Expression of mRNAs for Telomeric Repeat Binding Factor (TRF)-1 and TRF2 in Atypical Adenomatous Hyperplasia and Adenocarcinoma of the Lung

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ABSTRACT

Purpose and Experimental Design: It has been suggested that atypical adenomatous hyperplasia (AAH) may be a precursor of peripheral adenocarcinoma of the lung. Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric DNA onto chromosomal ends. Its activity is thought to participate in the development of most human cancers. Telomere-specific DNA-binding proteins, such as telomeric repeat binding factor 1 and telomeric repeat binding factor 2, also control telomere length in a complex interplay with telomerase. Here we investigated the expressions of the mRNAs encoded by the TERF1 and TERF2 genes using in situ hybridization in surgically resected specimens [28 AAHs (11 lesions were interpreted as low-grade AAH, and 17 were interpreted as high-grade AAH) and 40 peripherally located bronchioloalveolar carcinoma (BAC)].

Results: A clear overexpression of these mRNAs was recognized in low- and high-grade AAH and BAC samples (as compared with normal tissues) using in situ hybridization and these mRNAs were detected in normal AAH and BAC samples using reverse transcription-PCR. The expressions of TERF1 and TERF2 mRNA detected by in situ hybridization were scored positive in 36% and 82% of low-grade AAH, 65% and 83% of high-grade AAH, and 88% and 88% of BAC, respectively. Statistically significant differences in TERF1 mRNA expression could be shown between low-grade AAH and BAC and between high-grade AAH and BAC. There was no statistical difference in the positive expressions of TERF2 mRNA among low-grade AAH, high-grade AAH, and BAC.

Conclusions: These results are consistent with (but are not enough to confirm) the idea that high-grade AAH is closely related to BAC.

INTRODUCTION

AAH of the lung, a proliferation of alveolar epithelial cells, is usually found incidentally in lungs surgically resected for lung cancer, predominantly in patients with adenocarcinoma (1). In the 1999 WHO histological classification of lung and pleural tumors (2), AAH was classified among the preinvasive lesions along with squamous dysplasia, carcinoma in situ, and diffuse pulmonary neuroendocrine cell hyperplasia. However, the relationship between AAH and pulmonary adenocarcinoma is not well understood. Recently, attempts have been made to establish the pathogenesis of BAC by morphometric, immunohistochemical, and molecular studies (3, 4). The authors (3, 4) suggested that AAH should be regarded as representing a preneoplastic lesion or even an early-stage lesion of peripherally located BAC.

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric DNA at the end of chromosomes and compensates for the end replication problem, allowing cells to proliferate indefinitely (5–7). Using the PCR-based telomerase repeat amplification protocol assay, it has been shown that telomerase is activated in a large majority of human cancer tissues, but not in most normal tissues or in tissues adjacent to malignant or benign tumors (8). Therefore, it appears that telomerase activity is a useful marker for cancer detection.

Two major subunits of the human telomerase core complex, namely, hTERC and hTERT, have been identified. hTERC, which was identified first, functions as a template for telomere elongation by telomerase (9–11). hTERT contains a reverse transcriptase domain that catalyzes this reaction (12–14). Recent studies have demonstrated that hTERC and hTERT form the minimum complex needed for telomerase activity (13, 14). Therefore, up-regulation of hTERC and hTERT expressions could play an important role in human carcinogenesis. More recently, telomere-specific DNA-binding proteins such as TRF1 and TRF2 have been put forward as additional candidates for the role of molecules modifying telomerase activity, and they have been suggested to play key roles in the maintenance of telomere function (15–18). In fact, TRF1 negatively regulates...
The maintenance of telomere length (15, 16, 18). Although TRF2 was initially implicated in the protection of chromosome ends (15, 16), more recent reports show that TRF2 protein is a second negative regulator of telomere length (17) acting at least in part independently of telomerase by activating a degradation pathway (18).

