Telomerase Expression in Respiratory Epithelium during the Multistage Pathogenesis of Lung Carcinomas


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Abstract

To investigate the role of telomerase in the multistage pathogenesis of lung cancer, we examined 205 fresh and archival tissue samples obtained from 40 patients, 34 of whom had invasive lung carcinoma, 5 with carcinoma in situ (CIS) without invasion, and 1 without lung carcinoma. We analyzed samples for telomerase enzyme activity using the semi-quantitative PCR-based telomeric repeat amplification protocol assay (131 samples) or by a radioactive in situ hybridization method for expression of the RNA component of human telomerase (hTR; 74 samples). A subset of samples was assayed by both methods, and the correlation was excellent (30 of 36; 83%). With the exception of a carcinoid tumor and a necrotic squamous cell carcinoma, all tumor cells were moderate to strongly positive for both hTR and telomerase activity, except for foci of keratinization in squamous cell carcinomas. Telomerase positivity, with weak enzyme activity and/or low hTR expression, was present in basal epithelial cells of large bronchi, both histologically normal (26%) and hyperplastic (71%), and in 23% of peripheral lung samples (in epithelium of small bronchi and bronchioles or lymphoid aggregates). More advanced epithelial changes (metaplasia, dysplasia, and CIS) were associated with telomerase dysregulation. Dysregulation in preneoplasia was manifested in three ways: almost all such lesions expressed hTR, although enzyme activity levels were several-fold lower than in the corresponding invasive tumors; cells throughout these multilayered processes expressed hTR; and, intense, focal up-regulation of hTR occurred in CIS foci in the vicinity of invasive cancers. Alveolar cells and areas of atypical adenomatous hyperplasia (possible precursor lesions for peripheral adenocarcinomas) were negative. Our studies demonstrate that dysregulation of telomerase occurs early in the multistage pathogenesis of bronchogenic lung carcinomas and that intense focal localized hTR expression in CIS may indicate imminent invasion.

Introduction

Carcinogenesis is a multistep process characterized by multiple genetic changes, and epithelial cancers in adults are preceded by a series of morphologically recognizable peneplastic lesions that develop and progress over a period of several years. For NSCLCs,

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4 The abbreviations used are: NSCLC, non-small cell lung carcinoma; CIS, carcinoma in situ; TRAP, telomeric repeat amplification protocol; ISH, in situ hybridization; hTR, human telomerase RNA; IPF, idiopathic pulmonary fibrosis; SCLC, small cell lung carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITAS, internal telomerase assay standard; AAH, atypical adenomatous hyperplasia.

in particular squamous cell carcinomas, the morphological steps may involve hyperplasia, metaplasia, dysplasia, CIS, invasive carcinoma, and finally metastatic carcinoma (1, 2). We have determined that loss of heterozygosity at chromosome 3p occurs very early (at the stage of hyperplasia) in the pathogenesis of most NSCLCs (3, 4).

Progressive telomere shortening has been proposed to be the major timing mechanism that determines in vitro cellular senescence (5). In tumor-derived cell lines and most cells immortalized in vitro, telomere length is stabilized, probably due to the activation of telomerase, an enzyme that synthesizes TTAGGG telomeric DNA onto chromosomal ends de novo (6, 7). Recently, it has been reported that telomerase is expressed in most human cancers, including lung cancers and immortal cell lines (8, 9), but is inactive in adult somatic cells, except for testis and stem cells of regenerative tissues (10). Thus, it has been postulated that reactivation of telomerase expression is necessary for the continuous proliferation of most cancerous cells to attain immortality (8).

Telomerase is a ribonucleoprotein, and the usual method of determination is the PCR-based TRAP assay for enzyme activity (11). Although the TRAP assay has provided many insights into the role of telomerase in tumor biology, it fails to identify whether all cells express telomerase or whether expression is limited to certain subtypes. In an effort to overcome this limitation, we have developed an ISH method for identifying the cellular expression of hTR in archival paraffin-embedded tissues (12).

In this study, we analyzed telomerase activity and hTR expression in lung tissue of normal, preneoplastic, CIS, and invasive tumors and in regional lymph nodes. We demonstrate that telomerase is dysregulated during the multistage pathogenesis of lung cancer.

Materials and Methods

Patients and Tissue Samples. Tissue samples (n = 205; normal, preneoplastic, and malignant) were obtained for telomerase assays from 40 patients, 34 with invasive lung cancer, 5 with CIS, and 1 with nonmalignant disease (IPF). Of the patients with invasive cancer, 14 with squamous cell carcinoma, 14 with adenocarcinoma, 3 with SCLC, 1 with large cell neuroendocrine carcinoma, 1 with adenosquamous carcinoma, and 1 with a typical bronchial carcinoid. Of 36 cancer/CIS patients whose smoking histories were available, all but one were current or former smokers.

