Telomere Length Abnormalities Occur Early in the Initiation of Epithelial Carcinogenesis

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ABSTRACT

Purpose: Telomeres help maintain chromosomal integrity. Dysfunctional telomeres can cause genetic instability in vitro and an increased cancer incidence in telomerase knock out mouse models. We recently reported that telomere shortening was a prevalent alteration in human prostate, pancreas, and breast cancer precursor lesions. In the present study, we address whether the previous findings are broadly applicable to human epithelial cancer precursors in general.

Experimental Design: Surgical specimens of epithelial cancer precursor lesions from the urinary bladder, esophagus, large intestine, oral cavity, and uterine cervix were examined using a recently developed technique for direct in situ telomere length assessment in formalin-fixed human tissue specimens.

Results: Widespread telomere length abnormalities were nearly universal (97.1% of cases) in the preinvasive stages of human epithelial carcinogenesis in all sites examined in this series, with telomere shortening the predominant abnormality (88.6% of cases).

Conclusions: Telomere length abnormalities appear to be one of the earliest and most prevalent genetic alterations acquired in the multistep process of malignant transformation. These findings support a model whereby telomere dysfunction induces chromosomal instability as an initiating event in many, perhaps most, human epithelial cancers. Together with previous findings from the prostate and pancreas, the percentage of intraepithelial neoplasia lesions showing telomere length abnormalities is 95.6%. The implications of these findings include the potential that telomere length assessment in situ may be a widely useful biomarker for monitoring disease prevention strategies and for improved early diagnosis.

INTRODUCTION

Grossly abnormal karyotypes, displaying both numerical and structural chromosomal changes, are a nearly universal finding in mismatch repair-proficient human epithelial malignancies, reflecting either a transient or ongoing state of chromosomal instability (1–3). This observation may be interpreted as a manifestation of a mutator phenotype acting at chromosomal and subchromosomal levels and may appear early in tumorigenesis (4–6). Several genes involved in the maintenance of chromosomal stability have been identified, representing candidate mutational targets for chromosome destabilization (7, 8). However, defects in such genes have thus far been implicated in only a small subset of human cancer cases, and these primarily affect chromosome number (9). Thus, the molecular mechanisms underlying chromosomal instability in the majority of human cancers remain a mystery. Likewise, the timing of chromosomal instability remains a critical question (10). One path to chromosomal instability is via telomere dysfunction. Telomeres are composed of specialized DNA sequence repeats complexed with telomere-binding proteins, located at the ends of linear chromosomes. Telomeres stabilize chromosomes by preventing deleterious recombinations and fusions and also keep cells from recognizing their chromosomal termini as DNA double-strand breaks (11). Telomeric DNA tracts are dynamic entities, subject to shortening during cell division because of their incomplete replication (referred to as the end-replication problem; Ref. 12). In addition, telomeres may shorten as a result of oxidative damage (13). Conversely, telomeres may be elongated through action of the ribonucleoprotein enzyme telomerase (14) or genetic recombination (15, 16).

Critically short telomeres become dysfunctional, and as demonstrated >50 years ago, loss of telomere function can be a major mechanism for the generation of chromosomal abnormalities (17, 18). In normal human cells, an incompletely characterized telomere length monitoring system responds to short telomeres by initiating either apoptosis or an irreversible cell cycle arrest, termed replicative senescence, responses proposed to have evolved as tumor-suppressive mechanisms in large, long-lived organisms (19–21). Inactivation of these checkpoints allows the development and tolerance of severe telomere shortening, such that one or more telomeres no longer perform their protective capping function. Chromosome end-to-end fusions ensue, producing dicentric, multicentric, and ring chromosomes that missegregate or break during mitosis, leading to a series of so-called Breakage-Fusion-Bridge cycles capable of generating...
aneusomies, as well as the various types of structural abnormalities typically seen in human solid tumor karyotypes (17, 22).

