Cellular Immunology 336 (2019) 66–74

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Epigenetic modulation enhances immunotherapy for hepatocellular carcinoma

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ARTICLE INFO

Presented at Clinical Congress Meeting sponsored by the American College of Surgeons in San Diego, October 2017

Keywords:
Epigenetic modulation
Checkpoint inhibitors
Immunotherapy
HCC

ABSTRACT

Background: Anti-PDL-1 immunotherapy for Hepatocellular Carcinoma (HCC) demonstrated a mixed response. Polycomb Repressor Complex 2(PRC2) contributes to the initiation and progression of HCC by suppressing tumor antigens and inhibiting an immune response. Two components of epigenetic modulation are Enhancer of Zeste Homolog 2 (EZH2, the catalytic component of PRC2) and DNA Methyltransferase 1 (DNMT1). We aim to investigate the potential role of epigenetic therapy targeting EZH2 and DNMT1 as a novel strategy to modulate immunotherapy response in HCC.

Methods: HepG2, Hep3B, and Hepa1-6 HCC cell lines were treated with EZH2 inhibitor (DZNep) and DNMT1 inhibitor (5-Azacytidine) with and without anti-PDL-1. Quantitative RT-PCR and immunohistochemistry were performed to evaluate the expression of tumor suppressors, tumor antigens, and Th1 chemokines. In-vivo C57/LJ immunocompetent mice model with subcutaneous tumor inoculation was performed with intraperitoneal drug injections.

Results: There was a significant upregulation of Th1 chemokines in HepG2 (CXCL9 5.5 ± 0.2 relative fold change; CXCL10 1.44 × 103 ± 37 relative fold change) and Hep3B (CXCL 9 6.85 × 103 ± 1.3 × 103 relative fold change; CXCL 10 2.15 × 103 ± 3.1 × 102 relative fold change). Additionally, there was a significant induction of cancer testis antigens NY-ESO-1 (3.6–3.7 ± 0.3 relative fold change) and LAGE (8.3–11.7 ± 1.9 relative fold change). In vivo model demonstrated statistically significant tumor regression in the combination treatment group (0.02 g ± 0.02) compared to epigenetic therapy (0.63 g ± 0.61) or immunotherapy alone (0.15 g ± 0.21) with untreated control (2.4 g ± 0.71). There was significantly increased trafficking of cytotoxic T- lymphocytes and associated apoptosis for the combination treatment group compared to epigenetic or immunotherapy alone.

Conclusions: This study demonstrates that epigenetic modulation could be a novel potential strategy to augment immunotherapy for HCC by stimulating T cell trafficking into tumor microenvironment via activation of transcriptionally repressed chemokine genes responsible for *T*-cell trafficking, inducing previously silent neoantigens for immune targets, and allowing tumor regression as a result. A clinical trial of this feasible combination therapy of these clinically available agents is warranted.

1. Introduction

Hepatocellular carcinoma (HCC) affects both men and women with over 4,292,000 estimated new cases and an estimated 2,814,000 deaths in China alone in 2015 [1]. In the United States, there has been a rise in the incidence of HCC from 2.6 per 100,000 in 1975 to 8.6 per 100,000 in 2011 due to increasing Hepatitis C virus infections and rising morbid obesity [2]. Treatment options, thus far, are limited in advanced HCC

Abbreviations: CXCL, chemokine (C-X-C motif) ligand; DNMT1, DNA Methyltransferase 1; EZH2, Enhancer of Zeste Homolog 2; HCC, hepatocellular carcinoma; PRC2, Polycomb Repressor Complex 2; RT-PCR, reverse transcription polymerase chain reaction

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https://doi.org/10.1016/j.cellimm.2018.12.010
Received 4 October 2018; Received in revised form 28 December 2018; Accepted 31 December 2018
Available online 02 January 2019
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with a marginal 2- to 3-month survival benefit associated with tyrosine kinase inhibitors (TKI's) such as sorafenib [3] or second-line regorafenib [4]. Furthermore, of the patients who progress on first-line therapy of sorafenib, only 30% proceed to receive second-line regorafenib [5], because morbidity related to disease progression often limits them.

Given the lack of effective alternative systemic therapy for HCC, the Food and Drug Administration (FDA) recently approved nivolumab, a PD1 inhibitor, for patients who progressed or were intolerant of first-line therapy with sorafenib for HCC as a result of Phase I/II Checkmate despite a marginal objective response rate of 20% [6]. Checkpoint inhibitors have historically have been ineffective for solid organ gastrointestinal malignancies due to the low rate of somatic mutations compared to melanoma or non-small cell lung cancer [7,8]. However, given the modest efficacy in HCC, strategies to improve its potential response rate is critical.

Epigenetic aberrations have been previously shown to contribute directly to the poor prognosis and survival outcome for many malignancies, including HCC [9]. Some of the critical epigenetic drivers are Enhancer of Zeste Homolog 2 (EZH2), a catalytic member of Polycomb Repressor Complex 2 (PRC2), and DNA methyltransferase (DNMT1). These proteins cause aberrant methylations of various histone tails in chromatin and promoter region in malignancies that cause transcriptional silencing of various gene expression, including those that affect immune function [10,11], resulting in the favorable tumor microenvironment.

2. Methods

The objective of the study is to demonstrate a potential therapeutic strategy to augment checkpoint immunotherapy by epigenetic modulation in hepatocellular carcinoma via combining epigenetic therapies targeting EZH2 and DNMT1 with anti-PDL-1 monoclonal antibodies. We aim to investigate the potential role of epigenetic therapy targeting EZH2 and DNMT1 as a novel strategy to modulate immunotherapy response in HCC by increasing tumor antigen expression, facilitating *T*-cell trafficking, and improving tumor regression.

2.1. Cell lines, drug treatments, and animals

Hepatocellular carcinoma cell lines HepG2, Hep3B (human cell lines) and Hepa1-6 (murine cell line), were obtained from the American Type Culture Collection (ATCC). The cell lines were maintained in DMEM media (Corning Cellgro) with 10% FBS (Sigma Aldrich, MO) and Pen/Strep (Corning Cellgro). NCI-DCTD provided validation of the cell lines upon purchase. The 5-azacytidine (5-AZA) drug was obtained from Cayman Chemicals; 3-Deazaneplanocin A (DZNep) from Sigma Aldrich, MO; interferon-gamma (IFN-y) from Millipore; and murine anti-PD-L1 antibody (#BE0101; clone 10F.9G2) from BioXcell, NH.

2.2. Cell culture and treatments

For in vitro experiments, 1.0 × 105 cells were seeded in 6-well tissue culture plates overnight and replaced with fresh DMEM culture media the next day. These cells were treated with individual and combined doses of 5 uM of 5-azacytidine, 5 uM of 3-Deazaneplanocin A (DZNep), and 10 ng/ml IFN-y. The doses of 5-azacytidine and DZNep have been previously utilized in the literature and dose demonstrated to effectively inhibit DNMT1 and EZH2, respectively [12,13]. The cells were treated for 48 h, washed with PBS, and harvested for western blot and qRT-PCR analysis.

