Identification of Cytogenetic Subgroups and Karyotypic Pathways in Transitional Cell Carcinoma

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ABSTRACT

The clinical course in urinary bladder cancer is difficult or impossible to predict based on conventional disease parameters. It is a reasonable hypothesis that the genetic aberrations acquired by the tumor cells, being instrumental in bringing about the disease in the first place, may also hold the key to more reliable prognostication. However, though 200 transitional cell carcinomas (TCC), the most common bladder cancer in the Western world, with clonal chromosomal abnormalities have been reported, our knowledge about the karyotypic characteristics of these tumors remains insufficient. The aberration pattern is clearly nonrandom, but no completely specific primary or secondary karyotypic abnormality has been identified, and the chronological order in which the aberrations appear during disease progression is not well known. The high degree of karyotypic complexity in epithelial tumors like TCC is one reason why our picture of the sequential order of cytogenetic evolution is unclear. To overcome some of these difficulties we have used several statistical methods that allow analysis and interpretation of the relationship between cytogenetic aberrations in TCC. We show that there exists a temporal order with respect to the appearance of chromosomal imbalances and that this order is highly correlated with tumor stage and grade. Analyzing changes in the distribution of imbalances per tumor in G1, G2, and G3 tumors, we suggest that progression involves the acquisition of cytogenetically detectable and submicroscopic genetic changes at comparable frequencies. By means of computer simulations, we show that the imbalances −9, +7, and 1q+ appear earlier than expected from random events and that −6q, −5q, −18, +5p, −22p, and −15 appear later than expected. Using principal component analysis, we identify two cytogenetic pathways in TCC, one initiated by −9 and followed by −11p and 1q+, the other initiated by +7 and followed by 8p− and +8q. The −9 pathway was correlated with stage Ta-T2 tumors, whereas the +7 pathway was correlated with stage T1-T3 tumors, i.e., +7 tumors appeared to be more aggressive. Although these pathways are well separated at earlier stages, they later converge to contain a common set of imbalances.

INTRODUCTION

The past two decades have seen a rapid progress in our understanding of the biology of human cancers. A large body of evidence now supports the concept that cancer arises through a multistep process involving the accumulation of somatic mutations (1–3), a process which may proceed over many years. In many tumors, the mutations are microscopically visible as nonrandom and often disease-specific chromosomal aberrations. The cytogenetic aberrations may be either primary or secondary (4). Primary changes are believed to be essential in establishing neoplastic transformation. They are frequently found as sole anomalies and are usually specific for a certain type of tumor. Secondary changes arise in cells already carrying a primary aberration; they too are usually nonrandom and reflect the clonal evolution during tumor progression (5). In contrast with what is seen in leukemias, most solid tumors have already at the time of diagnosis accumulated many secondary chromosomal rearrangements and possibly an even larger number of changes at the molecular level. The high degree of karyotypic complexity, which in part may represent cytogenetic noise, makes it exceedingly difficult to determine both the primary anomalies and the elements of cytogenetic evolution. To overcome some of these difficulties, we have developed and adapted several statistical methods that allow the analysis and interpretation of complex genomic imbalances in solid tumors (6).

BC, mostly TCC, is a common malignancy, being the fourth most common cancer among men in Europe and the United States (7). The clinical course of BC is very difficult to predict based on prognostic factors such as tumor grade and stage, multifocality, tumor shape, location, and the presence of carcinoma in situ (8). The identification of novel progression markers associated with aggressive disease behavior might help design new therapeutic strategies to be tried out in progression-prone cases, and the acquired genetic aberrations of TCC would seem to be prime candidates for such a role. Although >200 cases of BC with clonal karyotypic abnormalities have been reported, knowledge about their cytogenetic profile remains meager. The aberrations are clearly nonrandom, but no completely specific primary or secondary karyotypic abnormality has been identified, and the chronological order of how chromosomal changes are acquired during disease progression is not well understood. In the present study, we constructed a genomic imbalance map from all of the reported BC cases with abnormal karyotypes and used this map to identify the most frequent imbalances. Tumors were then classified with respect to the presence or absence of these imbalances and statistically analyzed to identify and assess karyotypic profiles, correlations between imbalances and the stage and grade of the tumor, and the chronological order of appearance of the chromosomal imbalances.

