New Immunotherapy Biomarker N-Cadherin Rescue Immunosuppress Caused by PD-L1/IDO-1 in Prostate Cancer

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Research

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Abstract

Background: N-cadherin is a major regulatory factor of epithelial-to-mesenchymal transition (EMT), and the association and coordination of EMT and neuroendocrine differentiation (NED) with immunosuppression have been reported in several tumor types. Therefore, we hypothesize that N-cadherin may play vital role in this process.

Methods: We performed human tissue studies, cell-level explorations and in vivo experiments to determine the statuses of N-cadherin and immune checkpoint proteins (PD-L1 and IDO-1) to verify the co-expression of these biomarkers. Then, we used tumor-bearing mice to test the treatment effect of N-cadherin deletion. Moreover, the mechanism by which N-cadherin regulates PD-L1/IDO-1 expression was also assessed.

Results: According to our data, N-cadherin expression is necessary for PD-L1 expression and dramatically increases the expression of IDO-1 through an IFN- γ response. On the contrary, IDO-1 and PD-L1 can prompt EMT. N-cadherin knockout completely reverses this process. The core mechanism underlying these phenomena is that N-cadherin causes JAK1 to be re-expressed in cells with genomic JAK1 loss via the active EMT/immunosuppression positive feedback loop.

Conclusion: These data indicate that N-cadherin can regulate the EMT/immunosuppression positive feed loop. Preclinical research has shown that N-cadherin deletion promotes the development of antitumor responses by decreasing immunosuppression.

Background

Hormone treatment can induce the conversion of androgen-dependent prostate tumors into androgen-independent tumors, and a high proportion of castration-resistant prostate cancer (CRPC) cases eventually progresses to highly aggressive small cell neuroendocrine prostate cancer (SCNC) with a poor prognosis [1, 2, 3]. Moreover, immunotherapy has emerged as a valuable strategy for several tumor types [4, 5], and thus, researchers have attempted to apply immunotherapy in prostate cancer especially in CRPC and SCNC; however, it is first necessary to find a treatment-related biomarker. The immune checkpoint protein programmed death ligand-1 (PD-L1) has been shown to induce T lymphocyte anergy and/or apoptosis by binding to its receptor PD-1, and some research has indicated that PD-L1 expression may be a reason tumor cells can escape immune surveillance, which leads to tumor development and metastasis [6]. In addition to PD-L1, indole amine 2,3-dioxygenase (IDO-1) is another checkpoint protein that contributes to cancer immune evasion. IDO-1 is a tryptophan-catabolizing enzyme that can induce the rapid conversion of tryptophan (Try) into kynurenine (Kyn) [7], and can activate regulatory T (Treg) cells to suppress effector T cells [8]. Both PD-L1 and IDO-1 can contribute to immune system impairment in individuals with cancer. Thus, inhibition of the immunosuppressive factors IDO-1 and PD-L1 has been proposed as an immunotherapy strategy.

In addition, the mutual positive feedback between epithelial-mesenchymal transition (EMT) and immunosuppression has also been demonstrated in several cancers [9, 10, 11, 12, 13, 14, 15]. EMT refers to the loss of polarity, tight junctions and adhesion between epithelial cells and the acquisition of infiltration and migration abilities [16]. Tumor cells, which undergo EMT, can invade normal tissues and induce metastasis, and a recent study demonstrated that N-cadherin can regulate EMT status [12]. Therefore, we proposed the hypothesis that N-cadherin can improve EMT and then enhance the positive feedback loop of EMT/immunosuppression, and that N-cadherin can be considered a therapeutic target. In this study, we attempted to address this issue and propose a potential new treatment strategy for advanced prostate cancer.

Methods

Patients and tissue samples

Several tissue microarrays (TMAs) were constructed and have been previously reported [17]. TMAs containing both benign and cancer tissues were constructed with prostatectomy specimens from 30 patients with localized prostate adenocarcinoma. Three cores were obtained from the benign and tumor areas of each prostatectomy specimen and were incorporated into the TMA. A CRPC TMA was constructed using CRPC tissue samples from 18 patients who were histologically diagnosed with adenocarcinoma but failed to respond to hormonal therapy. These tissue samples were derived from tissue collected during transurethral resection for the relief of obstructive symptoms and were incorporated into the CRPC TMA. A small cell prostate cancer (SCC) TMA was constructed from 16 primary SCC cases collected at Duke University Hospital. All samples were collected from patients who provided informed consent, and all related procedures were performed with the approval of the internal review and ethics boards of the indicated hospitals.

