

**Figure 2.**

Combined M-MDSC, CD4⁺T_{EM}, and CD8⁺T_{EM} assessments. A, correlations between each pair of the proportions of M-MDSCs, CD4⁺T_{EM}, and CD8⁺T_{EM} cells are shown in dot plots. Pearson correlation coefficient is indicated as "r." B, analysis of patient groups with different numbers of the adverse factors of high M-MDSC, low CD4⁺T_{EM}, and low CD8⁺T_{EM}, which we identified as adverse immunologic factors as shown in Fig. 1C and Table 2. Patients were divided into four groups based on the number of adverse factors of each patient: Group 1, no adverse factor ($n = 11$); Group 2, one ($n = 8$); Group 3, two ($n = 11$); Group 4, three ($n = 10$). Blue and red colors in the heat map indicate that a patient has low (<median value) or high (>median value) quantities, respectively, of the corresponding immune cell. C and D, Kaplan-Meier curves for PFS of the four different immunologic groups are shown in C. Because the curve of Group 1 overlapped with that of Group 2, and the curve of Group 3 overlapped with that of Group 4, Groups 1 and 2 were combined, as were Groups 3 and 4. The Kaplan-Meier curves of Groups 1 and 2 and Groups 3 and 4 are shown in D. P values were calculated by the log-rank test. E, values of VEGF-A and IL6 were compared between Groups 1 and 2 and 3 and 4 with a t test. The long and short horizontal lines in the figure indicate the means and SDs, respectively. Comparison of other cytokine values are shown in Supplementary Table S4.

increased vs. decreased M-MDSC, CD4⁺T_{EM}, and CD8⁺T_{EM}; $P = 0.3$, 0.9 , and 0.3 , respectively; Supplementary Fig. S2B). Comparison of PFS between before chemotherapy and the third blood sample was not performed because patients who underwent a third blood collection were selected patients who could continue initial chemotherapy for up to 6 months.

Discussion

The present study demonstrated that pretreatment immune status correlates with the PFS of patients with unresectable MCRC given first-line chemotherapy. We analyzed 25 immune cell subsets and identified high M-MDSC, low CD4⁺T_{EM}, and low CD8⁺T_{EM} values as adverse prognostic factors for PFS. In addition, combined assessment of all three adverse factors

showed the outcomes of patients who had two or three of these factors (Groups 3 and 4) to be significantly poorer than those of patients who had zero or one adverse factor (Groups 1 and 2). This negative impact remained statistically significant in multivariate analysis. Although many retrospective studies have already shown that the quantity of TILs in surgically resected specimens correlates with the outcomes of patients with resectable colorectal cancer (1, 3–13, 26), this prospective study has demonstrated that the quantity of immune cells in peripheral blood correlates with the outcomes of those with unresectable tumors.

Approximately 27.5% of the patients in this study had low M-MDSC, high CD4⁺T_{EM}, and high CD8⁺T_{EM} values (Group 1), whereas 25% of patients had high M-MDSC, low CD4⁺T_{EM}, and low CD8⁺T_{EM} values (Group 4). This inverse correlation

Tada et al.

Table 3. Multivariate analysis for PFS

Covariates	Multivariate analysis (N = 40)	
	HR (95% CI)	P value
Group		
1/2	Reference	
3/4	9.2 (2.5–34.2)	<0.001
Use of bevacizumab		
Used	Reference	
Not used	2.5 (0.7–9.3)	0.2
Primary lesion		
Right hemicolon	Reference	
Left hemicolon	0.5 (0.1–1.7)	0.3
Rectum	0.5 (0.2–1.7)	0.3
IFN γ		
Low	Reference	
High	1.6 (0.6–4.6)	0.4
IL8		
Low	Reference	
High	2.7 (0.9–8.7)	0.09

NOTE: Multivariate analysis for PFS was performed on different immune groups, patient characteristics, and cytokine values by using a Cox proportional hazards model. Covariates were chosen based on the criteria described in Materials and Methods.

between M-MDSCs and effector memory T cells is reasonable because MDSCs are immune-suppressive cells that inhibit the proliferation and activation of T cells. However, the remaining 47.5% of patients (Groups 2 and 3) showed discrepant results for the quantities of M-MDSCs, CD4⁺T_{EM}, and CD8⁺T_{EM}. These results suggest that the quantities of M-MDSCs, CD4⁺T_{EM}, and CD8⁺T_{EM} are specific for each patient (Fig. 2B and Supplementary Fig. S2). Therefore, combined assessment of the immune-suppressive cells (M-MDSC) and the cytotoxic effector cells (CD4⁺T_{EM} and CD8⁺T_{EM}) may provide a more appropriate reflection of the immune status of each patient and would also, presumably, illustrate the correlation between immune status and prognosis more accurately than would individual assessments of these cell subsets. For example, a patient with a high quantity of M-MDSCs would generally have a short PFS, but the negative impact might be canceled out in the presence of high quantities of CD4⁺T_{EM} and/or CD8⁺T_{EM}. Similarly, although a patient with a low quantity of CD8⁺T_{EM} might be expected to have a short PFS, the negative impact could be canceled out in the presence of a low M-MDSC and a high CD4⁺T_{EM} value. In fact, we demonstrated that PFS in Group 2, which consisted of such patients, is equivalent to that in Group 1, comprised of patients with low M-MDSC, high CD4⁺T_{EM}, and high CD8⁺T_{EM} values (Fig. 2C). Our results demonstrate that individual assessments of M-MDSC and effector memory T cells have potential prognostic value for PFS and that the combined assessment of these cell subsets predicts PFS with greater accuracy than that of any one cell subset alone.

