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ORIGINAL ARTICLE

Dickkopf-1 autoantibody is a novel serological biomarker for non-small cell lung cancer

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Abstract

Objective: We investigated whether or not there are autoantibodies for DKK1 (Dickkopf-1) in patients with non-small cell lung cancer (NSCLC) and whether this autoantibody can be used for cancer detection.

Methods: The levels of DKK1 autoantibodies were determined in 93 NSCLC patients and 87 healthy controls.

Results: We found that, in the sera, the presence of autoantibody against DKK1 was highly correlated with NSCLC. High anti-DKK1 autoantibody titres were found in the sera of NSCLC patients, whereas low or negative titres were found in the control group. The ROC curve results showed that autoantibody immunoassay exhibited 62% sensitivity and 84% specificity. The sensitivity for the detection of NSCLC in stage I also reach 64.3%. Furthermore, a combined ELISA assays for both DKK1 and autoantibody DKK1 increased sensitivity and classified 81.7% (76/93) of the NSCLC patients as positive, whereas only 13.8 % (12/87) of healthy volunteers were falsely diagnosed as positive.

Conclusions: Our results suggest that the detection of circulating DKK1 autoantibody could potentially serve as a useful non-invasive marker for determining lung cancer status.

Keywords: *DKK1; autoantibody; biomarker; non-small cell lung cancer; molecular diagnosis*

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (Parkin et al. 2001). The current stage classification of lung cancer is based on clinicopathological features (Mountain 1997). However, such clinical information can sometimes be incomplete or misleading for determining the prognosis of patients (Martini et al. 1995, Strauss et al. 1995). Molecular diagnostics may offer precise, objective and systematic human cancer classifications. However, standard molecular markers for the prognostic classification of most solid tumours have yet to be identified. The potential prognostic implications of several biological and molecular parameters, including K-ras mutation (Fukuyama et al. 1997, Sugio et al. 1992, 1993), c-erbB2 overexpression (Osaki et al. 1995) and p53 mutation (Dobashi et al. 1997, Oyama et al. 1998), have been reported for patients with non-small cell lung cancer (NSCLC).

DKK1 (Dickkopf-1) encodes a secreted protein and is a negative regulator of the Wnt signalling pathway in colon cancer cells (Gonzalez-Sancho et al. 2005, Niida et al. 2004). Some studies have shown that *DKK1* is overexpressed in lung cancer, esophageal cancer, Wilms' tumour, hepatoblastoma and hepatocellular carcinoma (HCC), suggesting that *DKK1* is oncogenic (Patil et al. 2005, Wirths et al. 2003, Yamabuki et al. 2007). These results indicate that DKK1 might be a useful cancer-specific antigen.

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There is increasing evidence that patients with cancer can produce autoantibodies against antigens in their tumours (Ben-Mahrez et al. 1990, Crawford et al. 1982, Lubin et al. 1993, Winter et al. 1992), suggesting that such autoantibodies may have diagnostic value. We hypothesized that *DKK1* overexpression elicits the induction of serum autoantibodies and that the presence of such autoantibodies could be used for cancer detection. To test the hypothesis, we employed the enzyme-linked immunosorbent assay (ELISA) method to measure the level of DKK1 autoantibody in lung cancer serum samples.

Materials and methods

Cell lines and tissue samples

The human lung cancer cell lines used in this study included: SPC-A1, NCI-H446, A549, NCI-H460 and NCI-H1299. All cells were grown in monolayers in appropriate medium supplemented with 10% fetal calf serum (FCS) and maintained at 37°C in humidified air with 5% CO_2. Primary lung tumours and adjacent normal lung tissue samples used for Western blot analysis were obtained from patients undergoing curative surgery at Shanghai Chest Hospital, affiliated with Shanghai Jiaotong University. Primary lung cancer samples were obtained earlier with informed consent. This study, as well as the use of all clinical materials within, has been approved by an individual institutional Ethical Committees. The clinical stage of each cancer patient was judged according to the International Union Against Cancer TNM classification.

Serum samples

Serum samples were obtained with written informed consent from 87 healthy control individuals (57 men, 30 women, median age 62.5 years, range 30–81 years) and from 93 lung cancer patients admitted to Shanghai Chest Hospital affiliated with Shanghai Jiaotong University (61 men, 32 women, median age 60.3 years, range 33–79 years). The samples from the lung cancer patients included 53 adenocarcinomas, 23 SCCs, five adenosquamous carcinomas, eight bronchial carcinomas and four large cell carcinomas. The serum samples of cancer patients were selected for the study based on the following criteria: (1) patients were newly diagnosed and previously untreated and (2) their tumours were pathologically diagnosed as lung cancers (stages I–IV). Serum was obtained at the time of diagnosis, aliquoted (10 µl volume) and kept frozen at -70°C until use. The serum samples were thawed only once before use and the diluted serum samples were never used more than once.

