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 dysregulation of transcription factors and constitutive activation of tyrosine kinases, 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 started clinical sequencing in tight collaboration with Human Genome Center and Health Intelligence Center to establish a platform for precision medicine.

(2) Development of anti-cancer therapy using recombinant vaccinia virus:
Vaccinia virus is a promising vector for oncolytic immunovirotherapy of cancer. For cancer specificity and safety, we introduced some genetic modifications into the viral genome by recombinant DNA technique. These include miRNA-regulated expression of B5R, an essential component for virus spreading, and deletion of thymidine kinase. We are now trying to apply MDVV (miRNA-regulated and thymidine kinase-deleted vaccinia virus) to a preclinical model of multiple myeloma.

(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.

(5) Translational research on tissue engineering:
To accomplish this goal, we are focusing on the issues including a) investigation on the environmental effect on tissue regeneration, b) search for molecules to affect the growth and differentiation of stem cells, c) clinical studies on bone tissue engineering, and d) development of fundamental technologies for the widespread use of tissue-engineered products.

1. BRAF-V600E mutation in circulating cell-free DNA is a promising biomarker of high-risk adult Langerhans cell histiocytosis.

Kobayashi M[1], Ohno N[2], Fukuyama T[2], Kawamata T[2], Uchimaru K[2], Tojo A[1,2].: [1]Division of Molecular Therapy, The Advanced Clinical Research Center, [2]Department of Hematology/Oncology, IMSUT Hospital

Langerhans cell histiocytosis (LCH) and Erdheim-Chester disease (ECD) are rare clonal disorders of a histiocyte/dendritic cell lineage, and oncogenic BRAF-V600E mutation could be detected in both lesions from most patients. In patients with various kinds of cancers, circulating cell-free DNA (cfDNA) contains cancer-derived genomic DNA and has been applied to non-invasive diagnostic procedure. Then, we evaluated BRAF mutation in cfDNA as a potential biomarker of LCH/ECD using allele-specific quantitative polymerase chain reaction (ASQ-PCR). cfDNA was prepared from plasma of 16 adult LCH patients including 2 patients complicated with ECD, and was subjected to genotyping BRAF alleles by ASQ-PCR, which was specifically designed for detection of BRAF-V600E with a 3'-phosphate-modified oligonucleotide blocker. Mutant BRAF load was expressed as the percentage of mutant alleles to total number of alleles. Five out of 8 high-risk patients with active lesions were positive for BRAF-V600E, and the mean ratio of mutant alleles to total alleles was 2.43% (median, 2.01%). Eight pts with inactive lesions and 8 normal participants were negative. Then, in a BRAF-V600E-positive patient, we followed the mutant load during the course of initial chemotherapy and confirmed that the mutant load correlated with the disease activity. Taken together, ASQ-PCR of BRAF-V600E in cfDNA may contribute to planning of risk-based treatment as well as monitoring of treatment efficacy in LCH/ECD, especially in an active, high-risk group.

2. Impact of Ph+ stem cell burden on clinical findings and nilotinib responses in *de novo* CML-CP: the results from the interim analysis of N-road, a multi-center phase II study.

Tsuda M[1], Tojo A[1].: [1]Division of Molecular Therapy, The Advanced Clinical Research Center

A previous study showed that Ph+ stem cell burden at diagnosis is a prognostic marker of molecular responses (MR) in CML-CP on dasatinib or imatinib. Here, we examined whether this would apply to *de novo* CML-CP in a phase II clinical study (N-road), in which first-line nilotinib is given for 24 M. By July 2014, 48 pts were enrolled and BM CD34+ cell fractions could be evaluated by FACS-FISH analysis at diagnosis in 43 pts, among those 39 pts passed 3 M, 39 pts 6 M and 23 pts 12 M, respectively. Absolute Ph+ cell counts in CD34+CD38− fraction were estimated in each patient by combining CD34 cell counts, proportion of CD38− fraction and percentage of Ph+ cells. Ph+ CD34+CD38− cell counts significantly correlated with WBC and inversely with RBC, Hb and percentage of lymphocytes. Between the 2 groups divided by the median cell counts, there were significant differences in RBC, Hb, Hct, and when divided by the mean cell counts, there was a significant difference in WBC. $MR^{3.0}$ rate was 9/39 at 3M, 23/39 at 6M and 14/23 at 12M. Although we could not find significant differences in MR rate at any check points according to the initial Ph+ stem cell burden, patients with lower number of Ph+CD34+ CD38− cells were likely to achieve $MR^{3.0}$ faster than those with higher number of cells. Increased Ph+ stem cell burden apparently affects the level of leukocytosis and anemia at diagnosis, but not $MR^{3.0}$ rate by 12 M on nilotinib, although it is likely to extend time to achieve $MR^{3.0}$.

