High-Resolution Array-Based Comparative Genomic Hybridization of Bladder Cancers Identifies Mouse Double Minute 4 (MDM4) as an Amplification Target Exclusive of MDM2 and TP53

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Abstract

Purpose: Loss of p53 function in urothelial cell carcinoma (UCC) by mutation or inactivation disrupts normal cell cycle checkpoints, generating a favorable milieu for genomic instability, a hallmark of UCC. The aim of this study was to characterize novel DNA copy number changes to identify putative therapeutic targets.

Experimental Design: We report our findings using array comparative genomic hybridization on a whole-genome BAC/PAC/cosmid array with a median clone interval of 0.97 Mb to study a series of UCC cases. TP53 status was determined by direct sequencing, and an in-house tissue microarray was constructed to identify protein expression of target genes.

Results: Array comparative genomic hybridization allowed identification of novel regions of copy number changes in addition to those already known from previous studies. A novel amplification previously unreported in UCC was identified at 1q32. A chromosome 1 tile path array was used to analyze tumors that showed gains and amplification; the mouse double minute 4 (MDM4) homologue was identified as the amplified gene. MDM4 mRNA expression correlated with copy number and tumor grade. Copy number changes of MDM4 and MDM2 occurred exclusively in tumors with wild-type p53. Overexpression of MDM4 corresponded to disruption of p53 transcriptional activity. Immunohistochemistry on an independent series by tissue microarray identified an inverse relationship between Mdm4 and Mdm2, with Mdm4 expression highest in invasive UCC.

Conclusion: The data indicate that gain/amplification and overexpression of MDM4 is a novel molecular mechanism by which a subset of UCC escapes p53-dependent growth control, thus providing new avenues for therapeutic intervention.

Genomic alterations within urothelial cell carcinoma (UCC) characterize divergent pathways of superficial and muscle invasive disease. They reflect the multistep molecular mechanisms that occur in the progression from superficial to muscle invasive disease (1–3). Genomic instability assessed by DNA copy number changes is a hallmark of aggressive UCC; losses of RB1 (13q) and amplification being overexpressed (7–9), for example, gains of ⑦p, ⑧q, ⑩p, ⑪p, and ⑬⑲ (⑳q) and gains of ⑤p, EGFR (⑳p), ⑳q21, ①⑳q13, ERBB2 (⑳q), ②q, and E2F3 (⑳p) are commonly reported (4–6).

Chromosomal regions that have more than two copies (gain and amplification), one copy (heterozygous deletion), or zero copy (homzygous deletion) potentially harbor oncogenes or tumor suppressor genes. DNA copy number has been shown to consistently correlate with gene expression level of a significant fraction of genes, with ⑳% to 62% of genes within a region of amplification being overexpressed (7–9), for example, gains of ①⑳q13 and ⑱q21 harboring CCND1 and ERBB2 oncogenes in UCC (10, 11). Frequently, copy number loss accompanies loss of heterozygosity where mutation of the retained copy results in likely biallelic inactivation of a target gene. In superficial UCC, loss of heterozygosity of chromosome ⑨ is frequent in which the CDKN2A/ARF and CDKN2B loci at ⑨p21 are common targets for deletion (12–14).

The influence of DNA copy number on global transcriptional pattern enforces the importance of the detection and mapping of copy number abnormalities to provide an approach for associating aberrations with disease phenotype and for localizing critical genes that would be therapeutic targets. For instance, the identification of a commonly amplified region that harbors overexpressed ERBB2 and EGFR resulted in the design of successful new clinical therapies for some solid tumors (15, 16).

Conventional cytogenetic methods do not provide a global view of the myriad of genetic events within a single tumor.
However, array comparative genomic hybridization addresses this predicament by allowing a genome-wide high-resolution mapping of DNA copy number changes. The patterns of copy number alterations identified by array comparative genomic hybridization in UCC have been reported to aid in differentiating tumors into more biologically and clinically relevant subtypes as well as genotyping cell lines for better informed in vitro studies (6, 17–19). In addition, the resolution has led to precise mapping of the boundaries of amplified and deleted regions indicating candidate genes relevant to cellular control pathways. However, these studies do not involve a large cohort of samples; thus, the high probability that novel DNA copy number changes remains to be discovered.

