Original Article
Circulating IgG antibody to ABCC3 in gynecological cancers

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Received November 29, 2016; Accepted November 30, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Recent work revealed an increase in circulating anti-ABCC3 antibodies in patients with lung cancer and esophageal cancer. To confirm whether the alteration of anti-ABCC3 antibodies occurs in other types of malignancies, the present work was undertaken to test circulating anti-ABCC3 antibodies in patients with gynecological cancers. An in-house enzyme-linked immunosorbent assay (ELISA) was applied to detect plasma anti-ABCC3 IgG among 148 patients with breast cancer (BC), 97 patients with cervical cancer (CC) and 109 control subjects. One-way analysis of variance (ANOVA) showed significant differences in plasma anti-ABCC3 IgG levels among BC patients, CC patients and control subjects (F=7.75, df=2, 351, P=0.0005). Binary logistic regression showed that anti-ABCC3 IgG levels were significantly lower in BC patients than CC patients (adjusted P=0.009) and control subjects (adjusted P=0.0003) but there was no significant difference in anti-ABCC3 IgG levels between CC patients and control subjects (adjusted P=0.286). Moreover, patients with ductal carcinoma but not those with lobular carcinoma had a decreased anti-ABCC3 IgG level compared with control subjects (adjusted P=0.0006); the levels of circulating anti-ABCC3 IgG appeared to decrease with stages of breast cancer. In conclusion, circulating anti-ABCC3 IgG may be a specific biomarker for prognosis of breast cancer although more tests are needed to confirm this initial finding.

Keywords: ABCC3 transporter, autoantibodies, breast cancer, cervical cancer, ELISA, tumor immunity

Introduction
Resistance of tumor cells to chemotherapy has been reported in a large body of publications and different tumor cells may display simultaneous resistance to multiple structurally unrelated antitumor agents, so-called multidrug resistance (MDR). Convincing evidence suggests that the development of MDR is likely to result from the overexpression of ATP-binding cassette (ABC) transporters [1-3]. There are 49 distinct genes identified to encode ABC transporters in humans. These ABC transporters are classified into 7 families (A to G), of which the roles of ABCB1, ABCC1-3 and ABCG2 in developing MDR have been frequently reported in study of therapeutic failure for many types of malignant tumors [1, 2]. These transporters function as an energy-driven pump to maintain intracellular drug concentrations below a toxic level. As a result, the tumor cells survive and chemotherapy becomes ineffective. It has been noted that the overexpression of some ABC transporters by tumors may trigger the secretion of anti-ABC antibodies based on the work on tumor-associated antigens (TAAs) [4]. Recent studies demonstrated that the levels of circulating antibodies to ABCB3 transporter were found to be significantly higher in patients with esophageal cancer and lung cancer than control subjects [5, 6]. To confirm whether circulating antibodies to ABCC3 may be a novel biomarker specific for other types of malignancies, the present work was undertaken to test circulating IgG antibodies to ABCC3 transporter in gynecological cancers, including breast cancer (BC) and cervical cancer (CC).

Subjects and methods

Subjects
A total of 148 female patients aged 50.2±9.1 years, who were newly diagnosed as having
Clinical interview and radiographic examination were applied to rule out the control subjects who had history of breast cancer, cervical cancer or any other malignant tumors; the Pap smear was applied to rule out the control subjects who had suffered from cervical cancer. All the subjects were of Chinese Han origin and all gave written informed consent to participate in this study. This work was approved by the Ethics Committee of Second Hospital of Jilin University and performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Autoantibody testing

