Abstract

During the past decades, the complexity of the bladder cancer genome has become evident. Early cytogenetic studies identified several patterns of chromosomal changes, particularly the frequent loss of chromosome 9. The cytogenetic approach was replaced by molecular methods, such as comparative genome hybridization (CGH) and loss of heterozygosity (LOH) analyses that describe genomic changes at a molecular and higher resolution. With these methods, the full complexity of the bladder cancer genome has been better appreciated. Using CGH and LOH analyses, it also became apparent that premalignant lesions of the bladder, such as hyperplasia and dysplasia, as well as carcinoma in situ (CIS), showed genomic changes. Whole genome analyses showed that low stage, low grade tumors generally show fewer changes than tumors of higher stage and grade. In addition, several genomic alterations were shown to be highly specific for more aggressive and invasive tumors. Based on the general association between complex genomic changes and tumor behavior, several investigations have been directed towards the identification of prognostic genomic markers for urothelial cancer. A complicating factor in the analysis and understanding of bladder cancer genomic progression is that recurring and, hence, chronologically later tumors may show genomes less rearranged than preceding tumors. Furthermore, morphologically normal urothelium in patients with bladder cancer frequently show the same type of genomic alterations as the tumor proper. This makes an issue of to what extent information on genomic changes will produce reliable prognostic information when limited to the tumor proper. © 2012 Elsevier Inc. All rights reserved.

Keywords: Bladder cancer; Chromosomal changes; Prognostic markers
will be described that may reduce the power of genomic changes detected in tumor biopsies as prognostic markers.

**Cytogenetic analyses of bladder cancer genome**

Early cytogenetic studies of bladder cancer demonstrated that losses of chromosome 9, or parts of chromosome 9, are the most frequently seen genomic alterations in UC. Loss of chromosome 9 is recurrently seen as the sole aberration, but also concomitant with complex changes in more advanced tumors. This pattern indicated loss of chromosome 9 material as an early and pathogenetically important event in UC. Other frequent chromosomal changes include loss of the short arm of chromosome 11 (11p), loss of the short arm of chromosome 8 (8p), and gain of the long arm of chromosome 1 (1q) [2]. By statistical analyses of the accumulated cytogenetic data kept at the Mitelman Database of Cytogenetic Aberrations [3], it has been possible to establish a temporal order for the appearance changes as well as possible pathways of UC genome development [4]. Taken together, the early cytogenetic investigations of UC could establish some fundamental characteristics of the UC genome. These included that chromosomal changes resulted in either losses or gains of genetic material. This latter finding shows that UC development is governed by inactivation of tumor suppressor genes and activation of oncogenes. The resolution of the cytogenetic approach is, however, too low to direct the identification of tumor related genes. In addition, many complex changes may not be clarified by chromosomal banding. The dependence on in vitro culturing of tumor cells is an additional limitation; it may limit the analysis to cells that have the capacity to grow in vitro. The introduction of comparative genome hybridization solved some of these problems.

**Molecular analysis of bladder cancer genome, comparative genome hybridization analysis**

In contrast to cytogenetic analysis, comparative genome hybridization (CGH) is not dependent on in vitro cultured cells. Instead, DNA is extracted from the tumor biopsy and chemically labeled with a fluorescent dye. In parallel, DNA from a normal tissue (e.g., blood) is labeled with a different dye. These DNAs are then denatured and hybridized simultaneously to normal human chromosomes. The intensity of the fluorescent dyes hybridized to the chromosomes will be proportional to the gene copy number in the original DNAs. Thus, by analyzing the proportions of the two dyes, it is possible to determine if a specific region in the tumor genome is lost or gained. Using this method, lost and gained regions are determined directly on normal chromosomes and not indirectly as in the case of cytogenetics. In this way, very complex chromosomal changes may be resolved in terms of gained and lost regions, and there is no need to clarify highly rearranged chromosomes as in the case of cytogenetics.