TP53 is the most frequently inactivated tumor suppressor gene within high-grade UCC. MDM2 has been previously reported to be gained and amplified in UCC, which we confirm in this study (20–23). Mouse double minute 4 (Mdm4) homologue, a non-ubiquitin ligase binding partner of TP53, and Mdm2 have been shown to negatively regulate the p53 pathway. Its contribution to wild-type TP53 cancers has been suggested (24, 25). Mice knockout studies suggest that Mdm4 and Mdm2 act in exclusive pathways, regulating p53 function in different ways (26–28). Under stress conditions, Mdm4 overexpression increases cell death, in conjunction with the induction and enhanced p53 activity, by increased dissociation of p53 from Mdm2. This apparent positive regulation of p53 in nontumoral cells is achieved through the stabilization of p53 and not through the inhibition of Mdm2 activity (29, 30).

Since the identification of the MDM4 locus amplification in glioblastomas, overexpression of the protein has been reported in a significant number of tumor cell lines and tumors (31–33). Artificial overexpression of MDM4 leading to immortalization of primary mouse embryonic fibroblast and neoplastic transformation in combination with HRas V12 corroborates the tumorigenic role of MDM4 (32). Hence, the oncogenic role of MDM4 in tumorigenesis remains undisputed but the divergent role of Mdm4 suggests that there is a greater relevance in comparing MDM4 and MDM2 levels than just the sole overexpression of Mdm2 in the regulation of p53.

In this study, we interrogated a cohort of UCC tumors, representative of the clinicopathologic spectrum, using 1-Mb array comparative genomic hybridization. We identified and mapped numerous novel, small regions of copy number gain as well as the highly frequent alterations previously documented in UCC. We report a novel amplification at 1q32 and, using a chromosome 1 tile path array, show gains and amplification of MDM4 as the candidate gene. We determined the TP53 mutational status in Mdm2- and Mdm4-expressing tumors and report the exclusive relationship between gains of MDM4, MDM2, and TP53 mutation. We further show an association between Mdm4 overexpression and reduced p53 transcriptional activity.

Fig. 1. A, genome-wide frequency of copy number alterations in all tumors. B, example of low-grade tumor whole-genome profile. C, example of high-grade tumor whole-genome profile. The whole-genome profile summary shows the frequency of genomic copy number changes of each clone throughout the array order from chromosome 1 to 22 based on National Center for Biotechnology Information 35 mapping position. Red, copy number gains; yellow, amplifications; green, hemizygous deletions; blue, homozygous deletion. A, composite of all the individually derived whole-genome profiles (e.g., B and C). The log2 ratios of test/reference fluorescence of the clones are plotted with clones arranged in genomic order frompter (left) to qter (right). The log2 ratios are replaced with modal copy number through confirmation by I-FISH.
Materials and Methods

Human bladder cancer specimens. Tumor samples from 109 patients of primary UCC were collected at cystectomy or transurethral resection, and snap frozen in liquid nitrogen. Use of tissues for this study was approved by Cambridgeshire Local Research Ethics Committee (Ref 03/018). A total of 30 sections of 30 µm of fresh frozen tissue were homogenized for DNA and RNA extraction. “Sandwich” H&E sections were prepared for cellularity and grade assessment by a reference urohistopathologist (A.W.). Tumors were staged and graded according to the American Joint Committee on Cancer and WHO/International Society of Urologic Pathology classifications (34, 35). Samples showing tumor cellularity of <70% and significant inflammatory cell contamination were excluded.

