Amplification and overexpression of the ID4 gene at 6p22.3 in bladder cancer

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Abstract

Background: Amplifications at 6p22.3 are prevalent in advanced stage bladder cancer (TCC). Previous studies have identified SOX4, CDKAL, and E2F3 as targets of this amplification and therefore potential oncogenes, but the more telomeric DEK gene too has been reported as overexpressed and amplified. We have therefore investigated whether the intermediate region harboring the oncogene candidate ID4 is also part of the amplicon.

Results: Expression of E2F3, DEK, and ID4 was investigated by real-time RT-PCR in 28 TCC compared to 6 normal bladder tissues and in 15 TCC cell lines compared to cultured normal urothelial cells. Expression of E2F3 as well as DEK increased on average in tumor vs. normal tissues (3-fold and 2.5-fold, resp.), but only the increase for E2F3 was statistically significant (p = 0.039). ID4 overexpression was observed in selected specimens. Each of the three genes was overexpressed in several cell lines, up to 150-fold (ID4), 30-fold (E2F3), and 9-fold (DEK), but these increases were not correlated to each other. Instead, moderate (DEK) to excellent (ID4) correlations were observed with copy number increases of microsatellites near each gene. Microsatellite copy number increases were highly heterogeneous across the investigated several Mb region revealing at least three subregions of amplification.

Conclusion: Extending previous reports, our data indicate that the 6p22.3 amplicon in TCC is highly heterogeneous and targets several genes in a variable fashion. Among these, expression of E2F3 and DEK appear to be generally increased in TCC, with additional increases caused by amplifications. In contrast, over-expression of ID4, which is normally predominantly expressed in testes and brain, appears to depend more strictly on gene amplification. Accordingly, the effect of amplifications at 6p22.3 in bladder cancer is expected to be non-uniform, thereby contributing to the highly variable biological and clinical behavior of advanced stage tumors. ID4 is a potential oncogene in a small subset of bladder cancers.

Background

Urothelial carcinoma, which is commonly called bladder cancer, occurs in two forms, a more prevalent papillary subtype and a rarer, but much more invasive subtype
[1,2]. Invasive bladder cancers usually develop from highly dysplastic carcinoma in situ, but some papillary tumors also progress to an invasive form. While papillary cancers often contain a limited number of chromosomal alterations, invasive cancers are characterized by a high degree of chromosomal instability [3,4]. Even T1 stage cancers, which have only invaded the lamina propria underlying the urothelium, often exhibit multiple chromosomal changes. Cancers at more advanced stages accumulate further chromosomal alterations. In particular, they harbor amplifications, e.g. of regions from chromosomes 5p, 6p, 8q, 11q, and 20q [4-7]. It is generally assumed that chromosomal segments consistently amplified in a cancer contain oncogenes [8]. Accordingly, genes amplified in advanced bladder cancers would be expected to contribute to the progression of this cancer.

One of the most consistently amplified region in advanced bladder cancers is located at 6p22.3 [5-7,9-12]. This amplification is detected in up to 25% of advanced stage bladder cancers and is present in many bladder cancer cell lines. The cell lines harboring this amplification provide a convenient experimental access to map the amplified region precisely and identify potential urothelial carcinoma oncogenes. Mapping of the 6p22.3 ampli-con has been performed by several groups who have identified different genes as potential targets of the amplification (Figure 1). In a first study [5], SOX4 was identified as a frequent, but not entirely consistent amplification target. Further studies revealed that many amplifications also included E2F3 and the encoded protein was overexpressed, particularly in high stage and high grade urothelial cancers [6,11,12]. A high resolution analysis by microarray-based comparative genomic hybridization identified CDKAL1 located between SOX4 and E2F3 as the most commonly amplified gene [7]. Another study indicated that DEK located further telomerically (Figure 1) may be amplified in a substantial proportion of bladder cancer tissues [10]. DEK was also found to be overexpressed in a cDNA microarray study, albeit predominantly in early stage tumors [12].

