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A somatic *TSHR* mutation in a patient with lung adenocarcinoma with bronchioloalveolar carcinoma, coronary artery disease and severe chronic obstructive pulmonary disease

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Abstract. In a screen for thoracic malignancy-associated markers, thyroid stimulating hormone receptor (TSHR) was identified as a candidate as it binds to the previously-characterized lung cancer marker NKX2-1. We screened for mutations in all coding regions of the *TSHR* gene in 96 lung adenocarcinoma samples and their matched adjacent normal lung samples. We found one patient with a somatic mutation at codon 458 (exon 10), which is located at the transmembrane domain where most *TSHR* mutations have been found in thyroid-related diseases. This patient had lung adenocarcinoma with BAC (bronchioloalveolar carcinoma) features in the setting of a prior medical history significant for carotid stenosis and severe chronic obstructive pulmonary disease (COPD). In order to characterize the genetic features of *TSHR* in lung cancer, we checked for *TSHR* expression and copy number in the 96 lung cancer tissues. TSHR protein expression was generally overexpressed in multiple thoracic malignancies (adenocarcinoma, squamous cell carcinoma and malignant pleural mesothelioma) by immunohistochemistry. Our data suggest that aberrant TSHR function may contribute to lung cancer development or a subgroup of lung cancer with specific clinical phenotypes.

Introduction

Lung cancer is one of the worst prognostic human cancers, taking more lives than any other human cancer worldwide (1). Progress in therapy for lung cancer has made recent progress

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with the identification of molecular markers such as *EGFR* and *EML4-ALK* (2-4). Gefitinib (Tarceva) targeting *EGFR* and Crizotinib (Xalkori) targeting *EML4-ALK* are leading examples of molecular targeting in lung cancer therapeutics (2,4). Patients with *EGFR* mutations have responded well to Tarceva. However, many patients later relapse due to a secondary *EGFR* mutation (T790M), decreasing the advantage of Tarceva treatment with respect to patient prognosis and survival (5). In 2007, a new fusion protein, *EML4-ALK*, was identified in a Japanese lung cancer patient without *EGFR* or K-*ras* mutations (3). The incidence of this fusion protein is greater in Asian patients without *EGFR* or K-*ras* mutations (6). Encouragingly, patients with *EML4-ALK* showed a dramatic response to an ALK inhibitor (7) and in 2011, the FDA approved Crizotinib for the treatment of non-small-cell lung cancer (NSCLC) in patients with *EML4-ALK* fusion and aberrant ALK expression. Overall mutation frequencies of *EGFR* and *EML4-ALK* are ~25% and 1-7% (3,6), respectively, which suggests that >65% of lung cancer patients lack specific molecular targets for treatment. Identification of new targets either for diagnosis or therapy is important in the effort to reduce mortality by lung cancer.

NKX2-1 (*TTF1* or *TITF1* or thyroid transcription factor-1) was originally identified in thyroid and lung (8). Many efforts have been made to identify characteristics of *NKX2-1* not only in thyroid but also in other organs, including the lung. *NKX2-1* was first thought to act as a lung oncogene (9,10), but later study suggested that it may act as a tumor suppressor in lung adenocarcinoma (11). Notwithstanding the clear contribution of NKX2-1 to lung development and homeostasis, it is not clear how to inhibit or boost NKX2-1 for the treatment of lung cancer. This gene is not clearly amplified, overexpressed, or mutated and as such no clear agonistic or antagonistic molecular targets of NKX2-1 have been developed for lung cancer.

In order to find potential lung cancer or general thoracic malignancy-related markers associated with NKX2-1, we screened other binding partners of NKX2-1, filtering out known type II pneumocyte and clara cell markers. One of the interesting binding partners of NKX2-1 is *TSHR* (12). NKX2-1 binds the promoter of *TSHR* and promotes constitutive TSHR expression and TSH/cAMP-induced negative regulation of TSHR (12,13). Activating and inactivating mutations have

Table I. Past medical and surgical history of case 611 with *TSHR* mutation.

