FEBS Open Bio 5 (2015) 809–812

journal homepage: www.elsevier.com/locate/febsopenbio

Study of circulating IgG antibodies to BIRC5 and MYC in non-small cell lung cancer

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ARTICLE INFO

Article history:
Received 9 August 2015
Revised 11 September 2015
Accepted 18 September 2015

Keywords:
Lung cancer
BIRC5
MYC
Autoantibodies
ELISA
Tumor immunity

ABSTRACT

An in-house enzyme-linked immunosorbent assay (ELISA) was developed in this study to detect circulating IgG antibodies to peptide antigens derived from baculoviral IAP repeat-containing protein 5 isoform 2 (BIRC5) and myc proto-oncogene protein (MYC) in non-small cell lung cancer (NSCLC). Student's *t*-test revealed that circulating anti-MYC IgG levels were significantly increased in patients with NSCLC compared with control subjects in the discovery sample ($t = 3.96$, $P = 0.0001$) but not in the validation sample ($t = 1.24$, $P = 0.217$), generating a combined *P*-value of 0.0003. Neither the discovery sample nor the validation sample showed a significant change in anti-BIRC5 IgG levels in NSCLC. Further analysis was performed to investigate whether circulating IgG antibodies to these two tumor-associated antigens (TAAs) significantly changed with early (stages I + II) and late (stages III + IV) NSCLC stages. The results showed that neither anti-MYC IgG nor anti-BIRC5 IgG levels significantly changed in patients with early stage NSCLC, while patients with late stage NSCLC had higher levels of circulating anti-MYC IgG than control subjects in the discovery sample ($t = 4.74$, $P < 0.0001$) but not in the validation sample ($t = 0.80$, $P = 0.423$), generating a combined *P*-value of 0.00003 ($X^2 = 26.13$, $df = 4$). In conclusion, circulating IgG antibodies to MYC and BIRC5 do not appear to serve as biomarkers for early diagnosis of lung cancer but anti-MYC IgG might have a prognostic value.

1. Introduction

Lung cancer is the most frequently diagnosed malignant tumor and the leading cause of cancer-related deaths for both men and women. An estimated 1.8 million new cases with lung cancer occurred in 2012, accounting for about 13% of total cancer diagnoses worldwide [6,20]. In China, the incidence of lung cancer is approximately 19% of all cancers diagnosed in recent years [2]. Of all lung cancer cases, more than 80% are likely to suffer from non-small cell lung cancer (NSCLC), including squamous cell cancer, adenocarcinoma and large cell cancer. Patients with early stage lung cancer may be curable so that there is an urgent need to develop early diagnosis tool for screening of the individuals at a high risk.

It has been suggested that circulating autoantibodies to tumor-associated antigens (TAAs) can serve as potential biomarkers for early diagnosis of malignant tumors [15,19,9,5,12,13]. A successful test has been developed for early diagnosis of lung cancer although the improvement of its sensitivity and specificity remains needed [10,1,7]. Identification of more TAAs will be the solution to enhance the panel positivity in early stage lung cancer. Increased levels of circulating antibodies to survivin, also called baculoviral IAP repeat-containing protein 5 (BIRC5), and to myc proto-oncogene protein (MYC) have been reported in lung cancer [14,8]. A recent study tested the levels circulating IgG against BIRC5 and MYC in breast cancer and revealed that the levels of these two autoantibodies were significantly higher in patients with early stage breast cancer than control subjects [22]. The present study was then designed to detect circulating IgG antibodies to BIRC5 and MYC among patients with non-small cell lung cancer (NSCLC) and control subjects in a Chinese population.

Abbreviations: cAg, control antigen; ELISA, enzyme-linked immunosorbent assay; hAgs, human antigens; NC, negative control; NSCLC, non-small cell lung cancer; OD, optical density; QC, quality control; SBI, specific binding index; TAAs, tumor-associated antigens

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http://dx.doi.org/10.1016/j.fob.2015.09.007
2211-5463/

2. Methods

2.1. Subjects

A total of 109 patients who were newly diagnosed as having NSCLC were recruited by the Fourth Affiliated Hospital of China Medical University, Shenyang, China, in the period between March 2013 and November 2014. Of these 109 patients aged 62.1 ± 10.4 years, 70 were male and 39 were female; they were divided into the discovery samples ($n = 49$) that were collected during 2013 and the validation samples ($n = 60$) that were collected during 2014. Their diagnosis and tumor staging were made based on radiographic examination and histological confirmation; inclusion of patients was restricted to those with adenocarcinoma and squamous carcinoma only. Based on NSCLC staging information, these patients were divided into two subgroups, the early stage group (stages I + II) and the late stage group (stages III + IV). Blood samples were taken prior to any anticancer treatment. A total of 216 healthy subjects, well matched in age (59.1 ± 3.5 years) and smoking history, were also recruited from local communities, 108 of whom were used as controls for the discovery samples and 108 for the validation samples. Clinical interview and radiographic examination were applied to rule out the control subjects who had history of lung cancer or any other malignant tumors. All the subjects were of Chinese Han origin and they all gave informed written consent to participate in this study as approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University, and conformed to the requirements of the Declaration of Helsinki.

