Recurrence of Urothelial Carcinoma of the Bladder: A Role for Insulin-Like Growth Factor-II Loss of Imprinting and Cytoplasmic E-Cadherin Immunolocalization

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Abstract

Purpose: This study documents the frequency of insulin-like growth factor-II (IGF-II) loss of imprinting (LOI) in a series of 87 bladder tissues. E-cadherin (CDH1) immunolocalization was also investigated due to the known redistribution of this adherence protein to the cytoplasm following exogenous exposure to IGF-II.

Experimental Design: Informative IGF-II cases were identified following DNA-PCR amplification and subsequent sequencing of the transcribable Apa I RFLP in exon 9 of IGF-II. Similar approaches using primer-specific cDNA templates identified the imprinting status of IGF-II in these informative cases. CDH1 cellular localization was assessed on a tissue microarray platform of 114 urothelial carcinoma of the bladder (UCB) cases (70 pTa noninvasive and 44 pT1 lamina propria invasive) using the commercially available Novocastra antibody.

Results: IGF-II LOI was evident in 7 of 17 (41%) UCB tumors and 4 of 11 (36%) tumor-associated normal urothelial samples. Two of four pT1 grade 3 tumors, the subject of much debate concerning their suitability for radical cystectomy, showed LOI at the IGF-II locus. In those tumors showing IGF-II LOI, 4 of 7 (57%) displayed concomitant CDH1 cytoplasmic staining. In contrast, only 3 of 10 (30%) IGF-II maintenance of imprinting tumors had concomitant CDH1 cytoplasmic localization. UCB cell lines displaying cytoplasmic CDH1 immunolocalization expressed significantly higher levels of IGF-II (CAL29, HT1376, and RT112) compared with RT4, a cell line displaying crisp membranous CDH1 staining. Finally, cytoplasmic CDH1 staining was an independent predictor of a shorter time to recurrence independent of tumor grade and stage.

Conclusions: We suggest that CDH1 cytoplasmic immunolocalization as a result of increased IGF-II levels identifies those nonmuscle invasive presentations most likely to recur and therefore might benefit from more radical nonconserving bladder surgery.

To date, insulin like growth factor-II (IGF-II) loss of imprinting (LOI) has been reported in most histologic types of sporadic embryonal, solid, and hematologic malignancies (reviewed in ref. 1). Moreover, identifying IGF-II LOI as a predictive marker of cancer risk has been highlighted for colorectal cancer, where IGF-II LOI in lymphocytes identified patients at risk of developing this malignancy (2) and, when detected in normal colonic mucosa, identified patients with a 5-fold increased chance of developing adenomas (3). In normal subjects, IGF-II LOI frequencies of approximately 10% have been reported in peripheral blood lymphocytes (2, 4) and range from 12% to 17% in normal tissues (3, 5, 6). Importantly, IGF-II LOI has been shown to result in increased IGF-II mRNA and protein expression (3, 7) as a result of the abnormal reactivation of the normally silent maternal allele.

In vivo mouse studies provide compelling evidence supporting the functional significance of IGF-II overexpression in tumor development. Specifically, transgenic overexpression of IGF-II induces spontaneous lung and mammary tumors (8, 9). Furthermore, IGF-II LOI engineered mice have increased expression of proliferation-specific genes (Cdc6, Mcm5, Mcm3, Chaf1a, Lgi1, and Ccne1), enhanced sensitivity to IGF-II signaling (10), and develop twice as many intestinal tumors as their control littermates (7). This supports the suggestion that the altered maturation of
Translational Relevance
Currently, there is no way of predicting which nonmuscle invasive UCB tumors (pT1 and pT2) will recur. Additionally, there is much controversy concerning the pT1 grade 3 cohort, where bladder conserving surgery would be advocated by some but not others. We have shown that immunohistochemical assessment of cytoplastic CDH1 staining can independently predict time to recurrence in the non-muscle invasive pTa and pT1 tumors over and above stage and grade. Moreover, CDH1 cytoplasmic staining is predictive of recurrence in the controversial pT1 grade 3 group. Future clinical practice to include specific assessment of cytoplastic CDH1 staining might be warranted in those cases where there is controversy regarding whether the bladder should be conserved. Our study also identifies a possible mechanism underlying CDH1 relocalization to the cytoplasm, that is, the presence of aberrant levels of IGF-II due in some cases to IGF-II LOI. IGF-II LOI has already been identified as an epimarker of colorectal cancer development, and we suggest that the finding of LOI in the TAN samples of our UCB tumors holds promise as a predictor of tumor development, playing a significant role in compromising cellular adherence through internalization of CDH1.

