Human Molecular Genetics, 2000, Vol. 9, No. 20 **2973–2980**

Molecular evolution of multiple recurrent cancers of the bladder

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Received 21 June 2000; Revised and Accepted 9 October 2000

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

INTRODUCTION

Tumorigenesis is a process that is largely occult. It is generally accepted that most cancers will develop through an accumulation of mutations in oncogenes and tumor suppressor genes, a process which precedes clinical detection of the tumor. The tumor that is finally clinically detected is more often than not a heterogeneous mixture of cancer cell subclones, which makes it difficult to establish the order of the genetic insults. The postulated steps in tumorigenesis are almost invariably based on a retrospective comparison of genomic alterations in tumors from different patients of different stage and grade. Early steps are then defined as genetic alterations that are present in all grades and stages, whereas later steps are detected solely in the higher stages and grades. The prototype for genetic evolution in cancer is presented by the colon cancer model that describes the distinctive stages from benign adenoma through to carcinoma by successive alterations in *APC*, *KRAS*, *TP53* and a gene on chromosome 18q, respectively (1). The only example so far, in which neoplastic development was monitored in time in one and the same patient by repeated biopsies, is Barrett's esophagus, a premalignant condition that predisposes to esophageal adenocarcinoma. In these patients regions of metaplasia, low- and high-grade dysplasia and adenocarcinoma can be distinguished in the same area. These histologically distinct stages are clonally related and presumably derived from a single precursor and a model for the genetic evolution of these different stages has been designed (2,3).

Bladder cancer is a disease that presents as superficial in ~75% of patients. Although these papillary tumors that extend into the lumen of the bladder are easily removed by transurethral resection (TUR), as many as 60–80% of patients will eventually develop one or more recurrences (4). New tumors arise most of the time at a different location and are not regrowths of an incompletely removed tumor (5). The multiple recurrences are most probably clonally related as appears from X chromosome inactivation studies and genetic and cytogenetic analyses (6,7). Therefore, these tumors are the result of dissemination and re-implantation of tumor cells in the bladder wall and/or the spreading of tumor cells via expansion within the urothelium. Due to this rather unique property, bladder cancer provides the opportunity to study the genetic relation and evolution of the different tumor subclones over long periods of time in one and the same patient because of their separate locations. Previous genetic studies of bladder cancer established that the most frequent alterations represented by loss of heterozygosity (LOH) are on chromosomes 4p, 8p, 9p, 9q, 11p and 17p (8–11). Furthermore, it has been found that recurrent tumors may have both concordant and discordant genetic alterations, suggesting that genetic evolution is an ongoing process in tumor development (12). However, a thorough description of the tumor evolution process is still lacking. In this study, we explored the unique possibilities of bladder cancer as a model for cancer evolution in general. To this end, we systematically mapped the individual tumor genotypes of 11 patients with 104 recurrent bladder cancers.

RESULTS

A total of 48 microsatellite markers were used to determine a genotype for each tumor based on the number and nature of markers with LOH. In addition, the *FGFR3* gene was screened for specific point mutations. The LOH data were re-interpreted to be used for a one-dimensional hierarchical cluster analysis as described by Eisen *et al.* (13). Figure 1 shows the results of such an analysis when all 104 tumors are used for clustering

