News Release

Title

An oncogenic alteration creates a tumor microenvironment that promotes tumor progression by conferring a metabolic advantage to regulatory T cells

Key Points

- **PD-1 blockade therapy for gastric cancer has been scientifically proven to be effective and is being used in clinical practice. However, the efficacy is not satisfactory, and treatment based on predictive biomarkers is needed for developing the optimal cancer therapy.**
- **We found that *RHOA*-mutated gastric cancer is resistant to cancer immunotherapy by suppressing the infiltration of effector $CD8^+$ T cells into cancer tissues via the PI3K/AKT pathway and by creating a microenvironment metabolically favorable to the survival of regulatory T cells, which are responsible for suppressing the immune response.**
- **The elucidation of the immunosuppressive mechanism of *RHOA*-mutated gastric cancer has revealed the potential for PI3K inhibitors in combination with cancer immunotherapy to improve treatment efficacy.**

Summary

Only a small percentage of patients afflicted with gastric cancer (GC) respond to immune checkpoint blockade (ICB). To explore the mechanism(s) underlying this resistance, Hiroyoshi Nishikawa, MD PhD. (Department of Immunology, Nagoya University Graduate School of Medicine) and his colleagues examined the immune landscape of GC. A subset of these tumors was characterized by high frequencies of regulatory T (Treg) cells and low numbers of effector T cells. Genomic characterization revealed that these tumors bore mutations in *RHOA* that are known to drive tumorigenesis. *RHOA* mutations in cancer cells increased production of free fatty acids, which are more effectively consumed by Treg cells than by effector T cells, and reduced effector T cell-recruiting chemokines by activating PI3K-AKT signaling pathways. Consequently, *RHOA*-mutated cancers developed a robust immunosuppressive tumor microenvironment (TME) characterized by high Treg cell accumulation despite the noninflamed status. The combination of PI3K-specific blockade and PD-1 blockade improved the immunosuppressive TME and augmented the antitumor effect, overcoming the resistance of *RHOA*-mutated tumors to PD-1 blockade in mouse models. We propose that the metabolic advantage conferred by *RHOA* enables Treg cell accumulation within the tumor, generating an immunosuppressive TME that underlies resistance to ICB. The findings were published in electronic edition of the American scientific journal “Immunity” on July 7th, 2020.

Research Background

PD-1 blockade therapy has been reported to be effective to various types of cancer, including gastric cancer. Considering that the inferior response rate to anti-PD-1 antibody therapy in gastric cancer compared to that in malignant melanoma and lung cancer, gastric cancer is supposed to have a stronger immunosuppressive tumor microenvironment. In this study, we therefore elucidated the immunological landscape in the TME of GC, such as the different frequencies of Treg cells and effector T cells, and the immunosuppressive function of Treg cells, proposing a potential new combination cancer immunotherapy.

Research Results

We first analyzed the immunological status (inflamed or noninflamed) and cell types in tumors from 23 GC patients who had undergone surgical resection. According to immune-related gene expression evaluated by RNA sequencing (RNA-seq), eight tumor samples had a noninflamed TME. From the flow-cytometry (FCM) and RNA-seq data, four patients bore an immunosuppressive TME that was characterized by Treg cell accumulation regardless of the noninflamed status of the TME (Figure 1), although Treg cells are generally accompanied by effector T cell infiltration in the inflamed TME. The WES analysis revealed that two of these patients possessed the *RHOA* Y42C mutation.