History	Age
Coronary artery disease, with history of myocardial infarction, status post percutaneous coronary intervention with stent placement	66
Chronic obstructive pulmonary disease (COPD)	
Bilateral severe carotid stenosis, status post bilateral carotid endarterectomies	66
Peripheral vascular disease	
Hypertension	
Hyperlipidemia	
Hiatal hernia	
Gastroesophageal reflux disease	
Diverticulosis	
Primary hyperparathyroidism, status post parathyroidectomy	33
Nephrolithiasis, status post bilateral nephrolithotomies	33
Pertussis, causing right pneumothorax, status post right thoracotomy	24
Bilateral cataracts, status post bilateral cataract repair	62

been reported in many thyroid-related diseases, such as hyper/hypothyroidism and thyroid cancer (14,15). Most activating mutations are known to be associated with autoimmune diseases of the thyroid (14,15). Epigenetic changes in *TSHR* have been reported to cause a significant down-regulation and decreased expression of *TSHR* in thyroid cancer (16). *TSHR* has not been studied in lung cancer or thoracic malignancies except in a mutation screening of a lung metastasis from thyroid cancer (17). No *TSHR* mutation was found in the pulmonary metastasis of thyroid cancer (17). As such, to date it is not clear whether *TSHR* is genetically involved in primary lung cancer development. Thus, we screened for *TSHR* mutation, DNA copy number, and mRNA expression in 96 lung adenocarcinoma and matched normal tissues. In addition to lung adenocarcinoma, other thoracic malignancies including squamous cell carcinoma (SCC) and malignant pleural mesothelioma (MPM) were measured for TSHR protein expression by immunohistochemistry.

Materials and methods

Patient samples. The Committee on Human Research (CHR) of UCSF reviewed and approved the application for the collection of tissue samples from patients with thoracic malignancies (approval number: 10-03352). All samples were collected under the IRB approval and written informed consents were obtained from all patients in this study.

TSHR mutation screening. Ninety-six lung adenocarcinoma tissue samples screened for our previous study (18) were used for *TSHR* mutation screening. All coding regions of *TSHR* were screened using PCR primers as previously described (19). Amplification was performed at 58-62˚C using 20 ng of genomic DNA in 96 sample pairs, 10X PCR buffer supplemented with 1.5 mM MgCl$_2$, 10 pmol of each primer, 50 mM of each dNTP, and 0.2 units of Taq polymerase (Qiagen) in a total

reaction volume of 25 μl. The PCR products were cleaned up using 1 μl of SAP (1 U/μl), 0.1 μl of Exo1 enzyme (10 U/μl), 1 μl of 1X PCR buffer, 2.4 μl of 10X NEB buffer (type 3), 5 μl of the PCR product, and 15 μl of water. Bi-directional sequencing was performed using the Taq dideoxy-terminator cycle sequencing kit and an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

TSHR mRNA expression analysis in 96 lung adenocarcinoma and matched normal tissues. RNA from 96 snap-frozen lung adenocarcinoma and matched normal tissues was extracted with TRIzol (Invitrogen), treated with DNase I (New England Biolabs), and purified with the RNeasy RNA purification kit (Qiagen). cDNA was synthesized using a reverse transcript RT III (Invitrogen). *TSHR* expression was quantified by Taqman real-time PCR (ABI, 7900HT) using 96 lung adenocarcinoma and matched normal tissues in triplicate (*TSHR* expression probe: 4331182, ABI). *GAPDH* was selected to normalize cDNA input. ΔCT 2 was calculated from an expression difference between *TSHR* and *GAPDH*. ΔCT values were then used to compare *TSHR* expression between tumor and normal samples.

