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IGFBP2 regulates PD-L1 expression by activating the EGFR-STAT3 signaling pathway in malignant melanoma

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Abstract

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Declaration of competing interest

The authors declare no conflicts of interest.

Immunotherapy targeting the PD-1/PD-L1 receptor has achieved great success in melanoma patients. Although many studies have addressed the underlying mechanisms involved in the blockade of PD-1/PD-L1 and the consequent modulation of the immune system, the mechanisms of PD-L1 upregulation and reliable biomarkers to predict the efficacy of anti-PD-1/PD-L1 therapy remain unknown. The present study demonstrates the correlation between IGFBP2 and PD-L1, revealing a novel immune-associated tumor function of IGFBP2 in facilitating nuclear accumulation of EGFR and activation of the EGFR/STAT3/PD-L1 signaling pathway in melanoma cells. Our results also suggest that combined IGFBP2 and PD-L1 expression has the potential to predict the efficacy of anti-PD-1 treatment for malignant melanoma; because the combination of high IGFBP2 and PD-L1 expression characterizes melanoma patients with worse overall survival and is associated with a better immune ecosystem. These characteristics have been confirmed by both in vitro and in vivo data. Consequently, IGFBP2 regulates PD-L1 expression by activating the EGFR-STAT3 signaling pathway and its function as a PD-L1 regulator might suggest novel therapeutic approach for melanoma.

Keywords

Melanoma; IGFBP2; PD-L1; Anti-PD-1; Immunotherapy

1. Introduction

Programmed cell death 1 (PD-1) is an immune checkpoint that promotes tumor development by driving immune escape via binding to its ligand, programmed cell death-ligand 1 (PD-L1) [1,2], which is a T cell co-inhibitory receptor with a structure similar to that of the CTLA-4 immune checkpoint receptor [3]. Unlike CTLA-4 ligands, PD-L1 is highly expressed in solid tumors [4]. Furthermore, the expression of PD-L1 on the tumor tissues of patients with renal cell carcinoma [5], esophageal cancer [6], gastric cancer [7] and ovarian cancer [8] indicates a poor prognosis.

Immune checkpoint inhibitors, including antibodies against CTLA-4 and PD-1/PD-L1, have provided unprecedented clinical benefits in the treatment of various cancers [9–11]. In particular, several studies have demonstrated the effectiveness of anti-PD-1/PD-L1 treatment in patients with advanced malignant melanoma, which resulted in the rapid emergence of PD-1/PD-L1 inhibitors as a central therapeutic modality for patients with advanced melanoma [12–14]. Although the results are encouraging [15–17], the high number of non-responders prevents these agents from being used practically. The reality that we are still far from completely understanding the events underlying tumor immune resistance.

Studies on the pathways underlying elevated PD-L1 in tumors have revealed different mechanisms in various cancers [18–20]. For example, activation of PI (3) kinase or the loss of the tumor suppressor PTEN were shown to upregulate PD-L1 expression in breast, prostate, colorectal and glioma cancer cells [21–23]. Regarding the pathways underlying elevated PD-L1 in melanoma, studies have confirmed that the activation of MAPK signaling pathway and treatment with INF γ treatment both promote PD-L1 expression [19,24,25]. PD-L1 expression is also transcriptionally modulated by c-Jun [24].

Insulin like growth factor binding protein 2 (IGFBP2) was originally identified as a protein that binds and modulates the activity of IGF-I and IGF-II growth hormones. By binding to integrins, IGFBP2 activates the PI3K/AKT [26], NF κ B [27] and ERK [28] signaling pathways, leading to increased cell proliferation, invasion, and drug resistance in many tumor types [28]. In addition, IGFBP2 and epidermal growth factor receptor (EGFR) are functionally related [29], and their nuclear co-localization was shown in glioblastoma and astrocytoma cells [29]. Other studies have confirmed that mutations in EGFR lead to its constitutive activation and stimulation of downstream signaling pathways, including upregulation of the STAT3 [30]. A previous study also showed that IGFBP2 potentiates nuclear EGFR/STAT3 signaling [19]. However, whether IGFBP2 is involved in PD-L1 expression is not clear.

In our study, we sought to determine whether IGFBP2 regulates the expression of PD-L1 and contributes to the evasion of cancer cells from host immunosurveillance. The results may help to develop a new therapeutic strategy to potentiate PD-L1-targeted immunotherapy in melanoma patients.

2. Materials and methods

2.1. Patients, tissue microarrays (TMAs) and immunohistochemistry (IHC)

All the procedures of the study were approved by the Institute Research Medical Ethics Committee of Tianjin Medical University Cancer Institute & Hospital. All the patients signed a fully written, informed consent form at the time of admission; this form explained that the tissues and other samples might be used for scientific research but would not compromise patient privacy. A cohort of 667 patients with histologically confirmed melanoma at Tianjin Medical University Cancer Institute & Hospital from February 1981 to May 2013 was included in this study [31]. TMAs were constructed from 127 formalin-fixed, paraffin-embedded tissues, patients who did not receive anti-PD-1 therapy. IHC was performed using rabbit antibodies against human IGFBP2 (1:200; ab190072, Abcam, USA), EGFR (1:200; ab137660; Abcam, USA) and PD-L1 [28–8] (1:200; ab205921, Abcam, USA). IGFBP2 and EGFR staining were scored as described previously [29]. For cytoplasmic staining, the staining intensity in tumor cells ranged from 0 to 3 (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining), and the percentage of positive cells was calculated as follows: 0, no staining; 1, < 25%; 2, 25 but < 50%; 3, 50 but < 75%; and 4, 75%. For the nucleus, the staining intensity in tumor cells ranged from 0 to 2 (0 = no staining, 1 = weak staining, and 2 = strong staining) whereas the percentage of positive cells was calculated as 0, no staining; 1, < 10%; 2, 10–30%; and 3, 30%; the final score was determined by the sum of the staining intensity and positive percentage scores. PD-L1 expression was considered positive when 5% of tumor cells were positive, as described in previous studies [21,32,33]. The TMAs were examined and scored by two pathologists.

