Advanced Clinical Research Center

Division of Molecular Therapy
分子療法分野

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The main theme of our research is toward the development of novel therapeutic options against intractable malignant disorders including leukemia, lymphoma and various cancers. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic stem cells on the basis of molecular and cellular biology as well as medical informatics. We also try to develop novel therapies in the field of regenerative medicine using bone marrow-derived mesenchymal stromal cells.

(1) Molecular and cellular analysis of hematological malignancies:
Tumor-specific genetic alterations often result in transcriptional dysregulation and activation of signal transduction pathways as well as defective tumor suppressors, which appear to be the primary cause of those tumors. We are studying the molecular and cellular aspects of hematological malignancies as a model system. Furthermore, we performed clinical sequencing in tight collaboration with Human Genome Center and Health Intelligence Center to establish a platform for precision medicine.

(2) Development of immunomodulatory therapy using genetically engineered cells with or without recombinant virus:
Either activation or suppression of immune system is critical for disease control, depending on the context of disease. We used genetically-engineered cancer cells (cancer cell vaccine) and mesenchymal stromal cells for activation of anti-cancer immunity and attenuation of graft versus host disease, respectively. Furthermore, recombinant vaccinia virus is used with cancer cell vaccine for oncolytic immunotherapy of cancer. We applied the miRNA-regulated and thymidine kinase-deleted vaccinia virus to a preclinical model of multiple myeloma. Additional modifications to stimulate immune response against tumor are currently under investigation using immunocompetent mouse tumor models.

(3) Investigation of cancer stem cells and search for molecular targets for their elimination:
We are focusing on cancer, stem cells, and cancer stem cells. We aim to elucidate molecular mechanisms how growth factor signaling regulates tumorigenesis and maintenance of stem cells and cancer stem cells. Moreover, by taking not only molecular biology but also new bioinformatics approaches, we aim to identify novel cancer biomarkers and molecular targets for cancer therapy. Our ultimate goal is to translate them into clinic.

(4) Clinical study of clonal evolution of HTLV-1-infected T cells into leukemia:
Adult T-cell leukemia is a T cell malignancy which develops in HTLV-1 infected

individuals after long latency period. HTLV-1 infected cells are regarded to transform through multi-step oncogenesis process. We are analyzing HTLV-1 infected cells in different stages of transformation whose phenotypes such as CD7 and CADM1 expression vary in each stage by sorting them using flow cytometer. These analyses will provide useful information regarding molecular mechanism to develop ATL.

1. Preclinical study of recombinant vaccinia viruses in mouse xenograft and immunocompetent syngeneic tumor models

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Vaccinia virus (VV) is a useful tool for oncolytic virotherapy. To make it more effective and safe, we modified the VV by deleting thymidine kinase and introducing miRNA target sequences in the genome. The doubly-regulated VV (DRVV) efficiently infected and killed myeloma cells in vitro, and intravenous injection of DRVV successfully infected subcutaneous myeloma xenografts in SCID mice, leading to a significant prolongation of overall survival. However, the DRVV failed to reduce tumor burden in an immunocompetent, B6/myeloma syngeneic model; probably due to its poor penetrance to the tumor or early clearance of the virus. To improve its efficacy in immunocompetent settings, we made further modifications in the DRVV genome in several ways. These include (1) stimulation of cytotoxic T lymphocytes against tumor by a tumor-associated antigen EphA2 and a pro-inflammatory cytokine IL-12 (2) stimulation of natural killer T cells by CD1d and αGalCel, and (3) cancellation of immunosuppressive environment due to myeloid-derived suppressor cells in the tumor by introducing HPGD, an anti-prostaglandin E2. Using B6/3LL tumor mice, we are currently accessing the efficacy of these revised versions of VVs.

2. Proportion of $CD4^+CADM1^+$ population predicts clinical progression in HTLV-1 asymptomatic carrier and indolent ATL

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In HTLV-1-infected patient samples, $CD4^+$ population includes $CADM1^-CD7^{pos}$(P), $CADM1^+CD7^{dim}$ (D) and $CADM^+CD7^{neg}$(N) subpopulations. HTLV-1 infected cells and clones are efficiently enriched in the $CADM1^+$ subpopulations (D and N)(Kobayashi et al, Clinical Cancer Research 2014). In HTLV-1 asypmptomatic carrier (AC) and indolent ATL phase, disease progression are reflected in increase of the D + N(%). In aggressive ATL phase, loss of CD7 occurs in many cases and $CD4^+$ population is occupied by the $CADM^+CD7^{neg}$(N). We next analysed many AC / indolent ATL cases using this flow cytometry combined with HTLV-1 clonality and clinical data (abnormal lymphocytes (%), proviral load (PVL), etc) (Kobayashi et al, Cancer Science 2015). We categorised these cases into the following groups. G1(D + N≤10%) includes low PVL ACs. G2(10%<D + N≤25%) mainly includes ACs with oligoclonal HTLV-1 clones. G3(25%<D + N≤50%) mainly includes ACs and smoldering ATLs. Many of these cases had major clones. G4(50%<D + N) mainly includes smoldering and chronic ATLs. We are following these cases to see how the flow cytometric profile (D + N(%)) predicts future risk. In this study, follow-up clinical and flow cytometric data (CADM1 vs CD7 plot in $CD4^+$ cells) were obtained in the previously analysed 74 cases of ACs and indolent ATLs. In cases in G1(D + N≤10%) and G2(10%<D + N≤25%), apparent clinical and flow cytometric progression were not observed in 7 years follow-up (at the time of this writing). In G3 (25%<D + N≤50%), one case (of 18 in total G3) received clinical trial, and three cases progressed from AC to smoldering ATL. In G4(50%<D + N), 8 cases (of 19 in total G4) remained clinically stable. Probability of (systemic) chemotherapy-free patients at 5 years estimated by Kaplan-Meier method was approximately 40%. In this group, in all evaluable cases except one, progression of flow cyometric profile was observed.

In conclusion, proportion of the $CD4^+CADM1^+$ population (D + N(%)) predicts clinical progression in HTLV-1 ACs and indolent ATLs. Cases in G1(D + N≤10%) and G2(10%<D + N≤25%), including ACs, are stable and considered low-risk. Four cases in G3(25%<D + N≤50%) clinically progressed. Cases in this group, including advanced ACs and smoldering ATLs in Shimoyama criteria, are therefore considered to form an intermediate-risk group. Cases in G4(50%<D + N), mainly including indolent ATLs, are unstable and high-risk for acute transformation. Efficient clinical intervention should be established in this group.

3. Artificial intelligence (AI)-guided precision medicine approach to hematological malignancies

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Next generation sequencing (NGS) of cancer genome is now becoming prerequisite for accurate diagnosis and proper treatment in clinical oncology (Precision oncology). While the genomic regions for NGS expand from a certain set of genes to whole exome or whole genome, the resulting sequence data becomes incredibly enormous, and then makes it quite laborious to translate the genomic data into medicine, so-called annotation and curation. We organized a clinical sequencing team and established a bidirectional (bed to bench and bench to bed) system to integrate clinical and genomic data in blood cancers. We also started a collaborative research with IBM Japan to adopt artificial or augmented intelligence (AI), Watson for Genomics (WG), to the pipeline of medical informatics. Genomic DNA was prepared from cancer cells as well as normal tissues (buccal swab) in each patient, and subjected to NGS. Sequence data was analyzed using an in-house semi-automated pipeline in combination with WG, which was used to identify candidate driver mutations and relevant pathways, from which applicable drug information was deduced. Until now, we have analyzed as many as 200 patients with hematological malignancies including AML, MDS, MPN, et al., and could obtain many informative findings. Although actionable mutations are quite insufficient for clinical practice mainly due to the lack of available molecular-targeted agents, our preliminary results indicate that AI can be a promising support tool for precision medicine.