Although several investigations have been made of telomerase activity and/or the subunits of human telomerase in lung carcinomas and AAH (11, 19–24), no studies have investigated the expressions of *TERF1* and *TERF2* mRNAs in AAH. We therefore examined their expressions in the AAHs and peripherally located BACs of the lung resected from 40 patients. In this study, we defined AAH lesions according to the following criteria: (a) AAH is composed of columnar or cuboidal cells arranged in a single row along the alveolar wall; (b) AAH cells are distinct from ciliated cells of the terminal bronchiolar epithelium; and (c) lung tissue surrounding AAH does not exhibit a chronic inflammatory reaction in the interstitial alveolar wall (Fig. 1).

*In situ* hybridization was performed essentially as described previously (25). Briefly, sections were treated with 0.2 N HCl for 20 min and then incubated in 2× SSC for 10 min at 37°C and incubated in 5 μg/ml proteinase K for 10 min at 37°C. Sections were subsequently postfixed in 4% paraformaldehyde for 5 min and then incubated in 0.1 M triethanolamine buffer (pH 8.0) containing 0.25% (v/v) acetic anhydride for 10 min to prevent nonspecific binding due to oxidation of the tissue. Hybridization was carried out overnight at 42°C in 50% (v/v) deionized formamide, 5× Denhardt’s solution, 5% (w/v) dextran sulfate, 2× SSC, 0.3 mg/ml salmon sperm DNA, 5 mM EDTA, and 10 ng/ml biotin-labeled probes. After performing a final stringent wash at 55°C for 20 min, hybridization was detected immunologically.

pfTRF1 (26) was digested by restriction enzymes *EcoRI* and *XhoI*, ligated between the *EcoRI* and *XhoI* cloning sites of pGEMT7Zf (Promega), and then labeled with biotin using a RNA labeling kit (Boehringer Mannheim). The antisense probe was a 247-bp segment of *TERF1* cDNA under a SP6 promoter, and the corresponding sense probe was a 1007-bp segment of *TERF1* cDNA under a SP6 promoter. Lung carcinomas with telomerase activity served as a positive control.

The corresponding sense probe was used for the negative control. In *in situ* hybridization of *hTERC* and *hTERT* mRNA was determined as described previously; the technique used and the results in these same patients have been reported elsewhere (28). For analysis of reactivity, the extent of staining was scored as follows: (a) −, a negative reaction of tumor cells; (b) +, ≤10% of tumor area stained; (c) +, 11–25% of tumor area stained; (d) +, 26–50% of tumor area stained; and (e) +, ≥51% of tumor area stained. Those tumors in which the stained tumor cells made up >10% of the tumor were graded as positive.

For examination of *TERF1* and *TERF2* mRNAs by RT-PCR, total mRNAs were obtained from six normal lung tissues, one high-grade AAH, and six BACs (the only suitable materials available from our stock). The total RNA was isolated using acid guanidinium isothiocyanate-phenol-chloroform extraction and ethanol precipitation (29). RT-PCR was performed using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied Biosystems, Alameda, CA) with *TERF1*, *TERF2*, and glyceraldehyde-3-phosphate dehydrogenase primers. Six primers were synthesized using an automated DNA synthesizer. Sequence information for all of the PCR primers used is listed in Table 1, together with the thermocycling conditions. The reaction master mix was prepared according to the manufacturer’s protocol to give final concentrations of 1× reaction buffer, 300 μM dATP, 300 μM dCTP, 300 μM dGTP, 600 μM dUTP, 3 mM Mg(OAc)₂, 0.1 unit/μl *rTth* DNA polymerase, 0.01 unit/μl AmpErase.
UNG, 900 nm primers, and 200 nm TaqMan probe. To perform PCR, the reverse transcription reaction was incubated at 60°C for 30 min, followed by incubation at 95°C for 5 min to deactivate AmpErase UNG. PCR was performed using an ABI PRISM 9600 Sequence Detector (Applied Biosystems). PCR products were separated by electrophoresis in a 3% agarose gel and stained with ethidium bromide.