MATERIALS AND METHODS

Selection of Material. The present study was based on the cases of BC with acquired clonal chromosome aberrations included in the Mitelman Database of Chromosome Aberrations in Cancer. Tumors with loss of the Y chromosome as the sole anomaly were excluded, and multifocal tumors were considered as one tumor. A total of 200 tumors met the inclusion criteria. When constructing the imbalance map, only the basic clone for each tumor was entered, i.e., subclones or duplicated aberrations acquired during clonal evolution were not registered. When the same chromosome was involved in both numerical and structural aberrations, only the total net imbalance was recorded. The frequencies for the individual imbalances were calculated by dividing the number of cases with loss or gain of a particular chromosome band with the total number of cases; for the Y chromosome the frequencies were based on male patients only. Imbalances (involving whole chromosomes, chromosome segments, or chromosome bands) found in ≥10% of the cases were selected for the present study (Table 1). When two or more neighboring bands exceeded the 10% limit, they were combined to form a contiguous region of imbalance. Each case was then evaluated with regard to involvement...
Multivariate Analyses of BC Karyotypes

Table 1 Frequency of imbalances

<table>
<thead>
<tr>
<th>Imbalancea</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1p22-pter (1p−)</td>
<td>15</td>
</tr>
<tr>
<td>+1q21-pter (1q+)</td>
<td>16</td>
</tr>
<tr>
<td>−2p10-pter (−2p)</td>
<td>15</td>
</tr>
<tr>
<td>−3p13-14 (3p−)</td>
<td>13</td>
</tr>
<tr>
<td>+4p10-pter (+4p)</td>
<td>14</td>
</tr>
<tr>
<td>−5q11-pter (−5q)</td>
<td>19</td>
</tr>
<tr>
<td>+5p11-pter (+5p)</td>
<td>12</td>
</tr>
<tr>
<td>−6q10-pter (−6q)</td>
<td>19</td>
</tr>
<tr>
<td>+7</td>
<td>19</td>
</tr>
<tr>
<td>−8p21-pter (−8p)</td>
<td>23</td>
</tr>
<tr>
<td>+8q10-pter (+8q)</td>
<td>17</td>
</tr>
<tr>
<td>−9</td>
<td>12</td>
</tr>
<tr>
<td>−10</td>
<td>12</td>
</tr>
<tr>
<td>−11p11-pter (−11p)</td>
<td>27</td>
</tr>
<tr>
<td>−15</td>
<td>12</td>
</tr>
<tr>
<td>−16</td>
<td>12</td>
</tr>
<tr>
<td>−17p11-pter (−17p)</td>
<td>17</td>
</tr>
<tr>
<td>−18</td>
<td>14</td>
</tr>
<tr>
<td>−22p10-pter (−22p)</td>
<td>12</td>
</tr>
</tbody>
</table>

* Abbreviations in parenthesis.

MULTIVARIATE ANALYSES OF BC KARYOTYPES

Of each of the selected regions, treating each imbalance as a binary variable, i.e., present or absent. The initial analyses included calculations of basic statistics, such as relative frequencies, correlation coefficients, and distributions of the NIPT.

To investigate if any temporal pattern could be identified, we defined early and late imbalances as those predominantly present in tumors with few and many imbalances, respectively. To this end, all of the tumors with a given imbalance were selected, and the distributions of NIPT were plotted. The modes of these distributions were used as a value for lateness and referred to as the TO. Thus, TO is a function of karyotypic complexity and when imbalances are referred to as late or early it is in this specific sense. The TOs for the different imbalances were tested by a resampling method in which the variability. In Fig. 3, one can discern two imbalance clusters, a large one consisting of 1p−, −2p, −4p, −10, −15, −16−18, and −22p, and a minor one including 3p−, +8q, 8p−, and −17p. Separated from these clusters, on the right side, are located the early imbalances −9, 1q+, and +7, and also −11p. The late imbalances −5q, +5p, and −6q were located between the two clusters on the opposite side of the early imbalances. Furthermore, the early imbalance −9 was negatively correlated with +7 (P = 0.002), 3p− (P = 0.022), 8p− (P = 0.025), and +8q (P = 0.046) but positively correlated with −11p (P = 0.002), whereas +7 was positively correlated with 3p− (P = 0.013). The first principal component corre-