Purification of DKK1 and ECPKA

The *DKK1* encoding sequence was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR). The primers used were 5′ GGAATTCCATATGATGGCTCTGGGCGCAG 3′ and 5′ CGGGATCCTTAGTGTCTCTGACAAGTGTGAAGC 3′. The PCR products were then digested with *Nde*I and *Bam*HI restriction enzymes and inserted into the *Nde*I/*Bam*HI backbone of pMalC2X (New England Biolabs, Inc., Ipswich, MA, USA). The *DKK1*-MBP fusions were expressed in BL21(DE3) cells. The recombinant DKK1 protein was purified on an Amylose Resin affinity column (New England Biolabs, Inc.) and then fractionated by SDS-PAGE (Supplementary Figure S1A, see online version of this article).

The ECPKA (extracellular proteinase A) encoding sequence was cloned by RT-PCR. The sequences of the primers used were 5′-CATATGGGCAACGCCGCCGCCGC-3′ and 5′ -GGATCCTAAAACTCAGAAAACTCCTTGCCAC-3′. The PCR product was first inserted into the pGEM-T easy vector (Promega, Madison, WI, USA). The resulting vector was then digested with *Nde*I and *Bam*HI restriction enzymes. The ECPKA cDNA was then inserted into the *Nde*I/*Bam*HI backbone of pET-28a (+) (Novagen, Madison, WI, USA). Recombinant ECPKA protein was expressed in BL21(DE3) cells, purified on a Ni-NTA His-Bind resin column (GE Healthcare UK Ltd, Amersham, UK), and fractionated by SDS-PAGE (Supplementary Figure S1B, see online version of this article).

ELISA method

Because DKK1 recombinant protein is not commercially available, we used our recombinant DKK1 fusions to detect the presence of autoantibody against DKK1 in the serum samples by ELISA assay. Each well of a microtitre plate was coated with 100 µl of diluted purified recombinant human DKK1 protein (1 µg ml^{-1} concentration in PBS) and incubated overnight at 4°C. After washing away unbound antigen with washing buffer (0.01 M PBS, 0.1% Tween-20, pH 7.4), the wells were blocked with 5% milk for 2 h at room temperature. To remove any potential antibody against maltose-binding protein (MBP) in the serum samples, 100 µl of 400-fold diluted serum samples (dilution buffer, 0.01 M PBS, pH 7.4, 5% non-fat dry milk, 0.5% Tween-20) were pre-incubated with MBP coated wells for 30 min. The serum samples were then added to wells coated with recombinant human DKK1 protein and incubated for 1 h at 3°C. After three washes with washing buffer (0.01 M PBS, pH 7.4, 0.5% Tween-20), 100 µl of 3000-fold diluted antihuman IgG–horseradish peroxidase (HRP) antibody enzyme conjugate (Proteintech group Inc., Chicago, IL, USA) in 0.01 M PBS, 5% milk, 0.1% Tween-20 was added to each well and

incubated for 2 h at room temperature. The wells were then washed five times in washing buffer before adding 100 μl of ABTS (2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid) substrate solution (1 mM ABTS, 29 mM anhydrous citric acid, 41 mM dibasic Na phosphate, pH 4.2, 0.03% H_2O_2) to each well. The plates were incubated at 37°C for 15 min and absorbance at 410 nm of each well was recorded using an ELISA reader (microplate reader benchmark; Bio-Rad, Hercules, CA, USA). Autoantibody titres in serum are represented as ratios to the mean absorbance of the normal control sera. A control serum was included on all plates to monitor plate-to-plate variation. The differences between groups (patients versus controls) were calculated for statistical analysis.

Serum levels of DKK1 protein were also measured by ELISA assay. First, a 100-fold dilution of commercially available rabbit polyclonal antibody specific to human DKK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to a 96-well microplate and incubated overnight at 4°C. After washing away any unbound antibody, blocking buffer (0.01 M PBS, pH 7.4, 5% non-fat dry milk, 0.5% Tween 20) was added to the wells and incubated for 1 h at 37°C. After washing, 50-fold diluted sera were added to the wells and incubated for 1 h at 37°C. After washing away any unbound substances, a 50-fold diluted mouse polyclonal antibody (see supplementary Figure S2 for antibody specificity, in online version of this article) raised against our recombinant DKK1 protein was added to the wells as a detection antibody and incubated for 1 h at 37°C. After washing to remove any unbound antibody-enzyme reagent, 1000-fold diluted HRP-conjugated goat antimouse IgG secondary antibody was added to the wells and incubated for 1 h at 37°C. After washing, ABTS substrate solution (Sigma) was added to the wells. The plates were incubated at 37°C for 15 min and absorbance at 410 nm was recorded using an ELISA reader. Concentration of DKK1 in serum is represented as ratios to the mean absorbance of the normal control sera. A control serum was included on all plates to monitor plate-to-plate variation. The differences between groups (patients versus controls) were calculated for statistical analysis.