In an early use of comparative genome hybridization, Kallioniemi et al. [5] could validate several of the findings obtained by cytogenetic investigations (e.g., the frequent losses of chromosome 9 and chromosome arm 11p) and were able to delineate target regions for genomic amplifications to the level of individual chromosomal bands. The efficiency by which CGH may identify genomic amplifications in highly rearranged cancer genomes was shown shortly after when Voorter et al. [6] identified previously unknown genomic amplifications in bladder (e.g., chromosomal bands 3p22-24, 10p13-14, 12q13-15, and 17q22-23). These findings show the strength of the CGH approach to identify recurrent genomic amplifications. With the aim to clarify any genomic differences between Ta and T1 tumors, Richter et al. [7] analyzed 28 cases of each category using CGH. One of their major findings was that T1 tumor in general contains a larger number of genomic alterations and, thus, has a more evolved genome; T1 tumors harbored about 3 times as many changes as Ta tumors. In addition, grade 3 tumors showed a remarkable difference compared with low grade tumors (i.e., G1 and G2 tumors). A large number of genomic amplifications were exclusively seen in T1 high grade tumors. These findings suggest that some of the differences in clinical behavior of T1 compared with Ta tumors are also reflected by differences at the genomic level. Zhao et al. [8] investigated the relationship between tumor grade and genomic alterations further. These authors could show that the major division with regard genomic changes occurred between high grade (G3) and low grade (G1 and G2) tumors. However, other investigations have emphasized a difference between tumor stages. Simon et al. [9] compared Ta/G2 with T1/G2 cases using CGH and found a significant higher number of aberrations in the T1/G2 tumors. Most likely, both tumor stage and tumor grade are affected by the complexity of genomic rearrangements. Richter et al. [10] compared T1 with muscle invasive (≥T2) tumors using CGH. Even though some differences were seen, only two changes reached close to statistical significance, gain of the short arm of chromosome 5 (5p) and loss of the long arm of chromosome 6 (6q). Important though, Richter, et al. identified a total 23 different genomic alterations occurring in bladder cancer highlighting the genetic complexity of advanced tumors. The influence of genomic alterations on progression of T1 tumors to muscle invasive growth was later shown by the same group [11]. The authors could show that cases with a large number of deletions, specifically deletions of the long arm of chromosome 18 and gains of 5p, showed a high propensity for tumor progression. The association between chromosomal losses and progression was recently corroborated by Prat et al. [12] also applying CGH. These authors could show that patients with tumors dominated by chromosomal losses have a worse survival rate than patients carrying tumors dominated by gains. These results, and the results of Richter et al. [11],
thus suggest that it may be the overall patterns of genomic changes that might determine the biological and clinical behavior of the tumor rather than presence or absence of specific genomic changes.

Even though conventional CGH resolved many of the complex features of tumor genomes, the obtained resolution was not enough to pinpoint possible target genes for the observed changes. Furthermore, small changes, such as homozygous deletions, were not detectable using conventional CGH. However, the genome initiative and the development of microarray technology changed the options radically. A prerequisite for genome sequencing was the cloning of the human genome into smaller tractable pieces, so called BAC-clones. Hence, an outcome of the genome initiative was the possibility to arrange the whole human genome in about 30,000 ordered BAC clones. For each BAC clone, the complete sequence was known and consequently also the genes located within the clone. Microarray technology made it possible to analyze all clones simultaneously in one experiment. In a microarray, all 30,000 clones are positioned on a glass slide in a known order. By applying the CGH methodology directly to such slides and by measuring the fluorescence intensity from the tumor and the reference DNA in each BAC clone, the resolution is increased from 300 cytogenetic bands, in which each band correspond to about 10 million bp, to 30,000 BAC clones, in which each clone represents about 200,000 bp.

