs showed homozygous losses with exception of chromosome 9p region. Homozygous losses at chromosome 9p were seen in 28 of 32 multiplex UCs, whereas only 5 of the 24 solitary UCs displayed similar changes. Loss of 2 Mb sequence at chromosome 9p24.1-23 harbouring only the PTPRD gene occurred in 6 multiplex and one solitary

UCs. We found the loss of a 1.54 Mb smallest overlapping region at chromosome 9p22.3-22.2 including the C9orf93, BNC2 and CNTLN genes in 8 multiplex UCs. The most frequent homozygous loss of 0.417 Mb harbouring the MTAP, CDKN2A and CDKN2B genes at 9p21.3 occurred in 13 multiplex and 4 solitary UCs.

Homozygous losses at 4 regions of chromosome 10q were seen in 14 multiplex tumours. In 13 of the 14 tumours a large heterozygous deletion between chromosomal bands 10q23.1 and 10q25.1 was seen, which included the small regions of homozygous deletions affecting the NRG3, PTEN and SORCS1 genes . Homozygous deletion was also seen at the smallest overlapping region of 6.7 Mb including 65 genes at chromosome 11q23.3 in 8 multiplex UCs. Homozygous loss of a 1.67 Mb region at chromosome 18q12.1 including the DSC1-3, DSG1-4 genes was detected in seven tumours. Finally, a loss 5.64 Mb at 21q21.3-22.11 including the JAM2, CLDN8 and CLDN17 genes and the loss of a 7.5 Mb at chromosome 21q22.13-22.3 including the CLDN14, ERG, ETS and UMODL1 was seen in 7 multiplex tumours each.

Amplifications occurs exclusively in G3 tumours

Altogether, 32 amplified regions were detected in UCs of high malignancy . Eight of the 11 G3 solitary UCs showed amplifications of DNA sequences at least at one chromosomal region . The number of genes in the amplified regions varied between 2 and over 100 including E2F2, E2F3, CCND1, FGF19, L3MBTL and YWHAB among others, which are known to be frequently amplified in UCs. Amplifications occurred only in invasive G3 UCs irrespectively of staging. Of interest, the 8 synchronous multiplex pT2,G3 tumours obtained from patient 5 did not showed any amplification but homozygous deletions at chromosome 9p22.3, 10q25.1 and 18q12.1 regions.

DISCUSSION

Alteration of chromosome 9 is probably the primary genetic change in the development of papillary and solid UCs

The present detailed allelotyping study delineated three new putative tumour gene loci at chromosome 9p24.1, 9p22.3 and 9q21.32 regions increasing the number of small genomic deletions at the chromosome 9 in UC up to seven. Allelic changes at one or more of these loci (PTPRD, BCN2, CDKN2A/B, DAPK1, PTCH, DBC1 and TSC1) in 88% of the tumours suggest that alteration of chromosome 9 plays a crucial role in the development of UCs. Although a correlation between 9p LOH and clinical outcome of the disease have been proposed, and others suggested that chromosome 9 alterations are secondary changes, we consider the genomic changes at chromosome 9 as initial events in the UC tumorigenesis. This hypothesis is supported by the finding presented in this thesis that LOH at the seven gene regions occurs at nearly similar frequency in all stages and grades of UCs and none of the alterations is associated with the TNM classification.

Mapping studies have long identified the cell-cycle regulator tumour suppressor genes CDKN2A (encoding the p16 and p14[ARF]) and CDKN2B (encoding p15) as a target of chromosome 9p deletions. Silencing of p16 gene by promoter methylation has been described in 30-60% of UCs and therefore a heterozygous deletion and mutation or hypermethylation of the other allele may result in the functional inactivation of the gene. The lack of p16 protein expression may lead to loss of its cell cycle regulatory function and increased proliferation of tumour cells.

