Title

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Journal

Head & Neck, 43(11)

ISSN

1043-3074

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Publication Date

2021-11-01

DOI

10.1002/hed.26827

Peer reviewed

HHS Public Access

Author manuscript

Head Neck. Author manuscript; available in PMC 2021 December 10.

Published in final edited form as:

Head Neck. 2021 November ; 43(11): 3364–3373. doi:10.1002/hed.26827.

PD-L1 is upregulated via BRD2 in head and neck squamous cell carcinoma models of acquired cetuximab resistance

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Abstract

Background: Tumor models resistant to EGFR tyrosine kinase inhibitors or cisplatin express higher levels of the immune checkpoint molecule PD-L1. We sought to determine whether PD-L1 expression is elevated in head and neck squamous cell carcinoma (HNSCC) models of acquired cetuximab resistance and whether the expression is regulated by bromodomain and extraterminal domain (BET) proteins.

Methods: Expression of PD-L1 was assessed in HNSCC cell line models of acquired cetuximab resistance. Proteolysis targeting chimera (PROTAC)- and RNAi-mediated targeting were used to assess the role of BET proteins.

Results: Cetuximab resistant HNSCC cells expressed elevated PD-L1 compared to cetuximab sensitive controls. Treatment with the BET inhibitor JQ1, the BET PROTAC MZ1, or RNAi-mediated knockdown of BRD2 decreased PD-L1 expression. Knockdown of BRD2 also reduced the elevated levels of PD-L1 seen in a model of acquired cisplatin resistance.

Conclusions: PD-L1 is significantly elevated in HNSCC models of acquired cetuximab and cisplatin resistance where BRD2 is the primary regulator.

Keywords

cetuximab resistance; PD-L1 upregulation; BRD2; BET

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cause of cancer worldwide, affecting 600,000 people annually.[1,2] Current treatment strategies, including targeted therapy or conventional chemotherapy, are limited by associated toxicities

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Competing Interests: The authors declare no competing interests.

and/or lack of efficacy. The aggressive and complex biology of HNSCCs has also challenged the clinical development of viable therapeutic options. In 2006, the Food and Drug Administration (FDA) approved cetuximab, a chimeric monoclonal antibody (mAb) targeting the epidermal growth factor receptor (EGFR), for the treatment of HNSCC, but only a subset of patients initially respond to cetuximab.[3] Further, most of the individuals who initially demonstrate a cetuximab response will ultimately succumb to disease progression, highlighting the need to identify underlying mechanisms of drug resistance to improve treatment response.

We previously demonstrated that JQ1, a bromodomain and extraterminal protein (BET) inhibitor, potentiated the anti-tumor effects of cetuximab and prevented tumor regrowth in cetuximab-treated patient-derived xenografts (PDXs).[4] The anti-tumor effect elicited by cetuximab plus JQ1 was accompanied by downregulation of receptor tyrosine kinases (RTKs), including HER3 and AXL. BET proteins are characterized by two tandem bromodomains (BRDs) at the N-terminus and an exclusive Extra Terminal (ET) domain at the C-terminus.[5] BET proteins bind to acetylated promoter and enhancer regions of chromatin via their BRD domains, and facilitate increased gene expression. Selective knockdown of BRD4 using siRNA downmodulated AXL expression in HNSCC and breast cancer cell lines, implicating BRD4 as a key mediator of upregulated RTK expression in cetuximab resistance.[4,6] These results demonstrated *in vitro* and *in vivo* role for BET proteins in mediating cetuximab resistance by highlighting the importance of BRD4, but the potential role of BRD3 and BRD2 in driving cetuximab resistance remains unclear.

Emerging evidence suggests BET proteins regulate the expression of “immune checkpoint” proteins.[7,8] Zhu and colleagues reported that BRD4 directly binds to the promoter region of *CD274*, the gene encoding PD-L1, a ligand for the checkpoint receptor protein PD-1 expressed by T-lymphocytes.[9] Activation of the PD-L1/PD-1 signaling axis in T-lymphocytes is known to impair T-cell-mediated immune surveillance in HNSCC. Similarly, the PD-L1/PD-1 axis negatively regulates the cytotoxic activity of NK cells.[10,11] Thus, PD-L1 expression levels on tumor cells may play a key role in modulating anti-tumor immunity in the HNSCC tumor microenvironment (TME) and drive drug resistance.