This somewhat confusing state may owe partly to the fact that many studies were performed prior to the publication of the finished sequence of chromosome 6 in October 2003 [14] and partly to the use of different techniques. Alternatively, the differences between the studies could...
also mean that the region of amplification is not uniform and that multiple genes might be targets.

Most previous studies have focussed on a more centromeric region within 6p22.3 containing SOX4, CDKAL1, and E2F3 (Figure 1). DEK is located about 2 Mb more telomeric of these genes. The interval framed by E2F3 and DEK contains another plausible oncogene candidate, i.e. ID4. The ID proteins ('inhibitor of differentiation') are named for their ability to bind and inhibit cell-type specific helix-loop-helix transcriptional activators inducing cell differentiation. Accordingly, they tend to stimulate cell proliferation, and have been implicated in various cancers [15-18]. Compared to ID1 and ID2, ID4 is a less well characterized member of the family. It is expressed in a tissue-specific manner, with the highest levels in testes and brain [19]. In the present study, we have therefore investigated to which extent ID4 gene copy numbers and expression are affected by 6p22 amplifications in bladder cancer, in comparison to E2F3 and DEK.

Results
Expression of 6p22 genes in bladder cancer cell lines
First, expression of ID4 mRNA in comparison to E2F3 and DEK mRNAs was investigated by real-time PCR in 16 TCC cell lines (Figure 2). Normal urothelial cells (UP) proliferating in culture and testicular tissue samples served as controls. Expression at least twice as strong as in normal urothelial cells was considered as over-expression. According to this criterion, twelve cell lines over-expressed ID4, with a maximum >150fold increase in HT1376. In 6 cell lines, E2F3 was over-expressed. The E2F3 over-expressing cell lines included HT1376 and 5637 in line with previous reports [7,11]. 5637 displayed a >30fold increased level of E2F3 mRNA. Six cell lines over-expressed DEK, although the relative increases were in general more moderate, with a maximum 9fold increase in RT112. Inspecting Figure 2 may suggest that cell lines over-expressing one gene also tended to over-express one or both others. However, this tendency was not reflected in a statistically significant correlation. Specifically, expression did not significantly correlate for any pair of genes, the best correlation coefficient reaching 0.39 between ID4 and DEK. The divergence is strikingly illustrated by the cell lines HT1376 and 5637, which presented increased expression levels for all three genes, but with either ID4 or E2F3 displaying particularly pronounced increases (Figure 2).

Gene amplification analysis
To determine whether the increases in mRNA expression were due to gene amplification, copy numbers were inves-
tigated for eight microsatellite loci located in 6p22.3. Five were located around ID4, from D6S422 close to E2F3 to DS1946, and three were located around DEK, including the intragenic marker D6S2051 (Figure 1). The results (Figure 3) demonstrate a considerable variation in the microsatellite copy numbers across the region, even within the same cell line. For instance, in HT1376 the copy numbers of the eight microsatellites ranged from approximately 0.5 to 13 normal genome equivalents, which would correspond to 1 – 26 copies in a diploid cell. As all bladder cancer cell lines are aneuploid, typically hypo- or hypertriploid with a modal distribution, between 2 – 40 copies would be present in a single cell. In HT1376 specifically, two different segments of amplification are discernible, one telomeric to DEK and one around ID4. In fact, amplification of the region including E2F3 and of SOX4 has been shown previously in this line [7,9,11]. Thus, there are at least three distinct regions of amplification in this cell line. In contrast, no amplification of microsatellites telomeric of D6S422 was evident in 5637, which contains increased copy numbers of E2F3 and SOX4 [7,9,11]. A more homogeneous increase of all more telomeric markers including those close to ID4 and DEK was seen in J82. RT112 contained a selective amplification of the D6S2051 marker located in the DEK gene in accord with the maximum expression of this gene in this cell line. None of the microsatellites showed increased copy numbers in T24 or SD, although E2F3 as well as DEK, but not ID4 were overexpressed in T24.
Expression of *ID4* correlated excellently with the copy numbers of each of the microsatellites around the gene, yielding coefficients between 0.89 and 0.95 (Figure 4A). A closer inspection shows however that this strong correlation is primarily caused by the cell lines with clear-cut amplifications of the region, i.e. J82 and HT1376 while at lower copy numbers the relationship is essentially random. *DEK* expression correlated moderately well with the copy number of the intragenic D6S2051 marker (Figure 4B). *E2F3* expression showed the best correlation with the microsatellite marker D6S422 located most closely to the gene (Figure 4C). However, this apparent correlation was mostly due to the strongly increased copy number of D6S422 in the 5637 cell line; if this data point is removed, the relationship is essentially random.