In the present study, we found two small regions of LOH at chromosome 9p apart of the CDKN2A/B loci. One of them at chromosome 9p24.1 harbours the receptor protein tyrosine phosphatase delta, PTPRD gene. The smallest overlapping deletion involving only the PTPRD genomic sequences in our series suggest that this gene might have a tumour suppressor function in UCs as well. Another new locus of selective LOH was found at chromosome 9p22.3 region harbouring BNC2 gene encoding a DNA-binding zinc-finger protein. Homozygous loss of the BNC2

locus was seen in oesophageal cancer and the stable expression of the BNC2 resulted in growth arrest of oesophageal carcinoma cell line.

Loss of chromosome 9q regions has already been implicated in the genetics of UCs and in the high risk of tumour recurrence. Deletion and decreased expression of PTCH and homozygous deletion or methylation of DBC1 genes suggest their possible role in UC biology. Mutation of the TSC1 gene in approx. 10% of the UCs but not in other types of cancers suggests that mutational and deletional inactivation of TSC1 may contribute to the development of bladder cancer. In our study, LOH at the genomic sequences of the DAPK1 gene and the LOH was associated with the multifocal growth and intravesical recurrence of UCs. Loss of expression of the DAPK1 through promoter methylation has earlier been demonstrated in 29-88% of UCs and also other cancer cells. Most importantly, methylation of the DAPK1 gene was significantly associated with the high rate and short time of recurrence of UCs. DAPK1 is a positive mediator of IFNA induced apoptosis and it was also shown that DAPK1 is involved in the p53-dependent apoptosis pathway. The association of methylation and LOH at the DAPK1 with the recurrence of UCs suggests, that the DAPK1 gene is one of the main target of chromosome 9q deletion. However, it is not yet clear how the loss of function of DAPK1 contributes to the high frequency of intravesical spreading of UCs.

Chromosome 8p changes are associated with the staging and grading of UCs

This study confirmed the correlation between allelic changes at chromosome 8p and grading/staging of UCs and delineated a breakpoint region at chromosome 8p12-p11.2. Recently, Veltman et al. have identified recurrent break points within a 9 Mb region at chromosome 8p12 in UCs. They suggested one breakpoint at the large genomic region of 2.3 Mb around the NRG1 and a second one around the FGFR1. Adelaide et al. have also detected a breakpoint cluster stretched over 1.1 Mb within the NRG1 in breast and pancreas cancer cell lines, some of them within the extremely large (900 kb) intron between the alternative first exon and exon 2. High level amplification at this chromosomal region was found in UCs and breast cancers. The amplified region included the putative cancer genes FGFR1, SFRP1 and TACC1, whereas the NRG1 was rarely included in the amplicon. These data suggest that chromosome 8p11.2-12 region harbour instable DNA sequences, which are frequently involved in translocation, deletion or amplification affecting several genes. In our study, the most frequent break occurred proximal to the NRG1 leading to the loss of one allele of this gene.

We found the downregulation of NRG1 in all stages and grades of UCs irrespectively of the allelic changes at the corresponding gene locus. Adalaide et al. also showed that the expression pattern of NRG1 isoforms in breast cancers is not correlated with the genomic alterations suggesting the lack of transcriptional consequences of the breaks within one allele of the NRG1. The NRG1 is a member of the cell-cell signalling protein neuregulins that are ligands for receptor tyrosine kinases of the ERBB family. Corresponding to the several isoforms of NRG1 the cellular responses include stimulation or inhibition of proliferation, apoptosis, migration, differentiation and adhesion in distinct cell types and cancers. The role of the NRG1 in UCs, however, is not yet established.