RESULTS

Frequencies, Distribution, and Temporal Order of Imbalances.

Of the selected 19 imbalances, the majority showed frequencies of 12–19% (Table 1). The most frequent imbalance was −9 (42% of the cases) followed by −11p (27%) and 8p− (23%). The distribution of the NIPT showed a geometrical distribution with very few tumors having >10 imbalances (Fig. 1A). The same type of distribution was obtained when all of the aberrations, including also imbalances less frequent than 10%, balanced translocations, and marker chromosomes, were included (Fig. 1A). The temporal analysis revealed loss of chromosome 9 to be the earliest imbalance followed by gain of chromosome 7 (Fig. 2). Both these imbalances appeared earlier (TO = 1) than expected from the simulation. Among the moderately early imbalances (TO = 2–4), 1q+ appeared earlier than expected from the simulation, whereas of the late imbalances (TO > 4), −5q, +5p, −6q, −15, −18, and −22p appeared later than expected from the simulation.

Correlations among Imbalances. The relationships between the imbalances were investigated by PCA. The three first principal components explained 36%, whereas the two first ones explained 28% of the variability. In Fig. 3, one can discern two imbalance clusters, a large one consisting of 1p−, −2p, −4p, −10, −15, −16−18, and −22p, and a minor one including 3p−, +8q, 8p−, and −17p. Separated from these clusters, on the right side, are located the early imbalances −9, 1q+, and +7, and also −11p. The late imbalances −5q, +5p, and −6q were located between the two clusters on the opposite side of the early imbalances. Furthermore, the early imbalance −9 was negatively correlated with +7 (P = 0.002), 3p− (P = 0.022), 8p− (P = 0.025), and +8q (P = 0.046) but positively correlated with −11p (P = 0.002), whereas +7 was positively correlated with 3p− (P = 0.013). The first principal component corre-

Fig. 1. Distributions of the number of imbalances and aberrations per tumor. A, the distribution of imbalances present in more than 10% of the tumors (NIPT) and the distribution of the number of aberrations per tumor (NAPT) including imbalances less frequent than 10%, balanced aberrations, and marker chromosomes. B, the distribution of NIPT in G1, G2, and G3 tumors.
lated well with the TA (r = 0.84; P < 0.001). By combining the data obtained from the TA and the PCA, two cytogenetic pathways emerge. One is characterized by −9 as the initiating imbalance, has −11p and/or 1q as frequent secondary events, and 1p−, −4p, −6q, −5q, +5p, −10, −15, −16, −17p, and −18 as late changes. In the second pathway, the early imbalance +7 is followed by 3p−, 8p−, +6q, and −6q, −5q, and +5p as late changes.

**Clinical Correlations.** The average NIPT increased with stage (2.1, 3.3, 4.5, and 5.4 for Ta, T1, T2, and T3 tumors, respectively) and with grade (1.9, 3.2, and 5.2 for G1, G2 and G3 tumors, respectively). Grade and stage were strongly correlated (P = 0.001). An analysis of the distributions of NIPT in G1, G2, and G3 tumors (Fig. 1B) revealed geometrically shaped distributions with increasing averages, which in G3 tumors produced an almost flat distribution. Thus, although stage and grade are correlated with complex karyotypes, many of the high stage and grade tumors have simple karyotypes.

