Telomere Length and Telomerase Expression in Atypical Adenomatous Hyperplasia and Small Bronchioloalveolar Carcinoma of the Lung

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Key Words: Telomere length; Telomerase; Telomerase reverse transcriptase; hTERT; Atypical adenomatous hyperplasia; Bronchioloalveolar carcinoma; Pulmonary adenocarcinoma

Abstract

Telomeres are located at the ends of every human chromosome and are subject to shortening at each cycle of cell division in cell senescence and early carcinogenesis. We examined the expression of telomeric DNA in 21 atypical adenomatous hyperplasias (AAHs) and 40 bronchioloalveolar carcinomas (BACs) measuring 2 cm or less in greatest diameter using fluorescent in situ hybridization and the expression of human telomerase reverse transcriptase (hTERT) messenger RNA (mRNA) in 35 AAHs and 37 BACs. The mean numbers of telomeric signals per nucleus were 5.0 in AAH and 7.4 in BAC, each significantly less than for normal cells (14.7; \(P < .0001\)), but the mean number of telomeric signals for AAH and BAC was not statistically different (\(P = .22\)).

In “benign” lung samples, the pattern of expression of hTERT mRNA was barely detected in the nonciliated cells of the bronchioles and alveolar type II cells. Positive expression of hTERT mRNA was recognized in 66% of AAHs and 97% of BACs. Our results demonstrate telomere shortening, indicating its presence in the earliest phase of pulmonary carcinogenesis. Telomere length and telomerase may be involved in carcinogenesis in the lung.

In the 2004 World Health Organization classification of tumors of the lung, pleura, thymus, and heart, 3 types of preinvasive epithelial lesions are recognized1: (1) squamous dysplasia and carcinoma in situ, (2) atypical adenomatous hyperplasia (AAH), and (3) diffuse idiopathic pulmonary cell hyperplasia. Squamous dysplasia and carcinoma in situ are associated with the development of squamous cell carcinoma. Previous studies have implicated AAH as a precursor of adenocarcinoma, and this lesion can be difficult to separate from the nonmucinous variant of bronchioloalveolar carcinoma (BAC). Neuroendocrine cell hyperplasia seems to be a precursor of the development of multiple tumorlets and typical or atypical carcinoids.2

AAH is a focal lesion, often 5 mm or less in diameter with a distinct border within otherwise normal alveolar tissue, and defined as consisting of columnar or cuboidal cells arranged in a single row along the alveolar wall. AAH cells are distinct from the ciliated cells of the terminal bronchiolar epithelium, and the lung tissue surrounding AAH does not display a chronic inflammatory reaction in the interstitial alveolar wall.1,3

BAC exhibits growth of neoplastic cells along preexisting alveolar structures (lepidic growth) without evidence of stromal, vascular, or pleural invasion.1,2 Differentiation between nonmucinous BAC and AAH may be difficult. During pulmonary adenocarcinoma carcinogenesis, AAH accumulates certain genetic disorders and oncoproteins such as carcinoembryonic antigen,3,4 p53 oncoprotein,5,6 survivin,7 and telomerase component,8-10 and then expresses \(K\)-ras mutation,11 3p loss of heterozygosity (LOH), 9p LOH, 17p LOH,12 and LOH of tuberous sclerosis complex–associated regions.13

Telomeres are composed of hexanucleotide units, TTAGGG, and have a total length of 10 to 15 kilobases (kb).
They seem to have a key role in stabilizing chromosomes during replication. In all normal somatic cells, each cycle of cell division and DNA replication results in a loss of 50 to 200 terminal nucleotides from each chromosome. In contrast, immortalized cell lines and cancer cells show no net loss of telomere length on cell division, suggesting that telomere maintenance is essential for cellular immortality. In humans, telomere length in the articular cartilage gradually decreases during aging; indeed, the recent study by Morita et al using Southern blot analysis revealed a statistical correlation between telomere length and aging.

If critical telomere shortening indeed contributes to the genetic instability thought to underlie human epithelial neoplasia, it would be expected to occur during or before the invasive phase of carcinogenesis. Unfortunately, studies of telomere length in preinvasive lesions have been impeded because of the technical requirement for fairly large, unixed tissue samples and because of the cell-type heterogeneity typical of such samples.

Recently, a new technique for measuring telomere length has been developed using fluorescent in situ hybridization (FISH) on archival tissue sections of prostate, pancreas, and breast. This in situ method shows good agreement with the commonly used telomere repeat–Southern blot method. In an attempt to clarify the issue of telomere length in AAH and BAC, we performed FISH for telomere length and ISH for human telomerase reverse transcriptase (hTERT) messenger RNA (mRNA).