4. Phenotype-based gene analysis allowed successful diagnosis of X-linked neutropenia associated with a novel WASP mutation in a Japanese adult patient

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X-linked neutropenia (XLN) is a congenital neutropenia caused by gain-of-function mutation in Wiskott-Aldrich syndrome protein (WASP). To our knowledge, only 3 cases of XLN have been described thus far. Here we report a first Japanese case of XLN, who was successfully diagnosed by next generation sequencing (NGS). A 32-year-old male had repeated bacterial infections during his childhood and adolescence, and was clinically diagnosed as severe congenital neutropenia (SCN) when 6-year-old. Since adulthood, he had been free from regular medical follow-up with much less infectious episodes until when 29-year-old, he was admitted to our hospital for febrile neutropenia. His family history showed no significant evidence for inheritance of SCN. His peripheral blood showed severe neutropenia (ANC<200) and slight thrombocytopenia. Bone marrow examination revealed marked myeloid hypoplasia with a slight dysplastic change. *ELANE* gene mutation, a major cause of SCN, was kindly examined by the Department of Pediatrics at Hiroshima University School of Medicine, but was negative. Then, we performed NGS analysis of genomic DNA from the patient and his mother. Sequence data was subjected to medical informatics using in-house pipeline as well as phenotype-based gene analyzer (Yang H, et al. Nat Methods 12: 841-3, 2015), resulting in identification of a missense mutation in exon 9 of *WAS* gene (c. T869C, p.I290T), and his mother was a heterozygous carrier. This mutation is located in the GTPase binding domain of WASP like other mutations in 3 XLN patients (L270P, S272P, I294T). Based on this close similarity, we tentatively conclude that this is the 4th case of XLN caused by a novel WASP mutation. The pathogenesis of XLN is still to be elucidated.

5. HDAC inhibitors activate anti-tumor immunity of monoclonal antibody for myeloma by regulation of immunosurveillance-related antigens in tumors

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Monoclonal antibody therapy including anti-CD38 (daratumumab) or SLAMF7 (elotuzumab) antibody containing regimens is well tolerated and has high activity in relapse and refractory multiple myeloma (MM). However, some patients eventually become resistant to antibody and this resistance is supposed to be due to reduced anti-tumor immunity in these patients. This study will examine the hypothesis that HDAC inhibitors could enhance the anti-myeloma effects of monoclonal antibodies by regulation of immunosurveillance-related antigen expression in myeloma cells. We examined whether pan HDAC (panobinostat), HDAC1-3 (romidepsin), and HDAC6 (ACY1215 and ACY241) inhibitors could change expression of these antigens in myeloma cells. In antibody therapy, NK cells show anti-myeloma effects via binding to Fc region of monoclonal antibodies attached to myeloma cells (antibody-dependent cell-mediated cytotoxicity: ADCC). We found that treatment of myeloma cells with the examined HDAC inhibitors upregulates expression of the ligands of NK cell receptors for activation (MICA/MICB and ULBP-2/5/6) in several myeloma cell lines. In addition to ADCC, complement-dependent cytotoxicity (CDC) plays an important role in anti-tumor effects of antibody therapy for MM. We revealed that expression of CD55/CD59 which protect myeloma cells from accidental complement attack is downregulated by HDAC inhibition. These results suggest the possibility that combination of monoclonal antibody with HDAC inhibitors could enhance ADCC as well as CDC activity. In fact, the enhancement of ADCC or CDC of daratumumab by HDAC inhibitors was displayed in several myeloma cell lines in accordance with expression change of MICA/MICB or CD55/CD59. Thus, reversal of resistance to daratumumab or elotuzumab may be expected through modified expression of immunosurveillance-related antigens by HDAC inhibitors. The obtained results here will be helpful to develop novel therapies to overcome the resistance of relapsed and refractory MM patients.

6. Semaphorin signaling via MICAL3 induces symmetric cell division of breast cancer stem-like cells

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Cancer stem-like cells (CSCs) are maintained in the CSC niche by increased frequency of symmetric cell division at the expense of asymmetric cell division, although the mechanisms remain largely unknown. Here, we show that CSCs utilize some neuronal signaling components for inducing symmetric cell division. Semaphorin 3 (Sema3), known as a neuronal repulsion cue, produced by niche breast cancer cells induced interaction among molecules interacting with CasL 3 (MICAL3), collapsin response mediator protein 2 (CRMP2), and Numb. Knockdown of *MICAL3*, *CRMP2*, or *Numb* decreased sphere formation in breast cancer cells. Neuropilin-1, a Sema3 receptor, and Numb were specifically co-expressed at high levels in patient-derived breast CSCs (BCSCs). *MICAL3* knockdown significantly decreased symmetric cell division in BCSCs and tumor-initiating activity in a patient-derived xenograft (PDX) model. Hence, the niche factor Sema3-stimulated MICAL3/CRMP2/Numb axis, which is common to some neuronal signals, appears to play a critical role in the symmetric division of CSCs.

7. Cancer stem-like properties and drug resistance are dependent on purine synthetic metabolism mediated by the mitochondrial enzyme MTHFD2

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Tumor recurrence is attributable to cancer stem-like cells (CSCs), the metabolic mechanisms of which currently remain obscure. Here, we uncovered the critical role of folate-mediated one-carbon (1C) metabolism involving mitochondrial methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) and its downstream purine synthesis pathway. *MTHFD2* knockdown greatly reduced tumorigenesis and stem-like properties, which were associated with purine nucleotide deficiency, and caused marked accumulation of 5-aminoimidazole carboxamide ribonucleotide (AICAR)—the final intermediate of the purine synthesis pathway. Lung cancer cells with acquired resistance to the targeted drug gefitinib exhibited increased stem-like properties and enhanced expression of MTHFD2. *MTHFD2* knockdown or treatment with AICAR reduced the stem-like properties and restored gefitinib sensitivity in gefitinib-resistant cancer cells. Thus, MTHFD2-mediated mitochondrial 1C metabolism appears critical for cancer stem-like properties and resistance to drugs including gefitinib through con-

sumption of AICAR, leading to depletion of the intracellular pool of AICAR. Because CSCs are dependent on MTHFD2, therapies targeting MTHFD2 may eradicate tumors and prevent recurrence.

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Our major projects are (1) Co-ordinate control of cell division and differentiation by a crosstalk between JAK/STAT and small GTPases, (2) Molecular targeted therapies, and (3) Elucidation of molecular basis of leukemia, hematological malignancioes.

1. Co-ordinate control of cell division and cell differentiation of by the Rho family small GTPases.

Takeshi Fukushima, Yosuke Tanaka, Toshihiko Oki, Toshiyuki Kawashima, Kohtaro Nishimura, Susumu Goyama, and Toshio Kitamura.

In search for key molecules that prevent murine M1 leukemic cells from undergoing IL-6-induced differentiation into macrophages, we previously isolated an antisense cDNA that encodes full-length mouse MgcRacGAP through functional cloning. In human HL-60 leukemic cells, overexpression of the human MgcRacGAP induced differentiation to macrophage. Interestingly, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. Moreover, the GAP activity of MgcRacGAP was required for completion of cytokinesis. We also found that MgcRacGAP is phosphorylated by Aurora B at the midbody. Intriguingly, this phosphorylation induced the Rho-GAP activity of MgcRacGAP, which was critical for completion of cytokinesis. We identified S387 as a phosphorylation site responsible for the acquirement of Rho-GAP activity during cytokinesis at the midbody. On the other hand, MgcRacGAP mainly localizes in the nucleus in the interphase. We demonstrated that MgcRacGAP directly bound transcription factors STAT3 and STAT5, and enhanced transcriptional activation of STAT proteins as a Rac GAP. MgcRacGAP was found to harbor functional NLS and works as a nuclear chaperon together with Rac1.

We found using an MgcRacGAP-GFP fusion protein that MgcRacGAP expression increased in the early G1 phase in parallel with or even earlier than Geminin, suggesting that MgcRacGAP may play roles in G1 check point. MgcRacGAP accumulates to the midbody during cytokinesis, and the midbody is included in one of the daughter cells after cell division. It was suggested by some researchers that the midbody is frequently released from the cells in stem cells. We therefore hypothesized that the cells with midbody tend to differentiate and the cells without midbody tend to self-renew or enter G0 phase. To test this hypothesis, we have recently generated a transgenic mouse expressing the MgcRacGAP-mVenus fusion protein in hematopoietic stem cells and/or progenitors.

2. Molecular targeting therapies using small molecule compounds

Akiho Tsuchiya, Reina Nagase, Toshiyuki Kawashima, Yukinori Minoshima, Susumu Goyama, and Toshio Kitamura:

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We established an efficient screening protocol for identification of STAT3 inhibitors. Through the screening of a library of small molecule compounds, we found the compounds RJSI-1 and RJSI-2 that inhibited STAT3 activation. RJSI-2 also inhibited activation of STAT1, STAT5, JAK1 and JAK2. On the other hand, RJSI-1 inhibited nuclear transport of phosphorylated STAT proteins, implicating a novel mechanism in inhibiting STAT proteins. We have also shown that these compounds are effective in a tumor-burden mouse model. In addition, we collaborated with a US-based biotech company in modification of RSJI-1 for optimization to develop anti-cancer drugs, and have developed JP1156 that kills the tumor cells more efficiently both in vitro and in vivo with much lower IC50.

