Combination Immunotherapy with Anti-PD-1/PD-L1 Antibody plus Anti-VEGF Antibody May Promote Cytotoxic T Lymphocyte Infiltration in Hepatocellular Carcinoma, Including in the Noninflamed Subclass

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Noninflamed Tumors

The most recent immunogenomic classification of hepatocellular carcinoma (HCC) was published in 2022 [1]. According to this report, 65% of HCC cases are of the noninflamed class, while 35% are of the inflamed class, the latter of which potentially respond well to immune checkpoint inhibitors. The inflamed class can be further classified into the active subclass, the exhausted subclass, and the immune-like subclass. The inflamed class is characterized by strong interferon (IFN) signaling and cytolytic activity; upregulation of effector molecules of cytotoxic T cells; and increases in checkpoint molecules and CD8+ T cells (Fig. 1). The noninflamed class can be further classified into the intermediate subclass and the excluded subclass. The excluded subclass is characterized by CTNNB1 mutations and an extremely low level of tumor-infiltrating lymphocytes, while the intermediate class is characterized by TP53 mutations. The common features of both noninflamed subclasses include increases in M2 macrophages and regulatory T cells; decreases in checkpoint molecules and CD8+ T cells; reductions in IFN-γ signature, GZMB, PRF1, and PD-1 signaling; and decreases in CXCR3 ligands (CXCL9, CXCL10, and CXCL11) and CCL5 (Fig. 1). Interestingly, although CTNNB1 mutation was usually known to cause immune exclusion in other cancer types, only HCCs with CTNNB1 mutations are found to be classified into two distinct types, which are novel findings [1]: one in which PTK2 is hypomethylated and MHC class 1-related genes are hypermethylated, which leads to impaired antigen-presenting ability, and one in which MHC class 1-related genes are hypomethylated, indicating that HCC with CTNNB1 mutations can be either inflamed or noninflamed tumors [1] (Fig. 1).
Blocking Mechanism of Cytotoxic T Lymphocyte Infiltration into the Tumor

Adhesion molecules are necessary for activated cytotoxic T lymphocytes (CTLs) to infiltrate into tumors after priming in lymph node [2]. Upon binding of integrins on CTLs to adhesion molecules on endothelial cells, CTLs migrate into tissues through a process involving tethering, rolling, arresting, and crawling [2], but in the presence of VEGF, this integrin-mediated adhesion and migration of CTLs is known to be impaired due to reduced levels of adhesion molecules such as ICAM-1 and VCAM-1 [2]. In contrast, administration of anti-VEGF antibodies increases the level of intratumoral infiltrating CD8+ T cells [3]. VEGF also upregulates Fas ligands on endothelial cells in the presence of IL-10 and prostaglandin E2, thereby inducing CTL apoptosis and consequently decreasing the level of intratumoral CTLs [4], thereby inducing CTL apoptosis and consequently decreasing the level of CTLs. Conversely, inhibition of VEGF is known to suppress Fas ligand expression, thereby promoting CTL infiltration [5]. It is also known that even when CTLs are infiltrating, VEGF increases the proliferation and expansion of regulatory T cells and myeloid-derived sup-

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**Table 1.** Immunogenomic classification. Modified from ref. [1].

|                  | Inflamed class (~35%) | Non-inflamed class (~65%) |
|------------------|-----------------------|---------------------------|
|                  | Active                | Exhausted                | Immune-like               | Intermediate | Excluded |
|                  | +                     |                          |                          |              |
|                  | Increased TIL infiltrate |                         | Decreased TIL infiltrate |
| CD8 T cells      | ↑                     | M1 macrophage            | ↑ CD8 T cells            | ↑ M2 macrophage |
| M1 macrophage    | ↑                     | T cell exhaustion        | M1 macrophage            | T regulatory cell |
|                  | Increased checkpoint molecules/CD8 | Decreased checkpoint molecules/CD8 |
| IFN-γ, GZMB, PRF1, PD-1 signaling | ↑ IFN-γ, GZMB, PRF1, PD-1 signaling |
| CXCL9, CXCL10, CXCL11, CCL5 | ↑ CXCL9, CXCL10, CXCL11, CCL5 |
| Hoshida S1       | ↑                     | Hoshida S2               | Hoshida S2/S3            |
| Chiang IFN       | ↑                     | Chiang CTNNB1            | Chiang poly7             |
| Chiang CTNNB1    | ↑                     | Chiang poly7             | Chiang CTNNB1            |
| CTNNB1 mutation  | ↑                     | TP53 mutation            | CTNNB1 mutation          |
| Hypomethylation of MHC-1 related genes | Hypomethylation of MHC-1 related genes |
| FDG-PET positive |                       | Iso-High intense on HBP of EOB-MRI |

