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ARTICLE

Morphology of tumor and nontumor tissue in liver resection specimens for hepatocellular carcinoma following nivolumab therapy

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Abstract

Nivolumab is an immune checkpoint inhibitor (ICI) approved for treatment of many cancers, including hepatocellular carcinoma (HCC). Liver injury is a known complication in patients treated with nivolumab for nonliver tumors. To date, the morphologic changes to tumor and nontumor liver have not been well-characterized in HCC patients. We identified 20 patients who underwent partial hepatectomy or liver transplantation after receiving nivolumab for HCC. Demographics, laboratory values, and imaging results were obtained from medical records. All available slides from resection specimens were evaluated for tumor necrosis, tumor-infiltrating lymphocytes (TILs), and features of liver injury. Patients in the study included 16 males and 4 females with median age of 56 years. The underlying liver disease was HBV in 10, HCV in 6, and unknown/other in 4. Twelve patients were treated with nivolumab in the neoadjuvant setting, whereas eight were treated with nivolumab, usually along with other therapies, before undergoing liver transplantation. On review of resection specimens, three patients (all from the neoadjuvant group) demonstrated marked treatment response attributable to nivolumab. TILs were present in 17/20 cases. One case that showed treatment response in the neoadjuvant group demonstrated non-necrotizing granulomas and prominent bile duct intraepithelial lymphocytes (IELs) in the nontumor liver. One case from the transplant group showed bile duct damage and prominent ductular reaction after long-term nivolumab therapy (32 doses). Our findings indicate that nivolumab is effective in a subset of patients, including in the neoadjuvant setting. Granulomas and bile duct IELs are rare findings in cases treated with nivolumab but, when seen, may indicate potential response to therapy. Bile duct damage and ductular reaction may be manifestations of long-term nivolumab therapy. Future prospective and longitudinal studies with pretreatment tumor biopsies may help identify patients apt to respond to ICI therapy and further characterize patterns of ICI-related liver injury.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and is estimated to be the fourth most common cause of cancer-related death overall [1–4]. Despite efforts over the past decade to improve survival with systemic therapy for advanced-stage HCC, the overall survival with current therapy remains dismal [5]. The first effective systemic therapy was the oral kinase inhibitor sorafanib and more recently immune checkpoint inhibitors (ICIs) have entered the field. ICIs are a promising class of oncological therapy that has been proven effective in the treatment of many cancers [6–9]. Specifically for HCC, the Food and Drug Administration (FDA) has approved ICIs, including the programmed cell death 1 (PD-1) inhibitors nivolumab and pembrolizumab, and the combination of the cytotoxic T-lymphocyte-associate antigen 4 (CTLA-4) inhibitor ipilimumab and nivolumab [9–13]. In the clinical trial CheckMate-040, nivolumab therapy resulted in promising survival benefit in patients who had disease progression or unacceptable side effects with first-line therapy sorafenib, which prompted an accelerated FDA approval of nivolumab as second-line therapy for HCC in 2017 [10]. This trial was followed by a phase 3 randomized trial of sorafenib versus nivolumab (CheckMate-459) in

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which nivolumab failed to improve overall survival over sorafenib and, therefore, the drug has not yet been approved as first-line therapy for HCC [13]. ICIs continue to be an active area of research in treatment for HCC, and a recently published phase III trial found that atezolizumab [a programed cell death ligand 1 (PD-L1) inhibitor] in combination with bevacizumab (a vascular endothelial growth factor inhibitor) is superior to sorafenib as first-line therapy for HCC [14].

PD-1 is an immune checkpoint molecule expressed on the surface of T cells, dendritic cells, and macrophages. This molecule provides inhibitory signals to the immune system in order to modulate the activity of T cells in peripheral tissues and maintain self-tolerance in the setting of infection and inflammation. In cancer, when PD-1 expressed on activated T cells binds to its ligand PD-L1 on the tumor cells, there is inactivation of the cytotoxic T cells, resulting in suppression of the host immune response [15, 16]. Nivolumab is a fully human immunoglobulin G4 monoclonal antibody that targets PD-1, blocking the interaction between PD-1 and PD-L1, and consequently enhancing the host immune response against tumor cells promoting an antitumor response [17].

Given how checkpoint molecules regulate the immune system function, it is not surprising that ICIs can lead to over-activation of the immune system and subsequent immune-related adverse events (IRAEs). As these therapies do not target any specific antigen, IRAEs can involve dysfunction and inflammation of a single organ or multiple organ systems. The most commonly-affected organ systems are skin (34% of cases) and gastrointestinal tract (13% of cases) [18–20]. These adverse effects are potentially fatal, but deaths are rare, reported in <1% of IRAE cases [21, 22].