In addition to STAT3 inhibitors, we have recently started a new project to develop STAT5 inhibitors in collaboration with a pharmaceutical company. To this end, we have developed a screening method to search for STAT5 inhibitors. In addition to STAT3/5 inhibitors, we have started several collaborations with several domestic and global pharmaceutical companies to evaluate the efficacies of a variety of molecular targeted therapies in our established mouse MDS/AML/MPN models.

3. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative neoplasms (MPN).

Daichi Inoue, Reina Nagase, Takeshi Fujino, Yasutaka Hayashi, Shuji Asada, Reina Takeda, Kojin C Kawabata, Naoko Watanabe, Makoto Saika, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Yuka Harada[1], Hironori Harada[2], Tetsuya Nosaka[3], Jiro Kitaura[4], Yosuke Tanaka, Tomofusa Fukuyama, Susumu, Goyama, and Toshio Kitamura: [1]Department of Clinical Laboratory Medicine, Bunkyo Gakuin University, Laboratory of Oncology, [2]Department of Medical Science Tokyo University of Pharmacy and Life Sciences, [3]Mie University School of Medicine, and [4]Allergy Center, Juntendo University.

Recent progress using high-speed sequencing has identified mutations in genes that are not categorized to class I and class II mutations, including epigenetic factors, and splicing factors. We have recently established two MDS models induced by ASXL1 mutations and EZH2 mutations; mice transplanted with bone marrow cells expressing C-terminal truncating mutants of ASXL1 or EZH2 derived from MDS patients developed MDS-like diseases in a year or two. Concerning the molecular mechanisms, the ASXL1 mutant (ASXL1-MT) suppressed PRC2 functions, leading to the derepression of posterior HoxA genes and miR125a via inhibition of H3K27 trimethylation. While expression of posterior HoxAs is known to contribute transformation of hematopoietic cells, miR125a is a well-known oncogenic micro RNA, in particular for hematological malignancies. In addition to known target genes of miR125a, we have identified Clec5a/MDL1. We have also found that Clec5a is required for granulocytic differentiation of 32D cells, implicating its downregulation in the pathogenesis of MDS. ASXL1 mutations are frequently associated with SETBP1 mutations (SETBP1-MT) that stabilize SETBP1 and SET oncoprotein, leading to activation of the PI3K/Akt pathway. In the BMT model, combination of ASXL1-MT and SETBP1-MT induced AML with much shorter latencies. GSEA indicated that the TGF beta pathway was profoundly inhibited, implying the inhibition of the TGF beta pathway in leukemic transformation of MDS. Further experiment is now under way to clarify the molecular mechanisms by which the TGF beta pathway was inhibited.

We have recently established Rosa26-knock-in mice for ASXL1-MT and the EZH2 mutant. These KI mice did not develop MDS in a year, but presented disturbed differentiation of erythroid cells and mild macrocytic anemia.

4. Investigating molecular pathogenesis of AML1-MTG8/ETO and MLL-fusion acute myeloid leukemias.

Tomofusa Fukuyama, Susumu Goyama, Keita Yamamoto, Taishi Yonezawa, Joseph S. Palumbo[5], James C. Mulloy[5], Toshio Kitamura: [5]Cincinnati Children's Hospital Medical Center

Using human and mouse models for AML1-MTG8/ETO and MLL-fusion leukemias, we have been elucidating new molecular aspects in the pathogenesis and progression of acute myeloid leukemia (AML).

t(8; 21) AML is the most common cytogenetic subtype of AML, and the resultant AML1-MTG8 chimeric protein is believed to play an important role for leukemogenesis. However, the role of AML1-MTG8 is still unclear because persistent existence of chimeric gene including chimeric fusion point detected by PCR is observed in complete remission or healthy persons, even in utero. In addi-

tion, full length of AML1-MTG8 by itself cannot cause leukemia in mouse models, suggesting that additional "events" should be required for leukemogenesis. Interestingly, AML1-ETO9a (AE9a) that lacks c-terminus of AML1-MTG8 is shown to possess leukemogenic potential in a mouse model of retroviral transduction-transplantation. Nonetheless, AE9a protein is barely expressed in t(8; 21) cells and a recent report suggested that there was no impact on clinical outcome by AE9a. Now we have identified a new splicing variant that has significant ability to induce leukemia in mouse model. Mechanisms of leukemogenesis by it as well as clinical significance are currently under investigation.

MLL-fusion leukemia is an aggressive form of leukemia carrying chimeric fusion of the MLL gene. We previously showed that the combined loss of Runx1/Cbfb inhibited the development of MLL-AF9-induced leukemia. However, c-Kit + /Gr-1 − cells remained viable in Runx1/Cbfb-deleted cells, indicating that suppressing RUNX activity may not eradicate the most immature LSCs. We found upregulation of several hemostasis-related genes, including the thrombin-activatable receptor PAR-1 (protease-activated receptor-1), in Runx1/Cbfb-deleted MLL-AF9 cells. Similar to the effect of Runx1/Cbfb deletion, PAR-1 overexpression induced CDKN1A/p21 expression and attenuated proliferation in MLL-AF9 cells. To our surprise, PAR-1 deficiency also prevented leukemia development induced by a small number of MLL-AF9 leukemia stem cells (LSCs) in vivo. PAR-1 deficiency also reduced leukemogenicity of AML1-ETO-induced leukemia. Re-expression of PAR-1 in PAR-1-deficient cells combined with a limiting-dilution transplantation assay demonstrated the cell-dose-dependent role of PAR-1 in MLL-AF9 leukemia: PAR-1 inhibited rapid leukemic proliferation when there were a large number of LSCs, while a small number of LSCs required PAR-1 for their efficient growth. Mechanistically, PAR-1 increased the adherence properties of MLL-AF9 cells and promoted their engraftment to bone marrow. Taken together, these data revealed a multifaceted role for PAR-1 in leukemogenesis, and highlight this receptor as a potential target to eradicate primitive LSCs in AML.

5. Identification of E3 ubiquitin ligases for RUNX1 and RUNX1-RUNX1T1

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RUNX1 is a member of RUNX transcription factors and plays important roles in hematopoiesis. Disruption of RUNX1activity has been implicated in the development of hematopoietic neoplasms. Chromosomal translocations involving the RUNX1 gene are associated with several types of leukemia, including acute myeloid leukemia driven by a leukemogenic fusion protein RUNX1-RUNX1T1. Previous studies have shown that RUNX1 is an unstable protein and is subjected to proteolytic degradation mediated by the ubiquitin-proteasome pathway. However, the precise mechanisms of RUNX1 ubiquitination have not been fully understood. Furthermore, much less is known about the mechanisms to regulate the stability of RUNX1-RUNX1T1. In this study, we identified several RUNX1-interacting E3 ubiquitin ligases using a novel highthroughput binding assay. Among them, we found that STUB1 bound to RUNX1 and induced its ubiquitination and degradation mainly in the nucleus. Immunofluorescence analyses revealed that the STUB1-induced ubiquitination also promoted nuclear export of RUNX1, which probably contributes to the reduced transcriptional activity of RUNX1 in STUB1-overexpressing cells. STUB1 also induced ubiquitination of RUNX1-RUNX1T1 and downregulated its expression. Importantly, STUB1 overexpression showed a substantial growth-inhibitory effect in myeloid leukemia cells that harbor RUNX1-RUNX1T1, whereas it showed only a marginal effect in other non-RUNX1-RUNX1T1 leukemia cells and normal human cord blood cells. Taken together, these data suggest that the E3 ubiquitin ligase STUB1 is a negative regulator of both RUNX1 and RUNX1-RUNX1T1. Activation of STUB1 could be a promising therapeutic strategy for RUNX1-RUNX1T1 leukemia.

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Our overall goal is medical sciences on infectious diseases in two directions, from clinic to bench and from bench to clinic. Our current main subject is immunopathogenesis of HIV-1 infection. We are focusing on how cellular immune responses fight against to HIV-1 and how immune system is disrupted and develops AIDS. We are also working on viral pathogenesis in HIV-infected patients. We work together with the staffs in the Department of Infectious Diseases and Applied Immunology in the IMSUT hospital and apply the research results to the people living with HIV-1/AIDS. We are extending our research project to other viral diseases including viral hepatitis and associated morbidities, especially pathogenesis of co-infection with HIV and hepatitis B virus (HBV) or hepatitis C virus (HCV).