The first study that applied array-CGH to bladder cancer was performed by Veltman et al. [13]. In this study, the arrays were limited to contain a little more than 2,000 BAC clones, with one section particular selected to cover known tumor suppressor and oncogenes. Irrespectively, this study showed in more detail the complexity of chromosomal rearrangements in bladder cancer. Several small genomic changes were identified and recurrent homozygous deletions were detected in 3 locations, 8p23.1, 9p21.3, and 11p13. Based on the sequence data for the involved BAC clones, possible target genes could be identified for 2 of the regions, the previously known CDKN2A (p16) in 9p21, and TRAF6 and RAG1 in 11p13. In addition, seven high-level amplifications were identified to which possible target genes were assigned to five, including, among others, E2F3 in 6p22, CCND1 in 11q13, and CCNE1 in 19q13, all 3 genes regulate the cell cycle in a positive fashion. Hurst et al. performed a similar study on UC derived cell lines and identified in addition to homozygous deletions in 9p21 (CDKN2A), homozygous deletions in 9q33, including the gene DBC1, and in 10q23, including the known tumor suppressor gene PTEN [14]. These authors further characterized the 6p22 amplification and could associate increased expression of the E2F3 gene with an increased gene copy number, making E2F3 a likely target for 6p22 amplifications. Blaveri et al. [15] corroborated many of the previously found genomic amplifications by a study that included tumors derived from 98 patients. Apart from validating previously amplified regions and possible target genes in 6p22 (E2F3) and 11q13 (CCND1), these authors also reported the genomic amplification of MDM2, a TP53 antagonist. Heidenblad et al. [16] used a high resolution BAC array platform to verify the association between 6p22 amplification and increased $E2F3$ expression and to delineate the extension of 8 additional genomic amplifications. In addition, this author noted a remarkable clustering of homozygous deletions to chromosome 9. Blaveri and coworkers [15] also analyzed their data from the perspective of genomic instability. They confirmed previous studies using conventional CGH that Ta tumors generally show very few genomic changes compared with T1 and \( \geq T2 \) cases, and that G3 tumors showed the most rearranged genomes, irrespective of stage. By hierarchical clustering of the muscle-invasive tumors, from which survival data were available, it was possible to identify three classes of tumors based on the number of changes. The group of patients with the less rearranged genomes in their tumors showed a significantly better survival than patients with moderate and highly rearranged tumor genomes. The influence of the extent of genomic rearrangement on overall survival was also shown by Cox proportional hazards analysis. Single aberrations could, however, not be associated with survival. A similar strong association between high grade and high stage, and signs of genomic instability was observed by Heidenblad et al. [16]. Hence, both these studies point to the possible role of genomic instability in UC tumor progression.

**Molecular analysis of the bladder cancer genome, loss of heterozygosity analysis**

Comparative genome hybridization, and particularly array based CGH, is a superior method for identification and molecular mapping of amplified genomic regions. The method is, however, less sensitive to losses. There are mainly two reasons for this. Losses of genomic material generally comprise the loss of 1 copy and only in rare occasions of two copies (i.e., homozygous deletions) and the resulting 50% reduction in signal intensity may be hard to detect in a heterogeneous material. In addition, lost material may be reduplicated. In this latter scenario, no changes will be observed at the chromosomal level. However, such a loss and duplication process may have an adverse effect at the genetic level. A typical scenario for the inactivation of a tumor suppressor gene is that the normal diploid cell (wt/wt) acquires a gene mutation in a tumor suppressor gene (wt/mut). To fully inactivate the tumor suppressor gene, an inactivation of the remaining wt allele is required, frequently accomplished by chromosomal deletions resulting in a \(-/mut\) genotype. If the remaining chromosome with the mutated version of the gene is reduplicated the cell will obtain two seemingly normal chromosomes but the cells will be mut/mut at the genotype level. Both the \(-/mut\) and the mut/mut situations may be detected by a loss of heterozygosity analysis (LOH), but
only the formed with CGH. A LOH analysis requires that the patient is heterozygous (A/a) for a number of genetic markers covering a region of interest. If such a region has been lost in a tumor, an analysis of the patient’s blood will result in A/a genotype, whereas only one of the alleles, A or a, will be detected in the tumor biopsy. The tumor has lost one of the original alleles and hence the term loss of heterozygosity.