2.3. Generation of stable cells expressing shRNA constructs

Commercially available fourth-generation lentivirus packing system (Lenti-X, Takara-Clontech) was used for generating stable cell lines. Blasticidin-resistant – shDNMT1 (Vectorbuilder, CA) and puromycin-resistant shEZH2 (Sigma-Aldrich, MO) vectors were obtained, amplified, and purified for packing. For generating lentivirus, the Lenti-X reagent was mixed with 6 μg of shRNA plasmid and transfected in 293 T cell line for packing. The harvested lentivirus was used to transduce target cell lines using polybrene with established protocol. Hepa1-6 was transduced with shRNA targeting EZH2 (shEZH2), DNMT1 (shDNMT1), or both sham sequences (shControl) (Sigma-Aldrich) according to manufacturer's instructions. Cell lines were selected with puromycin (Sigma-Aldrich) and expanded after confirmation of knockdown by qRT-PCR and immunoblot.

2.4. In vivo experiments

All animal procedures were approved by IACUC and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals to establish the orthotopic immunocompetent HCC murine model. Male C57/LJ mice were injected in the subcutaneous flank with 5×10^5 Hepa1-6 cells suspended in sterile PBS. After 7–10 days, all mice were randomly assigned to receive either saline, drugs alone (0.2 mg/kg 5-AZA, 2.5 mg/kg DZNEP), with or without 10 mg/kg anti-PD-L1 monoclonal antibody (Bio X Cell #BE0101; clone 10F.9G2) via 250 μL intraperitoneal injection every Monday, Wednesday, and Friday for 3 weeks. There were 5 mice per each treatment group that was performed. Tumor size and mouse weights were measured by calipers weekly for 3 weeks at which time the tumors were excised and processed.

2.5. RNA isolation, real-time quantitative reverse transcription PCR

RNA isolation was performed using the commercially available kit (Qiagen RNEasy kit, Qiagen, Japan) per manufacturer instructions. RNA levels were quantified using nano-drop (Thermo Fisher), and 1 μg RNA was used to prepare cDNA using the commercially available kit (QScript, Quanta Biosciences). The qRT-PCR was performed using primers C-X-C motif chemokine ligand 9 &10 (CXCL9, CXCL10) (Sigma-Aldrich) to analyze chemokine response after treatment with of 10 ng/ml IFN-y with or without 5-AZA and DZNep. Cancer-testis antigen expression was analyzed for LAGE, MAGE A3, NY-ESO-1, and WT1 using DNA primers (Sigma-Aldrich). The housekeeping control gene for all qRT-PCR experiments was performed with the GAPDH gene.

2.6. Immunohistochemistry

The immunohistochemistry staining was performed by using the DoubleStain IHC Kit (Abcam) after performing IHC antigen retrieval protocol per manufacturer's instructions. The dilution titration of the CD8 + antibody (Abcam) was 1/200 and FOXP3 (Abcam) was 1/100. Hematoxylin and eosin staining were performed in standard fashion.

2.7. Statistical analysis

SEM is indicated by bars on all figures and was calculated using Microsoft Office Excel 2018. $P < 0.05$ was considered significant. All experiments were done with a minimum of triplicate samples and performed at least three times. Dependent on data distribution and experimental design, paired or unpaired Student *t*-test and Wilcoxon rank-sum tests were used.

2.8. NCBI-Geo-Affymetrix Databank

NCBI-Geo-Affymetrix Databank of 28 patients with metastatic HCC was analyzed from the tumor samples submitted for microarray with multiple gene expression performed. The tumor samples were accompanied by 22 patients with primary HCC. The EZH2 expression in metastatic HCC tumor compared to primary HCC revealed that there is

elevated EZH2 expression in metastatic tumor compared to primary HCC as a sign of increased aggressiveness and invasion ($p < 0.01$) (https://www.ncbi.nlm.nih.gov/geoprofiles/1515128). Additionally, NCBI-Geo-Affymetrix Databank of 10 patients with HCC was analyzed from PBMC samples submitted for microarray with multiple gene expression performed. The PBMC samples were accompanied by the normal patient samples to compare the gene expression differences of the HCC. The PDL-1 expression in samples of those with HCC compared to normal PBMC samples reveal that there is elevated PDL-1 expression in the sample with an HCC tumor compared to normal patient PBMC (https://www.ncbi.nlm.nih.gov/geoprofiles/106262214). Given that sample records are submitted by the various scientific community and reflect a wide variety of data types that are processed and normalized using a wide variety of methods, there is no standard unit for gene expression in the Geo DataSet. They are considered arbitrary units per given sample clusters and is not appropriate to make direct comparisons of values between different DataSets.

3. Results

3.1. Upregulation of EZH2 and PD-L1 in HCC

To demonstrate the rationale for epigenetic therapy in HCC initially, we analyzed the submitted HCC tissue samples for microarray from the Geo-Affymetrix database, which demonstrated a higher EZH2 expression in metastatic HCC compared to primary HCC. The higher level of EZH2 expression was associated with an increased level of invasion in the 28 patient samples with HCC who had metastatic disease compared to those limited only to their primary HCC disease (Fig. 1, top).

In addition to the aberrant epigenetic expression of EZH2 in HCC, we evaluated the potential immunosuppressive molecule in the tumor microenvironment of HCC compared to normal hepatocytes. Comparison of the peripheral blood samples of 10 normal healthy patients to those with HCC in the Geo-Affymetrix database demonstrated there were increased levels of PD-L1 expression in the HCC patient samples compared to normal healthy control patient samples (Fig. 1, bottom). PD-L1 was well established as a critical factor contributing to the immunosuppression in clinical cancer patients [14,15]. The current result suggested there is an immunosuppressive tumor microenvironment in HCC compared to healthy patients and supported the current role of using an anti-PDL-1 monoclonal antibody for HCC despite its limited clinical objective response thus far.