2.2. Bioinformatic analysis of RNA sequencing data in US metastatic melanoma with anti-PD-1 therapy (GSE78220)

RNA sequencing data from 28 patients with malignant melanoma who received anti-PD-1 therapy were analyzed from the GEO database (GSE78220) [34]. According to the response to anti-PD-1 treatment, patients were divided into two groups: response and non-response groups. Cluster analysis of RNA expression levels in two groups was performed using R (package pheatmap). The difference in the level of PD-L1 was analyzed by the Boxplot (R ggplot2 package). Patients were classified as high or low expression based on the median mRNA level of IGFBP2 and PD-L1, and the 28 patients were divided into four groups (high IGFBP2+high PDL1, high IGFBP2+low PDL1, low IGFBP2+high PDL1 and low IGFBP2+low PDL1). Differences among the four groups were analyzed by Fisher exacttest.

2.3. Anti-PD-1 treatment efficacy and assessment

Information from 13 patients who were diagnosed with melanoma, failed prior chemotherapy, and treated at the Tianjin Medical University Cancer Institute & Hospital between July 2015 and December 2018 was collected. These are stage IV and III patients with unresectable malignant melanoma. The treatment dose of Keytruda (pembrolizumab) was 2 mg/kg, once every three weeks, and the dose of Opdivo (nivolumab) was 3 mg/kg, once every two weeks. The cancer immunotherapy response was captured by Response Evaluation Criteria in Solid Tumors (RECIST) [35,36]. Partial response (PR) means the tumor was reduced by at least 30% for at least 4 weeks. Progression of disease (PD) means that the maximum diameter of the target lesion increased by at least 20% or new lesions occurred. Stable disease (SD) means the sum of the maximum diameter of the target lesion reduced to less than PR or increased to less than PD.

2.4. Bioinformatic analysis of RNA TCGA malignant melanoma gene expression data

According to the mRNA levels of PD-L1 and IGFBP2, 443 patients with malignant melanoma from TCGA datasets were divided into three groups by the quartile method, which leads to 111 patients (over 75%) in the PD-L1/IGFBP2-high group, 111 patients (under 25%) in the PDL1/IGFBP2-low group and the other 221 in the middle group. The CIBERSORT platform (Stanford University) was used to analyse the differences in immune cells between the groups (both from GEO and TCGA datasets) [37].

2.5. Cell culture, treatments and transfections

A875 and A375 melanoma cells (Beijing Cellular Research Institute, China) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and incubated under 5% CO₂ at 37 °C. To establish stable cell lines that overexpress IGFBP2, A375 and A875 were transfected with an LV5-IGFBP2 viral expression vector (GenePharma, Shanghai, China) in the presence of 5 µg/mL polybrene. In some experiments, A875 cells were incubated overnight in serum-deprived media and then treated with exogenous recombinant IGFBP2 (ab63223; Abcam, USA). Depletion of IGFBP2 in A375 and A875 cells was achieved by transfection with two different pools of siRNAs targeting IGFBP2 from Thermo Fisher Scientific (ID: #1-45934 and #2-HSS142627) using Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY, USA)

for 24 h, according to the manufacturer's protocol. For inhibition of EGFR, cells were treated with 2 nM erlotinib (S1023, Selleck) for 48 h [38,39].

2.6. Immunoprecipitation (IP) and Western blot

Cells were lysed in cell lysis buffer (P0013, Beyotime Biotechnology). After the cell lysate was precleared for 1 h at 4 °C with protein A + G agarose beads (P2012, Beyotime Biotechnology) and the corresponding IgG antibody (A7016, Beyotime Biotechnology), it was incubated with protein A + G agarose beads and antibodies against IGFBP2 (1:50; #3922, Cell Signaling Technology, USA) or EGFR (1:100; #4267; Cell Signaling Technology, USA) at 4 °C overnight. The immunoprecipitate was eluted, and the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon TM-PVDF membrane (Millipore, Billerica, MA) for 2 h at 0.25 mA in transfer buffer (24 mM Tris base, 191 mM glycine and 20% [v/v] methanol). The membrane was blocked for 1 h at room temperature with 5% BSA in TBST containing 0.1% Tween-20 and incubated overnight at 4 °C with primary antibodies against: IGFBP2 (1:1000; #3922, Cell Signaling Technology, USA), EGFR (1:1000; #4267; Cell Signaling Technology, USA), phosphoEGFR-Y1068 (1:1000; #2236; Cell Signaling Technology, USA), STAT3 (1:1000; ab68153; Abcam, USA), phospho-STAT3-Y705 (1:2000; #9145; Cell Signaling Technology, USA) and PD-L1 (1:1000; #13684, Cell Signaling Technology, USA) in blocking solution. After the membrane was washed in TBST, it was incubated for 1 h at room temperature in 1% BSA with the horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG (1:5000; Santa Cruz Biotechnology) or anti-mouse IgG (1:3000; Santa Cruz Biotechnology). The protein bands were quantified by determining the ratio of each protein band with its corresponding actin protein level using ImageJ. Data were collected from three independent experiments.