Immunohistochemistry in tissue samples

To prepare them for immunohistochemistry (IHC), all the slides (including the target sample slides and the positive and negative control slides) were deparaffinized, rehydrated, and boiled in a water bath for 40 min in citrate buffer (pH 6.0) before antibody staining. Then, the slides were incubated with primary antibodies (the optimized dilution was previously determined) for 1 h at room temperature. Horseradish peroxidase-conjugated secondary antibodies (Dako EnVision+Kit) were applied for 30 min and visualized with diaminobenzidine (DAB) after incubation for 30 min at room temperature.

Cell culture and cell treatment

The benign prostate cell line BPH-1 and the prostate cancer cell lines LNCap, C4-2, CWRR-1, PC3 and NC-IH660 were obtained from ATCC. The LASCPC-01 cell line was obtained from Owen N. Witte's laboratory [18]. The NC-IH660 and LASCPC-01 cells were cultured in HITES medium, while the LNCap, C4-2, CWRR-1 and PC3 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin, and BPH-1 cells were cultured in RPMI medium supplemented with 20% FBS and 1% penicillin. The LNCap C1/C2/C3/LC1 cell lines and the N-cad knockdown PC3 cell line (PC3/N-cad KD) were obtained from Robert E Reiter's laboratory. These cell lines were also cultured in RPMI medium supplemented with 10% FBS and 1% penicillin. All cell lines were maintained at 37°C in 5% CO₂. When we tested the effects of interferon gamma (IFN- γ), a JAK/STAT inhibitor and Kyn on the cells, we treated cells with 50 mg/mL IFN- γ for 24 h, 250 nM of a JAK/STAT inhibitor (Santa Cruz CAS 457081-03-7) or 100 μ M/L Kyn in the medium for 48 h. We also treated the cells with a PD-L1 neutralizing antibody (10 μ g/mL) (Invitrogen, 16–5982-82) for 24 h to inhibit PD-L1 expression [19]. IL-8 inhibition was previously described [20], briefly, cells were transfected with 100 nM DNA plasmids with a short hairpin RNA to inhibit IL-8, (category number C01001; GenePharma, Shanghai, China) using the transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Protein was extracted with RIPA buffer, and RNA was extracted with TRIzol.

Antibodies

The antibodies used for both IHC and Western blotting were anti-IDO-1 (CST-51851), anti-interferon gamma receptor 1 (IFNGR1; Invitrogen A5-27841), anti-p-IFNGR1 (Invitrogen PA5-38504), and anti-N-cad (Thermo Fisher MA1-91128 CH-19). The PD-L1 antibody used for Western blotting and flow cytometry was CST-13684, and the one used for IHC was Dako Phe19-Thr239. The secondary antibody used for flow cytometry was conjugated with Alexa Fluor 488. Western blotting for JAK1, p-JAK1, STAT1, p-STAT1, STAT3 p-STAT3, E-cadherin, and Vimentin was performed using the following antibodies: Santa Cruz SC-1677, PA5-104554, Santa Cruz SC-464, Invitrogen KIKSI0803, Santa Cruz SC-8019, CST-9145, Sigma SAB2104222 and Sigma V6389, respectively. IHC for IFN- γ was performed with the A259336 antibody purchased from Invitrogen. The PCR primers are shown in the Supplemental Materials.