3.2. Upregulation of chemokines CXCL9 and CXCL10

Given the increased prognostic variable of higher tumor-infiltrating lymphocytes (TIL) cells in the tumor microenvironment, we focused on the etiology of the barriers for cytotoxic *T*-cell trafficking. One of the known barriers for lack of TIL in the tumor microenvironment is the lack of CXCL9 and CXCL10 chemokine expression via epigenetic repression, which is responsible for *T*-cell trafficking [16,17]. To overcome this immune trafficking inhibition, we utilized two epigenetic drugs, 5-AZA and DZNep, to unmethylate the transcriptional repression of these chemokines caused by abnormal promotor region methylation and heterochromatin formation of histones by DNMT1 and EZH2, respectively. To demonstrate this concept, we used two types of human HCC cell lines, Hep G2 and Hep3B, which were treated with 5-Aza and DZNep along with IFN-y for induction of CXCL9 and CXCL10 to

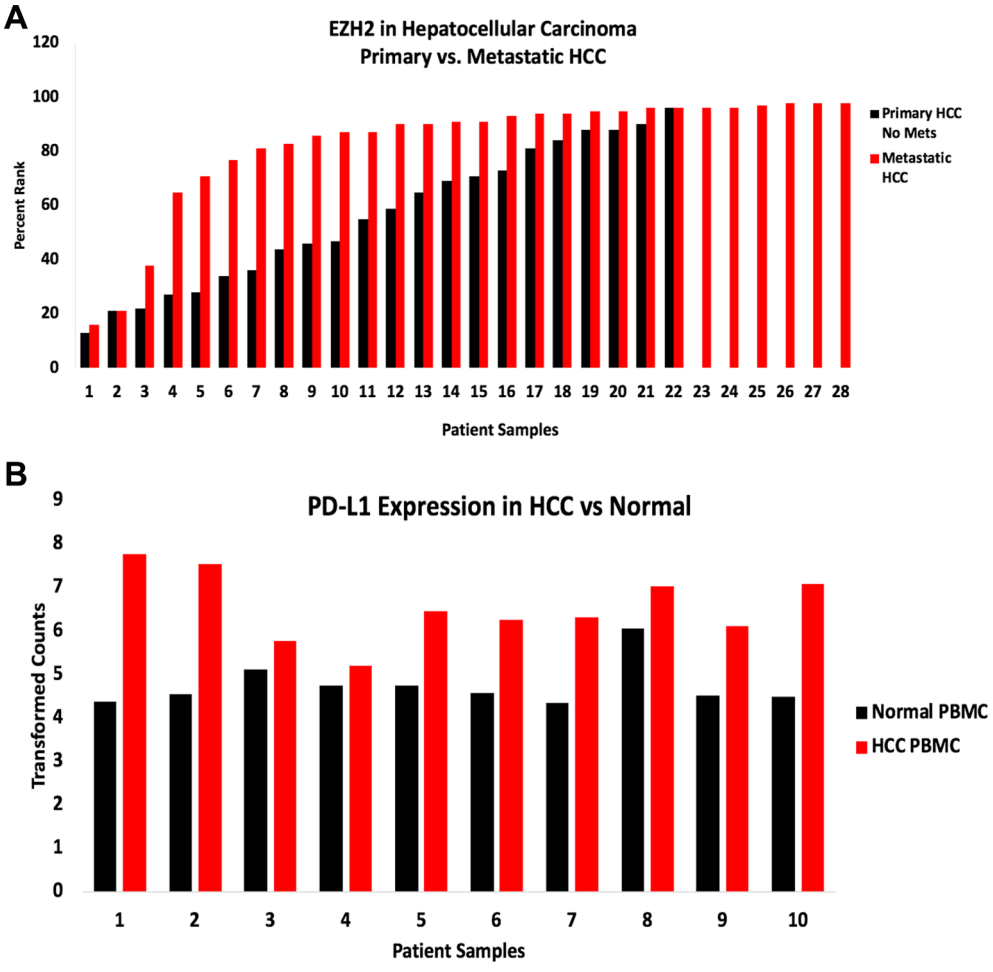

Fig. 1. Top: NCBI-Geo-Affymetrix Databank of 28 patients with metastatic HCC was analyzed from the tumor samples submitted for microarray with multiple gene expression performed. The tumor samples were accompanied by 22 patients with primary HCC. The EZH2 expression in metastatic HCC tumor compared to primary HCC revealed that there is elevated EZH2 expression in metastatic tumor compared to primary HCC as a sign of increased aggressiveness and invasion ($p < 0.01$). https://www.ncbi.nlm.nih.gov/geoprofiles/1515128. Bottom: NCBI-Geo-Affymetrix Databank of 10 patients with HCC was analyzed from PBMC samples submitted for microarray with multiple gene expression performed. The PBMC samples were accompanied by the normal patient sample to compare the gene expression differences of the HCC. The PDL-1 expression in samples of those with HCC compared to normal PBMC samples reveal that there is elevated PDL-1 expression in the sample with an HCC tumor compared to normal patient PBMC. https://www.ncbi.nlm.nih.gov/geoprofiles/106262214.