Liver injury is a known complication in patients treated with ICIs for nonliver tumors and overall occurs in 5–30% of patients [7, 23]. The incidence of hepatic injury associated with nivolumab for the treatment of nonliver cancers is 5–10% and often becomes clinically evident 8–12 weeks after initiation of therapy [24, 25]. Histologically, most published studies report nonspecific features of a panlobular hepatitic process [25–30] with a cytotoxic T-cell infiltrate showing an increased number of CD3+ and CD8+ lymphocytes and decreased CD20+ B cells and CD4+ T cells compared with autoimmune hepatitis and drug-induced liver injury [27]. In addition, other patterns of injury have been reported, such as cholestatic [31–34], mixed hepatitic and cholestatic [35], and granulomatous [36]. Prominent sinusoidal lymphohistiocytic infiltrates and central vein endotheliitis also have been reported; however, these findings were seen with the CTLA-4 inhibitor ipilimumab [27, 37].

Little is known about the histological findings in tumor and nontumor tissue in patients treated with nivolumab for HCC. Evaluation of liver toxicity of therapies for HCC is complicated by the fact that most tumors arise in a cirrhotic liver in the setting of an underlying liver disease. Further, the tumor nodules themselves may affect the surrounding liver tissue, causing compression and other local effects. With these caveats in mind, our aim is to characterize morphologic changes in the tumor and nontumor liver tissue from patients who underwent partial or total hepatectomy following nivolumab therapy for HCC.

Materials and methods

Study population

In this retrospective study, we identified patients at our institution with HCC, who were treated with nivolumab followed by partial hepatectomy or liver transplantation between June 2018 and March 2020. The study was approved by the Ethics Committee and Institutional Review Board.

Patient data

The following data were collected from our pathology database and electronic medical records: age, sex, underlying liver disease, previous cancer treatments, nivolumab therapy history (number of doses and duration of therapy), laboratory data [hepatitis B and hepatitis C viral loads, alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin, alkaline phosphatase, and α-fetoprotein levels (AFP)], and imaging findings. Viral loads were recorded as the most recent level prior to surgery. AST, ALT, alkaline phosphatase, total bilirubin, and AFP were recorded at two time points per patient: pre-nivolumab (within one month prior to first dose of nivolumab) and post-nivolumab (1–2 months after first nivolumab dose). Results from two imaging studies were reviewed for each patient when available: pre-nivolumab (most recent imaging study prior to first nivolumab dose) and post-nivolumab (most recent imaging study prior to surgery). Nivolumab was administered intravenously at a dose of 240 mg, generally every 2 weeks until time of surgery.

Liver surgical specimen evaluation

All available slides from each surgical specimen were reviewed by two liver pathologists (CCS and SCW) for evaluation of tumor and nontumor tissue. Eighteen of 20 cases had representative sections of liver taken away from the tumor available for review while in two cases only slides containing both tumor and nontumor tissue were available for evaluation. Tissue immediately adjacent to the tumor was avoided in evaluation for nontumor changes.

Regarding the tumor tissue, presence and percentage of necrosis, presence of tumor-infiltrating lymphocytes (TILs), and tumor differentiation were recorded. For the nontumor tissue, biliary and vascular changes, and the presence and types of granulomas were assessed. Biliary changes included prominent ductular reaction, periductal fibrosis, intraepithelial lymphocytes (IELs) within bile ducts, bile duct damage, and bile duct loss. Vascular changes included features of obliterative portal venopathy (attenuation or loss of portal vein branches, herniation of portal vein branches into the parenchyma) and zone 3 necrosis. Grade [38] and stage [39] were recorded for patients with underlying chronic viral hepatitis.

Statistical analysis

Descriptive statistics (mean, median, SD) were calculated. Two-tailed Student's *t*-test was used to compare means, whereas Fisher's exact test was used to compare categorical variables. A *P*-value of <0.05 was considered statistically significant.

Results

Table 1 summarizes the clinical characteristics, prior treatment history, nivolumab doses, radiologic response, pathologic evaluation of tumor and nontumor liver, and follow-up, whereas Table 2 summarizes the laboratory findings for our cohort of patients with HCC who received nivolumab therapy followed by surgery.

Demographics and underlying liver disease

Twenty patients were identified, who underwent nivolumab treatment prior to surgery. Cases included 16 males and 4 females with a mean age of 60.5 years (SD 13.1). The most common liver disease was chronic viral hepatitis (ten chronic hepatitis B and six chronic hepatitis C), whereas one patient had hemochromatosis and one had malignant transformation of a hepatocellular adenoma with hepatocyte nuclear factor-1α mutation. The underlying liver disease was unknown in two cases.

Treatment history

Ten patients had no treatment for HCC prior to nivolumab therapy, whereas seven had prior resection plus locoregional therapy [transarterial chemoembolization (TACE), y90 radioembolization (y90), and/or radiofrequency ablation (RFA)] and three had prior locoregional therapy only (TACE or y90). Two patients had received sorafenib prior to nivolumab therapy. Twelve patients underwent partial hepatectomy following neoadjuvant nivolumab therapy (2–10 doses, median 2.5 doses), whereas 8 patients underwent liver transplantation while on nivolumab therapy (3–32 doses, median 17.5 doses), often in combination with locoregional therapies.