Materials and Methods

Clinical UCB samples
Fresh-frozen UCB samples. Twenty-four paired UCB tumors and grossly normal urothelial bladder (tumor-associated normal [TAN]) tissues were obtained sequentially from patients undergoing bladder cystectomies from the period of 2001 to 2002. Normal urothelium was acquired distant from the tumor site with the exclusion of muscle. Twenty-one of these were primary tumors and 3 were recurrent lesions (cases A134R, A198R, and A78R, Table 1). Tissues were snap-frozen in liquid nitrogen before DNA and RNA extraction. Following homogenization, high molecular weight DNA was extracted using a phenol/chloroform-based methodology, and total RNA was extracted using the guandine based TRIzol reagent.

Full-face formalin-fixed, paraffin-embedded UCB sections. To examine more closely the frequency of IGF-II LOI in nonmuscle invasive primary presentations, 20 pTa (noninvasive) and 19 pT1 (lamina propria invasion) formalin-fixed, paraffin-embedded (FFPE) UCB lesions were sequently identified from patients on file (1991-2002) in the Mater Hospital Dublin, which form part of our TMA platform of 114 UCB cases (70 pTa, noninvasive and 44 pT1, lamina propria invasion). H&E-stained sections were reviewed by one pathologist and epithelial areas were macrodissected before nucleic acid extraction. DNA and RNA extractions were carried out using the commercially available (Qiagen) QIAmp DNA Mini Kit and RNeasy FFPE Kit, respectively.

UCB TMA cohort. A total of 114 primary UCB tumors were constructed into a TMA platform of which 70 (61%) were pTa, and 44 (39%) were pT1. There were 37 (32.5%) grade 1 tumors, 52 (45.6%) grade 2 tumors, and 25 (21.9%) grade 3 tumors. From this cohort, there were 20 pTa grade 3 tumors for analysis. The bladder tumors were staged according to the tumor-node-metastasis staging protocol of the American Joint Committee on Cancer (20) and graded according to the WHO classification (21). Ethical approval was obtained for the study through the Hospital Ethics Committee of the Mater Misericordiae Hospital. Cause of death was verified by general practitioner and hospital records and/or through the National Death Registry. Follow-up times ranged from <1 to 159 months with a mean follow-up time of 60 months. Recurrence was defined as any tumor identified 3 months after initial biopsy. This included grossly identifiable tumor treated by cautery and histologically confirmed tumor. The study population were Caucasian and included 78 (68.4%) men and 36 (31.6%) women; 69 (41.6%) had at least one recurrence within their follow-up period. FFPE blocks from all 114 patient samples were sectioned and stained with H&E and graded by two pathologists (M.H. and E.W.K.) to confirm pathologic stage and grade of the bladder tumors. The relevant tumor areas were marked and used as the donor cores for TMA construction. TMAs were constructed using the Beecher Instruments Tissue Microarrayer as described originally by Kononen et al. (22). Cores (2 mm) were sampled in quadruplicate for each case where tissue allowed. Two locator cores, consisting of a liver core and a normal ureter core, were placed in each array.

UCB cell line panel. Six UCB cell lines of varying stages and grades (TCCSUP, CAL29, HT1376, RT112, T24, and RT4) were obtained from the German Collection of Microorganisms and Cell Cultures. Cell cultures were grown and maintained at 37 °C and 5% CO2 in DMEM (TCCSUP, CAL29, and HT1376), RPMI 1640 (RT112 and RT4), and McCoy’s 5A (T24) nutrient medium supplemented with FCS, 4 mmol/L L-glutamine and 0.06 mg/mL benzylpenicillin/0.1 mg/mL streptomycin.
Identification of informative cases for IGF-II in clinical samples
DNA-PCR was performed on 0.25 μg genomic DNA using published primers A (5'-cttgacctgtagcatatgg-3') and B (5'-gcttggcctaatcatcttca-3'), which encompass the transcribable ApaI single nucleotide polymorphisms (SNP; ref. 23). Following DNA-PCR amplification of a 292-bp amplicon, subsequent commercial sequencing in the reverse orientation identified informative cases.

Identification of informative cell lines for IGF-II
Six transcribable SNPs located within the 3'-untranslated region of the IGF-II transcript were analyzed in the bladder cell line cohort. Two of the six SNPs, ApaI (A1876G) and C2207T (24), yielded informative cases following DNA-PCR amplification using published primers (23, 24) and commercial sequencing.

Determination of allelic usage in informative samples
ApaI RT-PCR was performed on 1 μl primer B-specific cDNA using published primers B and C (5'-cttgacctgtagcatatgg-3'; ref. 23). To the weakness of the generated 1.12-kb RT-PCR product in some samples, nested PCR was carried out on the 1.12-kb PCR product using primers A and R (5'-ctctctttggtcttactggg-3'; ref. 23). IGF-II imprinting phenotypes were assessed following commercial sequencing in the reverse orientation. Allelic usage was similarly assessed within UCB cell lines following RT-PCR generation of amplicons encompassing the ApaI and C2207T SNPs.