Western blot analysis of DKK1 expression in lung cancer tissues and lung cancer cell lines

Tumour tissues or cells were lysed in lysis buffer (50 mmol l^{-1} Tris–HCl (pH 8.0), 150 mmol l^{-1} NaCl, 0.5% NP40, 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail; Pierce, Rockford, IL, USA). The protein content of each lysate was determined using a BCA protein assay kit (Pierce) with bovine serum albumin as a standard. Each lysate (30 μg) was fractionated on a 12% denaturing polyacrylamide gel (with 4% polyacrylamide stacking gel) and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad). After blocking with 5% non-fat dry milk in PBS-Tween 20 (PBST), the membrane was incubated with a commercially available rabbit polyclonal antibody against human DKK1 (hDKK1; Santa Cruz Biotechnology) at 4°C, overnight. Immunoreactive proteins were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing with PBST, the reactants were developed using the enhanced chemiluminescence kit (Pierce).

Western blot analysis of anti-DKK1 antibody in serum

The purified, recombinant human DKK1 fusion protein (100 ng/lane) was fractionated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked, washed, and blot strips were incubated with pre-cleared serum from either lung cancer patients or normal controls (1:100) in 5% non-fat dry milk in PBST. The western blot strips were then washed with PBST three times, incubated with antihuman IgG–HRP conjugate, and the chemiluminescent reactions were developed using the enhanced chemiluminescence kit (Pierce).

Statistical analysis

Graphpad prism v4.03 software was used for statistical analysis. Means, SD and confidence intervals (CI) were calculated where appropriate. A p-value <0.05 was considered significant throughout. Receiver-operating characteristic (ROC) curves (Zweig & Campbell 1993) were used to calculate cut-off values for optimal sensitivity and specificity.

Results

DKK1 autoantibody detection in the sera of patients with lung cancer

To test our hypothesis that DKK1 excretion elicits the induction of serum autoantibodies, we employed a modified ELISA method to measure the levels of anti-IgG autoantibodies against DKK1 in the sera of normal individuals (n = 87) and lung cancer patients (n = 93). Recombinant DKK1 was expressed and purified as a MBP-fusion protein and used as an antigen in the ELISA assay (see Materials and methods). In Figure 1A, anti-DKK1 autoantibody titres are represented as arbitrary ratios to the mean absorbance of the normal control sera. The mean (F1 SD) level of DKK1 autoantibody detected in the sera of 93 lung cancer patients was 1.57 ± 0.53. In contrast, the mean (F1 SD) level of DKK1 autoantibody in 87 healthy individuals was 1 ± 0.42. The difference in the

