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研究課題名（和文）Colony stimulating factor 1 receptor (CSF1R) and immune regulatory M2c-like tumor associated macrophages (TAMs) in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma transformation (tFL)

研究課題名（英文）Colony stimulating factor 1 receptor (CSF1R) and immune regulatory M2c-like tumor associated macrophages (TAMs) in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma transformation (tFL)

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研究成果の概要（和文）：リンパ腫の治療法はまだありません。Colony stimulating factor 1 receptor (CSF1R)は腫瘍関連マクロファージのバイオマーカーである。腫瘍関連マクロファージは病気の病因にとって重要である。CSF1R発現が高いとびまん性大細胞型B細胞リンパ腫・濾胞性リンパ腫の予後が不良です。薬物を用いてCSF1R陽性腫関連マクロファージを標的とすることは、潜在的な新しい治療戦略である。

研究成果の概要（英文）：We successfully analyzed CSF1R [pro-tumoral M2-like tumor associated macrophages(TAMs)], immune regulatory M2c-like TAMs (CD163, PTX3, IL10, TGFB) and other markers of immune microenvironment in Diffuser Large B-cell Lymphoma (DLBCL, 240 cases, 29 sinonasal) and Follicular Lymphoma (FL, 100 Japan, 88 Europe). We performed (1) Immunohistochemistry and digital image quantification (2) Immunofluorescence, optical, confocal and atomic force microscopy (3) Genome-wide copy number and loss-of-heterozygosity (LOH) profiling (4) In vitro analysis of M2 macrophages + FL with gene expression profiling, including GSEA and network analysis (5) Correlation with histological and clinical features. We concluded that high CSF1R and M2c-like markers correlated with FL progression and DLBCL transformation; TAMs created 3D networks, TAMs induced changes in gene expression of immune response type II, high TAMs associated with poor prognosis in DLBCL and TAMs correlated with high copy No changes and RGS1.

研究分野：　医歯薬学 人体病理学

キーワード：　CSF1R　びまん性大細胞型B細胞リンパ腫　濾胞性リンパ腫　腫瘍関連マクロファージ　血液病理学　免疫組織化学　全ゲノムのコピー数解析　CD163

１．研究開始当初の背景

Scientific background:

(1) The frequency and prevalence of the diseases of the immune system seems to be increasing in the last decades, the reasons are still not clarified but it may be related to several factors including:

① Changes in the lifestyle, i.e. work related stress.

② Food quality, safety and type of diet.

③ Pollution of the environment, use of drugs and secondary effects of medication with immunomodulatory effects, etc.

These factors will be added to the disease susceptibility of the person that is mainly determined by the genomic characteristics.

(2) An immune disorder is a dysfunction of the immune system. These disorders can be characterized in several diverse ways:

① By the component(s) of the immune system affected.

② By whether the immune system is overactive or underactive.

③ By whether the condition is congenital or acquired.

(3) The list of non-tumoral diseases of the immune system includes:

① Autoimmune disorders such as lupus, scleroderma, hemolytic anemia, vasculitis, diabetes type I, Graves disease, rheumatoid arthritis, multiple sclerosis and Goodpasture's syndrome.

② Primary immune deficiencies such as severe combined immunodeficiency (SCID), DiGeorge syndrome, common variable immunodeficiency (CVID) and Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [IPEX is characterized by mutations in the FOXP3 gene that impair the normal function of regulatory T cells (Tregs). Tregs play a significant role in controlling immune responses and preventing autoimmune disorders].

③ Secondary immune deficiencies such as Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS).

④ Allergies such as seasonal allergy, mastocytosis, perennial allergy, food allergy, allergic rhinitis and atopic dermatitis.

⑤ Others such as inflammatory bowel disease (IBD). IBD is a chronic inflammation of all or part of your digestive tract. IBD primarily includes ulcerative colitis, Crohn's disease and collagenous colitis and lymphocytic colitis

(4) Neoplasia of the immune system.