Statistical analysis was performed using χ² analysis. Regression analysis among TERF1, TERF2, hTERC, and hTERT mRNA expressions was performed using Spearman’s correlation coefficient by rank. *P* < 0.05 was considered significant.

**RESULTS**

Of the 28 AAH lesions, 17 were interpreted as high-grade lesions on the basis of the finding of increased cellularity and cytologic pleomorphism (Fig. 1B). In detail, low-grade AAH showed a low proliferating cell density, and the proliferating cells formed a single row along the alveolar wall, with the cells arranged either intermittently or continuously. The nuclei were small and showed minimal variations in size and shape. In high-grade AAH, the density of the proliferating cells was increased, and their nuclei were larger and exhibited greater variation in terms of size, shape, and hyperchromasia. However, none of the cells in the 17 high-grade AAH lesions showed mitotic figures. By comparison with high-grade AAH, low-grade BAC exhibited significantly greater nuclear atypia, a greatly increased nuclear:cytoplasmic ratio, and hyperchromasia, as well as a higher proliferating cell density.

Expression of TERF1 mRNA was confined to the cytoplasm of tumor cells (Fig. 2). In the normal lung, TERF1 mRNA was detected focally as weak to moderate staining of the bronchial and ductal cells within bronchial glands and in nonciliated cells within bronchioles. Alveolar type I and type II cells were negative. A positive TERF1 mRNA expression was recognized in 36% of low-grade AAH (4 of 11 lesions), 65% of high-grade AAH (11 of 17 lesions), and 88% of BAC (35 of 40 lesions). Statistically, the differences between low-grade AAH and BAC and between high-grade AAH and BAC were significant (*P* = 0.0004 and *P* = 0.046, respectively). Expression of TERF2 mRNA was also confined to the cytoplasm of tumor cells (Fig. 3). In the normal lung, the pattern of expression of TERF2 mRNA was essentially the same as that of TERF1 mRNA. That is to say, it was represented focally as weak to moderate staining in serous cells and ductal cells within bronchial glands and in nonciliated cells within bronchioles, and alveolar type I and type II cells were always negative. The detection rate for positive TERF2 mRNA expression was 82% in low-grade AAH (9 of 11 lesions), 83% in high-grade AAH (15 of 18 lesions), and 88% in BAC (35 of 40 lesions). In this regard, there was no statistical difference among low-grade AAH, high-grade AAH, and BAC.

When Spearman’s correlation coefficient by rank was used, TERF1 mRNA staining scores showed a significant correlation with hTERC and hTERT mRNA staining scores (*P* < 0.0001 and *P* < 0.0001, respectively). However, there was no significant correlation between TERF2 mRNA staining scores and either hTERC or hTERT mRNA staining scores.

When RT-PCR was used, TERF1 and TERF2 mRNAs were detected in all samples examined (six normal lung tissues, one high-grade AAH, and six BACs), although the strength of their expressions varied (Fig. 4).

**DISCUSSION**

The purpose of our investigation was to seek a better understanding of the relation of AAH to the pathogenesis of peripherally located BAC. We found previously that overexpressions of both hTERC and hTERT mRNAs increased significantly from low-grade AAH through high-grade AAH to BAC, which we felt supported the idea that high-grade AAH is a precursor of peripherally located BAC (28). In the present study, we observed that the incidence of positive expression for TERF1 mRNA using in situ hybridization also increased significantly from low-grade AAH through high-grade AAH to BAC. This finding of an increasing incidence of TERF1 mRNA from low-grade AAH through high-grade AAH to BAC is not, by itself, sufficient evidence to confirm the above idea (although it may be consistent with it).