2.7. Immunofluorescence (IF) and immunocytochemistry (ICC)

Cells on chamber slides were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with primary antibodies against EGFR (1:200; ab137660, Abcam, USA), IGFBP2 (1:500; SC-25285) and PD-L1 (1:100; ab213524, Abcam, USA) at 4 °C overnight. Cells were then incubated with a secondary antibody (Alexa Fluor; 1:500; Life Technologies) in PBS containing 1% goat serum for 1 h at room temperature in the dark. The nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole, dihydrochloride). Images were acquired using a Leica confocal microscope at 40 × /NA.

2.8. Statistical analysis

General statistical analyses and graphing were performed using SPSS version 20.0 software for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism 6 (Graph Pad, La Jolla, CA, USA). Patient survival curves were plotted via the Kaplan-Meier plot and a log-rank test. The statistical significance of associations was evaluated using the Pearson chi-square test. Overall survival (OS) was defined as the duration from the date of diagnosis to the date of death or the last follow-up. The statistical significance of protein associations in the TMA data set was evaluated using the Pearson chi-square test. Student's t-tests were applied for paired comparisons where variances were estimated to be similar. A two-sided p-value <

0.05 was considered statistically significant. The indicated annotations correspond to the following p-values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. IGFBP2 expression is positively correlated with PD-L1 and the high expression of these two proteins is associated with worse OS in melanoma patients

IGFBP2 is highly expressed in a variety of tumors and is associated with poor prognosis [40]. High expression of the PD-L1 protein also indicates a poor prognosis for multiple tumor types, including lung, head, neck, and gastric cancers. To investigate the role of IGFBP2 and PD-L1 in melanoma tumorigenesis and progression, we evaluated IGFBP2 and PD-L1 protein expression in melanoma tissues by performing IHC on TMAs containing histologically confirmed melanoma from 127 patients who did not receive anti-PD-1 therapy. From our results, high expression levels of IGFBP2 and PD-L1 were detected (Fig. 1A). Furthermore, we found a positive correlation between IGFBP2 and PD-L1 with an odds ratio (OR) of 2.35, a chi-square value of 4.731 and a p-value of 0.03.

According to the result of Kaplan-Meier analysis, melanoma patients with high IGFBP2 expression levels had worse OS than those with low IGFBP2 levels (Fig. 1B), and melanoma patients with high expression levels of PD-L1 also had worse OS than those with low PD-L1 levels (Fig. 1C). Furthermore, patients with high expression levels of both IGFBP2 and PD-L1 showed worse OS than other groups (Fig. 1D, Table 1 in Data in Brief).

3.2. IGFBP2 combined with PD-L1 may better predict the efficacy of anti-PD-1 treatment in melanoma patients

RNA sequencing data from 28 patients with malignant melanoma who received anti-PD-1 therapy were analyzed from the GEO database (GSE78220), which was published by Hugo et al. [34]. The patients were divided into two groups according to response to anti-PD-1 therapy: response and non-response groups (13 patients in no response group and 15 patients in response group). We evaluated the expression levels of PD-L1-related genes, including PD-L1 itself, with hypothetical roles in modulating the response to anti-PD-1 therapy, but no significant difference was observed between the two groups (Fig. 1A in Data in Brief). The bioinformatic analysis of GSE78220 demonstrated that higher mRNA expression levels of PD-L1 were positively correlated with the response to anti-PD-1 treatment, but without statistical significance (Fig. 1B in Data in Brief). When divided the patients into high and low expression groups according to the median mRNA expression of PD-L1 and IGFBP2, we found that 5 patients with high levels of both PD-L1 and IGFBP2 mRNA responded better to PD-1 blockers than the others, indicating that both high PD-L1 and IGFBP2 mRNA levels may predict the efficacy of anti-PD-1 therapy (Fig. 1C in Data in Brief). Compared with the high expression of either IGFBP2 or PD-L1, the receiver operating characteristic (ROC) curve showed a larger area under the curve (AUC) in patients with both high mRNA levels (0.536 vs. 0.667) (Fig. 1D in Data in Brief; Table 2 in Data in Brief), which indicated that patients with high IGFBP2 and high PD-L1 may have better therapeutic results than other groups. These results suggest that malignant melanoma patients with high mRNA expression of IGFBP2 and PD-L1 may respond better to anti-PD-1 treatment. However, the

relationship between the protein expression of IGFBP2 and PD-L1 and the therapeutic effect for the patient is not clear.

3.3. Melanoma patients with both high IGFBP2 and PD-L1 expression might potentially exhibit a better immune ecosystem and benefit from anti-PD-1 treatment

The efficacy of immunotherapy, such as anti-PD-1/PD-L1 and anti-CTLA 4, might be determined by the balance of tumor cells and the immune system. In general, the activation or depression of the immune system would determine the response of immunotherapy. Based on the results above, high IGFBP2 and high PD-L1 expression was associated with worse OS and high IGFBP2 and high PD-L1 mRNA levels may better predict the efficacy of anti-PD-1 treatment in melanoma patients, we investigated the immune status of patients to determine whether the immune ecosystem is involved in the mechanism of this phenomenon.