Neoplasm is an abnormal growth of tissue, which if it forms a mass, is commonly referred to as a tumor. ICD-10 classifies neoplasms into four main groups: benign neoplasms, *in situ* neoplasms and malignant neoplasms

A neoplasm of the immune system is called lymphoma if affects lymph nodes and leukemia if affects the blood. Among the most frequent lymphomas in Western countries including Japan, in the non-Hodgkin lymphoma subtype (NHL), there is the Diffuse Large B-cell Lymphoma (DLBCL; **びまん性大細胞型 B 細胞リンパ腫**) and Follicular Lymphoma (FL; **濾胞性リンパ腫**).

Of note, it is known that deregulations of the immune system including infection can lead to the development of lymphoid neoplasms:

① Background Helicobacter pylori infection is the main cause of gastric mucosa-associated lymphoid tissue (MALT) lymphoma *(Ponzoni M. et al. Best Pract Res Clin Haematol. 2017 Mar - Jun;30(1-2):32-40).*

② Hepatitis C virus is associated B-NHL subtypes include Splenic marginal zone lymphoma (SMZL) and DLBCL lymphomas (*Szynglarewicz B. et al. Pathol Oncol Res. 2007;13(4):382-4*).

③ Infection with the Epstein-Barr virus (EBV) is an important risk factor for Burkitt lymphoma in some parts of Africa. EBV is also related to Hodgkin lymphoma, Extranodal NK/T-cell lymphoma, nasal type (ENKL) and other several EBV-Associated

Lymphoproliferative Disorders *(Linke-Serinsöz E. et al. Semin Diagn Pathol. 2017 Apr 7. pii: S0740-2570(17)30044-8).*

④ IPEX and IBD have also been associated to the development of lymphoma although the associated risk is still not well defined *(Lucas KG. et al. Pediatr Blood Cancer. 2008 May;50(5):1056-7).*

(5) Tumor immune microenvironment.

The tumor microenvironment is the cellular environment in which the tumor exists, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix. In case of the neoplasms of the immune system, for example FL or DLBCL, the tumoral cell is the B lymphocyte and the immune microenvironment is comprised of reactive (non-tumoral) B lymphocytes, T lymphocytes, macrophages, dendritic cells, vessels, fibroblasts and extracellular matrix. Nowadays we know that the relationship between the microenvironment and the tumoral cells will influence the generation, maintenance and final evolution (outcome) of the tumor.

For instance, in case of FL we know that high numbers of tumor-infiltrating FOXP3-positive regulatory T cells (Tregs) and high numbers of tumor-infiltrating programmed cell death 1-positive regulatory lymphocytes (PD-1) are associated with improved overall survival *(Carreras J. et al. Blood. 2006 Nov 1;108(9):2957-64; Carreras J. et al. Journal of Clinical Oncology. 2009 Mar 20;27(9):1470-6).*

(6) Tumor associated macrophages (TAMs) and CSF1R.

During tumor progression, circulating monocytes and macrophages are actively recruited into tumors where they alter the tumor microenvironment to accelerate tumor progression. Macrophages shift their functional phenotypes in response to various microenvironmental signals generated from tumor and stromal cells.

Based on their function, macrophages are divided broadly into two categories: classical M1 and alternative M2 macrophages. The M1 macrophage is involved in the inflammatory response, pathogen clearance, and antitumor immunity. In contrast, the M2 macrophage influences an anti-inflammatory response, wound healing, and pro-tumorigenic properties.

M1 and M2 macrophages have distinct chemokine and chemokine receptor profiles. M1 markers are CD86, CD80, IL1R, TLR2, TLR4, iNOS, TNF, IL6, IL1B (among others). M2 markers are CD163, CD204, CD206, IL10, TGFB, CCR2, CCL1, CCL17, CCL22, CCL24 and VEGF (among others). M2c subtype is characterized by having immune modulatory properties and are characterized by IL10, TGFB, PTX3 and CCR2. M2c macrophages by producing IL10 increase the numbers of FOXP3+Tregs *(Macrophage Polarization Mini Review. Published by Bio-Rad Laboratories, Inc. LIT.NMR.2016.1).*