In the present study, TERF1 mRNA was detected only focally in normal lung tissues (in the nonciliated cells of the bronchioles and in both the serous cells and ductal cells of the bronchial glands) using in situ hybridization, whereas using RT-PCR, it was detected in all of the total RNA samples obtained from six normal lung tissues. Although the reason for this discrepancy is unclear, one possibility is that the probes used for in situ hybridization may be of lower sensitivity than the primers used for RT-PCR.

On the basis of the evidence obtained using in situ hybridization in the present study, overexpression of TERF1 mRNA is found in both AAH and BAC: we found it in 54% of all AAH (15 of 28 lesions), but in 88% of BAC (35 of 40 lesions). These figures accord well with the figures obtained for the incidence of overexpression of hTERC and hTERT mRNAs in our previous study (55% and 59% in AAH and 98% and 98% in BAC, respectively; Ref. 28). Furthermore, we found that the staining scores obtained for TERF1 mRNA paralleled the staining scores obtained for hTERC and hTERT mRNAs in these same patients (Spearman’s correlation coefficient by rank). Recent studies have established that hTERC and hTERT form the minimum

**Table 1** Primer sequences and thermocycle conditions used for RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense primer (5’-3’)</th>
<th>Antisense primer (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
<th>Sizes of PCR products (bp)</th>
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<tr>
<td>TERF1</td>
<td>ATGCCGACCCCTACTGAGGAG</td>
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<td>301</td>
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<tr>
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<td>CTTCGAAATTCCTGGACCA</td>
<td>64</td>
<td>40</td>
<td>301</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGTGTTAAAGTGGGAGTC</td>
<td>GAAGATGGTGATGGGATTTC</td>
<td>60</td>
<td>40</td>
<td>226</td>
</tr>
</tbody>
</table>

* TERF1, telomeric repeat binding factor 1; TERF2, telomeric repeat binding factor 2; GAPDH, rat glyceraldehyde-3-phosphate dehydrogenase.
complex required for telomerase activity (13, 14). Despite the up-regulation of telomerase activity that occurs in activated normal lymphocytes, however, telomeres still shortened to some extent, as observed when the culture was continued for many weeks (30, 31). Furthermore, overexpression of TRF1 in the tetracyclin-responsive human fibrosarcoma cell line HTC75 resulted in a gradual decline in telomere length at a rate of $\sim 10$ bp/population doubling (17), and the forced tethering of a large number of TRF1 molecules to a single telomere induced a shortening rate of $\sim 80$ bp/population doubling (18). TRF1 does not affect telomerase activity globally within the cell, but clearly an excess of TRF1 inhibits $cis$ telomerase activity at the telomere itself (18). Therefore, in AAH and BAC, too, overexpression of $TERF1$ mRNA may partially counteract telomerase activation, limiting telomere elongation. However, these findings may not necessarily indicate an acquisition of malignant potential. Confirmation of a role for TRF1 in the carcinogenesis of BAC would require a large-scale study using molecular techniques showing that this parameter does indeed influence the carcinoma sequence.

It has been shown that telomerase activity is not present in most normal tissues (8) and that the expression of hTERT correlates closely with telomerase activity both in vitro and in vivo (12). Furthermore, the transcription of hTERT may play a part in the mechanism by which telomerase regulation is achieved. In several studies, telomerase activity was detected during the early phase (intestinal metaplasia to adenoma in colon and stomach and dysplasia to carcinoma in situ in uterine cervix) of carcinogenesis (11, 32–34). Those authors (11, 32–34) suggested that $hTERT$ mRNA expression is up-regulated, that telomerase activation is a critical step during tumor carcinogenesis, and that both can be recognized at an early stage in the oncogenic process. In our previous study, we found $hTERT$ mRNA expression not only in BAC but also in AAH using in situ hybridization. The incidence of $hTERT$ mRNA expression was higher in low-grade AAH than in normal lung tissues. Likewise, in the present study using in situ hybridization, $TERF1$ and $TERF2$ mRNAs each showed an increased incidence in low-grade AAH (compared with normal lung tissues), and this was particularly marked in the case of $TERF2$ mRNA, although both mRNAs were detected using RT-PCR in all of the total RNA samples obtained from six normal lung tissues, one high-grade AAH, and six BACs. We could not detect $TERF2$ mRNA at all in normal lung alveolar cells, and it was detected only focally as weak to moderate staining in nonciliated bronchiolar cells using in situ hybridization. Recently, Smogorzewska et al. (17) reported that an overexpression of TRF2 in HCT75 cell lines was accompanied by a progressive shortening