**Fig. 1.** Immunogenomic classification. Modified from ref. [1].

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**Fig. 2.** Decreased CD8+ T cell infiltration mechanism and increased CD8+ T cell infiltration mechanism by blocking VEGF both in inflamed and non-inflamed type tumor microenvironment. **a** Decreased CD8+ T cell infiltration mechanism in tumor microenvironment before conversion to inflamed tumor. Adhesion molecules are necessary for activated CTL to infiltrate into tumors after priming in lymph node. Upon binding of integrins on CTLs to adhesion molecules on endothelial cells, CTLs migrate into tissues through a process involving tethering, rolling, arresting, and crawling, but in the presence of VEGF, this integrin-mediated adhesion and migration of CTLs is impaired due to reduced levels of adhesion molecules such as ICAM-1 and VCAM-1. VEGF also upregulates Fas ligands on endothelial cells inducing CTL apoptosis and consequently decreasing the level of intratumoral CTLs. **b** Possible mechanism of CD8+ T cell recruitment by anti-PD-L1 plus anti-VEGF combination therapy in inflamed (immune hot)-type tumor microenvironment. Inhibition of VEGF suppresses Fas ligand expression, thereby promoting CTL infiltration into the tumor. Furthermore, inhibition of VEGF decreases Treg proliferation and increase IFN-γ production from CTL, resulting in production of CXCR3 ligands, which further increase CTL infiltration into the tumor. (For figure see next page.)
pressor cells to counteract CTLs and their effects. VEGF also increases the expression of immune checkpoint molecules such as PD-1, CTLA-4, LAG-3, and TIM-3 on the surfaces of CTLs [6] that have already been infiltrated, and thus, single-agent immune checkpoint inhibitor treatment is not effective in such an immunosuppressive microenvironment. However, basic research to date has demonstrated that inhibition of VEGF skews the immune

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**Diagram a**

- Weak recognition between CXCR3-CXCL9
- VEGF reduce the ICAM and VCAM expression
- CTL
- CXCR3
- Selectin ligands
- Selectins
- Endothelial cells
- Lower density of CXCL9, 10, 11
- CXCL-9
- PD-L1
- PD-1
- DC
- VEGF
- VEGFR
- Cancer cells
- Treg
- IFNγ

**Diagram b**

- Vascular normalization → ICAM, VCAM↑
- Cytotoxicity
- CTL
- CXCR3
- Selectin ligands
- Selectins
- Endothelial cells
- CXCL-9
- PD-L1
- PD-1
- DC
- Anti-PD-L1
- Anti-VEGF
- IFNγ
- VEGF
- VEGFR
- Cancer cells
- Treg

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microenvironment toward an immune-responsive microenvironment by inhibiting apoptosis and enhancing infiltration of activated CTLs by suppressing Fas ligand expression; increasing ICAM-1 and VCAM-1 expression; suppressing the expression of PD-1, CTLA-4, LAG-3, and TIM-3 on CTLs; and upregulating the expression of MHC class 1 molecules, while decreasing T reg cells and myeloid-derived suppressor cells [4, 7]. However, it should be noted that these phenomena are the effects of VEGF inhibition not only in noninflamed tumors but also in inflamed tumors (Fig. 2).