Enzyme-linked immune-sorbent assay (ELISA) was developed in-house using two linear peptide antigens as described in previous studies [5-9]. In brief, these 2 linear peptide antigens were synthesized by a solid-phase chemical method. The synthetic peptides were dissolved in 67% acetic acid to obtain a concentration of 5 mg/ml as stock solution; the working solution was made by diluting the stock solution with phosphate-buffered saline (PBS)-based coating buffer (P4417, Sigma-Aldrich) to 10 µg/ml for the ABCC3 antigen and to 20 µg/ml for the control antigen. Costar 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, USA) were half-coated in 0.1 ml/well of the ABCC3 antigen and half-coated in 0.1 ml/well of the control antigen. The antigen-coated 96-well plate was incubated at 4°C overnight. 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 hour incubation at room temperature, 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 hour incubation at room temperature, the plate was washed 3 times and 100 µl peroxidase-conjugated goat antibody to human IgG (A8667, Sigma-Aldrich) diluted 1:30000 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 hour incubation at room temperature, the plate was washed 3 times and 100 µl peroxidase-conjugated goat antibody to human IgG (A8667, Sigma-Aldrich) diluted 1:30000 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 minutes later by adding 50 µl Stop Solution (SS04, Life Technologies). The measurement of the optical density (OD) was completed within 10 minutes at 450 nm with a reference wavelength of 620 nm. To reduce the interference from a non-spe-
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 anti-ABCC3 IgG antibodies. Each sample was tested in duplicate and SBI was calculated as follows:

$$\text{SBI} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{NC}})}{(\text{OD}_{\text{control}} - \text{OD}_{\text{NC}})}$$

To minimize an intra-assay deviation, the ratio of the difference between duplicate sample OD values to their sum was used to assess the assay accuracy. If the ratio was >10%, 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 pooled plasma samples, namely quality control (QC) sample, which were randomly collected from >100 healthy subjects and tested on every 96-well plate.

**Data analysis**

All the data were expressed as mean ± standard deviation (SD) in SBI. One-way analysis of variance (ANOVA) was applied to examine the differences in circulating IgG levels between patients with breast cancer, those with cervical cancer and control subjects. Binary logistic regression was applied to examine the differences in SBI between different groups that were classified based on health conditions, types of tumors and clinical stages, with adjustment for age.

**Results**

ANOVA showed significant differences in plasma anti-ABCC3 IgG levels among patients with breast cancer, those with cervical and control subjects \((F=7.75, \text{df}=2, 351, P=0.0005)\). As shown in Figure 1, binary logistic regression showed that plasma anti-ABCC3 IgG levels were significantly lower in BC patients than CC patients \((P=0.009)\) and control subjects \((P=0.0003)\) but there was no significant difference in SBI between CC patients and control subjects \((P=0.286)\).

As shown in Table 1, patients with ductal carcinoma were more likely to have a decreased level of anti-ABCC3 IgG as compared with control subjects \((P=0.006)\) but anti-ABCC3 IgG levels did not show a significant change in patients with lobular carcinoma \((P=0.094)\). Plasma anti-ABCC3 IgG levels appeared to be decreased with stages of breast cancer (Table 2), and patients at stage I did not show a significant low level of anti-ABCC3 IgG as compared with control subjects \((P=0.217)\), but a significant low level of this antibody was observed in those at stages II \((P=0.0017)\) and III \((P=0.0083)\). While patients at stage IV also had a lower anti-ABCC3 IgG level than control subjects (Table 2), this did not achieve a statistical significance due to a small sample size \((P=0.219)\).

Of 97 patients with cervical cancer, 84 suffered from SCC), neither patients with SCC nor those with other types of CC showed significant
changes in plasma anti-ABCC3 IgG levels as compared with control subjects (Table 3). As shown in Table 4, there was no significant change in plasma anti-ABCC3 IgG levels among patients at stage I (adjusted $P=0.579$) and those at stage II (adjusted $P=0.245$) as compared with control subjects.