IGF-II promoter 1-specific expression analysis
Primer B-specific cDNA was subjected to transcript specific amplification using primer B (23) and a promoter 1-specific primer P1F (5'-ctcgccacggtagagacagg-3'; ref. 25). Nested PCR using 0.5 μl starting template generated a shorter 346-bp amplicon, employing P1F and Ex3R (5'-gcttggcctaatcatcttca-3') primers (25). Promoter 1-derived transcripts are usually restricted to the adult liver; therefore, normal liver tissue was used for primer optimization and served as a positive control. As a further control for the presence of amplifiable cDNA within the RT-PCR, the region encompassing the ApaI SNP in the common 3'-untranslated region of all five transcripts was also RT-PCR amplified (23).

Immunohistochemical localization of CDH1
Each of the tumors used in the IGF-II imprinting analysis formed part of the UCB TMA. Therefore, all of the CDH1 scoring and evaluation of membranous versus cytoplasmic staining detailed in Table 1 were determined from this TMA platform. TMAs were sectioned and subjected to 1× Trilogy (Cell Marque)/pressure cooker antigen retrieval. Immunohistochemical staining was carried out using standard approaches with the CDH1 primary antibody (Novocastra) and Vexation ABV (avidin/biotin; Vector Laboratories) detection system. Immunohistochemical staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride. All sections were counterstained with hematoxylin. Negative controls were included for all sections prepared by omission of primary antibody. Normal tonsil epithelium was used as a positive control.

CDH1 membranous staining was quantified for each core and divided into groups as follows: 0% positive cells, 1% to 30% positive cells, 31% to 50% positive cells, and >50% positive cells. CDH1 cytoplasmic staining was also scored independently of the membranous staining and grouped as follows: 0% staining, 1% to 10% positive, 11% to 30% positive, 31% to 50% positive, and >50% positive. All scores from multiple cores were averaged. All cores were examined by one independent observer (E.K.).

CDH1 immunofluorescence
Cells were fixed in ethanol for 1 h at -20°C and blocked in 1% bovine serum albumin. Staining was done using 5 μg/mL mouse monoclonal anti-CDH1 (BD Biosciences) and polyclonal goat anti-mouse FITC conjugated secondary antibody (DAKO). Cells were counterstained with 4',6-diamidino-2-phenylindole and subsequently mounted using p-phenylenediamine antifade mounting medium. Cells

Table 1. Immunohistochemical localization of CDH1, imprinting phenotype (MOI/LOI), and LOH of IGF-II within informative UCB cases with histologic profiling, grading, and staging

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specimen</th>
<th>Sex</th>
<th>Stage</th>
<th>Grade</th>
<th>Membrane</th>
<th>Cytoplasm</th>
<th>IGF-II imprinting phenotype normal</th>
<th>IGF-II imprinting phenotype tumor</th>
<th>LOH</th>
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<tr>
<td>A260</td>
<td>FFPE</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>N/A</td>
<td>MOI</td>
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<td>--</td>
</tr>
<tr>
<td>A276</td>
<td>FFPE</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>1-10</td>
<td>N/A</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A58</td>
<td>FFPE</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>N/A</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A225</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>LOI</td>
<td>--</td>
<td>LOH</td>
<td>--</td>
</tr>
<tr>
<td>A223</td>
<td>Frozen</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>31-50</td>
<td>Negative</td>
<td>MOI</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>FFPE</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
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<td>N/A</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
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<tr>
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<td>FFPE</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
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<td>Negative</td>
<td>N/A</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A261</td>
<td>FFPE</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>N/A</td>
<td>MOI</td>
<td>--</td>
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<td>Frozen</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>MOI</td>
<td>--</td>
<td>LOH</td>
<td>--</td>
</tr>
<tr>
<td>A226</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1-30</td>
<td>Negative</td>
<td>MOI</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A55</td>
<td>FFPE</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
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<td>1-10</td>
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<td>MOI</td>
<td>--</td>
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<tr>
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<td>FFPE</td>
<td>M</td>
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<td>11-30</td>
<td>N/A</td>
<td>MOI</td>
<td>--</td>
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<tr>
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<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>LOI</td>
<td>LOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A192</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>Negative</td>
<td>MOI</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A204</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>31-50</td>
<td>Negative</td>
<td>MOI</td>
<td>LOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A220</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>31-50</td>
<td>Negative</td>
<td>MOI</td>
<td>MOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A233</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1-30</td>
<td>11-30</td>
<td>LOI</td>
<td>MOI</td>
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<td>--</td>
</tr>
<tr>
<td>A198&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Frozen</td>
<td>M</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td>LOI</td>
<td>LOI</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Frozen</td>
<td>F</td>
<td>pT&lt;sub&gt;a&lt;/sub&gt;</td>
<td>2/3</td>
<td>&gt;50</td>
<td>Negative</td>
<td>MOI</td>
<td>--</td>
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</tr>
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</table>

Abbreviations: NNA, normal not available; LOI, loss of imprinting; MOI, maintenance of imprinting; LOH, loss of heterozygosity.