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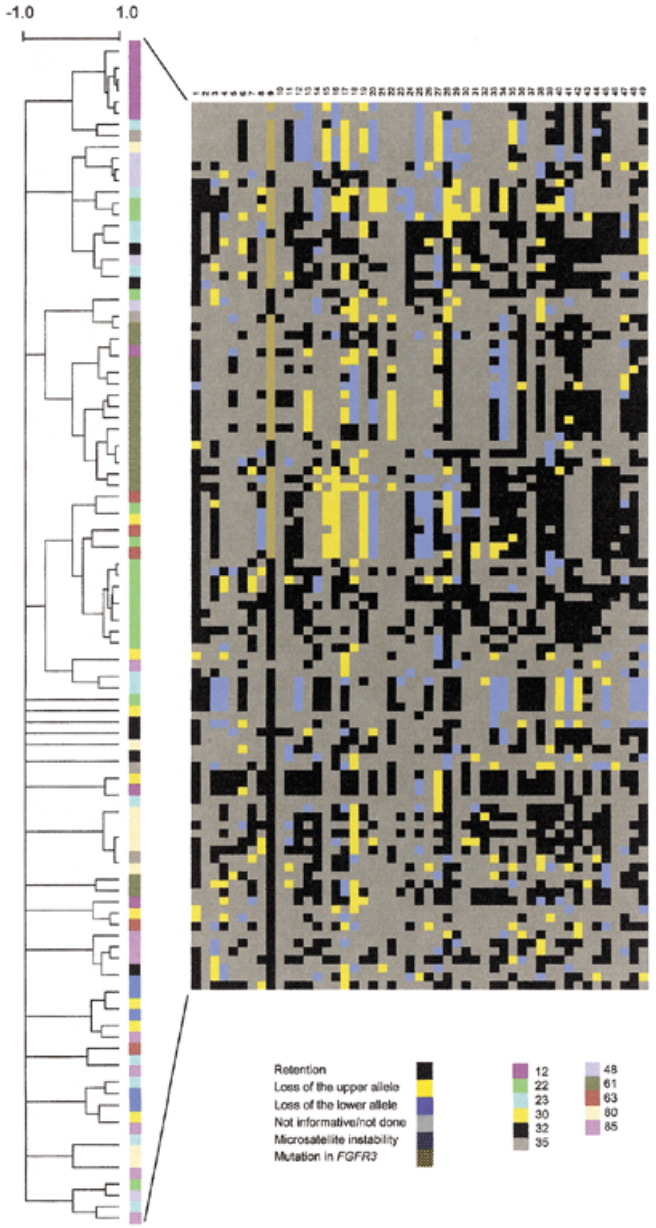

Figure 1. Cluster analysis of 104 tumors from 11 patients shows that the different tumors from one patient are clonally related. The color bar underneath the dendrogram depicts the different patients. These are represented by different colors (patient number and color are indicated on the lower right).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

based on the LOH and mutation analysis results. Patients in the figure are identified by colors next to the dendrogram. From this figure it is evident that the tumors from one patient tend to cluster together. For instance, 13 of the 15 tumors from patient 61 cluster in one sub-branch of the cluster dendrogram. Therefore these results suggest that tumors from one patient are more related to each other than tumors between patients, providing further support for a monoclonal process of tumorigenesis.

Subsequently, we assessed the relationships between the different tumors of single patients. Figure 2A shows the number, stage and grade of the 15 bladder tumors from patient 22. These tumors were removed between 1977 and 1990. Identification number, stage and grade of the tumors are indicated. Tumors are ordered in a chronological order, i.e. tumor A was removed before tumor B and so on. Twenty-nine microsatellite markers were informative for this patient and 17 showed LOH in one or more tumors, ranging from no loss to a loss of 11 markers. The extent of loss is indicated by the ratio between upper and lower alleles as calculated by the Phosphor Imager. An identical point mutation in the *FGFR3* gene was detected in 10 of 15 tumors. When the genetic aberrations seen in the individual tumors of this patient are compared, it is clear that these cannot be explained by a linear model based on the chronology of appearance, simply because consecutive tumors have genotypes of different complexity. For example, no genetic alteration was seen in tumor I, removed in 1985, whereas previously resected tumors A–H all displayed loss of one or more markers and/or had a mutation in the *FGFR3* gene. Note also that these losses do not appear to be random and unrelated, since for most markers LOH in different tumors concerns the same allele. We then reordered the tumors with respect to genetic events. A representation of the data based on a one-dimensional cluster analysis is given in Figure 2B. The scale next to the dendrogram indicates the correlation coefficient calculated by the program. From this calculation it appears that all tumors except I are considered to be highly related. Because the cluster analysis does not provide a direction to the tumor evolution process, we then reordered the tumors based on the cluster data but with the assumption that a tumor with no or little genetic damage will have evolved before a tumor with extensive damage. In addition, this handmade reconstruction allows the introduction of hypothetical steps in the evolution process. The resulting evolutionary tree of the tumors in patient 22 is depicted in Figure 2C. Tumor I is considered to be the primary tumor and, for instance, tumor B, which was removed 7 years before I, to be a descendant from tumor I. As can be seen in Figure 2B and C, B is several genetic steps removed from I. Based on this analysis, we propose that the genetic tree reflects the development of, and relationships between, the different tumors from this patient better than the linear chronological order in which the tumors were removed.