Tumor beard mice experiments

Immunocompromised NSG (NOD.Cg-Prkdc Il2rg/SzJ) and nude (nu/nu) mice were obtained from The Jackson Laboratory. In all, 2 $\times$ 10⁶ LNCap (and LNCap C1) or 1 $\times$ 10⁶ PC3 (and PC3 N-cad KO) cells were suspended in 0.1 mL 1 $\times$ RPMI 1640 medium and 10% FBS with 50% Matrigel (Corning) and then inoculated subcutaneously into the bilateral flanks of 3- to 6-week-old male NSG mice. After the LNCap and PC3 xenografts grew to 1 cm³ (tumor volume was calculated using the formula volume (mm³) = length $\times$ height²/2), the mice were divided into two groups. Each mouse in one group was treated with 10 μ g IFN- γ in 200 μ L Iscove's Modified Dulbecco's Medium (Life Technologies, Grand Island, NY) via intraperitoneal injection once per day for two consecutive days. Each mouse in the other group received DMSO as a control. Forty-eight hours after IFN- γ or DMSO injection, the mice were sacrificed and the xenografts were harvested, after which they were fixed in 10% formalin and embedded in paraffin for subsequent analysis. Single-cell isolation was performed with the xenografts; the tissue samples were completely minced and incubated in an enzymatic mixture (80 mg collagenase type II in 40 mL advanced DMEM with 1/1000 ROCK inhibitor and 1/1000 1.0 M DHT) for overnight digestion. The cells were washed with fresh advanced DMEM before culture in 5 mL TrypLE+5 μ L ROCK inhibitor in a 37°C

incubator for 5 min. The cells were repeatedly pipetted up and down and cultured again to efficiently separate the clumps. The cells were then centrifuged and stored in PBS for flow cytometric analysis or RNA extraction.

Quantification and statistical analysis

Statistical analyses were performed using R Project for Statistical Computing, Statistica, GraphPad, and MATLAB. The details and analytical methods are indicated in the figure legends, Results, or Methods sections. The immunohistochemically stained slides were scanned using an Aperio AT2 microscope (Leica Biosystems) at 20× magnification and were analyzed with Aperio software. Quantification was performed by pathologists who were blinded to the diagnosis of the tissue cores. Flow cytometric analysis was performed using BD FACSDiva software, and the results were analyzed using FlowJo 10.0. A human cytokine array was quantified using ImageJ.

Results

Expression of the immunosuppressive factors PD-L1/IDO-1 is associated with N-cadherin expression

Some studies have demonstrated that neuroendocrine differentiation (NED) and EMT share similar properties and are always co-expressed in advanced cancer [21, 22, 23, 24, 25]. To determine whether the EMT marker N-cadherin is also specifically expressed in NE cells in human prostate cancer and to address the role of N-cadherin in the expression of immunosuppressive factors, we performed immunohistochemistry (IHC) on TMAs containing human prostate cancer samples of different stages using antibodies against N-cadherin. Scattered or clustered NE cells expressing N-cadherin were identified (Fig. 1a), and according to the IHC score (Fig. 1b), the expression of N-cad was found only in NE tissue samples, but not in benign, adenocarcinoma or CRPC tissue samples. We also performed a cell-level experiment for which we selected different cell lines to represent the different types of prostate cancer. BPH-1 is a benign prostate cell line, and LNCap is an early-stage tumor cell line that is usually representative of adenocarcinoma. C4-2 and CWRR1 cells are commonly used to represent the CRPC phenotype. PC3, LASCPC-01 and NCI0H660 cells were selected as NE cell lines. As shown in the results in Fig. 1c, the cell lines with N-cadherin expression (PC3, LASCPC and NCI-H600) also expressed low levels of E-cadherin, which is in agreement with published data; this demonstrates that N-cadherin can enhance EMT [12]. Interestingly, as shown in Fig. 1d, we confirmed that PD-L1 was observed only in tissue with N-cadherin positivity (SCC group). The analysis of the PD-L1 expression signature is shown in Fig. 1e, which indicates that the IHC score for PD-L1 in the SCC group was much higher than the scores in the other groups. Fig. 1f and S-Fig. 1a and b also show the protein and mRNA data for both PD-L1 and IDO-1; only in the cell lines in which N-cadherin is activated was PD-L1 expression detectable, and IDO-1 expression was also increased in those cells. In addition, when the expression of IDO-1 was compared among the different tissue groups according to the IHC results, the highest level of IDO-1 was found in the N-cadherin-positive group (Fig. 1g and h). Fig. 1i shows the synergistic association between PD-L1 and IDO-1. Fig. 1j and k provide more evidence that the expression of PD-L1 and IDO-1 was higher in the N-

cadherin- positive group than in the N-cadherin-negative group. We also confirmed that IFN- γ expression was higher in tumor tissue than in benign tissue and that IFN- γ also accumulates in lymphocytes (S-Fig. 1c), where it simulates the tumor microenvironment. This explains why we selected IFN- γ to determine the mechanism of N-cadherin mediation of PD-L1/IDO-1 production.