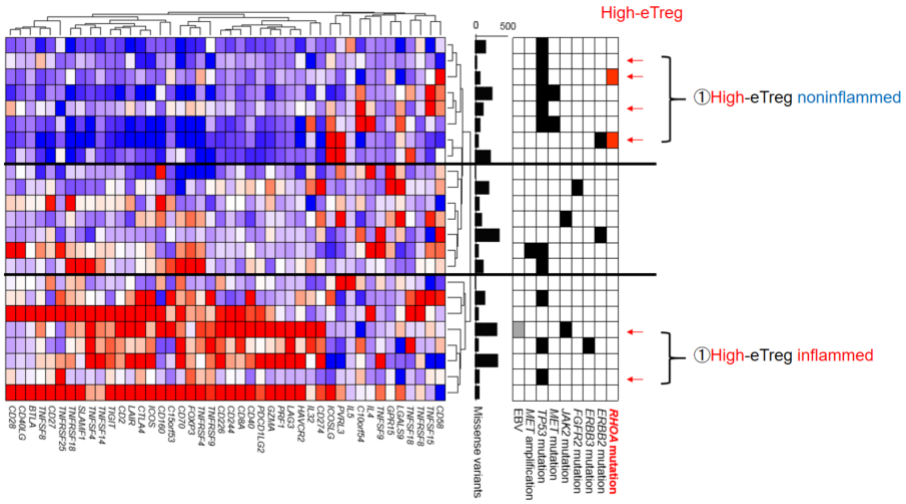

Figure 1. Integrated analysis of immune-related gene expression and flow cytometric data of gastric cancer surgical specimens.
Gene expression data from surgically resected gastric cancer specimens were clustered using gene sets involved in the immune response and integrated with data from flow cytometric analyses with TILs.

Additionally, eighty-five advanced GC patients' samples from another cohort were analyzed. *RHOA* Y42 mutations were detected in seven GC samples with digital polymerase chain reaction (PCR). The frequency of eTreg cells, the ratio of eTreg cells/$CD8^+$ T cells, and the expression of CTLA-4 by eTreg cells in the TME were significantly higher in *RHOA* Y42-mutated GCs than in *RHOA*-wild type (WT) GCs (Figure 2).

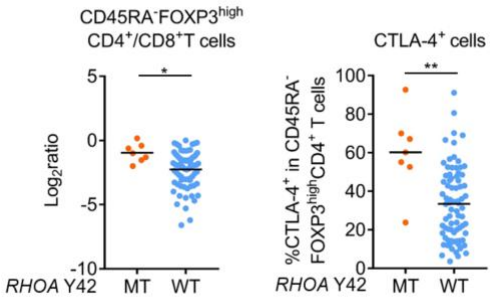

Figure 2. Comparison of TILs from gastric cancers according to *RHOA* gene status.
TILs from gastric cancers were analyzed using flow cytometry to evaluate the ratio of $CD8^+$ T cells to Treg cells and CTLA-4 expression by Treg cells according to *RHOA* gene status.

To explore how the noninflamed TME was developed by *RHOA* Y42 mutations, comprehensive gene expression was examined with microarray analyses using *RHOA* WT- or Y42C-overexpressing GC cell lines ($MKN1^{WT}$ and $MKN1^{Y42C}$, respectively). When we focused on cytokine or chemokine gene expression, the expression of CXCL10 and CXCL11, which recruit effector $CD8^+$ T cells, was lower in $MKN1^{Y42C}$ cells than in $MKN1^{WT}$ cells. In addition, differential analyses of transcription factor expression between $MKN1^{WT}$ and

MKN1^{Y42C} cells uncovered the concurrent reduction in the expression of IRF1, which reportedly regulates CXCL10 and CXCL11 expression. Similar trends were observed in gene expression of clinical GC samples (Figure 3). Overall, the *RHOA* Y42 mutations decrease the levels of effector T cell-recruiting chemokines such as CXCL10 and CXCL11 via the reduction of IRF1 expression.