Due to the necessary individual handling of DNA markers most LOH studies has been directed to limited regions of the genome. However, by the advent of microarray technology and the use of single nucleotide polymorphism (SNP) whole genome analyses has been made possible. Primdahl et al. [17] analyzed UC tumors with a panel of 1,494 SNP markers. Muscle-invasive cases showed a higher frequency of LOH but the difference was not statistically significant. A significant association between inactivation the TP53 gene was however established. Five chromosomes 6, 8, 9, 11, and 17 demonstrated hot spots for LOH indicating the presence of candidate tumor suppressor genes. Three regions were observed on chromosomes 6, 8, and 9, respectively, and single hotspots on chromosomes 11 and 17. Many of the hotspots coincided with regions previously detected by array-CGH. Hoque et al. [18] performed both SNP and microsatellite analyses. In their analysis, 25 out of 39 chromosome arms showed allelic imbalances in at least 30% of the cases, indicating a remarkable heterogeneity in UC genomes. LOH in 9p was seen in 70% and in 9q in 77% of the cases indicating chromosome 9 as the most frequently affected chromosome in UC, followed by LOH at 8p (50%), 20q (43%), and 8q (43%). The frequency of LOH at 9p and 9q was equally distributed among the tumor stages, whereas allelic losses at the remaining chromosomes correlated with higher stages. A similarly high frequency of allelic loss of chromosome 9 was reported by Koed et al. [19], with LOH at 9p amounting to 42% and in 9q to 67%. Almost all chromosome arms showed instances of allelic losses, except 16p, 17q, and 19p, which seemed to be remarkable stable. Both Hoque et al. [18] and Koed et al. [19] also noted that Ta tumors show a significantly lower frequency of LOH than T1 tumors, whereas T1 and ≥T2 cases were similar in this respect. Also Chan et al. [20] demonstrated an association of LOH with high grade and high stage. These authors also found a high incidence of LOH at 9p and 9q, 76% and 67%, respectively. Hence, LOH analyses of UC have revealed a number of genomic regions of possible importance for UC development.

Genomic alterations in carcinoma in situ (CIS), dysplasia, and hyperplasia

Genomic analyses of CIS, dysplasia, and hyperplasia have been hampered by the scarcity of the material and the need for micro dissection. Despite this, some important information on genomic changes in these lesions has been presented. Hartmann et al. [21] showed that CIS demonstrated high frequencies of chromosome 9 and chromosome arm 17p losses. Cases with moderate dysplasia also showed losses of chromosome 9 and chromosome arm 17p, albeit at somewhat lower frequencies. The finding of similar changes in CIS and various stages of dysplasia were corroborated by Krause et al. [22]. Zieger et al. [23] analyzed 12 samples of CIS and 4 samples of low grade dysplasia. Of the 12 CIS cases, 9 showed several genomic alterations reminiscent of advanced muscle invasive UC, whereas 3 showed no changes. None of the low grade dysplasia showed any changes. Even though the data are scarce, one may conclude that genomic alterations may already occur in dysplasia, and that carcinoma in situ may show complex genomic rearrangements.

Hyperplasias have also been found to harbor genomic alterations. Hartmann et al. [24] showed by fluorescent in situ hybridization (FISH) analysis that 9 out of 12 cases showed losses at the CDKN2A locus, of which one showed a homozygous deletion. Chow et al. [25] verified the frequent chromosome 9 losses in hyperplasia by using LOH analyses that also indicated losses of 11p and 17p. Oberman et al. [26] used both CGH and LOH to analyze hyperplasias from 10 patients. The CGH analysis revealed losses of chromosome 9 in 5 out of 10 cases and LOH in 8 cases, indicating losses of chromosome 9 as an important event in this precancerous lesion. One case showed a seemingly homozygous deletion of 9p21, the CDKN2A locus. In many cases, additional alterations were detected, such as losses of 11p and gains of 11q13. In the same study, papillary tumors were analyzed by the same methods and the authors conclude that the number of chromosomal abnormalities in hyperplasia does not differ from what is detected in papillary tumors. Also van Oers et al. [27] detected frequent losses of chromosome 9 by LOH, seen in 7 out of 24 patients. These investigators also detected FGFR3 mutations in hyperplasia, a frequent and characteristic mutation in papillary UC of low grade and stage. Taken together, hyperplasia shows a spectrum of genomic alterations that is similar to papillary tumor.