Materials and Methods

We collected specimens of 35 AAH lesions of the lung and 40 nonmucinous BACs of the lung measuring 2 cm or less in greatest diameter, as stated previously. We had previously divided AAH lesions into low-grade and high-grade, but we now follow the 2004 World Health Organization classification and study both grades combined. AAH is composed of columnar or cuboidal cells arranged in a single row along the alveolar wall. None of the cells in AAH showed mitotic figures. Intranuclear inclusions, usually considered the histologic hallmark of the type II alveolar epithelial cell, were seen in more than 25% of AAH cells. As for BAC, the nonmucinous type is defined as a growth of neoplastic cells along preexisting alveolar structures without evidence of stromal, vascular, or pleural invasion. However, some cases contain moderate alveolar collapse with an increase in elastic tissue in the thickened alveolar septa and are termed sclerosing BAC.

The project was approved by the ethical review committee of the National Defense Medical College, Tokorozawa, Japan.

Telomere FISH

Deparaffinized slides (21 AAHs and 40 BACs) were hydrated through a graded ethanol series. Slides were placed in citrate buffer (pH 6.0; 0.01 mol/L) and heated in a microwave processor (Azumaya, Tokyo, Japan) for 20 minutes at 95°C and then allowed to cool at room temperature for 30 minutes. They were then washed with deionized distilled water and placed in a Proteinase K solution. After a wash with deionized water, the slides were postfixed in 4% paraformaldehyde for 5 minutes, washed with 0.1 mol/L of phosphate buffer followed by cold ethanol, and then air dried.

After 0.1% Nonidet P-40 (Sigma, St Louis, MO) in 2× sodium chloride sodium citrate buffer (SSC) had been applied to the samples for 30 minutes at 37°C, they were washed with 0.1 mol/L of phosphate buffer. Slides were incubated with 70% formamide in 2× SSC for 10 minutes at 85°C, followed by cold ethanol, and then air dried. Denaturation was carried out for 6 minutes at 83°C in a dark room with 35 µL of a fluorescein isothiocyanate (FITC)-labeled telomere-specific peptide nucleic acid probe (0.3 µg/mL of peptide nucleic acid in 70% formamide, 10 mmol/L of Tris, pH 7.5, and 0.5% B/M blocking reagent [Boehringer Mannheim, Indianapolis, IN]). Slides were microwaved intermittently every 4 seconds for 1 hour at 42°C, and then hybridization was performed overnight at 37°C in a dark room, followed by washing with 2× SSC for 30 minutes at 45°C. To amplify the signals, we used rabbit anti-FITC antibody (DakoCytomation, Glostrup, Denmark), biotinylated antirabbit immunoglobulin antibodies (Nichirei, Tokyo, Japan), and, finally, FITC avidin (Vector Labs, Burlingame, CA). Slides were counterstained with 4′,6-diamidino-2-phenylindole and then mounted.

Ten or more nuclei from single representative samples of AAH or small BAC were quantified for telomeric-repeat length, in conjunction with 10 or more nuclei from normal cells from the same case, all by telomere FISH.

By using this technique for telomere quantification, a previous study showed that the number of telomere signals is linearly proportional to average telomere length, as assessed by Southern blotting.

ISH for hTERT

Samples (35 AAHs and 37 BACs for hTERT mRNA) were formalin fixed and paraffin embedded and then examined by ISH for hTERT mRNA. ISH was performed essentially as previously described. Briefly, sections were treated with 0.2N hydrochloric acid for 20 minutes, then incubated in 2× SSC for 10 minutes at 37°C, and, finally, incubated for 10 minutes at 37°C in 1 µg/mL of Proteinase K (Merck, Darmstadt, Germany; in phosphate-buffered saline [PBS]). Sections were subsequently postfixed in 4% paraformaldehyde in PBS for 5 minutes, washed with 0.2% glycine for 10 minutes, and then incubated for 10 minutes in 0.1 mol/L of...
triethanolamine buffer, pH 8.0, containing 0.25% (vol/vol) acetic anhydride to prevent nonspecific binding due to oxidation of the tissue. Finally, they were washed with 95% ethanol and dried.