Modulation of the Wnt signalling has been implicated in the development in several types of tumours including the UCs. The secreted frizzled-related proteins (sFRPs) contain an N-terminal domain homologous to the cysteine-rich domain of the frizzled family of Wnt receptors. They compete with the frizzled receptors for Wnt binding and act as soluble modulator of Wnt signalling. Loss of function of the SFRP1 therefore may lead to an increased signalling in the Wnt pathway and to an increased cell proliferation. Ugolini et al. described markedly reduced or absent SFRP1 transcript in invasive ductal breast cancers, but correlation between tumour grade and SFRP1 expression status was not found. Other studies indicated a correlation between upregulation of SFRP1 and apoptosis. Recently, it was suggested that loss of function of SFRP1 is an independent indicator for poor survival of invasive bladder cancer. We found downregulation of the SFRP1 in all stages and grades of UCs suggesting that loss or decrease of the SFRP1 function is associated with

the development (cell proliferation) rather than with the progression (invasive growth) of this type of tumour.

Loss of one allele and mutational or methylational silencing of the remaining allele are implicated in the inactivation of most tumour genes. Recently, haploinsufficiency has also been suggested as a possible mechanism leading to inactivation of tumour genes. Our LOH, methylation and expression study on the SFRP1 gene suggests a more complex mechanism. For example, the SFRP1 was completely silenced by loss of one allele and methylation of the remaining allele in an UC or exclusively by methylation without allelic changes in a dysplasia. In some cases, however there was no difference in the methylation status in tumour and corresponding normal DNA, nor allelic changes at the gene locus were detected, but the SFRP1 was downregulated. The mechanism of deletional/mutational inactivation of the SFRP1 has been excluded by others. Thus, in addition to known genetic (allelic loss and haploinsufficiency) and epigenetic (methylation) changes other mechanism may also be involved in the silencing of the SFRP1 in UCs.

Chromosome 11p changes do not associated with tumour progression

Allelotyping of chromosome 11 revealed a frequent LOH within the imprinted region of chromosome 11p15.5 band. There are 12 genes annotated at this region including the H19 and IGF2 genes. The IGF2 (insuline-like growth factor 2), which expressed exclusively from the paternal allele, belongs to the insuline family of growth factors. The IGF2 does not expressed in normal urothelial cells, but highly expressed in urothelial cancers, probably due to loss of imprinting. The H19 is another imprinted gene, which expresses a non-coding RNA from the maternally inherited chromosome. The H19 is expressed in normal urothelial cells but not in UCs. Epigenetic changes at the IGF2 and H19 are associated with Wilms' tumour and Beckwith-Wiedemann syndrome. Alterations in this region are associated with rhabdomyosarcoma, adrenocortical carcinoma, lung, ovarian, and breast cancer. Loss of imprinting of IGF2 is implicated in the development of prostate cancer and other tumours.

The other region of allelic imbalance at chromosome 11p15.5 region harbours the RHOG, STIM1, RRM1 and TRIM21 genes. There is no expression of these genes, with exception of the RRM1 (ribonucleotid reductase M1) gene, in normal urothelial cells. The third region of LOH harbours the SSRP1, PRG3, CTNND1 and FAM11A genes. The CTNND1 gene has been involved in deletions as well as in amplifications in some cases. In the present study, amplification involving the CTNND1 gene was seen in one solitary UC by array-CGH. CTNND1 encodes a member of the Armadillo protein family, which has a function in cell adhesion and in signal transduction.

It remains unclear from this study (as well as from other analyses), which genes from chromosome 11 are involved in the development and progression of UCs. There is no association between the allelic changes at any site of the chromosome 11 and the staging of UCs. A slight association can be seen between grading and allelic changes.

Mutation of the p53 gene is associated with high-grade malignancy but not with tumour type

We found mutations in exons 5-8 of the p53 gene in 21% of consecutively operated UCs of the bladder. Although LOH at the p53 gene locus occurred in all grade and stages, mutation of p53 gene was detected exclusively in high grade invasive UCs. These data suggest that allelic change at chromosome 17p13 is only a rate limiting step towards an aggressive growth and proliferation of UCs. We found p53 mutations not only in CIS, solid growing pT1,G3 and pT2-4 UCs but also in papillary pT1,G3 UCs indicating that p53 mutation is not strictly pathway correlated genetic change but associated with the high malignancy of tumours. We found a high frequency (90%) of LOH at one or more tumour gene locus on chromosome 9 in the UCs with p53 mutation including homozygous losses at the CDKN2A/B, DAPK1 and PTCH gene regions. Thus, we found a concomitant mutation of p53 and LOH at chromosome 9 in both papillary and solid growing invasive UCs.