The cases were divided into Ta, T1, T2, and T3 tumors, and the frequency for each imbalance was calculated and plotted (Fig. 4). The frequencies for 11 of the 19 selected imbalances showed significant dependence on stage. The early imbalance −9 was most frequent in Ta and T1 tumors, 49% and 54%, respectively, but showed decreased frequencies in T2 (25%) and T3 (10%) tumors. Loss of chromosome 9 was positively correlated with T1 but negatively correlated with T3 tumors. Gain of chromosome 7, the second early imbalance, was seen in 7% of Ta but in 26% of T1 tumors. The frequencies declined slightly in T2 (17%) and T3 (21%). Gain of chromosome 7 was positively correlated with T1 but negatively correlated with Ta tumors.

The imbalances 1p−, 1q+, −2p, 3p−, −4p, +5p, −5q, −6q, 8p−, −8q, −18, and −22p increased in frequency with increasing tumor stage. Imbalances seen at particularly high frequencies (>35%) in T3 tumors were −2p, 3p−, −4p, −5q, −6q, 8p−, and +8q. Of these, −2p, −4p, −5q, and −6q were positively correlated with T3 tumors but negatively correlated with Ta tumors. Loss of 3p showed a sharp increase in frequency in T3 tumors, from 10% in Ta-T2 tumors to 37% in T3 tumors. Another group of imbalances, including −10, −11p, −15, and −16, showed higher frequencies in T1 and/or T2 but lower frequencies in T3 tumors. Taken together, the data indicate that −9 is associated with Ta-T2 tumors and tends to be accompanied by −10, −11p, −15, and −16 as possible secondary changes, whereas +7 is associated with T1-T3 tumors and often has −2p, 3p−, −4p, −5q, −6q, 8p−, and +8q as possible later imbalances.

As the imbalances +5p, −5q, and −6q were well separated from the remaining imbalances in the PCA of the imbalances, tumors containing these three imbalances were analyzed separately. This group of tumors was found to have +7 in 20% and −9 in 40% of the cases and consisted only of T2 and T3 tumors. However, when these tumors were grouped according to stages T2 (n = 8) and T3 (n = 9), +7 was found to be absent from the T2 but present in 3/9 of the T3 tumors, whereas −9 was seen in 4/8 of the T2 and in none of the T3 tumors. Thus, although −5q, +5p, and −6q are late imbalances, they appear at different tumor stages depending on the previous imbalances.

**Analysis of the Tumor Population.** A PCA was performed in which the tumors were the variables and the imbalances the cases. The number of imbalances present in each individual tumor was also included as a parameter, in addition to the presence of specific imbalances; this procedure groups tumors with similar complements of imbalances and number of imbalances close together in the resulting PCA diagram. In Fig. 5A, six clusters of tumors are seen in which each tumor is organized in order of karyotypic complexity, i.e., less complex tumors in the periphery and more complex tumors toward the center. The obtained structure in Fig. 5A was systematically analyzed for imbalances that organized the individual clusters, i.e., they were present in all of the tumors encompassing one or more of the identified subgroups. It was found that all of the subgroups could be characterized by the presence or absence of +7, −9, −11p, or combinations thereof. In Fig. 5B, seven clusters were defined with respect to the presence or absence of +7, −9, or −11p. The tumors...
located between the +7 and −11p tumors, brown in Fig. 5B, were characterized by the absence of all three imbalances.

In the latter group of tumors, characterized by the absence of +7, −9, and −11p, the NIPT (2.9) was lower than that of the tumors with −9 or +7, i.e., 3.7 and 4.2, respectively. The distribution of NIPT in this group was similar to the overall distribution of the tumors and ranged from 1 to 12, with 95% of the tumors having less than seven imbalances. The frequencies for superficial (Ta and T1) and invasive tumors (T2 and T3) as well as for G1, G2, and G3 tumors were calculated for this tumor group and compared with the remaining group of tumors, i.e., those with +7, −9, or −11p with NIPT below 7. The frequency of invasive tumors was significantly higher \( p < 0.015 \) as was the frequency of G3 relative to G2 \( p < 0.012 \) among tumors without +7, −9, or −11p. Thus, this group of tumors is characterized by the absence of the early changes +7, −9, and −11p, a lower NIPT, and by being more advanced.