**6p22 gene expression in bladder cancer tissues**

To determine whether the results from the cell lines can be extended to bladder cancer tissues, the expression of the three genes was determined in 28 tumor tissue samples and 6 morphological normal samples from cystectomy specimens by real-time RT-PCR (Figure 5, Table 1). In accord with previous reports, expression of *E2F3* mRNA and *DEK* mRNA were often increased in tumor compared to normal tissues. Median *E2F3* mRNA expression was 2.24 arbitrary units in cancers compared to 0.72 in normal tissues, i.e. about threefold higher. This difference

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**Figure 4**

Relationship of 6p22 gene expression changes to copy number changes of adjacent microsatellites in bladder cancer cell lines. A: *ID4* mRNA vs. D6S1128E (r = 0.95), B: *DEK* mRNA vs. D6S2051 (r = 0.57), C: *E2F3* mRNA vs. D6S422 (r = 0.79),
was statistically significant \( (p = 0.039) \). Median DEK mRNA expression was 1.26 compared to 0.52, i.e. 2.5fold higher, but the difference did not reach statistical significance. In contrast, ID4 expression essentially did not differ between normal and cancer tissues. Instead, individual cancer specimens showed strongly increased expression beyond the range of normal tissues.

**Discussion**

Taken together with previous analyses of 6p22.3 amplifications in bladder cancer, the present study has implications concerning the structure of 6p22.3 amplicons, the effect of the amplification on gene expression, and more generally, concepts of the significance of gene amplification in human cancers. Specifically, the findings raise interesting aspects with regard to ID4.

Our findings indicate that 6p22.3 amplifications in bladder cancer are even more heterogeneous than hitherto assumed. Previous studies have identified the centromeric region around CDKAL1 as part of an amplicon that often, but not consistently included SOX4 and E2F3 \([7,9,11,12]\). The telomeric region around DEK had not been investigated as well yet \([10]\). Our study confirms that this region is also subject to copy number gains and amplifications. Specifically, our findings are in good accord with ref. \([10]\) describing frequent copy numbers gains of microsatellites around DEK, but at highly variable frequencies. The three

Figure 5

Expression of genes at 6p22.3 in bladder cancer tissues. Box plot representation of ID4 (left), E2F3 (center), and DEK (right) mRNA expression relative to β-actin mRNA as determined by real-time RT-PCR in 28 bladder cancer tissues (see Table 1) and 6 morphologically normal bladder tissues. Note the different scales in the three figure parts.
markers investigated in that study were located within 0.5 Mb and were amplified contiguously in 24% of the specimens, whereas each of these three markers individually was amplified in 45%, 56%, and 64% of the cases. Thus, upon closer analysis, this region of amplification appears also heterogeneous in itself. The intermediate region harboring \textit{ID4} had been more or less disregarded in previous studies, but our data indicate that it is clearly gained or even amplified in a certain number of cases, sometimes concomitantly with, and sometimes independent of the other two regions. In summary, therefore, one might discern three main segments of amplification, which split up into further subregions in individual cancers. The 6p22.3 amplicon therefore seems to belong to a class which is characterized by pronounced heterogeneity and great structural complexity (see below).