In the present study, we hypothesized that drug resistant HNSCC preclinical models would exhibit BET-dependent upregulation of PD-L1. In support of this hypothesis, we observed higher PD-L1 levels in a panel of cetuximab and cisplatin resistant HNSCC cell lines. Using isogenic HNSCC cell line models of acquired cetuximab resistance, pharmacologic inhibition of BET proteins decreased PD-L1 expression. Genetic knockdown of BRD2, but not BRD4, suppressed PD-L1 expression indicating that BRD2 is the primary BET protein mediating expression of PD-L1 in CTXR cells. Regulation of PD-L1 in cisplatin resistant cell lines was also BRD2-dependent. Our collective findings suggest that targeting the BRD2/PD-L1 pathway could reverse cetuximab resistance by both tumor intrinsic and extrinsic mechanisms.

MATERIALS AND METHODS

Cell Lines and Reagents

All cell lines were cultured in DMEM (Fisher), 10% fetal bovine serum (Gemini Bio products) and 1% penicillin-streptomycin (Life Technologies). PE/CA-PJ49 and FaDu were purchased from Sigma-Aldrich and American Type Culture Collection (ATCC; Manassas, VA), respectively. All cell lines were authenticated periodically (at least every 6 months) via STR profiling (at the UC Berkeley Sequencing Core). Mycoplasma testing was performed periodically on all cell lines used in this study. Cetuximab resistant clones of PE/CA-PJ49 and FaDu were generated previously.[4] PE/CA-PJ49 and CAL33 cell lines were treated with 500nM cisplatin and surviving clones were selected and continually cultured in increasing doses up to 5uM. CAL33 cells were previously derived from a tongue SCC and were kindly provided by Dr. Gerard Milano (Centre Antoine-Lacassagne, Nice, France). Cisplatin resistance was confirmed by performing viability assays in comparison to their respective parental lines. JQ1 was provided by Dr. James Bradner (Dana Farber Institute) for *in vitro* studies and MZ1 was purchased from Tocris Bioscience (Bristol, UK).

Crystal violet viability assay

Cell Lines were seeded in 96 well plates followed by cetuximab or cisplatin exposure for 96 hours. Viability was assessed after staining the cells with crystal violet for 30 minutes. The crystal violet solution was rinsed off with tap water, the plate was dried overnight. Crystal violet was dissolved with 5% SDS solution and the absorbance was determined using a colorimetric plate reader as previously described.[4]

Flow Cytometry

Parental and CTXR clones were seeded in 6 cm dishes and treated in the absence or presence of inhibitors for 72 hours. Cells were detached with 0.05% trypsin, counted and incubated with PD-L1/PE-Cy7 antibody or PE-Cy7 isotype control (1:300; Biolegend) for 15 minutes in the dark on ice. Cells were washed and resuspended in FACS buffer and analyzed on a FACS Calibur Dx instrument. Data was analyzed using FlowJo Software.

Immunoblotting

Cells were washed with cold PBS and lysed with RIPA lysis buffer. Protein was quantified and lysates were resolved by SDS-PAGE, transferred to PVDF membranes, before an overnight incubation with primary antibodies. Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (BioRad) for 1 hour followed by visualization of immunoreactive bands by chemiluminescence (Santa Cruz Biotechnology). Antibodies used for immunoblotting included: PD-L1 (CST;#13684; 1:1000), β-actin (Abcam; ab6276. 1:5000), BRD4 (Abcam; 128874. 1:1000), BRD2 (CST; #5848. 1:1000).

To measure PD-L1 glycosylation, PE/CA-PJ49 and FaDu parental and CTXR lysates were treated with or without PNGaseF (New England BioLabs) according to the manufacturer's instructions. The lysates were then resolved by SDS-PAGE as described earlier.

RT-PCR

Messenger RNA was isolated from cells using the RNAeasy Kit (Qiagen), followed by cDNA synthesis using the iSCRIPT cDNA Synthesis Kit (Bio-Rad). PCR reactions were executed using the SYBR Green Master Mix (Bio-Rad) and the Bio-Rad CFX96 cycler. Relative mRNA levels were quantified following normalization to the housekeeping gene, *Tata-binding protein* (*TBP*). Primers for *CD274* were: forward: 5'- TGGCATTTGCTGAACGCATTT-3' and reverse: 5'-TGCAGCCAGGTCTAATTGTTTT-3'.

RNA interference

Parental and cetuximab-resistant clones were transfected with 50 pmol of pooled siRNA oligonucleotides and Lipofectamine RNAiMAX (Life Technologies). BRD4- and BRD2-specific siRNAs were ordered through Sigma-Aldrich. Sequences for BRD4 siRNAs have been described previously.[8] Sequences for BRD2 siRNA were: BRD2 siRNA#1: 5'-CAAGAAAGCGAAUGAGAAA-3', and BRD2 siRNA#2: 5'-CAGAAGAGAUUGAGAUUGA-3'.

