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BRIEF REPORT

Peripheral blood telomere alterations in ground glass opacity (GGO) lesions may suggest malignancy

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Abstract

A ground glass opacity (GGO) lung lesion may represent early stage adenocarcinoma, which has an excellent prognosis upon prompt surgical resection. However, GGO lesions have broad differential diagnoses, including both benign and malignant lesions. Our objective was to study telomere length and telomerase activity in patients with suspected lung cancer in which GGO was the predominant radiographic feature. Knowledge of telomere biology may help distinguish malignant from benign radiographic lesions and guide risk assessment of these lesions. Peripheral blood samples were taken from 22 patients with suspected adenocarcinoma with the GGO radiographic presentation. Multidisciplinary discussion confirmed the need for surgery in all cases. We used an age and gender-matched group without known lung disease as a control. Telomere length and aggregates were assessed by quantitative fluorescence in situ hybridization (QFISH) and quantitative PCR. Cell senescence was evaluated by senescence-associated heterochromatin foci. Subjects with GGO lesions had a higher percentage of lymphocytes with shorter telomeres (Q-FISH, $P = 0.003$). Furthermore, relative telomere length was also reduced among the GGO cases (qPCR, $P < 0.05$). Increased senescence was observed in the GGO group compared to controls ($P < 0.001$), with significant correlation between the senescence-associated heterochromatin foci and aggregate formation ($r = -0.7$ and $r = -0.44$ for cases and controls, respectively). In conclusion, patients with resectable early adenocarcinoma demonstrate abnormal telomere length and cell senescence in peripheral blood leukocytes compared to control subjects. Abnormal telomere biology in the peripheral blood may increase suspicion of early adenocarcinoma among patients with GGO lesions.

Introduction

Early adenocarcinoma (previously bronchoalveolar carcinoma or BAC) is a subset of adenocarcinoma distinguished by its peripheral location, "lepidic" growth pattern, and tendency for both bronchogenic and lymphatic spread. Recent reclassification of adenocarcinoma by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society replaced the term BAC with subtypes based on invasive potential.[1] The radiographic presentation of these early adenocarcinoma subtypes is highly variable, ranging from a small solitary nodule to extensive lobar consolidation.

Ground glass opacity (GGO) lung lesions are challenging for clinicians because of the extensive deferential diagnosis, frequent occurrence, and the concern of lung cancer.[2] However, the GGO radiographic pattern presumably represents a subset of patients with a more indolent growth pattern and improved prognosis.[3–6] These early lesions may represent a large number of lung cancers with excellent prognosis after early resection.[7,8] Therefore, the determination of preoperative features is needed to help distinguish benign from malignant lesions.

Telomeres are essential for the stability, identity, and function of chromosomes. The shortest telomeres are expected to generate a cellular response leading to senescence and apoptosis.[9] When critically short, telomeres become dysfunctional and activate DNA damage responses. Recent studies have shown accelerated telomere shortening in cancer[10] and malignancy associated conditions, such as hepatitis C[11] and trisomy 21,[12] but not in early stage adenocarcinoma presenting as GGO.

In this study, we examined telomere characteristics and evaluated cell senescence in the peripheral leukocytes of patients with GGO lesions suspected as lung malignancy.

Methods

Patient selection

Patients with suspected early adenocarcinoma with the radiographic presentation of GGO or subsolid (as defined by the guidelines) were identified.[13] All were recommended by multidisciplinary discussion for histologic evaluation. Following informed consent, a peripheral blood sample was taken prior to resection or biopsy. The GGO patient group (i.e. cases) was compared to an age and gender-matched control group without known lung/other malignancy.

Lymphocyte extraction from peripheral blood samples

Blood samples were immediately treated with a hypotonic solution (0.075-M potassium chloride at 37°C for 15 minutes) and washed four times with fresh, cold 3:1 methanol: acetic acid solution. The lymphocytes were stored at -4°C. DNA was extracted using the 5 Prime Kit (5 Prime GmbH, Hamburg, Germany) according to the manufacturers' instructions.