**DISCUSSION**

**Mode of Karyotypic Evolution in TCC.** The distribution of NIPT for BC was geometrical, similar to what has been described previously for breast and colon cancer, renal cell carcinomas, and neuroglial tumors (6). A biological interpretation of such a distribution could be that the acquisition of changes is relatively rare, that the various aberrations have the same probability of occurring, and that the rate of acquisition is independent of earlier changes. As no subpeak was seen in the distribution, we conclude that all of the tumors developed by the same mode of karyotypic evolution, i.e., no tumors with a relative increase in karyotypic instability, seem to be present. A similar distribution was obtained when all of the aberrations were included in the analysis, indicating that the acquisition of all of the cytogenetically detectable aberrations followed the same mode. Progression from G1 to G3 was associated with the acquisition of additional imbalances, although a large proportion of G2 and G3 tumors contained only one imbalance. The change in the NIPT distributions from G1 to G3 conforms to a model in which progression involves the acquisition of both cytogenetically detectable and undetectable genetic changes at comparable probabilities. Given a multistep model of tumor progression, this means that not all of the genetic steps in progression are accounted for by visible chromosome aberrations.

The TA revealed a distinct order of appearance of the common imbalances. Loss of chromosome 9 and gain of chromosome 7 both appeared earlier than expected from the simulations indicating that they are important in the first stages of tumorigenesis. Of the nine imbalances having TOs of 5 or more, six (+5q, +5p, −4q, −15, −18, and −22p) appeared later than expected from the simulations, indicating that they are important in tumor progression. Their selective value may be dependent on the presence of other earlier imbalances. The TA showed a good correspondence to tumor stage. The two early imbalances, −9 and +7, were positively correlated with T1. Of the four imbalances (−10, −11p, −15, and −16) showing positive correlation to T2, only two (−15 and −16) had TOs larger than 5, and only one (−15) appeared later than expected from the simulation.
whereas six (3p−, −4p, −5q, +5p, −6q, and −22p) of the nine imbalances showing positive correlation to T3 had TOs of 5 or more, and four (−5q, −6q, +5p, and −22p) appeared later than expected from the simulations. Furthermore, many of the later imbalances, which showed a positive correlation with stages T2 and T3, showed a negative correlation to Ta. Thus, the temporal appearance of imbalances in BC is highly ordered and correlated with stage.

Two Cytogenetic Pathways in TCC. In the PCA of the imbalances, the first principal component was found to correspond well to the TA and, thus, approximated a time axis within the representation. The two early imbalances −9 and +7 were well separated in the PCA and also showed negative correlation. This shows that −9 and +7 represent alternative early steps in the karyotypic evolution of BC. Three imbalances, 3p−, 8p−, and +8q, were positioned close to +7 in the PCA diagram and were also negatively correlated with −9, which suggests a cytogenetic pathway with +7 as an early imbalance followed by 8p−, +8q, and 3p−. Loss of chromosome 9, on the other hand, was located close to the imbalances −11p and 1q+, suggesting that these, particularly −11p, are secondary changes to the loss of chromosome 9.

To expand the analysis of the cytogenetic events, the tumors were subjected to PCA. To resolve the complex PCA pattern obtained, the NIPT was added as an observation for each individual tumor. This procedure produced a characteristic structure with seven clusters of tumors. By systematically analyzing these clusters for imbalances that were present in all of the tumors in a given subgroup, three cluster-determining imbalances were identified: +7, −9, and −11p. Either the presence or absence of these three imbalances could describe all of the potential subgroups. This adds weight to the interpretation that −9, −11p, and +7 are important in the development of BC.