Tissue microarray. For our custom-made tissue microarray (TMA), an independent cohort of 123 formalin-fixed, paraffin-embedded tissue samples of primary UCC was obtained from the pathology archives of Addenbrooke's Hospital, Cambridge University Trust. Normal and carcinoma in situ samples were included. The TMA was constructed as previously described (36).

RNA extraction and reverse transcription-PCR analysis. Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) following the manufacturer’s protocol precedent to the DNA extraction step. Inclusion of a DNase step was used to optimize RNA purity. Isolated RNA was analyzed following the manufacturer’s protocol. Concurrently, 1 µL of the back extraction buffer per 1 mL of sample was run on a 1% agarose gel to check the quality of the DNA.

Five micrograms of total RNA were reverse transcribed in a final volume of 40 µL using SuperScript kit (Invitrogen). MDM4 and TP53 target gene 1 (TP53TGG) transcript levels were analyzed and SDH, UBC, GAPDH, and Actin were used as housekeeping genes. Quantitative reverse transcription-PCR assays were done on the ABI Prism 7700 (Applied Biosystems) following the manufacturer’s recommendation. Each sample was analyzed in triplicate.

DNA extraction, purification, qualification, and quantification. DNA was extracted from stored interphase layer from the RNA extraction protocol. One-half milliliter of the back extraction buffer per 1 mL of Tri-Reagent used for the RNA extraction was added to each tube and mixed intensively for at least 3 min by inversion or put on a shaker for 10 min. The tubes were spun at 12,000 × g for 30 min at room temperature and the upper aqueous layer was removed. To 1 mL of Tri-Reagent used for original RNA isolation, 400 µL isopropanol was added, mixed, and incubated for 5 min at room temperature. Samples were spun at 12,000 × g for 15 min at 4°C. The supernatant was discarded and the resulting DNA pellet was washed by inversion with 500 µL of 70% ethanol. The tubes were then spun down at 12,000 × g for 15 min at 4°C. The ethanol was discarded and the pellet was dissolved in 400 µL of 1× Tris-EDTA buffer and stored at 20°C. DNA was then purified using the phenol-chloroform-isooamylalcohol method. The resulting washed, precipitated DNA was resuspended in 100 µL of nuclease-free water and stored at -20°C. DNA was quantified and qualified using the NanoDrop ND-1000 Spectrophotometer following the manufacturer’s protocol. Concurrently, 1 µL of sample was run on a 1% agarose gel to check the quality of the DNA.

Construction of genomic arrays. The 1-Mb array clone set was obtained from the Wellcome Trust Sanger Institute and the 1-Mb array was constructed as described previously (37). Clone positions quoted were according to the National Center for Biotechnology Information 35 assembly of the human genome. The chromosome 1 tile path array covering 97.9% of the sequenced regions of chromosome 1 was used to refine the boundaries of amplification (38).

Labeling and hybridization to microarrays. Labeling and hybridizations were done as described previously (37). Briefly, 400 ng of test and reference DNA were labeled using a BioPrime Labeling kit (Invitrogen)
with a modified deoxynucleotide triphosphate reaction mixture. Test DNA was hybridized with sex-mismatched reference DNA from samples of pooled blood from 20 normal males or 20 normal females. The labeled and purified test and reference DNA were mixed and coprecipitated with 45 μg Cot1 DNA (Roche Diagnostics). The precipitated DNA was dissolved in hybridization buffer, incubated at 37°C for 2 h, and hybridized to the array that had been prehybridized with 400 μg herring sperm DNA (Sigma-Aldrich) and 80 μg Cot1 DNA. Arrays were allowed to hybridize for up to 24 h at 37°C, then washed and dried.