3. Efficacy and safety of doubly-regulated recombinant vaccinia virus in a mouse xenograft model of multiple myeloma

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Multiple myeloma is a malignancy of plasma cells of the bone marrow. Although the prognosis is variable, no curative therapy has been defined. Vaccinia virus is a member of the poxvirus family and is best known as the vaccine that eradicated smallpox in the 1970s. Vaccinia virus infects cancer cells and kills such cells in a variety of ways. These include direct infection, triggering of immunomediated cell death, and vascular collapse. The potential of the vaccinia virus as an anti-tumor therapy has attracted the attention of oncologists. Interestingly, our preliminary experiments revealed that myeloma cells were particularly susceptible to vaccinia virus. To exploit this susceptibility and to render vaccinia more myeloma-specific, we generated thymidine kinase-deleted microRNA (miRNA)-regulated vaccinia viruses in which the essential viral gene B5R was regulated by miRNAs of normal human cells. Of the miRNAs examined, let-7a was found to be the most reliable in terms of regulating viral transmission. Exposure to unregulated vaccinia virus killed myeloma-transplanted SCID mice; the animals succumbed to viral toxicity. In contrast, the thymidine kinase-deleted let7a-regulated virus remained localized within myeloma cells, triggering tumor regression and improving overall survival. In conclusion, a thymidine kinase-deleted let-7a-regulated vaccinia virus was safe and effective when used to treat myeloma. Such recombinant vaccinia viruses are therefore excellent novel candidates in the context of anti-myeloma therapy.

4. Advanced HTLV-1 carriers and early-stage indolent ATLs are indistinguishable based on the CADM1 vs. CD7 plot in flow cytometry.

Kobayashi S[1], Watanabe E[2], Ishigaki T[3], Ohno N[4], Yuji K[4], Nakano K[5], Yamochi T[5], Watanabe N[2], Tojo A[1,4], Watanabe T[5], Uchimaru K[4].: [1]Division of Molecular Therapy, The Advanced Clinical Research Center, [2]Laboratory of Diagnostic Medicine, IMSUT Hospital, [3]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, [4]Department of Hematology/Oncology, IMSUT Hospital, [5]Graduate School of Frontier Sciences, The University of Tokyo

In CD4(+) cells from peripheral blood of in human T-cell leukemia virus type 1 (HTLV-1)-infected subjects, CADM1(−) CD7(+) (P), CADM1(+) CD7 (dim) (D) and CADM1(+) CD7(−) (N) subpopulations are observed. The D and N subpopulations increase as asymptomatic HTLV-1 carriers (AC) progress to indolent adult T-cell leukemia-lymphoma (ATL) and the N subpopulation then expands in aggressive ATL. In the present study we examined whether the analysis can estimate the risk of developing ATL in advanced AC. Peripheral blood samples from AC (N = 41) and indolent ATL patients (N = 19) were analyzed by flow cytometry using the CADM1 versus CD7 plot for CD4(+) cells and inverse long PCR (clonality analysis) of FACS-sorted subpopulations. Almost all AC with a high HTLV-1 proviral load (>4 copies/100 cells) had a CADM1 (+) (D + N) frequency of >10%. AC with 25% < CADM1(+) ≤50% contained expanded clones similar to smoldering-type ATL. In many patients in the 25% < CADM1(+) ≤50% group, the proportion of abnormal lymphocytes was distributed around the 5% line, which divides AC and smoldering-type ATL in Shimoyama's classification. In conclusion, the CADM1 versus CD7 plot is useful for selection of putative high-risk AC. The characteristics of some AC and smoldering ATL are said to be similar; however, long-term follow up is required and the clinical outcome (e.g. rate of transformation) of these cases should be used to determine whether to include them in the same clinical category.