Previous studies have variously identified \textit{SOX4}, \textit{E2F3}, \textit{CDKAL1}, and \textit{DEK} as potential targets of the 6p22.3 amplification; the present study adds \textit{ID4} to this list. In this regard, it is interesting to compare the cell line data, where \textit{ID4} emerged as the most frequently over-expressed gene with the tissue data which showed generalized increases in expression of \textit{E2F3} and of \textit{DEK} (Figure 1 vs. Figure 5). This apparent discrepancy can be relatively simply resolved by two plausible assumptions. Normal bladder tissue is largely quiescent, albeit proliferation increases strongly in response to tissue damage [20]. Thus, urothelial cancers are distinguished from normal tissue not only by expression of cancer-specific genes, but also by generalized over-expression of genes associated with cell proliferation. The generally increased expression of \textit{E2F3} and \textit{DEK} in the cancer tissues may reflect the latter effect, with further increases in individual cases due to deregulation and copy number gains of these genes. In contrast, early passage cultured urothelial cells proliferate as rapidly as cancer cell lines [21-23]. Therefore, the increases in \textit{E2F3} and \textit{DEK} in cultured cancer vs. normal cells may turn out as comparatively moderate. In contrast, \textit{ID4} expression has probably to be considered as ectopic in bladder cancer, since it is normally restricted to other tissues including testes and brain [19]. Thus, overexpression may more strictly depend on amplification of the gene, particularly in tumor tissues.

Table 1: Tumor tissues investigated

| No | Sex | Age | Stage | Lymph node status | Metastasis status | Grade | E2F3 | ID4 | DEK |
|----|-----|-----|-------|------------------|------------------|-------|------|-----|-----|
| 3  | m   | 75  | pT3b  | N0               | M0               | G2    | 5.59 | 0.48| 4.88|
| 6  | m   | 76  | pT3b  | pN2              | M0               | G3    | 10.37| 0.36| 1.37|
| 12 | m   | 70  | pT3a  | N0               | M0               | G3    | 1.73 | 0.04| 1.99|
| 28 | m   | 72  | pT3b  | N0               | M0               | G3    | 3.73 | 0.24| 2.27|
| 41 | f   | 54  | pTa   | Ns               | M0               | G2    | 11.69| 0.49| 3.29|
| 47 | m   | 76  | pT3b  | N0               | M0               | G3    | 3.75 | 0.19| 1.84|
| 52 | f   | 87  | pT3b  | N0               | M0               | G3    | 2.86 | 0.25| 1.43|
| 55 | m   | 83  | pT4a  | N1               | M0               | G3    | 1.25 | 0.18| 0.52|
| 61 | m   | 75  | pT3b  | N0               | M0               | G3    | 1.98 | 0.12| 1.48|
| 62 | f   | 78  | pT3b  | N0               | M0               | G2    | 1.60 | 0.54| 0.72|
| 64 | m   | 74  | pT3b  | N2               | M0               | G3    | 3.34 | 0.11| 1.26|
| 67 | f   | 94  | pT2   | Ns               | Mx               | G3    | 4.38 | 0.33| 1.50|
| 69 | m   | 77  | pT3b  | N0               | M0               | G3    | 2.17 | 0.14| 1.19|
| 104| m   | 61  | pT1   | N0               | M0               | G2    | 1.49 | 0.24| 0.72|
| 105| m   | 83  | pTa   | Ns               | M0               | G2    | 3.80 | 0.27| 1.88|
| 109| m   | 68  | pT4a  | N0               | M0               | G3    | 2.14 | 0.23| 0.30|
| 111| m   | 81  | pT3b  | N0               | M0               | G2-3  | 2.24 | 0.52| 0.61|
| 115| m   | 74  | pT3a  | N2               | M0               | G3    | 1.74 | 0.12| 0.84|
| 120| m   | 66  | pT2   | N0               | Mx               | G2    | 3.12 | 0.15| 0.66|
| 150| f   | 78  | pT3a  | N2               | M0               | G3    | 0.54 | 0.20| 0.86|
| 168| m   | 64  | pT3a  | N2               | M0               | G3    | 1.68 | 0.12| 1.42|
| 170| f   | 63  | pT2   | N0               | M0               | G2    | 0.58 | 0.08| 0.27|
| 172| m   | 72  | pT3a  | N0               | M0               | G3    | 1.73 | 0.13| 0.85|
| 205| f   | 65  | pT2   | N0               | M0               | G2    | 0.36 | 0.10| 0.17|
| 212| m   | 73  | pT2a  | N1               | M0               | G2    | 0.62 | 0.16| 0.79|
| 224| m   | 65  | pT2a  | N0               | M0               | G2    | 4.29 | 3.39| 1.87|
| 231| m   | 95  | pT2   | Ns               | M0               | G2    | 13.04| 1.11| 1.59|
| 246| m   | 67  | pT4a  | N2               | M0               | G3    | 3.07 | 0.27| 1.29|
It is commonly assumed that regions in the genome that are amplified in cancers harbor proto-oncogenes that are activated by overexpression as a consequence of increased gene dosage. Indeed, several bona fide oncogenes have been found in amplified regions and some have even been identified by cloning from amplicons. In such cases, amplicons consistently contain one particular gene, alone or together with a limited number of others, e.g. ERBB2 only or together with TOP2A. Interestingly, the structure of such amplicons can be quite simple [24-26]. The mechanism underlying such amplifications is not understood in detail, but appears to involve the re-replication of a single chromosome fragment, most likely via a circular double-minute intermediate [26].