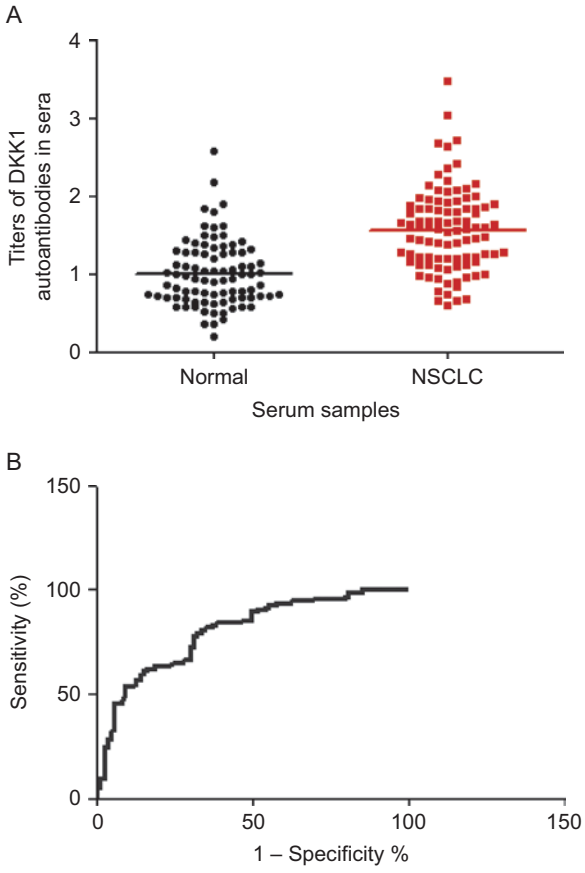

Figure 1. Serum titres of DKK1 autoantibody and receiver operating characteristic (ROC) plot of DKK1 autoantibody enzyme-linked immunosorbent assay (ELISA) assay in patients with cancer and controls. (A) Titres of DKK1 autoantibody in the sera. Autoantibody titres are represented as ratios to the mean absorbance of the normal control sera. Values >1.38 were considered positive. A control serum was included on all plates to monitor plate-to-plate variation. Variations never exceeded 5%, so values were not corrected. The differences between groups (patients versus controls) were calculated to have significance at p <0.05. (B) ROC plot of DKK1 autoantibody ELISA assay: the sensitivity and specificity of the DKK 1 autoantibody ELISA (from the data of Figure 1A) is presented in ROC curves. NSCLC, non-small cell lung cancer.

levels of DKK1 autoantibody measured in healthy individuals and NSCLC patients was significant (p <0.001, Mann–Whitney U test). Using ROC curves (shown in Figure 1B) drawn from the data of these 93 cancer patients and 87 healthy controls (Figure 1A), a cut-off level was set for this assay to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for DKK1 autoantibody (with a sensitivity of 62% (57 of 93) and a specificity of 84% (73 of 87)).

We also examined whether or not the DKK1 autoantibody levels correlate with cancer stage. However, no significant difference was found between the four stages of NSCLC. The diagnostic sensitivity of DKK1 autoantibody for stages I–IV was 64.3% (18 of 28), 70% (7 of 10), 57.4% (27 of 47) and 62.5% (5 of 8), respectively.

Immunological identification of DKK1 autoantibody

To confirm the presence of DKK1 autoantibody, we tested the immunological detection capability of DKK1 autoantibody present in the sera of patients with cancer (Figure 2). Sera selected from patients carrying high levels of DKK1 autoantibodies exhibited immuno-cross-reactivity toward the purified recombinant DKK1 protein (70 kDa; Figure 2, strips 6–9), whereas no such immuno-cross-reactivity for DKK1 protein was observed using normal sera (Figure 2, lanes 2–5). The detected bands (lanes 6–9) are of the same molecular weight as the positive control (lane 1).

Expression of DKK1 in NSCLC tissues and lung cancer cell lines

We used western blot analysis to investigate the expression of DKK1 protein in clinical samples and lung cancer cell lines. The results show that the 35 kDa DKK1 protein is expressed in five lung cancer cell lines, as detected by anti-DKK1 antibody (Figure 3A). The western blot analysis of clinical samples revealed that DKK1 can be expressed in tumour tissues, tumour-adjacent tissues, or both. No significant differences in DKK1 expression levels were found between tumour tissues and tumour-adjacent tissues (Figure 3B).

Levels of DKK1 in patients with lung cancer

To examine whether or not there is a correlation between the serum levels of DKK1 protein and its autoantibody, ELISA assays were done to detect DKK1 protein in serologic samples from 93 patients with NSCLC and 87 normal controls. As shown in Figure 4A, the average level of DKK1 protein was higher for the patient group than in the control group. The mean (F1 SD) serum level of DKK1 in 93 lung cancer patients was 3.23 ± 0.73. In contrast, the mean (F1 SD) serum level of DKK1 in 87 healthy individuals was 1 ± 0.54. This difference between healthy individuals and NSCLC patients is significant (p <0.0001, Mann–Whitney U test). Using ROC curves drawn from the data of these 93 cancer patients and 87 healthy controls (Figure 4B), the cut-off level for this assay was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for DKK1 (with a sensitivity of 75.3% (70 of 93) and a specificity of 86.2% (75 of 87)).