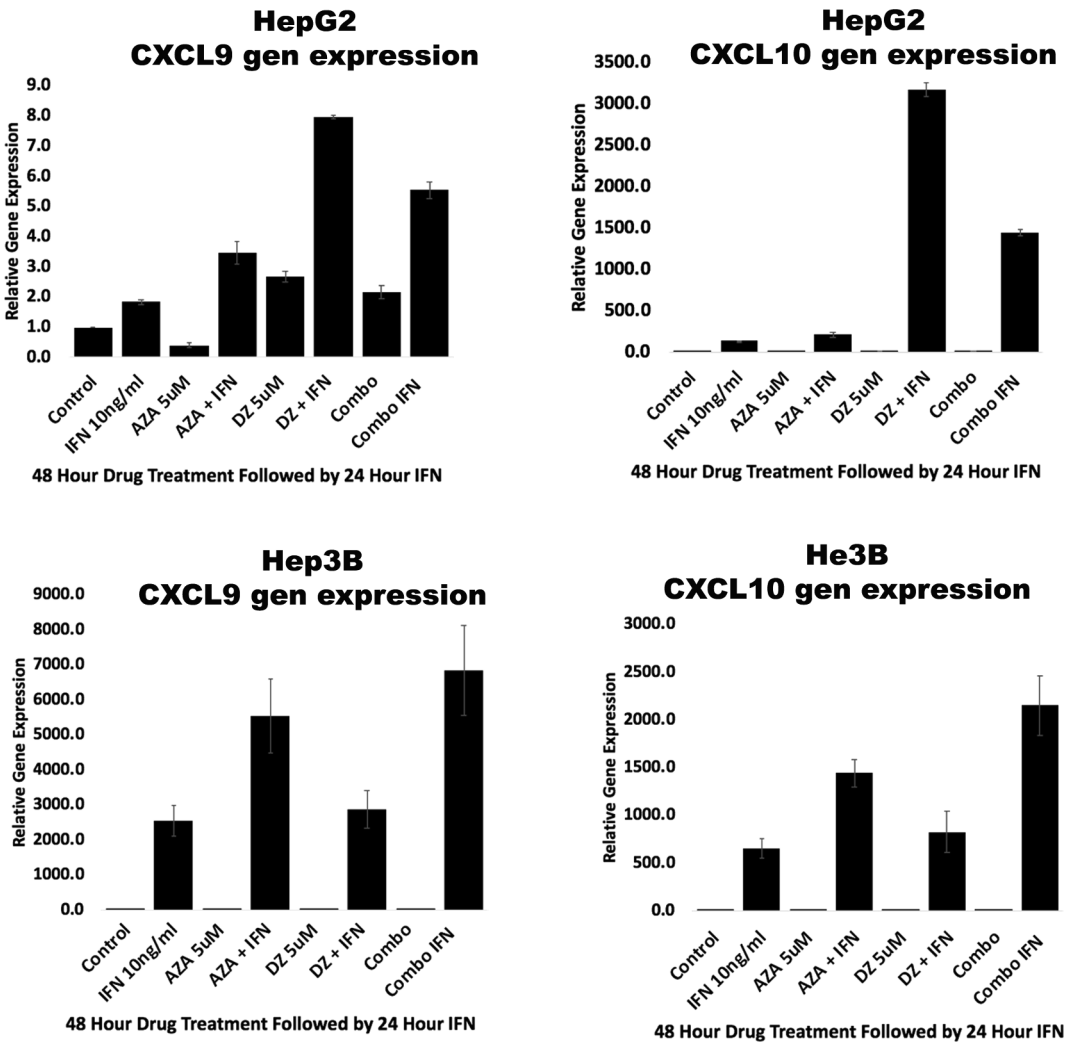

Fig. 2. A) There was a synergistic upregulation of chemokines CXCL9 and CXCL10 for the combination group of DZNep, 5-Aza, and IFN-y in human hepatocellular cancer cell lines HepG2 (CXCL9 5.5 ± 0.2 relative fold change; CXCL10 1.44×10^3 ± 37 relative fold change) and Hep3B (CXCL 9 6.85×10^3 ± 1.3×10^3 relative fold change; CXCL 10 2.15×10^3 ± 3.1×10^2 relative fold change ($p < 0.01$). B) The effect of 5-Aza, DZNep with and without IFN-y demonstrated decreased cell proliferation after 48 h drug treatment of 5-Aza with and without DZNep followed by 24 h drug treatment with and without IFN-y (HepG2 IFN-y alone 0%, AZA 65% ± 5.5, AZA + IFN 58% ± 0.5, DZNep 73% ± 1.5, DZNep + IFN 74% ± 5%, AZA + DZNep 77% ± 1.5, AZA + DZNep + IFN 78% ± 1; Hep3B IFN-y alone 0%, AZA 58% ± 4, AZA + IFN 66% ± 0.5, DZNep 44% ± 0.5, DZNep + IFN 47% ± 1, AZA + DZNep 66% ± 0.5, AZA + DZNep + IFN 68% ± 3.5; $p < 0.01$).

evaluate the effect on the CXCL9 and CXCL10 chemokine expression. In both cell lines, inhibition of EZH2 and DNMT1 demonstrated significant upregulation of chemokines CXCL9 and CXCL10 (Fig. 2). This demonstrated that chemokine expression was epigenetically repressed by EZH2 and DNMT1, and can be activated by epigenetic modulation in our HCC cell lines.

3.3. Upregulation of neoantigens

The critical component of an effective anti-tumor immune response is the tumor antigen that the immune *T*-cell major histocompatibility complex (MHC) complex recognizes; therefore, one of the current limitations of immunotherapy for GI malignancies is their low rate of neoantigen expression from their low rate of somatic mutations. However, the cancer testis antigens (CTAs) are an alternative type of neoantigen that provides an attractive target for immunotherapy. While CTAs are rarely expressed in normal testes and placenta, they lack the MHC required for antigen presentation on cell surfaces for an immunogenic response. However, CTAs are expressed in variable levels in malignant lesions but are limited in clinical use due to their low level of expression because of epigenetic repression. Thus, we have aimed to demonstrate that the epigenetic repression of such cancer testis antigens can be reversed via epigenetic therapy with the induction of neoantigens, thereby making an immunosilent tumor more immunogenic by presenting antigen expression on its cell surface for immune recognition. Thus, treatment of human HCC cell lines Hep G2 and Hep 3B with 5uM 5-Aza and 5uM DZNep has allowed the induction of previously low-expressed cancer testis antigens NY-ESO-1 and LAGE that was statistically significant ($p < 0.01$) (Fig. 3).

3.4. Increased T-cell infiltration into the tumor microenvironment

We further wanted to demonstrate that the induction of a synergistic response in chemokines CXCL9 and CXCL10 expression could be translated into increased *T*-lymphocyte trafficking into the tumor microenvironment. Thus, we used a syngeneic subcutaneous tumor model using the C56L/LJ immunocompetent murine model with intraperitoneal injections of 5-Aza, DZNep, with or without PDL-1 inhibitor. Our model demonstrated that the level of tumor-infiltrating lymphocytes within the tumor microenvironment was significantly