Genomic alterations as prognostic tools in bladder cancer

The accumulated genomic data have revealed several features of the bladder cancer genome. Frequent changes, such as loss of chromosome 9 and loss of 9p21, the CDKN2A loci, in particular, have been identified. It has also become well established that high grade tumors show an increased number of genomic alterations, many of which are only detected in the more aggressive forms. The observed strong association between genomic profiles and tumor behavior has initiated several studies with the aim to investigate chromosomal aberrations as possible prognostic markers. Very early, the UroVysion system for bladder cancer
surveillance was approved by the Food and Drug Administration (FDA) and has been used in several investigations since (Table 1). The UroVysion system is based on in situ hybridization to human urine specimens and scores the number of chromosomes 3, 7, and 17, as well as the number of copies of the CDKN2A locus. It has, however, been debated to what extent the UroVysion assay may replace standard cytoscopy for tumor surveillance [28]. Zellweger et al. [29] modified the criteria for tumor classification using UroVysion and obtained improved results. This may indicate that the assay may need to be adjusted. One may also question the set of markers used in this assay. It was approved by FDA in 2001 and, after this, several large-scale whole genome array analyses has been performed. A critical evaluation of the more recent data may change the genomic regions to be included, as well as the exact loci that should be targeted. For instance, the UroVysion probe set includes a marker for chromosome 7. Gains of chromosome 7 were frequently seen in the earlier cytogenetic analyses but are less frequently seen in later CGH analyses. Additional genomic regions that are altered preferentially in aggressive tumors include gains of the short arms of chromosomes 5 (5p) and 6 (6p), and losses of the short chromosome arm of 17 (17p) and the long arm of chromosome 13 (13q). Detailed mapping of these regions has made it possible to link these changes with alterations of particular genes. Hence, losses of 17p and 13q have been associated with the tumor suppressor genes TP53 and RB1, respectively, and amplifications of 6p with the oncogene E2F3, which would make it possible to design more specific genomic markers for these regions.

That more precise markers selected by biological knowledge may improve the power was shown by Cai et al. [30]. These investigators examined the prognostic use of 9p21 deletions in connection with bacillus Calmette-Guerin (BCG) treatment. The authors chose to use the gene IFN-α (interferon alpha) as a marker for 9p21 deletion, and not the more commonly used CDKN2A located very closely. The rationale was that IFN-α is involved in the immune response elicited by the BCG treatment and that deletions of CDKN2A do not always include deletions of IFN-α. Loss of IFN-α locus was shown to have a significant impact on the response to BCG treatment. This is one of few investigations that link outcome with the alteration of a genomic

