Telomere Shortening and Chromosomal Abnormalities in Intestinal Metaplasia of the Urinary Bladder

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Abstract Purpose: Although intestinal metaplasia is often found in association with adenocarcinoma of the urinary bladder, it is unclear whether intestinal metaplasia of the bladder is a premalignant lesion. Telomere shortening has recently been implicated in epithelial carcinogenesis. We used quantitative fluorescent in situ hybridization (FISH) to measure telomere length and UroVysion FISH to detect cytogenetic abnormalities in urinary bladder specimens with intestinal metaplasia.

Experimental Design: Paraffin-embedded tissue blocks from 34 patients with intestinal metaplasia of the urinary bladder were evaluated. Twelve of the 34 patients had coexistent cystitis glandularis, and telomere length was measured in these lesions for comparison. Tissue sections were prepared and hybridized with a telomere-specific peptide nucleic acid probe. Quantitative FISH on interphase nuclei was used to assess telomere signal intensity. Additional sections were hybridized with centromeric probes for chromosomes 3, 7, and 17 and a locus-specific probe 9p21. Multicolor FISH was used to analyze for cytogenic abnormalities in the interphase nuclei of intestinal metaplasia.

Results: In all 34 cases, reduced average telomere signal intensity was observed in the nuclei of intestinal metaplasia cells compared with adjacent control nuclei to produce a mean relative intensity of 48.5% (P < 0.0001). When cystitis glandularis was present, significant differences in the telomere-specific signal intensity existed between cystitis glandularis and normal cells (P = 0.0005) and between cystitis glandularis and intestinal metaplasia cells (P = 0.0015). Three of the 34 cases showed chromosomal gains in the UroVysion FISH assay.

Conclusions: Our findings indicate that intestinal metaplasia in the urinary bladder is associated with significant telomere shortening relative to telomere length in adjacent normal urothelial cells. These lesions also occasionally showed cytogenetic abnormalities associated with telomere shortening. Our findings support the hypothesis that intestinal metaplasia of the urinary bladder is a precursor lesion to and could be a marker in the development of adenocarcinoma of the urinary bladder.
on the frequent coexistence of intestinal metaplasia and adenocarcinoma of the bladder; a large series of primary enteric-type adenocarcinoma of the urinary bladder showed the association in 32% of the cases (6). The actual pathologic demonstration of adenocarcinoma developing after a diagnosis of intestinal metaplasia is limited to a small number of case reports (7–12).

Given the low incidence of intestinal metaplasia in the bladder and the lack of long-term follow-up, molecular analysis may provide useful information about the malignant potential of this lesion. A number of recent studies have focused on the role of telomeres in epithelial carcinogenesis. These repetitive DNA sequences associate with a number of proteins to protect the ends of linear chromosomes from degradation, recombination, and end-joining reactions (13). Dysfunctional or shortened telomeres have been shown to cause chromosomal instability leading to epithelial cancers in mouse models (14) and immortal human cells in vitro (15). Furthermore, fluorescence in situ hybridization (FISH) analysis with a telomere-specific probe has shown that telomere shortening is present in a wide variety of human epithelial cancer precursors, supporting the idea that telomere dysfunction plays an important role in epithelial carcinogenesis (16, 17). FISH has also been used with multiple centromeric and locus-specific probes to detect cytogenetic abnormalities in urine specimens, providing a sensitive and specific method for the detection of urothelial carcinoma (18). In this study, we used FISH to assess telomere length and the cytogenetic abnormalities in bladder specimens with intestinal metaplasia in an attempt to learn more about the malignant potential of this lesion.

### Materials and Methods

**Tissue samples.** Tissues from 34 patients with the diagnosis of intestinal metaplasia of the urinary bladder from 1989 to 2005 were obtained from the Surgical Pathology archives of the participating institutions. All cases were reviewed and diagnosed according to well-accepted criteria (5, 19). The diagnosis of cystitis glandularis was reserved for luminal structures within the lamina propria with peripheral transitional cells and an innermost lining of columnar or cuboidal cells. When the innermost lining contained goblet cells, the diagnosis of intestinal metaplasia was used (5, 19).