Data normalization and analysis. Arrays were scanned and analyzed as described previously (37, 38). Briefly, local background intensity was subtracted from the median foreground intensity for each channel. Fluorescence ratios were calculated for each spot, and the ratios of duplicate spots were averaged. Before replicate spot ratios were averaged, to control for spatial variation across the slides, print-tip normalization was done by calculating the median test/reference spot intensity ratio for each block and dividing individual clone test/reference ratios by this value. Decisions on copy number change were based on intensity of staining as low, moderate, or high (none (0%), low (>0% and <20%), moderate (20% and <50%), and high (>50%)). Nuclear expression. Cytoplasmic staining was qualified as low (<0%), moderate (>0% and <20%), and high (>20%).

Table 2. Homozygous deleted clones and genes that reside within the boundaries of flanking clones

<table>
<thead>
<tr>
<th>International ID</th>
<th>Homozygous loss (%)</th>
<th>Hemizygous loss (%)</th>
<th>Locus</th>
<th>Genes</th>
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<td>17.3</td>
<td>9p21.3</td>
<td>CDKN2B</td>
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<tr>
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<td>17.3</td>
<td>9p21.3</td>
<td>MTAP, CDKN2A, CDKN2B</td>
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<td>17.3</td>
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<tr>
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<td>7</td>
<td>6q15</td>
<td>MDM1</td>
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<tr>
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<td>1.2</td>
<td>12.3</td>
<td>3p14.2</td>
<td>FHIT</td>
</tr>
<tr>
<td>RP11-464I4</td>
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<td>11.1</td>
<td>13q31.1</td>
<td>Q8BUTS_HUMAN</td>
</tr>
<tr>
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<td>8.5</td>
<td>11p15.1</td>
<td>PLEKH7</td>
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<tr>
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<td>2.4</td>
<td>8q21.11</td>
<td>JPH, GDP1, PI15</td>
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<td>18q22.3</td>
<td>Q96HYO, ZNF07</td>
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<td>SOF12, Q6PV4</td>
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<td>4.1</td>
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</table>

Results

UICC whole-genome profile summary. A total of 109 UCC tumors were processed for this study. After normalization and quality assessment, 98 tumors were included (Supplementary Table S1). Frequency plots summarizing copy number gains and losses for all clones are shown in Fig. 1. Amplifications and homozygous deletions were identified in high-grade superficial and invasive tumors in a background of high levels of copy number gains and hemizygous deletions. Although copy number gain and loss was evident in TaG1 and TaG2 tumors, no amplifications were identified. There

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was a considerable difference in the magnitude of genomic alterations across tumor grade when expressed as fraction of genome altered, as previously described (Supplementary Fig. S1; ref. 18).

Copy number gain and loss was detected across the entire genome. However, the distribution of alterations was non-uniform and regions of high-frequency change were evident. In addition to recurrent alteration of specific loci, there was a high incidence of alteration of neighboring clones, whole chromosome arms, and entire chromosomes. Tables 1 and 2 present the most frequently amplified and deleted clones, respectively. Using the 1-Mb array, we narrowed down previously identified regions of gains as well as novel hemizygous and homozygous deletions. We found several novel homozygous deletions in single cases in the regions where hemizygous deletions frequently occurred in this series. For instance, we identified a single homozygous loss and multiple hemizygous deletions at 12q24.33. The locus harbors FZD10, a member of frizzled family of receptors in the WNT/FZD signaling cascade, and we confirmed the loss of the particular locus by I-FISH (Supplementary Fig. S2). There was also a high frequency of loss on chromosome 19q13.31. Among the genes that reside within the boundaries of the clone is CBLC, a member of the Cbl family of ubiquitin ligases. The Cbl family of genes targets the degradation of protein tyrosine kinases, including EGFR, and as such may be biologically relevant to UCC.

