Elevated serum antibodies against insulin-like growth factor-binding protein-2 allow detecting early-stage cancers: evidences from glioma and colorectal carcinoma studies

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Background: Tumor-specific immunity of insulin-like growth factor-binding protein-2 (IGFBP-2) has been reported in several cancers. We aimed to assess the role of serum IGFBP-2 antibodies (IGFBP-2 Abs) in early cancer detection.

Patients and methods: Glioma and colorectal carcinoma (CRC) were used as models. Serum IGFBP-2 and IGFBP-2 Abs were measured in 260 tumor patients (145 gliomas, 45 colorectal polyps, and 70 CRCs) and 141 controls. Receiver operating characteristic curves were applied.

Results: Serum IGFBP-2 Ab levels were significantly elevated in tumors (mean: 82 ng/ml, median: 17 ng/ml, range: 0–1387 ng/ml) compared with controls (11, 0, 0–212 ng/ml) (P < 0.0001) and higher in early than advanced cancers opposite of serum IGFBP-2 levels. IGFBP-2 Abs effectively discriminated between controls and grade II and III gliomas [area under the curve (AUC): 0.821–0.864; 95% confidence interval (CI) = 0.762–0.936; P < 0.0001], and CRC I–II (AUC: 0.668; 95% CI = 0.566–0.770; P = 0.002) as well as indicative of advanced polyps at high risk of CRC (AUC: 0.72; 95% CI = 0.630–0.811; P < 0.0001). The sensitivity and specificity for diagnosing grade II–III gliomas reached 66%–84% and 81%. Combined serum IGFBP-2 and IGFBP-2 Abs augmented the discriminative power of all stage tumors (AUC: 0.823), gliomas (AUC: 0.800), and CRCs (AUC = 0.917).

Conclusion: Our results first demonstrate IGFBP-2 Abs for early cancer detection and in combination of serum IGFBP-2 for improved cancer diagnosis.

Key words: colorectal cancer, early cancer diagnosis, glioma, IGFBP-2, serum antibodies

introduction

Patients with early cancers are offered the greatest potential for a cure by a complete surgical resection. Due to the asymptomatic nature of the early disease and the lack of effective screening methods, most patients present with unresectable incurable cancer when in clinics. Imaging and endoscopy technologies may improve early detection, but the high cost and technical complexity limit their applications. A blood test, on the other hand, could be administered easily and cost-effective for screening and diagnosis.

The findings that patients with malignant tumors produce autoantibodies against tumor-associated antigens (TAAs) in their tumors suggest that such autoantibodies have cancer diagnostic values especially for early cancers because circulating TAA autoantibodies can report malignant transformation before standard clinical examination [1–4]. The best studied is p53 autoantibodies (p53Abs), which was found to predate cancer diagnosis in subjects at high risk for cancers such as heavy smokers and individuals with chronic obstructive pulmonary disease for lung cancer [5], patients with Barrett’s esophagus for esophageal cancer [6] and individuals with premalignant oral lesions for oral cancer [7]. Patients with liver cirrhosis who later progressed to hepatocellular carcinoma were found to develop anticellular autoantibodies [8]. Recently, autoantibodies to annexin I, 14-3-3 theta, and LAMR1 were found in prediagnostic lung cancer sera, which preceded onset of symptoms and diagnosis [9].
Insulin-like growth factor-binding protein-2 (IGFBP-2) is a secreted protein and recently reported to be a human tumor antigen, eliciting T-cell and B-cell immunity in patients with breast, ovarian, and colon cancers [10, 11]. IGFBP-2 is overexpressed in a majority (30% to >80%) of many malignant tumors, including glioma and colorectal, prostate, ovarian, breast, and adrenocortical cancers, and overexpressed IGFBP-2 is associated with increased tumor stages and grades [12–17]. It stimulates cancer cell migration and invasion and as a regulator of phosphatidylinositol 3-kinase (PI3K)/Akt/PTEN promotes tumor progression [18–21]. Circulating IGFBP-2 levels are often reported to be associated with relapse, metastasis, and prognosis in advanced cancers but found less satisfactory for diagnosing early cancers such as gliomas [22–25]. Considering the ability of TAA autoantibodies to report malignant transformation, circulating IGFBP-2 Abs may provide a potential approach for diagnosing early cancers in a broad population of patients.