Chromatin Immunoprecipitation

FaDu parental and the isogenic cetuximab-resistant subline CTXR#3 cells were cultured to 70 – 80% confluency, and 24 hours after seeding the cells, freshly prepared complete cell fixation solution (CFS) was used to fix the cells according to the manufacturer's instruction (Active Motif #53040). The Active Motif Kit was used to perform chromatin immunoprecipitation (ChIP). Following cell lysis, chromatin was sonicated and prepared for immunoprecipitation. Sheared chromatin was immunoprecipitated with normal IgG (1:200; CST, #3900), BRD2 (1:200; Active Motif, #61797), BRD3 (1:200; Active Motif, #61489), or BRD4 (1:200; Active Motif, #39909) antibodies. Prior to immunoprecipitation, 500 ng of the sheared chromatin from each treatment group was removed and used as the input. After precipitating the antibody-bound protein/DNA complexes, they were incubated with Protein G agarose beads at 4°C for 3 hours. Beads were washed and the protein DNA complexes were eluted according to manufacturer's protocol. The protein-DNA crosslinks were reversed via proteinase K digestion at 55 °C for 30 minutes followed by a 2 hour incubation at 80°C. DNA was purified according to the manufacturer's protocol and prepared for real-time quantitative PCR. PCR reactions were performed using SYBR Green Master Mix and a primer set for the *CD274* promoter region. *CD274* promoter primers were: forward, 5'-AAGCCATATGGGTCTGCTC-3' reverse, 5'-TTATCAGAAAGGCGTCCCCC-3'. Fold enrichment was calculated using the formula: The cycle number derived from qPCR analysis from pulling down either BRD2, BRD3, or BRD4, normalized to IgG, and converted to percent enrichment.

Statistics

To assess significance between treatment groups for the *in vitro* studies, a Student *t*-test (two tailed) or a One-way ANOVA (two-tailed) with Dunnett's post-hoc test was used to determine significance with P values at least < 0.05 categorized as statistically

significant. Error bars for all figures represent SEM and unless otherwise stated, figures are representative of at least three independent experiments.

RESULTS

Cetuximab-resistant HNSCC models express elevated PD-L1 levels

We previously generated two isogenic HNSCC cell line models (PE/CA-PJ49 and FaDu) of acquired cetuximab resistance.[4] To determine the expression of PD-L1 in these models we performed qRT-PCR, immunoblotting and flow cytometry in CTXR clones of PE/CA-PJ49 (CTXR1, CTXR3, CTXR4) and FaDu (CTXR2, CTXR3). Immunoblotting analysis illustrated elevated PD-L1 levels in CTXR cell lines compared to their parental counterparts (Fig. 1A). In all cases, CTXR clones expressed ~2-3 fold higher *CD274* mRNA levels (Fig. 1B). PD-L1 glycosylation stabilizes PD-L1 against proteosomal degradation and is indicative of membranous expression.[12] We confirmed that the elevated PD-L1 observed in the CTXR clones was glycosylated. Parental and CTXR lysates were pre-treated with PNGaseF, which cleaves and removes N-linked oligosaccharides from glycoproteins.[13] PNGaseF-treated CTXR lysates displayed diminished expression of the higher molecular weight PD-L1 leaving a single band at approximately 36 kDa which represents native PD-L1(Suppl. Fig 1).[14]

To confirm the elevated expression of membranous PD-L1, flow cytometric analysis of surface PD-L1 was performed. Similar to results observed from immunoblotting, increased expression of PD-L1 was observed in CTXR cells compared to their parental counterparts (Figs. 1C, D). Collectively, these findings illustrate that acquisition of cetuximab resistance in these two preclinical models is accompanied by upregulation of the immune checkpoint ligand PD-L1.