Laboratory data

One patient (patient 13) with chronic hepatitis C, who underwent liver transplantation, had a high HCV viral load of 263,000 IU/ml at time of surgery, whereas the other 15 patients with either chronic hepatitis B or C had either low (<25 IU/ml) or undetectable viral load. AST, ALT, alkaline phosphatase, and total bilirubin levels from before (within 1 month prior to first nivolumab dose) and after nivolumab treatment (1–2 months after first nivolumab dose, but prior to surgery) were compared. An increase was defined as an increase of 50% from before nivolumab therapy levels and an abnormal post-nivolumab therapy level. Six patients (patients 2, 3, 7, 12, 13, and 17) showed an increase in either AST or ALT, one patient (patient 12) showed an increase in alkaline phosphatase, and three patients (patients 2, 3, and 7) showed an increase in total bilirubin. Two of the six patients showing increase in these laboratory values had also undergone locoregional therapy within the same timeframe—patient 12 underwent y90 therapy 2 weeks prior to first nivolumab dose and patient 13 underwent TACE 2 months prior to first nivolumab dose. When taken as a group, there was no significant difference in mean AST, ALT, alkaline phosphatase, or total bilirubin between the pre- and post-nivolumab time points, respectively (AST 62.7 vs. 68.4 U/L, $p = 0.79$; ALT 61.6 vs. 73.0 U/L, $p = 0.54$; alkaline phosphatase 126.2 vs. 126.5 U/L, $p = 0.99$; total bilirubin 1.3 vs. 1.5 mg/dL, $p = 0.77$). Serum AFP levels were elevated in 12 patients within the month prior to first nivolumab therapy. Seven of these 12 patients showed a reduction in serum AFP by at least 50% following 1–2 months of nivolumab treatment.

Imaging studies

Review of imaging reports pre- and post-nivolumab therapy showed three patients with partial response, ten with stable disease, and six with progressive disease (RECIST 1.1 [40]). One patient did not undergo post-nivolumab treatment imaging prior to surgery, so radiologic treatment response could not be evaluated.

Pathologic evaluation

Three patients showed treatment response that could be attributed to nivolumab therapy on pathologic evaluation, two with no residual viable tumor in the specimen, and one

Table 1 Summary of clinical data, treatment history, pathology of tumor and nontumor liver, and follow-up for patients treated for hepatocellular carcinoma with nivolumab followed by surgery.

Patient	Age at surgery (years)	Sex	Liver disease	Fibrosis stage [39]	Prior therapy	Nivolumab doses	Radiologic response	Tumor differentiation	Tumor necrosis[a]	Tumor pathology	Nontumor pathology	Follow-up	Follow-up (months)
Nivolumab as neoadjuvant therapy prior to partial hepatectomy													
1	41	M	HBV	3	RES	4	PD	Poor	None	TILs	Focal PN	Recurrence	23
2	61	M	HCV	5	None	2	SD	Mod	None	TILs	Focal PN	Died MI, NED	17
3	56	F	HBV	1	RES × 2	2	SD	Mod	None	TILs	Focal PN, PDF	Recurrence	9
4	35	M	HBV	6	None	2	PD	Poor	None	TILs		NED	10
5	38	M	HBV	3	None	4	PD	Poor	None	TILs		NED	7
6	65	M	HCV	6	None	10	SD	Mod	None	TILs	PDF	NED	7
7	29	M	HBV	6	None	2	PD	Mod	0–15%	TILs		Recurrence	14
8	60	M	C282Y	NA	None	3	PD	Poor	10%	TILs		NED	5
9	62	M	HBV	3	None	2	PR	Poor	95%	Marked TILs	OPV	NED	28
10	63	M	HCV	6	None	2	Not done	NT	100%	Marked TILs	Granulomas, BD IELs	Recurrence	12
11	67	F	HCV	5	None	4	PR	NT	100%	TILs	PDF	NED	6
12	78	M	Unknown	NA	Y90 × 2	6	PR	NT	100%	TILs, granulomas		NED	1
Nivolumab as first- or second-line therapy followed by liver transplantation													
13	56	F	HCV[a]	6	TACE × 3	7	SD	Mod	50%			NED	10
14	69	M	Unknown	NA	RES, Y90 × 2	32	SD	Mod	0–100%	TILs	OPV	NED	12
15	57	M	HBV	2	RES, sorafenib, TACE × 4, RFA × 2	32	SD	Mod	0–100%		BDD, PDR, PDF, OPV	NED	12
16	29	M	HBV	3	TACE × 3	25	SD	Poor	0–100%	TILs		NED	10
17	66	M	HBV	6	RES × 2, sorafenib, TACE	10	SD	Mod	0–100%			NED	7
18	64	F	MTA	NA	RES, Y90	8	PD	Well	0–75%	TILs		NED	7
19	53	M	HCV	6	Y90 × 2	25	SD	NT	100%	TILs		NED	1
20	62	M	HBV	6	none	3	SD	Poor	<5%	Tumor fibrosis, TILs		NED	7

BDD bile duct damage, *BD IELs* bile duct intraepithelial lymphocytes, *C282Y* hereditary hemochromatosis, *F* female, *HBV* chronic hepatitis B, *HCV* chronic hepatitis C, *M* male, *Mod* moderately differentiated, *MI* myocardial infarction, *MTA* malignant transformation of an adenoma, *NA* not applicable, *NED* no evidence of disease, *NT* no residual tumor, *OPV* obliterative portal venopathy, *Poor* poorly differentiated, *PD* progression of disease, *PDF* periductal fibrosis, *PDR* prominent ductular reaction, *PN* perivenular necrosis, *PR* partial response, *RES* resection, *SD* stable disease, *TACE* transarterial chemoembolization, *TILs* tumor-infiltrating lymphocytes, *Well* well differentiated, *Y90* yttrium 90 radioembolization.