We used CIBERSORT, a method for characterizing the cell composition of complex tissues from their gene expression profiles [37], to analyse the TCGA melanoma gene expression data. More than 547 gene matrices representing the expression signatures of 22 types of immune cells were used to evaluate the immune ecosystem based on the proportions of immune cells [37]. Patients with IGFBP2 or PD-L1 expression > 75% were categorized as the high group, < 25% were categorized as the low group and others were categorized as the middle group. According to the statistical analysis of the median of different groups, patients in the high PD-L1 group presented increased proportions of naïve B cells, CD8 T cells, CD4 memory activated T cells, T follicular helper cells, activated NK cells and M1 macrophages, and decreased proportions of CD4 memory resting T cells, CD4 naïve T cells, CD4 memory resting T cells, NK cell resting cells and M0/M2 macrophages (Fig. 2A). Patients in the high IGFBP2 group showed decreased T follicular helper cells and increased M0 macrophages (Fig. 2B). Patients with both high PD-L1 and high IGFBP2 showed increased CD8 T cells, CD4 memory activated T cells, activated NK cells and M0 macrophages, which represent a high level of activation of the immune ecosystem; the patients with low PD-L1 and low IGFBP2 showed increased CD4 memory resting T cells and resting NK cells (Fig. 2C). These data indicate that the immune system was activated in patients with high IGFBP2 and high PD-L1 expression, which might explain why high IGFBP2 and PD-L1 expression is not only associated with worse OS but also better predicts the efficacy of anti-PD-1 treatment.

3.4. Potential role of IGFBP2 combined with PD-L1 in anti-PD-1 treatment

Thirteen patients with malignant melanoma who received anti-PD-1 treatment were included to perform a preliminary associative study of the role of PD-L1 and IGFBP2 (Table 3 in Data in Brief). Seven patients were able to undergo the final efficacy evaluation, six of whom had at least one extracranial measurable site of disease. One of the patients has unresectable stage III disease with no measurement lesion, but no progression has occurred since treatment. The change in the target lesions and maximum change in the target lesions were evaluated according to RECIST 1.1 (Fig. 2A and B in Data in Brief). As a result, one patient achieved PR, three patients achieved SD and three patients suffered from PD at the last time point evaluation. In addition, immunohistochemical staining of the melanoma patients who responded well to anti-PD-1 treatment showed high levels of IGFBP2, EGFR

and PD-L1 expression (Fig. 2C, D, E in Data in Brief). The imaging data of the patient with PR are shown and presented as follows. At the beginning of the treatment, the patient was administered pembrolizumab, and the CT review found a metastatic lesion of approximately 4×2.9 cm in the right upper lobe (Fig. 2F in Data in Brief). After 2.6 months, the volume of the metastatic lesion was slightly larger than before (Fig. 2G in Data in Brief) but then gradually decreased by 67.5% (Fig. 2H in Data in Brief). Among the 13 Chinese patients enrolled in our study, we could only obtain 6 formalin-fixed, paraffin-embedded patient tissues. The expression levels of IGFBP2 and PD-L1 were shown in Fig. 3 in Data in Brief. Our preliminary results showed that IGFBP2, in combination with PD-L1, may have the potential to predict the efficacy of anti-PD-1 treatment in melanoma. However, an independent large cohort study is needed to validate this finding.

3.5. IGFBP2 regulates PD-L1 expression by activating the EGFR/STAT3 pathway

Our current results demonstrated that IGFBP2 expression is positively correlated with PD-L1 expression and combined IGFBP2 and PD-L1 expression is associated with worse OS and better predicts the efficacy of anti-PD-1 treatment. To explore the mechanism of this phenomenon and whether IGFBP2 regulates PD-L1 expression, human melanoma cell lines A875 and A375 that expressed moderate levels of IGFBP2 were used as an in vitro model.

First, we examined PD-L1 levels in A875 cells treated with various concentrations of exogenous recombinant IGFBP2 for 1 h (Fig. 3A). When the concentration of recombinant IGFBP2 was 100 ng/mL, increased PD-L1 expression, as well as increased levels of pEGFR (Y1068) and pSTAT3 (Y705) were observed compared with those in the control cells.

Second, cells were treated with 100 ng/mL exogenous IGFBP2 protein at different time points. As a result, the expression levels of phosphorylated (activated) forms of pSTAT3 and pEGFR increased at 10 min (Fig. 3B), while PD-L1 expression levels did not detectably increase until 20 min. Forty-five minutes after adding exogenous IGFBP2 protein, the expression of PD-L1 was at high levels along with the increase in EGFR/STAT3 expression. Based on these results, we speculated that PD-L1 expression in melanoma cells might be regulated by IGFBP2 through the EGFR/STAT3 pathway.

To further confirm our speculation, A875 and A375 cells were transfected with an IGFBP2 overexpression vector and stable cell lines were established. In cells that constitutively overexpressed IGFBP2, we observed higher levels of pEGFR (Y1068) and pSTAT3 (Y705), as well as an increased expression of PD-L1 (Fig. 3C). By contrast, to avoid off-target effects, we used two different targets siRNAs as off-target controls for simultaneous experiments. As a result, knocking down IGFBP2 led to decreased EGFR and STAT3 activation and decreased PD-L1 expression (Fig. 3D).