A proposal for a simple pathway of UC development

Based on data of the present study and those from the literature we propose a genetic model of UC development. The very first genetic change in the development of UCs is a monosomy or uniparental isodisomy of chromosome 9 or LOH at distinct chromosome 9 regions harbouring the tumour suppressor genes PTPRD, BNC2, CDKN2A/B, DAPK1, PTCH, DBC1 and TSC1. Hemi or homozygous deletion and mutation of the PTPRD occurs in distinct types of cancer and the reconstitution of PTPRD expression in tumour cells led to growth suppression and apoptosis. The functional inactivation of BNC2 or CDKN2A/B results in growth advantage. Loss of expression of the DAPK1 through promoter methylation has been demonstrated in 29-88% of UCs. DAPK1 is a positive mediator of IFNA induced apoptosis and it was shown that DAPK1 is also involved in the p53-dependent apoptosis pathway. Deletion and decreased expression of PTCH and homozygous deletion or methylation of DBC1 genes indicates their possible role in UC biology. Mutation of the TSC1 gene in approx. 10% of the UCs but not in other types of cancers suggests that mutational and deletional inactivation of TSC1 may also contribute to the development of bladder cancer. Therefore, at least seven genes controlling different cellular functions such as cell proliferation and apoptosis might be inactivated along the chromosome 9 by hemi- or homozygous deletions, mutation and methylation. It is likely, that the initial genetic changes at one or more of these regions of chromosome 9 lead by chance to hyperplasia or dysplasia, and determine the subsequent events in the molecular pathology of UCs.
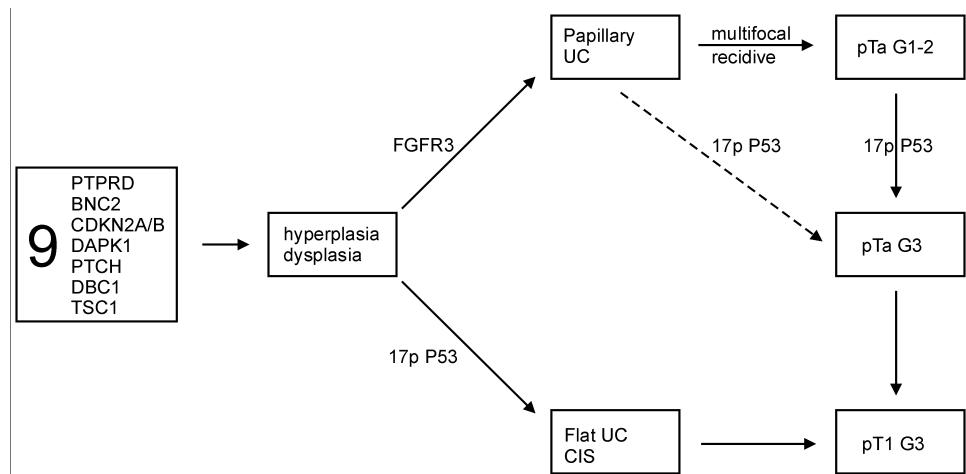

Figure: A proposed, simple pathway of the development and progression of urothelial cancers.