It has been postulated that dysfunctional telomeres could play a causal role in carcinogenesis by instigating chromosomal instability, thus promoting neoplastic transformation (19, 23–26). Results from telomerase-knockout mouse models in which animals possessing critically short telomeres exhibit an increased cancer incidence support this concept (27, 28). In particular, Artandi et al. (29) demonstrated that crossing telomerase-knockout mice with p53+/– mice resulted in a shift in the spectrum of tumors normally seen in the p53-defective background (primarily lymphomas and sarcomas) to one dominated by carcinomas displaying the types of karyotypic aberrations (e.g., nonreciprocal translocations) often observed in human epithelial cancers. This is a significant finding because most mouse models of human cancer involve overexpression of oncogenic transgenes or the knocking out of tumor suppressor genes, and tumors arising in these models generally show only simple chromosomal gains and losses rather than the more complex chromosomal aberrations that typify human carcinomas.

When examined by Southern blot analysis, the telomeres of invasive human cancers often appear shorter than their normal tissue counterparts (24). The combined observations of short telomeres, plus the frequent activation of telomerase in human epithelial cancers, suggest that the majority of tumors undergo critical telomere shortening at some point during their development. This could simply be a consequence of the end-replication problem combined with extensive cell turnover occurring during tumor expansion. On the other hand, if telomere shortening occurs early, it could be playing an important role during the initiation stage of tumorigenesis. Thus, the timing of the occurrence of telomere shortening during human cancer development is a critical question.

The vast majority of epithelial malignancies appear to develop from morphologically defined precursor lesions termed intraepithelial neoplasia (IEN; Ref. 30). Examinations to date have revealed evidence of genetic instability in IEN lesions, suggesting an early role for genetic changes in malignant transformation (5, 6, 31, 32). If telomere dysfunction is a major cause of this genetic instability, then signs of this dysfunction should likewise be evident in these early premalignant lesions. In support of this hypothesis, previous work investigating telomere lengths using a high-resolution in situ method for telomere length assessment in IEN lesions of the prostate and pancreas revealed dramatic telomere shortening in >90% of lesions examined (33–36).

In the current study, we used this method to test the hypothesis that telomere shortening is a widespread early contributor to human epithelial tumorigenesis in general. To this end, we probed telomere lengths in well-characterized preinvasive precursor lesions of several human epithelial cancers, including those of the large intestine, bladder, uterine cervix, esophagus, and oral cavity. We found clear evidence of telomere length abnormalities, primarily telomere shortening, as well as surprising telomere length heterogeneity, in the majority of IEN lesions from these human epithelial tissues, which represent a large proportion (~400,000 cases/year in the United States) of clinically relevant carcinoma sites.

### MATERIALS AND METHODS

#### Tissue Samples

Human tissues were obtained from the Department of Surgical Pathology at the Johns Hopkins University School of Medicine. The study was approved by the Johns Hopkins Internal Review Board. Biopsies and surgical specimens were routinely fixed in 10% neutral-buffered formalin and subjected to standard processing and paraffin embedding. For tissue microarray construction, representative areas containing morphologically defined lesions, normal tissues, or both were identified on H&E-stained sections, circled on the glass slides, and used as a template. Tissue microarrays were constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD). The tissue microarray blocks were sectioned at 4 μm and stained by telomere-fluorescence in situ hybridization (FISH) with or without immunofluorescence, with adjacent sections used for H&E staining. In total, 35 separate IEN lesions from 25 cases were examined, including 11 lesions from 8 bladder cases [2 low-grade papillary carcinoma in situ (CIS), 4 high-grade papillary CIS, 5 high-grade flat CIS], 3 squamous intraepithelial lesions (SILs) from 3 uterine cervix cases (1 low-grade SIL, 2 high-grade SILs), 7 lesions from 5 large intestine cases (5 adenomatous polyps, 2 high-grade dysplasias), 6 lesions from 3 esophageal cases (2 Barrett’s esophagi with low-grade dysplasia, 4 with high-grade dysplasia), and 8 lesions from 6 oral cavity cases (1 mild dysplasia, 4 moderate dysplasias, and 3 high-grade dysplasias).