Patient 61 also developed 15 tumors between 1976 and 1990. Twenty-four markers were informative and of these 16 showed LOH in one or more tumors, with a maximum of eight markers with LOH in a single tumor (Fig. 2D). Again, the genotypes of the tumors suggest a different order in genetic events than their chronological appearance. The strikingly consistent loss of the lower allele of *D10S169* in all tumors indicates that loss of this marker is the first or a very early event and that a clonal relationship between recurrences is very likely. An identical *FGFR3* mutation was observed in 14 of 15 tumors. In Figure 2E the clustered analysis is shown. In this patient, the correlation coefficient between tumors ranges from 0.05 to ~1, again suggesting an intimate relationship between these tumors. As for the previous case, we then reconstructed the tumor clus-

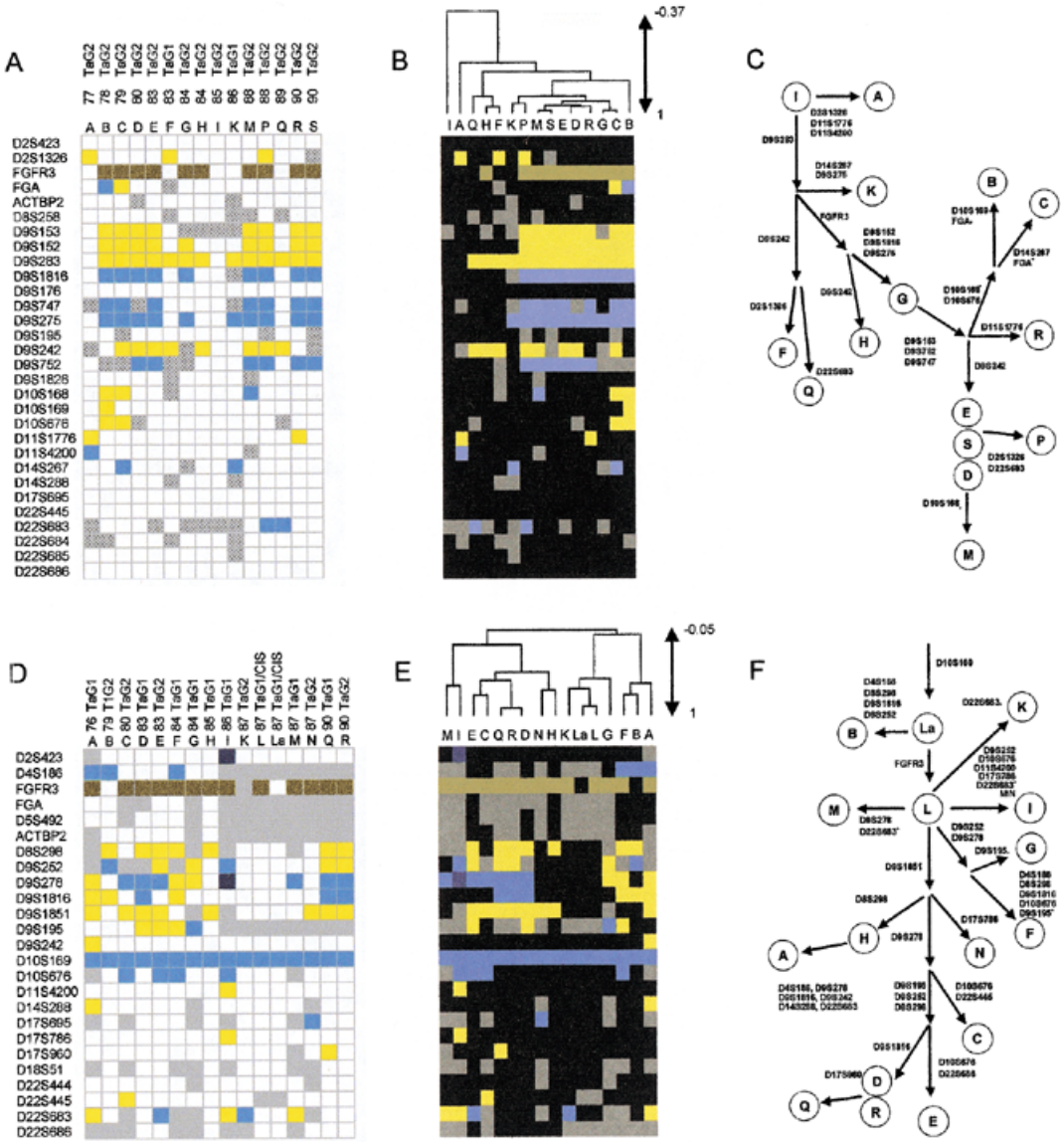

Figure 2. Genotypes, hierarchical tree clustering and deduced evolutionary trees for the multiple tumor recurrences from patient 22 and 61. For an explanation of colors, see Figure 1, with the exception that in Figure 2A and D retention is indicated by white cells. (**A**) LOH analysis and genotypes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

tering assuming a direction in the genetic build-up and by the introduction of hypothetical genetic steps. The adjusted tumor tree representing the genetic pathways along which the recurrences have developed in patient 61 is shown in Figure 2F. All tumors derive from tumor L, since in this tumor only one genetic insult was detected. Tumor A from patient 61 was removed in 1976 and tumor L in 1987. Tumor A has many additional genetic aberrations that are lacking in L. Thus, in the genetic tree, L precedes A. It can be seen in Figure 2D that tumor L is a mixture of CIS and Ta. Considering the extent of LOH, loss of *D10S169* is most probably present in all cells of this tumor. The intensity of the single-stranded conformation polymorphism (SSCP) signal, however, suggested that the *FGFR3* mutation was restricted to a fraction of the tumor (data not shown). We therefore divided tumor L in the fraction with mutation (L) and the fraction without receptor mutation (La). In the model, La is the founding tumor moiety, giving rise to B, the only tumor without the *FGFR3* mutation and L, from which the remaining tumors derive (Fig. 2F).

We were able to establish such a representation of sequential events, linking the tumors to one or more common precursor clones, for all patients, except one. In the six tumors from this