LOH at chromosome 9 occurs at high frequency in urothelial hyperplasia and dysplasia. This finding also supports the key role of chromosome 9 in pre-neoplastic and neoplastic cell proliferation. If such cells acquire an activating mutation of the FGFR3 gene, a papillary pTa,G1 UC will arise. These tumours may recur several times as solitary or multiplex pTa,G1 UCs. However, in some cases a G1 to G2-G3 transition may take place, presumably after acquiring a p53 mutation. In rare cases the inactivation of p53 gene may occur at a very early stage of papillary UC development resulting in a primary pTa,G3 papillary UC. Some of the papillary pTa,G3 UCs turn to be invasive before detected and therefore, such tumours carry both FGFR3 and p53 mutations and LOH of chromosome 9.

If hyperplastic or dysplastic cells with chromosome 9 alteration acquire p53 mutation (or these cells carry already a p53 mutation due to environmental carcinogenesis as a rate limiting step), they start to grow as a cytologically "wild" group of aneuploid tumour cells, e.g. CIS, which after selection of the fittest cell clone grow as an invasive pT1,G3 UC (Figure). These tumours carry only the p53 mutation and LOH at chromosome 9 but not FGFR3 mutation. Thus, the pT1,G3 UCs are at the crossroad of the two divergent pathways and progress by time towards muscle invasive tumour.

Several other genetic alterations at distinct chromosomal regions and genes are involved in the genetics of UCs, most of which can be observed in later stage of tumour development. These genes may modify the differentiation, morphology or growth capacity of tumour cells, may trigger the invasive growth or determine other biological and morphological characteristics of UCs, but they are secondary to the alteration of chromosome 9. Previously, by exploiting the mechanism of probabilistic reasoning in Bayesian Networks and reconstructing the possible flow of progression of allelic changes, Bulashevska et al. have suggested that LOH at chromosome 9 is a primary event in UC pathogenesis.

Recently, Goebell and Knowles proposed that mutation of the FGFR3 marks low grade papillary UCs and deletion and/or mutation of the TP53 gene the pathway of CIS, pT1, muscle invasive carcinoma. If we accept this hypothesis, the FGFR3 and p53 mutations display a mutually exclusive pattern only pTa,G1 vs. CIS whereas pT1,G3 (and also pT2-4) UCs may display both p53 and FGFR3 mutations. Indeed, FGFR3 and p53 mutations were described in 9% of a series of pT1,G3 UCs indicating that papillary UC with FGFR3 mutation progressed into invasive tumour by acquiring p53 mutations. The p53 mutation and LOH of chromosome occurred in 9 in papillary pT1,G3 UCs in the present study. The high frequency of chromosome 9 alteration in association with p53 mutation in invasive G3 UCs indicates that LOH at chromosome 9 occurs at high frequency in solid growing tumours as well.

Microsatellite analysis might be used for monitoring the disease but with restrictions

Several studies suggested the use of microsatellite analysis of urine for diagnosing and or monitoring UCs. However, this technique has no place in today's routine diagnosis. Although the pilot study presented here has a higher sensitivity then cytology, it could not reach the 93% sensitivity of the gold-standard cystoscopy. The main problem of microsatellite analysis of urine samples that first, one should search for something, which was lost and second, the DNA extracted from the urine is contaminated or highly contaminated with normal DNA and therefore a loss of signal cannot be detected unequivocally. In general, advanced tumours contain more genetic alterations, which might be quantitative (deletion, amplification) and qualitative (mutation). To detect tumours with lower stage is more difficult due to the low number or the lack of detectable genetic changes.

Homozygous losses associated with multiplex urothelial carcinoma of the bladder

The high resolution array-CGH profiling disclosed nearly all DNA changes known to be generally associated with development and progression of UCs. Copy number losses at chromosomes 2q, 8p and 18p, which are associated with tumour progression, occurred preferentially in solitary UCs. The higher frequency of copy number losses of chromosomes 9q, 10q, 11q, 18q and 21q regions in multiplex UCs together with the frequent homozygous losses of small genomic sequences at the same regions suggest, that alteration of genes at these genomic sequences might be associated with the intravesical spreading of UCs.