**Telomere-FISH and Telomere/Immunostaining-FISH.** The protocol for combined staining of telomeric DNA (FISH probe) and immunostaining was performed without protease digestion, as described previously (33). Briefly, 4-μm thick sections from formalin-fixed, paraffin-embedded tissues were deparaffinized, hydrated through a graded ethanol series, and underwent heat-induced antigen retrieval for 14 min in citrate buffer in a steamer, followed by application of a Cy3-labeled, telomere-specific, peptide nucleic acid (PNA) probe (0.3 μg/ml) complementary to the mammalian telomere repeat sequence [custom synthesized by Applied Biosystems (Framingham, MA)] and having the sequence (NH2 terminus to COOH terminus) CCCTAAACCCTAACCTAA with a NH2-terminal covalently linked Cy3 fluorescent dye. Denaturation was conducted for 4 min at 83°C, followed by a 2-h room temperature hybridization step. Slides were then washed and counterstained with the DNA-binding dye 4’,6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO).

**Hybridization Probe Access Control.** To rule out differences in probe penetration or target accessibility as potential sources of observed differences in fluorescent telomere signal intensities in fixed tissue samples, we used a second fluorescently labeled PNA probe with specificity for centromeric DNA repeats (5’-Cy3-labeled PNA probe having the sequence ATTCGTTGAAAACGGGA synthesized by Applied Biosystems; Ref. 37). Using this probe, independent hybridizations were performed on tissue samples of several IEN lesions that displayed significant diminution of telomere hybridization signals in this study. Directly adjacent tissue sections were hybridized in parallel with either the centromere-specific PNA probe or the telomere-specific probe as described above.
Microscopy and Image Assessment. Areas containing normal epithelium and IEN lesions were identified on H&E stained slides prior to telomere length assessment on adjacent tissue sections by fluorescence microscopy as previously described (33). In the case of tissue microarray slides, such regions had been previously identified during array construction. During telomere length assessment, directly adjacent H&E reference slides were examined simultaneously with the telomere-FISH slides with a pathologist specializing in the particular tissue under study. In epithelial cells, telomeric staining produced a speckled pattern of widely distributed nuclear signals in all cases examined, with no evidence of significant peripheral or other nuclear sublocalization—in keeping with results previously reported for mammalian somatic cells (34, 36, 38, 39). For all tissues, the intensity of telomere staining, previously shown to be linearly related to telomere length, was assessed visually, with staining in IEN lesions compared qualitatively to that found in either adjacent normal-appearing epithelial cells or, where unavailable, to normal adjacent stromal cells (33, 40). IEN telomeres were scored as either very short (nearly unde-

Table 1 Results of telomere length survey on IEN lesions

<table>
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<tr>
<th>Site</th>
<th>Case</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>IEN lesion</th>
<th>Grade</th>
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<th>Relative telomere length</th>
<th>Heterogeneity</th>
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<td>HG</td>
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<tr>
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<tr>
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* IEN, intraepithelial neoplasia; CIS, carcinoma in situ; LG, low grade; HG, high grade; SIL, squamous intraepithelial neoplasia; Mod., moderate.
tectable), short (fluorescent signal intensity easily recognizable as less intense than normal-appearing epithelium or nonlymphocytic stroma), normal (signals equivalent to normal epithelium or stroma), long (signals significantly greater than normal), or very long (signals markedly brighter than normal epithelium or stroma; comparable with or greater than lymphocytes, which invariably exhibit robust telomere signals).

Anaphase bridges were defined as one or more clearly intact 4',6-diamidino-2-phenylindole-stained chromatin strands connecting and perpendicular to well-separated anaphase mitotic nuclei.

RESULTS

Microscopic examination of tissue sections containing IEN lesions hybridized with a telomere-specific fluorescent PNA probe revealed the presence of telomere length abnormalities in 34 of 35 (97%) of IEN lesions assayed. These included premalignant, preinvasive lesions of the large intestine, bladder, uterine cervix, esophagus, and oral cavity (Table 1, Figs. 1 and 2).

In agreement with previous studies, bright fluorescent telomere signals were observed in fibroblasts, smooth muscle cells, and endothelial cells of the stroma surrounding both premalignant lesions and normal-appearing epithelia, whereas lymphocytes exhibited very strong signals, typically stronger than those of other stromal cell types (Figs. 1 and 2; Refs. 34, 36, 41).

**Esophagus.** Six lesions from 3 cases were examined, including 2 Barrett’s esophagi with low-grade dysplasia and 4 with high-grade dysplasia. All dysplastic lesions displayed short or very short telomeres (Fig. 2, A and B). In 2 cases, regions of Barrett’s metaplasia were also observed on the biopsy speci-
mens, and these exhibited telomere lengths that were intermediate between normal esophagus and dysplasia.