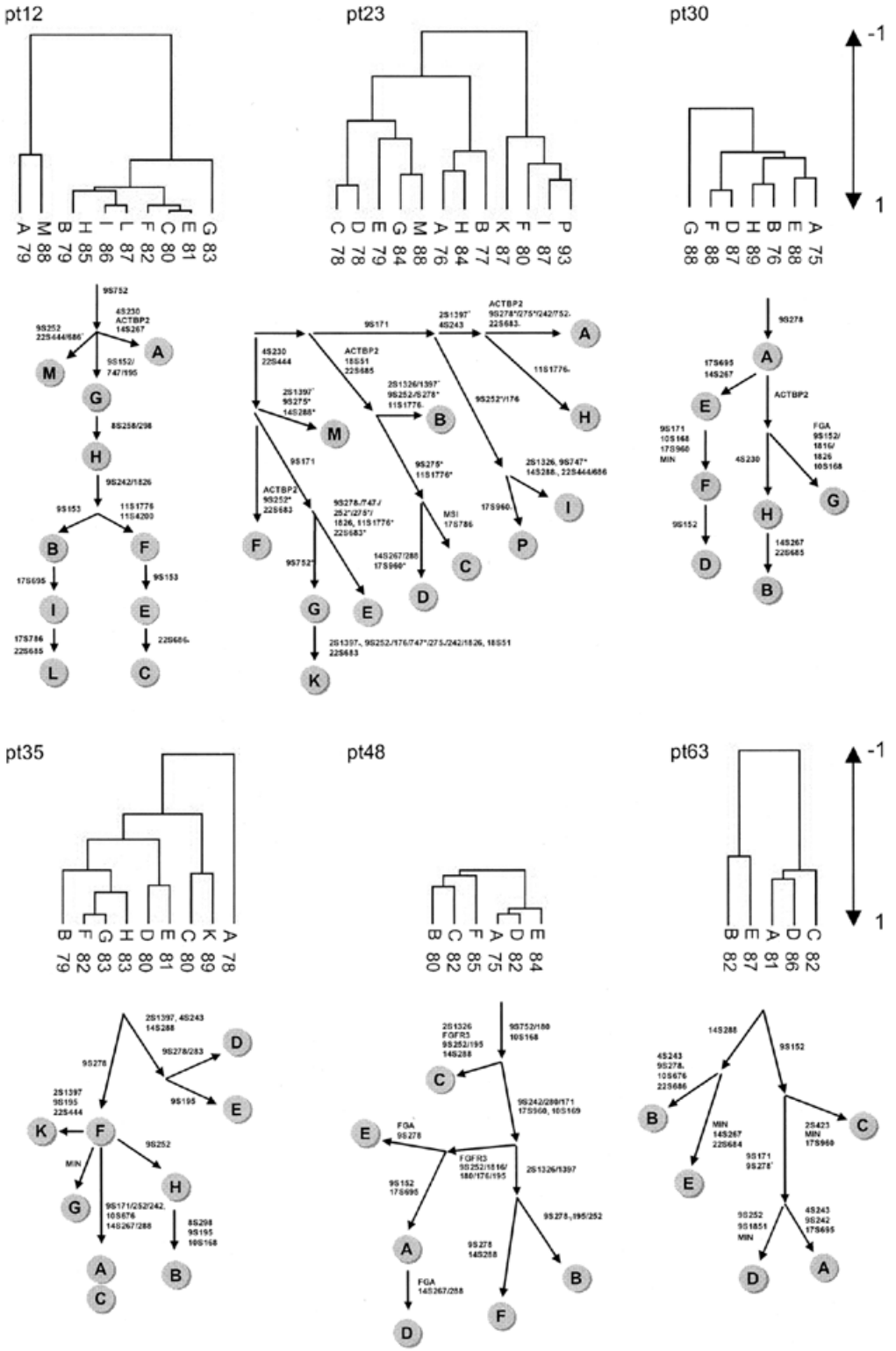

Figure 3. Cluster dendrograms and reconstructed genetic trees of the remaining eight patients. For reasons of clarity, markers are depicted without the initial letter D. Because of the alternating losses observed for some markers, asterisks indicate whether the upper or lower allele is lost. The scale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

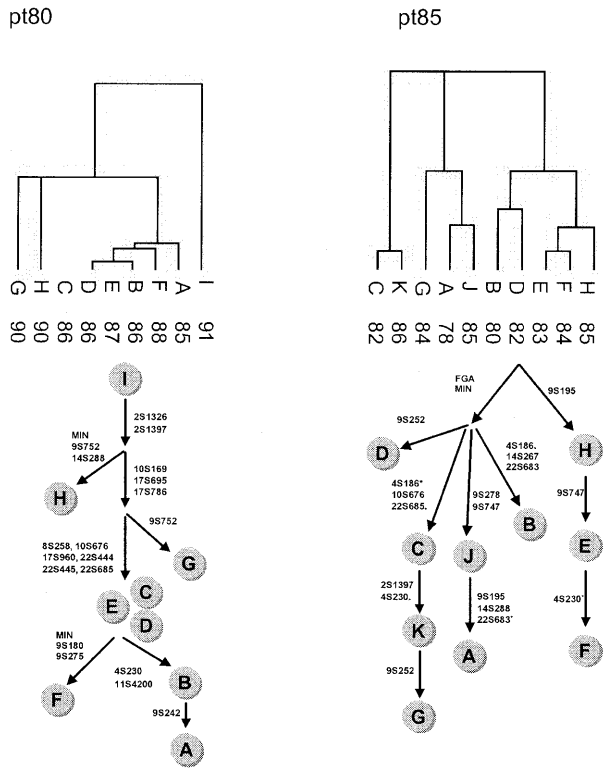

Figure 3. Continued.