1. Identification of preS/S sequence of HBV derived from patients co-infected with HIV and HBV.

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The number of patients infected with HBV dramatically decreased by successful prevention of mother-to-child transmission and post-transfusion infection, but horizontal transmission, especially of genotype A, is recently increasing. HBV vaccine is very useful for preventing infection, but about 10% of vaccinated adults cannot get an adequate neutralizing antibody. In addition, some vaccine-escape mutants (VEM) which cannot be controlled by vaccination have been identified. Considering these situations, a novel and more effective HBV vaccine are necessary and expected to be developed. In fact, some candidates which include preS as well as S region of HBV are under study. To develop a more effective vaccine, it is necessary to search sequence of this region of HBV derived from current patients with acute infection and also immune-compromised patients such as HIV co-infected patients. Therefore we extracted DNA from sera of HIV/HBV co-infected patients who visited IMSUT hospital, and cloned preS/S region of HBV for direct sequencing. Among 18 patients with successful analysis, G145A mutation in HBV-S region, which is reported to be VEM, is detected in 2 patients infected with genotype C. Both patients had no vaccination and were homosexual, but considering their background, it is not possible that they directly transmitted HBV. The results suggest that VEM actually exist in some patients co-infected with HIV. We will analyze more patients and determine the presence of other VEM and also sequence candidates for an effective new vaccine.

2. Suppression of mitophagy by HCV and exploration of drugs to restore mitophagy

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HCV infection is closely associated with hepatocellular carcinoma (HCC) development, and dysfunction of mitochondria and subsequent reactive oxygen species (ROS) accumulation by HCV, especially the core protein, may contribute to the pathogenesis. In hepatocytes of transgenic mice harboring the core protein, accumulation of morphologically abnormal mitochondria is observed, suggesting that mitophagy is suppressed by the core protein, leading to ROS accumulation. We examined the expression of several mitophagy-related proteins in core-expressing HepG2 cells and found that expression of Bnip3 (BCL2 and adenovirus E1B 19 kDa-interacting protein 3), localized to outermembrane of mitochondria as a mitophagy receptor, is decreased in core-expressing cells. Further analysis revealed that the core protein interacts with Bnip3 and impairs Bnip3 homodimerization and Bnip3-LC3 interaction (manuscript in preparation). These results suggest that the core protein disrupts Bnip3 function which contributes to suppression of mitophagy, therefore it is possible that if Bnip3 function is restored by some chemicals, mitophagy will be recovered and ROS accumulation will be attenuated. For a screening we used NanoBiT system which detects protein-protein interaction quantitatively. We examined the effect of chemicals (provided as autophagy-related library) on Bnip3-Bnip3 interaction, and found some chemicals increased this interaction in the core-expressing cells. We also observed increased homodimerization of endogenous Bnip3 in the cells incubated with some effective chemicals. Further analysis and screening of other libraries are necessary for the determination of more effective and nontoxic chemicals, but this study will lead to the future development of drugs for the prevention of HCC accompanied in HCV-infected patients.

3. Characteristics of Transmitted Drug-Resistant HIV-1 in Recently Infected Treatment-Naive Patients in Japan.

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Progress in antiretroviral treatment has led to fewer virological failure cases, but 10%-20% of treatment-naive HIV/AIDS cases are reported to harbor drug-resistant strains (RS), suggesting transmission of drug-resistant HIV. We have determined the trend in prevalence of transmitted drug-resistant (TDR) HIV in Japan from 2003.

Drug-resistance test had been performed on national-wide HIV-1-infected cases newly diagnosed. The overall prevalence of TDR was about 9%, ranging from 5.2% in 2004 to 13.2% in 2017. The prevalence of RS was significantly higher among cases who were male, Japanese, and men who have sex with men. Common mutations in both groups were M46I/L and T215 revertants. Furthermore, sequences with these mutations, K103N and D30N/N88D formed clusters on phylogenetic trees. It was suggested that HIV with these mutations have become circulating strains.

4. Delineation of autoantibody repertoire through differential proteogenomics in hepatitis C virus-induced cryoglobulinemia.

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Antibodies cross-reactive to pathogens and autoantigens are considered pivotal in both infection control and accompanying autoimmunity. However, the pathogenic roles of autoantibodies largely remain elusive without a priori knowledge of disease-specific autoantigens. Here, through a novel quantitative proteogenomics approach, we demonstrated a successful identification of immunoglobulin variable heavy chain (VH) sequences highly enriched in pathological immune complex from clinical specimens obtained from a patient with hepatitis C virus-induced cryoglobulinemia (HCV-CG). Reconstructed single-domain antibodies were reactive to both HCV antigens and potentially liver-derived human proteins. Moreover, over the course of antiviral therapy, a substantial "de-evolution" of a distinct sub-repertoire was discovered, to which proteomically identified cryoprecipitation-prone autoantibodies belonged. This sub-repertoire was characterized by IGHJ6*03-derived, long, hydrophobic complementarity determining region (CDR-H3). This study provides a proof-of-concept of *de novo* mining of autoantibodies and corresponding autoantigen candidates in a disease-spe-

cific context in human, thus facilitating future reverse-translational research for the discovery of novel biomarkers and the development of antigen-specific immunotherapy against various autoantibody-related disorders.

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Advanced Clinical Research Center

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Our division has been conducting basic research projects for development of innovative cancer therapy using immunologic and gene therapy approaches. The reagents, modalities, and concepts developed in this division have been clinically applied as translational research projects. We believe that bidirectional information exchange between the bench and the bedside would be one of the most important requirements for the successful development of novel and effective therapies.

I. Clinical development of anti-programmed death 1 (PD-1) Ab in melanoma patients

Hideaki Tahara

Check-point blockades, which block the regulatory pathways of CTL activation with antagonistic antibodies to promote immunological responses, have been shown to be effective for various types of cancer in the clinical trials. Among them, anti-PD-1 antibodies have been particularly drawing attention of the oncologists.

Nivolumab (ONO-4538/BMS-936558/MDX-1106) is a fully human monoclonal IgG4 antibody (HuMAb) against PD-1 which has high affinity for PD-1 (Kd 2.6 nM) and block cross-linkage to both PD-L1 (B7-H1) and L2. Based on good safety profiles and promising anti-tumor effects in phase I trial for recurrent solid tumor patients, we initiated phase II study of nivolumab as a pharmaceutical-supported trial to treat melanoma patients in Japan. The results of such trial have shown the significant anti-tumor effects and manageable side-effects, and nivolumab has become the first government-approved drug in the world as a PD-1 related drug. We are now analyzing the immunological parameters to further develop this powerful agent. As a result of such efforts, we have found that the levels of the serum cytokines in the melanoma patients before the treatment could be the biomarkers to predict the response to nivolumab.

II. Development of cancer immunotherapy using the blockade of MFG-E8

Yu Mizote, Mika Uematsu-Hamada, Miho Kudo, Keito Inaba, Hiroaki Uchida, Hideaki Tahara

The secreted protein, milk fat globule-EGF factor 8 (MFG-E8), stimulates disease progression through coordinated αvβ3 integrin signaling in tumor and host cells. MFG-E8 enhances tumor cell survival, invasion, and angiogenesis, and contributes to local immune suppression.

We have shown that systemic MFG-E8 blockade cooperates with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumors. The combination treatments evoke extensive tumor cell apoptosis that is coupled to efficient dendritic cell cross-presentation of dying tumor cells. Our previous findings suggest that systemic MFG-E8 blockade might intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms also in human. In order to

apply these findings to treat cancer patients, we have developed antibodies specific to the human MFG-E8. These antibodies include the one with blocking activity on MFG-E8 functions and the one suitable for immune-staining of human tissue. We are currently investigating the human situations related to MFG-E8 and have found that strong expression of MFG-E8 in the tumor cells has significant impact on the survival of certain types of cancer patients (manuscript in preparation). Furthermore, we are now in the process of developing this agent for clinical application.

III. Development of novel gene and cell therapy against cancer via T-cell immune checkpoint blockade

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We have reported for anti-tumor effects and mechanisms of IL-23, which is a cytokine secreted by dendritic cells, and have been trying to develop novel and effective cancer immunotherapy. Recently, we have been focused on T-cell suppressing pathway of immune responses against cancer including CTLA-4, PD-1, and TIM-3. These immune checkpoints have been blocked using antagonistic antibodies against them to enhance the anti-tumor immune response of gene therapy using cytokines with or without dendritic cell administration. At the same time, the mechanisms of such combination therapies have been investigated.