For this purpose, we examined 401 sera from 141 controls and 260 patients with gliomas, colorectal polyps, or colorectal carcinomas (CRCs) and presented the first evidence for IGFBP-2 Abs as early diagnostic biomarkers and in combination of serum IGFBP-2 for cancer detection.

patients and methods

patients and samples
Blood samples (N = 401) were prospectively collected from consented individuals under an Institutional Ethics Committee-approved study from Beijing Tiantan Hospital for patients with gliomas (N = 145) [78 World Health Organization (WHO) grade II astrocytomas (As), 37 grade III anaplastic astrocytomas (AAs), and 30 grade IV glioblastomas (GBMs)] from 2008 to 2010 and from Beijing Shijitan Hospital for volunteer controls (N = 141) and patients with colorectal polyps (N = 45) (nine multiple hyperplastic polyps, seven nonadvanced and 29 advanced adenomas) and CRC (N = 70) (38 CRC stage I–II and 32 CRC stage III–IV) from 2009 to 2010 (supplemental Table S1, available at Annals of Oncology online). To avoid selection bias, the recruitment was essentially unimpeded by selection criteria, except for the requirement that either an individual be a healthy with no history of any cancer or newly diagnosed unimpeded by selection criteria, except for the requirement that either an individual be a healthy with no history of any cancer or newly diagnosed.

The blood was drawn before surgery or the colonoscopy and centrifuged at 250 g for 10 min, and the serum was then collected and stored as aliquots at −70°C until use. An adenoma was defined as advanced if it contained villous components, was large (≥1 cm), or had high-grade dysplasia based on the histological results of the colonoscopy.

Tumor tissues of 63 astrocytomas and 32 advanced polyps (23 advanced adenomas and nine multiple hyperplastic polyps) were obtained by surgery and the colonoscopy, respectively, and then formalin-fixed, paraffin-embedded for immunohistochemical analysis (IHC).

indirect ELISA for serum IgG antibodies against IGFBP-2
Serum IgG antibodies against IGFBP-2 were assessed by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, immunol 4HBX microtiter plates with extra-high binding surface (Dynex Technologies Inc., Chantilly, VA) were coated overnight with 50 μl of human recombinant IGFBP-2 protein (R&D Systems Inc., Minneapolis, MN) diluted in 50 mM carbonate buffer (Sigma-Aldrich Corp., St Louis, MO) to a concentration of 1.0 μg/ml or carbonate buffer alone in alternating columns. The last column of wells was added with 50 μl per well of serially diluted human IgG (25, 100, 250, 500, 1000 ng/ml) (Invitrogen Ltd., Paisley, UK) to generate a standard curve. The coefficient of variation among the assays using the human IgG samples was 0.0998–0.012 corresponding to 25–1000 ng/ml of the human IgG standards.

Plates were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 50 μl per well, for 2 h at room temperature, washed four times with 0.1% tween-20 in PBS, and then incubated with diluted (1/10) serum in 1% BSA/PBS, 50 μl/well, for 2 h at room temperature with gentle shaking. Plates were washed again and 50 μl per well of goat anti-human IgG-HRP (Invitrogen Ltd) diluted 1: 30 000 in 1% BSA was added and incubated for 1 h at room temperature. Following four washes, plates were developed with 100 μl 3’,5’,5’-tetramethyl benzidine (Invitrogen Ltd) and read at 640 nm. Reaction was stopped by adding 100 μl 1N hydrochloric acid (HCl) when the 250 ng/ml standard reached approximately an OD of 0.35. Plates were then read at 450 nm. The OD value of each serum was calculated as the OD of the recombinant human IGFBP-2-coated wells minus the OD of the carbonate buffer-coated wells. The concentration of serum IGFBP-2 Abs was calculated based on the standard curve. Every serum was duplicated in each assay and repeated twice.