In the analysis of the frequencies of imbalances with respect to tumor stage, −9 behaved in an extraordinary way, being very frequent in Ta and T1 tumors, 49% and 52%, respectively, but rare in T3 tumors (10%). Furthermore, −9 showed a positive correlation with T1 tumors but a negative one to T3 tumors. Thus, the low frequency of −9 in high-stage tumors is highly significant. The imbalances −10, −11p, −15, and −16, shown to be associated with −9 in the PCA, all showed a positive correlation with T2 tumors and a decreased frequency in T3 compared with T2 tumors. This suggests the existence of a cytogenetic pathway through −9 with −11p and 1q+ as secondary changes followed later by −15, −16, and −10. The −9 tumors seem to progress from stage Ta to T1 and possibly to T2, but very rarely to T3. Although the frequency of 9p (9%) and 9q (6%) losses did not reach the inclusion criteria, the importance of −9 in BC (11) motivated an analysis also of these partial imbalances. Loss of 9q was found to appear relatively early (TO = 3) and was seen earlier than expected from the simulation. Furthermore, the frequency of −9q declined with stage, being 10% in Ta and absent in T3 tumors. Losses of 9p material, on the other hand, increased in frequency with stage, from 5% in Ta to 11% in T3, and appeared later than −9q (data not shown). Thus, loss of 9q material behaved similarly to loss of the entire chromosome 9, being an early imbalance predominantly present in low-stage tumors, whereas −9p appeared to be a rather late event.

Gain of chromosome 7 was seen in 7% of the Ta tumors but in 26% of the T1 tumors. Gain of chromosome 7 was also negatively correlated with Ta tumors and positively correlated with T1 tumors. Furthermore, the frequency of +7 was maintained (~20%) in T2 and T3 tumors. This, together with the previous results, suggests the existence of a second cytogenetic pathway occurring in more aggressive tumors than those of the −9 pathway characterized by the changes +7, 8p−, +8q, and 3p−. The specificity of the −9 pathway for Ta-T2 tumors and the +7 pathway for T1-T3 tumors was particularly apparent in tumors with −6q, −5q, and +5p. The frequencies of −9 and +7 in this advanced group of tumors, which were either T2 or T3, were 40% and 20%, respectively. However, when this group was divided into T2 and T3 tumors, half of the T2 tumors contained −9 and none +7, whereas one-third of the T3 tumors contained +7 and none −9.

A large subgroup of TCCs showed absence of the imbalances −9, +7, and −11p. These tumors also demonstrated a lower NIPT. Despite this, a higher frequency of advanced tumors, invasive versus noninvasive and G3 versus G2, was seen in this class of tumors. As the TA showed a highly ordered temporal appearance of genetic events in TCCs, leading to the more advanced stages, this suggests that these tumors may have cytogenetically cryptic changes equivalent to the early events and, therefore, show a lower NIPT. Thus, given a multistep model of tumorigenesis, the initiating events of TCC may not always be detectable as cytogenic changes.

Final Conclusions. The presented data indicate the presence of at least two cytogenetic pathways in BC, the first characterized by the loss of chromosome 9, the second by the gain of chromosome 7 (Fig. 6). Loss of chromosome 9 was predominantly followed by −11p and 1q+ and then by −17p. Of the later imbalances, −10, −15, and −16 were predominantly associated with −9. Gain of 7 was followed by 8p− and +8q, both of which were highly specific for the +7 path-
way, and by −17p. Furthermore, 3p− was a specific late event in the +7 pathway. Although these events, except for −17p, had the character of being pathway-specific, the two well-separated cytogenetic routes converged at subsequent stages as both accrued the imbalances -2p, -4p, -5q, +5p, -6q, -5q, +5p, and −2p, −4p, −18, −22p. Richter et al. (12) has presented a similar but not converging pathway of chromosomal events. One interesting feature of the identified pathways is that the −9 pathway predominately leads to Ta-T2 tumors, whereas the +7 pathway leads to T1-T3 tumors. The fact that these two pathways are associated with different levels of tumor aggressiveness but still have many of the very late imbalances in common, indicates that the progression to an aggressive tumor may already be predetermined by the early events in the pathway, something that could possibly be used as a prognostic tool in the clinical management of BC patients.

REFERENCES

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