TSHR DNA copy number analysis in 96 lung adenocarcinoma and matched normal tissues. DNA copy number analysis of *TSHR* was done in the same 96 pairs of samples (*TSHR* DNA probe: Hs01214599_cn). DNA copy number assay for *TSHR* was performed using Taqman real-time PCR. All samples were analyzed in triplicate and RNase P gene (VIC/Tamra probe) was used for internal control to normalize DNA quantity. Taqman probes were labeled by FAM/non-fluorescent quencher. Sample DNA (10 ng) were analyzed on a 384-well plate using 2X Taqman Universal PCR master mix with no AmpErase UNG. Deletion or gain was determined by ΔCT calculation and analyzed by software provided by ABI. Further data analysis was also performed manually. After average ΔCT values of normal controls were calculated, each ΔCT value of samples
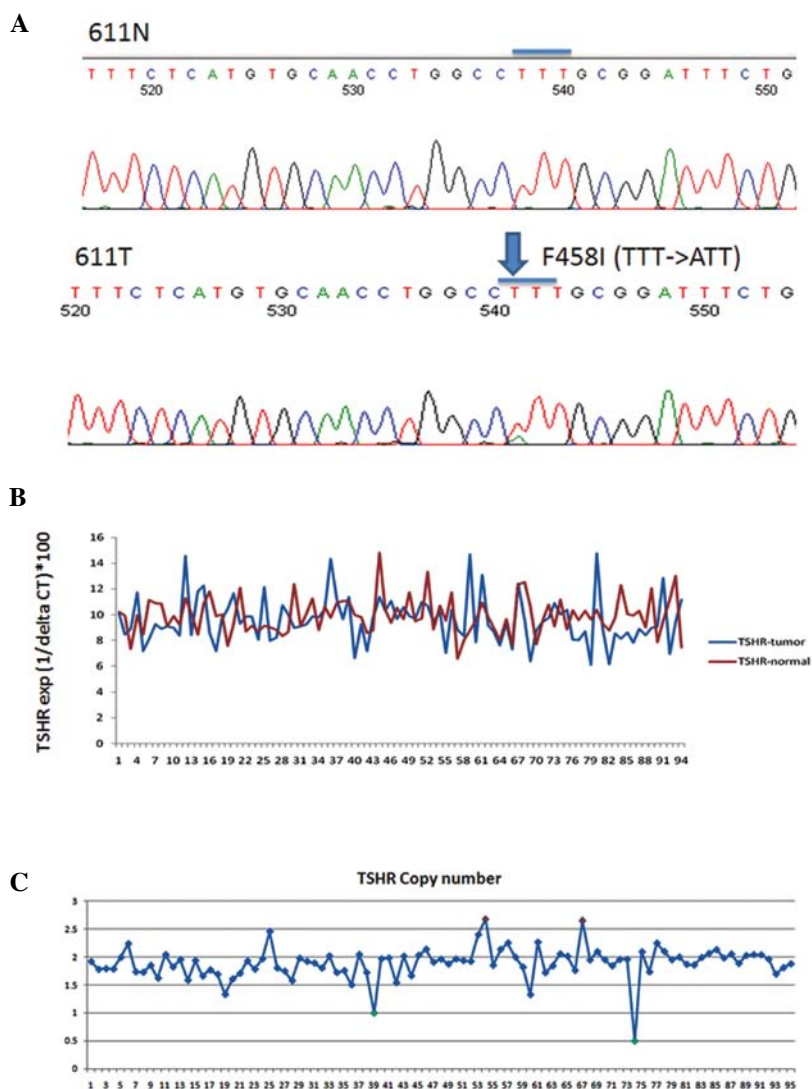
Figure 1. Somatic mutation of *TSHR* in lung adenocarcinoma patient (ID: 611) and *TSHR* mRNA expression and DNA copy number in lung adenocarcinoma and matched normal tissues. A missense mutation of *TSHR* at exon 10 (codon 458) was found in a primary lung cancer sample and not its matched normal tissue. The mutation is located at the transmembrane domain where most *TSHR* mutations have been reported in hyper/hypothyroidism, thyroid cancer and other thyroid-related diseases. *TSHR* expression was checked by Taqman using 96 lung adenocarcinoma and matched normal tissues (*TSHR* expression probe: 4331182, ABI). There was no statistically significant mRNA expression change between normal and matched tumor tissues (paired t-test p=0.118). Y axis: TSHR expression [(1/ΔCT)*100] (A) DNA copy number analysis of *TSHR* was done in the same 96 pairs of samples (*TSHR* DNA probe: Hs01214599_cn) (B) Deletion or gain was determined by ΔCT calculation and analyzed by software provided by ABI. Two potential amplifications (red) and two deletions (green) were identified in 96 AD samples. Most of the samples except these four showed normal ranges of DNA copy number, which means LOH or amplification are not major genetic events of TSHR contributing lung tumor development.

was divided by this normal value. $\Delta\Delta$CT values were then calculated and the threshold level for copy number was set as <1.2 for deletion and >2.6 for gain.