![Fig. 2](https://example.com/image2.png)

Fig. 2 Expression of gene encoding TRF1 ($TERF1$ mRNA) in (A) low-grade AAH, (B) high-grade AAH, and (C) peripherally located BAC. *In situ* hybridization showed $TERF1$ mRNA to be localized to the cytoplasm of tumor cells. *D*, a sense probe was not detected in $TERF1$ mRNA-positive adenocarcinoma. Scale bar, 200 μm.
of telomere length, a phenotype similar to that observed with TRF1. They suggested that overexpression of TRF2 may play a regulatory role in the maintenance of telomerase length in these lesions, as does overexpression of TRF1. Furthermore, this notion was supported when Matsutani et al. (35), who dealt with 20 gastric carcinomas using RT-PCR, showed that carcinomas with a short telomere length (shorter than 2 kb) exhibited a higher TRF1 expression and tended to express TRF2 more strongly than those with a long telomere length. Therefore, we suspect that in AAH, too, overexpression of TRF2 mRNA may exert a negative regulatory influence over telomere length. The fact that TRF2 mRNA expression does not correlate with telomerase expression is in agreement with the recent finding that TRF2 can shorten telomeres without inactivating telomerase (18). The important overexpression of TRF2 in precursor lesions, even in low-grade AAH, suggests that TRF2 plays a key role in the early steps of carcinogenesis, for instance by inducing telomere dysfunction and/or by altering checkpoint controls. However, these findings, as well as the expression of TRF1, may not necessarily indicate the acquisition of malignant potential. Confirmation of a role for TRF2 in the carcinogenesis of BAC also needs further study using molecular techniques.

In conclusion, although our finding of an increasing incidence of TERF1 mRNA from low-grade AAH through high-grade AAH to BAC (using in situ hybridization) may be consistent with the idea that high-grade AAH is a lesion closely associated with BAC of the lung, it is not, by itself, sufficient to confirm the validity of that idea. However, the incidence of TERF1 mRNA and, even more markedly, that of TERF2 mRNA were found to be above normal in low-

Fig. 3 Expression of the gene encoding TRF2 (TERF2 mRNA) in (A) low-grade AAH, (B) high-grade AAH, and (C) peripherally located BAC. In situ hybridization showed TERF2 mRNA to be localized to the cytoplasm of tumor cells. D, a sense probe was not detected in TERF2 mRNA-positive adenocarcinoma. Scale bar, 200 μm.

Fig. 4 RT-PCR results for the mRNAs for (A) TERF1 and (B) TERF2 in normal lung tissue, high-grade AAH, and peripherally located BAC.

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grade AAH using in situ hybridization. Interestingly, overexpression of TRF1, but not of TRF2, appears to correlate with telomerase activation, suggesting distinct effects of TRF1 and TRF2 during carcinogenesis in BAC of the lung. TRF1 and TRF2 interact with other factors that regulate telomere length [reviewed in Mergny et al. (36)]. These include a second enzyme, tankyrase (which binds TRF1), PinX1 and TIN2 (two negative regulators of telomerase’s interaction with TRF1), and hRAP1 (which interacts with TRF2). Clearly, additional studies will be required to examine the alterations in the complete set of telomere-associated proteins in the course of neoplastic transformation in high-grade AAH.

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