3.6. EGFR is required for the IGFBP2-mediated upregulation of PD-L1 expression, and IGFBP2 co-immunoprecipitates and co-localizes with EGFR

To examine the role of EGFR in IGFBP2-regulated PD-L1 expression, 2 nM erlotinib was added to the culture medium to treat A375 cells with stable IGFBP2 overexpression to inhibit EGFR expression [41]. After 48 h, we observed that inhibition of EGFR led to decreased levels of pSTAT3 and PD-L1 (Fig. 4A). A similar result was obtained when A875

cells were treated with 100 ng/mL exogenous recombinant IGFBP2 for 45 min in the presence of 2 nM erlotinib (Fig. 4B). These data indicate that EGFR is necessary for the IGFBP2-mediated activation of STAT3 and PD-L1 expression.

Furthermore, we examined the potential interactions between IGFBP2 and the EGFR proteins. A875 cells were cultured in serum-deprived medium overnight and then incubated with 100 ng/mL exogenous IGFBP2 for 45 min. Co-immunoprecipitated experiments revealed co-precipitation of IGFBP2 and EGFR. The association of the two proteins increased in cells in the presence of 100 ng/mL exogenous IGFBP2 compared with that in the control cells (Fig. 4C). This experiment was repeated with A375 cells stably overexpressing IGFBP2, and the results showed a similar association and interaction of EGFR with IGFBP2 (Fig. 4D).

To further evaluate the functional relationship between IGFBP2 and EGFR, IF was performed to examine localization of the EGFR/IGFBP2 interaction in the cell. In Ad-BP2 cells, both proteins increased and translocated to the nuclei compared with their quantities and localizations in Ad-vec cells (Fig. 4E). ICC for PD-L1 expression in Ad-BP2 and Ad-vec confirmed that the level of PD-L1 expression was higher in Ad-BP2 cells than in the control cells (Fig. 4F).

3.7. IGFBP2 facilitates EGFR nuclear accumulation and activates the EGFR/STAT3 pathway

Since IGFBP2 and EGFR co-localize in both the cytoplasm and nucleus, especially in the nucleus, A375 and A875 cells stably overexpressing IGFBP2 were fractionated into cytoplasmic and nuclear fractions; Western blotting was used to investigate whether the nuclear IGFBP2 and EGFR complex can induce STAT3 transcriptional activation. In both cell lines, higher IGFBP2 levels and increases in pEGFR were observed in the nuclear fractions from Ad-BP2 cells than those from Ad-vec cells (Fig. 5A, D). Moreover, we observed increased level of pSTAT3 in the nuclear fraction and increased protein expression of PD-L1 in the cytoplasm (Fig. 5A, D). Therefore, we suggest that IGFBP2 facilitates the accumulation and activation of EGFR in the nucleus where it activates STAT3, which in turn, induces PD-L1 expression. To confirm this, IGFBP2 was knocked down in A375 and A875 cells using two different pools of siRNAs, and Western blot analysis was performed on the fractionated cell lysates. IGFBP2 depletion led to impaired EGFR nuclear localization along with a decreased nuclear pSTAT3 and a decreased cytoplasmic PD-L1 (Fig. 5B, E). These results confirm that IGFBP2 facilitates the nuclear accumulation of EGFR, which promotes STAT3 transcriptional activity, resulting in increased PD-L1 expression.

To further decipher the role of EGFR in this pathway, we inhibited EGFR activation by adding 2 nM erlotinib to A375 and A875 cells. Decreased activation of EGFR and STAT3 in the nuclei was detected along with decreased expression of PD-L1 in the cytoplasm in cells treated with erlotinib (Fig. 5C, F).

3.8. Nuclear expression of IGFBP2 and EGFR shows a correlation with PDL1 expression in human melanoma tissues

To validate the above results in vivo, TMAs constructed from 127 melanoma patient tissues were used to assess the protein expression levels of nuclear IGFBP2 and EGFR. IHC analyses showed that high nuclear IGFBP2 expression concomitant with high nuclear EGFR expression (Fig. 6A). When nuclear IGFBP2 was undetectable, nuclear EGFR staining was also dim. Melanoma cases with high EGFR showed worse OS (Fig. 6B).

Statistical analysis of EGFR nuclear staining showed that elevated nuclear EGFR expression was detected in melanoma tissues with positive nuclear IGFBP2 expression (Fig. 6C). This suggests that the nuclear accumulation of EGFR may be enhanced by increased nuclear IGFBP2 localization. Nuclear IGFBP2 staining intensity showed a significant positive correlation with nuclear EGFR staining ($p < 0.001$). These in vivo data provide further evidence that IGFBP2 facilitates EGFR nuclear accumulation and activates EGFR/STAT3 pathway.

4. Discussion

Melanoma is an aggressive cutaneous cancer with high mortality and poor prognosis [42,43]. In advanced and metastatic stages, the outcome for melanoma patients remains poor, with a median survival ranging from 8 to 16 months and an OS of 5 years in less than 10% of the cases [20]. Treatment of melanoma by immunotherapy using the checkpoint inhibitors ipilimumab [44] or blockers of PD-1/PD-L1, e.g., nivolumab and pembrolizumab, has shown significant promise [45]. However, the mechanism of upregulation of PD-L1 and reliable biomarkers to predict the efficacy of anti-PD-1/PD-L1 therapy remain unknown. Increased PD-L1 expression is affected by many factors, such as gene transcription, DNA copy number alteration as well as epigenetic modification status. Indeed, studies have shown increased PD-L1 expression on the cell membrane due to reduced proteasomal degradation [1]. STAT3 affects the expression of PD-L1 via transcriptional regulation and bypasses any potential posttranslational regulatory mechanisms that influence PD-L1 expression; that is, the changes in PD-L1 mRNA, total protein and membrane protein were consistent with each other [20,46,47]. In our study, IGFBP2 regulated PD-L1 expression through the EGFR/STAT3 signaling axis. Therefore, we used total PDL1 expression as a surrogate for the cell surface abundance of PD-L1 protein.