**Discussion**

Breast cancer is the most common malignancy diagnosed in women worldwide [10]. Based on population-based cancer registration data collected by the National Central Cancer Registry in China, 248620 females were diagnosed as having breast cancer in 2011 [11]. Identification of powerful biomarkers for early diagnosis can help reduce morbidity of this malignancy. Despite several screening modalities have been applied for clinical diagnosis of breast cancer, there is still an urgent need to develop an alternative modality of screening for early diagnosis [12]. Circulating antibodies to TAAs are likely to serve as biomarkers for early diagnosis and prognostic evaluation as they can be detectable several years before identification of tumors by radiographic detection or incidence screening [13-16].

Cervical cancer is the third leading cause of cancer death in females worldwide [10]. While this type of cancer is radiosensitive, it is resistant to chemotherapy such as platinum anticancer drugs [17, 18]. The ABC transporter system may be involved in mediating the efflux of anticancer drugs from cervical cancer cells [17]. However, the present work failed to show a significant change of circulating anti-ABCC3 IgG levels in cervical cancer. Possibly, such antibody is not a specific biomarker for cervical cancer.

ABCC3 is one of the MDR-associated proteins encoded by ABC transporter genes and is highly expressed in several tumor types, including breast cancer [19-21]. A recent study revealed that 28.1% of HER2-positive tumor samples from patients with primary breast cancer overexpressed ABCC3 transporter compared with 7.3% of HER2-negative tumor samples [21], suggesting that ABCC3 may be a novel biomarker for breast cancer. With respect to circulating IgG antibodies to ABCC3, two previous studies made analyses in lung cancer [5] and esophageal cancer [6]; they showed that the levels of circulating antibodies to ABCC3 transporter were significantly higher in patients with these two types of thoracic cancer. However, data from our study showed that the individuals with breast cancer had a significant low level of anti-ABCC3 IgG antibody, especially those with ductal carcinoma (Table 2). These results suggest that circulating anti-ABCC3 IgG may be a useful biomarker for prognosis of breast cancer.

Theoretically, the overexpression of ABCC3 in primary breast tumors may result in elevated level of circulating anti-ABCC3 IgG antibody as reported in thoracic cancers. It raises a question of why circulating anti-ABCC3 IgG antibody is decreased in patients with breast cancer. One possible explanation is the antigen-specific immunologic high-dose tolerance. A previous report showed a decrease in immune response to TAAs as the malignancy became disseminate [22]. In addition, it has been reported that insufficiency in immune response is often correlated with poor prognosis [23]. This observation is consistent with our data showing that plasma anti-ABCC3 IgG levels were decreased with stages of breast cancer (Table 2). Moreover, it was considered that sex hormones may significantly affect the humoral immune responses to TAAs [24]. Therefore, we speculate that ABCC3-specific immunologic high-dose tolerance may occur in patients with breast cancer.

In conclusion, our study provided evidence that circulating anti-ABCC3 IgG may have prognostic values for breast cancer but not for cervical cancer, although further validation remains needed to confirm this initial finding.

**Acknowledgements**

We thank the patients and healthy volunteers for their support and participation. This work

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**Table 4. The effect of stages in levels of IgG autoantibodies to ABCC3 transporter in cervical cancer**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Patient (n)</th>
<th>Control (n)</th>
<th>Adjusted $R^2$</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.44±0.28 (38)</td>
<td>1.50±0.43 (109)</td>
<td>0.088</td>
<td>0.579</td>
</tr>
<tr>
<td>II</td>
<td>1.42±0.29 (45)</td>
<td>1.50±0.43 (109)</td>
<td>0.023</td>
<td>0.245</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.53±0.43 (14)</td>
<td>1.50±0.43 (109)</td>
<td>-0.013</td>
<td>0.814</td>
</tr>
</tbody>
</table>

The antibody levels are expressed as mean ± SD in SBI. *Adjusted $P$ for age.
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was supported by the Young Scientist Research Award funded by the Natural Science Foundation of Heilongjiang Province (Grant number QC2014C092) and by the Research Fund granted by the Health Bureau of Heilongjiang Province (Grant number 20130370). All the subjects gave written informed consent to participate in this study.

Disclosure of conflict of interest

None.

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