IV. NK cells control tumor-promoting function of neutrophils in mice

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Although the importance of NK cells as a direct anti-tumor effector is well appreciated, the immuno-regulatory function of NK cells to control cancer-associated inflammation, which facilitate tumor progression, remains unknown. In this study, we demonstrate that the novel function of NK cells to control tumor-promoting inflammation through the functional modification of neutrophils. NK cells control the tumor-promoting function of neutrophils via an IFN-γ-dependent mechanism and the tumor progression in an NK cell-depleted host is totally diminished when the IL-17A-neutrophils axis is absent. In NK cell-depleted mice, neutrophils acquire the tumor-promoting phenotype as seen in the up-regulation of VEGF-A expression to promote tumor growth and angiogenesis. Importantly, a VEGFR inhibitor preferentially suppressed tumor growth in NK cell-depleted mice and such a selective anti-tumor effect in NK cell-depleted mice was a neutrophil-dependent. Furthermore, the systemic neutropenia by an antimetabolite treatment shows a significant anti-cancer effect only in mice with no NK cells. Thus, NK cells likely play an important role in controlling the tumor-promoting and angiogenic function of neutrophils.

V. Treatment of malignant pleural mesothelioma using replication-defective recombinant adenoviral vector expressing the suppressor of cytokine signaling 3 (SOCS3). (Manufacture of the viral vector for preclinical studies in non-human primates)

Tetsuji Naka[2], Hiroyuki Mizuguchi[3], Takafumi Nakamura[4], Hisako Katano[5], Hiroaki Uchida, Takuma Suzuki, Hideaki Tahara: [2]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan, [3]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, [4]Tottori University, [5]University of Tokyo

In collaboration with the research team, we have prepared the replication-defective recombinant adenoviral vector expressing the suppressor of cytokine signaling 3 (SOCS3), AdSOCS3 for treatment of malignant pleural mesothelioma. We have supported the vector production using Vector Facility in IMSUT utilizing the master and working cell banks of 293 cells, which we established previously. The purified final products have been used for preclinical study in monkey. We are also carrying out the safety and biodistribution studies for AdSOCS3 in the context of intrapleural or intravenous administration in a mouse model. Based on the results of these studies, we are in the phase of preparing the phase I study for the patients with malignant pleural mesothelioma using this strategy.

VI. Development of fully retargeted herpes simplex virus (HSV) vectors for oncolytic virotherapy

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Herpes simplex virus (HSV) vectors are promising agents for oncolytic virotherapy. Uchida established a fully retargeted HSV platform that mediates virus entry exclusively via tumor-associated antigens in the lab of Prof. Joseph Glorioso at the

University of Pittsburgh. Entry of HSV is initiated by the binding of glycoprotein D (gD) to one of its receptors, herpesvirus entry mediator (HVEM) or nectin-1. This interaction results in a conformational change in gD, triggering sequential activation of gH and gB to execute fusion between the viral envelope and cell membranes. We inserted single-chain antibodies (scFv) against a number of different cell surface molecules such as epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), and epithelial cell adhesion molecule (EpCAM), into the retargeted HSV platform that encodes a gD ablated for binding to natural receptors and a gB containing entry-enhancing mutations we previously identified. As a result, we observed specific virus entry into cells expressing the cognate target antigen for each of the retargeted constructs. Our results indicate the adaptability of our system to different targeting ligands, leading to a new generation of broadly applicable and effective oncolytic HSV vectors. Furthermore, we introduced syncytial mutations into the gB and/or gK genes of gD-retargeted HSVs and found that gD retargeting does not abolish the hyperfusogenic activity of syncytial mutations and that these mutations do not eliminate the dependence of HSV entry and spread on a specific gD-receptor interaction. These observations suggest that syncytial mutations may be valuable for increasing the tumor-specific spreading of retargeted oncolytic HSV vectors. We are now testing whether syncytium formation in tumors would be associated with more potent antitumor effects *in vivo*.

VII. Establishment of highly functional monoclonal antibodies through novel screening methods for targeted cancer therapy

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Monoclonal antibodies (mAbs) have become an established therapeutic modality in clinical oncology. In order to identify cell-surface molecules that may be useful for targeting various types of cancers, our group established a unique screening approach that employs an adenoviral vector harboring fiber proteins engineered to bind antibodies, Adv-FZ33. This approach led to the successful identification of an array of potential target molecules for cancer treatment. Immunotoxins (antibody-drug conjugates; ADC) are a promising class of cancer therapeutics composed of a cytotoxic agent linked covalently to a cancer-targeted antibody. To systematically hunt for cell-surface molecules that may be efficiently targeted by immunotoxins, our group created another method for screening highly functional cancer-targeted mAbs and cognate antigens. The receptor-binding domain of the Diphtheria toxin (DT) was replaced with the antibody-binding domain (3C) derived from the Streptococcal protein G. The resultant mutated toxin protein (DT-3C) was used for selection of mAbs for specific cell killing activity as components of immunotoxins. Our novel screening system is advantageous in that the selected antibodies bind to intact cancer cells and get internalized efficiently, which has been critically required for therapeutic applications but elusive thus far. Furthermore, we have successfully taken advantage of some of these in-house monoclonal antibodies for development of novel fully retargeted HSV vectors. Additionally, we have created an HSV-based probe for screening of Abs that could mediate HSV entry by recognition of unknown receptors. We expect that this novel Ab-screening system may lead to a new generation of RR-oHSV vectors.

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臨床ゲノム腫瘍学分野

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Associate Professor	Tsuneo Ikenoue M.D., Ph.D.		准教授	博士(医学)	池　上　恒　雄
Project Senior Assistant Professor	Kiyoshi Yamaguchi Ph.D.		特任講師	博士(薬学)	山　口　貴世志

Research Projects
We have been working on the following five projects, 1) understanding the role of Wnt/β-catenin signaling pathway in colorectal carcinogenesis, 2) discovery of molecular targeted anticancer drugs through a screening of large-scale chemical libraries, 3) establishment and investigation of mouse models of human cancer, 4) elucidation of genetic characteristics of human tumors and mechanisms of their development, and 5) clinical sequencing for the implementation of genomic medicine. These projects are aimed to develop strategies for better diagnosis, effective treatment, and prevention of human cancer.

1. Understanding the role of Wnt/β-catenin signaling pathway in colorectal carcinogenesis

Kiyoshi Yamaguchi, Yoichi Furukawa

Constitutive activation of Wnt signaling pathway plays a crucial role in the development of colorectal cancer through a bipartite β-catenin/TCF transcriptional activator. Genes up-regulated by Wnt/β-catenin signaling include *MYC*, *CCND1*, and *LGR5*, well-known direct targets of β-catenin/TCF7L2 complex. In our laboratory, we previously identified target genes such as *RNF43*, *SP5*, *CLDN1*, *ENC1*, *APCDD1*, and *FRMD5*, which are up-regulated by the signaling. On the other hand, roles of down-regulated genes by the signaling remain largely unknown. Thus, we performed transcriptome analysis of colorectal cancer cells introduced with β-catenin siRNAs or a dominant negative form of TCF7L2 (dnTCF7L2), and searched for genes commonly up-regulated by the treatment with β-catenin siRNAs or dnTCF7L2. As a result, we identified interferon-induced protein with tetratricopeptide repeats 2 (*IFIT2*), whose promoter activity was up-regulated by either transduction of β-catenin siRNA or dnTCF7L2. In agreement with the results, expression of *IFIT2* was significantly lower in colorectal cancer tissues compared with normal colonic tissues. Interestingly, its overexpression decreased cell proliferation and increased apoptosis of colorectal cancer cells. These data suggested that the down-regulation of IFIT2 by Wnt/β-catenin signaling might play an important role in human carcinogenesis. Further studies of its regulatory mechanism will contribute to a better understanding of colorectal carcinogenesis and the development of strategies to enhance apoptotic effect of anti-cancer drugs.