2.2. Antibody testing

An enzyme-linked immunosorbent assay (ELISA) was developed in-house using linear peptide antigens derived from human BIRC5 and MYC proteins as described in a previous study [22]; a peptide fragment derived from a maize protein (NCBI: 1BFA_A) was used as the control antigen (cAg). The sequence information of these three peptides is given in Table 1. Briefly, both hAgs and cAg were synthesized by solid-phase chemistry with purity of >95%, and then applied to develop a relative ELISA test for detection of circulating IgG antibodies to ANXA1 and FOXP3. Synthetic peptides were dissolved in 67% acetic acid to obtain a concentration of 5 mg/ml (stock solution kept at −20 °C), and were diluted with phosphate-buffered saline (PBS)-based coating buffer (P4417, Sigma–Aldrich) containing 0.1% sodium azide. Coaster 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, USA) were half-coated in 0.1 ml/well of each hAg and half-coated in 0.1 ml/well of cAg. The antigen-coated 96-well microplate was incubated overnight at 4 °C. After the plate was washed 3 times with wash buffer made from Tris-buffered saline with Tween®20 (T9039, Sigma–Aldrich), 100 µl plasma sample diluted 1:150 in assay buffer (PBS containing 1.5% BSA) was added and 100 µl assay buffer was also added to the negative control (NC) wells. Following 2 h incubation at room temperature, the plate was washed 3 times and 100 µl peroxidase-conjugated goat antibody to human IgG (A8667, Sigma–Aldrich) diluted 1:30,000 in assay buffer was added to each well. After incubation at room temperature for an hour, color development was initiated by adding 100 µl Stabilized Chromogen (SB02, Life Technologies) and terminated 25 min later by adding 50 µl Stop Solution (SS04, Life Technologies). The measurement of the optical density (OD) was completed within 10 min at 450 nm with a reference wavelength of 620 nm. To reduce the interference from a non-specific signal produced by passive absorption of various IgG antibodies in plasma to the surface of 96-well microplate, a specific binding index (SBI) was used to express the levels of circulating antibodies to BIRC5 and MYC. Each sample was tested in duplicate and SBI was calculated as follows:

$$\mathrm{SBI} = (\mathrm{OD}_{\mathrm{hAg}} - \mathrm{OD}_{\mathrm{NC}})/(\mathrm{OD}_{\mathrm{control\ antigen}} - \mathrm{OD}_{\mathrm{NC}}).$$

2.3. Data analysis

Antibody-testing data were expressed in mean ± standard deviation (SD) in SBI. Student's *t*-test was applied to examine the difference in SBI between the patient group and the control group, and the Fisher's method of combining *P*-value as introduced by Elston [4] was applied to work out combined *P*-values based on 2 *t*-tests performed on the discovery samples and the validation samples and on the early stage group and the late stage group.

To minimize intra-assay deviation, the ratio of the difference between duplicated OD values to their sum was used to assess the precision for assay of each sample. If the ratio was found to be >15%, the test of this sample was treated as being invalid and would not be used for data analysis. The inter-assay deviation was estimated using a pooled plasma sample, namely quality control (QC) sample, which was randomly collected from >100 unrelated healthy subjects.

3. Results

Student's *t*-test revealed that circulating anti-MYC IgG levels were significantly increased in patients with NSCLC compared with control subjects in the discovery sample ($t = 3.96$, $P = 0.0001$) but not in the validation sample ($t = 1.24$, $P = 0.217$), generating a combined *P*-value of 0.0003 ($X^2 = 21.19$, $df = 4$). Neither the discovery sample nor the validation sample showed a significant change in circulating anti-BIRC5 IgG levels in NSCLC (Table 2).

As shown in Table 3, neither anti-MYC IgG nor anti-BIRC5 IgG levels significantly changed in the patients with early stage NSCLC whereas patients with late stage NSCLC had higher levels of circulating anti-MYC IgG than control subjects in the discovery sample ($t = 4.74$, $P < 0.0001$) but not in the validation sample ($t = 0.80$, $P = 0.423$), generating a combined *P*-value of 0.00003 ($X^2 = 26.13$, $df = 4$). Neither the discovery sample nor the validation sample showed a significant change in circulating anti-BIRC5 IgG levels in late stage NSCLC (Table 4).

4. Discussion

This study suggests that circulating anti-MYC IgG instead of anti-BIRC5 IgG levels were significantly increased in NSCLC (Table 2), but the altered anti-MYC IgG levels were observed only in patients with late stage NSCLC (Table 4). Whether circulating

Table 1
Information of peptide antigens used for development of ELISA antibody test.

Antigen	Sequence (N → C)	NCBI accession	Working solution (µg/ml)
BIRC5	H-dflkdhristfknwlhhfqglfpgatslpv-OH	NP_001012270	10
MYC	H-rvkldsvrvlrqisnnrkcfellptpplsps-OH	NP_002458	10
Control	H-haqlegrlhdlpgcprevqrgfaatlvtn-OH	1BFA_A	10