Combination Immunotherapy with Anti-PD-L1 Antibody plus Anti-VEGF Antibody Promotes CTL Infiltration into the Tumor Tissue

A recent study showed that the presence of dendritic cells and ligands of CXCR3 (CXCL9, CXCL10, and CXCL11) is crucial for infiltration of CTLs into tumors [8]. Indeed, infiltration of CD8+ T cells into the tumor tissue was attenuated in noninflamed tumors where expression of CXCR3 ligands was low [8]. Ishikura et al. [9] showed that even in such a noninflamed mouse model with PD-L1low and CD88low phenotypes, although anti-PD-L1 antibody or anti-VEGF antibody monotherapy did not increase the infiltration of CTLs into tumors, combination therapy with anti-PD-L1 and anti-VEGF antibodies increased infiltration of CD8+ T cells into tumors and consequently significantly enhanced antitumor effects. Furthermore, combination therapy significantly induced IFN-γ, leading to changes in downstream events such as increases in CXCL9 expression as well as the infiltration of activated CD8+ T cells [9]. It was discussed that even in the noninflamed microenvironment where CD8+ T cells, PD-L1, and CXCR3 ligands are low, anti-PD-L1 and anti-VEGF antibodies together induce first IFN-γ and then CXCR3 ligands, thereby promoting the infiltration of CTLs in tumors [9]. In addition to the widely accepted mechanism of CD8+ T cell activation by VEGF inhibition, which induces tumor vessel normalization and following improvement in the immune microenvironment [10], another possible mechanism that has been suggested for IFN-γ induction by anti-VEGF antibodies is that anti-VEGF antibody-induced hypoxia induces IFN-γ production by sparsely existing intratumoral CD8+ T cells, resulting in the release of CXCR3 ligands from dendritic cells [11, 12] (Fig. 3). In addition, anti-VEGF antibodies were shown to increase the hypoxic area as well as the number of IFN-γ+ CD8+ T cells there [9]. Furthermore, because inhibition of HIF-1α suppresses IFN-γ+ CD8+ T cells, it was speculated that increases in the level of IFN-γ+ CD8+ T cells, which have an antitumor effect, were the result of hypoxia and depended on HIF-1α [13] (Fig. 3). In the noninflamed mouse model, anti-VEGF monotherapy did not increase CD8+ T cells in tumor tissue, but combination with anti-VEGF plus anti-PD-L1 antibody enhanced CXCL9 levels and CD8+ T cell infiltration, indicating that hypoxia-induced activation of tumor-infiltrating CD8+ T cells may need to block PD-1/PD-L1 signaling in the noninflamed tumor microenvironment. Therefore, combination therapy is likely to trigger the immune cycle via IFNγ-CXCL9 production and exert a therapeutic effect at least in a part of noninflamed tumors with a low level of intratumoral CD8+ T cells and dendritic cells. In addition, since anti-VEGF antibody has a direct anti-tumor effect leading to cancer antigen release and thus, activating cancer immunity cycle, the combination of anti-PD-L1 antibody and anti-VEGF antibody is likely to exert a therapeutic effect in a part of noninflamed tumors having even a low level of intratumoral CD8+ T cells and dendritic cells.

The combination of TACE with anti-PD-L1 plus anti-VEGF immunotherapy is likely to be an extremely effective therapy because TACE causes tumor destruction and the release of large amounts of cancer antigens, thereby activating the cancer immunity cycle, and in conjunction with anti-PD-L1 and anti-VEGF antibodies, exerts a mass-reducing effect. Thus, the combination of TACE with anti-VEGF plus anti-PD-1/PD-L1 antibodies is anticipated to be an extremely promising therapeutic strategy for intermediate- and advanced-stage HCC. Indeed, in the clinical setting, many patients have achieved cancer-free drug-free status by using the ABC-TACE sandwich therapy wherein anti-VEGF plus anti-PD-1/PD-L1 (atezolizumab plus bevacizumab) therapy and TACE are performed sequentially, or by using the ABC LEN-TACE sandwich therapy [14, 15], indicating that these results support preclinical results and theories.

Fig. 3. CD8+ T cell transportation/infiltration mechanism in noninflamed (immune cold)-type tumor microenvironment. a Infiltration of CD8+ T cells into the tumor tissue was attenuated in noninflamed tumors where expression of CXCR3 ligands is low. b Double block of PD-1/PD-L1 and VEGF induces hypoxic state, which further increases IFN-γ from CTL, resulting in increased production of CXCR3 ligands, which further increase CTL infiltration into the tumor.