To better characterize the impact of N-cadherin on immunosuppression, we also performed an in vivo experiment to test the association between N-cadherin and immunosuppression. We first implanted tumor cells in an inguinal site (Fig. 2a): PC3 cells were used in one group, while LNCap cells were used in the other group. Each group received IFN- γ as a treatment or DMSO as a control after the tumors grew to 1 cm³; we collected the tumors 48 h after drug injection (the animals were sacrificed by CO₂ inhalation). Some tissue was used for paraffin embedding, some was dissociated into single cells for flow cytometric analysis, while other tissue was used for RNA extraction for PCR. The expression of PD-L1 was examined by flow cytometry and was inducible only in the PC3 group (Fig. 2b). Evaluation by IHC revealed that only the PC3 tumor-bearing mice could produce PD-L1/IDO-1 under IFN- γ stimulation (Fig. 2c and d). The same results were obtained with PCR (Fig. 2e and f). Interestingly, as shown in Fig. 2g, N-cadherin expression was found only in the PC3 group and was enriched after response to IFN- γ . Additionally, Fig. 2h shows hematoxylin and eosin (H&E) staining of mouse tissue.

Overall, we reported that the enhanced accumulation of N-cadherin is linked with higher expression of the checkpoints molecules PD-L1/IDO-1. Confirmation that EMT and immunosuppression are able to mutually modulate each other was our next area of focus.

How EMT and immunosuppression mutually regulate each other

The increased production of N-cadherin under IFN- γ stimulation was identified by IHC (Fig. 2g). To dissect the mechanism, we used IFN- γ to treat cell lines that represent benign prostate tissue and different prostate cancer types, and then evaluated the expression of the EMT markers N-cadherin, E-cadherin and Vimentin. N-cadherin and Vimentin were up-regulated according to IFN- γ in the N-cad-activated cell lines (Fig. 3a), while E-cadherin expression was down-regulated. However, IFN- γ did not cause N-cad-inactivated cells to express N-cadherin. However, the mechanism by which IFN- γ mediates EMT is still unclear and whether N-cadherin is able to regulate the PD-L1 and IDO-1 expression signature is unknown. Therefore, we tested whether IDO-1 can change the EMT status in these cells. IDO-1 is an enzyme involved in Try metabolism that improves Try conversion into Kyn. Hence, using Western blot, we detected whether local accumulation of Kyn enhances the EMT process. Stimulation with 100 mmol/L Kyn significantly increased the expression of N-cadherin and Vimentin, while the expression of E-cadherin decreased (Fig. 3b). This result is in agreement with the findings reported by Kolijn et al [26]. Additionally, we found that Kyn and IFN- γ treatment failed to accelerate cell growth (S-Fig-2a), and thus the phenotypic change resulted directly from stimulation instead of rapid cell growth. These results may also explain why IDO-1 and PD-L1 expression was simultaneously enriched (Fig. 1i). Another interesting finding is that PD-L1 expression also improved after Kyn treatment, while IDO-1 production did not significantly change (Fig. 3b), which contrasts with the results reported by Kolijn et al. [26]. Indeed, after treatment with a PD-L1

neutralizing antibody, we tested the EMT status according to N-cadherin and Snail expression. The EMT activation level was reduced after PD-L1 attenuation (Fig. 3c). These data indicated that the IFN- γ response can not only induce immunosuppression but can also promote EMT.

Then, we compared the phenotypes between N-cadherin-overexpressing and N-cadherin knockout cells. As shown in Fig. 3d, LNCap C1 and C2 cells concomitantly lost E-cadherin expression and gained Vimentin expression, although one cell line with low expression (LNCap subline (C3)) retained E-cadherin expression and did not change morphologically (although we observed N-cadherin expression in LNCap C3 cells, E-cadherin was also expressed and appeared to exhibit a stronger expression than N-cad); thus, only LNCap C1 and LNCap C2 cells secreted PD-L1 and IDO-1 in response to IFN- γ (Fig. 3e, S-Fig. 2b and c). The LNCap N-cad-activated cell lines LNCap C1 and C2 were characterized by a significant up-regulation in growth compared with the C3 cell line (S-Fig. 3a). This phenomenon also reminds us of the accelerated growth in N-cadherin activated cells. At the same time, N-cadherin knockout reversed NED and EMT to some degree, and IL-8 expression disappeared, which can explain this phenomenon seen in our data (S-Fig. 3b) and agrees with findings of previous studies [12, 13, 15]. After N-cadherin was attenuated, PD-L1 and IDO-1 expression almost disappeared (Fig. 3e and S-Fig. 2b and c). Additionally, we did not observe Vimentin expression in LNCap C3 cells, and thus, we still did not consider this cell line to be an EMT-activated cell line. This may be the reason that PD-L1 and IDO-1 failed to be induced in LNCap C3 cells (Fig. 3e and S-Fig. 2b and c).