We demonstrated that the immune status at pretreatment correlated with PFS; however, changes of those cells after chemotherapy did not correlate with PFS. It is very likely that change of immune status after chemotherapy is influenced by several factors, such as direct cytotoxicity from therapeutic agents, disease progression or regression, incidence of adverse event, and so on. These various factors may make it difficult to interpret the correlation between change of immune status and PFS.

We also analyzed plasma cytokines that affect the formation of immune cell subsets. We found that VEGF-A and IL6 were

significantly higher in Groups 3 and 4 than in Groups 1 and 2. VEGF-A contributes not only to tumor angiogenesis but also to formation of the immunosuppressive microenvironment in tumors (27). VEGF-A augments MDSCs (28, 29) and inhibits DC maturation (30, 31), directly inhibits the activation and proliferation of T cells (32), and upregulates expression of the programmed death-1 molecule on T cells (33). IL6 is a multi-functional cytokine with pro- and anti-inflammatory activity. Under certain pathologic circumstances, IL6 augments MDSCs. Based on these findings, increased VEGF-A and IL6 concentrations in our cohort may have contributed to the adverse immune status, which resulted in shorter PFS.

Our present prospective study included a rather small number of patients. Nevertheless, we identified statistically significant prognostic factors for PFS. Assessment of the impact on overall survival requires an additional follow-up period because only 8 of our patients did not survive. Despite this limitation, our results have meaningful clinical implications: Antitumor immunity may be helpful for the effects of chemotherapy and thus provide a rationale for developing a regimen combining chemotherapy with immunotherapy. An immunotherapeutic approach that reduces M-MDSCs or increases effector memory T cells might overcome immunologically mediated adverse impacts on prognosis.

In conclusion, we analyzed 25 immune cell subsets in peripheral blood from patients with unresectable MCRC before first-line chemotherapy and identified high M-MDSC, low CD4⁺T_{EM}, and CD8⁺T_{EM} quantities as significant adverse factors for PFS. Combining the assessment of these three adverse factors gave greater accuracy of PFS prediction for the immunologically different patient subgroups. These results suggest that pretreatment peripheral immune status correlates with the outcomes of patients with unresectable MCRCs receiving first-line chemotherapy. Further studies involving patients with other types of cancer are warranted to assess our results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Tada, S. Kitano, Y. Shimada, K. Aoki, N. Okita, Y. Yamada, Y. Heike, T. Hamaguchi

Development of methodology: K. Tada, S. Kitano, Y. Heike

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Tada, H. Shoji, T. Nishimura, N. Hiraoka, S. Iwasa, A. Takashima, Y. Heike, T. Hamaguchi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Tada, K. Nagashima, K. Aoki, Y. Yamada, Y. Heike, T. Hamaguchi

Writing, review, and/or revision of the manuscript: K. Tada, S. Kitano, Y. Shimada, K. Nagashima, Y. Honma, N. Okita, Y. Yamada, N. Katayama, N. Boku, Y. Heike, T. Hamaguchi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Tada, S. Kitano, K. Kato, Y. Heike, T. Hamaguchi

Study supervision: S. Kitano, Y. Shimada, N. Katayama, Y. Heike, T. Hamaguchi

Acknowledgments

The authors thank Kei Nakano-Miura and Tsukasa Shinohara, who provided invaluable technical support. The authors also thank Dr. Yoichi Takaue for reviewing the article.

Grant Support

This work was supported in part by JSPS KAKENHI (Grants-in-Aid for Scientific Research), a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, and the National Cancer Center Research and Development Fund (23-A-44 and 26-A-11).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 8, 2015; revised March 2, 2016; accepted March 18, 2016; published OnlineFirst April 28, 2016.