**Large Intestine.** Seven lesions (5 adenomatous polyps, 2 high-grade dysplasias) from 5 cases were examined, all of which possessed short or very short telomeres compared with those within the normal-appearing epithelium (Fig. 1). In addition, anaphase bridges were observed in colonic adenomas where telomeres were found to be short (Fig. 3E). One adenoma (without associated high-grade dysplasia) and one high-grade dysplasia exhibited striking regional heterogeneity in which abrupt transitions were seen between large areas having very short telomeres and those possessing normal or longer than normal telomeres (Fig. 3, B–D). A detailed examination of these same cells poststained with H&E after telomere-FISH revealed no discernable differences in either cell or nuclear architecture between cells with short telomeres and those with elongated telomeres.

Invasive cancers, all of which contained short telomeres, were present in 3 cases. In 1 of these, the corresponding adenoma was of mixed telomere length phenotype, with the short component comparable with the cancer with respect to telomere length.

Although telomere lengths appeared uniform between cells within individual normal-appearing colonic crypts (Fig. 1, A and B), significant crypt-crypt variation was occasionally seen in 2 of 5 cases (Fig. 3F).

**Bladder.** In bladder specimens, 8 of 11 (72.7%) IEN lesions contained cells with short or very short telomeres. Of the epithelial sites examined in this study, telomere length variability was most prevalent in IEN lesions of the bladder, where 7 of 11 lesions displayed mixtures of cells with either abnormally short or abnormally long telomeres (Fig. 4). These mixed telomere length phenotype IEN lesions exhibited either regional, cell-cell intralesional variability, or both.
In 1 heterogeneous lesion, anaphase bridges were observed in the component with short telomeres, whereas, in contrast, cleanly separated anaphases were observed in the component having long telomeres.

In 1 case, invasive cancer was also present and exhibited very short telomeres. The flat CIS lesion (mixed telomere length phenotype) associated with this cancer displayed an equivalent level of telomere shortening in the component with short telomeres.

In 1 of 5 flat CIS lesions, very long telomeres were the sole abnormality seen (Fig. 4B).

**Uterine Cervix.** One low-grade SIL and 2 high-grade SILs from separate cases all displayed shorter telomeres than adjacent normal-appearing squamous epithelium, with 1 of the high-grade lesions also containing cells with normal length telomeres (Fig. 2, E and F).

**Oral Cavity.** Eight lesions from 6 cases were examined, all but one of which had short or very short telomeres (Fig. 2, C and D). In the case that did not display abnormal telomeres in the IEN lesion, invasive cancer was also present, and it exhibited telomere shortening.

**Confirmation of Hybridization Probe Access.** To rule out differences in either probe penetration or DNA accessibility as potential sources of the differences we observed in fluorescent-telomeric signal intensities in fixed tissue samples, we used a second fluorescently labeled PNA probe with different target specificity, namely centromeric DNA repeats (37). Using this probe, which is similar in size to the telomere-specific probe, independent hybridizations were performed on serial sections of tissue samples of several IEN lesions that displayed significant telomere shortening in this study. These adjacent sections were hybridized in parallel with either the centromere-specific PNA
probe or the telomere-specific probe, under identical hybridization conditions. In each case, the centromere-specific probe gave robust signals in all cells, including those in which telomeric signals were dim or undetectable (Fig. 5).

DISCUSSION

In previous work applying high-resolution fluorescence in situ telomere length assessment to human clinical specimens, evidence of marked telomere shortening was found in >90% of premalignant cancer precursor lesions of both the prostate and pancreas (34–36). In the current study, to determine how widespread this somatic DNA alteration is in human epithelial cancer precursor lesions, we surveyed 35 IEN lesions from an array of epithelial tissues, including the bladder, esophagus, large intestine, oral cavity, and uterine cervix, representing many clinically important human carcinoma target sites.

In this study, we found that in the vast majority (97.1%) of IEN lesions examined, the earliest identifiable epithelial cancer precursors are composed of cells possessing telomere length abnormalities, with most lesions (88.6%) displaying abnormally short telomeres (Table 1). It therefore appears that the telomere shortening frequently observed in malignant epithelial tumors has already occurred by the preinvasive stage (Table 2). Indeed, when both were present, invasive cancers and accompanying IEN lesions typically exhibited similar degrees of telomeric shortening in agreement with previous results in the prostate and pancreas (34–36).