latter patient (no. 32) in total only seven genetic hits were scored and this number is too low for a reliable cluster analysis. A representation of the cluster data and the deduced genetic trees for the other eight patients is given in Figure 3. In two of these patients, a first tumor was detected from which all other tumors developed. For the others, a hypothetical first tumor or tumor cell has been assumed. It also appears that for only one patient (patient 30) the first clinically presenting tumor (tumor A) is also the founding tumor in the genetic pedigree (Fig. 3). When the positions of the tumors in all trees are compared with their clinical manifestation, it is apparent that the chronology of tumor presentation does not parallel the genetic evolution of the tumors at all. Thus, this appears the leading principle rather than an exception.

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

DISCUSSION

An accumulation of mutations in essential genes can transform a normal cell into a cancer cell. This transformed cell may then grow out to form a tumor with additional mutations occurring during this process. The tumor that is finally clinically detected is more often than not a heterogeneous mixture of cancer cell subclones, which makes it difficult to establish the order of the genetic insults. Bladder cancer, however, provides the unique property that different tumor subclones grow at separate sites and thus can be studied independently. The presence of identical alterations in different bladder tumors from one patient and the increase in the number of genetic alterations allowed us to order the multiple tumors in each patient in the form of evolutionary genetic trees or pedigrees. In such a model, an original transformed cell grows out and sheds cells into the lumen of the bladder. Some of these cells will have acquired additional genetic damage. They attach to the bladder wall, grow out and can themselves lead to secondary disseminations and so on, thus creating the different branches of the tree. This model resembles the evolution of cell lineages in Barrett esophagus (2). In their model esophageal adenocarcinoma evolves from premalignant conditions such as metaplasia and dysplasia. Their results also indicate that this clonal evolution is more complex than predicted by a linear model. Here we show that bladder cancer cell lineages evolve, like in Barrett's model, over a period of many years, giving rise to clonal expansion and outgrowth due to newly acquired aberrations and continue to do so after the emergence of recurrent tumors.

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

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

A surprising finding is the sheer number of alterations in some tumors. There is a great variability in the number of LOH events that were observed per tumor, ranging from none or a few alterations in the early steps of the genetic trees to LOH of

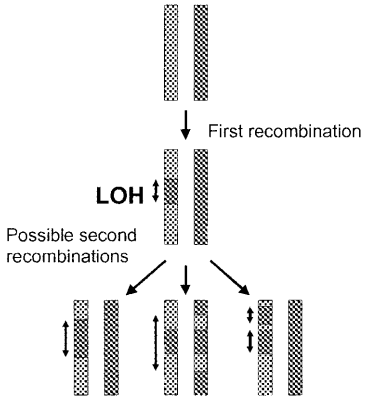

Figure 4. Enhanced rates of mitotic recombination may create multiple regions of LOH and expand existing LOH. The recombination takes place between homologous chromatids. For reasons of clarity, sister chromatids are not included. The arrows next to the chromosomes indicate the extent of LOH.

65% of the informative markers. When these numbers are extrapolated it appears that a large part of the genome may be affected. A related extrapolation has recently been presented by Stoler *et al.* (14). In their paper the authors show that colonic polyps, representing early steps in the tumor progression pathway, have a mean number of 11 000 genomic events per cell. Our findings also illustrate that the number of genomic alterations even in early tumor stages is already astoundingly complex. These findings can best be explained by assuming that genomic instability is already present early in tumorigenesis.