Our study not only suggests that combined IGFBP2 and PD-L1 expression might serve as an efficient predictor of anti-PD-1 immunotherapy, but also reveals that IGFBP2 has a novel tumor-promoting function in facilitating EGFR nuclear accumulation and in activating the EGFR/STAT3/PD-L1 signaling pathway in melanoma cells, as illustrated in our model (Fig. 7). The combined application of these signaling pathway inhibitors may improve the prognosis of patients, thus providing a strong basis for the combination of anti-PD-L1 therapy and anti-IGFBP2 therapy in the future. This model is a simplified version that will likely be modified in future studies.

In the present study, patients with both increased PD-L1 and IGFBP2 protein expression had worse OS. Owing to the small number of melanoma patients who received anti-PD-1

treatment in our study, we cannot be certain that the high protein expression of IGFBP2 and PD-L1 is related to the response of anti-PD-1 treatment. Therefore, we will continue to include patients to validate our hypothesis. Analyses of the transcriptome RNA sequencing data of melanoma patients who received anti-PD-1 treatment also suggested that the simultaneous high expression of IGFBP2 and PD-L1 is an important factor affecting the anti-PD-1 response. Patients with high IGFBP2 and PD-L1 expression demonstrated activation of the immune ecosystem according to the TCGA database, which may determine the response to immunotherapy. Thus, we confirmed that both IGFBP2 and PD-L1 expression levels might predict anti-PD-1/PD-L1 efficacy in malignant melanoma.

There was also a positive correlation between the expression of IGFBP2 and PD-L1 in our study. Based on these findings, we speculated that PD-L1 expression might be regulated by IGFBP2. To validate our hypothesis, we constructed cell lines stably overexpressing IGFBP2. The results show that IGFBP2 expression leads to the activation of EGFR and STAT3 and upregulation PD-L1. By contrast, we knocked down IGFBP2 and observed a concomitant decrease in the activation of EGFR and STAT3 as well as reduced PD-L1 expression. As a key point in the regulatory process of the IGFBP2/EGFR/STAT3/PD-L1 signaling pathway, blocking EGFR activation inhibited STAT3 activation and reduced PD-L1 expression. Therefore, our results demonstrate a novel pathway by which IGFBP2 co-localizes with EGFR and facilitates EGFR nuclear accumulation as well as the activation of the EGFR/STAT3/PD-L1 signaling pathway in melanoma cells.

EGFR signaling is an important pathway that is involved in diverse cellular processes, and aberrant EGFR activation occurs in a wide variety of tumors. Therefore, targeting the EGFR signaling pathway has been a focus of many drug development efforts in cancer therapy, such as in epithelial cancer [48]. Many clinical trials have investigated EGFR-mediated tumor immune escape as a target for immunotherapy using immune checkpoint inhibitors, especially for the treatment of lung cancer [49]. The role of EGFR in melanoma is less clear since data on EGFR expression in melanoma are conflicting [50]. Additionally, EGFR staining did not demonstrate any prognostic value in melanoma [50].

Therefore, the detection of the downstream markers of EGFR may be more useful as predictive markers for aberrant EGFR activation and signaling. The role of EGFR in the progression and metastasis of at least a subset of melanomas has been shown [50], and one report showed that activation of the oncogenic EGFR pathway enhances the susceptibility of lung tumors to PD-1/PD-L1 blockade, suggesting that EGFR mediates tumor immune escape [49]. Several studies have suggested different mechanisms of PD-L1 upregulation by EGFR; for example, Concha-Benavente et al. (2013) found that overexpression of EGFR in response to IFN- γ through the JAK2/STAT1 pathway upregulated PD-L1 expression and that specific inhibition of JAK2 abolished PD-L1 upregulation in head and neck cancer. In another study, the mutated and constitutively active EGFR/KRAS-MAPK pathway was suggested to cause the upregulation of PD-L1 in non-small-cell lung cancer [51]. In our study, we demonstrated that EGFR upregulated PD-L1 through the EGFR/STAT3 pathway, which is consistent with other reports that show the activation of STAT3 by AKT/mTOR [52] or PI3K-mediated upregulation of PD-L1 in BRAF mutant melanoma cells [53]. A previous report showing the direct binding of STAT3 to the PD-L1 promoter in antigen-

presenting cells [54] suggests a direct regulation of PD-L1 gene expression by STAT3 at the transcriptional level. Altogether, upregulation of PD-L1 in different cancer cells may occur through different mechanisms, and PD-L1 or PD-1 as a marker of the EGFR signaling, can be used as a predictive marker for aberrant EGFR-mediated immune evasion.

The underlying mechanism of the upregulation of PD-L1 expression in tumor cells is not clear. Concha-Benavente et al. (2013) demonstrated that PD-L1 expression may be regulated by two major mechanisms: the “extrinsic” or “intrinsic” mechanisms [55]. IFN γ , an “extrinsic” factor produced by CD8⁺ tumor infiltrate, activates the “intrinsic” EGFR/STAT1 pathway, which then leads to an increase in PD-L1 expression. Interestingly, in our study, IGFBP2 was observed to be an “extrinsic” factor that switches on the “intrinsic” EGFR/STAT3 pathway. Our data also show that IGFBP2 recruits EGFR to the nucleus to initiate EGFR/STAT3-mediated PD-L1 expression.