Interestingly, when comparing the results of DKK1 and its autoantibody, we found only 58.6% (41 of 70) of the patients with high levels of DKK1 protein expression

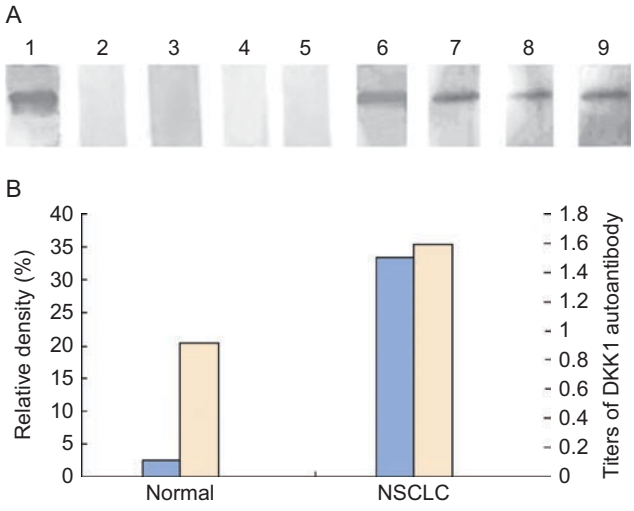

Figure 2. Western blot analysis of DKK1 autoantibody in sera from lung cancer patients and controls. (A) Lane 1 was blotted with DKK1 polyclonal anti-DKK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); lanes 2–5 were blotted with normal control sera (100-fold dilution); lanes 6–9 were blotted with sera from patients with lung cancer (100-fold dilution). (B) The results of densitometric quantification of the Western blot and titres of DKK1 autoantibody in sera of enzyme-linked immunosorbent assay. NSCLC, non-small cell lung cancer.

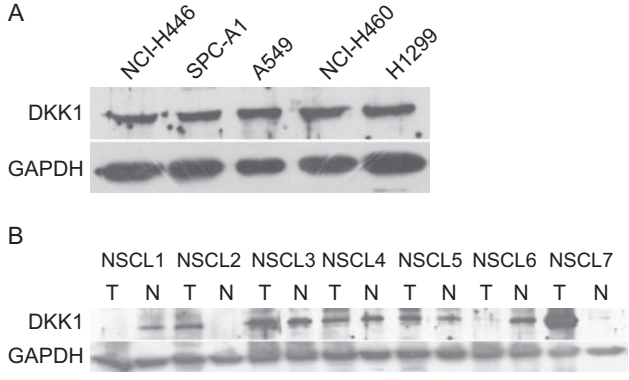

Figure 3. Expression of DKK1 in lung cancer cell lines and tumour tissues. (A) Expression of DKK1 in five lung cancer cell lines detected by western blot. Lanes 1–5 represent NCI-H446, SPC-A1, A549, NCI-H460 and NCI-H1299, respectively. (B) Expression of DKK1 in representative pairs of seven non-small cell lung cancer (NSCLC) samples detected by western blot. T and N denote tumour tissue and tumor-adjacent tissues, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a loading control.

have high levels of DKK1 autoantibodies. The discrepancy between the level of DKK1 protein and its autoantibody led us to try both ELISA assays in combination to distinguish the patient group and the control group. When the same ROC cut-offs were used in combination, the sensitivity increased to 81.7% and the specificity remained 86.2%.

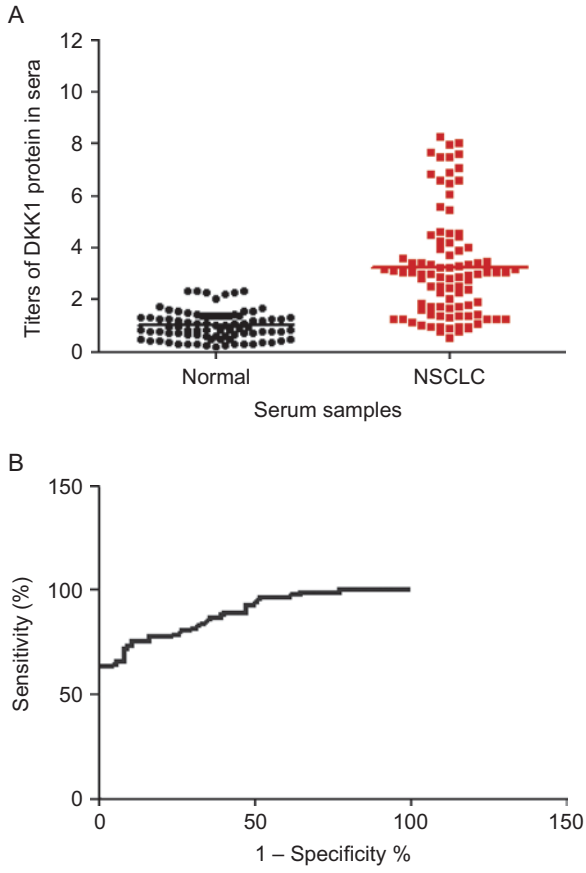

Figure 4. Serum concentration of DKK1 and the receiver operating characteristic (ROC) plot of DKK1 enzyme-linked immunosorbent assay (ELISA) in patients with non-small cell lung cancer (NSCLC) and control sera. (A) Serum concentration of DKK1 expressed as ratios to the mean absorbance of the normal control sera. Values >1.55 were considered positive. (B) ROC plot of DKK1 ELISA assay. The sensitivity and specificity of the DKK1 ELISA is presented in ROC curves.