**Telomere FISH.** From each specimen, multiple 4-μm unstained sections were prepared from buffered formalin-fixed, paraffin-embedded tissue blocks. The unstained slides were deparaffinized with two changes of xylene, 15 min for each. Following the xylene, the slides were treated with two changes of absolute ethanol, 10 min each. The slides were washed with TBS (Tris 1.4 g, NaCl 8.75 g (pH 7.5)) for 2 min then fixed with 3.7% formaldehyde in TBS for 2 min. The slides were air dried in a hood and then boiled in a glass staining jar with 1× citrate buffer (pH 6.0; Zymed) within a beaker filled with distilled water on a hot block for 10 min. The boiled slides were kept in the citrate buffer until they cooled down to room temperature. The slides were removed from the staining jar, washed with distilled water for 3 min, and then transferred to 2× SSC for 5 min. The slides were air-dried and digested with 0.75 mL pepsin [5 mg/mL in 0.9% NaCl and 0.01N HCl; Sigma] at 37°C for 30 min. The slides were then washed with distilled water for 3 min; with TBS for 5 min twice; and then with 70%, 85%, and 96% ethanol, 2 min for each. They were then air-dried. The telomere-specific PNA probe (DAKO) was diluted with 70% formamide in TBS solution (1:50). Ten microliters of diluted probe were applied followed by 22 × 22 mm coverslips sealed with rubber cement. The slides were put into an opaque plastic box wrapped with aluminum foil, denatured at 80°C for 10 min, and hybridized at 37°C overnight.

**Analysis of telomere signal intensity.** The slides were examined using a Zeiss Axiosplan 2 microscope (Zeiss) with the following filters: SP-100 DAPI, FITC MF-101 from Chroma. The images were captured with Isis software. The images were adjusted to be seen clearly. The target cell nuclei were encircled, and the background was subtracted automatically. The total FITC pixel numbers and the percent saturation were then measured.

The percentage saturation is a term in light intensity measurement. The image is taken by the charged coupled device and processed as pixel number (for the area the signal covers) and the percentage of saturation (the light intensity of signal pixels). The signal saturation is divided into 256 grades and each pixel is assigned a grade according to the measurement. Then, the computer generates an average intensity for the overall pixels within the signal. This represents the average signal level compared with the 100% saturated signal pixels (light intensity of each pixel would be 256). Using these variables, the computer generates a result, percentage saturation, that indicates how bright the signal is, and thereby indicates how long the telomeres are. The basis of this formula is that the fluorescence intensity is positively correlated with the telomere length (16, 17). DAPI was used in each case for counterstaining to recognize the lesion and to pick the right cells to measure. Only the telomere signals located within the DAPI defined, nonoverlapping nuclei were measured. DAPI would not generate any signal because the DAPI light channel is not measured.

Telomere signal intensity was calculated by pixel numbers × % saturation for the individual cells. A total of 25 nonoverlapping cells from each target area were measured. The normal urothelium on the same slide was used as a control. The relative telomere intensities for intestinal metaplasia and for cystitis glandularis were calculated by dividing their average values by the average control value. The values for relative telomere intensity were used to compare telomere lengths as the two have been shown to be linearly related (16).

**UroVision FISH.** Additional unstained slides were prepared as previously described (20–25) for multitarget, multicolor FISH. The centromeric probes (CEP) were fluorescence labeled as follows: CEP3 probe Spectrum Red, CEP7 probe Spectrum Green, and CEP17 probe Spectrum Aqua. The locus-specific probe 3p21 was labeled with Spectrum Gold (Vysis). The probes were diluted with DenHyb2 (Insitus) in a ratio of 1:10. Five microliters of diluted probes were added to the slides in the reduced light condition. The slides were then covered with a 22 × 22 coverslip and the edge was sealed with rubber cement. The slides were put into an opaque plastic box wrapped with aluminum foil. The slides were denatured at 83°C for 12 min and hybridized at 37°C overnight. After hybridization, the slides were washed at 45°C with prewarmed 0.1× SSC with 1.5 mol/L urea twice, 20 min each. They were then washed with 2× SSC for 20 min and a 2× SSC/0.1% NP40 for 10 min at 45°C. The slides were further washed with room temperature 2× SSC for 5 min. The slides were counterstained with 10 μL DAPI (Insitus) for 2 min and then covered with a 50 × 22 coverslip with the edge sealed with nail polish liquid.

The stained slides were observed and documented using a Metasystem under ×100 oil objective. The areas of intestinal metaplasia were assessed for chromosomal abnormalities. The following filters were used: SP-100 for DAPI, FITC MF-101 for Spectrum Green, Gold 31003 for Spectrum Gold, Aqua 31036V2 for Spectrum Aqua, and TriRed Sp-103 for Spectrum Red signals. Signals from each probe were counted under false color, with which the computer will show each color channels into red, green, gold, and aqua color. Five sequential
focus stacks with 0.4-μm intervals were acquired and then integrated into a single image to reduce thickness-related artifacts. For each case, 50 nuclei were examined for genetic alterations. The signal numbers from each probe were documented for further analysis.