2. Cancer drug discovery through a large chemical library screening

Kiyoshi Yamaguchi, Yoichi Furukawa, Satoru Nagatoishi[1], Kohei Tsumoto[2]: [1]Project Division of Advanced Biopharmaceutical Science, [2]Medical Proteomics Laboratory, IMSUT

Establishment of well-designed high-throughput screening system is an essential for the identification of small molecules that inhibit a signaling pathway or a molecule of interest. Cell-based as-

says using TCF/LEF reporter (TOPFLASH), as readout of β-catenin/TCF-dependent transcriptional activity, have contributed to the discovery of small molecules that modulate Wnt signaling. Recently, we developed an efficient cell-based reporter assay system named "bidirectional reporter assay". Integrated transcriptome analysis identified a histidine ammonia-lyase gene (*HAL*) that was negatively regulated by β-catenin/TCF-dependent transcriptional activity. We leveraged a promoter region of *HAL* as another transcriptional readout of Wnt signaling. Cells stably expressing both an optimized HAL reporter and the TOPFLASH enabled bidirectional reporter activities in response to Wnt signaling. Indeed, increased HAL reporter activity and decreased TOPFLASH activity were observed simultaneously in the cells when β-catenin/TCF7L2 was functionally blocked. Most importantly, this method could decrease the number of false positives observed when screening an inhibitor library compared with the conventional TOPFLASH assay. Applying this assay system, we performed a high-throughput screening of Wnt inhibitors using a commercially available library containing 20,000 compounds. Consequently, we have successfully identified several candidate small molecules that suppress Wnt signaling. We are currently working to elucidate the mode of action of these chemicals using approaches of chemical biology.

3. Establishment and investigation of novel mouse models of human cancer

Tsuneo Ikenoue, Yoichi Furukawa

Genetically engineered mice are useful tools for studying human diseases, including cancer. In this project, we have established a mouse model of intrahepatic cholangiocarcinoma (ICC) by liver-specific *Kras* activation and *Pten* deletion. Using a lineage tracing system, we have demonstrated that ICCs originate from cholangiocytes, but not from hepatocytes in this model. We are now studying the molecular mechanisms how *Kras* activation and *Pten* deletion induce ICC.

In addition, we are trying to establish novel cancer mouse models using mice carrying a conditional mutant allele of *Fbxw7* or *Idh1/2* genes, which are frequently mutated in human gastrointestinal and liver cancers. Intensive analysis of these models should provide better understanding of their carcinogenesis and facilitate the development of new therapies to these cancers.

4. Elucidation of genetic characteristics of human tumors and mechanisms of their development

Rei Noguchi, Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Atsushi Niida[1], Rui Yamaguchi[3], Seiya Imoto[2], Satoru Miyano[1,3]: [1]Division of Health Medical Computational Science, [2]Division of Health Medical Data Science, Health Intelligence Center, [3]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT.

Pseudomyxoma peritonei (PMP) is a rare disorder, and characterized by the accumulation of abundant mucinous or gelatinous fluid that is produced from tumorous cells disseminated in the abdominal cavity and pelvis. Our previous analysis of 18 PMPs containing 10 low-grade tumors and 8 high-grade tumors determined that *KRAS* and/or *GNAS* mutations are common genetic features of PMP. Furthermore, we suggested that mutations in *TP53* and/or genes related to the PI3K-AKT pathway might provide malignant properties to PMP. To comprehensively understand genetic alterations in PMP, we extensively analyzed PMP tumors and matched normal colonic mucosa by the whole-genome sequencing and RNA sequencing. Ongoing analysis of genetic and transcriptome data will provide the better understanding of tumor characteristics, and facilitate the development of personalized medicine for PMP.

5. Clinical sequencing for the implementation of genomic medicine

Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Eigo Shimizu[1], Mitsuhiro Komura[1], Rui Yamaguchi[1], Tetsuo Shibuya[2], Satoru Miyano[1,2], Takanori Hasegawa[3], Seiya Imoto[3], Kazuaki Yokoyama[4], Arinobu Tojyo[4], Koichiro Yuji[5]: [1]Laboratory of DNA Information Analysis, [2]Laboratory of Sequence Analysis, Human Genome Center, [3]Division of Health Medical Data Science, Health Intelligence Center, [4]Division of Molecular Therapy, [5]Division of International Advanced Medical Research, Advanced Clinical Research Center, IMSUT.

Cancer cells accumulate multiple genetic and epigenetic changes in the genome. Next-generation sequencing (NGS) allowed us to analyze the comprehensive human genome, and facilitated the identification of germline changes responsible for hereditary diseases and somatic alterations in human neoplasms. In collaboration with Human Genome Center, Health Intelligence Center, and Advanced Clinical Research Center, we have been working on the determination of germline mutations in patients suspected of hereditary colon tumor and application of a cognitive computing system for the personalized medicine. These projects are aimed to use the information of personal genome and/or cancer genome in clinic, and apply the data for their diagnosis and treatment.

In the first project, we have applied NGS technology for unexplained cases with familial polyposis. For example, we had a patient with synchronous carcinomas and oligo-polyps in the colon. Although we suspected Lynch syndrome on the basis of the patient's family history, any pathogenic mutations in the mismatch repair genes including *MSH2*, *MLH1*, and *MSH6* were not found in the patient. Subsequently, whole-genome sequencing of the peripheral blood DNA identified a frameshift mutation in the *POLE* gene. Recently, mutations in the polymerase genes have been identified as rare cause of multiple early-onset adenomas and carcinomas, a condition termed polymerase proofreading-associated polyposis (PPAP). These data indicated the patient with PPAP, and demonstrated the usefulness of NGS in clinical diagnosis of cancer.

In the second project, we have been testing interpretation of genomic data using IBM Watson for Genomics (WfG). After written informed consent was obtained from the patients with colorectal, breast, pancreatic, tongue cancer, lymphoma, and hepatoblastoma, they were enrolled in this study. Genetic alterations in their tumors were determined by NGS and the data were subsequently analyzed by WfG. The results of WfG including predicted driver mutations and suggested actionable drugs were discussed in the Tumor Board meeting of this project, which is held every two weeks.

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Advanced Clinical Research Center

Division of Innovative Cancer Therapy
先端がん治療分野

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The major research topic of our laboratory is to develop oncolytic virus therapies for various malignant tumors. Oncolytic viruses are designed so that they can infect, replicate selectively within, and destroy tumor cells. G47Δ, a recombinant, triple-mutated oncolytic herpes simplex virus type 1 (HSV-1), exhibits potent anti-tumor efficacy while maintaining safety. Two clinical trials using G47Δ are currently being conducted at IMSUT Hospital.

Creation of novel recombinant oncolytic HSV-1

The use of genetically-engineered oncolytic viruses is a novel therapeutic strategy for cancer. Various kinds of virus have been studied worldwide as oncolytic viruses, but genetically engineered HSV-1 is particularly useful because of following favorable characteristics: (1) It shows little toxicity to normal tissues, and there exist theoretical backgrounds for tumor cell selectivity. (2) The viral genome is stable. (3) It can efficiently infect wide range of tumor types and exhibits a potent oncolytic activity. (4) Cell-to-cell spread is minimally affected by circulating antiviral antibodies. (5) Inflammatory reactions to the virus are generally mild and repeated administrations are possible. (6) There are antiviral drugs available to terminate viral replication when undesired events occur. (7) Antitumor immune responses are elicited in the course of oncolytic activities by the virus. (8) The large size of HSV-1 genome (～152kb) allows insertion of large or multiple foreign genes.

Conventional homologous recombination techniques had required time-consuming processes to create new recombinant oncolytic HSV-1. We have established an innovative recombinant HSV-1 construction system using bacterial artificial chromosome and two sets of recombinases (Cre/loxP and FLP/FRT). This system allows rapid generation of multiple new recombinant HSV-1 with desired sequences inserted into a specific locus.

Application of oclolytic HSV-1 for malignant glioma is a major study interest in our laboratory. In addition, *in vitro* and *in vivo* tumor models of other cancers, including renal cancer, prostate cancer, bladder cancer, malignant mesothelioma, tongue cancer, esophageal cancer, gastric cancer, colon cancer, lung cancer, breast cancer, nasopharyngeal cancer, cholangiocarcinoma, hepatic cancer, pancreatic cancer, malignant melanoma, and malignant lymphoma have also been used for testing efficacy and safety.

Studies using cancer stem cells derived from surgical specimen

There exists a small population of tumor-initiating, stem-like cells within the tumor. Because cancer stem-like cells (CSC) are reported to be resistant to current therapies and responsible for recurrence, a novel approach that can eliminate CSCs is needed to cure the disease. We currently use glioma-de-

rived CSCs to study new therapeutic approaches including oncolytic virus therapy using genetically engineered HSV-1. G47Δ has been shown to kill CSCs efficiently. Novel oncolytic HSV-1 that exhibit high efficacy for tumors rich in CSCs have been created and are being evaluated.