Since we proposed that N-cadherin can be considered a therapeutic target, we wanted to acquire more in vivo evidence, and thus, we constructed a new tumor-bearing mouse model. We implanted PC3 N-cad KO cells and LNCap N-cad-activated cells into NOD scid gamma (NSG) mice. The tumor growth status was monitored following a 4-5-week schedule with two injections of IFN- γ , as previously described. Tumors were excised after IFN- γ treatment and processed for IHC detection of PD-L1 and IDO-1 expression. As shown in Fig. 3f, Fig. 3g and Fig. 3h, for the LNCap C1 tumor sample, under stimulation with IFN- γ , the expression of PD-L1 and IDO-1 was detectable, while after N-cadherin knockout, PC3-derived tumors were no longer able to express PD-L1 and IDO-1. The anti-immunosuppressive effect of N-cadherin was identified by comparison of those models. Indeed, the results of S-Fig. 3c suggest rapid tumor growth in the N-cadherin-activated LNCap mouse model, whereas in contrast, the N-cad KO PC3 model was characterized by significant down-regulation of tumor growth.

The IFNGR/JAK/STAT pathway is responsible for the expression of PD-L1 and IDO-1

We examined the mechanism of PD-L1 and IDO-1 expression as well as the role of N-cadherin in this process. First, we focused on IFNGR1 because IFN- γ primarily regulates PD-L1 and IDO-1 expression through IFNGR1 [27]. The IHC results verified our hypothesis, as IFNGR1 expression was significantly higher in the SCC group, even though some staining was also seen in the CRPC group (Fig. 4a and b). We also compared IFNGR1 expression in N-cadherin-positive cases and N-cadherin-negative cases, and the results showed IFNGR1 enrichment in N-cadherin-positive tissues (Fig. 4c). Similar results were observed for p-IFNGR1, which is the activated form of IFNGR1, with the highest level in the SCC group (Fig. 4d and

e). p-IFNGR1 was also up-regulated in the N-cadherin-positive cases (Fig. 4f). Additionally, IHC in tissue from tumor-bearing mice showed that IFNGR1 staining was found only in the PC3 group, and only in this group was IFNGR1 able to be activated by IFN- γ (Fig. 4g and h). Fig. 4i and Supplemental Fig-4a demonstrated that at the cellular level, the expression of IFNGR1 is higher in N-cadherin-activated cell lines and is able to be activated by IFN- γ only in the CRPC and SCC cell lines (N-cadherin-activated cell lines). All cell lines phosphorylated by IFN- γ are induced to secrete IDO-1, but only N-cadherin-activated cell lines, which contain higher levels of p-IFNGR1, are able to produce PD-L1 (Fig. 1f).