Assessment of telomere length

Quantitative fluorescence in situ (Q-FISH) protocol was used to assess telomere length, as previously described.[14,15] A protocol for telomere FISH was applied using a CY3-labeled telomere-specific peptide nucleic acid probe (catalog No. 5326; DAKO, Glostrup, Denmark). The slides were counter stained with 4′,6-diamidino-2-phenylindole (DAPI, 1000 ng/mL, Abbott Molecular, Abbot Park, IL, USA) for observation (Olympus AX-70, Olympus Corporation, Tokyo, Japan). Quantitative FISH of telomere repeats has been previously used to assess telomere length in both metaphase and interphase nuclei and has been proven a valid and accurate method.[16]

Telomere length measurement with quantitative PCR

Relative mean telomere length was measured using a modified version of a multiplex monochrome qPCR method, first described by Cawthon[17] and later by Thorvaldsdottir *et al.*[18] Telomere length was calculated as a ratio (T/S) between detected fluorescence from telomere repeat copy number (T) and a single copy reference sequence (S) from the *HBG* gene.

The following primer sequences were used: telg: ACAC TAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT and telc: TGTTAGGTATCCCTATCCCTATCCCTATCCC TATCCCTAACA; hbgu: CGGCGGCGGGCGGCGCGGG CTGGGCGGcttcatccacgttcaccttg and hbgd: GCCCGGCCC GCCGCGCCCGTCCCGCCGgaggagaagtctgccgtt.

All experimental DNA samples were assayed in triplicate, with 5 ng of DNA for each reaction, using PowerUP SYBR Green (Applied Biosystems, Foster City, CA, USA) for a 10 μL reaction volume. The thermal cycling profile was: stage 1: 2 minutes at 50°C and 5 minutes at 95°C; stage 2: cycles of 15 seconds at 94°C and 15 seconds at 49°C; and stage 3: 40 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 74°C with signal acquisition, 10 seconds at 84°C, and 15 seconds at 88°C with signal acquisition.

Telomere aggregate count

We have previously described our two dimensional method of measuring telomere length and aggregate formation.[11] For each specimen, 100–200 cells were counted.

Senescence-associated heterochromatin foci

Slides from the FISH analysis were stained with DAPI. Cells were analyzed to determine senescence-associated heterochromatin foci (SAHF) status based on the number of cells with fragments (SAHF) and the number of SAHF fragments in each cell, as previously described.[19]

Statistical analysis

Data was analyzed using SPSS version 21.0. Plots were drawn using GraphPad Prism version 7. Data is presented as the mean and standard error of the mean (SEM) for continuous variables and as the frequency and percentage for categorical variables. Comparison between the study groups was conducted using *t*-test, chi-square/Fisher's exact tests, or Pearson correlation analysis according to the scale measured variables. The correlation coefficient (r) was calculated for each group. The significance levels were set at 0.05.

Ethics, consent, and permissions

The institutional ethics committee of Meir Medical Center (MMC-0067-14) approved the study. Informed consent was obtained from all participants. NIH study number NCT02239432.

Results

Data from 20 GGO cases and from 18 healthy age and gender-matched controls was analyzed. The baseline clinical and pathological characteristics are presented in Table 1. Because telomere length has previously been associated with diabetes,[20] we also verified that there were no differences between the groups regarding the percentage of diabetes and/or obese patients (Table 1).

Key radiographic findings included a mean lesion size of 18.6 ± 9 mm (range: 8–32 mm). All were solitary lesions: 12 (54%) demonstrated[18] fluorodeoxyglucose avidity and none demonstrated fluorodeoxyglucose avid draining lymphadenopathy. Of the GGO cases, 20 were diagnosed with early adenocarcinoma (8 lepidic-predominant, 3 invasive adenocarcinoma, and 9 well-differentiated adenocarcinoma). Two cases were malignancy free and were excluded from further analysis.