<sup>a</sup>pT<sub>a</sub> noninvasive; pT<sub>1</sub>, lamina propria invasive; pT<sub>3</sub> and greater, muscle invasive.

<sup>1</sup>Grade includes 2, 1+2, 1-2, moderate grade. 3 includes 3, 2+3, 2-3, high grade, poorly differentiated.

<sup>2</sup>Immunohistochemical scoring of CDH1 determined from the UCB TMA.

<sup>3</sup>Recurrence information only.
were visualized using an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and Axiovision 3.0.6 software (Carl Zeiss). Negative controls were conducted for each cell line by omission of primary antibody.

**Cytoskeletal-associated and cytoskeletal-unassociated CDH1 assessment**

Cytoskeletal-associated (TX-100-insoluble) and cytoskeletal-unassociated (TX-100-soluble) proteins were extracted from cell cultures following 24 h growth in serum-deprived medium. Cells were harvested by scraping into 300 μl buffer 1 [1% TX-100, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 2 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L Na 3VO4, 10 mmol/L sodium pyrophosphate, and 1× protease inhibitors]. The cell suspensions were then incubated at 4°C for 10 min with agitation followed by centrifugation at 20,000 × g for 15 min to separate the TX-100-soluble fraction. The remaining pellet was resuspended in 100 μl buffer 2 [1% TX-100, 0.5% deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 2 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L Na 3VO4, 10 mmol/L sodium pyrophosphate, and 1× protease inhibitors], subjected to 2 to 4 s of sonication, and incubated on ice for 15 min before the final centrifugation step at 20,000 × g at 4°C for 15 min and isolation of the TX-100-insoluble fraction. Protein content was quantified and normalized using the Bradford method (Bio-Rad Laboratories).

**Western blotting analysis.** Normalized quantities of TX-100-soluble and TX-100-insoluble protein fractions were incubated at 95°C in SDS sample buffer for 5 min before SDS-PAGE using 8% (w/v) acrylamide gels. Protein was subsequently transferred to nitrocellulose membranes and blocked in 5% skimmed milk followed by incubation overnight at 4°C with the mouse monoclonal anti-CDH1 (BD Biosciences). After washing with PBS containing 0.1% Tween 20, membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG (Pierce) and visualized by chemiluminescence using West-Dura (Pierce). Washing with PBS containing 0.1% Tween 20, membranes were blocked in 5% skimmed milk followed by incubation overnight at 4°C with the mouse monoclonal anti-CDH1 (BD Biosciences). After washing with PBS containing 0.1% Tween 20, membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG (Pierce) and visualized by chemiluminescence using West-Dura (Pierce). Densitometric analysis was carried out to quantify CDH1 expression using Scion image analysis software. Relative quantities of cytoskeletal-associated (TX-100-insoluble) and cytoskeletal-unassociated (TX-100-soluble) CDH1 within an individual cell line were calculated as a percentage of total expression.

**IGF-II QRT-PCR.** RNA was extracted from the cell cultures following 24-h growth in serum-deprived medium using the guanidine-based TRIzol reagent according to the manufacturer’s instructions. Random hexamer cDNA was synthesized and IGF-II expression levels were quantified using the TaqMan comparative Ct method of analysis (Hs.00171254 TaqMan Gene Expression Assay). β-Actin was employed as an endogenous control.

**Statistical methods**

The χ2 test was used for comparison of proportions. Kaplan-Meier methods were used to construct disease-free interval curves and Cox proportional hazards models assessed CDH1 staining categories for recurrence. Patients who died of other causes or were still alive were censored at the last date of follow-up. Patients who did not recur were also censored at last date of follow-up. Multifactorial models including age, gender, smoking grade, and stage were constructed to test for independence of effect of CDH1 immunolocalization on outcome. CDH1 TMA statistical analysis was conducted using SAS version 9. CDH1 cytoskeletal-associated and cytoskeletal-unassociated expression and TaqMan IGF-II QRT-PCR statistical analyses were carried out using the Student’s t test (GraphPad InStat version 3.06 for Windows). Results were considered to be significant for P values < 0.05.9