Clearly, the 6p22 amplicon in bladder cancer belongs to a different class of amplicons which are characterized by great heterogeneity and instability. Such amplicons often contain different segments and accordingly different genes from one region and even sequences from different chromosomes [27-30]. The mechanism causing these amplifications is considered to be most likely breakage-fusion-bridge cycles initiated e.g. by hypoxia [31] or breakage at fragile sites [32].

Considering this background, the question which is the oncogene targeted by 6p22 amplifications in bladder cancer and the specific issue of the role of ID4 have to be approached with due caution, since in each individual case the amplicon may be influenced by random factors such as the location of an initiating double-strand break and structural factors such as preferred sites of breakage of dicentric chromosomes arising during breakage-fusion-bridge cycles. Nevertheless, the relatively high prevalence of 6p22 amplifications in bladder cancer and the relative specificity of this amplification for this cancer type argue for a functional selection as well.

For several 6p22 genes subject to amplification, it is plausible to assume that their overexpression may confer a more aggressive phenotype to bladder cancer cells. SOX factors determine cell fate and cell differentiation [33], so SOX4 overexpression might lead to further dedifferentiation. E2F transcription factors activate the transcription of genes required for DNA synthesis and E2F3 appears to repress some promoters that are activated by E2F1, including the ARF promoter in CDKN2A [34]. DEK is part of a oncogenic fusion protein resulting from t(6;9)(p23;q34) translocations in acute myeloid leukemia [35] and is implicated in regulation of chromatin structure, which is evidently aberrant in invasive bladder cancers. However, specific functional studies on the role of these proteins in urothelial cells are lacking. The function of CDKAL1 is entirely unknown.

ID4 belongs to a protein family whose members have been shown to interfere with cell differentiation by blocking the effects of HLH transcription factors and pocket proteins, including RB1. Several members have been reported to be over-expressed in human cancers [15-18]. ID4 in particular has been shown to be overexpressed in rat mammary carcinomas. Accordingly, overexpression of ID4 blocked the differentiation of HC11 mammary epithelial cells and stimulated their proliferation [36]. It is also the target of a specific chromosomal translocation in some cases of B-cell acute lymphoblastic leukemia [37]. Conversely, ID4 expression has been reported to be down-regulated by promoter hypermethylation in colon carcinomas [38]. Evidently, as for the other oncogene candidates on 6p22, more detailed studies are required on the biochemical and functional properties of ID4 in normal and cancerous urothelial tissue.