Grayed rows indicate cases with treatment response to nivolumab.

[a]Cases with a range of percent tumor necrosis indicate multiple tumors with differing degrees of tumor necrosis.

Table 2 Summary of laboratory data for patients treated for hepatocellular carcinoma with nivolumab followed by surgery.

	AST (U/L)		ALT (U/L)		Alk Phos (U/L)		tbili (mg/dL)		AFP (ng/mL)	
Normal range ->	1–35		1–49		38–126		0.1–1.2		0–9	
Patient #	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Nivolumab as neoadjuvant therapy prior to partial hepatectomy										
1	32	29	31	31	87	78	**1.9**	**1.3**	2.4	<2
2	31	**87**	38	**126**	82	53	0.6	**1.5**	3.3	<2
3	25	**242**	13	**237**	80	57	0.8	**1.6**	**283.4**	**485.9**
4	26	19	27	22	93	84	0.8	0.5	**16.6**	**12.3**
5	ND	**45**	47	**50**	110	118	0.4	0.6	**>20,000**	**24,768**
6	**43**	**37**	39	42	105	119	0.6	0.4	**15.5**	7.3
7	**69**	**212**	42	**198**	111	54	**1.5**	**4.2**	**730,069**	ND
8	**60**	**51**	**57**	**48**	**174**	**146**	0.5	0.4	**30.1**	ND
9	30	29	27	29	95	105	0.8	0.8	**3,861**	**534**
10	**45**	**43**	**66**	**67**	69	83	0.3	0.4	**245**	218
11	**66**	34	30	23	107	107	0.6	0.7	**86,221**	**31,537**
12[a]	**69**	**80**	**58**	**102**	125	**212**	0.8	0.7	**220**	**41**
Nivolumab as first- or second-line therapy followed by liver transplantation										
13[a]	**90**	**156**	40	**74**	94	126	**7.3**	**8.6**	2.3	2
14[a]	34	27	**68**	49	82	62	0.6	0.9	3.5	3.5
15[a]	**316**	**58**	**277**	**82**	**270**	**232**	**1.3**	1.0	<2	<2
16[a]	11	30	**137**	**70**	103	122	0.9	0.7	**313**	5.5
17	**36**	25	36	**63**	120	90	0.7	0.9	1.3	7.7
18[a]	23	34	26	36	**188**	**174**	0.3	0.3	8.5	8.7
19[a]	**39**	34	32	31	**145**	**139**	0.7	0.6	2.8	2.7
20	**147**	**96**	**140**	**80**	**283**	**368**	**5.1**	**3.9**	**17.5**	**110.7**

AFP α-fetoprotein, *Alk* Phos alkaline phosphatase, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *tbili* total bilirubin, *ND* not done.

Grayed rows indicate cases with treatment response to nivolumab.

Bolded values indicate levels above the normal range.

[a]Cases indicate patients who underwent locoregional therapy within 2 months prior to first nivolumab dose (cases 12 and 16 within 2 weeks, case 15 within 2–4 weeks, and cases 13, 14, and 18 within 1–2 months).

with 95% tumor necrosis (Fig. 1a–d). These three cases included two of the three cases showing partial response by imaging and one case in which post-nivolumab imaging was not performed prior to surgery. The third case showing partial response by imaging (patient 12) also received y90 therapy within 2 weeks of initiation of nivolumab therapy, making it difficult to determine the contributions of each treatment. Three of the 12 patients treated with nivolumab in the neoadjuvant setting showed treatment response, whereas none of the patients treated with nivolumab while awaiting liver transplant showed definitive nivolumab-related treatment response. Some degree of tumor necrosis was seen in 14 of 20 cases, but in 11 cases these changes were attributed to prior locoregional therapy by comparison of serial imaging reports and/or identification of embolic beads and/or y90 spheres in the vicinity of the necrotic lesion.

TILs, characterized by clusters of lymphocytes within the tumor and/or at the tumor–nontumor interface, were identified in 15 of 20 cases, while two additional cases demonstrated sheets of lymphocytes and associated tumor necrosis, and were designated as marked TILs (Fig. 1). Resections from all three patients with nivolumab-related treatment response demonstrated TILs, including both patients showing marked TILs. The tumor that demonstrated nivolumab-related treatment response, but contained residual tumor, was poorly differentiated. In patients without treatment response, all degrees of differentiation were seen.

Nontumor liver was evaluated for biliary changes, vascular/perivascular changes, and granulomas. Grading and staging of viral hepatitis was also performed when appropriate. Cases 3 and 6 only had slides containing both tumor and nontumor tissue for evaluation, whereas the remaining 18 cases included sections of liver taken away from the tumor.