BET inhibition suppresses PD-L1 expression

We previously reported that BET inhibition sensitized cetuximab-resistant cell lines and HNSCC PDXs to cetuximab treatment by abrogating the transcriptional upregulation of RTKs.[4] Additionally, studies have indicated that BRD4 occupies the *CD274* promoter region and regulates its transcription.[9,15] Therefore, we investigated the potential role of BET proteins in regulating PD-L1 expression levels by first employing the pharmacologic inhibitor JQ1. JQ1, an inhibitor of BET proteins (BRD2, BRD3 and BRD4), exhibits its highest affinity for BRD4 and its lowest to BRD2.[5,16-18] Hence, low concentrations (50nM) of JQ1 can be used to selectively inhibit BRD4, while higher concentrations (500nM) exhibit pan-inhibitory activity.[19] Parental and CTXR clones were treated with 50 or 500nM JQ1 for 72 hours. Treatment with 500nM JQ1 resulted in decreased PD-L1 expression in both PE/CA-PJ49 and FaDu CTXR clones, while 50nM JQ1 had little, if any, effect. (Figure 2A, B). This observation indicates that upregulation of PD-L1 in the acquired cetuximab resistance models is not mediated by BRD4, but is dependent on BRD2 or BRD3.

To further interrogate the contribution of individual BET family members on PD-L1 expression, we used MZ1, a JQ1-based Proteolysis Targeting Chimera (PROTAC) degrader of BET proteins.[5,17,20] MZ1 degrades only BRD4 at lower concentrations and all 3 BET

proteins at higher concentrations. In the FaDu parental and CTXR#3 cell line, 50nM MZ1 downregulated only BRD4 expression while 500nM MZ1 reduced expression of both BRD4 and BRD2 (Fig. 2C). Experiments were focused on BRD4 and BRD2, the BET proteins with the highest or lowest affinity for JQ1, respectively. Expression of BRD3 was not assessed. In the FaDu CTXR cell line, PD-L1 expression was only downregulated with 500nM MZ1, but was unchanged with 50nM MZ1 treatment. These results confirm a role for BRD proteins in the elevated expression of PD-L1 in our CTXR HNSCC models. Moreover, these observations indicate that PD-L1 expression is regulated by either BRD3, BRD2 or, potentially, a combination of BRD4, BRD3 and BRD2.

Knockdown of BRD2 reduces PD-L1 levels in cetuximab resistant HNSCC models

The observation that 500nM MZ1 decreased PD-L1 expression indicated that BRD2 or BRD3 alone or some combination of BRD4, BRD3 and BRD2 regulates PD-L1 expression. To address the individual contribution of specific BET proteins on PD-L1 expression, we performed RNAi-mediated knockdown of BRD4 or BRD2 in the CTXR clones (BRD3 was not assessed). FaDu parental and CTXR clones were transfected with either non-targeting control (NTC), BRD4, or BRD2 siRNAs for 72 hours and knockdown efficiency was confirmed by immunoblotting (Fig. 3A). As previously described by others ([21]) and shown in Figure 2C, the BRD4 antibody primarily detects BRD4 at ~115kd and a shorter isoform at ~100kd. Both isoforms were observed at longer exposures by immunoblotting and downmodulated with siRNA or MZ1. Interestingly, immunoblotting analysis showed that BRD4 knockdown did not suppress PD-L1 expression but instead appeared to modestly increase its expression. However, knockdown of BRD2 was sufficient to decrease PD-L1 protein expression in CTXR clones (Fig. 3A; Suppl. Fig. 2). These findings were confirmed using flow cytometry (Fig. 3B). Specifically, in the PE/CA-PJ49 CTXR#4 and FaDu CTXR#3 clones, an approximate 20% decrease in PD-L1 positivity was observed with BRD2 siRNA vs NTC siRNA. Additionally, treatment with BRD2 siRNA decreased *CD274* mRNA by more than 50% compared to NTC siRNA across the CTXR clones (Fig. 3C).

We next investigated the transcriptional role of BRD2 compared to BRD4 and BRD3 in the cetuximab-resistant clones. We examined the enrichment of BET family members on the *CD274* promoter in the FaDu parental and CTXR#3 cells. We performed a chromatin immunoprecipitation (ChIP) assay and observed significant enrichment of BRD2 at the *CD274* promoter in the CTXR#3 versus parental cells (Fig 3D). Consistent with previous reports, BRD4 was also observed on the *CD274* promoter but there was no significant difference in BRD4 or BRD3 occupancy on the *CD274* promoter in the parental versus CTXR#3 cells.[9,15] Overall, these studies indicate that BRD2, but not BRD4, plays a critical role in modulating PD-L1 expression in HNSCC models of acquired cetuximab resistance