IGFBP2 is an important driver in cancer malignancy, and accumulating evidence also suggests that IGFBP2 modulates the immune response in cancer patients and is a potential target for cancer immunotherapy [56]. Although the IGFBP2 vaccine was shown to be immunosuppressive, removing the IL-10-inducing T helper epitopes in the vaccine was suggested to ensure potent IGFBP2 anti-tumor activity [57]. Other modulators of the insulin pathway, such as klotho, not only inhibit the migration of clear cell renal cell carcinoma cells by inhibiting the EGFR-MAPK signaling pathway [58] but also regulate the function of dendritic cells by the insulin/IGF-1/PI3K/Akt signaling pathway [59]. At the same time, we attempted to verify the modulation of PD-L2 expression upon IGFBP2 signaling, but we did not obtain any viable results.

In summary, our study demonstrates the correlation between IGFBP2 and PD-L1 and reveals a novel tumor immune-associated function of IGFBP2 in facilitating EGFR nuclear accumulation and activation of the EGFR/STAT3/PD-L1 signaling pathway in melanoma cells. Our results also suggest that combined IGFBP2 and PD-L1 expression has the potential to predict the efficacy of anti-PD-1 treatment in malignant melanoma; because high IGFBP2 combined with high PD-L1 expression levels characterize melanoma patients with worse OS and exhibit a better immune ecosystem. Thus, our results expand our understanding of this signaling network by demonstrating that the differential IGFBP2 expression modulates EGFR-STAT3 activation, and ultimately, PD-L1 expression. The dynamic nature of these oncogenic signaling molecules may contribute to the ineffectiveness of EGFR-targeted therapy in melanoma, possibly because EGFR is constantly recruited to the nucleus by IGFBP2, which renders the cells resistant to therapies that target membrane-bound EGFR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

PD-1	programmed cell death 1
PD-L1	programmed cell death-ligand 1
STAT3	signal transducer and activator of transcription 3
IGFBP2	insulin like growth factor binding protein 2
EGFR	epidermal growth factor receptor
TMA	tissue microarray
IHC	immunohistochemistry
DMEM	Dulbecco-modified essential medium
FBS	fetal bovine serum
IP	immunoprecipitation
IF	immunofluorescence
OS	overall survival
ICC	immunocytochemistry
ROC	receiver operating characteristic
AUC	area under the curve

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Fig. 1. IGFBP2 expression is positively correlated with PD-L1, and the high expression of these two proteins is associated with worse OS in melanoma patients.

(A) High IGFBP2 and PD-L1 staining in melanoma patient tissue samples detected by IHC. Left panel: hematoxylin and eosin (H&E) staining; middle panel: high IGFBP2 expression; right panel: high PD-L1 expression; (B) TMA data from 127 melanoma patient tissues showed that a high level of IGFBP2 expression was associated with worse OS ($p < 0.05$); (C) TMA data from 127 melanoma patient tissues showed that a high level of PD-L1 expression was associated with worse OS ($p < 0.05$); (D) Patients were stratified into 4 groups according to the expression levels of IGFBP2 and PD-L1: IGFBP2 high + PD-L1 high group, IGFBP2 high + PD-L1 low group, IGFBP2 low + PD-L1 high group and IGFBP2 low + PD-L1 low group. The results showed that both high IGFBP2 and high PD-L1 expression patients had worse OS in the malignant melanoma patient cohort ($p < 0.05$).

Fig. 2. Melanoma patients with both high IGFBP2 and high PD-L1 expression may benefit from anti-PD-1 treatment.

Patients were categorized based on IGFBP2 or PD-L1 expression as follows: > 75%, high group, < 25%, low group and remaining patients, middle group. (A) According to the analysis of TCGA melanoma gene expression data, high PD-L1 patients showed increased proportions of naïve B cells, CD8 T cells, CD4 memory activated T cells, T follicular helper cells, activated NK cells and M1 macrophages and decreased proportions of CD4 naïve T cells, CD4 memory resting T cells, NK cell resting and M0/M2 macrophages; (B) The high IGFBP2 group showed decreased T follicular helper cells and increased M0 macrophages; (C) Patients with both high IGFBP2 and PD-L1 showed increased CD8 T cells, activated CD4 memory T cells, activated NK cells and M0 macrophages, which represent activation of the immune ecosystem; patients with low PD-L1 and IGFBP2 showed increased resting CD4 memory T cells and resting NK cells. (Statistical analysis was performed in the high, middle and low groups. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

Fig. 3. IGFBP2 regulates PD-L1 expression through the EGFR/STAT3 pathway.

(A) Increased IGFBP2 by exogenous IGFBP2 protein induced increased pEGFR, pSTAT3, and PD-L1 expression. A875 cells were treated with exogenous IGFBP2 protein at the indicated concentrations (0, 50, 100, and 200 ng/mL) for 60 min. When the concentration of recombinant IGFBP2 was 100 ng/mL, increased expression of PD-L1 and increased levels of pEGFR (Y1068) and pSTAT3 (Y705) (upper panel) were observed compared with those in the control cells. Quantitative analysis showed a significantly increased expression of PD-L1, pEGFR (Y1068) and pSTAT3 (Y705) (lower panel); (B) Along with the increased IGFBP2 expression, pEGFR, pSTAT3 and PD-L1 were induced. Western blot analysis of A875 cells treated with 100 ng/mL exogenous IGFBP2 protein for different time periods (0,