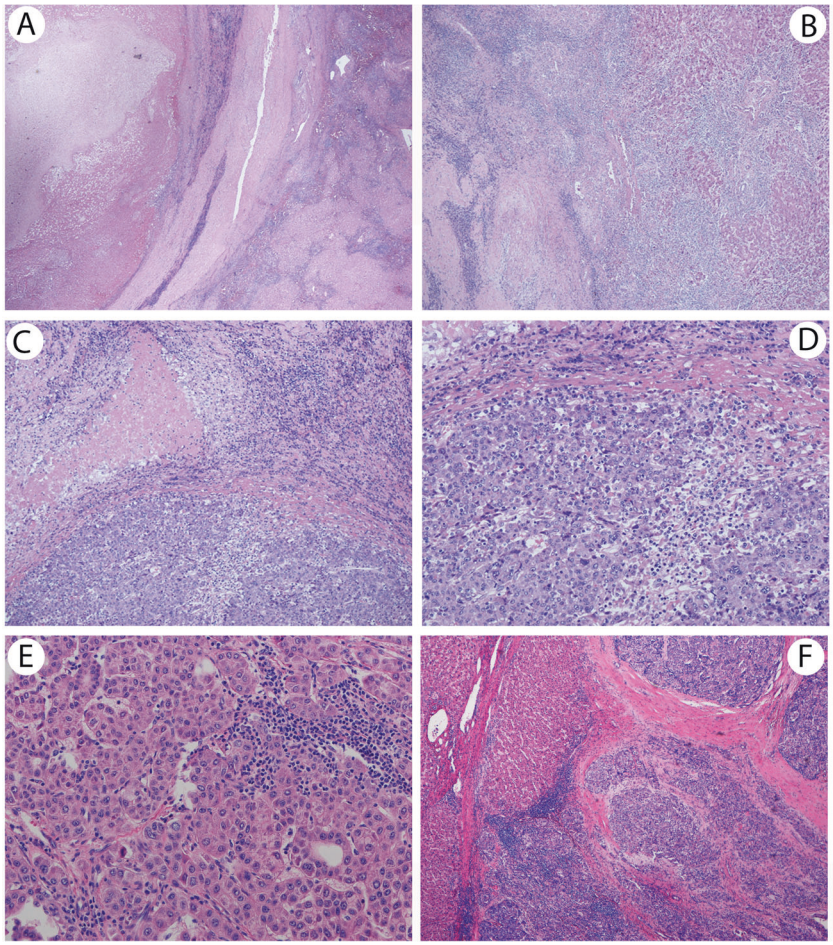

Fig. 1 Morphologic findings in tumor tissue following nivolumab therapy. **a–d** Slides from patient 9 show treatment response to nivolumab with extensive tumor necrosis and sheets of TILs (**a**, **b** hematoxylin and eosin ×20), and focal residual poorly differentiated HCC (**c** hematoxylin and eosin ×100; **d** hematoxylin and eosin ×200). **e** Slides from patient 2 shows intratumoral TILs (hematoxylin and eosin ×200). **f** Slides from patient 7 shows TILs at the tumor–nontumor interface (hematoxylin and eosin ×40).

Of the nontumor findings, biliary changes were the most commonly seen with four showing periductal fibrosis, one showing IELs within bile ducts, and one showing both bile duct damage and prominent ductular reaction. Vascular/perivascular changes included three cases showing features of obliterative portal venopathy and three cases showing focal perivenular necrosis. One case (patient 10), which also showed IELs within the bile ducts and nivolumab-related tumor response, contained numerous non-necrotizing granulomas without fibrin ring features (Fig. 2). The case demonstrating bile duct damage and prominent ductular reaction (patient 15) had long-term therapy with nivolumab (32 doses), and evaluation of nontumor liver tissue from the patient's previous liver resection 45 months earlier did not show any biliary changes (Fig. 3). Of the patients with underlying viral hepatitis, most showed mild or no activity (14/16 were grade 0 or 1) and half were cirrhotic at time of surgery.

Follow-up

Median follow-up for all patients was 9.4 months from time of surgery. There were no deaths due to tumor, although one patient who received nivolumab in the neoadjuvant setting died of a myocardial infarction without evidence of tumor recurrence 17 months after surgery. Of the 12 patients who received nivolumab in the neoadjuvant setting, 4 developed tumor recurrence and one patient showed stable disease of a separate unresected tumor nodule (median follow-up 9.9 months from time of surgery). No tumor recurrence was seen in allografts of the 8 patients who received nivolumab as first- or second-line therapy followed by liver transplantation (median follow-up 8.3 months from time of liver transplantation).