Telomere length was evaluated as previously described.[20] The percentage of cells with short telomeres was significantly higher among the GGO cases compared to the control ($P = 0.009$) (Fig 1a-c). Relative average telomere length was measured by qPCR using primers that hybridize the telomeric hexamer repeats.[17] Our findings were also validated using this method ($P < 0.05$) (Fig 1d).

Cytogenetically, SAHF appear as compacted punctuate DAPI-stained foci of DNA in senescent cell nuclei.[21] We evaluated signs of senescence in the peripheral blood lymphocytes by evaluating the amount of SAHF bodies. Senescence was significantly increased in the GGO cases versus the control ($P < 0.0001$) (Fig 2).

The negative telomere control slides that were not exposed to the CY3 labeled telomere-specific peptide nucleic acid probe did not show any telomere staining, therefore assuring that telomere length and aggregate evaluation reflected actual differences between the study groups.

The percentage of aggregates was not significantly different in the GGO group. However, a correlation was found between the senescence and the number of aggregates in both the case and the control groups ($P = 0.0002$, r = 0.64 and $P = 0.04$, r = 0.44 for cases and controls, respectively) (Fig 3).

Discussion

In this study, patients with biopsy-proven adenocarcinoma presenting radiographically with GGO lung lesions demonstrated decreased telomere length and increased cell senescence in peripheral blood leukocytes compared to healthy controls. All tumors were solitary stage 1A tumors and were successfully resected.

Table 1 Patient and control characteristics

Characteristic	GGO group (*n* = 20)	Control group (*n* = 18)	PV	OR (CI 95%)
Gender - male	7 (35%)	5 (38%)	1	0.82 [0.21–3.5]
Age ± SEM	70.9 ± 1.7	68.8 ± 1.9	0.48	
COPD	5 (25%)	2 (11%)	0.41	0.37 [0.07–2.3]
Diabetes	4 (20%)	4 (22%)	1	1.14 [0.29–4.5]
Obesity (BMI > 30)	4 (20%)	5 (27%)	0.71	1.5 [0.33–5.8]
Hypertension	11 (55%)	6 (33%)	0.21	0.4 [0.12–1.5]
Never smoker	6 (30%)	9 (50%)	0.32	2.33 [0.62–7.9]
FEV1 (% predicted) ± SEM	89.1 ± 5.6	110 ± 13.5	0.1	[–47.9–4.5]
FEV1 / FVC ± SEM	0.77 ± 0.03	0.78 ± 0.03	0.88	[–0.13–0.1]
Size of GGO mm, mean ± SEM	20.9 ± 8.3	Not relevant		

BMI, body mass index; CI, confidence interval; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GGO, ground glass opacity; OR, odds ratio; PV, P-value; SEM, standard error of the mean.