References

- House AK, Watt AG. Survival and the immune response in patients with carcinoma of the colorectum. *Gut* 1979;20:868-74.
- Kroemer G, Galluzzi L, Zitvogel L, Fridman WH. Colorectal cancer: The first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy? *Oncoimmunology* 2015;4:e1058597.
- Chiba T, Ohtani H, Mizoi T, Naito Y, Sato E, Nagura H, et al. Intraepithelial CD8+ T-cell-count becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: Possible association with suppression of micrometastasis. *Br J Cancer* 2004;91:1711-7.
- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Page C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960-4.
- Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. *J Clin Pathol* 1989;42:162-6.
- Jass JR. Lymphocytic infiltration and survival in rectal cancer. *J Clin Pathol* 1986;39:585-9.
- Menon AG, Janssen-van Rhijn CM, Morreau H, Putter H, Tollenaar RA, van de Velde CJ, et al. Immune system and prognosis in colorectal cancer: A detailed immunohistochemical analysis. *Lab Invest* 2004;84:493-501.
- Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* 2011;29:610-8.
- Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998;58:3491-4.
- Prall F, Duhrop T, Weirich V, Ostwald C, Lenz P, Nizze H, et al. Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. *Hum Pathol* 2004;35:808-16.
- Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 2011;71:1263-71.
- Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molitor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005;353:2654-66.
- Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009;27:5944-51.
- Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: Impact on clinical outcome. *Nat Rev Cancer* 2012;12:298-306.
- Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015;348:124-8.
- Arihara F, Mizukoshi E, Kitahara M, Takata Y, Arai K, Yamashita T, et al. Increase in CD14+HLA-DR⁻/low myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. *Cancer Immunol Immunother* 2013;62:1421-30.
- Dreus-Elger K, Iorns E, Dias A, Miller P, Ward TM, Dean S, et al. Infiltrating S100A8+ myeloid cells promote metastatic spread of human breast cancer and predict poor clinical outcome. *Breast Cancer Res Treat* 2014;148:41-59.
- Gabittas RF, Annels NE, Stocken DD, Pandha HA, Middleton GW. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer Immunol Immunother* 2011;60:1419-30.
- Kitano S, Postow MA, Ziegler CG, Kuk D, Panageas KS, Cortez C, et al. Computational algorithm-driven evaluation of monocytic myeloid-derived suppressor cell frequency for prediction of clinical outcomes. *Cancer Immunol Res* 2014;2:812-21.
- Walter S, Weinschenk T, Stenzl A, Zdrojow R, Pluzanska A, Szczylik C, et al. Multipptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med* 2012;18:1254-61.
- Hochster HS, Hart LL, Ramanathan RK, Childs BH, Hainsworth JD, Cohn AL, et al. Safety and efficacy of oxaliplatin and fluoropyrimidine regimens with or without bevacizumab as first-line treatment of metastatic colorectal cancer: Results of the TREE Study. *J Clin Oncol* 2008;26:3523-9.
- Cassidy J, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, et al. Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer. *J Clin Oncol* 2008;26:2006-12.
- Kotsakis A, Harasymczuk M, Schilling B, Georgoulas V, Argiris A, Whiteside TL. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. *J Immunol Methods* 2012;381:14-22.
- Grambsch PM, TM T. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika* 1994;81:515-26.
- Hosmer DW, Lemeshow S, May S. *Applied survival analysis: Regression modeling of time-to-event data*. 2nd ed. New York (NY): Wiley; 2008.
- Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186-92.
- Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer Res* 2013;73:2943-8.
- Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* 1998;92:4150-66.
- Huang Y, Chen X, Dikov MM, Novitskiy SV, Mosse CA, Yang L, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood* 2007;110:624-31.
- Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP, et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol* 1998;160:1224-32.
- Dikov MM, Ohm JE, Ray N, Tchekneva EE, Burlison J, Moganaki D, et al. Differential roles of vascular endothelial growth factor receptors 1 and 2 in dendritic cell differentiation. *J Immunol* 2005;174:215-22.
- Ziogas AC, Gavalas NG, Tsiatas M, Tsitsilonis O, Politi E, Terpos E, et al. VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. *Int J Cancer* 2012;130:857-64.
- Voron T, Colussi O, Marcheteau E, Pernot S, Nizard M, Pointet AL, et al. VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J Exp Med* 2015;212:139-48.

Cancer Immunology Research

Pretreatment Immune Status Correlates with Progression-Free Survival in Chemotherapy-Treated Metastatic Colorectal Cancer Patients

Kohei Tada, Shigehisa Kitano, Hirokazu Shoji, et al.

Cancer Immunol Res 2016;4:592-599. Published OnlineFirst April 28, 2016.

Updated version	Access the most recent version of this article at: doi: 10.1158/2326-6066.CIR-15-0298
Supplementary Material	Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2016/04/28/2326-6066.CIR-15-0298.DC1

Cited articles	This article cites 32 articles, 19 of which you can access for free at: http://cancerimmunolres.aacrjournals.org/content/4/7/592.full#ref-list-1
-----------------------	--

Citing articles	This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://cancerimmunolres.aacrjournals.org/content/4/7/592.full#related-urls
------------------------	---

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
----------------------	--

Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
-----------------------------------	--

Permissions	To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/4/7/592 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.
--------------------	--