Soluble IGFBP-2 and IGFBP-2 Abs could form complexes in sera, which might affect the measurement of IGFBP-2 Abs, although it could happen less likely because antibodies form much more stable complexes with immobilized antigens than with soluble antigens especially after sera are diluted [26]. To exclude the possibility, we carried out a pilot study by using a series of diluted sera (1/10, 1/50, 1/100, 1/200) from a polyp patient whose serum was detected positive for IGFBP-2 Abs by indirect ELISA assay. We found a linear negative relation between dilutions and OD values (supplemental Figure S1, available at Annals of Oncology online), indicating no effect of the presence of IGFBP-2 in sera on the measurement of IGFBP-2 Abs levels in the setting. Based on the dilution assay results, the analytical sensitivity of the method measuring IGFBP-2 Abs reached <2 ng/ml.

direct ELISA for serum IGFBP-2
Serum IGFBP-2 was measured with commercially available IGFBP-2 ELISA plates coated with anti-IGFBP-2 polyclonal antibodies (RapidBio Laboratory, Calabasas, CA) by following the manufacturer’s instructions as we previously described [22]. The concentrations of the IGFBP-2 standards for building a standard curve were 0, 2.5, 10, 25, 50, and 100 ng/ml. The analytical sensitivity of the method should reach 2.5 ng/ml. Each serum was diluted by 1/40 and assayed in duplicate and all assays were repeated twice. An averaged concentration of IGFBP-2 was used for statistical analysis.

IHC analysis
IGFBP-2 immunostaining was carried out as we previously described [22]. The sections of tumor tissues were immunostained with an IGFBP-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Five most heavily stained fields were chosen to determine the percentage of positive cells. Zero represents no positive, + represents <30%, + + represents 31%–60%, and ++ + represents >60% positive staining cells.

statistical analysis
Statistical analyses were carried out using SPSS 13.0. Demographic data were analyzed with chi-square test. The differences of IGFBP-2 Ab and serum IGFBP-2 levels between controls and patients were assessed using a standard nonparametric Mann–Whitney U test. The relationship between
IGFBP-2 Ab levels and clinical information (age and gender) were evaluated by univariate linear regression analysis. Receiver operating characteristic (ROC) curves were constructed to determine the discriminatory capacity of IGFBP-2 Abs for diagnosis, and the area under the curve (AUC) was analyzed by chi-square test. The multi-sensor neural network analysis followed by ROC curve analysis was used to evaluate the discriminatory capacity of combined IGFBP-2 Abs and IGFBP-2 for diagnosis. Correlations of serum IGFBP-2 Ab levels with tumor tissue IGFBP-2 expression levels were assessed by Pearson correlation analysis. All P values were two-tailed with 0.05 specified as statistical significance.

results

elevated serum IGFBP-2 Ab levels in tumors and higher in early than advanced cancers

The study population was summarized in supplemental Table S1, available at *Annals of Oncology* online. Because of the recruitment strategy, some differences in gender and age were observed in the subject groups. However, the regression analyses of effects of gender and age on IGFBP-2 Ab levels showed that the serum IGFBP-2 Ab levels did not vary significantly with gender and age in the groups (supplemental Table S1, available at *Annals of Oncology* online), suggesting no significant roles of gender and age in antibody response to IGFBP-2.