(For figure see next page.)
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Dual Aspect of the WNT/β-Catenin Mutation (CTNNB1 Mutation)

Different from previous reports in other solid tumors in which immune exclusion is usually caused by CTNNB1 mutation, in the immunogenomic classification proposed by Montironi et al. [1], novel findings that there are 2 types of HCCs with CTNNB1 mutations: one is the immune excluded subclass in the noninflamed class and the other is the immune-like subclass in the inflamed class. Although it is unclear at present, hypermethylation and hypomethylation of MHC class 1-related genes may be involved in the immune-excluded subclass and the immune-like subclass, respectively. Immunotherapy may have completely opposite effects in these two distinct subclasses with CTNNB1 mutations. In other words, the immune-like subclass with the phenotype characterized by prominent infiltration of immune cells (e.g., infiltrations of CD8+ T cells, increases in M1 macrophages, and IFN-γ signature), and the immune-excluded subclass with the completely opposite phenotype will respond to immunotherapy very differently [16].

It is suggested that there might be two phenotypes in cancer in the context of CTNNB1 mutations: one is a mild phenotype associated with the expression of HNF4α and the other is a more aggressive phenotype characterized by stem cell feature and epithelial-to-mesenchymal transition (EMT). It is suggested that the Wnt/β-catenin pathway is involved in EMT that should be associated with aggressive tumor phenotype [17]. Activation of the Wnt/β-catenin pathway could induce the EMT effectors such as Snail and N-Cadherin [18]. Coordination of the phosphoinositide 3-kinase/Akt and Wnt/β-catenin signaling pathway is known to be contributed to EMT by controlling histone acetylation [19, 20].

Meanwhile, HNF4α induces OATP1B3 expression, which is associated with iso-to-higher intensity in the hepatobiliary phase of Gd-EOB-DTPA MRI [21–23], while the other phenotype should be completely different and associated with positive findings in FDG-PET images. Although the former does not respond to immunotherapy, the latter does respond, and this is consistent with the findings of Montironi et al. [1].

Conclusion

Preclinical studies have recently demonstrated that a combination of anti-PD-L1 antibody and anti-VEGF antibody can be effective for treating even noninflamed tumors by inducing CXCR3 ligands such as CXCL9 and CXCL10. It was confirmed clinically that the effect of this combination therapy was further enhanced by the addition of TACE [14, 15], which increases immunogenicity. In that sense, the results of currently ongoing clinical trials are eagerly awaited to confirm the efficacy of TACE plus combination immunotherapy (anti-PD-L1 antibody plus anti-VEGF antibody) in patients even with noninflamed tumor microenvironment.

Conflict of Interest Statement

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M. Kudo conceived, wrote, and approved the final manuscript.

References

1 Montironi C, Castet F, Haber PK, Pinyol R, Torres-Martín M, Torrens L, et al. Inflamed and non-inflamed classes of HCC: a revised immunogenomic classification. Gut. 2022.
2 Huinen ZR, Huijbers EJM, van Beijnum JR, Nowak-Sliwinska P, Griffioen AW. Anti-angiogenic agents: overcoming tumour endothelial cell anergy and improving immunotherapy outcomes. Nat Rev Clin Oncol. 2021; 18(8):527–40.
3 Boucher Y, Kumar AS, Posada JM, Gjini E, Pfaff K, Lipschitz M, et al. Bevacizumab improves tumor infiltration of mature dendritic cells and effector T-cells in triple-negative breast cancer patients. NPJ Precis Oncol. 2021;5(1):62.
4 Li SJ, Chen JX, Sun ZJ. Improving antitumor immunity using antiangiogenic agents: Mechanistic insights, current progress, and clinical challenges. Cancer Commun. 2021; 41(9):830–50.
5 Motz GT, Santoro SP, Wang LP, Garrabrants T, Lastra RR, Hagemann IS, et al. Tumor endothelium FaS-L establishes a selective immune barrier promoting tolerance in tumors. Nat Med. 2014;20:607–15.
6 Voron T, Colussi O, Marchetieu E, Pernot S, Nizard M, Poinet AL, et al. VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. J Exp Med. 2015;212:139–48.
Anti-PD-1/PD-L1 plus Anti-VEGF Promotes CTL Infiltration in HCC