**Results**

**IGF-II imprinting analysis**

Identification of informative/heterozygous cases for IGF-II. Heterozygosity for the transcribable Apal polymorphism (23) was investigated in a cohort of 24 frozen UCB tumors on which paired TAN tissue was also available. In addition, 20 pTa (noninvasive) and 19 pT1 (lamina propria invasive) FFPE UCB tumors were also screened. This totaled 63 tumor samples and 24 TAN samples. In the combined tumor cohort, 17 of 63...
(27%) UCB retained heterozygosity for the ApaI polymorphism, whereas 2 tumors on which tumors and TAN were available displayed loss of heterozygosity (LOH) for IGF-II (A225 and A197; Fig. 1). Of the remaining 44 tumors, 40 of 63 (64%) displayed the c allele, whereas 4 of 63 (6%) showed the t allele. In the 24 TAN frozen cases from patients with UCB, 11 (46%) were heterozygous (A78N, A134N, A192N, A197N, A198N, A204N, A220N, A223N, A225N, A226N, and A233N; Table 1). Therefore, the imprinting analysis presented in Table 1 represents results for 29 informative tissues in total (11 paired tumors and TAN frozen samples and 7 FFPE unpaired tumors; Table 1). In the case of the UCB cell lines, RT4 was informative for the previously described ApaI SNP (23), and the TCCSUP cell line was informative for the alternative SNP (a3; C3253T). The remaining cell lines, CAL29, RT112, T24, and HT1376, were uninformative for all six SNPs analyzed.

IGF-II imprinting patterns in informative/heterozygous UCB/TAN paired samples. Figure 2 shows the four imprinting patterns identified in this study for the paired UCB and TAN samples, represented by cases A226, A233, A204, and A198. The four profiles consist of (a) MOI in both tumor and TAN, 5 of 9 (56%; cases A78, A192, A220, A223, and A226; Fig. 2A); (b) MOI in the tumor concomitant with LOI in the paired TAN, 1 of 9 (11%; case A233; Fig. 2B); (c) LOI in the tumor with MOI in the paired normal, 1 of 9 (11% case A204; Fig. 2C); and (d) LOI in both tumor and paired normal, 2 of 9 (22%; cases A134 and A198; both recurrent tumors; Fig. 2D). In the case of sample A226 (Fig. 2A), despite the clear representation of both c and t alleles at the DNA level in both tumor and TAN samples, only the c allele is evident at the cDNA level in both samples, indicating an IGF-II MOI profile. Alternatively, case A198 (Fig. 2D) displays clear IGF-II (LOI) at the cDNA level, with the c and t alleles represented in both A198R and A198N. In the current study, 4 of 11 (36%) normal urothelial tissues (A225N, A134N, A198N, and A233N) and 7 of 17 (41%) urothelial carcinomas amenable to imprinting analysis showed LOI of IGF-II (A276T, A16T, A134R, A55T, A140T, A204T, and A198R; Table 1). Of the informative cell lines, TCCSUP displayed IGF-II LOI, whereas RT4 showed IGF-II MOI.

Of the two tumors displaying LOH A225T (pTa grade 2) and A197T (pT1 grade 3), the TAN of case 225 displayed concomitant LOI for IGF-II representing both structural and imprinting alteration at the IGF-II locus within the same
individual patient (Fig. 1A), whereas, in case A197, the TAN displayed IGF-II MOI (Fig. 1B). For A197T and A225T, both tumors have lost the c allele (Fig. 1; Table 1) with expression from the remaining t allele clearly shown in both cases (Fig. 1).

**IGF-II LOI and promoter usage**

The biallelic IGF-II expression evident in our UCB cases represents altered imprinting and was not due to a switch in promoter usage to the normally biallelic promoter 1, corroborating previous findings in bladder cancer (17).
Cytoplasmic CDH1 staining and IGF-II imprinting status

Table 1 summarizes CDH1 staining and immunolocalization within informative IGF-II cases. All cases examined retained CDH1 membranous staining. Concomitant CDH1 cytoplasmic staining was evident in 7 tumors (A276T, A55T, A140T, A198R, A58T, A261T, and A233T; Fig. 3A; Table 1). Four of these 7 (57%) cases showed IGF-II LOI in the tumor (A276T, A55T, A140T, and A198R). A further tumor A233T displayed cytoplasmic CDH1 staining (Table 1) while displaying MOI IGF-II in the tumor and IGF-II LOI in the TAN, suggesting a possible paracrine effect in the redistribution of CDH1 to the cytoplasm as a result of the increased levels of IGF-II in the surrounding normal tissue (Table 1). The remaining 2 cases (cases A58 and A261) displayed an IGF-II MOI profile in the tumor. There was no available TAN in these cases to establish if IGF-II LOI may be evident in the surrounding tissue similar to that found in case A233N, which may account for the cytoplasmic CDH1 localization.