Discussion

In spite of improved modern surgical techniques and adjuvant chemoradiotherapy, lung cancer still has one of the worst prognoses among all malignant tumours. Therefore, the development of novel diagnostic biomarkers for early detection of cancer and for the better choice of adjuvant treatment modalities to individual patients is urgently required.

The prolonged time course of carcinogenesis (Wang et al. 2005) opens the possibility for a very small tumour or a subtle biochemical change in a cell to produce a detectable level of antistimulant autoantibody in response to chemical or viral carcinogens. These autoantibody markers could be detectable well before the released tumour antigen reaches a detectable level.

Recent studies demonstrated that serum levels of DKK1 are elevated in patients with multiple myeloma (Politou et al. 2006), lung cancer and esophageal

squamous cell carcinomas (Yamabuki et al. 2007). Therefore, in this study, we examined whether or not there are DKK1 autoantibodies in the sera of patients with lung cancer. In this study, we first detected the presence of DKK1 autoantibody in NSCLC cancer patients and determined that the levels of DKK 1 autoantibody in the sera of NSCLC patients were higher than normal controls. Although the clinical value of DKK1 autoantibody remains to be elucidated, our results suggest that the detection of circulating DKK1 autoantibody could serve as a useful non-invasive marker for determining lung cancer status. Using DKK1 protein as an antigen to monitor the development of DKK1 autoantibody during oncogenesis may be advantageous for screening healthy individuals or patients with lung cancer. Importantly, higher DKK1 autoantibody levels were found in 64.3% of patients with early stages of NSCLC (stage I). Although further validation using a larger sample set covering various clinical stages will be required, an assay based on the DKK1 autoantibodies in the sera of cancer patients, as described here, may be of great importance for early diagnosis of NSCLC.

It has been reported that DKK1 is widely overexpressed in lung cancer, esophageal cancer, Wilms' tumour, hepatoblastoma, HCC and multiple myeloma (Patil et al. 2005, Qian et al. 2007, Wirths et al. 2003, Yamabuki et al. 2007). Exogenous expression of DKK1 enhances the cellular migration/invasive activity of mammalian cells. However, in the present study, we did not find that the expression of DKK1 is higher in lung tumour tissues than in tumour-adjacent tissues. The difference between our results and other studies may be ascribed to different sources of tissue samples.

In the present study, the levels of DKK1 protein in the sera of patients with NSCLC were also examined. DKK1 levels were also found to be higher in cancer patients. Unexpectedly, we found that only 58.6% (41 of 70) of the patients with higher levels of DKK1 protein expression have higher levels of DKK1 autoantibodies. Screening for a combination of DKK1 and its autoantibody increases the diagnostic sensitivity to 81.7%, while the specificity remains 86.2 %.

In a previous study, ECPKA autoantibody was proposed as a universal serum biomarker for cancers of various cell types (Nesterova et al. 2006). Therefore, we also examined ECPKA autoantibody in the serum samples used in this study. The average level of ECPKA autoantibody was also found to be higher in NSCLC patients than in controls. However, as shown in supplementary Figure S3 (see online version of this article), the diagnostic value of ECPKA autoantibody for NSCLC (with a sensitivity of 32.3 % (20 of 93) and a specificity of 84% (73 of 87)) is lower than that of DKK1 autoantibody. The discrepancy between our results and those of Nesterova et al. (90% sensitivity and 88% specificity) may be due to the small sample size ($n = 6$) used in their study (Nesterova et al. 2006). Our data suggest that for the diagnosis of NSCLC, DKK1 autoantibody is a better marker than ECPKA autoantibody.

One of the advantages of autoantibody biomarkers over antigen markers is that several kinds of autoantibodies can be easily combined together into a minichip to enhance the sensitivity and specificity of diagnosis. For example, a 22-phage-peptide autoantibody minichip has 88.2% specificity (95% CI 0.78–0.95) and 81.6% sensitivity (95%CI 0.70–0.90) in discriminating prostate cancer patients and control groups (Wang et al. 2005). From a list of cancer-associated antibodies that numbers in the thousands, fewer than 50 total antibodies have been described for NSCLC. However, antibodies to several NSCLC proteins have been evaluated as independent markers of disease, including p53, annexin I, annexin II, NY-ESO-1, L-myc, c-myc, and 90K/Mac-2 binding protein (M2BP); antibodies to each are found in 15–30%, 30%, 33%, 8%, 10%, 13% and 30% of NSCLC patients, respectively (Brichory et al. 2001a, b, Cioffi et al. 2001, Jager et al. 1999, Lubin et al. 1995, Ozaki et al. 2002, Yamamoto et al. 1996, 1999). Therefore, DKK1 autoantibody may be combined with other autoantibody markers to enhance the sensitivity and specificity of diagnosis of NSCLC.