10, 20, 30, 45, and 60 min). Quantitative analysis shown below the immunoblot indicates fold-change relative to the unstimulated control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin or total protein for phosphorylated proteins; (C) In the A875 and A375 cell lines, overexpression of IGFBP2 by transfecting Ad-IGFBP2 induced increases in pEGFR, pSTAT3, and PD-L1. Western blot of A875 and A375 cell lines with stable constitutive overexpression of IGFBP2 (Ad-BP2) and empty vector (Ad-vec). Quantitative analysis shown below the immunoblot indicates fold-change relative to the control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin or total protein for phosphorylated proteins; (D) Decreased IGFBP2 expression by IGFBP2 siRNA induced decreased pEGFR, pSTAT3, and PD-L1. Western blot analysis of cells after knockdown of IGFBP2 by two different siRNAs (si-BP#1 and si-BP#2) or transduction of control siRNAs (si-control) for 24 h. Quantitative analysis shown below the immunoblot indicates fold-change relative to the control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin or total protein for phosphorylated proteins.

Fig. 4. EGFR is required for IGFBP2-mediated PD-L1 expression and IGFBP2 co-immunoprecipitates and co-localizes with EGFR.

(A) Inhibition of EGFR led to a decrease in pSTAT3 levels as well as in PD-L1 expression in A375 cells. Western blot analysis of the indicated A375 cells treated with 2 nM erlotinib (EGFRi) for 48 h. Quantitative analysis shown below the immunoblot indicates the fold-change relative to the control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin or total protein for phosphorylated proteins; (B) Inhibition of EGFR led to a decrease in the pSTAT3 levels as well as PD-L1 expression in A875 cells. Western blot analysis of A875 cells treated with exogenous IGFBP2 protein at 100 ng/mL for 45 min in the presence or absence of 2

nM erlotinib. Quantitative analysis shown below the immunoblot indicates fold-change relative to the control. * $p < 0.05$, ** $p < 0.01$, compared with actin or total protein for phosphorylated proteins; (C) IGFBP2 co-immunoprecipitated with EGFR in A875 cells. Co-immunoprecipitation and Western blot analyses in lysates from A875 cells treated with 100 ng/mL exogenous IGFBP2 for 45 min; (D) IGFBP2 co-immunoprecipitated with EGFR in A375 cells. Co-immunoprecipitation and Western blot analyses in lysates from A375 cells that stably overexpressed IGFBP2 (Ad-BP2) or controls (Ad-vec); (E) The increased IGFBP2 undergoes nuclear translocation and interacts with the increased EGFR. Confocal microscopy images of IF staining for IGFBP2 (green) and EGFR (red) in A375 cells that stably overexpressed IGFBP2 (top panel) or controls (bottom panel); (F) Increased IGFBP2 induced PD-L1 expression. ICC (left columns) and IF (right columns) for PD-L1 expression in IGFBP2 stably overexpressing cells (Ad-BP2) and control cells (Ad-vec).

Fig. 5. IGFBP2 enhances the activation and accumulation of EGFR in the nucleus, followed by activation of the EGFR/STAT3 pathway.

(A, D) In A375 and A875 cells, increased IGFBP2 levels led to increased levels of pEGFR and pSTAT3 in the nucleus, as well as increased protein levels of PD-L1 in the cytoplasm.

(A): A375 cells; (D): A875 cells. Quantitative analysis shown below the immunoblot indicates fold-change relative to the control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin (cytoplasm marker), histone H3 (nuclear marker) or total protein for phosphorylated proteins; (B, E) In A375 and A875 cells, siRNA-mediated decreases in IGFBP2 levels led to lower levels of pEGFR and pSTAT3 in the nucleus, as well as decreased protein levels of

PD-L1 in the cytoplasm. (B) A375 cells; (E) A875 cells. Quantitative analysis shown below the immunoblot indicates fold-change relative to control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin, histone H3 or total protein for phosphorylated proteins; (C, F) Inhibited EGFR activation by erlotinib-induced inactivation of EGFR and STAT3 along with decreased PD-L1 in the cytoplasm. (C) A375 cells; (F) A875 cells. Quantitative analysis shown below the immunoblot indicates fold-change relative to control cells. * $p < 0.05$, ** $p < 0.01$, compared with actin, histone H3 or total protein for the phosphorylated proteins.

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Fig. 6. Nuclear expression of IGFBP2 is correlated with the nuclear accumulation of EGFR in melanoma patients.

(A) Representative immunohistochemical images of melanoma tissues. Nuclear EGFR was detected in nuclear IGFBP2-positive melanoma tissues. When IGFBP2 expression was negative, nuclear EGFR accumulation was decreased; (B) TMA data from 127 melanoma patient tissues showed that a high level of EGFR expression (blue line) was associated with worse OS in this patient cohort; (C) The 127 tissues were scored according to nuclear IGFBP2 expression, and positive or negative nuclear EGFR expression was described in the Methods section. With the increased nuclear IGFBP2 staining intensity, the positive nuclear staining of EGFR gradually increased.

Fig. 7. Summary of the role of IGFBP2 in regulating PD-L1 expression by activating the EGFR-STAT3 signaling pathway in malignant melanoma.

IGFBP2 activates STAT3 phosphorylation by co-localizing with EGFR and then facilitating EGFR nuclear accumulation and activating EGFR phosphorylation. The recruitment of STAT3 to the PD-L1 promoter precedes the transcriptional activation of PD-L1. As a result, IGFBP2 promotes the expression of PD-L1 in malignant melanoma.