5. Clinical sequencing of malignant as well as rare and undiagnosed hematological disorders

Yokoyama K[1,7], Yokoyama N[2], Kobayashi M[3], Yamaguchi K[4], Furukawa Y[2,4], Yamaguchi R[5], Imoto S[6], Miyano S[5], Tojo A[1,3].: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Department of Applied Genomics, IMSUT Hospital, [3]Division of Molecular Therapy, Advanced Clinical Research Center, [4]Division of Clinical Genome Research, Advanced Clinical Research Center, [5]Laboratory of Genome Database, Human Genome Center, [6]Division of Health Medical Data Science, Health Intelligence Center, [7]Center for Gene and Cell Thrapy

Sequensing of the genome or exome for clinical applications such as precise diagnosis and therapeutic intervention, referred to as clinical sequencing (CS), has now entered medical practice. We organized IMSUT CS team in tight collaboration with Human Genome Center and Health Intelligence Center, and have been establishing a bidirectional (bed to bench and bench to bed) system to integrate clinical and genomic information in hematological disorders, especially in malignant as well as rare and undiagnosed diseases. We mainly performed target sequencing on a series of clinical samples by operating next generation sequencers (NGS) with primer sets from a disease-oriented and/or more extended panel of genes, and intensively evaluated those CS results for identification of driver and causative genes with the aid of artificial intelligence (AI), IBM-Watson. We have successfully delineated

the pathogenesis in several tough cases which could not be rationally interpreted if CS is not available.

6. Maintenance of stemness of breast cancer cells by FRS2beta, a feedback inhibitor for HER2-ERK pathway, during mammary tumorigenesis

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Inflammatory microenvironment contributes to tumorigenesis. Although it is thought that breast cancer stem cells (CSCs) appear and grow in the inflammatory microenvironment that is called CSC niche, it remains largely unclear how it occurs. Here, we uncovered FRS2beta, a feed-back inhibitor of ERK activity in progenitor cells, plays critical roles for various cytokine production to increase progenitor cell- and CSC- state, and create the CSC niche. Expression levels of FRS2beta were increased in mammary tumors of MMTV-ErbB2 mice than in normal mammary tissues. Deficiency of FRS2beta greatly delayed mammary tumorigenesis, decreased mammosphere-forming ability and tumor stroma, a component of the CSC niche. Expression levels of various cytokines, including IGF1 and CXCL12, stemness- and stroma-inducing cytokines, respectively, were reduced in FRS2beta-mutant mammospheres, pre-cancerous mammary and tumor tissues. Furthermore, expression levels of FRS2beta were higher in $CD44^{high}CD24^{low}$ CSCs-enriched population in patient-derived breast cancer tissues. Thus the mechanisms to maintain progenitor cell-state by prevention of excess ERK activity may permit production of various cytokines to create CSC niche, and allow appearance and growth of CSCs. These results suggest that treatment with MEK inhibitors may allow survival of CSCs, raising the risk of recurrence. We provide a rationale of combination therapy targeting both FRS2β and MEK inhibitors to eradicate tumors.

7. Semaphorin/MICAL3/CRMP2 regulates tumor initiating activity in human breast cancer stem cells

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The breast cancer stem cells (BCSCs) are thought be a source of tumor cells of breast cancer tissues. We previously examined heregulin /ErbB3/PI3K signaling by using DNA microarray and identified many candidate molecules that potentially function in cancer stem cells. Among them, we focused on MICAL3, a multidomain signal transduction protein, since it is unknown whether MICAL3 functions in cancer stem cells. Knockdown of MICAL3 by using shRNA reduced tumor sphere forming efficiency in breast cancer cell lines and patient-derived primary breast cancer cells. We also found that tumor initiating activity was greatly reduced in cells expressing shRNA for MICAL3 by using patient-derived xenograft models (PDXs). Overexpression of the MICAL3 mutant in the FAD-containing monooxygenase domain reduced tumor sphere forming efficiency. Moreover, upon semaphorine 3A stimulation, MICAL3-mediated generation of H_2O_2 induced homodimer formation of CRMP2. Thus semaphorin/MICAL3/CRMP2 may play important roles for BCSCs maintenance through generation of H_2O_2 produced by MICAL3.

8. Clinical study on bone tissue engineering

Kagami H[1,2], Inoue M[1,2], Nagamura-Inoue T[3], Chinzei M[4], Yuji K[5], Nobuhiro Ohno[5], Uchimaru K[5], Tojo A[1,5].: [1]Division of Molecular Therapy, The Advanced Clinical Research Center, IMSUT, [2]Department of Oral and Maxillofacial Surgery, Matsumoto Dental University, [3]Department of Cell Processing and Blood Transfusion, IMSUT Hospital, [4]Department of Anesthesia and Surgical Center, IMSUT Hospital, [5]Department of Hematology/ Oncology, IMSUT Hospital