We found that serum IGFBP-2 Ab levels were significantly elevated in tumor patients (mean: 82 ng/ml, median: 17 ng/ml, range: 0–1387 ng/ml) compared with controls (11, 0, 0–212 ng/ml) (P < 0.0001, Figure 1A). Patients with gliomas (mean: 128 ng/ml, median: 24 ng/ml, range: 0–1359 ng/ml, P < 0.0001), colorectal polyps (32, 14, 0–212 ng/ml, P < 0.0001), or CRCs (22, 1.0, 0–161 ng/ml, P = 0.001) had significantly increased IGFBP-2 Abs compared with controls (Figure 1B). Serum IGFBP-2 Ab levels were obviously higher in glioma patients than polyp or CRC patients.

When stratifying the analysis by WHO 2007 classification of central nervous system tumors and American Joint Committee on Cancer stage for CRC, we surprisingly found that IGFBP-2 Ab levels were higher in early cancers than advanced cancers (Figure 1C and D). Patients with A (mean: 171 ng/ml, median: 17 ng/ml, range: 0–1387 ng/ml) showed significantly increased IGFBP-2 Abs compared with controls (P < 0.0001, Figure 1A). Patients with gliomas (mean: 128 ng/ml, median: 24 ng/ml, range: 0–1359 ng/ml, P < 0.0001), colorectal polyps (32, 14, 0–212 ng/ml, P < 0.0001), or CRCs (22, 1.0, 0–161 ng/ml, P = 0.001) had significantly increased IGFBP-2 Abs compared with controls (Figure 1B). Serum IGFBP-2 Ab levels were obviously higher in glioma patients than polyp or CRC patients.

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serum IGFBP-2 Abs as early biomarkers for cancer diagnosis

We conducted ROC curve analyses using serum IGFBP-2 Ab levels in discriminating between patients and controls. The AUC of using IGFBP-2 Abs as diagnostic biomarkers for tumors, gliomas, and CRCs was 0.729 [95% confidence interval (CI) = 0.679–0.779; \( P < 0.0001 \)], 0.783 (95% CI = 0.729–0.836; \( P < 0.0001 \)), and 0.632 (95% CI = 0.550–0.774; \( P = 0.002 \)), respectively, (Figure 2A–C). When stratifying gliomas by tumor grade, the AUC for A, AA, and GBM was 0.82 (95% CI = 0.792–0.936;
When stratifying CRCs by tumor stage, the AUC for CRC I–II and CRC III–IV was 0.668 (95% CI = 0.566–0.770; \( P = 0.002 \)) and 0.590 (95% CI = 0.477–0.703; \( P = 0.113 \)) (Figure 2H–I), respectively, indicating some ability of IGFBP-2 Abs to diagnose CRC I–II but not CRC III–IV. The AUC value for polyps was 0.72 (95% CI = 0.630–0.811, \( P < 0.0001 \)) (Figure 2G), implicating that IGFBP-2 Abs may indicate the presence of some colorectal polyps at high risk for progressing to CRC.

An cut-off value of 11 ng/ml IGFBP-2 Abs (mean level of the controls) was associated with the sensitivity of 66%, specificity of 81%, positive and negative predictive values of 65% and 81% for A, 81%, 81%, 53%, and 94% for AA, and 40%, 81%, 39%, and 83% for CRC I–II. At the cut-off of 130 ng/ml IGFBP-2 Abs, the sensitivity, specificity, and positive and negative predictive values yielded 25%, 99%, 91%, and 71% for A and 32%, 99%, 86%, and 85% for AA.

**combined serum IGFBP-2 and IGFBP-2 Abs augment cancer diagnosis**

As mentioned previously, overexpressed IGFBP-2 was associated with increased tumor malignancy [12–17]. We, thus, measured IGFBP-2 levels in the 401 sera and found that serum IGFBP-2 levels were significantly higher in advanced cancers than early ones and controls (\( P \leq 0.005 \) for gliomas; \( P < 0.0001 \) for polyp and CRC) (Figure 3A and B). Therefore, combined serum IGFBP-2 and IGFBP-2 Abs could improve diagnosis of the cancers at different stages or grades in which IGFBP-2 plays a significant role in cancer initiation and progression.