PD-L1 is also upregulated in cisplatin-resistant HNSCC

Ock and colleagues previously observed that PD-L1-negative HNSCC patients displayed a significant increase in PD-L1 expression in the tumor following cisplatin treatment.[22] Others observed elevated PD-L1 expression levels in HNSCC cell lines with acquired resistance to cisplatin.[23,24] In addition, acquired resistance to cisplatin in small cell lung cancer (SCLC) was accompanied by elevated PD-L1 expression.[25] Based on these observations, we

sought to determine whether BET regulation of PD-L1 was observed in other drug-resistant models of HNSCC. First, we established cisplatin-resistant (CISR) cell lines. PE/CA-PJ49 and CAL33 parental cells were treated with increasing doses of cisplatin (500nM to 5μM) and surviving clones were selected over the course of four months and cultured in cisplatin until they displayed resistance to cisplatin compared to the parental line. In contrast to parental cells, the CISR clones demonstrated no decrease in viability following cisplatin treatment (Fig. 4A; Suppl. Fig. 3A). Similar to CTXR clones, PE/CA-PJ49 and CAL33 CISR clones also displayed elevated levels of PD-L1 (Fig. 4B; Suppl. Fig 3B). To determine whether BRD4 or BRD2 regulated PD-L1 expression in the CISR clones, we transfected PE/CA-PJ49 parental and CISR clones with NTC, BRD4 or BRD2 (but not BRD3) siRNA. Knockdown of BRD2, but not BRD4, decreased the elevated PD-L1 protein levels observed in two out of the three CISR clones (Fig. 4B). Interestingly, as in the CTXR cells, knockdown of BRD4 in the CISR clones resulted in unexpected upregulation of PD-L1. Treatment with 500nM JQ1 resulted in decreased expression of PD-L1 in CAL33 CISR clones, similar to what was seen in JQ1-treated CTXR cells (Suppl. Fig.3B). Collectively, these results indicated that, 1) BET inhibition decreases the elevated PD-L1 levels observed in cisplatin resistant HNSCC models, 2) BRD2 regulates expression of the immune checkpoint PD-L1 in the context of acquired drug resistance in HNSCC.

DISCUSSION

Mechanisms of drug resistance in cancer have mainly been investigated in the context of tumor intrinsic mechanisms. However, in view of the promising clinical results seen with immunotherapy in HNSCC, understanding the immune related mechanisms of drug resistance is also important. We previously demonstrated that resistance to the FDA-approved EGFR inhibitor cetuximab in HNSCC was driven by the upregulation of multiple RTKs which was abrogated by the pharmacological BET inhibitor JQ1.[4] With emerging literature highlighting the impact of cetuximab treatment on increased activity of PD1-expressing immunosuppressive cell populations, we sought to determine the impact of BET inhibition on expression of the PD1 ligand, PD-L1, in HNSCC models of acquired cetuximab resistance.[26,27] We generated these HNSCC models of acquired cetuximab resistance by isolating clones from HNSCC cell lines grown in media containing increasing concentrations of cetuximab (up to 500 nmol/L) over the course of 6 months.[4] These cells expressed elevated levels of the immune checkpoint PD-L1 compared with their isogenic, cetuximab-sensitive, parental controls. At higher concentrations, pharmacological inhibition using JQ1 and its PROTAC derivative, MZ1, decreased PD-L1 expression in the cetuximab-resistant cell lines. We also observed that RNAi-mediated knockdown of BRD2, but not BRD4, decreased PD-L1 expression in the CTXR cells. Cisplatin is the most commonly used chemotherapy agent for the treatment of HNSCC. In addition to CTXR models, we also detected elevated PD-L1 in HNSCC models of acquired cisplatin resistance. As seen with cetuximab-resistant cells, BRD2 knockdown decreased the elevated PD-L1 levels in the cisplatin-resistant cells, suggesting that BRD2-mediated upregulation of PD-L1 could represent a more general mechanism of acquired drug resistance *in vivo*.

Other studies have demonstrated that PD-L1 expression is induced by multiple therapeutic agents in breast and SCLC cancer models.[25,28] In breast cancer, PD-L1 expression was

transiently elevated by cellular stress pathway components upon treatment with multiple cytotoxic agents. Additionally, in SCLC models of acquired cisplatin resistance, PD-L1 expression was augmented and shown to be dependent on DNA methyltransferase 1 (DNMT1) and the RTK, KIT. However, the role of BET proteins was not evaluated in either of these previous studies. BET proteins are readers of acetylated histones found at enhancer and super enhancer chromatin regions associated with euchromatin.[29] Increased understanding of the dynamic chromatin landscape with acute and long-term drug treatment influenced by BET proteins will help elucidate the therapeutic implications of BET inhibition in overcoming cetuximab and/or cisplatin resistance in HNSCC.