**Statistical analysis.** The relative telomere signal intensity was defined as the ratio of signal intensity with respect to that of normal urothelial cells. The mean telomere signal intensities of intestinal metaplasia, cystitis glandularis, and normal urothelial cells were compared using Wilcoxon paired signed rank test. A *P* value <0.05 was considered significant, and all *P* values were two sided.

**Results**

All 34 specimens showed reduced average signal intensity in the intestinal metaplasia cells compared with the urothelial control cells. The ratio of signal intensity of intestinal metaplasia cells versus that of normal urothelial cells ranged from 20.6% to 90.8%, with an average of 48.5% (Fig. 1A-D; Table 1A). In the 12 specimens with coexisting cystitis glandularis and intestinal metaplasia, significant differences in the telomere-specific signal intensity existed between cystitis glandularis and normal cells (*P* = 0.0005) and between cystitis glandularis and intestinal metaplasia cells (*P* = 0.0015; Table 1B).

**Discussion**

When compared with urothelial controls, the cells within areas of intestinal metaplasia of the urinary bladder had significantly shortened telomeres. This finding may be an integral part of metaplastic change, a process that is believed to be induced by chronic irritation and inflammation. Bladder epithelium responds to inflammation with proliferation to replace damaged cells (1). With each round of replication, telomeres are expected to shorten due to the end-replication problem (26, 27). Inflammation also brings about oxidative damage, which seems to be poorly repaired in telomeric DNA (28). This damage has been shown to accelerate telomere shortening in replicating human fibroblasts (29). In support of our findings in the bladder, shortened telomeres have been found in other organs subject to chronic inflammation, such as in Barrett’s esophagus and ulcerative colitis (30, 31).

UroVysion FISH showed cytogenetic abnormalities in 3 of the 34 cases of intestinal metaplasia. All three cases showed significantly reduced telomere length. Among three cases, one showed a gain of chromosome 3 only. The second case showed a gain of chromosomes 3 and 7 (Fig. 1E). A gain of chromosomes 3, 7, and 9 was found in the third case.

**Fig. 1.** Telomere shortening in intestinal metaplasia of the urinary bladder. A, H&E staining of intestinal metaplasia characterized by glandular structures in the lamina propria, lined by columnar epithelium including goblet cells. B, FISH with telomere-specific probe of inset in A showing reduced telomere signal intensity in metaplastic cells (IntMet) compared with the adjacent stromal cells. Another example of intestinal metaplasia showed similar findings (C and D). E, representative hybridization in a case of intestinal metaplasia using the UroVysion probe set containing CEP3, CEP7, CEP17, and locus-specific probe 9p21. The metaplastic cell shows three CEP3 (red), three CEP7 (green), two CEP17 (aqua), and two locus-specific probe 9p21 signals, indicating a gain of chromosomes 3 and 7.
Table 1. Relative telomere signal intensity (%) with respect to normal urothelial cells in 34 cases of intestinal metaplasia (A) and 12 specimens with coexisting intestinal metaplasia and cystitis glandularis (B)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>N</th>
<th>Mean telomere length of cells ± SE</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal cells</td>
<td>34</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Intestinal metaplasia*</td>
<td>34</td>
<td>48.50 ± 3.33</td>
<td>20.58-90.76</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal cells</td>
<td>12</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cystitis glandularis †</td>
<td>12</td>
<td>81.51 ± 3.89</td>
<td>55.56-110.00</td>
</tr>
<tr>
<td>Intestinal metaplasia † ‡</td>
<td>12</td>
<td>46.10 ± 6.54</td>
<td>20.58-87.21</td>
</tr>
</tbody>
</table>

*Telomere intensity was statistically shorter compared with that of the normal cells with $P < 0.0001$ using Wilcoxon paired signed rank test.
†Telomere intensity was statistically shorter compared with that of the normal cells with $P = 0.0005$ using Wilcoxon signed rank test.
‡Telomere intensity was statistically shorter compared with that of the normal cells with $P = 0.0015$ using Wilcoxon signed rank test.
§The telomere intensity was significantly shorter compared with cystitis glandularis ($P = 0.0015$, Wilcoxon signed rank test).

Interestingly, the epithelial cells present in areas of cystitis glandularis possessed telomeres whose lengths were intermediate between those of normal urothelial cells and those of the cells in areas of intestinal metaplasia. This observation supports the idea that cystitis glandularis is a proliferative process with biological properties that are intermediate between those of normal urothelium and those of intestinal metaplasia. Perhaps the cells of cystitis glandularis are triggered to produce the intestinal transcription factors observed by Sung et al. (5) in an effort to ameliorate ongoing inflammation-induced damage and telomere loss.