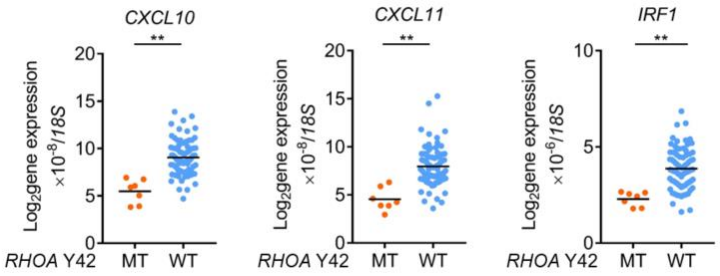

Figure 3. Comparative analysis of chemokine-related gene expression in gastric cancers according to *RHOA* gene status.
Quantitative real-time PCR was used to compare chemokine-related gene expression in gastric cancers according to *RHOA* gene status.

Gene alterations in the effector domain of *RHOA*, including Y42C decreased phosphorylation of PTEN and increased phosphorylation of AKT. Phosphorylated GSK3β, which is induced by the PI3K-AKT signaling pathways, reportedly suppresses STAT1, a well-known transcription factor of IRF1. In line with this, the increased phosphorylated GSK3β level induced by *RHOA* Y42C led to the suppression of STAT1, resulting in IRF1 expression reduction (Figure 4). Therefore, *RHOA* Y42C reduces the expression of CXCL10 and CXCL11 by upregulating the PI3K-AKT signaling pathways (Figure 4).

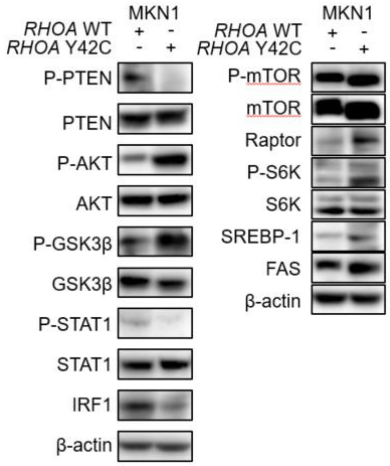

Figure 4. Comparative study of protein expression in *RHOA* WT- and Y42C- overexpressiong cell lines.
We compared protein expression of molecules in the PI3K/AKT pathway using *RHOA* WT- and Y42C- overexpressiong cell lines.

To elucidate the mechanism(s) of eTreg cell accumulation and immunosuppression by *RHOA* Y42 mutations, we employed Gene Set Enrichment Analysis (GSEA) of the RNA-seq, which revealed that the gene set related to fatty acid metabolism was significantly enriched in *RHOA*-Y42C GCs. Consistently, the expression of *FASN* (encoding FAS), which plays an important role in fatty acid synthesis, was significantly higher in *RHOA* Y42-mutated GCs than in RHOA-WT GCs. We hypothesized that *RHOA* Y42-mutated GCs produced larger amounts of FFAs via increased FAS expression, which contributed to the prolonged survival of eTreg cell subset in the TME compared with that of other effector T cell subsets. In line with this hypothesis, both *FASN* mRNA and FAS protein expression was significantly higher in *RHOA* Y42C-overexpressing cell lines than in *RHOA* WT-overexpressing cell lines via the activation of PI3K-AKT/mTOR/S6, as were observed by western blotting (Figure 4).

We next addressed the differences in fatty acid metabolism among T cell subsets in vivo using the murine MC-38 cell line. Lipid uptake and content, which were assessed with BODIPY FL C16 and BODIPY 493, respectively, were significantly higher in Foxp3$^+$CD4$^+$ cells (Treg cells) than in Foxp3$^-$CD4$^+$ T cells or CD8$^+$ T cells in the TME (Figure 5). Additionally, the expression of molecules related to FFA uptake and metabolism, including CD36 (a scavenger receptor mediating the uptake of FFAs), CPT1A (the rate-limiting enzyme in long-chain fatty acid oxidation), PPARα, and PPARγ in Treg cells was significantly higher than that in Foxp3$^-$CD4$^+$ T cells or CD8$^+$ T cells (Figure 5). Overall, Treg cells effectively utilize FFAs for their survival compared with the other T cell subsets in the TME, whereas conventional CD4$^+$ T cells and CD8$^+$ T cells are likely to depend on glucose consumption.