**Conclusion**

In conclusion, our study indicates that the 6p22.3 amplification prevalent in advanced bladder cancers is highly heterogeneous and contributes to the altered expression of several genes, including ID4, in a highly variable manner. Thus, this genetic change may contribute to the highly variable biological and clinical behaviour of invasive bladder cancers.

**Methods**

**Tissues**

Cancerous and normal bladder tissues were used from a previous study [39]. Normal tissues were identified by gross morphology, with microscopic verification in case of extended tumors. Important clinical parameters of the cancer tissues are summarized in Table 1. RNA from testicular normal and cancer tissues used as a control for ID4 expression was also prepared in the course of a former study [40].

**Cell lines and primary cultures**

The bladder cancer cell lines 253J, 639v, 5637, BFTC905, BFTC909, EJ, HT1376, J82, MGHU4, RT112, SD, SW1710, T24, UMUC3, VM5Cub1, and VM5Cub2 and primary urothelial cells were cultured as described previously [23].

**DNA and RNA extraction**

High-quality DNA and RNA were extracted by standard methods using commercial kits from Qiagen (Hilden, Germany) and Peqlab (Erlangen, Germany).

**RT-PCR**

Following photometric quantification, 2 μg mRNA were transcribed into first strand cDNA using SuperscriptII (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol with oligo-dT primers. Real-time RT-
PCR was carried out using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) and the primers indicated in Table 2. The amplification mixture consisted of 1x reaction mix (LightCycler-FastStart DNA Master PLUS SYBR Green I; Roche Diagnostics), 10 pmol (DEK, E2F3, ID4) or 5 pmol (β-actin) of each primer and 20 ng cDNA in a final volume of 10 µl. The reaction was monitored between the annealing and elongation steps at 640 nm. After the final cycle, melting-point analysis of the samples was performed over the range of 69°C – 99°C. Turning-point values for the specific genes were related to those for β-actin.

**Microsatellite analysis**

Microsatellites located at 6p22 (see Figure 1) were amplified using published primer sets (see the ensembl database for sequences and Tm’s) in duplex reactions with one microsatellite from chromosome 12 (D12S1650) or chromosome 15 (D15S127) as control. These chromosome microsatellites are rarely affected by allelic imbalances in bladder cancer [3]. One primer from each pair was labeled with IRD-800 fluorescence and the products were resolved and detected on a LiCOR 4200S automated sequencer. Reactions were carried out in the linear phase of PCR with 25–30 cycles, on a LiCOR 4200S automated sequencer. Reactions were performed as described (page number not for citation purposes)

| Designation | Sequence | Tm (°C) | Product size (bp) |
|-------------|----------|---------|------------------|
| GAPDH 350s  | TCCCATCACCATCTTCCA   | 62.3    | 379              |
| GAPDH 350as | CATCAGCCACAGTTTCC     | 61.9    |                  |
| Akin661S    | TGACGGGTCAC           | 72.2    | 661              |
| Akin661AS   | CTAAGCAGATT          | 70.9    |                  |
| ID4 fw      | CCGGCCCAACAAAGAAAGTCAG | 59.4    | 188              |
| ID4 rv      | GGTGTGACGCGGTCGAG     | 58.2    |                  |
| E2F3 fw     | AGCTCTTGGTGTCTCTAC    | 59.4    | 155              |
| E2F3 rv     | TCTTAAATGGGTGGATGCTCT | 55.3    |                  |
| DEK fw      | GTGGGTCAAGTCAGTGCCC   | 58.2    | 291              |
| DEK rv      | AGGACATTTGGTGCTTGA    | 55.3    |                  |

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