The significance of telomere shortening has been revealed by recent studies of epithelial carcinogenesis. Direct evidence linking telomere shortening to chromosomal instability has been provided in studies using human cell cultures (15) and mouse models (14). These studies have shown that replication in the setting of dysfunctional telomeres leads to chromosomal end-to-end fusions, anaphase bridging, and breakage, analogous to the breakage-fusion-bridge cycles originally described by McClintock (32). This chromosomal instability produces rare immortal cells in vitro and epithelial cancers in mice, and it is thought that this mechanism may account for a proportion of the abnormal karyotypes seen in human epithelial cancers. Of note, these studies used some degree of cell cycle checkpoint inactivation to bypass the cellular senescence that normally prevents replication with DNA damage. Taken together, these findings suggest that telomere shortening, as well as the loss of cell cycle control, are important in epithelial carcinogenesis.

In an attempt to better understand the timing of these events in malignant transformation, FISH analysis of telomeres in premalignant lesions has been very useful. Meeker et al. (17) showed telomere shortening in a wide variety of epithelial cancer precursors, indicating that telomeres play an early role in carcinogenesis. In a study specific to Barrett’s esophagus, telomeres were the shortest in the earliest stages of the metaplasia-dysplasia-carcinoma sequence (31). In contrast, the maximum level of chromosomal instability was observed in the specimens with high-grade dysplasia. In addition, this chromosomal instability was found to generate losses and gains of chromosomal arms specific for cell cycle regulatory proteins. These results indicate that telomeric dysfunction may precede and contribute to the loss of the cell cycle regulation and chromosomal instability thought to be necessary for malignant transformation.

Our findings of chromosomal gains in three cases of intestinal metaplasia may be examples of the chromosomal instability generated by shortened telomeres. All three of these cases showed significant telomere shortening in the nuclei of cells with the intestinal phenotype. As the UroVysion probes were developed for the sensitive and specific detection of urothelial carcinomas in urine cytology specimens, additional unrecognized chromosomal abnormalities may have been present in these tissue sections of metaplastic intestinal epithelium. We did not observe 9p21 deletion in any of the three cases of intestinal metaplasia that showed chromosomal abnormalities. This may be due to the small sample number or may indicate that the carcinogenic pathways that lead from intestinal metaplasia to adenocarcinoma are different from the pathways involved in the development of urothelial carcinoma. Additional studies with probes directed toward chromosomal abnormalities implicated in the development of adenocarcinoma may shed further light on this question.

In view of the previous studies linking telomere shortening to chromosomal instability and demonstrating shortened telomeres in known epithelial precursor lesions, we believe that our results are important for the current understanding of intestinal metaplasia of the urinary bladder. Intestinal metaplasia is not a well-established premalignant lesion likely due to its low incidence and a lack of long-term follow-up. As a result, we attempted to detect the presence of premalignant molecular traits in this condition. Our findings of shortened telomeres and chromosomal gains indicate that these metaplastic cells are progressing toward a state of chromosomal instability that may eventually lead to malignant transformation. An environment of continued inflammation and irritation would provide stimuli for ongoing epithelial proliferation and may eventually select for rare cells capable of bypassing cell cycle checkpoints (33–36). The resulting replication with dysfunctional telomeres could provide the additional chromosomal instability necessary for the development of adenocarcinoma.

Our results support the previous case studies documenting the coexistence of these lesions (6) and the progression from intestinal metaplasia to adenocarcinoma of the bladder (7–12). However, Corica et al. (37) found that none of 53 patients with intestinal metaplasia developed adenocarcinoma after more than 10 years. This may be explained by the patient population in their study. Over half of the patients were children with a history of repaired bladder extrophy, and nearly all of the patients underwent removal of the lesional epithelium. It would be expected that the metaplastic cells in these children would be early in their replicative life span. Furthermore, repair of the extrophy likely removed the stimulus for accelerated telomere shortening. The lack of follow-up in our study represents a limitation; however, molecular analysis provides a new way to evaluate this lesion
using material already available. Future molecular studies as well as long-term follow-up may be necessary to provide conclusive evidence about the malignant potential of intestinal metaplasia.

In summary, we have shown significant telomere shortening in intestinal metaplasia of the urinary bladder. Three cases with telomere shortening also showed cytogenetic abnormalities associated with urothelial carcinoma. Considering the fact that telomere shortening is a trait that is commonly observed in epithelial cancerous tumors, our findings suggest that intestinal metaplasia is a precursor to and a marker for adenocarcinoma of the bladder. This could have important implications in the clinical management of this lesion. It seems appropriate, especially in cases of long-standing chronic inflammation, that intestinal metaplasia should be treated with complete resection if possible, amelioration or elimination of the inflammatory stimulus, and long-term endoscopic surveillance for the development of malignancy.

References

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