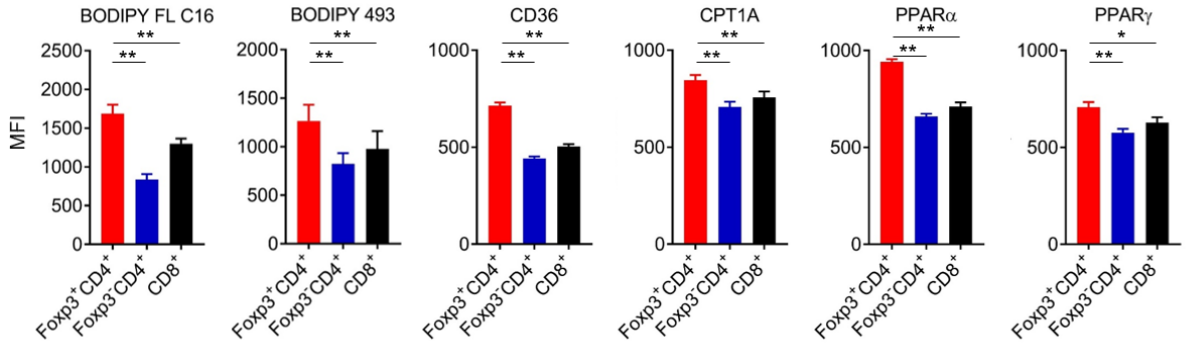

Figure 5. Comparative study of fatty acid metabolism-related molecular expression by each subset of TILs.
TILs were prepared from subcutaneously implanted MC-38 in immunocompetent mice, and the expression of fatty acid metabolism-related molecules and their fatty acid uptake (BODIPY FL C16) and fatty acid content (BODIPY 493) were compared in each subset of TILs using flow cytometry.

To validate the importance of this metabolic change for Treg cells, we investigated the effects of FFAs on tumor-infiltrating lymphocytes using *Fasn*-overexpressing MC-38 murine cell line (MC-38^{Fasn}) that produced FFAs. The frequency and the number (counts/tumor weight) of Treg cells, and the ratio of Treg cells/CD8$^+$ T cells in the TME were significantly higher in MC-38^{Fasn} tumors than in MC-38^{mock} tumors. CTLA-4 expression by Treg cells in the TME was significantly higher in the MC-38^{Fasn} tumors than in the MC-38^{mock} tumors, suggesting the importance of FFAs for the survival and immunosuppressive function of Treg cells. To assess the immunological effect of *RHOA* Y42C on the TME of GCs, we established *RHOA* WT- or *RHOA* Y42C-overexpressing MC-38. The frequency and the number of Treg cells and the ratio of Treg cells/CD8$^+$ T cells were significantly higher in *RHOA* Y42C-overexpressing tumors than in *RHOA* WT-overexpressing tumors, whereas the numbers of CD8$^+$ T cells and conv CD4$^+$ T cells were significantly lower. We then addressed the antitumor efficacy of anti-PD-1 mAb in vivo. Anti-PD-1 mAb failed to inhibit the growth of *RHOA* Y42C-overexpressing tumors compared with that of *RHOA* WT-overexpressing tumors (Figure 6). We evaluated the efficacy of anti-PD-1 mAb in three advanced GC patients with *RHOA* Y42 mutations and revealed that no patient responded to anti-PD-1 mAb.

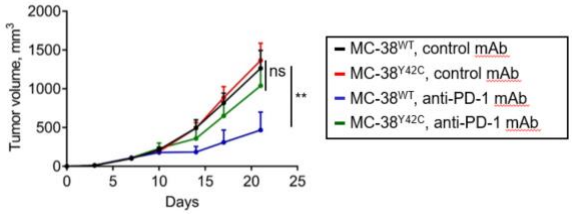

Figure 6. Comparative study of the effect of *RHOA* gene status on the response to anti-PD-1 antibody treatment.
RHOA WT-overexpressing cell line and *RHOA* Y42C-overexpressing cell line were subcutaneously transplanted into mice and the efficacies of anti-PD-1 mAb treatment on each tumor growth were evaluated.