TAMs closely resemble the M2-polarized macrophages and are critical modulators of the tumor microenvironment. Clinicopathological studies have suggested that TAM accumulation in tumors correlates with a poor clinical outcome and TAM-targeting therapy as a promising novel strategy for an indirect cancer therapy *[Chanmee T. et al. Cancers (Basel). 2014 Aug 13;6(3):1670-90].*

Macrophage colony-stimulating factor 1 receptor (CSF1R) is a tyrosine-protein kinase that acts as cell-surface receptor for CSF1 and IL34 and plays an essential role in the regulation of survival, proliferation and differentiation of hematopoietic precursor cells, especially mononuclear phagocytes, such as macrophages and monocytes. CSF1R promotes the release of proinflammatory chemokines in response to IL34 and CSF1, and thereby plays a significant role in innate immunity and in inflammatory processes. CSF1R promotes reorganization of the actin cytoskeleton, regulates formation of membrane ruffles, cell adhesion and cell migration, and promotes cancer cell invasion.

CSF1R is involved in disease:

① Aberrant expression of CSF1 or CSF1R can promote cancer cell proliferation, invasion and formation of

metastases. Overexpression of CSF1 or CSF1R is observed in a significant percentage of breast, ovarian, prostate, and endometrial cancers.

② Aberrant expression of CSF1 or CSF1R may play a role in inflammatory diseases, such as rheumatoid arthritis, glomerulonephritis, atherosclerosis, and allograft rejection. (*The UniProt Consortium. Nucleic Acids Res. 2017 Jan 4;45(D1): D158-D169*).

Due to the function, CSF1R could be considered as a M2-like macrophage polarization marker.

2．研究の目的

Purpose of the study:

We aimed to characterize the tumoral microenvironment of FL, transformed FL (tFL) and *de novo* DLBCL focusing on CSF1R marker and M2c-like immune regulatory macrophages.

3．研究の方法

Research method:

(1) Generation of series of FL, tFL and *de novo* DLBCL.
(2) Analysis of normal distribution of biomarkers in normal lymphoid tissue (reactive lymph node and tonsil) by immunohistochemistry (IHC).
(3) IHC staining of biomarkers in FL, tFL and *de novo* DLBCL and digital image quantification.
(4) Immunofluorescence (IF) staining of biomarkers and analysis using confocal microscopy.
(5) Study of the interphase between TAMs and B cells by atomic force microscope.
(6) Statistical correlation between histological and clinical variables.
(7) Analysis of the tumoral microenvironment and macrophages in a location subtype of DLBCL, the sinonasal DLBCL and correlation with the genomic profile in terms of copy number and LOH profiling.
(8) *In vitro* study using M2-like polarized macrophages and FL samples.

4．研究成果

Research results:

The achievements can be summarized as follows:
(1) We have focused on CSF1R marker, which identifies tumor associated macrophages, as well as a series of associated tumor microenvironment immune biomarkers.

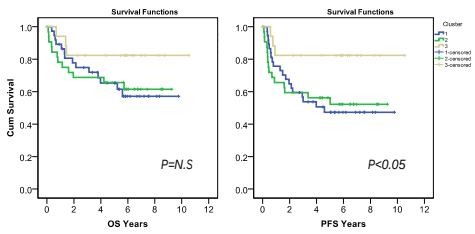
(2) The series of cases was comprised of DLBCL (n=240) and FL (n=100) from Japan and FL from Europe (n=88).

(3) IHC was successful in the FFPE tissues both in reactive lymphoid tissue and lymphoma (FL, tFL and DLBCL). We focused on CSF1-CSF1R pathway (CSF1R, CSF1, HIF1 and caspase3), immune regulatory M2-c like TAMs (CD163, MITF, PTX3, IL10, TGFB and CCR2), T helper 17 cells (IL17A), regulatory T lymphocytes (Tregs, FOXP3) and cytotoxic T lymphocytes (CD8), and additional markers of M1-like polarization (CD16, CD14), M2-like polarization (CD204 and CD306), RGS1 and CD169.

(4) In FL and DLBCL Japan:

① CSF1R M2-1ike macrophages associated with poor progression free survival (PFS) (P=0.016) in primary DLBCL.

CSF1R TAMs expression associates with poor prognosis in DLBCL



② High CD163 associated with poor overall survival (OS) and PFS (P=0.009 and P=0. 005) in DLBCL.