Most samples were from lobectomy specimens of the patients with invasive cancers and the patient with IPF. For the TRAP assay, we obtained fresh samples of tumor tissue, nontumorous peripheral lung, hilar and mediastinal lymph nodes, and bronchial epithelium distant from the tumor tissue. Each sample was bisected, and one half was processed for routine histopathological examination, and the other half was stored at —80°C until it was tested for telomerase enzyme activity. Multiple bronchial biopsies were obtained from five subjects with radiologically occult lung cancers by white light or fluorescence bronchoscopy (13), and the pathological diagnosis of CIS was confirmed by frozen section examination. For bronchial biopsies, two frozen sections (5
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TRAP were run blinded. Assays of telomerase activity were processed as described earlier (8, 11, 12). Archival paraffin-embedded samples were used for ISH. Of these samples, 36 of the samples, 13 fresh-frozen samples were used for TRAP assay, and 74 patients with lung cancer and the patient with IPF were selected for ISH. Of all obtained were expressed in arbitrary units. The activity of each sample was normalized to the signal obtained for the internal standard present in base repeat ladder was determined by area integration, and this value was tested by both methods.

**TRAP Assay for Telomerase Activity.** Extracts of tissue specimens and assays of telomerase activity were processed as described previously (8, 11, 12). For quantitation, the signal intensity of the telomerase-specific six-base repeat ladder was determined by area integration, and this value was normalized to the signal obtained for the internal standard present in analyzed sample (12, 14). The normalized values of telomerase activity so obtained were expressed in arbitrary units. The activity of each sample was normalized to that of 1 μg of total cellular protein. All samples analyzed for TRAP were run blinded.

**ISH for hTR.**ISH for hTR was performed as described previously (12). Briefly, paraffin sections (5 μm) were collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), and hybridization was performed using [35S]UTP-labeled single-stranded RNA probes. The plasmid pGEM-SZf(+) (Promega Corp., Madison, WI) containing a hTR cDNA (559 nucleotides), obtained from Geron Corp. (Menlo Park, CA), was used as a template to generate sense and antisense probes. The sense probe was used as a negative control. To confirm the presence of intact RNA, replicate slides from each sample used for hTR expression were also tested for expression of a housekeeping gene, GAPDH. The Zbal/HindIII fragment from the GAPDH cDNA in pBR322, obtained from the American Type Culture Collection (Rockville, MD), was subcloned into pBlue-script. Intense GAPDH hybridization signals were present in all sections studied.

**Results**

**Concordance between Enzymatic Activity and in Situ hTR Expression.** Data from both TRAP assays and ISH were available from 36 samples of normal, neoplastic, and neoplastic tissues. The concordance between the two methods was 30 of 36 (83%). Of the six discordant samples, hTR expression was negative in one sample with very low enzyme activity. In four samples of neoplastic tissues, weak ISH signals were present in a small subset of cells, whereas the TRAP assay was negative. In one sample of normal lymph node, enzymatic activity was relatively high (6.6 units/μg of protein), but ISH was negative. Histological examination of the sample processed for ISH failed to reveal germinal centers. It was presumed that germinal centers were present in the sample selected for enzyme activity. Because of the high degree of concordance, we pooled the results of the two methods for determining telomerase expression, as presented below. Discordant samples were scored as positive. We refer to the pooled data of the two assays as “telomerase positivity.”

**Telomerase Positivity in Lung Cancers and Lymph Nodes.** Of the 131 samples analyzed for telomerase activity, the ITAS was absent in 37 samples (44%) at the initial protein concentration (6 μg) tested, indicating high telomerase activity, the presence of inhibitors of PCR or Taq polymerase, or both. However, after serial dilution, all samples demonstrated an ITAS band and were interpretable.

Telomerase positivity was detected in 32 of 34 (94%) lung tumors (Figs. 1 and 2). The telomerase-negative tumors were a typical carcinoma and a necrotic squamous cell carcinoma containing very few viable tumor cells in the sample tested. The enzyme activity levels in the positive tumors were relatively high (mean 34.2 units/μg of protein; Figs. 1 and 2B). Relatively moderate or high hTR expression was present in most tumor cells except for differentiating, keratinized squamous cells, although some degree of heterogeneity was noted (Fig. 3D).