A more in-depth genomic analysis of acquired cetuximab resistance was performed and reported in the SCC25 HNSCC cell line.[30] Interestingly, in this acquired cetuximab resistance model, elevated PD-L1 mRNA levels were not observed by RNA-seq analysis, however a significant increase in epigenetic alterations was observed over time as the cell lines developed resistance to cetuximab. Further, single cell RNA-Seq and ATAC-seq analysis in this SCC25 model identified changes in chromatin accessibility after just 5 days of cetuximab treatment.[31] Moreover, this study found that JQ1 treatment overcame cetuximab resistance through decreasing EMT markers and consequently decreasing the migratory/invasive phenotype *in vitro*. PD-L1 has been previously shown to mediate increased proliferation, migration and EMT in lung cancer models.[32]

Multiple studies have indicated that BRD4 is bound to the *CD274* promoter and regulates its transcription.[9,15] Although, we also observed BRD4 occupancy on the *CD274* promoter in the FaDu parental and CTXR clones (Figure 3D), knocking down BRD4 by RNAi or MZ1 did not decrease PD-L1 expression. Interestingly, unlike BRD3 and BRD4, we observed elevated occupancy of the *CD274* promoter only with BRD2 in CTXR cells. These results indicate that BET proteins may differentially regulate the transcriptional landscape that mediates drug resistance. BRD2 and BRD4 have previously been reported to have distinct transcriptional regulatory roles.[33,34] BRD2 has been shown to interact with transcription factors resulting in transcriptional regulation of specific oncogenic genes including STAT3 and NF-κB.[33,35,36] Both STAT3 and NF-κB have been shown to be key transcriptional regulators of PD-L1 in cancer.[37] It was also reported that elevated PD-L1 expression observed in HNSCC models of acquired cisplatin resistance was dependent on IL-6.[23] Our group recently demonstrated that IL-6 expression was elevated in HNSCC models of cetuximab resistance and the expression was abrogated by JQ1 treatment.[38] These combined reports indicate that in addition to reading acetylated histones, BRD2 may facilitate occupancy of promoter regions with key transcription factors and influence expression of key oncogenic genes indirectly.

In conclusion, blocking the key epigenetic BET family members in combination with cetuximab may have a very promising tumor intrinsic and extrinsic effect in HNSCC through decreased cellular growth and enhanced anti-tumor immunity. Further studies employing *in vitro* immune co-cultures and relevant immunocompetent HNSCC mouse models are required to strengthen the rationale and understanding of this therapeutic strategy. Clinical studies are needed to fully understand the impact of targeting BRD2 in conjunction with immune checkpoint inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

R35CA231998, U54CA209891, and T32DE007306.

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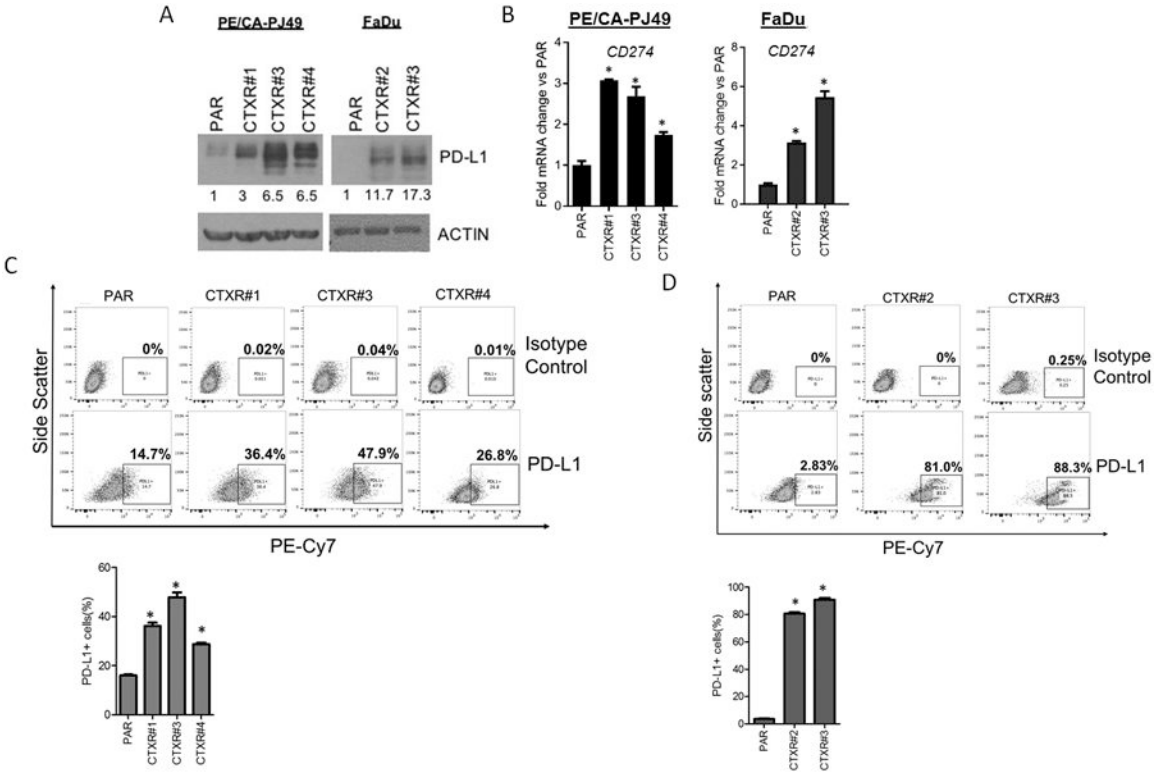