As shown in Figure 4, the combination of serum IGFBP-2 and IGFBP-2 Abs effectively grouped subjects into three clusters, low levels of IGFBP-2 and IGFBP-2 Abs, high levels of IGFBP-2 Abs, and high levels of IGFBP-2. The ROC curve of using combined IGFBP-2 and IGFBP-2 Abs for diagnosis showed greatly improved diagnostic values for tumors (AUC: 0.823; 95% CI = 0.78–0.866) (Figure 4A), gliomas (AUC: 0.800; 95% CI = 0.749–0.852) (Figure 4B), and CRCs (AUC: 0.917; 95% CI = 0.870–0.964) (Figure 4C) when compared with using IGFBP-2 or IGFBP-2 Abs alone for tumor (AUC: 0.672 or 0.730), gliomas (AUC: 0.551 or 0.784), and CRCs (AUC: 0.916 or 0.622).

**relation of serum IGFBP-2 Abs and tumor IGFBP-2 expression in astrocytomas and advanced polyps**

The presence of serum antibodies against IGFBP-2 should reflect antibody response to IGFBP-2 overexpressed in tumors especially for early cancer patients who have relatively strong immunocompetence. We, thus, examined IGFBP-2 tumor expression by IHC in 63 astrocytomas and 32 advanced polyps (supplemental Figure S2, available at *Annals of Oncology* online).

We found 73% (46/63) of the astrocytomas expressed low (46%) to high (27%) IGFBP-2, 71% (33/46) of which had IGFBP-2 Ab levels above the cut-off value of 11 ng/ml IGFBP-2 Abs. However, we did not find a correlation between serum IGFBP-2 Ab levels and tumor IGFBP-2 expression levels in astrocytomas. Similarly, 56% (18/32) of the advanced polyps expressed low (22%) to high (34%) IGFBP-2, 50% (9/18) (seven advanced adenomas and two multiple hyperplastic polyps) of which developed IGFBP-2 Abs >11 ng/ml cut-off value. The association between tumor IGFBP-2 expression levels and serum IGFBP-2 Ab levels was also not found in the advanced polyps.
Overexpressed IGFBP-2 is found in 30% to >80% of many malignant tumors [12–17] and predicts poor prognosis [22–25], indicating the need for diagnosing IGFBP-2-responsive malignant tumors. The study is the first to report the value of IGFBP-2 Abs for diagnosing early gliomas and colorectal cancers, and possibly precancerous polyps, and in combination of serum IGFBP-2 for detecting the examined cancers at different stages or grades.

Astrocytomas are diffusely infiltrating low-grade gliomas (LGGs) more aggressive than other two infiltrating LGGs, oligodendrogliomas, and oligoastrocytomas [27]. Magnetic resonance imaging remains the standard procedure for their diagnosis when clinical symptoms appear, although most
patients are asymptomatic. Our findings of the predominant presence of IGFBP-2 Abs in astrocytoma patients indicate a potential of IGFBP-2 Abs in diagnosing this disease by the blood test. Furthermore, continuously increased IGFBP-2 Abs in patients with AAs suggest that IGFBP-2 Abs may allow monitoring the progression of astrocytomas to AAs. Decreased IGFBP-2 Abs in GBM patients is probably resulted from increased immune suppression in those patients. Whether IGFBP-2 Abs have a potential for identifying individuals at high risk of LGGs need to evaluate when the high-risk population for LGGs is established.

Adenomatous polyps contribute to >95% of CRC development, and advanced adenomas and multiple hyperplastic polyps have a greater malignant potential [28, 29]. The study shows that IGFBP-2 Abs were significantly higher in patients with colorectal polyps than healthy controls. Fifty percentage of advanced polyps expressing low to high IGFBP-2 in tumors have IGFBP-2 Ab levels >11 ng/ml cut-off point (mean level of the controls). The findings indicate that IGFBP-2 Abs are not only applicable for early detection of gliomas but indicative of advanced adenomas and multiple hyperplastic polyps, well-known precancerous changes.