Comparison of patients with and without pathologic treatment response to nivolumab

Table 3 summarizes the clinical features and morphologic findings in tumor and nontumor liver in the 3 patients with pathologic nivolumab treatment response compared with the 17 patients without pathologic nivolumab treatment response. There was no significant difference in regards to age, sex, underlying liver disease, or pretreatment serum AFP levels between those with and those without pathologic response to nivolumab therapy. The patients demonstrating pathologic nivolumab response were more likely to

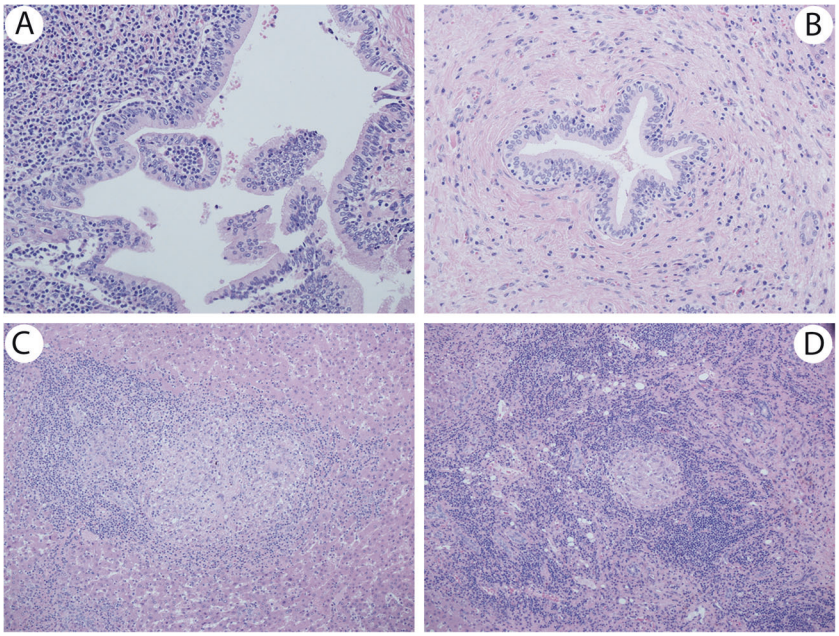

Fig. 2 Morphologic findings in nontumor liver following nivolumab therapy. Nontumor liver in a patient with complete nivolumab response (patient 10) shows prominent intraepithelial lymphocytes within bile duct epithelium (**a** hematoxylin and eosin ×100, **b** hematoxylin and eosin ×200) and several non-necrotizing granulomas (**c**, **d** hematoxylin and eosin ×100).

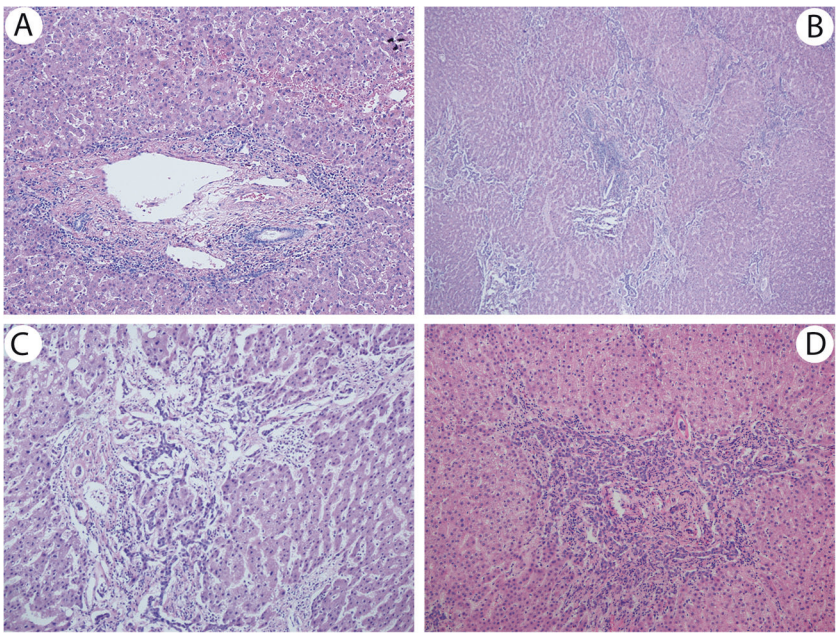

Fig. 3 Morphologic findings in nontumor liver following long-term nivolumab therapy. Nontumor liver from a prior resection specimen from patient 15 showing preserved bile duct and minimal ductular reaction (**a** hematoxylin and eosin ×100). The patient subsequently developed a new tumor, received 32 doses of nivolumab therapy over 16 months, and eventually underwent liver transplantation. Nontumor parenchyma from the explanted liver showed bile duct damage and marked ductular reaction (**b** hematoxylin and eosin ×40; **c**, **d** hematoxylin and eosin ×100).

show a reduction of AFP levels of at least 50% post-nivolumab therapy than those who did not show pathologic nivolumab treatment response ($p = 0.049$). With regard to pathologic findings, there was no difference in tumor grade, presence of TILs, or nontumor findings, although cases demonstrating pathologic treatment response were more likely to show marked TILs than those without pathologic treatment response ($p = 0.015$). There was no difference in rate of tumor recurrence between those with and without pathologic nivolumab treatment response.

Discussion

Despite the considerable progress that has been made in understanding the epidemiology, risk factors, and molecular profiles of HCC in the past few decades, treatment options are often limited due to advanced stage of tumor at diagnosis and median survival of patients with advanced HCC remains dismal at ~1 year [5]. The development of ICIs has led to dramatic advances in cancer therapy with remarkable response in many advanced malignancies [6–9]. The effect

Table 3 Clinical features and tumor and nontumor morphologic findings in patients with and without treatment response to nivolumab.