Immunohistochemistry (IHC). Ten pairs of matched normal and lung adenocarcinoma (AD) and squamous cell carcinoma (SCC), and five malignant pleural mesothelioma (MPM) samples were stained with anti-TSHR antibody (ab5492, Abcam). Sections (5 μm) were hydrated in xylene and graded concentrations of ethanol, then steamed in citrate (Biogenex) for 20 min. Slides were then incubated at room temperature with a blocking solution (10% goat serum in TBS and 1% BSA) for 1 h. Blocking solution was then removed and the primary antibody was applied. The slides were incubated at 4°C overnight. The slides were incubated using reagents from

the Invitrogen Histostain Plus Broad Spectrum kit (85-9643) according to the protocol provided by Invitrogen.

Results

Somatic TSHR mutation. Out of 96 lung adenocarcinoma samples, one mutation was identified in adenocarcinoma tissue (patient ID: 611) that did not appear in matched normal tissue (Fig. 1A). A missense mutation at exon 10 (codon 458, TTT→ATT, F458I) was identified at the transmembrane domain of *TSHR* (Fig. 1A). The results suggest an overall observed *TSHR* mutation frequency of 1% (one out of 96 AD). We investigated the prior clinical history of the *TSHR*-mutated patient (patient ID: 611) to identify unique clinical insight. The patient was a 67-year-old (at the time of her second operation, during
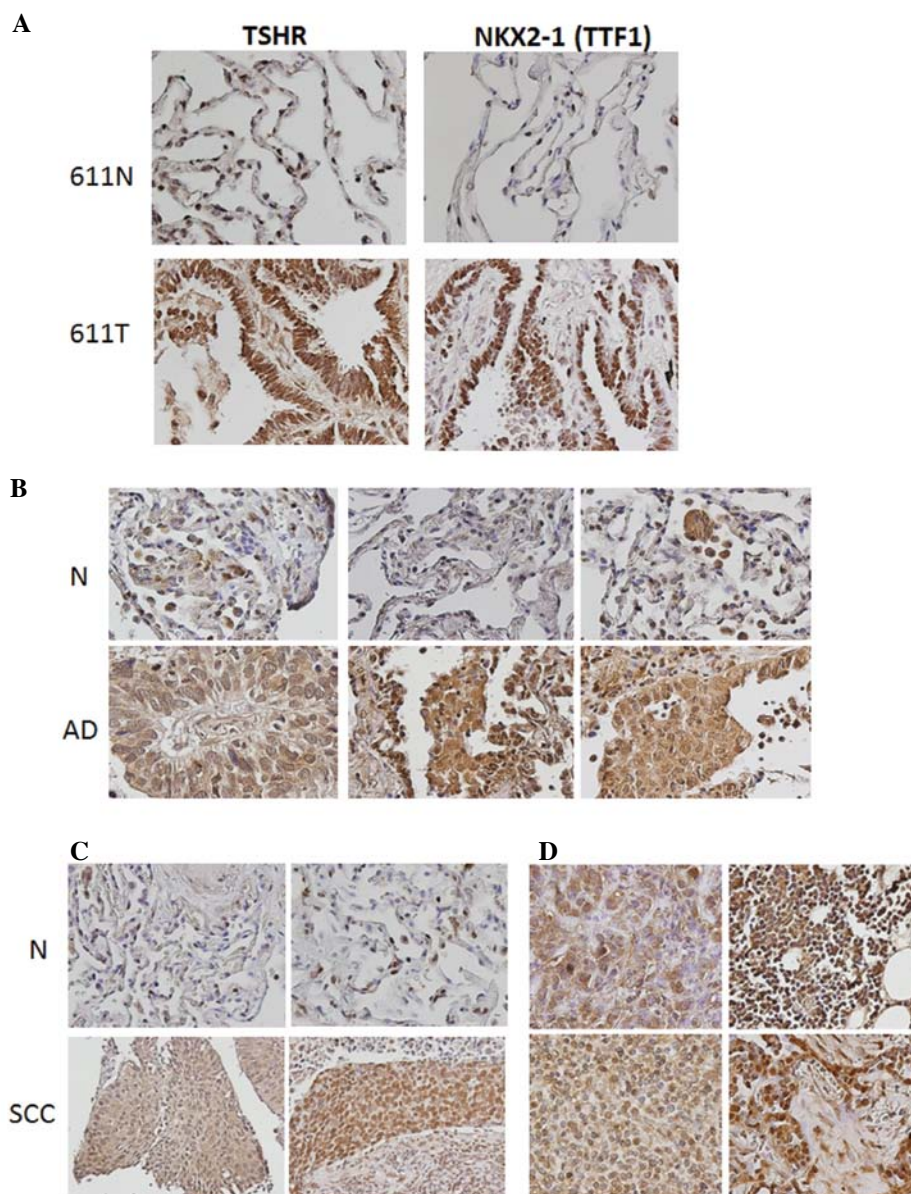
Figure 2. Immunohistochemistry of TSHR in lung adenocarcinoma (AD), squamous cell carcinoma (SCC), and malignant pleural mesothelioma (MPM). Overexpression of TSHR and NKX2-1 (TTF1) in tumor samples compared to matched normal tissues in patient 611 with TSHR somatic mutation (A). Representative examples of three pairs of matched normal and lung AD (B), two pairs of matched SCC (C), and four MPM (D) specimens were stained with anti-TSHR antibody (ab5492, Abcam). High TSHR expression was detected in tumor specimens, but only mild in the matched normal specimens (B-D). MPM specimens were also analyzed for TSHR protein level and high TSHR levels compatible with those of AD and SCC were detected (C). Microscope magnification: x20, x40.