There are two levels of genetic instability: at the nucleotide level [microsatellite instability (MIN)] and at the chromosome level [chromosome instability (CIN)], the latter being much more frequent in cancer (15). We found microsatellite instability in 19 tumors, but only few of these showed instability for several markers. In general, MIN is not considered to play a major role in bladder cancer (16), although it is reported to be more frequent in young patients with bladder cancer (17). What type of instability mechanism could best explain the findings presented here? It appears that the LOH events that we observe on the best studied chromosome, chromosome 9q, reveals what can best be described as a patchwork pattern of losses and retentions, rather than loss of an entire chromosome or chromosome arm. For instance, in patient 22 (Fig. 2C) loss of *D9S283* is followed by loss of *D9S275* and *D9S752*; moreover, all three areas of loss increase in size as is apparent from subsequent losses of adjacent markers. Likewise, in patient 61, LOH of the marker *D9S1851* is followed by losses of the adjacent markers *D9S1816* and *D9S278*. However, in the case of these latter markers loss of alternate alleles occurs in different tumors. To explain these findings, we suggest a model in which the losses of heterozygosity are caused by an increased rate of mitotic recombination. Recombination between two homologous chromatids during mitosis could result in multiple crossovers (18). As a consequence, the crossover region in the recipient chromosome becomes identical in sequence to the donor chromosome. When recombinations occur frequently this leads to an expansion of the region of loss of heterozygosity. A model to explain this mechanism is given in Figure 4. The consequence of mitotic recombination is that no actual loss of chromosome regions occurs; only the sequence of part of one chromosome is now an exact duplicate of the other. Thus, the tumor genome in later stages of the genetic tree becomes more and more homozygous. Such a mechanism would be compatible with the low-stage, low-grade phenotype of the papillary bladder tumors. Although some of the LOH events could perhaps be explained by tetraploidization followed by loss of a chromosome, we believe that this is not the major explanation for our findings for the following reasons. Firstly, this would not explain the patchwork nature of the losses; secondly, in >40% of the cases, the LOH is far too profound; and thirdly, flow cytometry of bladder cancers has shown that especially the low-stage, low-grade papillary tumors, like those in this study, are mostly diploid (19).

An enhanced rate of mitotic recombination is seen in hereditary syndromes like Bloom's syndrome, Fanconi anemia and Werner's syndrome (20–22). The pattern of chromosome instability in especially Bloom's syndrome is characterized by sister-chromatid exchanges and homologous chromatid interchanges reflected in a gain of homozygosity for polymorphic loci (23,24). Patients with these diseases have an increased risk of developing several cancers. The genes responsible for these syndromes have, in part, been cloned and the protein products of both the *BLM* and *WRN* genes are DNA helicases (25,26). Therefore, we reason that it is not unlikely that a gene that functions in these diseases or a gene with similar characteristics may play a role in bladder cancer pathogenesis. Because of the increase in LOH with each step in the genetic trees, we favor a model in which such a type of genomic instability, caused by an enhanced rate of mitotic recombination generating functional homozygosity, occurs early in tumor evolution and may even be the elusive first step.

MATERIALS AND METHODS

LOH analysis

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

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

Human tumor tissues

We selected 104 paraffin-embedded bladder tumor specimens from 11 different patients with five or more recurrences. Sections were examined microscopically by a pathologist (T.H.v.d.K.). Parts that represented tumor tissue were punched out of the original paraffin blocks. In general the percentage tumor tissue in the material dissected by this procedure was estimated to be >90%. Normal bladder epithelium of the same patient served as a constitutive control for each patient. A group of unrelated blood DNA samples was analyzed for all markers in order to correct for variation in ratio between allele-specific combinations and to serve as alternative control in those instances where the normal epithelium DNA was not reliable or unavailable. DNA isolation was done as described previously (27).

Mutation analysis

Patients were screened for the recently described mutations in the *FGFR3* gene (28) (exons 7, 10 and 15) with SSCP analysis at room temperature on 6% polyacrylamide gels (49:1; acryl:bisacryl) or amplification products were analyzed for heteroduplex formation using weakly denaturing polyacrylamide gels (29:1; acryl:bisacryl) (29). The nature of the mutation was confirmed by subsequent sequence analysis. Dr F. Radvanyi kindly provided primers for the *FGFR3* exons 7, 10 and 15.

Cluster analysis

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

SUPPLEMENTARY MATERIAL

Supplementary material relating to this paper is available at http://www.hmg.oupjournals.org .

ACKNOWLEDGEMENTS

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

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