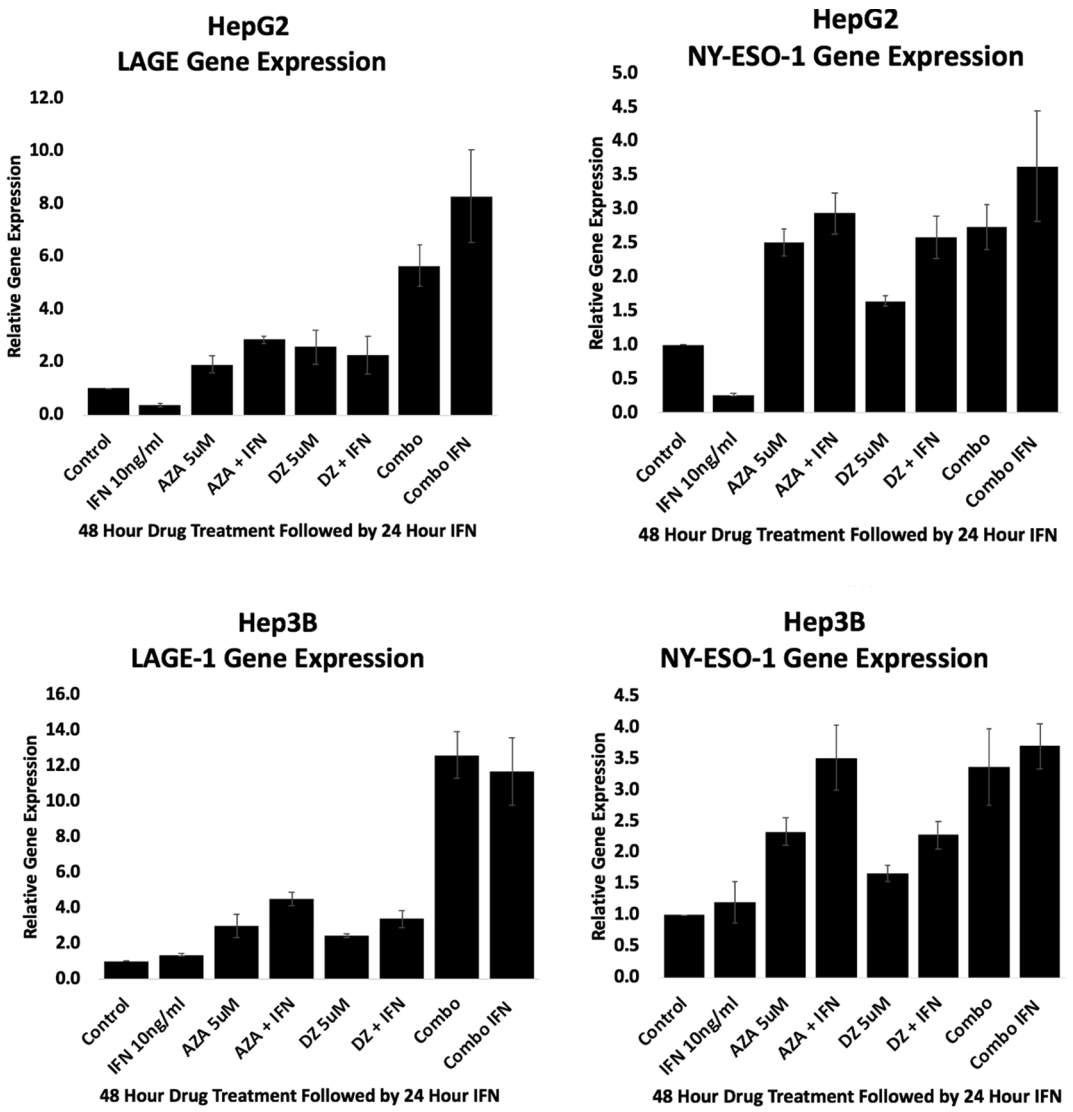

Fig. 3. A) There was an increase in antigen expression after treatment with DZNep with or without Azacytidine for both cancer-testis antigens, NY-ESO-1 (3.6–3.7 ± 0.3 relative fold change) and LAGE (8.3–11.7 ± 1.9 relative fold change) with the treatment of DZNep and 5-AZA ($p < 0.01$). B) PDL-1 expression was upregulated by treatment with DZNep with or without Azacytidine after induction with IFN-y 10 ng/ml in HepG2 and Hep3B cell lines (IFN-y alone 15.3 ± 0.9, Aza alone 35.8 ± 3.3, DZNep alone 45.4 ± 6.4, and combination 53.1 ± 5.5); ($p < 0.01$).

higher compared to control, epigenetic therapy alone, or anti-PDL-1 alone (Fig. 4). This result demonstrated that the synergistic upregulation was seen in CXCL9 and CXCL10 expression correlated similarly to the increased CD8 + cytotoxic *T*-cell infiltration to lower FOXP3 expression as a sign of decreased *T*-regulatory cells in the tumor microenvironment. The increased ratio of CD8 to FOXP3 has been associated with increased cytotoxic *T*-cell activation that is associated with increased CXCL9 and CXCL10 induction.

We further investigated whether the correlated tumor infiltration by the TIL cells was associated with increased cell death in our *in vivo* study. As shown by TUNEL assay, the level of apoptosis was highest in the combination group when compared to the control, epigenetic alone, or anti-PDL-1 group alone (Fig. 5).

3.5. Combination therapy caused tumor regression

Given the increased tumor infiltration of *T*-lymphocytes with corresponding cytolysis and increased neoantigen expression, we wanted to demonstrate overall tumor regression as a result of the epigenetic modulation by 5-AZA and DZNep. We demonstrated that there was a dramatic decrease in overall tumor weight with increased tumor reduction for the combined epigenetic and immunotherapy group (0.02 g ± 0.02) compared to either epigenetic therapy (0.63 g ± 0.61) alone, immunotherapy alone (0.15 g ± 0.21) or untreated control (2.4 g ± 0.71). To validate the drug model, we also performed a double knockdown model of EZH2 and DNMT1. Using the double EZH2 and DNMT1 knockdown in combination with anti-PDL-1 inhibitor, no tumor growth was detected in the mice (Fig. 6).

4. Discussion

4.1. Limitations of immunotherapy in HCC

Hepatocellular carcinoma is an aggressive cancer with limited systemic chemotherapy options and a need for more effective therapy than current standard options [3,4]. Immunotherapy has emerged as a promising therapy for melanoma, non-small cell lung cancer, head and neck cancers, and renal cell carcinoma [7]. However, the application of immunotherapy in hepatocellular carcinoma is still met with low response rates [6] compared to other immunogenic histologies and warrants strategies for improving the efficacy of therapy. Response to immunotherapy has been most successful for melanoma and lung cancer,

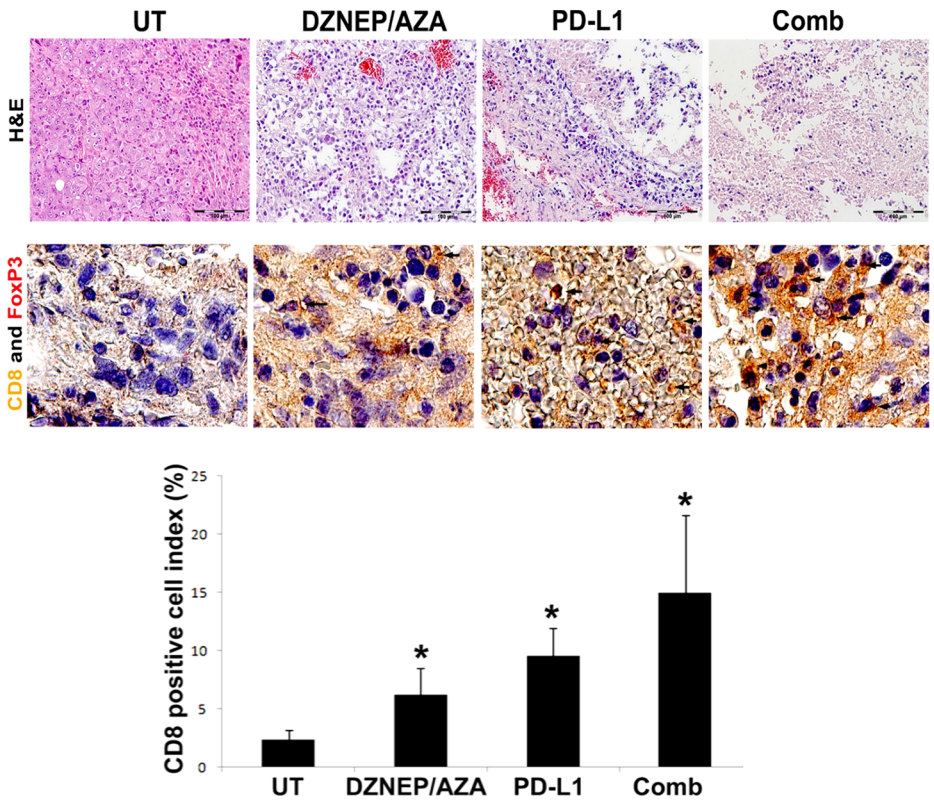

Fig. 4. Immunohistochemistry with hematoxylin and eosin demonstrating increased CD8 + (brown)/ FOXp3 (red) lymphocyte ratio with a combination treatment of epigenetic therapy (DZNEP/AZA) combined with PD-L1 inhibitor compared to control (UT) ($^{*}p < 0.05$). The same hematoxylin and eosin slides were used for Figs. 4 and 5.