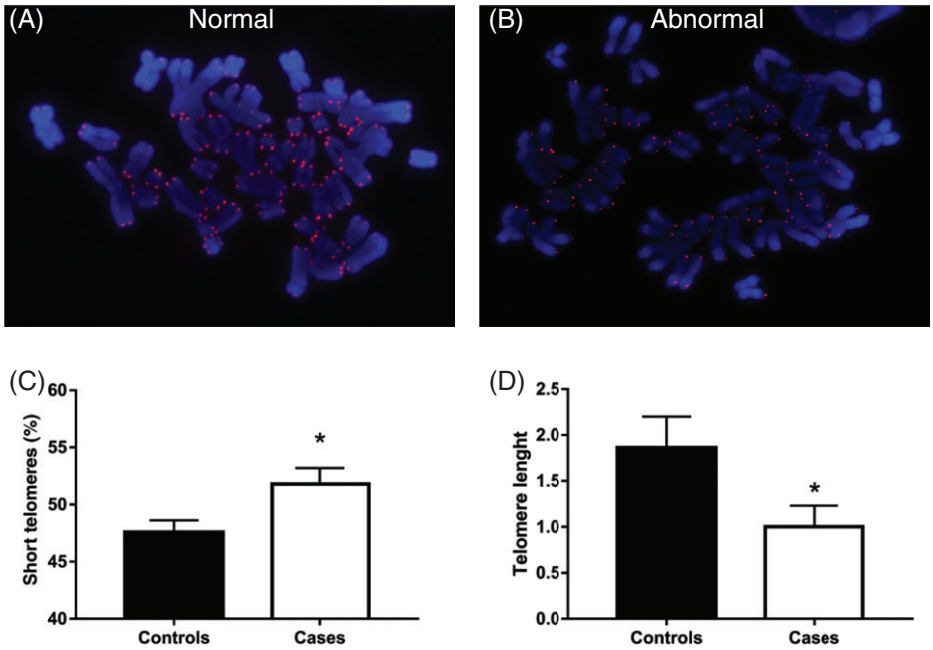

Figure 1 Ground glass opacity cases present with relatively shorter telomeres. Representative fluorescence in situ hybridization (FISH) images of (**a**) a control normal cell and (**b**) a cell with abnormal telomeres with a low signal (magnification x1000). Telomere length was measured by (**c**) quantitative FISH and (**d**) quantitative PCR. $*P < 0.05$, $**P < 0.01$.

Telomeres are considered a biomarker for aging and diseases, such as cancer,[22] heart disease, and osteoporosis.[23] Because of the semiconservative nature of DNA replication, telomeres shorten during each replication.[24] Telomerase activity is activated aberrantly in most lung cancers[9,25] and mutations in telomerase components

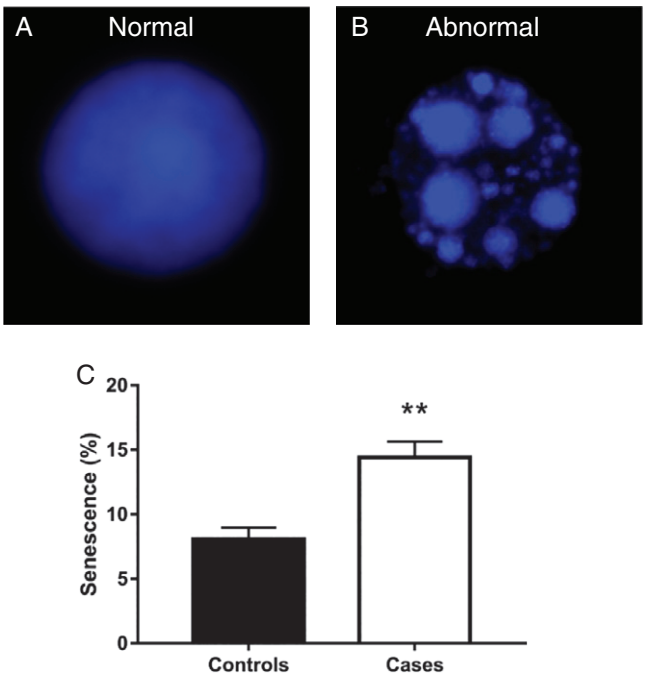

Figure 2 Ground glass opacity cases present with increased senescence. Representative fluorescence in situ hybridization (FISH) images of (**a**) a cell without senescence and (**b**) a senescent cell with several fragments (magnification x1000). (**c**) Senescence was measured by quantitative FISH, measuring the relative number of senescence-associated heterochromatin foci bodies. $**P < 0.01$.