which the specimen was collected) Caucasian woman with a history of coronary artery disease, carotid stenosis, chronic obstructive pulmonary disease (COPD) and a 50-pack-year smoking history who had presented 15 months earlier with a 3-year history of cough and 6-month history of hoarseness. CT scan showed bilateral upper lobe lung lesions, and fine needle aspiration suggested bilateral lung adenocarcinoma. Her case was discussed at our institution's thoracic oncology tumor board, and the lesions were thought likely to be two primary tumors. She first underwent left upper lobectomy, and pathology showed stage IIB moderately differentiated adeno-carcinoma. Postoperatively, she received carboplatin and gemcitabine for 2 cycles, then carboplatin and paclitaxel for 4 cycles. Imaging revealed stable disease, so she then under-

went right upper lobectomy, during which time our tumor and adjacent lung specimens were collected. Pathology showed stage IB adenocarcinoma with features of bronchioloalveolar carcinoma. This patient had no signs or symptoms associated with thyroid cancer or diseases (Table I). A systematic review of English-language manuscripts available in the PubMed database yielded no prior report that *TSHR* is associated with COPD or coronary artery disease.

TSHR mRNA expression and copy number. To determine if *TSHR* is amplified or overexpressed in lung AD samples, we measured *TSHR* gene expression in 96 AD and matched normal tissues using a real-time quantitative PCR (Fig. 1B). There was no clear up-regulation of *TSHR* in tumor samples

compared to the matched normal tissues. *TSHR* DNA copy number was then checked, and only two potential amplifications of *TSHR* in 96 AD samples were found (Fig. 1C), which suggests that *TSHR* amplification might not be a major genetic event in lung cancer. For deletion, only additional two samples showed clear deletions in 96 AD samples.

TSHR protein expression by immunohistochemistry. Next, we checked TSHR protein expression in tumor and matched normal samples of the patient harboring the *TSHR* mutation. Both TSHR and NKX2-1 (TTF1) were generally overexpressed in tumor samples compared to normal tissues (Fig. 2A). Additional samples of three different subtypes of thoracic malignancies, adenocarcinoma (AD), squamous cell carcinoma (SCC), and malignant pleural mesothelioma (MPM), were analyzed for TSHR expression by immunohistochemistry (IHC) (Fig. 2B-D). High TSHR protein expression was found in AD and SCC while low or mild expression was detected in matched normal samples (Fig. 2B and C). For MPM, only tumor samples were stained for TSHR and high TSHR expression was detected (Fig. 2D). Although overall mRNA expression of *TSHR* was low and not significantly different in normal and tumor tissues (Fig. 1B), the protein level of TSHR is meaningfully high in all three subtypes of thoracic malignancies, AD (Fig. 2B), SCC (C) and MPM (D).