③ The FL series was comprised of 26 FL (low grade, 13; high grade 3a, 7; 3b, 3; and transformed FL, 3). IHC focused on pan-TAMs (CD68), M1-like TAMs (CD16), M2-like TAMs (CSF1R, CD163, PTX3, MITF, CCR2 and CSF1) and FOXP3+Tregs.

In comparison to DLBCL, FL is characterized by lower expression of CD68, CD16, CD163, MITF and FOXP3 (P<0.05).

Progression of FL towards tFL is characterized by progressive increase of CD68, CSF1R, CD163 and MITF

(P<0.05).

(5) In FL Europe:

① High grade FL correlated with high follicular CSF1R (60. 7% vs. 5. 1%, P<0. 0001).

② High follicular CSF1R had trend correlation to poor OS (P= 0. 091).

③ High CD163 correlated with higher LDH (P= 0. 003).

④ COX regression between CSF1R and FLIPI showed only FLIPI significant for survival.

(6) Atomic force microscopy identified connection area between macrophages and B lymphocytes.

(7) In-vitro study of macrophages and FL showed increased gene expression of angiogenesis, matrix degradation and chemokine signaling; gene set enrichment analysis (GSEA) showed increased markers of host immune response type II, including macrophage markers.

(8) In the specific subtype of DLBCL, the primary sinonasal DLBCL from Japan, high macrophages associated to higher genomic copy number changes and RGS1 marker was identified having prognostic relevance.

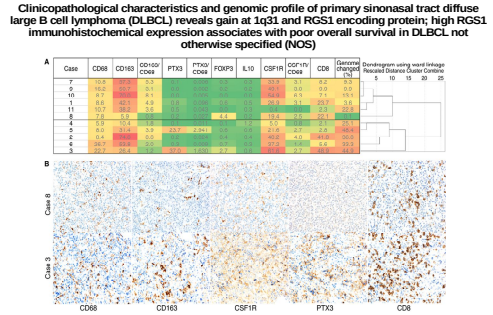
The abstract of the publication of sinonasal DLBCL is as follows:

"Aims: We aimed to define the clinicopathological characteristics of 29 primary sinonasal diffuse large B cell lymphoma ($DLBCL^{sn}$) in a series of 240 cases of DLBCL not otherwise specified [$DLBCL^{all\ (NOS)}$], including $DLBCL^{sn}$ training set (n = 11) and validation set (n = 18), and $DLBCL^{non\text{-}sn}$ (n = 211).

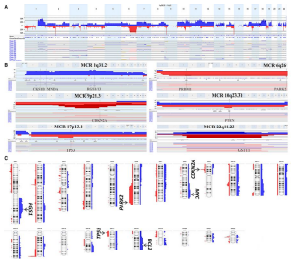
Methods and results: In the training set, 82% had a non-germinal center B-cell-like (Hans' Classifier) (non-GCB) phenotype and 18% were Epstein–Barr virus-encoded small RNAs $(EBER)^{+}$. The genomic profile showed $gains^{(+)}$ of 1q21.3q31.2 (55%), 10q24.1 (46%), 11q14.1 (46%) and 18q12.1q23 (46%); $losses^{(-)}$ of 6q26q27 (55%) and 9p21.3 (64%); and copy number neutral loss of heterozygosity (LOH) (acquired uniparental disomy, UPD) at 6p25.3p21.31 (36%). This profile is comparable to $DLBCL^{NOS}$ (GSE11318, n = 203.) and closer to non-GCB/activated B-cell-like subtype (ABC). Nevertheless, +1q31, −9p21.3 and −10q11.1q26.2 were more characteristic of $DLBCL^{sn}$ (P < 0.001). Array results were verified successfully by fluorescence in situ hybridization (FISH) on +1q21.3 (*CKS1B*), −6q26 (*PARK2*), +8q24.21 (*MYC*), −9p21.3 (*MTAP*, *CDKN2A/B*), −17p13.1 (*TP53*) and +18q21.33 (*BCL2*) with 82–91% agreement. Minimal common regions included biologically relevant genes of *MNDA* (+1q23.1), *RGS1* and *RGS13* (+1q31.2), *FOXP1* (+3p13), *PRDM1* (*BLIMP1*) and *PARK2* (−6q21q26), *MYC* (+8q24.21), *CDKN2A* (−9p21.3), *PTEN* (−10q23.31), *MDM2* (+12q15), *TP53* (−17p13.1) and *BCL2* (+18q21.33). Correlation between DNA copy number and protein immunohistochemistry was confirmed for RGS1, RGS13, FOXP1, PARK2 and BCL2. The microenvironment had high infiltration of M2-like tumour associated macrophages (TAMs) and CD8+T lymphocytes that associated with higher genomic instability. The $DLBCL^{sn}$ validation set confirmed the clinicopathological characteristics, all FISH loci and immunohistochemistry (IHC) for RGS1. *RGS1*, one of the most frequently altered genes, was analysed by IHC in $DLBCL^{all}$ and high RGS1 expression associated with non-GCB, EBER+ and unfavourable overall survival (hazard ratio = 1.794; P = 0.016).