Amplification and gain of 1q32.1 in UCC contains MDM4. We identified a novel region of amplification and high frequency of gain (17%) on chromosome 1q32 (Fig. 2A). We focused on this region for further evaluation using a chromosome 1 tile path array to define the location and the delimiting boundaries of amplification. In the sample that harbored the amplification, we identified two clones that were amplified: RP11-563I16 and a smaller clone, RP11-430C7, which almost completely resides within RP11-563I16. Gains, but no amplifications were identified in the overlapping clone RP11-739N20. Two genes are covered by RP11-430C: MDM4 (base pair 202752134 to 20279387) and the leucinerich repeats neuronal protein 5 precursor (glioma amplified on chromosome 1 protein/LRRN5). However, the overlapping clone RP11-23I7 corresponding to the region containing LRRN5 was gained and not amplified. These results indicate that MDM4 is the target of amplification in the chromosome 1q32.1 locus. The amplification of MDM4 was confirmed using I-FISH (Supplementary Fig. S3).

MDM4 expression is increased in high-grade/invasive UCC and is gene dosage dependent. We assessed MDM4 expression across the entire cohort. There was a positive correlation between transcript expression and tumor stage with significantly higher expression in muscle invasive tumors compared with superficial tumors (P < 0.001; Fig. 2B). Amplification and gain of copy number does not necessarily result in elevation of mRNA transcript level. To investigate this, we evaluated mRNA...
levels of MDM4 in 70 samples with known copy number by quantitative reverse transcription-PCR. Figure 2C shows the positive association between mRNA expression and copy number status for the samples tested. There was a direct relationship between copy gain and expression of MDM4; mRNA levels were increased almost 2- and 3-fold in tumors with gain and amplification \((P < 0.001)\). This correlation supports the concept that MDM4 is likely to be biologically significant in UCC and is overexpressed in high-grade and high-stage disease. Interestingly, there was no difference in MDM4 expression for the four samples in which we detected a hemizygous loss at 1q32.1 in comparison with tumors with normal copy number. Mdm4 nuclear expression, as assessed by immunohistochemistry, was higher in tumors with gain and amplification in keeping with a gene dosage effect (Supplementary Fig. S4).

**Gain of MDM4 is exclusive of MDM2 gain and TP53 mutation, and affects p53 transactivity.** Inactivation of p53 in cancers can result from the amplification or overexpression of MDM2. As MDM4 shares a high homology with MDM2, its overexpression represents an alternative mechanism by which p53 function may be inactivated \((39)\). To determine this relationship, we assessed the copy number status of MDM4 \((1q32.1)\) and MDM2 \((12q15)\) and the p53 mutation status of these tumors.

Table 3 illustrates almost mutual exclusivity between MDM4, MDM2 copy number gains/amplification, and mutation of p53. MDM2 was amplified in four samples and gained in four samples. In all but one case, MDM4 gain was exclusive of MDM2, and in all cases, p53 mutation was exclusive of either MDM4 or MDM2 copy number alteration.

To investigate the biological significance of MDM4 mRNA induction on p53 transactivity in wild-type tumors, we compared the mRNA levels of MDM4 against TP53TG1 in a subset of 60 wild-type and 10 mutant p53 UCC samples. TP53TG1 expression is induced in a wild-type p53-dependent manner and is postulated to play a role in DNA damage response \((40)\). Independent of histopathology, wild-type p53 tumors expressing high levels of MDM4 exhibited low levels of TP53TG1 in harmony to the postulated oncogenic role of MDM4 \((P < 0.001)\). The level of expression of the p53 target gene in these tumors was comparable with expression levels in mutant p53 tumors (Fig. 2D). These results suggest that, in addition to p53 mutation and MDM2, MDM4 plays an important role in the p53 pathway in UCC.

**Evaluation of MDM4 and MDM2 protein expression in UCC.** Based on these studies, we reasoned that an inverse relationship exists between MDM4 and MDM2 and tested this at a protein level on an independent TMA set. We detected Mdm4 and Mdm2 expression across superficial and invasive tumors with a higher proportion of late-stage tumors \((>T2)\) expressing high levels of Mdm4 compared with Ta tumors (Fig. 3A). However, Mdm4 expression was significantly higher in grade 3 tumors compared with grade 1 and 2 tumors \((P < 0.001)\). Comparative analysis of coexpression confirmed that, despite many tumors displaying moderate levels of both proteins, a larger proportion of high-expressing Mdm4 tumors expressed low levels of Mdm2 compared with low-level Mdm4-expressing tumors \((P < 0.005)\). In addition, there was a group of tumors that were negative or low for both proteins (Fig. 3B).