Figure 1. Cetuximab-resistant HNSCC cells display elevated PD-L1 levels.

(A) Immunoblot analysis of PE/CA-PJ49 and FaDu parental (PAR) and cetuximab-resistant clones (CTXR) for PD-L1 and Actin. Densitometric ratios of PD_L1 bands to Actin bands were determined by Image J and indicated as fold change to Parental. (B) Messenger RNA was extracted from PE/CA-PJ49 and FaDu PAR and CTXR clones, reverse transcribed and subjected to qRT-PCR for CD274 (n=3). *CD274* expression was normalized to the housekeeping gene TATA Binding protein (TBP). (C) Flow cytometric analysis for membrane PD-L1 was performed with PE/CA-PJ49 and (D) FaDu PAR and CTXR clones (n=3). *$p < 0.05$ (One-way ANOVA with Dunnett's post-hoc test). Error bars represent SEM.

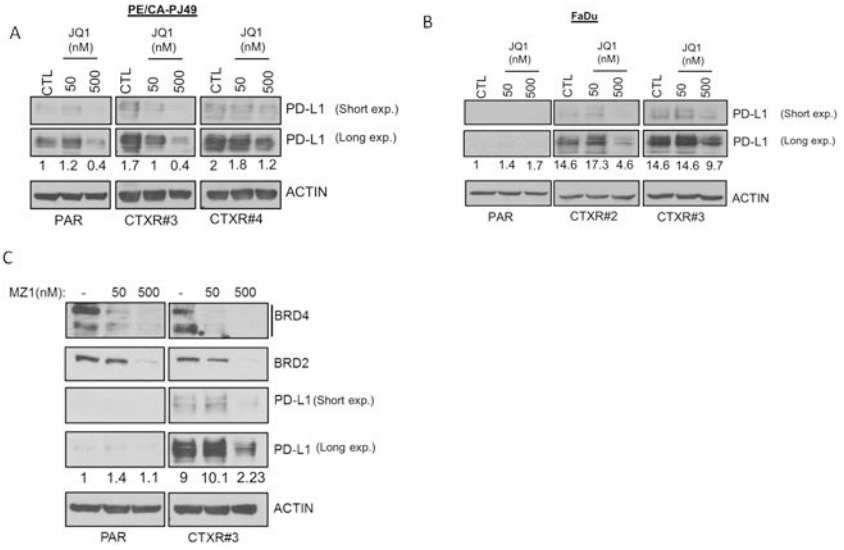

Figure 2. BET inhibition and downregulation decreases PD-L1 expression in CTXR HNSCC models.

(A) PE/CA-PJ49 and (B) FaDu parental and CTR clones were treated with vehicle, 50 and 500nM of JQ1 for 72 hours. Immunoblot analysis for PD-L1 and Actin was performed. (C) FaDu parental and CTXR clones were treated with vehicle, 50 and 500nM MZ1 for 72 hours. Immunoblotting analysis for BRD4, BRD2, PD-L1 and Beta-actin was evaluated. Densitometric ratios of PD-L1 bands to Actin bands were determined by ImageJ and represented as a fold change to the Parental control lanes.