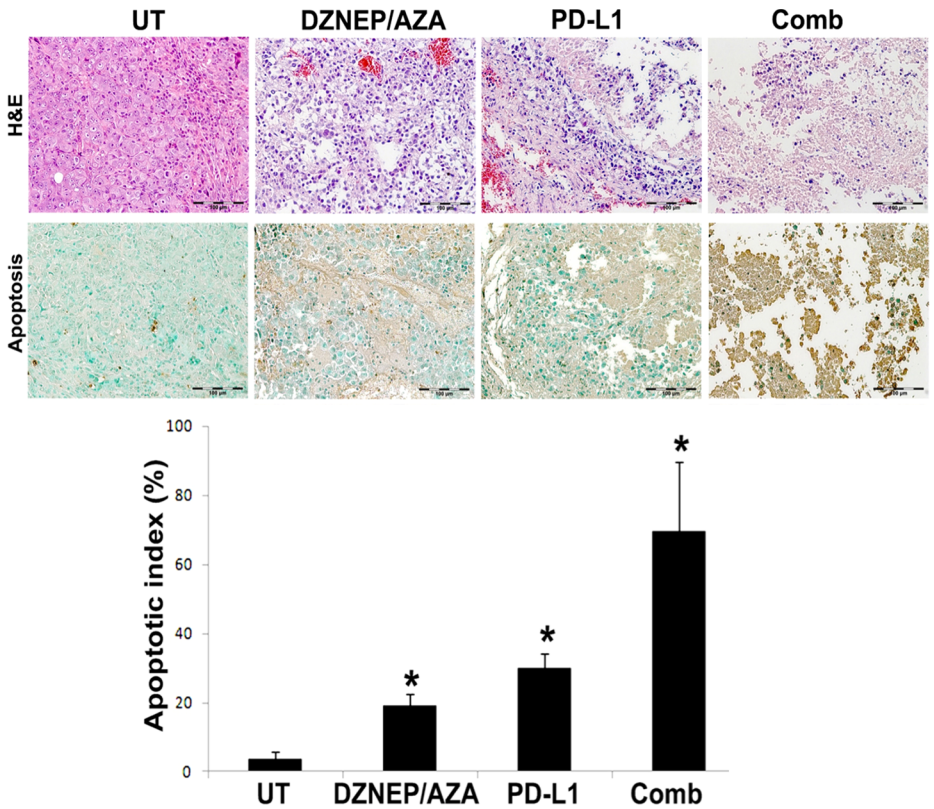

Fig. 5. Immunohistochemistry with hematoxylin and eosin demonstrating associated increased apoptosis (brown) that corresponded to the increased *T*-lymphocyte infiltration after combination treatment of epigenetic therapy (DZNEP/AZA) combined with PD-L1 inhibitor compared to control (UT) ($^{*}p < 0.05$). The same hematoxylin and eosin slides were used for Figs. 4 and 5.

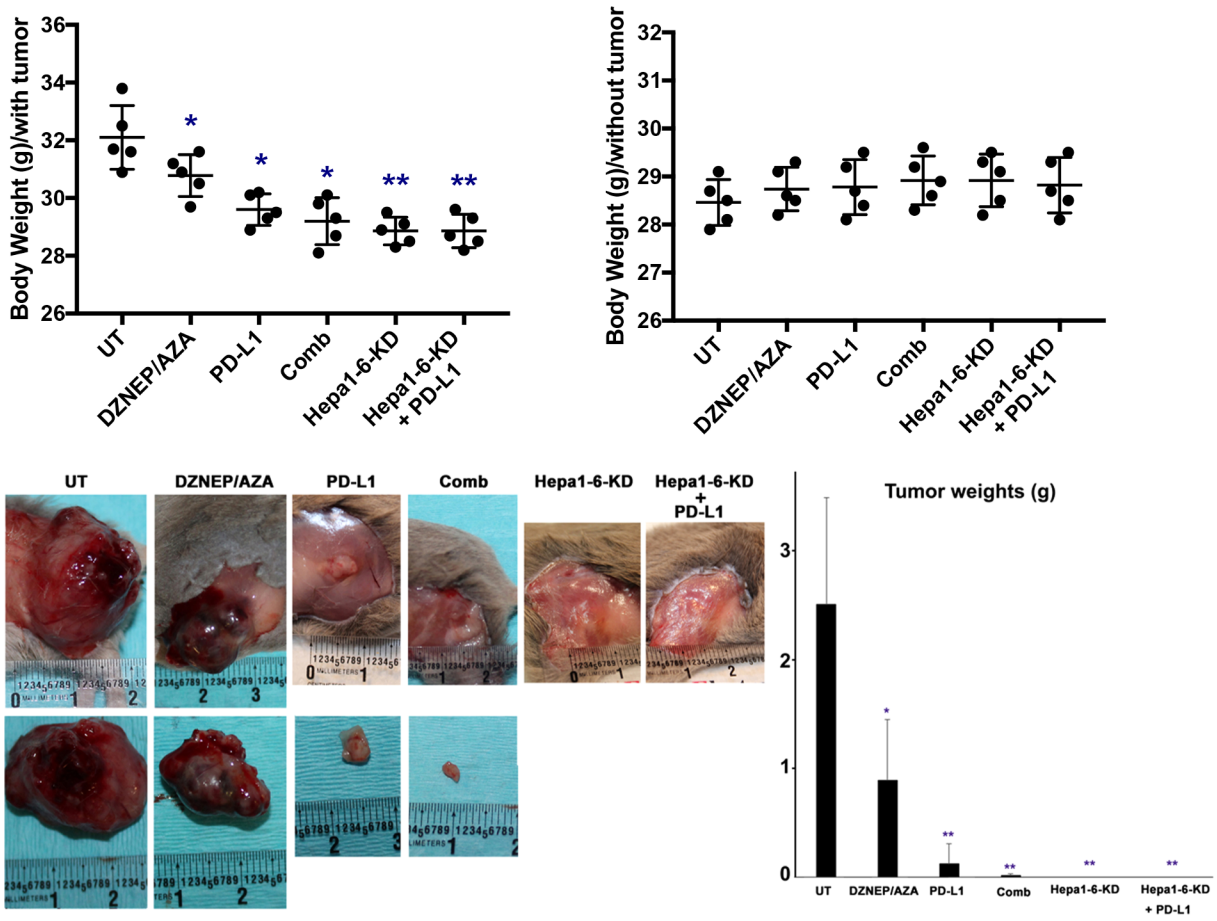

Fig. 6. To demonstrate epigenetic modulation by inhibition of EZH2 and DNMT1 in combination with immunotherapy using anti-PDL-1was responsible for the tumor regression, we performed flank injection of 5 × 105 Hepa1-6 cells in C56/LJ mice. After tumor inoculation was confirmed, we performed intraperitoneal injections of epigenetic drugs DZNep/AZA with or without anti-PDL-1 inhibitor three times per week (Monday, Wednesday, Friday). After 3 weeks of treatment, the animals were sacrificed and measured. There was a moderate response to the epigenetic drug but significant response to PDL-1 alone and further response in the combination of the epigenetic drug with PDL-1 antibody. In order to validate the drug model and specificity of the target inhibitions of EZH2 and DNMT1, we generated short hairpin lentiviral knockdown of EZH2 (shEZH2) and DNMT1 (shDNMT1). Hepa1-6 shEZH2/shDNMT1 knockdown (K_D) and anti-PDL-1 inhibitor alone demonstrated a complete decrease in tumor weight compared to control $^{*}(p < 0.05)$ $^{**}(p < 0.01)$. As a correlate of drug toxicity on the mice with the combination of treatments, the body weights of the mice were unchanged from control and were well tolerated.