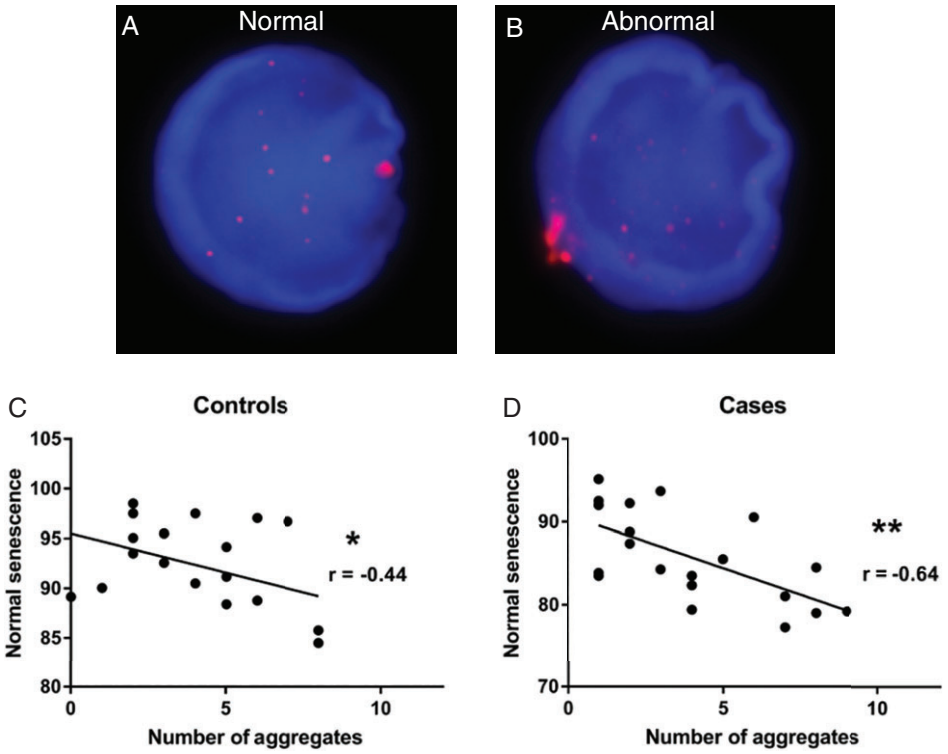

Figure 3 Telomere aggregate formation is correlated with the state on senescence. Representative fluorescence in situ hybridization images of aggregate formation: (**a**) normal telomere distribution; (**b**) abnormal telomere distribution (magnification x1000). (**c,d**). Pearson correlation analysis was applied to determine the relationship between senescence and the mean number of telomere aggregates for each of the study groups (n = 20 for cases, n = 18 for controls).

predispose to solid malignancies.[26] For patients with non-small cell lung cancer, numerous studies correlate increased tumor telomerase activity with an increased likelihood of stage IIIB and IV disease and/or reduced survival.[27–30]

The GGO lung lesion may represent early stage adenocarcinoma, which has excellent prognosis upon early diagnosis and prompt surgical resection.[7,8] However, preoperative diagnosis of such lesions is complicated by several limitations. The differential diagnosis is broad, including inflammatory and infectious processes. Positron emission tomography (PET) may be falsely negative because of the slow growth of early adenocarcinoma lesions. The potential for false negative PET scans was reported in a series of 83 proven malignancies in which PET detected only 10/29 (34%) early adenocarcinoma lesions.[31] Preoperative features favoring adenocarcinoma, such as thp\ose described above, may help to identify high-risk GGO lesions from those more likely to be benign.

Although GGO lesions are very premature (i.e. stage 1), abnormal telomere biology was already evident in our peripheral blood cell samples. To the best of our knowledge, this is the first study to describe such changes in early adenocarcinoma presenting as GGO lesions. Although the study included a relatively small sample size, significant results were obtained.

Our results have several potential clinical implications. Patients with known short telomere syndrome may indicate screening for early lung adenocarcinoma and prompt resection. Conversely, patients with coexisting underlying lung fibrosis, one of the most important manifestations of short telomere syndrome,[32] may be at an increased risk of postoperative pulmonary complications should they undergo lung resection for early adenocarcinoma.[33] Furthermore, medical therapy may play a role for such patients and those with non-operable disease.[34]

In conclusion, abnormal telomere biology was observed in the peripheral blood leukocytes of patients with GGO lesions, which were later identified as early adenocarcinoma. Although further study is required, such findings may increase suspicion of early adenocarcinoma among GGO lesions and have implications for treatment.

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Disclosure

No authors report any conflict of interest.

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