Fig. 3. Association of CDH1 cytoplasmic immunolocalization with IGF-II TaqMan QRT-PCR expression levels and imprinting phenotype. A, CDH1 immunolocalization in informative UCB cases showing IGF-II LOI (A140T) and IGF-II MOI (A226T) profiles. MOI of IGF-II is associated with membranous localization of CDH1 (A226T) and LOI of IGF-II is associated with the redistribution of CDH1 from the membrane to the cytoplasm (A140T). B, CDH1 mRNA expression within a panel of UCB cell lines (n = 6). CDH1-negative expression is evident within TCCSUP and T24 cells, whereas varying degrees of cytoskeletal-associated and cytoskeletal-unassociated CDH1 are present within CAL29, HT1376, RT112, and RT4 cells. 4′,6-Diamidino-2-phenylindole staining highlights the nuclei, whereas FITC highlights CDH1 immunolocalization. C, quantification of CDH1 cytoskeletal-associated and cytoskeletal-unassociated proteins within the UCB cell line panel. Following Western blotting analysis, densitometry allowed quantification of the percentage of cytoskeletal-associated and cytoskeletal-unassociated proteins relative to total protein within each cell line. A representative Western analysis for each fraction is shown. Significantly higher unassociated CDH1 is observed within HT1376 and RT112 compared with RT4 cell lines (P = 0.009 and 0.02, respectively, Student’s t test). D, basal IGF-II relative expression levels. Demonstration of the relative IGF-II QRT-PCR levels within a panel of UCB cell lines measured using the TaqMan comparative ΔCT method of analysis. Relative expression levels are shown as log values. The TCCSUP cell line is employed as a calibrator (1), to which all other cell lines are compared. β-Actin is used as an endogenous control. Significantly higher IGF-II expression is observed within CAL29, HT1376, and RT112 compared with RT4 UCB cell lines (P = 0.0003, 0.003, and 0.005, respectively, Student’s t test).
In those tumors displaying IGF-II MOI, only 3 of 10 (30%; A58T, A261T, and A233T) had concomitant CDH1 localization, with the remaining tumors showing exclusive membranous staining for CDH1 (Fig. 3A; Table 1).

**Subcellular localization of CDH1 and IGF-II relative expression levels in vitro**

Figure 3B shows the immunofluorescent localization of CDH1 in a panel of 6 UCB cell lines. Cytosplasmic staining is clearly evident in the CAL29, HT1376, and RT112 cell lines compared with the RT4 line, the latter displaying crisp CDH1 membranous staining. T24 and TCCSUP display CDH1-negative staining. Western blot analysis of cytoskeletal-unassociated (cytoplasmic) and cytoskeletal-associated (membranous) fractions shows significantly increased amounts of cytoskeletal-unassociated CDH1 in HT1376 and RT112 compared with RT4 (P = 0.009 and 0.02, respectively; Fig. 3C), corroborating the immunofluorescent findings (Fig. 3B). Furthermore, TaqMan QRT-PCR IGF-II levels were also significantly higher in CAL29, HT1376, and RT112 compared with RT4 (P = 0.003 and 0.005, respectively; Fig. 3D), mirroring the increased cytoskeletal-unassociated CDH1 localization in these cells.

**Significance of CDH1 cytoplasmic localization and disease-free interval/time to recurrence in nonmuscle invasive pT1 and pT1 UCB presentations**

Stage and tumor grade were unable to predict time to recurrence in univariate and Kaplan-Meier distributions for our cohort of pT1 (noninvasive) and pT1 (lamba propria invasive) UCB presentations (Fig. 4A and B; Table 2). However, CDH1 cytoplasmic staining present in >10% (Fig. 4C) or >30% (Fig. 4D) of cells was significantly associated with a shorter time to recurrence. Cytosplasmic staining in >50% of cells did not reach significance due to the smaller numbers. Importantly, these findings retained significance in multivariate analyses (Table 2) independent of sex, age at diagnosis, stage or grade, or smoking history. None of the differential CDH1 membrane staining patterns were associated with disease-free interval. In the analysis of the clinically problematic pT1 grade 3 tumors (n = 20), cytoplasmic staining in >30% of cells was a significant predictor of subsequent recurrence in both univariate and importantly multivariate analyses adjusted for age, sex, and smoking history (Table 2).

**Discussion**

In the present study, IGF-II LOI was shown in 7 of 17 (41%) informative UCB. In previous bladder cancer studies, IGF-II LOI was evident in 1 of 2 informative bladder tumors (14), a bladder cancer case with concomitant LOI in the IPW gene (15), and more recently 2 of 9 (22%) bladder cancers (17). In the current study, IGF-II LOH occurred in the tumors of 2 of 11 (18%) informative paired tumors and TAN cases (A197T and A225T), representing a noninvasive (pT1 grade 2) and lamina propria invasive (pT1 grade 3) early-stage UCB cases, respectively. This finding corroborates previous data where IGF-II LOH (frequently associated with a duplication event; ref. 26) was found in ~40% of bladder tumors, including pTa tumors, but was more common in tumors of higher grade and stage (11). Of the four IGF-II imprinting studies in bladder in the literature, LOH was only discussed in one (17) where 1 of 13 (8%) displayed IGF-II LOH.