7 Kudo M. Scientific rationale for combined immunotherapy with PD-1/PD-L1 antibodies and VEGF inhibitors in advanced hepatocellular carcinoma. *Cancers*. 2020;12(5):1089.
8 Iwai T, Sugimoto M, Patil NS, Bower D, Suzuki M, Kato C, et al. Both T cell priming in lymph node and CXCR3-dependent migration are the key events for predicting the response of atezolizumab. *Sci Rep*. 2021;11(1):13912.
9 Ishikura N, Sugimoto M, Yorozu K, Kurasawa M, Kondoh O. Anti-VEGF antibody triggers the effect of anti-PD-L1 antibody in PD-L1(low) and immune desert-like mouse tumors. *OncoRep*. 2022;47:32.
10 Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov*. 2011;10:417–27.
11 Roman J, Rangasamy T, Guo J, Sugunan S, Meednu N, Packirisamy G, et al. T-cell activation under hypoxic conditions enhances IFN-gamma secretion. *Am J Respir Cell Mol Biol*. 2010;42:123–8.
12 de Almeida PE, Mak J, Hernandez G, Jessadson R, Herault A, Javinal V, et al. Anti-VEGF treatment enhances CD8(+) T-cell antitumor activity by amplifying hypoxia. *Cancer Immunol Res*. 2020;8:806–18.
13 Gropper Y, Feferman T, Shalit T, Salame TM, Porat Z, Shakkhar G. Culturing CTLs under hypoxic conditions enhances their cytolysis and improves their anti-tumor function. *Cell Rep*. 2017;20:2547–55.
14 Kudo M. Curative conversion therapy after systemic therapy for hepatocellular carcinoma: ABC conversion therapy. *Kan-Tan-Sui (Hepato-Biliary-Pancreas)*. 2022. (in Japanese).
15 Kudo M. A novel treatment strategy for patients with intermediate-stage HCC who are not suitable for TACE: upfront systemic therapy followed by curative conversion. *Liver Cancer*. 2021;10(6):539–44.
16 Morita M, Nishida N, Sakai K, Aoki T, Chishina H, Takita M, et al. Immunological microenvironment predicts the survival of the patients with hepatocellular carcinoma treated with anti-PD-1 antibody. *Liver Cancer*. 2021;10(4):380–93.
17 Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. *Sci Signal*. 2014;7:re8.
18 Lv YF, Dai H, Yan GN, Meng G, Zhang X, Guo QN. Downregulation of tumor suppressor STF cDNA 3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-3β/β-catenin/Snail signaling pathway. *Cancer Lett*. 2016;373:164–73.
19 Song Y, Li ZX, Liu X, Wang R, Li LW, Zhang Q. The Wnt/β-catenin and PI3K/Akt signaling pathways promote EMT in gastric cancer by epigenetic regulation via H3 lysine 27 acetylation. *Tumour Biol*. 2017;39:1010428317712617.
20 Aoki T, Nishida N, Kudo M. Clinical significance of the duality of Wnt/β-catenin signaling in human hepatocellular carcinoma. *Cancers*. 2022;14(2):444.
21 Kudo M. Gd-EOB-DTPA-MRI could predict WNT/β-catenin mutation and resistance to immune checkpoint Inhibitor therapy in hepatocellular carcinoma. *Liver Cancer*. 2020;9(5):479–90.
22 Aoki T, Nishida N, Ueshima K, Morita M, Chishina H, Takita M, et al. Higher enhancement intrahepatic nodules on the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI as a poor responsive marker of anti-PD-1/PD-L1 monotherapy for unresectable hepatocellular carcinoma. *Liver Cancer*. 2021;10(6):615–28.
23 Ueno A, Masugi Y, Yamazaki K, Komuta M, Effendi K, Tanami Y, et al. OATP1B3 expression is strongly associated with Wnt/β-catenin signalling and represents the transporter of gadoxetic acid in hepatocellular carcinoma. *J Hepatol*. 2014;61:1080–7.