Although the cause of the shortened telomeres we observed in IEN lesions is unknown, research to date suggests three possibilities. The first is the end-replication problem, a result of the inability of DNA polymerases to completely replicate the termini of linear DNA molecules. Thus, if the initial target cell for malignant transformation was negative (or weakly positive) for the telomere length maintenance enzyme telomerase, then loss of telomeric DNA could take place during the clonal expansion/selection processes thought to occur during tumor development (42–44). Secondly, several proteins have been implicated in the formation of a protective higher-order capping structure at the telomeres, and experimental changes in the level of expression or function of several of these proteins have been shown to affect telomere length, both positively and negatively (45, 46). It remains to be seen, however, whether similar changes in telomeric capping proteins occur during tumorigenesis. Finally, telomeres may rapidly shorten as a result of inefficiently repaired DNA damage caused by oxidative stress (13).

In light of the above, it is noteworthy that several chronic inflammatory conditions, known to cause both increased cell turnover and oxidative damage, are positively correlated with the risk of epithelial cancer development (47). Intriguingly, inflammatory cells are frequently seen in association with IEN lesions, and telomere shortening has recently been reported in ulcerative colitis, a cancer-predisposing chronic inflammatory condition (41). However, further study is required to determine whether telomere shortening occurs in local settings of chronic or acute inflammation.

The presence of anaphase bridges in IEN lesions with short telomeres, plus the fact that the magnitude of telomere shortening we observe in IEN lesions is strikingly similar to that seen in invasive carcinomas within the same tissue section, suggests that many IEN lesions possess cells with telomeres that have
shortened well beyond the normal senescence checkpoint. These observations are in keeping with previous research, indicating that the expression of telomerase components, as well as telomerase enzymatic activity itself, often become pronounced at the IEN stage, as would be anticipated given that critical telomere shortening provides a strong selective pressure for the activation of telomere length maintenance mechanisms (48–55).

Although genetic instability is thought necessary for tumor initiation, increasing levels of instability present a problem for continued tumor growth because lethal genetic defects will accrue, creating a barrier to tumor expansion. Interestingly, not all IEN lesions are believed capable of fully advancing to the malignant state. It is, therefore, conceivable that unchecked telomere shortening at the IEN stage may represent a bottleneck to tumor development, with abortive IEN lesions either maintaining some checkpoint functionality or being ultimately self-limited because of an inability to activate telomere maintenance mechanisms. Consequently, only cells that find a way to maintain their telomeres, thus allowing unlimited cell division and some degree of genomic stabilization, will be able to pass through this bottleneck and progress to give rise to an invasive tumor.

In the current survey, one surprising finding was that 10 of 35 lesions (29%) displayed marked telomere length heterogeneity, particularly bladder IEN (Figs. 3 and 4). The variability in these mixed lesions consisted of cell-cell or regional differences between cells with normal to very long telomeres and cells with abnormally short telomeres. This heterogeneity may reflect differing rates of telomere dynamics in individual cells or in clonal outgrowths due, perhaps, to differences in proliferation rates, protection against oxidative damage, or to differences in telomere preservation/elongation mechanisms. Notably, intratumoral heterogeneity of telomerase activity has been reported in advanced cancers (56, 57). Once reliable methods for detection of telomerase activity in situ are available, it will be interesting to test whether areas in lesions with long telomeres correlate with regions of increased telomerase activity.

Another surprising finding of the current study was that several of the aforementioned heterogeneous lesions contained cells with telomeres that appeared much longer than those of adjacent normal-appearing epithelia, stroma, or lymphocytes—a phenotype reminiscent of the known telomerase-independent telomere elongation pathway termed alternating lengths of telomeres (ALT; Ref. 16). In 3 bladder lesions, cells with unusually long telomeres were the sole abnormality seen. However, and in keeping with the fact that the ALT pathway is seen predominately in tumors and in vitro-immortalized cells of mesenchymal rather than epithelial origin, we saw no evidence of the larger, very bright telomeric signals indicative of the presence of so-called ALT-associated promyelocytic leukemia nuclear bodies, a nearly universal finding in cell populations displaying the ALT phenotype (16, 58). The extraordinarily long telomeres seen in some of the IEN lesions surveyed here may, instead, be caused by telomerase-mediated telomere extension. On the other hand, they may be the result of an ALT-like telomere elongation pathway that does not involve the formation of ALT-associated promyelocytic leukemia bodies.