In addition to its potential diagnostic utility, DKK1 autoantibody may also make a contribution to the oncogenesis of NSCLC. In a recent study (Gonzalez-Gronow et al. 2006), antibodies against glucose-regulated protein of 78 kDa (GRP78) isolated from the serum of a prostate cancer patient recognized a similar epitope on GRP78. Interestingly, the autoantibody GRP78 was found to have proproliferative effects on several kinds of prostate cancer cell lines. Therefore, autoantibodies against DKK1 may have some effect on the oncogenesis of NSCLC. Further studies will need to be performed to elucidate the epitope of DKK1 recognized by its autoantibodies and the role of the autoantibodies in cancer progression.

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***Declaration of interest*:** The authors declare that there are no conflicts of interest.

References

Ben-Mahrez K, Sorokine I, Thierry D, Kawasumi T, Ishii S, Salmon R, Kohiyama M. (1990). Circulating antibodies against c-myc oncogene product in sera of colorectal cancer patients. *Int J Cancer* 46:35–8.

Brichory F, Beer D, Le Naour F, Giordano T, Hanash S. (2001a). Proteomics-based identification of protein gene product 9.5 as a tumor antigen that induces a humoral immune response in lung cancer. *Cancer Res* 61:7908–12.

Brichory FM, Misek DE, Yim AM, Krause MC, Giordano TJ, Beer DG, Hanash SM. (2001b). An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc Natl Acad Sci U S A* 98:9824–9.

Cioffi M, Vietri MT, Gazzerro P, Magnetta R, D'Auria A, Durante A, Nola E, Puca GA, Molinari AM. (2001). Serum anti-p53 antibodies in lung cancer: comparison with established tumor markers. *Lung Cancer* 33:163–9.

Crawford LV, Pim DC, Bulbrook RD. (1982). Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *Int J Cancer* 30:403–8.

Dobashi K, Sugio K, Osaki T, Oka T, Yasumoto K. (1997). Micrometastatic P53-positive cells in the lymph nodes of non-small-cell lung cancer: prognostic significance. *J Thorac Cardiovasc Surg* 114:339–46.

Fukuyama Y, Mitsudomi T, Sugio K, Ishida T, Akazawa K, Sugimachi K. (1997). K-ras and p53 mutations are an independent unfavourable prognostic indicator in patients with non-small-cell lung cancer. *Br J Cancer* 75:1125–30.

Gonzalez-Gronow M, Cuchacovich M, Llanos C, Urzua C, Gawdi G, Pizzo SV. (2006). Prostate cancer cell proliferation in vitro is modulated by antibodies against glucose-regulated protein 78 isolated from patient serum. *Cancer Res* 66:11424–31.

Gonzalez-Sancho JM, Aguilera O, Garcia JM, Pendas-Franco N, Pena C, Cal S, Garcia de Herreros A, Bonilla F, Munoz A. (2005). The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer. *Oncogene* 24:1098–103.

Jager E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jager D, Arand M, Ritter G, Old LJ, Knuth A. (1999). Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 84:506–10.

Lubin R, Schlichtholz B, Bengoufa D, Zalcman G, Tredaniel J, Hirsch A, de Fromentel CC, Preudhomme C, Fenaux P, Fournier G, Mangin P, Laurent-Puig P, Pelletier G, Schlumberger M, Desgrandchamps F, Le Duc A, Peyrat JP, Janin N, Bressac B, Soussi T et al. (1993). Analysis of p53 antibodies in patients with various cancers define B-cell epitopes of human p53: distribution on primary structure and exposure on protein surface. *Cancer Res* 53:5872–6.

Lubin R, Zalcman G, Bouchet L, Tredanel J, Legros Y, Cazals D, Hirsch A, Soussi T. (1995). Serum p53 antibodies as early markers of lung cancer. *Nat Med* 1:701–2.

Martini N, Bains MS, Burt ME, Zakowski MF, McCormack P, Rusch VW, Ginsberg RJ. (1995). Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg* 109:120–129.

Mountain CF (1997). Revisions in the International System for Staging Lung Cancer. *Chest* 111:1710–17.