We blocked this pathway to better understand the IFNGR-related regulatory process. Generally, IFNGR activates the JAK/STAT pathway, which leads to PD-L1 and IDO-1 production. Therefore, we blocked the entire JAK/STAT pathway to evaluate whether IDO-1 and PD-L1 could still be induced. As shown in Fig. 4a, PD-L1 and IDO-1 were no longer expressed when the JAK/STAT pathway was blocked. We confirmed that PD-L1 and IDO-1 are mediated by the JAK/STAT pathway, and therefore, we then explored the impact of different JAK/STAT pathway molecules on the expression of PD-L1 and IDO-1. Generally, PD-L1 and IDO-1 can be regulated by STAT1 or STAT3, and thus we tested the total and phosphorylated STAT1/3 levels. We found that JAK1 was expressed and was activated only in NE cell lines and that STAT1 was activated only in those cell lines (Fig. 5b and S-Fig. 4a), even though STAT1 is expressed in all cells (Fig. 5c and S-Fig. 4a). In addition, when IDO-1 was expressed in CRPC cell lines, it was not regulated by STAT1, but rather, by STAT3, as expected, which was confirmed by the STAT3 and p-STAT3 data; in CRPC cells, STAT3 was phosphorylated in response to IFN- γ (Fig. 5d). This also indicated that STAT3 cannot determine PD-L1 expression. Another interesting phenomenon is that although STAT3 is not expressed in PC3 cells, PC3 cells were still able to secrete IDO-1 and PD-L1 (Fig. 5d), which indicated that the JAK1/STAT1 pathway can regulate the expression of both PD-L1 and IDO-1. In addition, STAT3 is constitutively activated in the NCI-H660 cell line, which is why IDO-1 is expressed in these cells without any treatment, while PD-L1 is not. We also tested the impact of Kyn on the JAK/STAT pathway and found that Kyn can up-regulate the phosphorylation of JAK1, STAT1 and STAT3 even though the total protein levels did not change. These results indicated that IDO-1 can increase JAK/STAT pathway activation and the expression of PD-L1 (Fig. 5e). Based on this result and the data shown in Fig. 1i, this can be considered an explanation for the findings of other studies that demonstrated that PD-L1 and IDO-1 are always co-expressed in settings of immunosuppression [27]. Then, we compared JAK1 expression between N-cadherin-overexpressing and N-cadherin KO cell lines to explore why PD-L1/IDO-1 can be induced in LNCap C1 and LNCap C2 cells. Fig. 5f and S-Fig. 4b show that JAK1 was expressed in LNCap C1 and LNCap C2 cells but not in LNCap C3 cells, and that N-cadherin KO in PC3 cells caused a decrease in JAK1 and p-JAK1 expression. Examination of the phosphorylated protein levels (Fig. 5g) demonstrated that the JAK/STAT pathway in LNCap C1 and C2 cells can be activated by IFN- γ , which supports the data in Fig. 5f.

Discussion

We systematically profiled cells in vitro, cells in in vivo models, and preclinical therapy to reveal a potential new targeted treatment strategy involving N-cadherin and the molecular mechanism by which N-cadherin regulates immunosuppression. N-cadherin is a major EMT marker, and as stated in previously publications, the EMT process can be reversed when N-cadherin is deleted [12]. EMT activation is a common feature in advanced prostate cancer, is always involved in the neuroendocrine differentiation process, and is considered a marker of advanced cancer. EMT enforces immunosuppression and helps cancer cells escape immunosurveillance, which is known to play a major role in tumor invasion. Previous studies have suggested that EMT is activated in a number of malignant tumor cells, which results in the loss of typical characteristics of epithelial (Epi) cells (cell-cell junctions and apical-basal polarity) and the gain of mesenchymal (Mes) cell properties to promote migration and complete the invasion-metastasis cascade [28]. Therefore, whether immune checkpoint improvements simply occur as tumors become more malignant or whether they are regulated by EMT still requires further exploration. In our study, we demonstrated that N-cadherin is an important hurdle for tumors overcome in order to generate a response to immunotherapy, as it regulates the expression of PD-L1/IDO-1. Thus, N-cadherin can be considered a therapeutic target in immunosuppression. This is the core finding of our research.

Some studies have proposed that PD-1 blockade induces an incomplete rescue in cancers where its level is high [29]. Furthermore, other studies [30,31] have demonstrated that IDO-1 and PD-L1 exert synergistic effects on tumor immune invasion, which is also in agreement with our data. This is the reason why combination treatment targeting both IDO-1 and PD-L1 has been explored; however, the best method would be to block the upstream pathway that regulates the expression of immunosuppression-related factors. Therefore, the first step of our research was validation of the role of N-cadherin in the secretion of PD-L1/IDO-1.