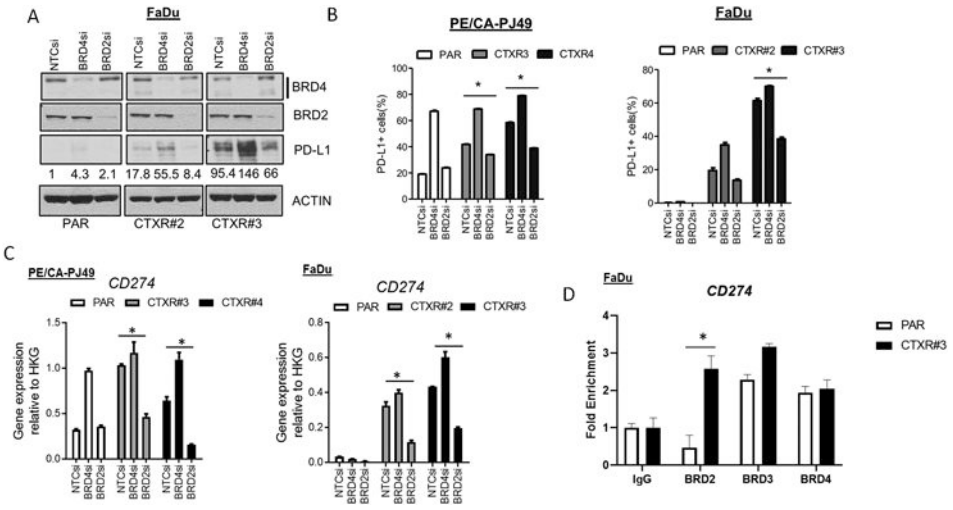

Figure 3. BRD2 knockdown decreases PD-L1 expression in HNSCC CTXR models.

(A) FaDu parental and CTXR cells were transfected with non-targeting control (NTC), BRD4 and BRD2 siRNAs for 72 hours. Protein expression levels of BRD4, BRD2, PD-L1 and beta-actin was determined by immunoblotting. Densitometric ratios of PD-L1 bands to Actin bands were determined by ImageJ and represented as a fold change to Parental NTC lane. (B) PE/CA-PJ49 and FaDu parental and CTXR clones were transiently transfected with siRNAs as described in (A). Flow cytometric analysis of PD-L1-positive cells was determined and (C) mRNA levels of CD274 was evaluated by qRT-PCR (n=3)* $p < 0.05$ (Student's t-test). (D) Chromatin immunoprecipitation analysis of IgG, BRD2, BRD3 and BRD4 proteins enriched on the CD274 promoter was assessed in the FaDu parental and CTXR#3 clone (n=2). * $p < 0.05$ (Student's t-test). Error bars represent SEM.

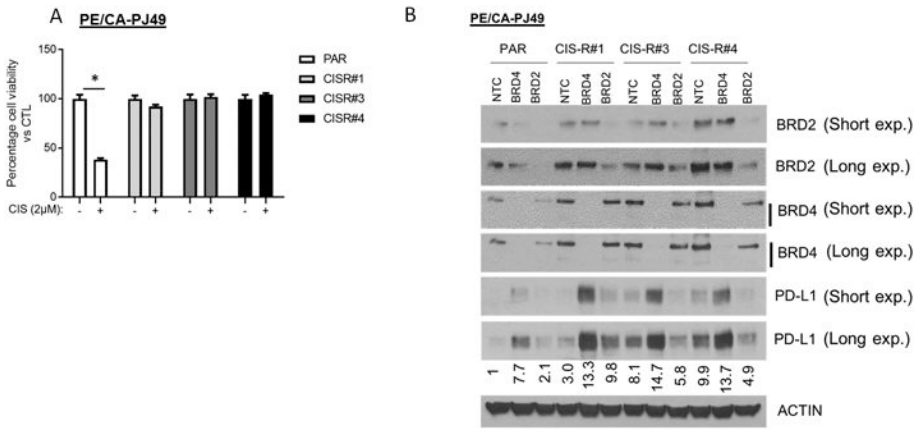

Figure 4. BRD2 knockdown decreases PD-L1 expression in cisplatin-resistant HNSCC model.

(A) PE/CA-PJ49 cells were exposed to a high concentration of cisplatin and the resistant clones were continually treated with cisplatin. Resistance to cisplatin in three different clones (#1, #3 and #4) was evaluated. Parental and CISR clones were treated with 2uM of cisplatin for 96 hours. Cell viability was determined by crystal violet assay (n=3) *$p<0.05$ (Student's t-test); Error bars represent SEM. (B) PE/CA-PJ49 PAR and CISR clones were transiently transfected with NTC, BRD4 and BRD2 siRNAs for 72 hours. Lysates were evaluated for BRD2, BRD4, PD-L1 and beta-Actin. Blots of short and long exposures for BRD2, BRD4 and PD-L1 are illustrated. Densitometric analysis of PD-L1 bands to Actin bands were determined by Image J and represented as a fold change to Parental NTC lane.