We then employed a selective PI3K small-molecule inhibitor (PI3Ki), to address whether modulating PI3K-AKT signaling pathways could overcome the immunosuppressive phenotype. PI3Ki inhibited AKT phosphorylation in a concentration-dependent manner, mainly in *RHOA* Y42C-overexpressing cancer cells, resulting in increased IRF1, CXCL10 and CXCL11 expression and decreased FAS expression in vitro. Next, the antitumor efficacy of anti-PD-1 mAb combined with PI3Ki was examined. Each single treatment hardly inhibited tumor growth, but combined treatment with PI3Ki and anti-PD-1 mAb significantly delayed MC-38^{Y42C} tumor growth (Figure 7).

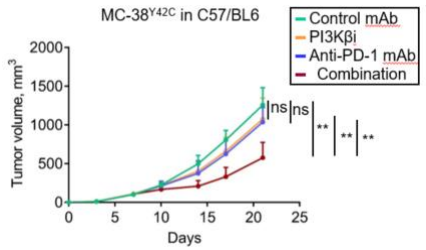

Figure 7. Analysis of efficacy of anti-PD-1 antibody in combination with PI3K inhibitor in *RHOA*-Y42C tumor.
RHOA Y42C-overexpressing cells were subcutaneously transplanted into immunocompetent mice and treated with anti-PD-1 mAb and/or PI3Ki, and each tumor growth curve was compared.

Research Summary and Future Perspective

The effects of specific gene mutations in gastric cancer on the anti-tumor immune response have not been elucidated. This study reveals that *RHOA*-mutated gastric cancer is characterized by high infiltration of regulatory T cells and a non-inflamed status. It has been found that downstream signaling pathways caused by *RHOA* mutations, one of the well-known driver gene mutations, are associated with cancer cell survival and proliferation. In addition to this, we newly propose that *RHOA* mutations make an immunosuppressive tumor microenvironment by producing free fatty acids which are more effectively utilized by regulatory T cells than effector CD8$^+$ T cells. Furthermore, the combination of PI3K-specific blockade and PD-1 blockade may improve the immunosuppressive TME and augment the antitumor effect, overcoming the resistance of *RHOA*-mutated tumors to PD-1 blockade.

Publication

Shogo Kumagai[1,2], Yosuke Togashi[1*], Chika Sakai[1], Akihito Kawazoe[3], Masahito Kawazu[4], Toshihide Ueno[4], Eiichi Sato[5], Takeshi Kuwata[6], Takahiro Kinoshita[7], Masami Yamamoto[8], Sachiyo Nomura[9], Tetsuya Tsukamoto[10], Hiroyuki Mano[4], Kohei Shitara[3] and Hiroyoshi Nishikawa[1,2*]

Institutional Affiliations

[1]Division of Cancer Immunology, Research Institute /Exploratory Oncology Research and Clinical Trial Center (EPOC), National Cancer Center, Tokyo/Chiba, Japan
[2]Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[3]Department of Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba, Japan
[4]Division of Cellular Signaling, Group for Cancer Development and Progression, National Cancer Center Research Institute, Tokyo, Japan
[5]Department of Pathology, Institute of Medical Science, Tokyo Medical University, Tokyo, Japan
[6]Department of Pathology and Clinical Laboratories, National Cancer Center Hospital East, Chiba, Japan
[7]Department of Gastric Surgery, National Cancer Center Hospital East, Chiba, Japan
[8]Division of Physiological Pathology, Department of Applied Science, School of Veterinary Nursing and Technology, Nippon Veterinary and Life Science University, Tokyo, Japan
[9]Department of Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo,

Japan
[10]Department of Pathology, Graduate School of Medicine, Fujita Health University, Aichi Japan.

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