Telomerase activity was present in almost all hilar lymph nodes, which were histologically positive as well as histologically negative, although the mean level of enzyme activity was 5-fold higher (43.5 units/μg of protein) in the tumor-positive group than in the tumor-negative group (8.8 units/μg of protein; Fig. 2). In lymph nodes and intrapulmonary lymphoid nodules, weak to moderate hTR expression was present in the germinal centers of secondary lymphoid follicles. These results are consistent with our previous findings that activated lymphocytes and the germinal centers of nonmalignant lymph nodes express modest levels of telomerase activity (12, 15).

![Telomerase activity in benign and malignant lung tissues. Telomerase assays were performed using extracts containing 0.06–6 μg of protein with (+) or without (−) RNase pretreatment. An extract of a human breast carcinoma cell line having telomerase activity was used as a positive control. The lysis buffer was used as a negative control. The ITAS is visualized as the uppermost single band. Telomerase activity is represented by a six-nucleotide (TTAGGG) RNase-sensitive repeat ladder. In Case 1, normal bronchial epithelium is telomerase negative, whereas nonmalignant (“normal”) peripheral lung and bronchial epithelial hyperplasia are weakly positive, and the tumor sample is strongly positive. In Case 2, the normal bronchial epithelium is telomerase negative, whereas the dysplastic bronchial epithelium is moderately positive, and the lung tumor sample is strongly positive because the ITAS band is only visualized after serial dilution. Two examples of CIS (CIS1 and CIS2) from two individuals are telomerase positive (one strongly positive and the other weakly positive). In all examples demonstrated, RNase treatment abolishes telomerase activity.](cancerres.aacrjournals.org>)}
Telomerase Positivity in Normal and Abnormal Respiratory Epithelium. Telomerase positivity was detected in histologically normal bronchial epithelium (7 of 27, 26%; Fig. 2A). Positivity was present in a higher fraction of abnormal epithelial samples, including hyperplasia (20 of 28, 71%), metaplasia (4 of 5, 80%), dysplasia (9 of 11, 82%), and CIS (11 of 11, 100%; Fig. 2A).

In the pseudostratified normal epithelium of large bronchi, low expression was limited to the basal cells (Fig. 3A). In hyperplastic...
foci, low expression was noted in basal cells and, occasionally, in parabasal cells (Fig. 3B). Hyperplastic cells of bronchi and bronchioles of the nonmalignant case also expressed low intensity of hTR. In most positive samples of multilayered metaplasia and in all positive examples of multilayered dysplasia and CIS, weak to moderate hTR expression was present, either focally or in all cells (Figs. 3C and 4). Telomerase positivity was also detected in 23% (14 of 60) samples of nontumorous peripheral lung tissues, although the enzyme activity was relatively low (0.3 units/µg of protein; Figs. 1 and 2). In samples of peripheral lung, hTR expression was present in basal cells of small bronchi and nonciliated cells of bronchioles. If intrapulmonary lymphoid follicles were present, germinal centers expressed telomerase, as described previously (12). Alveolar type I and type II cells were negative. Positivity was absent in six samples of AAH (Fig. 2A).

The relative levels of telomerase enzyme activities (0.2–0.6 units/µg of protein) in normal, preneoplastic, and CIS specimens were considerably lower than in the invasive carcinomas (34 units/µg of protein; Figs. 1 and 2B). We estimated the number of nonalveolar epithelial cells present in the normal and preneoplastic samples to be between 5 and 20% and the number of tumor cells in the tumor-containing samples to be between 20 and 60%. Even after adjustment for these estimated percentages, the levels of enzymatic activity in the normal, preneoplastic, and CIS samples were approximately 10-fold lower than in the invasive cancers.

**Focal Up-Regulation of hTR Expression in CIS Samples.** As described above, enzyme activity and hTR expression in normal, preneoplastic, and CIS samples were usually focal and relatively weak. However, in all seven foci (from five patients) of CIS adjacent to or overlying invasive or microinvasive carcinoma, considerable up-regulation of hTR was noted focally (Fig. 4). In the up-regulated foci, hTR expression was of a similar intensity to the underlying or adjacent invasive or microinvasive area and involved either the full thickness of the CIS or was focal, being more intense in the basal half. A similar level of hTR up-regulation was noted in two foci of CIS not in continuity with invasive areas and in a single focus of moderate dysplasia.