From our data, IGF-II imprinting alterations also occurred in adjacent TAN tissue in 4 of 11 (36%) paired UCB cases, with 2 cases showing coordinated IGF-II LOI in both the tumor and TAN, a novel finding for this tumor type but well documented for colorectal cancer where efforts have been taken to decipher the significance of IGF-II LOI in normal tissue (3, 27, 28). From these studies, it has been shown that IGF-II LOI in normal tissue is associated with a 5-fold increased risk of adenoma formation (3). Moreover, IGF-II LOI in normal mucosa correlated with an earlier onset of colorectal cancer by an average of 14 years compared with those whose normal mucosa showed IGF-II MOI (6). Importantly, individuals with colorectal neoplasia had a 5.1-fold increased incidence of IGF-II LOI in peripheral blood leukocytes (2), identifying IGF-II LOI as an “epi-risk” factor that can be detected using a blood test. The finding of IGF-II LOI in normal bladder

| Table 2. Univariate and multivariate analyses for disease-free interval in the total 114 cohort of UCB and disease-free interval survival for the pT1 grade 3 group (n = 20) |
|---|---|---|---|
| Full TMA cohort (n = 114) | **Univariate** | **Multivariate** |
| | Hazard ratio | P | Hazard ratio | P |
| Male sex | 0.99 | 0.96 | — | — |
| Age at diagnosis (y) | 1.01 | 0.62 | — | — |
| Ever smoked | 1.00 | 0.99 | — | — |
| Stage pT1 vs pTa | 1.33 | 0.26 | — | — |
| Grade 3 vs (1 + 2) | 1.27 | 0.40 | — | — |
| CDH1 cytoplasmic positive (n = 46; 40.7%) | 1.57 | 0.06 | — | — |
| CDH1 cytoplasmic >10 (n = 23; 20.4%) | 2.29 | 0.002 | 2.32 | 0.005 |
| CDH1 cytoplasmic >30 (n = 15; 13.3%) | 3.19 | 0.004 | 3.39 | 0.0004 |
| CDH1 cytoplasmic >50 (n = 9; 7.9%) | 2.02 | 0.08 | 2.29 | 0.057 |
| pT1 grade 3 cohort (n = 20) | — | — | — | — |
| CDH1 cytoplasmic positive | 2.79 | 0.12 | 3.01 | 0.11 |
| CDH1 cytoplasmic >10 | 1.96 | 0.23 | 5.82 | 0.03 |
| CDH1 cytoplasmic >30 | 34.27 | 0.002 | 48.04 | 0.004 |
| CDH1 cytoplasmic >50 | 17.49 | 0.04 | 8.21 | 0.15 |

* Adjusted for sex, age at diagnosis, stage, grade, and ever smoked.
tissue, at a similar frequency (36%) to these colorectal carcinoma studies, suggests that IGF-II LOI might play a similar role in predicting UCB occurrence.

In the current study, IGF-II LOI occurred at a similar frequency in patient cohorts with noninvasive, lamina propria invasive, and muscle invasive tumors (40% pT1a, 40% pT1b, and pT2 50%). With the exception of case A276T (pTa grade 1), the remaining IGF-II LOI tumors were either grade 2 or 3.

Of the 4 high-risk lamina propria pT1 grade 3 primary UCB tumors, 3 showed alterations, either epigenetic (2 of 4; A55T and A140T) or structural (1 of 4; A197T), at the IGF-II locus, suggesting that IGF-II may play an important role in the progression to muscle invasive UCB. This is supported by the fact that case A55 subsequently recurred with a pT2 grade 3 tumor, and in the case of A140T, this patient had evidence of disease at the last date of follow-up (7 years 10 months).

Importantly, the recurrent tumor (A134R pT2 grade 2) originally presented as a pT1 grade 3 tumor but eventually developed multiple recurrences (n = 7) with progression to muscle invasive UCB and metastatic disease. Therefore, pT1 grade 3 UCB patients known to be at risk of recurrence with possible progression to invasive and potentially metastatic disease may benefit from more radical surgical management following molecular analysis of the IGF-II locus.

The epithelial-to-mesenchymal transition provides epithelial cells with the plasticity required during tumor progression to allow invasion and metastasis (29). CDH1 is a key epithelial-to-mesenchymal transition marker integral to epithelial adhesion, alterations of which have been associated with tumor progression and metastasis (30). IGF-II has been shown to induce a rapid epithelial-to-mesenchymal transition in a rat bladder cell line NBT-II, where it caused the redistribution of CDH1 to the cytoplasm and &beta; catenin to the nucleus, inducing the transcription of &beta; catenin/TCF3 genes (19). Therefore, IGF-II LOI, potentially resulting in increased levels of IGF-II, could initiate this reported redistribution of CDH1/IGF-I receptor/ &beta; catenin complex (19), reinforcing the role of IGF-II in invasion and metastasis. In the present study, all informative IGF-II cases retained CDH1 membranous staining. However, in those tumors showing IGF-II LOI (A276T, A16T, A55T, A140T, A134R, A198R, and A204T), 4 of 7 (57%) cases A276T, A55T, A140T, and A198R) displayed concomitant CDH1 cytoplasmic staining. In contrast, in those tumors displaying IGF-II MOI, only 3 of 10 (30%; cases A58T, A261T, and A233T) had concomitant CDH1 cytoplasmic localization. Interestingly, A233T, although having an IGF-II MOI profile in the tumor, displayed IGF-II LOI in the TAN, suggesting a possible paracrine effect in the redistribution of CDH1 to the cytoplasm as a result of the increased levels of IGF-II in the surrounding normal tissue.