In addition to the variability described above for a subset of IEN lesions, intriguing differences in telomere lengths were also sometimes observed in normal-appearing epithelia of the large intestine. Although telomere lengths appeared uniform between cells within individual normal crypts, significant crypt-crypt variation was seen on occasion (2 of 5 cases; Fig. 3F). Because colonic crypts display evidence of clonality, this variability may represent telomere length mosaicism between the stem cells maintaining individual crypts. Telomere length variability is also observed in subsets of normal-appearing breast epithelium as well (59).

In summary, we report that a majority of IEN lesions are largely composed of cells with abnormal telomere lengths, primarily short telomeres, in keeping with previously published studies on IEN lesions of the prostate (34, 35) and pancreas (36),...
as well as results from breast (59) and biliary lesions. The results presented here support a model whereby telomere dysfunction induces chromosomal instability as an early initiating event in many, perhaps most, human epithelial cancers. Thus, intervention strategies aimed at preventing or even reversing telomere shortening may be effective in lowering cancer incidence. In addition, telomere length assessment by high-resolution in situ techniques may provide a novel end point for cancer chemoprevention studies and for improved early diagnosis of human cancer precursor lesions.

ACKNOWLEDGMENTS

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REFERENCES


Table 2 Summary of telomere length abnormalities found to date in intraepithelial neoplasia lesions by in situ telomere length assessment

<table>
<thead>
<tr>
<th>Site/Intraepithelial Neoplasia Lesion</th>
<th>Any Abnormalities (%)</th>
<th>Short (%)</th>
<th>Long (%)</th>
<th>Mixed (long &amp; short) (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder/carcinoma in situ</td>
<td>11 100</td>
<td>73 100</td>
<td>91 20</td>
<td>64 Current study</td>
<td></td>
</tr>
<tr>
<td>Large intestine/adenoma</td>
<td>5 100</td>
<td>100 20</td>
<td>0 20</td>
<td>20 Current study</td>
<td></td>
</tr>
<tr>
<td>Large intestine/high-grade dysplasia</td>
<td>2 100</td>
<td>100 50</td>
<td>0 0</td>
<td>50 Current study</td>
<td></td>
</tr>
<tr>
<td>Esophageal/dysplasia</td>
<td>6 100</td>
<td>100 0</td>
<td>0 0</td>
<td>0 Current study</td>
<td></td>
</tr>
<tr>
<td>Oral cavity/dysplasia</td>
<td>8 100</td>
<td>88 0</td>
<td>0 0</td>
<td>0 Current study</td>
<td></td>
</tr>
<tr>
<td>Uterine cervix/dysplasia</td>
<td>2 100</td>
<td>100 0</td>
<td>0 0</td>
<td>0 Current study</td>
<td></td>
</tr>
<tr>
<td>Prostate/prostatic intraepithelial neoplasia</td>
<td>45 96</td>
<td>96 0</td>
<td>0 0</td>
<td>0 0 34, 35</td>
<td></td>
</tr>
<tr>
<td>Pancreas/pancreatic intraepithelial neoplasia</td>
<td>82 96</td>
<td>96 0</td>
<td>0 0</td>
<td>0 0 36</td>
<td></td>
</tr>
<tr>
<td>Breast/ductal carcinoma in situ</td>
<td>23 83</td>
<td>83 4</td>
<td>4 4</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Bile duct/dysplasia</td>
<td>11 100</td>
<td>91 4</td>
<td>9 0</td>
<td>0 Footnote 4</td>
<td></td>
</tr>
</tbody>
</table>

Averages: – 95% 92% 7% 5%

Total number of lesions = 196

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Telomere Length Abnormalities Occur Early in the Initiation of Epithelial Carcinogenesis

Alan K. Meeker, Jessica L. Hicks, Christine A. Iacobuzio-Donahue, et al.


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