**Discussion**

In this study, we carried out a genome-wide survey to identify DNA copy number alterations using 1-Mb array comparative genomic hybridization. Genomic instability is a hallmark of UCC and high fraction of genome altered in muscle invasive disease, as previously reported, is evident in the cohort of samples we assessed. We confirmed previously documented changes as well as defined the borders at an increased resolution than previously reported in UCC.

A chromosome 1 array was successful in refining the amplification of the 1q32.1 locus, and we identified MDM4 as the gene located within the boundaries of the amplified clone(s), which we subsequently confirmed by I-FISH. We have shown that MDM4 mRNA and protein expression is related to gene dosage and up-regulated in high-stage and high-grade UCC. In tumors that harbored hemizygous deletion, mRNA expression level was similar to tumors with two intact alleles, suggesting a potential dosage compensation mechanism.

MDM4 gain and overexpression in UCC is likely to be biologically significant. A dysfunctional p53 pathway is a critical step in tumorigenesis of UCC. TP53 is mutated in up to 50% of invasive UCC and 11% of superficial disease. However,
in wild-type tumors, function can be compromised by inhibition of p53 protein, suggesting a larger role of p53 pathway inactivation (41–43). In our study, overexpression of MDM4 accompanied the down-regulation of TP53TG1, consistent with the postulated impairment of p53 transactivity. This reaffirms the oncogenic role of MDM4 in tumorigenesis in contrast to its proapoptotic role in normal cellular stress conditions (26–30).

Our findings add another dimension to the p53 regulatory network in UCC. Both Mdm2 and Mdm4 interact as p53 inhibitors during embryonic development. Mdm2-deficient and Mdm4-deficient mice die in utero but are viable in a p53-deficient background (44). Although both proteins are structurally related and both bind to p53, recent studies suggest divergent roles in p53 regulation. Mdm2 is thought to mainly regulate p53 stability, whereas Mdm4 regulates p53 activity (45). We found that MDM4, MDM2 copy number gain/amplification, and mutation of TP53 are mutually exclusive and present in 14% of the low-grade superficial tumors and in 59% of the high-grade invasive tumors.

Examination of protein expression by TMA on a larger independent cohort of samples confirms an inverse relationship between Mdm2 and Mdm4 in a subset of UCC tumors. As previously reported, Mdm4 staining was localized in the nuclear and cytoplasmic compartments concurrently/exclusively (46). Protein expression of Mdm2 and Mdm4 is not mutually exclusive, in contrast to the finding for DNA copy number gains, but is likely to be a realistic reflection of the dynamic interactions between Mdm4 and Mdm2 in UCC cells. As is the case for other oncogenes, such as EGFR amplification-associated overexpression, gene dosage may not be the only mechanism of overexpression (47). The effect of gene amplification/gain, mRNA overexpression of MDM4 in the presence of wild-type TP53, and the resulting loss of p53 transactivation, together with the inverse correlation to Mdm2 protein expression are important in UCC, providing an insight to an alternative tumorigenic mechanism to escape p53-dependent growth control.

Restoration of p53 function is an attractive therapeutic approach, and in this context, our novel finding is clinically
relevant. Recently, a class of small molecules, the nutlins, which inhibit the interaction between Mdm2 and p53, has shown activity in vitro and in vivo in tumors with wild-type p53 but not mutated p53. Activity of nutlin-3 is lost in MDM4 \(^{+/−}\) tumors.

However, complete ablation of MDM4 or the synergistic decrease of MDM2 and MDM4 gene dosage increased tumor suppression in vivo, confirming MDM4 as a potential therapeutic target in tumors expressing high Mdm4 (48–50).

References


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