Hybridization was carried out overnight at 42°C in 50% (vol/vol) deionized formamide, 10% Denhardt solution, 5% (wt/vol) dextran sulfate, 2× SSC, 0.2 mg/mL of human placental DNA, and 10 ng/mL of biotin-labeled antisense probe, which was 194 base pairs (bp) of human telomerase complementary DNA inserted at the Smal site of pBluescript II KS (+/–; a kind gift from SRL, Tokyo, Japan) under a T7 promoter. The corresponding sense probe was 194 bp of hTERT complementary DNA placed under a T3 promoter using an RNA labeling kit (Boehringer Mannheim).28 The sense probe was used as a negative control. After incubation, the sections were washed sequentially as follows: twice with 2× SSC at 42°C for 10 minutes, once with 2× SSC at room temperature for 10 minutes, and once with 0.2× SSC at room temperature for 10 minutes. Signal amplification was performed using Strept AB complex/AP (DAKO, Carpinteria, CA), and hTERT mRNA was detected using BCIP (5-bromo-4-chloro-3-indolyl phosphate; Sigma) and NBT (nitroblue tetrazolium; Sigma). For our analysis of reactivity, the extent of staining was scored as follows: −, a negative reaction; ±, 10% or less of tumor area stained; +, 11% to 25% stained; 2+, 26% to 50% stained; or 3+, 51% or more stained. Tumors in which the stained tumor cells made up more than 10% of the tumor were graded as positive.

**Analysis of Telomere Length**

Fresh-frozen samples from 9 adenocarcinomas, including 4 cases of small BAC, 2 cases of small mixed subtype, and 1 case each of papillary adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma were immersed in matrix...
Telomere–fluorescence in situ hybridization (FISH) performed, using a fluorescein isothiocyanate (FITC)-labeled telomere-specific peptide nucleic-acid probe on “benign” bronchiolar cells (D) and type II alveolar cells (E) (×100). Telomere signals can be recognized as green spots in these sections of formalin-fixed, paraffin-embedded tissues. The mean number of telomere signals per nucleus was 14.7 in the benign cells.

FISH was performed using an FITC-labeled probe on AAH. Almost all cells displayed a reduced number of green signals (compared with benign lymphocytes). The mean number of telomere signals per nucleus was 5.0 in AAH (×100).

Telomere-FISH performed using an FITC-labeled telomere-specific probe on BAC (×100). A moderately reduced number of telomere signals can be seen as green spots within the nuclei of tumor cells (H; in contrast with the many signals in the surrounding alveolar cells [left side]). The mean number of telomere signals per nucleus was 7.4 in BAC (×100).
digestion buffer (0.4% pronase, 0.125% collagenase, and 1× PBS) and incubated at 50°C for 3 hours for decomposition of the matrix. Then, cell-lysis solution (0.04% Proteinase K, 10 mmol/L of Tris-hydrochloride [pH 8.0], 1 mmol/L of EDTA, and 1% polysorbate-20) was added, and incubation was allowed to proceed at 60°C for 3 hours. The DNA was then extracted using a DNA extraction kit (Gentra Systems, Minneapolis, MN).

Analysis for telomere length was performed essentially as described elsewhere. Briefly, 1 µg of DNA digested with HindIII and RsaI at 37°C for 2 hours was loaded onto a 0.8% agarose gels and separated by electrophoresis at 50 V (5 V/cm²) for 3 hours. The 1-kb DNA ladder (GIBCO BRL, Karlsruhe, Germany) served as a molecular weight marker. The gels were denatured and vacuum blotted to a positively charged nylon membrane (Immobilon Ny+, Millipore, Bedford, MA). After cross-linking the DNA to the membrane with the aid of UV light (120 mJ), the filters were hybridized twice for 15 minutes in 0.2× SSC/0.1% sodium dodecyl sulfate at 42°C and twice for 15 minutes in 2× SSC/0.1% sodium dodecyl sulfate at 50°C. Detection of the DNA fragments that had hybridized to the digoxigenin-labeled (TTAGGG)₇ telomeric probe (Roche Diagnostics) was performed by chemiluminescence, according to the manufacturer’s instructions (Roche Diagnostics). Softex films (Fuji, Tokyo, Japan) were exposed within the linear range of the chemiluminescence reaction for 1 to 5 minutes. Exposed films were scanned and analyzed using National Institutes of Health (Bethesda, MD) Image 1.3.1. Mean length was defined as Σ(OD_i) / Σ(OD_i/L_i), where OD_i is the densitometer output over interval i, and L_i is the kilobase size at the middle of interval i.

Data Analysis

Results are expressed as mean ± SD. The Fisher protected least significant difference test or Scheffé test was applied to the data when significant F ratios were obtained in an analysis of variance. A P value less than .05 was considered significant.

Results

Quantitative Assessment of Telomere Signals

Telomere FISH (performed using an FITC-labeled telomere-specific peptide nucleic acid probe) revealed telomere DNA in the “benign” bronchiolar cells, type II alveolar cells, bronchial gland cells, fibroblasts, endothelial cells, and lymphocytes. Telomere signals are detected as green spots within the nuclei of the cells in sections of formalin-fixed, paraffin-embedded tissue. The mean number of telomere signals per nucleus was 14.7 ± 3.5, median 14.3 in “benign” cells such as bronchial cells and lymphocytes. There was no difference compared with benign cells adjacent to AAH and BAC.