One of the interesting finding was the high frequency of heterozygous loss of chromosome 9q in multiplex UCs. Loss of chromosome 9q has already been associated with high risk of tumour recurrence. Deletion and decreased expression of PTCH (9q22.32) and homozygous deletion or methylation of DBC1 (9q33.1) genes suggest their possible role in UC biology. Mutation of the TSC1 (9q34.2) gene in approx. 10% of the cases suggests that inactivation of TSC1 may also contribute to the development of bladder cancer. However, it is not yet clear how the loss of function of these genes might contribute to the intravesical spreading of UCs. Recently, it was shown that promoter methylation of the DAPK1 (9q21.33) was significantly associated with the high rate and short time of UC recurrence. Although we did not find a small deletion hitting only the DAPK1, one allele of the gene together with the chromosome 9q was deleted in multiplex UCs. These findings indicate that DAPK1 gene might be the main target of chromosome 9q deletion, especially in multiplex UCs.

To find the locus of genes associated with one of the two groups of UCs, the homozygous deletions and amplifications of small genomic sequences were analysed in detail. An overlapping homozygous deletion of 417 kb at chromosome 9p21.3 including the CDKN2A/B occurred in multiplex and solitary UCs. The cell-cycle regulator tumour suppressor genes CDKN2A (encoding the p16 and p14^{ARF}) and CDKN2B (encoding p15) have already been identified as a target of chromosome 9p deletions their role in the genetics of UCs discussed in detail earlier.

In the present study, two non-syntenic homozygous losses at chromosome 9p apart of the CDKN2A/B region were seen. One of them at chromosome 9p24.1-23 harbours the receptor proteine tyrosine phosphatase delta, PTPRD gene. The PTPRD is frequently deleted, mutated or methylated in glioblastoma multiforme, neuroblastoma and lung cancer. The smallest overlapping homozygous deletion involving only the PTPRD genomic sequences, e.g. biallelic inactivation of the PTPRD suggest that this gene might have a tumour suppressor function in UCs as well. Another new target region was found at chromosome 9p22.3-p22 including the C9orf03, BNC2 and CNTLN genes. Recently, a new ovarian cancer susceptibility locus has been localized to the BNC2 at chromosome 9p22. BNC2 encodes DNA-binding zinc-finger protein which is highly expressed in normal ovary and testis. Homozygous loss of the BNC2 locus was seen in esophageal cancer and the stable expression of the BNC2 resulted in growth arrest of esophageal carcinoma cell line.

The most striking observation of the present study was the association of small homozygous losses apart of the 9p region with multiplex UCs. The frequent homozygous losses at chromosomes 10q, 11q, 18q and 21 found exclusively in multiplex UCs suggests, that these regions may harbour genes involved in the intravesical seeding of tumour cells. The biallelic inactivation of such genes might be lead to detachment of tumour cells and homing at another region of the urinary bladder. For example, the homozygous loss at chromosome 18q12.1 removes completely the cluster of desmocollin (DSC) and desmoglein (DSG) genes, which are members of the cadherin cell adhesion molecule superfamily. DSC2 and DSG2 are expressed in normal urothel as well as in urothelial carcinomas. The lack of DSC2 and DSG2 in some multiplex UCs therefore, may trigger the loss of cellular adhesion and seeding of tumour cells at other localisation. Similarly, the homozygous loss at two chromosome 21q regions and the lack of CLDN8, CLDN14 and CLDN17, which are integral membrane proteins, components of the tight junctions may contribute to intravesical "metastasis" of tumour cells.

The amplifications of distinct genomic sequences have already been associated with high grade UCs. Some of them, such as amplification of E2F3, CCND1, ORAOV1, YWHAB, SDC4 has been described in different studies. In the present study, amplifications occurred exclusively in G3 UCs of high malignancy. Although Nord et al. found "overrepresentation of focal amplifications within high grade and recurrent cases", they have included several recurrent UCs of high grade malignancy in their series. The data presented here suggest that amplifications are generally associated with the high grade of UCs whereas homozygous deletions might be associated with the multiplex (synchronous or asynchronous) nature of tumours.