Tumor findings	All patients $N = 20$	Treatment response $N = 3$	No treatment response $N = 17$
Age, years—mean (SD)	56 (14)	64 (3)	54 (15)
Male (%)	16 (80%)	2 (67%)	14 (83%)
Underlying liver disease			
HBV (%)	10 (50%)	1 (33%)	9 (53%)
HCV (%)	6 (30%)	2 (67%)	4 (24%)
Other/unknown (%)	4 (20%)	0	4 (24%)
Cirrhotic (%)	8 (40%)	1 (33%)	6 (35%)
Elevated pretreatment AFP (%)	12 (60%)	3 (100%)	9 (53%)
Posttreatment decrease in AFP (%)	8 (40%)	3 (100%)*	5 (29%)*
Tumor findings			
Tumor grade			
No viable tumor (%)	4 (20%)	2 (67%)	2 (12%)
Well differentiated (%)	1 (5%)	0	1 (6%)
Moderately differentiated (%)	8 (40%)	0	8 (47%)
Poorly differentiated (%)	7 (35%)	1 (33%)	6 (35%)
TILs (%)	17 (85%)	3 (100%)	14 (82%)
Marked TILs (%)	2 (10%)	2 (67%)**	0**
Tumor recurrence	4 (20%)	1 (33%)	3 (18%)
Nontumor findings			
Non-necrotizing granulomas (%)	1 (5%)	1 (33%)	0
Periductal fibrosis (%)	5 (25%)	1 (33%)	3 (18%)
Bile duct IELs (%)	1 (5%)	1 (33%)	0
Bile duct loss (%)	1 (5%)	0	1 (6%)
Prominent ductular reaction (%)	2 (10%)	0	1 (6%)
Obliterative portal venopathy (%)	3 (15%)	1 (33%)	2 (12%)
Focal perivenular necrosis (%)	3 (15%)	0	3 (18%)

AFP serum α-fetoprotein, *HBV* chronic hepatitis B, *HCV* chronic hepatitis C, *IELs* intraepithelial lymphocytes, *TILs* tumor-infiltrating lymphocytes.

*$p = 0.049$; **$p = 0.015$.

on the liver of nivolumab treatment for HCC is not yet well-characterized. This patient population makes evaluating for liver toxicity difficult as these patients often have underlying hepatitis, are often cirrhotic and may demonstrate liver injury due to the intraparenchymal mass. To the best of our knowledge, this is the first study to systematically report the morphological changes in tumor and nontumor tissue in HCC patients treated with nivolumab as first- or second-line therapy.

Our study dealt with two patient populations, 12 patients who were administered nivolumab in a neoadjuvant setting prior to scheduled surgical resection, and 8 patients who were given nivolumab, usually in combination with locoregional therapies, while awaiting availability of an organ for liver transplantation. We found that 3 of 12 patients treated with nivolumab in the neoadjuvant setting showed treatment response that could be attributed to nivolumab therapy, while none of the 8 patients treated with nivolumab while awaiting liver transplantation showed definitive response to nivolumab therapy. An additional patient treated in the neoadjuvant setting also showed complete tumor necrosis, but this patient was also treated concurrently with y90, so the contribution of nivolumab could not be accurately assessed. Pretreatment clinical characteristics such as underlying liver disease, and serum AFP levels did not correlate with treatment response. Pretreatment tumor characteristics could not be assessed as the diagnosis of HCC is routinely made by imaging studies, so pretreatment biopsy material was not available in this retrospective study. Evaluation of the resection specimens following treatment revealed that most cases (17 of 20) contained TILs, while the presence of marked TILs was associated with pathologic treatment response to nivolumab ($p = 0.015$).

Our study had only limited follow-up data with a median follow-up period of 9.4 months after surgery. During our study, we report only one patient death at 17 months after surgery, but this patient was without evidence of recurrent tumor. Within the neoadjuvant cohort, one of three patients who showed treatment response with nivolumab developed tumor recurrence, whereas three of nine patients who did not show treatment response developed tumor recurrence ($p = 1.0$). None of the patients who underwent liver transplantation developed recurrence. Longer follow-up times will be needed to determine the true effect of nivolumab on tumor recurrence and survival.

We saw a variety of changes in the nontumor liver, including IELs within bile ducts, ductular reaction, bile duct damage, periductal fibrosis, granulomas, obliterative portal venopathy-type changes, and perivenular necrosis. As the majority of patients (16 of 20) had underlying viral hepatitis, it was not practical to evaluate for hepatitic changes related to nivolumab therapy. Some of the more intriguing findings are illustrated in the more detailed case descriptions below.