In contrast, almost all cells in AAH displayed a reduced number of green signals compared with the benign lymphocytes; the number of telomere signals per nucleus was 5.0 ± 3.6, and the median was 3.4 in AAH. There was a reduced number of telomeres compared with the surrounding benign cells in the same case in 18 of 21 cases of AAH. A moderate reduction in the number of telomere signals within the nuclei of tumor cells was observed in BAC, in contrast with the many signals in the surrounding alveolar cells. The number of telomere signals per nucleus was actually 7.4 ± 7.7, and the median was 4.2 in BAC. There were 36 of 40 cases of BAC exhibited reduced numbers of telomere signals.

Figure II summarizes the data for the mean numbers of telomeric signals per nucleus in benign cells (n = 61), AAH (n = 21), and small BAC (n = 40). Compared with normal cells, AAH and BAC displayed significantly smaller numbers of telomere signals; the P values all were less than .0001 (Figure 1).

ISH for hTERT mRNA

Expression of hTERT mRNA was confined to the cytoplasm of the benign serous cells of the bronchial glands, lymphoid follicles, and tumor cells, as described previously. Expression of hTERT mRNA was sometimes observed in hyperplastic nonciliated cells of the bronchioles and in
hyperplastic alveolar type II cells (both reactive against local inflammation). Detection of positive hTERT mRNA expression increased from AAH (23/35 lesions [66%]) to BAC (36/37 lesions [97%]). The mean ± SE staining scores obtained for AAH and BAC were 1.6 ± 1.3 and 2.24 ± 0.9, respectively. Statistically, the difference between AAH and BAC was significant (P = .02).

For the relationship between telomere FISH and hTERT mRNA, 3 cases of AAH that displayed no telomere shortening expressed hTERT, as did 2 cases of BAC that displayed no telomere shortening.

Analysis of Telomere Length

Telomere-length measurements were made using the Southern blotting technique, after extraction of DNA from fresh-frozen samples of various carcinomas. In benign tissue in 5 cases, the number of telomere signals tended to be linearly proportional to the average telomere length (P = .07). Numbers 545, 879, and 956 (BAC measuring 2 cm) and number 974 (moderately differentiated type measuring 1.9 cm) showed telomere-shortening in the tumor tissue but not in the nonneoplastic tissue [Image 2]. In addition, number 931 (moderately differentiated adenocarcinoma measuring 4 cm), number 603 (adenosquamous carcinoma measuring 9.5 cm), and number 605 (squamous cell carcinoma measuring 4 cm) revealed telomere shortening compared with nonneoplastic tissue. In contrast, there was no telomere shortening in number 852 (BAC measuring 2 cm) or in number 838 (poorly differentiated type measuring 1.7 cm).

Discussion

The pathogenesis of pulmonary adenocarcinoma remains obscure despite extensive studies carried out during many years. Epidemiologically, adenocarcinoma is the most common histologic subtype of lung cancer in Japan and other countries, and it develops in the peripheral portion of the lung, with pleural indentation. This type is associated with females and with nonsmokers. A recent article on non–small cell lung cancer noted that mutations in the tyrosine kinase domain of the epidermal growth factor receptor gene correlated with responsiveness to the tyrosine kinase inhibitor gefitinib.29 Tyrosine kinase domain mutations have been found in 21% of non–small cell lung cancers, and they are more frequent in “never smokers,” in adenocarcinomas, in patients of East Asian origin, and in females.29-32 AAH, as a preinvasive lesion, may progress and develop, through a BAC stage, into invasive adenocarcinoma, with the accumulation of several sequential molecular abnormalities.3-13,33-36 However, there are no data to suggest what percentage of AAH cases actually progress to invasive adenocarcinoma.36 The prognosis of resected lung carcinoma with AAH was not different from that without AAH.37,38

Previously, telomere lengths have been measured by Southern blotting.18,21,24 However, this technique needs relatively large, unfixed fresh samples and so is inappropriate for the study of a small piece of tissue like AAH. Moreover, the results obtained by examination of a band that includes benign tissue (such as connective tissue or lymphocytes) may not accurately reflect the status of the tumor parenchyma itself. Compared with Southern blot analysis, telomere FISH allows us to avoid the shortcomings due to the inclusion of unwanted tissue, and we can see the whole background more clearly. Of 17 cases of prostate cancer previously examined by telomere FISH, 10 had significantly shorter telomeres in the tumor than in the adjacent normal tissue.24 Concerning prostate cancer, application of telomere FISH to high-grade prostate intraepithelial neoplasia revealed that this lesion showed strikingly shorter telomeres than those of adjacent normal-looking epithelial cells in 28 (93%) of 30 lesions, suggesting that telomere shortening may have an early role in human prostate tumorigenesis.22