**Discussion**

We observed telomerase positivity in 3 of 3 SCLCs, 29 of 31 resected NSCLCs, and all 5 cases of squamous CIS. The only negative tumors were a necrotic squamous cell carcinoma with relatively few viable tumor cells in the sample selected and a typical carcinoid, a tumor of low malignant and metastatic potential. In our previous study using only TRAP (9), we did not examine the histopathology of the actual tumor samples selected for telomerase determination, and thus, we could not determine whether some tumors were truly telomerase negative and consisted of a population of possibly mortal cells. In the present study, uniformly moderate to high hTR expression was present in all tumor cells except for the differentiated cells in squamous cell carcinomas. As in normal squamous epithelia, heavily keratinized differentiated squamous tumor cells have low or absent replicative ability compared to the undifferentiated basal cells. Thus, in tumors,
as in normal epithelia, differentiation and lowered replicative capacity may be associated with decreased or absent telomerase expression. A similar phenomenon was not noted in adenocarcinomas and SCLCs. In these tumor types, undifferentiated precursor cells usually cannot be identified, and partially differentiated glandular cells and neuroendocrine cells, respectively, function as the tumor stem cells.

Telomerase activity was detected in almost all regional lymph nodes, irrespective of whether they contained or were free of detectable metastatic tumor. We (12, 15) and others have demonstrated that activated lymphocytes express modest levels of enzyme activity. In nonmalignant regional lymph nodes and intrapulmonary lymphoid nodules, hTR expression was detected only in the germinal centers of secondary follicles. Also, the intensities of the hTR expression signals in primary and metastatic tumor cells were similar. Although the levels of telomerase activity were higher in tumor-containing nodes, enzyme expression does not appear to be a useful marker for the detection of tumor micrometastases.

A subset of histologically normal bronchial epithelium and peripheral lung samples expressed low enzyme levels, approximately 10-fold lower than in the tumors (after correction for the relative number of epithelial cells in the samples). In the normal pseudostratified bronchial and bronchiolar epithelium, hTR expression was weak and limited to basal cells. Expression was absent in alveolar type I and type II cells and in stromal cells. In hyperplastic bronchial epithelium, a higher incidence of telomerase positivity was noted, but hTR expression was low and remained limited to basal or parabasal cells. Although telomerase positivity was noted in histologically normal and slightly abnormal bronchial epithelium, most samples were from current or former smokers. We have demonstrated that nearly 50% of histologically normal bronchial samples from lifetime smokers harbor multiple molecular abnormalities. Thus, whether telomerase positivity in these samples represents normal, physiological expression in regenerating stem cells or an abnormality in smoking-damaged epithelium remains to be determined.

In metaplastic, dysplastic, and CIS bronchial lesions, telomerase positivity was present in 80–100% of samples. Of interest, telomerase enzyme activity has been described in a high percentage of premalignant lesions adjacent to invasive squamous head and neck cancers (16). In preneoplastic bronchial lesions, hTR expression was focal but usually involved cells present throughout the entire thickness of these multilayered processes. In these lesions, enzyme activity was relatively low, comparable to the levels present in histologically normal lung samples. However, focal up-regulation of hTR expression was noted in all samples of CIS adjacent to or continuous with foci of microinvasive or invasive cancer. In these regions of CIS, the intensity of hTR expression was similar to those in the invasive foci. A similar level of hTR up-regulation was noted in two foci of CIS not in continuity with invasive areas and in a single focus of moderate dysplasia. The up-regulation did not appear to be related to cell proliferation, as the hTR-positive and -negative foci were histologically identical.

Whereas the preneoplastic changes in large bronchi associated with centrally arising squamous cell carcinomas are well documented, the preneoplastic changes associated with peripherally arising adenocarcinomas are poorly understood. A relatively recently described change, AAH, also known as bronchioloalveolar adenoma, has been suggested as a possible precursor of peripheral adenocarcinomas (17). The presence of morphometric changes, RAS gene mutations and p53 protein overexpression are further evidence that AAH lesions are true preneoplastic lesions (18, 19). Our failure to demonstrate telomerase positivity in a limited number of AAH lesions in the vicinity of peripheral adenocarcinomas suggests that telomerase activation (if it occurs) is a late or occasional event.

Thus, dysregulated expression of telomerase was noted in bronchial premalignant and CIS lesions. The dysregulation was manifested in three ways: almost all such lesions were telomerase positive; instead of being limited to the basal layer, cells throughout these multilayered processes expressed hTR; and intense, focal up-regulation of hTR expression occurred in CIS in the vicinity of invasive cancers. Telomerase dysregulation in adult tissues may represent a method to overcome or abrogate cellular senescence, resulting in a commitment to indefinite cell growth, eventually leading to malignancy. Because telomerase dysregulation in bronchial epithelium occurs at a very early stage in the pathogenesis of squamous carcinomas and because intense up-regulation of hTR precedes invasion, telomerase positivity may be useful as a marker for identifying smokers at an increased risk for the development of invasive carcinoma and as a surrogate end point for the monitoring of chemoprevention trials.

References

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