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Advanced Clinical Research Center

Division of Advanced Medicine Promotion
先端医療開発推進分野

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Associate Professor	Masanori Nojima, M.D., Ph.D., M.P.H.	准教授　博士(医学)	野　島　正　寛

Division of Advanced Medicine Promotion was established in 2011. Our mission is to assist the clinical development and the conduct of clinical trials, especially for translational researches. For this purpose, it is critical to discover the new "seeds" and to eradicate many blockades until the clinical utilization. In this sense, our role is the translation from the results of basic science of our Institute to the conduct of clinical trials in the IMSUT Hospital. In IMSUT Hospital, we work together with staffs of Center for Translational Research. Concurrently, for the reduction of blockades during translational researches, we engage in research on Regulatory Science.

1. Assistance of Clinical Trials/TRs at IMSUT Hospital

Masanori Nojima, Fumitaka Nagamura

At IMSUT Hospital, we work together with staffs of Center for Translational Research. The assistance of Translational (Clinical) Research Coordinators is indispensable for the conduct of clinical trials, especially for TR. The activities of Coordinators are results of the collaboration between Division of Advanced Medicine Promotion and Center for Translational Research. In 2017, we supported three investigator-sponsored clinical trials based on investigational new drug application (IND) and one non-IND clinical studies.

2. Scholastic Program for the Graduate Students of Nurses in the Area of Translational Research.

Minako Kouno, Riyo Owada, Fumitaka Nagamura

TR is the early phase of clinical trials, which applied the developments of basic researches for patients with incurable and/or life-threatening diseases. Highly educated nurses are indispensable for the conducts of TRs in terms of the protection of participants in TRs and the conducts of scientifically appropriate TRs. We developed the scholastic program for the graduate students of nurses in the area of TR. We planned and implemented the one-week program to foster the expert research nurse aimed at the graduate students. It consists of the lectures on the feature points of TR (e.g. ethical considerations of TR, and the role of research nurse), role-plays of TRC and obtaining Informed Consent, case conference, and the experience of the actual operations. We evaluated the reports and the questionnaires from the students to explore the degree of their understandings and satisfactions for this program. These reports and questionnaires were analyzed. Generally, our program meets the demands of the students, however, the improvement of the content on the experience of the actual operations is the next issue.

3. Management of "Translational Research Network Program" of Japan Agency for Medical Research and Development.

Maranori Nojima, Fumitaka Nagamura

In 2017, Japan Agency for Medical Research and Development has launched third program for TR core centers, and the University of Tokyo was designated as a core center, which was composed of IMSUT hospital and the University of Tokyo Hospital. In 2017, we supported 22 SEED A (basic research stage), 14 SEED B (preclinical study stage), and 9 SEED C (clinical study stage).

4. Approach for epigenome and multi-omics research by methodology of bioinformatics and biostatistics

Masanori Nojima

Epigenome and multi-omics research using clinical samples in collaborative study or public database of comprehensive omics-analysis. We are now focusing on the multi-omics approach integrating DNA methylation, mRNA expression, and miRNA, and building statistical models to assess functional linkage.

5. Statistical consulting for basic research

Masanori Nojima

For basic researchers, we suggest exploratory statistical approach and molecular epidemiological approach.

Publications

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Advanced Clinical Research Center

Division of Advanced Genome Medicine
先端ゲノム医学分野

Associate Professor Yoshihiro Hirata, M.D., Ph.D.
Project Assistant Professor Ryosuke Muroyama, M.D., Ph.D.

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The goals of our researches are to identify the mechanisms and to establish novel therapies especially for cancers and inflammatory diseases of the digestive system. One of the research fields is the inflammatory diseases, in which we investigated the molecular pathogenesis of cholangitis and inflammatory bowel disease. Another research fields is the malignancies. Using genetically engineered mice, we have elucidated the carcinogenic mechanisms driven by gene mutations.

1. Pathogenesis of squamo-columnar junction cancer of the stomach

Yoshihiro Hirata

Squamo-columnar junction (SCJ) is one of the transitional zones in body where two different cell types merge. Barrett's adenocarcinoma and squamous cell carcinoma are two major tumors found in human gastric SCJ. The origin of SCJ tumors and the process of tumorigenesis are largely unknown. We have established a transgenic mouse line, in which invasive gastric SCJ tumor was specifically generated by TAM treatment. Histological examination reveals tumors are consist of both squamous cell carcinoma and adenocarcinoma. IHC, q-PCR, and immunoblot showed the tumors are positive for squamous cell markers, columnar cell markers, and SCJ specific markers such as KRT 7 and MUC4. Using lineage tracing, we try to identify cancer initiating cells as well as stem cells specific to gastric SCJ.

2. Biliary epithelial injury-induced regenerative response by IL-33 promotes cholangiocarcinogenesis from peribiliary glands

Hayato Nakagawa[1], Nobumi Suzuki[2], Yoshihiro Hirata, Yohko Hikiba[2], Yoku Hayakawa[1], Hiroto Kinoshita[1], Sozaburo Ihara[1], Koji Uchino[1], Yuji Nishikawa[3], Hideaki Ijichi[1], Motoyuki Otsuka[1], Junichi Arita[4], Yoshihiro Sakamoto[4], Kiyoshi Hasegawa[4], Norihiro Kokudo[4], Keisuke Tateishi[1], and Kazuhiko Koike[1]; [1]Department of Gastroenterology, The University of Tokyo, [2]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation, [3]Division of Tumor Pathology, Department of Pathology, Asahikawa Medical University, [4]Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, The University of Tokyo

The carcinogenic mechanism of extrahepatic cholangiocarcinoma (ECC) is unclear, due at least in part to the lack of an appropriate mouse model. Mice with tamoxifen-inducible, duct-cell-specific Kras activation and a TGFβ receptor type 2 (TGFβR 2) deletion were first generated by crossing LSL-KrasG12D, Tgfbr2flox/flox, and K19CreERT mice (KT-K19CreERT). However, KT-K19CreERT mice showed only mild hyperplasia of biliary epithelial cells (BECs) in the extrahepatic bile duct (EHBD). To analyze the additional effect of E-cadherin loss, KT-K19CreERT mice were crossed with CDH1flox/

flox mice (KTCK19CreERT). Surprisingly, KTC-K19 CreERT mice exhibited a markedly thickened EHBD wall accompanied by a swollen gallbladder within 4 wk after tamoxifen administration. Histologically, invasive periductal infiltrating-type ECC with lymphatic metastasis was observed. Recombined BECs lining the bile duct lumen detached due to E-cadherin loss, whereas recombined cells could survive in the peribiliary glands (PBGs). Detached dying BECs released high levels of IL-33, as determined by microarray analysis using biliary organoids, and stimulated inflammation and a regenerative response by PBGs, leading eventually to ECC development. Cell lineage tracing suggested PBGs as the cellular origin of ECC. IL-33 cooperated with Kras and TGFβR2 mutations in the development of ECC, and anti-IL-33 treatment suppressed ECC development significantly. Thus, this mouse model provided insight into the carcinogenic mechanisms, cellular origin, and potential therapeutic targets of ECC.

3. Role of dendritic cells in inflammatory bowel diseases

Sozaburo Ihara, Yoshihiro Hirata, Yohko Hikiba, Aya Murakawa, Moyo Tsuboi, Masahiro Hata, Mitsuru Konishi, Nobumi Suzuki, Kosuke Sakitani, Hiroto Kinoshita, Yoku Hayakawa, Hayato Nakagawa, Hideaki Ijichi, Keisuke Tateishi, Kazuhiko Koike

Dendritic cells (DCs) mediate host immune responses to gut microbes and play critical roles in inflammatory bowel disease. We examined the role of TGF-b signaling in DCs in colonic homeostasis. CD11c-cre Tgfbr2fl/fl mice developed spontaneous colitis, and CD11c-cre Tgfbr2fl/+ mice exhibited susceptibility to dextran sulfate sodium-induced colitis. Colitis in these mice was characterized by goblet cell depletion and dysbiosis caused by Enterobacteriaceae enrichment. Wild-type mice gavaged with Enterobacteriaceae from CD11c-cre Tgfbr2fl/fl mice feces showed severe colitis after dextran sulfate sodium treatment, whereas those treated with Notch inhibitor exhibited attenuated colonic injury with increased goblet cell numbers, thickened mucus layer, and fewer fecal Enterobacteriaceae. Wild-type mice transplanted with CD11c-cre Tgfbr2fl/fl bone marrow developed colitis showing increased Jagged1 and Jagged2 in DCs, increased Hes1 levels in epithelium, and goblet cell depletion. These findings suggest that TGF-b signaling in DCs regulates intestinal homeostasis by modulating epithelial cell differentiation and fecal microbiota. To explore the molecular mechanism underlying DC and epithelial cell interaction, we currently developed in vitro experimental system using organoids. Abnormalities in epithelial differentiation and its molecular mechanisms are now under investigation.