In breast cancer, marked telomere shortening has been found in most invasive carcinomas (53%), with the majority of...
ductal carcinomas in situ (78%) demonstrating markedly or moderately shortened telomeres.26 Moreover, telomere signals have been reported to be reduced in pancreatic intraepithelial neoplasia (91%), bladder carcinoma (73%), large intestinal adenoma (100%), esophageal dysplasia (100%), oral cavity dysplasia (100%), and uterine cervical squamous intraepithelial neoplasia (100%).25,39 In lung lesions, telomere shortening has been observed in bronchial dysplastic lesions before telomerase expression (by ISH),40 but telomere abnormalities in AAH lesions have not previously been reported.

In our analysis of telomere length, AAH and BAC clearly displayed smaller numbers of telomere signals than benign bronchial or bronchiolar tissue or lymphocytes. However, no significant differences were seen in telomere length between AAH and BAC. So, telomere shortening would seem to be an early event among the many steps involved in pulmonary adenocarcinogenesis, as is k-ras oncogene activation.41 The number of telomere signals was 7.4 ± 7.7 in BAC, with a large SD, indicating the heterogeneity of telomere lengths (Figure 1). The reason was that the activity of proliferation before stabilization by telomerase might affect the degree of telomere shortening26 or different telomere lengths might arise owing to heterogeneity of molecular determinants of telomere length despite the fact that histologic features are monotonous.

We confirmed telomere shortening by using Southern blotting in BACs measuring 2 cm or less in diameter (Image 2).

In all normal somatic cells, each cycle of cell division and DNA replication results in a loss of 50 to 200 terminal nucleotides from each chromosome.18-20 In contrast, almost all cancer cell lines, as well as certain germline and stem cells, show no telomere shortening owing to the action of telomerase synthesized by themselves (telomerase being a ribonucleoprotein enzyme composed of at least hTERT, human telomerase RNA component, and human telomerase-associated protein). By reverse transcriptase activity, telomerase makes a DNA copy of its own RNA sequences, and this then fuses to the 3' terminus of the chromosome.42-44 Expression of hTERT mRNA has been found in 94% of lung carcinomas, being mainly detected in the cytoplasm like human telomerase RNA component, and the correlation between hTERT expression and telomerase activity was excellent (P < .001) in most cases.27 In the present study, positive expression of hTERT mRNA was recognized in 66% of AAH and 97% of nonmucinous BACs. Statistically, a significant difference in expression could be shown between AAH and BAC (P = .02).

Regarding the relationship between telomere length and telomerase in neoplastic and nonneoplastic tissues, telomere lengths in gastric and colonic cancers have been found to be shorter than in the adjacent mucosa.45 Telomerase activity was found to be positive in 86% of gastric carcinomas,45 whereas telomere length and hTERT expression correlated significantly in colorectal cancer tissue and adjacent mucosa samples.46 Furthermore, significantly longer telomeres were seen in advanced stages than in the early stage of colorectal cancer.46,47

Our study showed a significant difference in hTERT expression between AAH and BAC, but there was no statistically significant difference in telomere length. According to a recent study using transgenic mice, telomere length and telomerase activity are independent determinants of the proliferative capacity of epidermal stem cells.48 hTERT expression in the absence of changes in telomere length might promote stem cell proliferation in vivo, as well as in vitro.

We have provided evidence suggesting that 18 of 21 cases of AAH displayed telomere shortening, whereas 4 cases of BAC that displayed no telomere shortening expressed hTERT. Our study demonstrates a reduced number of telomere signals, representing what is called telomere shortening, in AAH and in small BAC, suggesting that this phenomenon (ie, telomere shortening) begins in the earliest phase of pulmonary carcinogenesis. On the other hand, hTERT mRNA expression increased from AAH (66% of lesions) to BAC (97%), and, statistically, the difference in staining scores between AAH and BAC was significant (P = .02). hTERT expression would, therefore, seem to reach a maximum late in the progression of cancers. Telomere length and telomerase may be involved in carcinogenesis in the lung.

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Acknowledgments: We thank Robert Timms, PhD, for correcting the English version of the manuscript and Susumu Tominaga, Department of Pathology & Laboratory Medicine, National Defense Medical College, for excellent technical assistance.

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