Conclusions: $DLBCL^{sn}$ has a characteristic genomic profile. High RGS1 IHC expression associates with poor overall survival in $DLBCL^{all\ (NOS)}$."

Histopathology. 2017 Mar;70(4):595-621. doi: 10.1111/his.13106. Epub 2017 Jan 9. PMID: 27775850



Clinicopathological characteristics and genomic profile of primary sinonasal tract diffuse large B cell lymphoma (DLBCL) reveals gain at 1q31 and RGS1 encoding protein; high RGS1 immunohistochemical expression associates with poor overall survival in DLBCL not otherwise specified (NOS)



(9) In summary, ours results are highlighting the relevance of CSF1R and immune microenvironment biomarkers in the poor prognosis and pathogenesis of two of the most frequent non-Hodgkin lymphomas of Western Countries as well as Japan (FL and DLBCL).

５．主な発表論文等

〔雑誌論文〕（計 1 件）

(1) Carreras J, Kikuti YY, Beà S, Miyaoka M, Hiraiwa S, Ikoma H, Nagao R, Tomita S, Martin-Garcia D, Salaverria I, Sato A, Ichiki A, Roncador G, Garcia JF, Ando K, Campo E, Nakamura N.
Clinicopathological characteristics and genomic profile of primary sinonasal tract diffuse large B cell lymphoma (DLBCL) reveals gain at 1q31 and RGS1 encoding protein; high RGS1 immunohistochemical expression associates with poor overall survival in DLBCL not otherwise specified (NOS).
Histopathology. 2017 Mar;70(4):595-621. doi: 10.1111/his.13106. Epub 2017 Jan 9.
PMID: 27775850.

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(2) カレーラス ジュアキム、菊池イアーラ幸江、宮岡雅、平岩信一郎、生駒悠、富田さくら、長尾涼子、中村直哉。日本語演題名: High grade 濾胞性リンパ腫における RGS1 の免疫組織化学的発現の検討。英語演題名: RGS1 immunohistochemical expression in high grade follicular lymphoma (FL)。第 106 回日本病理学会総会。2017 年 4 月 27 日（木）～29 日（土）。京王プラザホテル〒160-8330 東京都新宿区西新宿 2-2-1。

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(5) Carreras Joaquim, 菊池イアーラ幸江, 雅宮岡, 平岩真一郎, 生駒悠, 富田さくら, 中村直哉。 Relevance of macrophagic signature in follicular lymphoma (FL), transformation to diffuse large B-cell lymphoma (tFL), and comparison with de novo diffuse large B-cell lymphoma (DLBCL)。第 56 回日本リンパ網内系学会総会。２０１６年９月１日（木）～９月３日（土）。ホテル日航熊本。〒860-8536 熊本市中央区上通町 2-1。

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〔図書〕（計 0 件）
〔産業財産権〕
○出願状況（計 0 件）
○取得状況（計 0 件）

〔その他〕
ホームページ等
Research activities of Joaquim Carreras Esteban.
http://carreras.med.u-tokai.ac.jp/

６．研究組織

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