Several clinicopathological factors have been proposed for prediction of recurrence and progression for "superficial" (pTa and pT1) UCs, such as grade, multifocality, early recurrence, concomitant CIS or the size of tumour. However, only few genetic changes have been associated with the development of synchronous or asynchronous multiplex UCs. It was shown in the present study, that biallelic inactivation of cellular adhesion genes by homozygous deletions occurs preferentially in multiplex UCs and therefore may contribute to the intravesical spreading of tumour cells.

CONCLUSIONS

1. Allelotyping of the chromosome 8p yielded in the detection of small interstitial deletions marking the CSMD1 gene at chromosome 8p and NRG1 at chromosome 8p regions. These alterations are associated with the progression, e.g. grading and staging of UCs.

2. A high resolution allelotyping of chromosome 9 led to the detection of three new gene loci harbouring the PTPRD, BNC2 genes at the short arm and DAPK1 on the short arm in addition to the known genomic alterations at the CDKN2A/B, PTCH, DBC1 and TSC1 genes. These genetic changes occurred at the same frequency in all stages and grades of solid or papillary growth of the UCs. This finding together with the high frequency of alterations (88%) indicates that alterations at least at one of the seven genes are primary genetic changes in UCs.

3. Although, selected genomic changes were seen at the imprinted area of chromosome 11p15, the lack of strong association with the staging or grading of UCs do not allow any conclusion on the biological effect of these alterations.

4. Loss of chromosome 17p13 sequences with one allele of the p53 gene may occur in all grades and stages of UCs, but mutation of the p53 occurs exclusively in highly malignant G3 tumours.

5. Based on the results presented in this thesis and data from the literature, a simple, unifying pathway for the development of solid growing (dysplasia, CIS, invasive UC) and papillary growing (pTa, subsequent recurrencies and possible invasive growth) tumours has been suggested.

6. Multiplex and recurrent UCs, irrespectively of grade and stage display frequent copy number losses at chromosomes 9q, 10q, 11q, 18q and 21q and homozygous losses of genes involved in cellular adhesion, whereas solitary UCs show preferentially copy number losses at chromosomes 2q, 8p and 18p as well as gene amplifications at different chromosomes.

7. Microsatellite analysis might be used for detection of recurrent tumours in urine only after establishing the genetic profile of primary tumours, but even in this setting with limitations.

ABBREVIATIONS/RÖVIDÍTÉSEK

ACTB   beta-actin

AI   allelic imbalance

ASR   age standardized incidence rate

BCG   Bacillus Calmette-Guérin

cDNA   complementary DNA

CGH   comparative genomic hybridisation

CIS   carcinoma *in situ*

dNTP   deoxyribonucleotide triphosphate

EDTA   ethylenediaminetetraacetic acid

EMBL   European Molecular Biology Laboratory

FISH   fluorescence in situ hybridization

GDB   Human Genome Database

HG-CGH   humane genome comparative genomic hybridisations

HMM   hidden Markov model

ISUP   International Society of Urological Pathology

LOH   loss of heterozygosity

MSP   methylation specific polymerase chain reaction

NCBI   National Center for Biotechnology Information

PCR   polymerase chain reaction

PUNLMP   papillary urothelial neoplasm of low malignant potential

qRT-PCR   quantitative real-time polymerase chain reaction

SDS   sodium dodecyl sulfate

sFRP   secreted frizzled-related proteins

TBE   Tris/Borate/EDTA buffer

TE, TE9   Tris/Edta buffer

TNM   Tumour, Node, Metastasis classification

TUR   transurethral resection

UC   urothelial carcinomas

UICC   Union International Contre le Cancer

WHO   World Health Organization

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