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BCG = bacillus Calmette-Guerin; FISH = fluorescent in situ hybridization; LOH = loss of heterozygosity.
regions directly implicated in the response to treatment. There have been several attempts to link changes of specific genomic changes to prognosis. Eguchi et al. [31] showed an association between loss of 8p23 and prognosis and Tzai et al. [32] showed an association with concomitant LOH at 9p and 14q and poor prognosis. Uchida et al. [33] showed that LOH at 9p21 (the CDKN2A locus) concomitant with LOH at 18q21 were an independent prognostic marker, but not when the markers were treated separately. Even though significant associations have been obtained, these results need to be replicated in larger cohorts to prove useful. Furthermore, even though significant results may be obtained in univariate tests, the critical examination is in the multivariate situation. The results of Tzai et al. [32] and Uchida et al. [33] indicate that the status of more than one chromosomal region has to be scored to produce working prognostic markers (Table 1). This is in line with the results obtained by Prat et al. [12] and Blaveri et al. [15] using CGH, which emphasize the importance of the genomic profiles in contrast to individual chromosomal regions. An alternative approach would thus be to put more weight on the number and type of changes than on which particular changes are present. This leads to the question if genomic instability by itself is an informative marker. Jin et al. [34] followed this approach and scored the numbers of metaphase bridges in pathologic slides of UC. The presence of metaphase bridges is the consequence of chromosomal fusions caused by so called breakage-fusion-bridge cycles that is known to be a major cause for chromosomal changes [35]. Hence, the presence of metaphase bridges is a sign on ongoing genomic instability. Indeed, Jin et al. [34] could show that the presence of metaphase bridges has an influence on prognosis. Genomic stability is also influenced by the function of centrosomes. The centrosome is a major microtubule organizing center for the formation of the bipolar mitotic spindles and plays an important role in accurate chromosome segregation to daughter cells. Changes in the centrosome function may thus lead to missegregation of chromosomes to daughter cells and, hence, genomic instability. Yamamoto et al. [36] used immunohistochemistry to evaluate the status of centrosomes in pathologic slides of bladder cancer specimens. They could show a significant association between an increased number of centrosomes and shorter recurrence-free survival as well as shorter progression-free survival. Taken together, with the recent data on genomic alterations in UC, it would be possible to design a set of markers that better represents genomic segments important for UC development than the markers used so far. The results from whole genome analyses suggest that such a set of markers should be used to create genomic profiles, as genomic markers tend to show a low prognostic impact when used individually. Optionally, prognostic genomic profiles could be complemented with information on general genomic instability.

Genomic aberrations in normal mucosa of UC patients, implications for prognostication

It is generally accepted that tumors evolve by the accumulation of genetic changes. Many of these changes are irreversible or are very unlikely to revert by chance. Thus, by establishing the genetic changes in a series of tumors (e.g., from the same patient) it would be possible to determine a genetic order of the samples. If recurrences originate from the tumors previously resected and several recurrences are sampled from the same patient, it would be expected that the genetic order of the tumors determined by the acquired genetic changes would coincide with the chronology of tumor presentation. This is, however, not the case. Van Tilborg et al. [37] studied 11 patients with 5 or more recurrences using LOH and mutation analyses. For each patient a chronology of the recurrences based on genetic events was established. However, no correlation was found between the genetic chronology and the chronology of tumor appearance; recurrences with complex chromosomal changes could appear long before clonally related recurrences with simple chromosomal changes. Hence, a tumor with a bad prognosis genomic profile could very well be followed by a recurrence with a more favorable genomic profile. These findings have been corroborated in a recent study using CGH, LOH, and mutation analyses by Lindgren et al. [38] and suggest that the recurring tumor could not have evolved directly from the previous and resected tumor. It also suggests that the cells producing the late recurrences would have been present in the urothelium at the time of the previous resection. These findings imply that cells or segments of the urothelium, with differently evolved but clonally related genomes, may coexist and produce overt tumors independently.

This conclusion is substantiated by the observed presence of genomic alterations in both precancerous lesions and in morphologically normal urothelium in patients with UC. FISH studies has shown that morphologically normal urothelium from patients with UC demonstrate similar chromosomal changes as may be seen in the tumor proper [39,40]. Genomic imbalances in normal urothelium have also repeatedly been shown by CGH and LOH [41,42]. Hence, genetically aberrant but morphologically normal cells appear to surround the growth of carcinomas. Extensive histologic and genetic maps of cystectomized bladders have revealed that specific genetic alterations may be seen in the invasive and the pre-invasive phases of the urothelial neoplasia, as well as in the microscopically normal urothelium [43,44]. The detailed mapping of bladders from patients with UC has thus shown that areas with genetic alterations may cover large parts of the urothelium. Some alterations are present in all mapped segments, indicating large clonal segments, whereas others may be scattered and show “islands” of changes. These findings indicate that tumors arise locally in genetically heterogeneous fields of the urothelium. With this in mind, one may question the
possibility to predict, with good confidence, future recurrence and progression events from the characteristics of locally resected tumors. The information needed to establish a proper prognosis regarding recurrences and tumor progression might be in the extension and the level of heterogeneity in the genetically aberrant but morphologically normal urothelium.

References