In our study, we found that the highest levels of N-cadherin and PD-L1/IDO-1 expression are observed in small cell prostate cancer and that PD-L1/IDO-1 are expressed at higher levels in N-cadherin-positive tumors. Recently, a new molecular link between EMT-up-regulated PD-L1 expression and CD8+ tumor-infiltrating lymphocyte (TIL) immunosuppression was established in human lung cancer [32]. EMT directly regulates the expression of PD-L1 and is associated with several other checkpoint ligands [32,33]. However, our research is the first to demonstrate that PD-L1 expression can be increased by the EMT marker N-cadherin in prostate cancer. Kolijn et al. [26] also proposed that IDO-1 can regulate N-cadherin, which can prompt prostate cancer cells to secrete IDO-1, but they did not evaluate PD-L1 and provided only mRNA data, not protein data. We extended this finding, revealed the mutual regulation between EMT and immune checkpoint molecules, and verified this finding in patient-derived tissues, cells and a tumor-bearing mouse model. We also found a coordinate expression between IDO-1 and PD-L1. This result led to a new hypothesis that combination treatment that targets both IDO-1 and PD-L1 can abrogate the immunosuppressive effect elicited by IFN- γ pathways that are active in the tumor microenvironment. However, the optimal therapy would still target upstream factors, and thus we provide evidence that N-cadherin inhibition can disrupt PD-L1 and IDO-1 expression. We used a tumor-bearing mouse model to

perform the treatment experiment. Our data shows that N-cadherin deletion can terminate the immunosuppression induced by this PD-L1/IDO-1 in vivo model.

Furthermore, we also revealed how N-cadherin mediates the IFNGR/JAK1/STAT1 pathway, which decreases antitumor immunity by PD-L1/IDO-1 secretion. We also found that N-cadherin can enhance JAK1 expression. Moreover, we observed that JAK1/STAT1 pathway activation was associated with higher expression of both PD-L1 and IDO-1. In contrast, JAK2 STAT3 was linked only to IDO-1 expression. The genomic loss of JAK1 occurred in some adenocarcinoma and CRPC cell lines [34], which is the reason some cell lines, especially adenocarcinoma cell lines, with a deficient IFN response failed to produce PD-L1/IDO-1. The expression of JAK1/2, STAT3, and PD-L1 increases during EMT, which has already been reported in lung cancer [19], and in agreement with our research, JAK1 can be rescued in LNCap C1/C2 cell lines that express JAK1. Our data explain the active N-cadherin feedback loop between immunosuppression and EMT. These findings provide insights into the molecules and signaling pathways involved in the interaction between EMT and other immune processes, which will hopefully promote the development of different therapeutic strategies aimed at boosting or suppressing specific EMT functions, depending on the pathological context. Above all, we defined a positive feedback loop, which is initiated by N-cadherin, between EMT and immune checkpoint protein expression. Moreover, targeting N-cadherin can significantly reverse immunosuppression, which is a very innovative discovery.

Although we used multiple model systems and human tissue, our study still had limitations. For example, the tumor xenograft model established in NSG mice lacks a tumor microenvironment composed of not only tumor cells but also lymphocytes. Additionally, IDO2 and TDO2 are also involved in Try degradation, but the roles of these two enzymes in EMT remain to be elucidated. The PC3 cell line is also controversial. PC3 is a special cell line, and usually, we do not classify it as SCC, even though it possesses some NE phenotypic characteristics, such as the expression of neuron-specific enolase (NSE). However, we sought to explore the association between N-cadherin and immunosuppression, and thus we considered PC3 to be an N-cad-positive cell line that expresses some NE markers. We also used two verified NE cell lines, LASCPC-01 and NCI-H660, in this experiment to provide more evidence.

Conclusion

In summary, this study revealed a new immunotherapy biomarker in N-cadherin. N-cadherin knockout can significantly decrease the PD-L1 and IDO-1 expression induced by an IFN response. Moreover, N-cadherin can rescue the expression of JAK1, and the IFN response will up-regulate the immunosuppressive factors PD-L1/IDO-1; this finding reveals the molecular pathway of how N-cadherin initiates immune escape. In addition, our data also show how the an IFN response can enhance the EMT process through induction of PD-L1/IDO-1 expression.