which is speculated to be due to these two histologies having the highest rate of somatic mutation, increasing the probability of having foreign antigen expression on the cell surface for immunologic response [18,19]. Unfortunately, hepatopancreatobiliary tumors are often not immunogenic due to their low rate of somatic mutations [20] and have thus far been elusive in their response to these immunotherapies.

4.2. Addition of epigenetic therapy

Our study aimed to potentially improve the therapeutic efficacy of checkpoint immunotherapy by combining it with epigenetic therapy to increase chemokine release in hepatocellular carcinoma. In turn, higher chemokine levels have been associated with increased *T*-lymphocyte infiltration into the tumor microenvironment, increase previously silent neoantigen expression, and cause tumor regression with associated apoptosis in hepatocellular carcinoma. CXCL9 and CXCL10 are chemokines that have been associated to serve as chemotactic signaling for *T*-lymphocytes, specifically tumor-infiltrating lymphocytes, which has been correlated with improved survival [21]; however, they are often transcriptionally repressed by epigenetic aberrations on the promoter regions of the genes responsible for its expression [16,17]. Therefore, epigenetic modulation using 5-Aza and DZNep in our study to unmethylate at the gene promoter and chromatin level, respectively, was associated with reactivation of these chemokine expressions to increase cytotoxic *T*-cell infiltration in the tumor. Also, cancer testis antigen expression on malignant cells, which serves as possible neoantigens on malignant cells, express MHC complex for antigen presentation for immune cells compared to those naturally found in testes and ovaries that do not possess MHC expression. Historically, while they are susceptible to immune response, they are often limited in clinical significance due to their small percentage expression in the cancer cell surface of less than 5% [22]. However, these previously immune silent antigens can be upregulated to create an immunogenic microenvironment by epigenetic modulation that was performed in our study. Therefore, we have shown in both human cell lines, as well as in a syngeneic immunocompetent murine model, that combining readily available clinical-grade epigenetic therapy with checkpoint inhibitors argues for potential clinical relevance with the potential to increase tumor regression in hepatocellular carcinoma, which currently often lacks effective therapeutic options in clinic patients.

4.3. DNMT1, EZH2, and PDL-1 inhibitors

While the current first-line therapy for HCC is the tyrosine-kinase inhibitor sorafenib, the objective response and improvement in survival are minimal [3]. One possible cause of the therapeutic resistance mechanism is the subsequent elevation of PDL-1 in addition to the elevation of DNMT1 via the STAT3 pathway, leading to resistance to

sorafenib [23]. The potential shared common pathway of PDL-1 and DNMT1 elevation after sorafenib treatment further supports the current rationale for using second-line immunotherapy [6], in contrast to the perspective that the two treatments are mutually exclusive. Thus, the current study for combination DNMT1, EZH2, and PDL-1 inhibitors is a logical approach for patients with HCC, especially for those after sorafenib. While the anti-PD-1 therapy demonstrated an objective response rate of 15–20% that led to FDA approval for patients with HCC refractory to sorafenib treatments, there is a basis for potential improvement in achieving higher response rates and durability in immunotherapy for HCC by utilizing adjunctive therapy such as epigenetic drugs to augment its efficacy. Conversely, epigenetic therapy has historically been used for myelodysplastic syndromes with decitabine, but there is emerging data that demonstrates that the source of resistance may be related to induction of PD-1/PDL-1 in tumors [24]. This suggests that the epigenetic combination with immunotherapy is a logical therapeutic strategy whose components complement each other, rather than using each as a single agent, given that the resistance pathways are closely linked.

Aberrant EZH2 expression in various malignancies has been associated with worse prognosis, survival, and immunosuppression [16,25,26]. Similarly, tumor aggressiveness, invasiveness, and progression have also been associated with overexpression of EZH2 in HCC [27,28]. Therefore, the elevation in EZH2 marker in aggressive HCC was deemed to be a good target for inhibition in our study as it also may play a role in immunosuppression [16]. There have been further attempts at modifications to the current epigenetic drugs that have since been studied, including a second-generation DNMT1 inhibitor, (Guadecitabine) SGI-110, that has shown clinical efficacy in HCC with up-regulation of previously repressed genes [29,30] and in combination with oxaliplatin [31]. Similarly, another type of epigenetic drug targeting histone deacetylase (HDACi) alone has shown promising efficacy by up-regulating MHC class expression in tumors and raising the susceptibility of tumor cells to cytolysis [32].

We have demonstrated an increase in tumor-infiltrating lymphocytes in the tumor microenvironment which can be associated with a cytotoxic effect on tumor cells as one of the pathways that may be responsible for tumor regression [33]. There is a robust prognostic benefit from having TIL present in the tumor microenvironment because minimal TIL infiltration has been associated with poor HCC-specific survival [34,35], and thus poses theoretical advantages on future potential therapy, i.e., adoptive cell therapy [36]. Furthermore, preliminary evidence suggests that adoptive cell therapy for HCC therapy is promising [36], and the application of our epigenetic modulation can theoretically be helpful prior to TIL harvesting of a tumor to increase the *T*-lymphocyte migration into the tumor from which the *T*-cell repertoire will be selected and expanded for re-infusion for adoptive cell therapy.

There are several limitations to our study. First, the HCC model was established in mice with a normal murine immune system rather than a transgenic model with a humanized *T*-cell repertoire, which could have potentially a more accurate depiction of the human immune response. Secondly, we limited our neoantigen expression evaluation to cancer testis antigens and WT1 as the known antigens, but recognize other neoantigens can be modulated as well with further upregulation that was not captured in our study. However, despite these limitations, we demonstrated that the obstacles of immunotherapy with lack of neoantigen expression, lack of *T*-cell infiltration, and effective tumor regression could be modulated with epigenetic therapy. Our study utilized three different HCC cell lines with similar results and are reproducible. In addition to using an immunocompetent model to test our epigenetic and immunotherapy drugs, we performed a corresponding gene-knockdown in the HCC cell line to validate our target of DNMT1 and EZH2 as the sole modifiers in our experiments. Finally, we demonstrated that epigenetic modulation could upregulate previously repressed silent neoantigens in immunotherapy.