Discussion

Lung cancer is the leading cause of cancer death in men and women in the US and world-wide (1). Five-year survival has stubbornly remained at a dismal 15-17% for a decade, making it one of the most lethal cancers. Chronic respiratory diseases are another major cause of deaths. Worldwide, COPD (Chronic Obstructive Pulmonary Disease) is responsible for 3 million deaths annually (20,21). COPD is relatively irreversible and the rate of incidence is increasing, such that COPD is expected to be the 3rd or 4th leading cause of death by 2030 (20,21) as a result of increases in smoking and air pollution. However, as only 15-20% of smokers are estimated to develop either lung cancer or COPD, there must exist additional major genetic mechanisms driving the development of these diseases (22-24). Up to half of lung cancer patients suffer from COPD and ~1% of COPD patients develop lung cancer per year (24,25). The prognosis of lung cancer patients with COPD is worse than that of patients without COPD (24). Although several genetic and epigenetic mechanisms have been proposed as playing a role in lung cancer development in COPD patients, (22-26) the exact mechanisms by which some patients with COPD later develop lung cancer remains unclear.

We screened for mutations in *TSHR* based on its putative relationship with NKX2-1, a potential tumor suppressor and type II pneumocyte marker in lung (11). The mutation frequency of *TSHR* in lung cancer in our study is ~1%, similar to *EML4-ALK*, a fusion protein estimated to play a role in 1-7% of lung adenocarcinoma (3,5). When analyzing clinical characteristic of patient 611 with *TSHR* mutation, we found that this patient had a complicated prior medical history significant for carotid stenosis, coronary artery disease and COPD. It was reported that COPD is frequent in higher grade and non-BAC adenocarcinoma (24,27). Thus, it is possible that THSR may be involved in the development of a BAC-type lung adenocarcinoma arising from a patient

population with pre-existing COPD, although further systematic study with large patient number will be required to validate this.

TSHR has been reported to be mutated in many thyroid-related diseases (14). Activating *TSHR* mutations have been reported in congenital hyperthyroidism, toxic multinodular goiter and hot nodules. Inactivating *TSHR* mutations have been reported in congenital hypothyroidism, multinodular goiter and cold nodules (14). Although several studies have reported *TSHR* somatic mutations in thyroid cancer (15), epigenetic changes (methylation) mainly seem to cause reduced expression of *TSHR* in thyroid cancer (14,16). The novel mutation identified in this study is located at codon 458 (exon 10), close to a proven functional M453T *TSHR* transmembrane domain mutation, where most activating *TSHR* mutations occur (14,15). It was reported that an activating germline M453T *TSHR* mutation caused non-autoimmune hyperthyroidism (28,29).

We hypothesized that the identified *TSHR* mutation at the transmembrane domain would cause an overexpression of TSHR or its binding partner, NKX2-1. We quantified *TSHR* mRNA levels in both lung adenocarcinoma and matched normal tissues and did not find a statistically significant change (Fig. 1B). At the DNA level, two samples showed potential copy number amplifications while two other samples showed clear deletions (Fig. 1C). Next, we checked TSHR protein level by immunohistochemistry. A significant overexpression of both TSHR and NKX2-1 in patient 611's tumor samples was observed (Fig. 2A). We also checked other subtypes of thoracic malignancies, AD, SCC and MPM, for TSHR protein level. As shown in Fig. 2B-D, high TSHR expression was identified in tumor samples but not in the matched normal tissues. This indicates that TSHR protein expression, not mRNA expression, may potentially be a useful lung tumor marker for either diagnostic or therapeutic purposes. An anti-TSHR antibody-based ELISA assay performed on blood specimens may provide a simple and reliable method for earlier detection of lung cancer.

Taken together, we first identified a somatic mutation of *TSHR* in a patient presenting with lung adenocarcinoma with BAC features and a prior medical history significant for COPD and coronary artery disease. Our observed mutation frequency of *TSHR* is ~1% (1/96) in lung adenocarcinoma patients, which suggests that mutation of *TSHR* is not a major event in lung carcinogenesis generally. It does not appear that *TSHR* contributes to lung cancer development by classical genetic events such as mutation, amplification, or overexpression. When considering a significant overexpression of TSHR in tumor compared to matched normal samples, TSHR protein, not DNA or mRNA, may be used for either diagnostic or therapeutic purpose. Although further studies with larger numbers of samples are required, it appears possible that a subgroup of lung adenocarcinoma patients, such as those with BAC, COPD and/or cardiovascular diseases, may be susceptible to a *TSHR* mutation.

Acknowledgements

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