Nesterova MV, Johnson N, Cheadle C, Bates SE, Mani S, Stratakis CA, Khan IU, Gupta RK, Cho-Chung YS. (2006). Autoantibody cancer biomarker: extracellular protein kinase A. *Cancer Res* 66:8971–4.

Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, Sugano S, Akiyama T. (2004). DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. *Oncogene* 23:8520–6.

Osaki T, Mitsudomi T, Oyama T, Nakanishi R, Yasumoto K. (1995). Serum level and tissue expression of c-erbB-2 protein in lung adenocarcinoma. *Chest* 108:157–62.

Oyama T, Osaki T, Mitsudomi T, Ogawa R, Nakanishi R, Sugio K, Yasumoto K. (1998). p53 alteration, proliferating cell nuclear antigen, and nucleolar organizer regions in thymic epithelial tumors. *Int J Mol Med* 1:823–6.

Ozaki Y, Kontani K, Hanaoka J, Chano T, Teramoto K, Tezuka N, Sawai S, Fujino S, Yoshiki T, Okabe H, Ohkubo I. (2002). Expression and immunogenicity of a tumor-associated antigen, 90K/Mac-2 binding protein, in lung carcinoma. *Cancer* 95:1954–62.

Parkin DM, Bray FI, Devesa SS. (2001). Cancer burden in the year 2000. The global picture. *Eur J Cancer* 37 (Suppl. 8):S4–66.

Patil MA, Chua MS, Pan KH, Lin R, Lih CJ, Cheung ST, Ho C, Li R, Fan ST, Cohen SN, Chen X, So S. (2005). An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. *Oncogene* 24:3737–47.

Politou MC, Heath DJ, Rahemtulla A, Szydlo R, Anagnostopoulos A, Dimopoulos MA, Croucher PI, Terpos E. (2006). Serum concentrations of Dickkopf-1 protein are increased in patients with multiple myeloma and reduced after autologous stem cell transplantation. *Int J Cancer* 119:1728–31.

Qian J, Xie J, Hong S, Yang J, Zhang L, Han X, Wang M, Zhan F, Shaughnessy JD, Jr., Epstein J, Kwak LW, Yi Q. (2007). Dickkopf-1 (DKK1) is a widely expressed and potent tumor-associated antigen in multiple myeloma. *Blood* 110:1587–94.

Strauss GM, Kwiatkowski DJ, Harpole DH, Lynch TJ, Skarin AT, Sugarbaker DJ. (1995). Molecular and pathologic markers in stage I non-small-cell carcinoma of the lung. *J Clin Oncol* 13:1265–79.

Sugio K, Inoue T, Inoue K, Yaita H, Inuzuka S, Ishida T, Sugimachi K. (1993). Different site mutation of the K-ras gene in a patient with metachronous double lung cancers. *Anticancer Res* 13:2469–71.

Sugio K, Ishida T, Yokoyama H, Inoue T, Sugimachi K, Sasazuki T. (1992). Ras gene mutations as a prognostic marker in adenocarcinoma of the human lung without lymph node metastasis. *Cancer Res* 52:2903–6.

Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, Chinnaiyan AM. (2005). Autoantibody signatures in prostate cancer. *N Engl J Med* 353:1224–35.

Winter SF, Minna JD, Johnson BE, Takahashi T, Gazdar AF, Carbone DP. (1992). Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res* 52:4168–74.

Wirths O, Waha A, Weggen S, Schirmacher P, Kuhne T, Goodyer CG, Albrecht S, Von Schweinitz D, Pietsch T. (2003). Overexpression of human Dickkopf-1, an antagonist of wingless/WNT signaling, in human hepatoblastomas and Wilms' tumors. *Lab Invest* 83:429–34.

Yamabuki T, Takano A, Hayama S, Ishikawa N, Kato T, Miyamoto M, Ito T, Ito H, Miyagi Y, Nakayama H, Fujita M, Hosokawa M, Tsuchiya E, Kohno N, Kondo S, Nakamura Y, Daigo Y. (2007). Dikkopf-1 as a novel serologic and prognostic biomarker for lung and esophageal carcinomas. *Cancer Res* 67:2517–25.

Yamamoto A, Shimizu E, Ogura T, Sone S. (1996). Detection of autoantibodies against L-myc oncogene products in sera from lung cancer patients. *Int J Cancer* 69:283–9.

Yamamoto A, Shimizu E, Takeuchi E, Houchi H, Doi H, Bando H, Ogura T, Sone S. (1999). Infrequent presence of anti-c-Myc antibodies and absence of c-Myc oncoprotein in sera from lung cancer patients. *Oncology* 56:129–33.

Zweig MH, Campbell G. 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 39:561–77.