The first case (patient 10) is a 63-year-old man with a history of hepatitis C with cirrhosis, who developed a 4.5 cm HCC in the left lobe of the liver, which involved the left portal vein. He received two doses of nivolumab as neoadjuvant therapy before undergoing a left lobectomy. Examination of the resection specimen revealed complete necrosis of the tumor, while the nontumor liver showed lymphocytic

infiltration of the bile duct epithelium as well as non-necrotizing granulomas (Fig. 2). Several studies including, Everett et al. [36], Peeraphatdit et al. [41], and others [25, 30, 42] reported the presence of non-necrotizing granulomas, including fibrin ring granulomas; however, the granulomatous reaction, presented in their studies, was thought to be the result of toxicity from dual CTLA-4/PD-1 inhibitors in patients treated for nonliver tumors. The granulomas seen in our cases did not have fibrin ring features. This case appears to demonstrate a general upregulation of the immune response leading to both tumor necrosis and liver damage manifested by granulomas and IELs within the bile duct. The development of IRAEs has been associated with improved survival in patients treated with ICI for melanoma [43–47] and non-small-cell lung cancer [48]. Interestingly, this patient showed no significant increases in lab values (AST, ALT, alkaline phosphatase, total bilirubin) related to the nivolumab therapy, despite the striking histologic findings, suggesting that laboratory values may underestimate the true degree of liver injury due to ICI therapy.

The second case (patient 15) is a man with chronic hepatitis B, who underwent right lobectomy for a 20.1 cm HCC. The background liver showed chronic hepatitis B with stage 2 fibrosis, but, importantly, there were no significant biliary findings at this time (Fig. 3a). Thirteen months later, he developed multiple lesions in the left lobe and underwent several rounds of locoregional therapy with TACE and RFA. He was started on nivolumab 29 months after initial surgery. Subsequent imaging studies showed necrosis of lesions treated by locoregional therapies, but no significant effect of the nivolumab therapy. The patient eventually underwent liver transplantation 45 months after initial surgery after receiving 32 doses of nivolumab over a 16-month period. Pathologic examination of the liver showed multiple tumor nodules with complete necrosis and one tumor nodule with 50% necrosis, all attributed to the locoregional therapy, but the nontumor liver now showed bile duct damage and marked ductular reaction (Fig. 3b–d) in addition to chronic hepatitis B with stage 2 fibrosis. It is difficult to determine whether the bile duct damage and ductular reaction were due to the nivolumab, the locoregional therapy, or some combination, but it does not appear to be due to an underlying biliary disease as these findings were not seen in the patient's prior liver resection specimen. Laboratory values before and after initiation of nivolumab therapy showed a consistently elevated serum alkaline phosphatase, but a reduction in AST, ALT, and total bilirubin levels. This patient had undergone two locoregional therapies around the same time as initiation of nivolumab therapy, with a TACE procedure 1 month prior to and a RFA procedure 1 month after initiation of nivolumab therapy, further complicating interpretation of these laboratory results.

Considering these cases together, the IELs seen within the bile ducts in patient 10 may indicate an early effect of nivolumab, while the bile duct damage and prominent ductular reaction seen in patient 15, if in fact due to nivolumab therapy, may represent a late effect due to chronic administration of the drug. Prospective studies with liver biopsies at multiple time points during long-term nivolumab therapy would help to elucidate progression of liver injury.

In summary, to the best of our knowledge, our study is the first to report histologic changes in tumor and nontumor liver in patients with HCC treated with nivolumab followed by surgery. We further demonstrate that nivolumab can be effective in the neoadjuvant setting for HCC. We found that a brief neoadjuvant regimen (2–4 doses over 1 to 2 months) resulted in complete or near-complete tumor necrosis with associated TILs in 3 of 12 patients treated. This strongly suggests that a subset of patients may be particularly sensitive to ICI therapy. We also documented several histologic findings in the nontumor liver that may represent liver injury related to nivolumab therapy. The findings of granulomas and IELs within bile duct epithelium may represent early nivolumab-related liver injury while bile duct damage and associated ductular reaction warrant further investigation as possible later manifestations of nivolumab-related liver injury. We also acknowledge several significant limitations of our study. First, our small cohort consists of a heterogeneous group of patients who differ in underlying liver diseases, stage of tumor, prior therapies (locoregional, resections, and sorafenib), and nivolumab treatment regimens. The 12 cases treated in the neoadjuvant setting are somewhat more uniform, with all patients deemed potential surgical candidates and all but one without concurrent locoregional therapies, although it still remains difficult to rule out contributions of other variables. Unfortunately, our study was not large enough for further subgroup analysis. The second major limitation is that the patients in our retrospective study did not undergo pretreatment tumor biopsies, as this was not a standard procedure at the time these patients were diagnosed with HCC. Currently, there is a lack of identifiable marker to select for HCC patients that may respond to ICIs. Thus far, neither tumoral PD-L1 expression nor baseline AFP predicted response to nivolumab in HCC [17]. Without pretreatment biopsies, we were not able to assess histologic or molecular tumor characteristics that may have improved patient selection. Further, due to the lack of pretreatment biopsies, we are also not able to determine whether histologic features of tumor seen here (e.g., TILs) are a characteristic of the tumor, related to treatment, or a combination thereof. Future prospective studies with pretreatment tumor biopsies would be invaluable in evaluating markers to predict which patients would demonstrate the dramatic responses to nivolumab we saw in a subset of our patients.

Compliance with ethical standards

Conflict of interest MES is a principal investigator in a separate study funded by Bristol Myers Squibb evaluating nivolumab.

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