4. Molecular characterization of gastric metaplasia development

Hiroto Kinoshita, Yoku Hayakawa, Mitsuru Konishi, Masahiro Hata, Mayo Tsuboi, Yuki Hayata, Yohko Hikiba, Sozaburo Ihara, Hayato Nakagawa, Tsuneo Ikenoue, Yoshihiro Hirata, and Kazuhiko Koike

Chronic inflammation and metaplasia are strongly associated with gastric carcinogenesis. Although various mouse models of gastric carcinogenesis have been reported, there are few mouse lines which enable gene manipulation selectively in the stomach. We establish a *Tff1*-Cre BAC transgenic mouse line in order to induce gene modification specifically in gastric pit cells. *Tff1*-Cre mediated recombination was most evident in the gastric pit cells, while recombination was also observed in a few gastric chief and parietal cells, as well as in the duodenum and proximal colon. We are currently interested in the roles of several gastric oncogenes, therefore, we introduced these oncogenes into gastric epithelium using *Tff1*-Cre mice and examined the pathology. *Tff1*-Cre mice can be a useful tool for the studies of gastric carcinogenesis both *in vivo* and *in vitro*.

5. Pathogenesis of of primary biliary cholangitis and novel therapy development targeting T cells

Ryo Nakagawa, Yoshimi Kaise, Ryosuke Muroyama, Yasuo Matsubara, Naoya Kato, Yoshihiro Hirata

Primary biliary cholangitis (PBC) is an autoimmune liver disease, but the causes are unknown. We performed comprehensive expression analysis of mRNA and microRNA of T cells from PBC. Four microRNAs were identified as being decreased in PBC patients, leading to activation of T cell receptor signaling pathways, involved in inflammation. One particular target, N-Ras, could be an attractive and novel immunotherapeutic option for PBC. We are currently investigating the effect of Ras inhibitors as the potential novel therapy for PBC. We have screened the effect of Ras inhibitors using IL-2 promoter reporter cells. We also investigated cytokine production from T cells after Ras inhibitor treatment.

6. The role of fusion HBx from HBV integrant in the hepatocarcinogenesis

Ryosuke Muroyama

We identified fusion HBx translated from HBV integrant in Hep3B cells, and established stably HBx knocked-down (KD) cells by siRNA. The fusion HBx consisted of 1-140 amino acids of HBx followed by 61 amino acids from human genome. In KD cells, cell proliferation and invasion ability was significantly reduced compared to the parental cells. Moreover, KD cells could not develop any visible tumor in nude mice while parental cells could. The fusion HBx had anchorage-independent growth ability in soft agar although the fusion HBx completely abrogated its transactivation ability. In GSEA, the up-regulated genes in KD cells were significantly enriched in an endoplasmic reticulum (ER) stress response. In further analysis, it was revealed that the fusion HBx dysregulated ER stress response via the modification of ATF3, ATF4, and ATF6 transcription. Interestingly, the effects of the fusion HBx on ER stress signaling pathway was similar to those of C-terminal truncated HBx but significantly different from those of wild HBx.

7. Novel zinc finger protein in gastrointestinal tract

Yasuo Matsubara

The gastrointestinal tract has definite anatomical and functional boundaries between its contiguous segments. Because some human cancers arise in a background of tissue metaplasia, e.g. Barrett's esophagus and intestinal metaplasia of the stomach, it is important to clarify the molecular and cellular basis of region formation and preservation. Some genetic markers that delimit gastrointestinal boundaries have been reported, but it is still unknown how such boundaries are established and maintained. We identified novel zinc finger protein in the gastric biopsy specimen by mass spectrometry. Its mRNA sequence and other mRNAs with similar sequences were determined by RACE. We try to analyze the molecular function of this protein.

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Advanced Clinical Research Center

Division of Genetic Therapeutics
遺伝子治療開発分野

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Senior Assistant Professor	Sumimasa Nagai, M.D., Ph.D.		講　師	博士(医学)	永　井　純　正

The main project of our division is to promote clinical development of novel gene therapy for cancer and chronic intractable diseases. We are currently engaged in clinical development of immuno-gene therapy with chimeric antigen receptor (CAR)-modified T cells for relapsed and refractory hematological malignancies.

1. Immuno-gene therapy with CD19-directed CAR-modified T cells (CD19-CAR-T cells) for adult patients with relapsed and refractory B-precursor acute lymphoblastic leukemia (B-ALL)

Sumimasa Nagai and Keiya Ozawa

It has been reported that CD19-CAR-T gene therapy is highly effective for relapsed and refractory B cell malignancies, especially B-ALL. In order to develop this novel promising gene therapy in Japan, we prepared Japanese multicenter clinical trial of CD19-CAR-T cell therapy for adult patients with relapsed and refractory B-ALL. This trial has started since 2017. CD19-CAR-T gene therapy for malignant B-cell lymphoma was conducted in two patients at Jichi Medical University Hospital as Phase I/II clinical research.

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Advanced Clinical Research Center

Division of Bioethics
生命倫理研究分野

Associate Professor　Ayako Kamisato, Ph.D.

准教授　博士(法学)　　神　里　彩　子

Division of Bioethics is a new laboratory that was established in 2017. New ethical, legal and social issues (ELSI) may occur when conducting advanced clinical research or clinical practice. In our laboratory, we study how and what decisions should be made by a nation, society, or individual when such issues arise.

1. The REC Education project

Ayako Kamisato, Kaori Muto[1], Fumitaka Nagamura[2], Sachie Yoshida[1]; [1]Department of PublicPolicy, Human Genome Center, [2]Division of Advanced Medicine Promotion, Advanced Clinical Research Center

Currently, there are more than 1,800 institutional Research Ethics Committees (RECs) in Japan. Since 2010, cases of research fraud have come to light (e. g., the scandal around the Novartis drug Diovan) and improving the quality of reviews by REC has become the need of the hour. Therefore, Japanese ethical guidelines regarding medical studies involving humans now mandate that institutions with established RECs should offer education and training programs to REC members at least once a year. However, the guidelines do not make any provisions regarding the contents of programs and the way to deliver. As implementation of programs require manpower and economic resources, most institutions are unable to provide high-quality education and training. To address this situation, we launched the REC Education project with support from the Japan Agency for Medical Research and Development (AMED) from FY 2016.

Our programs have the following salient features: 1) programs are animated, 2) in order to offer the learners how to review from their place, we created four characters: two experts in natural science and law, a lay member, and a secretariat, 3) each program has a subject of discussion, 4) an external expert committee evaluates each program prior to release, 5) each program is about 20 minutes long, 6) the programs are offered at no charge on the website, 7) REC which successfully complete the program receive a certificate of completion.

We have produced and released the following video programs on our website:

- Module 1. Revision of the Privacy Act
- Module 2. Procedure of Informed Consent for using human samples and information
- Module 3. Why REC is necessary? What is the role of each REC member?
- Module 4. Checklist for Effective Reviewing
- Module 5. Invasive Research and Interventional Study
- Module 6. Basic knowledge of clinical trials

Currently, we have more than 250 members and 90 institutions registered with us. We constantly assess our programs through questionnaires to get user feedback on each program. We have consistently received high scores from our users. We are planning to produce totally 12 modules by the end of FY 2018.

2. Policy making of human-animal chimeric embryos research

Ayako Kamisato

In Japan, there are guidelines called "Guidelines on the Handling of Specified Embryos" based on the Act on Regulation of Human Cloning Techniques. In these guidelines, there are some limitation such as; 1) the production of animal-human chimeric embryos shall be carried out only for the purpose of basic research for the production of human cell-derived internal organs that can be transferred to human body, 2) animal-human chimeric embryos shall be carried out limited to a period until a primitive streak appears or 14 days from the date of the embryos being produced if such primitive streak does not appear, 3) chimeric embryos may not be transferred to human or animal uterus.

Following the recent great scientific achievements in this field, discussions have started in the the Ministry of Education, Culture, Sports, Science and Technology (MEXT) since 2013. Dr. Kamisato participated in these discussions as a member of the council and contributed for policy making.

3. Production of Common IC Form for "Center of Healthy Aging Innovation project"

Ayako Kamisato

"Center of Healthy Aging Innovation project" promoted by Hirosaki University is one of the projects of JST Center of Innovation (COI) Program. One goal of this project is to build a platform of big data on medical and health. In order to achieve this goal, it is necessary to integrate data obtained from multiple cohort studies. To accelerate data integration, Dr. Kamisato produced a common IC form.

Publications

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