Our results show that IGFBP-2 Ab levels are obviously higher in patients with glioma than with CRC, although serum IGFBP-2 levels are oppositely higher in CRC patients than glioma patients. The findings indicate a preferential immunogenicity of IGFBP-2 in gliomas compared with CRCs, which may be related to the microenvironment of tumors and immune suppressive mechanisms induced by tumor cells [30], and possibly different roles of TAAs in tumor development of different tumor types. p53 Abs have been reported in breast, colon, oral, lung, and gastric cancers and are associated with high-grade tumors and poor survival [31]. However, p53 Abs were absent in sera of the patients with grade III and IV gliomas [32]. This could be explained by brain–blood barrier and majorly intracellular localization of p53 protein, whereas secreted IGFBP-2 is more likely recognized by the immune system.

Relative low sensitivity is a concern in utilizing autoantibodies to TAAs for tumor diagnosis [31]. In our case of astrocytomas, 71% of astrocytoma patients with overexpressed IGFBP-2 in tumors have IGFBP-2 Abs >11 ng/ml cut-off and 73% of astrocytomas overexpress IGFBP-2, suggesting that IGFBP-2 Abs effectively cover the majority of astrocytomas for diagnosis. The specificity of the autoantibodies to TAAs is generally high because such autoantibodies are rare in the normal population, making them dependable markers for cancer [31]. At the cut-offs of 11 ng/ml and 130 ng/ml IGFBP-2 Abs, the specificity for astrocytomas reached 81% and 99%, respectively. Our literature search did not find consistent reports of overexpressed IGFBP-2 in other diseases rather than tumors.

We previously reported that plasma IGFBP-2 levels decreased after tumor resection but reincreased after recurrence in GBM patients [22]. Therefore, the combined measurement of serum IGFBP-2 and IGFBP-2 Abs not only augments diagnostic power for the examined tumors at different stages but may allow monitoring their recurrence and progression.

The major weaknesses of the study are that the healthy controls did not receive a colonoscopy, possibly leading to misclassification of the study subjects, and the relatively small sample size. Further studies with larger sample size in different tumor types are necessary to validate the findings of the IGFBP-2-based seroreactivity assay for tumor diagnosis. Prospective studies are needed to confirm whether the nine advanced polyp patients with high IGFBP-2 Abs have a higher incidence of CRC.

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**Disclosure**

The authors declare no conflicts of interest.

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The role of comorbidities on the uptake of systemic treatment and 3-year survival in older cancer patients

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Background: Older patients are notably absent from clinical trials. Thus, observational studies are the primary avenue for understanding the role of comorbidity in cancer care and survival. We examined the impact of comorbidity on systemic treatment initiation and 3-year survival in a cohort of older cancer patients.

Patients and Methods: Our cohort comprised 2753 Australian veterans aged ≥65 years with full health coverage and a cancer registry notification for colorectal (CRC), breast, prostate or non-small-cell lung cancer (NSCLC). We established comorbidities based on drugs prescribed in the 6 months prior to cancer diagnosis.

Results: Patients with higher comorbidity burden were more likely to receive systemic treatment for prostate cancer [adjusted odds ratio 1.21, 95% confidence interval (CI) 1.05–1.39] but less likely for NSCLC (0.63, 95% CI 0.45–0.86). After adjusting for receipt of treatment, increased comorbidity resulted in shorter survival for CRC [adjusted hazard ratio (aHR) 1.16, 95% CI 1.07–1.26] and breast cancer (aHR 1.23, 95% CI 1.02–1.48). However, we did not demonstrate significant improvements in 3-year survival for patients receiving systemic treatment.

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