5. Conclusions

The addition of epigenetic therapy can potentiate immunotherapy for HCC by increasing neoantigen expression, increasing cytotoxic *T*-cell infiltration across the immunosuppressive tumor microenvironment, and is associated with increased apoptosis with subsequent effective tumor regression when compared to immunotherapy or epigenetic therapy alone. Clinical trials of multi-modality treatment for patients with locally advanced, recurrent and metastatic HCC cancer may be warranted.

6. Declarations

Ethics approval: The animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care. were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. (Consent to participate is not applicable; there were no human subjects.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: Research reported in this publication was supported by an Institutional Development Award (IDeA) from the NIGMS of the National Institutes of Health under grant number P20GM113226. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authors' contributions: In vitro, in vivo, qRT-PCR, and immunohistochemistry contributions were shared equally amongst the group. Everyone read and approved the submission of the manuscript.

Acknowledgments: None.

Authors' information: None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.12.010.

References

[1] W. Chen, et al., Cancer statistics in China, 2015, CA Cancer J. Clin. 66 (2) (2016) 115–132.
[2] L.A. Torre, et al., Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2) (2015) 87–108.
[3] J.M. Llovet, et al., Sorafenib in advanced hepatocellular carcinoma, N. Engl. J. Med. 359 (4) (2008) 378–390.
[4] J. Bruix, et al., Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESOURCE): a randomised, double-blind, placebo-controlled, phase 3 trial, Lancet 389 (10064) (2017) 56–66.
[5] U. Shinsuke, et al., Clinical outcomes of sorafenib treatment failure for advanced hepatocellular carcinoma and candidates for regorafenib treatment in real-world practice, Hepatol. Res. (2018).
[6] A.B. El-Khoueiry, et al., Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial, Lancet 389 (10088) (2017) 2492–2502.
[7] J.R. Brahmer, et al., Safety and activity of anti-PD-L1 antibody in patients with advanced cancer, N. Engl. J. Med. 366 (26) (2012) 2455–2465.
[8] T.N. Schumacher, R.D. Schreiber, Neoantigens in cancer immunotherapy, Science 348 (6230) (2015) 69–74.
[9] A. Villanueva, et al., DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma, Hepatology 61 (6) (2015) 1945–1956.
[10] J. Yin, et al., Ezh2 regulates differentiation and function of natural killer cells through histone methyltransferase activity, Proc. Natl. Acad. Sci. U.S.A 112 (52) (2015) 15988–15993.
[11] S. Bugide, M.R. Green, N. Wajapeyee, Inhibition of Enhancer of zeste homolog 2 (EZH2) induces natural killer cell-mediated eradication of hepatocellular carcinoma cells, Proc. Natl. Acad. Sci. U.S.A 115 (15) (2018) E3509–E3518.
[12] C.D. Kemp, et al., Polycomb repressor complex-2 is a novel target for mesothelioma therapy, Clin. Cancer Res. 18 (1) (2012) 77–90.

[13] S. Landman, et al., DNA methyltransferase inhibition promotes Th1 polarization in human CD4(+)CD25(high) FOXP3(+) regulatory T cells but does not affect their suppressive capacity, J. Immunol. Res. 2018 (2018) 4973964.
[14] G. He, et al., Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma, J. Exp. Clin. Cancer Res. 34 (2015) 141.
[15] Y. Cheng, et al., Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma, Cell Death Dis. 9 (4) (2018) 422.
[16] D. Peng, et al., Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy, Nature 527 (7577) (2015) 249–253.
[17] N. Nagarsheth, et al., PRC2 epigenetically silences Th1-type chemokines to suppress effector T-cell trafficking in colon cancer, Cancer Res. 76 (2) (2016) 275–282.
[18] E. Tran, et al., Immunogenicity of somatic mutations in human gastrointestinal cancers, Science 350 (6266) (2015) 1387–1390.
[19] E. Tran, P.F. Robbins, S.A. Rosenberg, 'Final common pathway' of human cancer immunotherapy: targeting random somatic mutations, Nat. Immunol. 18 (3) (2017) 255–262.
[20] M.S. Lawrence, et al., Mutational heterogeneity in cancer and the search for new cancer-associated genes, Nature 499 (7457) (2013) 214–218.
[21] H. Bronger, et al., CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer, Br. J. Cancer 115 (5) (2016) 553–563.
[22] T.S. Park, et al., Expression of MAGE-A and NY-ESO-1 in primary and metastatic cancers, J. Immunother. 39 (1) (2016) 1–7.
[23] J. Liu, et al., Targeting the PD-L1/DNMT1 axis in acquired resistance to sorafenib in human hepatocellular carcinoma, Oncol. Rep. 38 (2) (2017) 899–907.
[24] H. Yang, et al., Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents, Leukemia 28 (6) (2014) 1280–1288.
[25] Y. Liu, et al., Increased EZH2 expression is associated with proliferation and progression of cervical cancer and indicates a poor prognosis, Int. J. Gynecol. Pathol. 33 (3) (2014) 218–224.
[26] Y. Wang, et al., Overexpression of YB1 and EZH2 are associated with cancer metastasis and poor prognosis in renal cell carcinomas, Tumour Biol. 36 (9) (2015) 7159–7166.
[27] T. Sudo, et al., Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma, Br. J. Cancer 92 (9) (2005) 1754–1758.
[28] M. Sasaki, et al., The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma, Lab. Invest. 88 (8) (2008) 873–882.
[29] S. Jueliger, et al., Efficacy and epigenetic interactions of novel DNA hypomethylating agent guadecitabine (SGI-110) in preclinical models of hepatocellular carcinoma, Epigenetics (2016) 1–12.
[30] M. Liu, et al., Integrative epigenetic analysis reveals therapeutic targets to the DNA methyltransferase inhibitor SGI-110 in hepatocellular carcinoma, Hepatology (2018).
[31] Y. Kuang, et al., Guadecitabine (SGI-110) priming sensitizes hepatocellular carcinoma cells to oxaliplatin, Mol. Oncol. 9 (9) (2015) 1799–1814.
[32] H. Yang, et al., Histone deacetylase inhibitor SAHA epigenetically regulates miR-17-92 cluster and MCM7 to upregulate MICA expression in hepatoma, Br. J. Cancer 112 (1) (2015) 112–121.
[33] J.A. Trapani, Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B, Aust. N. Z. J. Med. 25 (6) (1995) 793–799.
[34] A. Kawata, et al., Tumor-infiltrating lymphocytes and prognosis of hepatocellular carcinoma, Jpn. J. Clin. Oncol. 22 (4) (1992) 256–263.
[35] K. Sideras, et al., PD-L1, Galectin-9 and CD8(+) tumor-infiltrating lymphocytes are associated with survival in hepatocellular carcinoma, Oncoimmunology 6 (2) (2017) e1273309.
[36] S.S. Jiang, et al., A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma, Oncotarget 6 (38) (2015) 41339–41349.