Abbreviations

EMT: epithelial-to-mesenchymal transition

SCC: small cell prostate cancer

Kyn: kynurenine

SCNC: small cell neuroendocrine prostate cancer

NED: neuroendocrine differentiation

PCa: prostate cancer

PD-L1: Programmed death ligand-1

IDO-1: indole amine 2,3-dioxygenase

NSE: neuron-specific enolase

H&E: hematoxylin and eosin

Declarations

Ethical Approval and Consent to participate: The mice experiment was approved by Duke ethical association

Consent for publication: Not applicable

Availability of supporting data: The raw data can be acquired from corresponding author Qiang Wei (arfuzinss@sina.com)

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Authors' contributions: Y.S., L.Y., H. X. and Q.W. contributed to the conception of the study; Y.S., L.Y., R.R., J. H. and J.G. performed the experiment; Y.S., L.Y., R.R., J. H. and J.G. contributed significantly to analysis and manuscript preparation; Y.S., J.H., R.R. and J.G. performed the data analyses and wrote the manuscript; Y.S., R.R., H.X., J.G. and Q.W. helped perform the analysis with constructive discussions.

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Figures

Figure 1

Expression of N-cadherin (N-cad), indoleamine-2,3-dioxygenase (IDO-1) and programmed death receptor-1 (PD-L1) in patient tissue samples and in cell lines. (a) Immunohistochemical staining for N-cad expression in benign, adenocarcinoma, castration-resistant prostate cancer (CRPC) and small cell

prostate cancer (SCC) tissue samples. (b) Immunohistochemistry (IHC) scores for N-cad expression in different tumors. (c) Expression of N-cad and E-cadherin (E-cad) in different cell lines. (d) Immunohistochemical staining for PD-L1 expression in different tumors. (e) IHC scores for PD-L1 expression in different tumors. (f) Expression of PD-L1 in different cell lines. (g) Immunohistochemical staining for PD-L1 expression in different tumors. (h) IHC scores of different tumors. (i) Linear relationship analysis of IDO-1 expression and PD-L1 expression. (j, k) Expression of PD-L1 and IDO-1 in N-cad-positive and N-cad-negative samples. (l) Expression of Chromogranin-A (Cg A) and neuron-specific enolase (NSE) in different cell lines.

Figure 2

Expression of N-cad, IDO-1 and PD-L1 in mouse tumors. (a) Tumor implantation site. (b) Flow cytometric analysis of mouse tumor cells after tissue dissociation. (c) Immunohistochemical staining for PD-L1 expression in different tumors. (d) Immunohistochemical staining for IDO-1 expression in different tumors. (e) mRNA expression of PD-L1 in different tumors after tissue dissociation and RNA extraction. (f) mRNA expression of IDO-1 in different tumors. (g) Immunohistochemical staining for IDO-1 expression in different tumors. (h) Hematoxylin and eosin (H&E) staining of mouse tissues.

overexpressing cells and N-cad KO cells after treatment with IFN- γ . (f) Immunohistochemical staining for PD-L1 expression in different tumors. (g) Immunohistochemical staining for IDO-1 expression in different tumors. (h) Hematoxylin and eosin (H&E) staining of mouse tissues.

Figure 4

Exploration of PD-L1 and IDO-1 expression regulated by interferon gamma receptor 1 (IFNGR1). (a) Immunohistochemical staining for IFNGR1 expression in benign, adenocarcinoma, CRPC and SCC tissue samples. (b) IHC scores for IFNGR1 expression in different tumors. (c) IFNGR1 expression in N-cad-positive and N-cad-negative cases. (d) Immunohistochemical staining for p-IFNGR1 expression in benign, adenocarcinoma, CRPC and SCC tissue samples. (e) IHC scores for p-IFNGR1 expression in different tumors. (f) p-IFNGR1 expression in N-cad-positive and N-cad-negative cases. (g) Immunohistochemical staining for IFNGR1 expression in mouse tissue samples. (h) Immunohistochemical staining for p-IFNGR1 expression in mouse tissue samples. (i) Expression of IFNGR1/p-IFNGR1 in different cell lines.

Figure 5

N-cadherin regulates PD-L1 and IDO-1 expression by impacting the JAK/STAT pathway. (a) Expression of PD-L1 and IDO-1 after JAK/STAT pathway blockade. (b, c, d) JAK/STAT pathway activation and PD-L1/IDO-1 expression. (e) Activation of the IFNGR/JAK/STAT pathway after treatment with Kyn. (f) The expression of JAK1/JAK3 in N-cad-overexpressing cells and N-cad KO cells. (g) The activation of the IFNGR/JAK/STAT pathway in N-cad-overexpressing cells and N-cad KO cells after treatment with IFN- γ .

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