Varying IGF-II expression levels were observed within our panel of UCB cell lines (RT4, TCCSUP, RT112, T24, HT1376, and CAL29), confirming previous reports (16, 31). The epithelial RT112 cell line, which has been shown previously to undergo 11p15.5 LOH (32), displayed the highest IGF-II expression. Due to down-regulation of the maternally expressed imprinted gene CDKN1C in RT112 cells, it has been concluded that the LOH of 11p15.5 within this cell line involves the maternal allele (32). Therefore, IGF-II LOH in combination with possible paternal allele duplication would explain the high IGF-II levels within the RT112 cell line. Although it is difficult to directly compare between cell lines due to karyotypic variability, it was surprising that IGF-II expression levels were lowest in the mesenchymal IGF-II LOI TCCSUP cell line, with higher levels being observed in the epithelial IGF-II MOI RT4 cell line. The IGF-I receptor has been shown to be significantly up-regulated in superficial and invasive UCB compared with normal urothelium (33), with a close relationship existing between IGF-I receptor and stage, grade, and recurrence (34). Reinforcing the suggestion that IGF-II LOI may effect IGF-I receptor expression (10), the undifferentiated TCCSUP IGF-II LOI cell line has been shown to have significantly more IGF-I receptor than the differentiated RT4 IGF-II MOI cell line (35). Therefore, the low IGF-II expression levels within the highly proliferating LOI TCCSUP cell line may induce a strong biological effect in this enhanced state.

A significant association between CDH1 cytoplasmic staining and time to recurrence in UCB was associated with cytoplasmic immunolocalization on a TMA UCB platform of 114 patient samples (70 pT3 and 41 pT1). Stage and grade were not predictive of this outcome in this cohort, highlighting the significance of CDH1 localization. The inability of stage and grade to predict disease-free interval in the pT3 and pT1 groupings has been reported by others (36). However, other studies have shown a significance for grade and stage in univariate but not multivariate analyses (37), with others reporting independent significance for stage but not grade (38). Importantly, in the pT1 grade 3 subcohort of 20 tumors, CDH1 cytoplasmic localization was an independent predictor of time to recurrence, highlighting a possible role for this evaluation in identifying the more unpredictable tumors that may indicate more surgical intervention than TURBT alone. The altered expression of CDH1 has been reported previously in bladder cancer, with much of the focus being on loss of membrane expression. CDH1 cytoplasmic localization has been either ignored or included as fully functional (39). A correlation has been reported between early tumor recurrence and abnormal CDH1 staining in terms of loss of membrane staining (40). However, there has been no previous report of any observed cytoplasmic staining as documented in this study. Our findings would suggest that it is the increase in cytoplasmic CDH1 that is associated with tumor recurrence and death from bladder cancer rather than the loss of membrane staining and that the redistribution of CDH1 from the membrane to the cytoplasm may reflect the role IGF-II plays in the intracellular sequestration and degradation of CDH1 during the epithelial-to-mesenchymal transition (19), identifying a novel role for aberrant IGF-II levels in compromising cellular adherence.

In summary, the finding of LOI of IGF-II in both tumor and adjacent normal tissue may be associated with an increased risk of UCB. Normal tissue showing this altered phenotype retained by nonradical surgical regimens may maintain an environment favorable for the initiation of future recurrences and warrants further investigation. Therefore, IGF-II imprinting analysis of paired UCB cases, along with other prognostic factors, for example CDH1 cytoplasmic staining, may aid in the identification of patients who should receive more radical surgery. Moreover, the degree of IGF-II alterations, both epigenetic and structural, evident within the high risk pT1 grade 3 subgroup, coupled with the presence of cytoplasmic CDH1 localization may represent those patients most likely to subsequently recur with muscle invasive and metastatic disease potentially
identifying patients for whom bladder conserving surgical management may not be appropriate. If aberrant clinical levels of IGF-II could be restored to normal, then this could hypothetically impede tumorigenic progression. Excitingly, in support of this, two in vivo studies have shown rescue of the transformed phenotype in mice as a result of sequestration of IGF-II by its degradative receptor IGF-IIR (41, 42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to acknowledge the Irish Cancer Society for their financial support of this work.

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Recurrence of Urothelial Carcinoma of the Bladder: A Role for Insulin-Like Growth Factor-II Loss of Imprinting and Cytoplasmic E-Cadherin Immunolocalization

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