Atrophic maxilla or mandible are major obstacles for dental implant therapy. For example, severe periodontitis, which is a leading cause of tooth loss in the elderly, accompanied by significant bone absorption, makes dental implant therapy very difficult if not impossible to perform. Furthermore, alveolar bone regeneration is also required to improve the functional and esthetic aspects of treatment outcome. Although use of dental implants is already an established clinical procedure, there are a large number of patients without adequate bone volume for placement of dental implants. For patients with severe atrophy of alveolar bone, autologous bone grafts from iliac bone, tibial bone, or mandible have been performed. However, these destructive procedures may not be feasible for all patients. Even when the amount of harvested bone is small, the procedure is inevitably accompanied by swelling and pain at the donor site. Although bioartificial bone substitutes have been frequently used, even with biological materials such as demineralized freeze-dried allografts or xenogeneic bone substitutes, the ability to induce bone regeneration is considered less efficient than native bone. Thus,

the application is limited. We are carrying out a clinical study of alveolar bone tissue engineering for dental implant therapy using bone marrow stromal cells (BMSCs), with a goal of eventual commercialization. The study has been approved by the institutional committee and by the Minister of Health, Labour and Welfare of Japan. The clinical study was sucdesfully carried out for 15 cases.

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Division of Cellular Therapy
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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.

Kohtaro Nishimura, Takeshi Fukushima, Toshihiko Oki, Toshiyuki Kawashima, Yukinori Minoshima, Yosuke Tanaka, 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 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. In addition, we have recently identified that APCCDH1 targets MgcRacGAP for destruction by ubiquitination. In summary, our results indicate that MgcRacGAP plays distinct roles depending on the cell cycle thereby co-ordinating control of cell division and determination of cell fate, implicating multiple levels of regulation of MgcRacGAP including phosphorylation and ubiquitination in distinct biological roles in different cell cycles. Now we plan to generate a transgenic mouse expressing MgcRacGAP-GFP (or -mVenus) fusion protein in hematopoietic

stem cells and/or progenitors to examine the relationship between cell division and cell fate.

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, Makoto Saika, Takeshi Fujino, Yasutaka Hayashi, Shuji Asada, Kojin C Kawabata, Naoko Watanabe, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Yuka Harada[1], Hironori Harada[1], Tetsuya Nosaka[2], Jiro Kitaura[3], Yosuke Tanaka, Tomofusa Fukuyama, Susumu, Goyama, and Toshio Kitamura: [1]Department of Hematology/Oncology, Juntendo University, [2]Mie University School of Medicine, and [3]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, and are now characterizing them. We have also started a new project on AML1-ETO where we have identified a new isoform with stronger leukemogenic activities.

4. Molecular pathogenesis of AML1-ETO and MLL-fusion leukemias.

Susumu Goyama, Toshio Kitamura, Nicolas N. Nassar[4], Joseph S. Palumbo[4], James C. Mulloy[4]: [4]Cincinnati Children's Hospital Medical Center

Using human and mouse models for AML1-ETO and MLL-fusion leukemias, we have elucidated new molecular aspects in pathogenesis and progression of acute myeloid leukemia (AML). The t(8;21)-related AML1-ETO fusion protein is one of the most common genetic aberrations in AML. Clinical data suggest that CBL mutations are a frequent event in t(8;21) AML, but the role of CBL in AML1-ETO-induced leukemia has not been elucidated. We demonstrate that CBL mutations collaborate with AML1-ETO to expand human CD34+ cells both in vitro and in a xenograft model. CBL depletion by shRNA also promotes the growth of AML1-ETO cells, demonstrating the inhibitory function of en-

dogenous CBL in t(8;21) AML. Mechanistically, loss of CBL function confers hyper-responsiveness to thrombopoietin and enhances STAT5/AKT/ERK/Src signaling in AML1-ETO cells. Interestingly, we found the protein tyrosine phosphatase UBASH3B/Sts-1, which is known to inhibit CBL function, is upregulated by AML1-ETO through transcriptional and miR-9-mediated regulation. UBASH3B/Sts-1 depletion induces an aberrant pattern of CBL phosphorylation and impairs proliferation in AML1-ETO cells. The growth inhibition caused by UBASH3B/Sts-1 depletion can be rescued by ectopic expression of CBL mutants, suggesting that UBASH3B/Sts-1 supports the growth of AML1-ETO cells partly through modulation of CBL function. Thus, our study reveals a role of CBL in restricting myeloid proliferation of human AML1-ETO-induced leukemia, and identifies UBASH3B/Sts-1 as a potential target for pharmaceutical intervention.

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 MLL-AF9 cells, indicating that suppressing RUNX activity may not eradicate the most immature leukemia stem cells (LSCs). We found upregulation of several hemostasis-related genes, including the thrombin-activatable receptor PAR-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 LSCs in vivo. 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 inhibits rapid leukemic proliferation when there are a large number of LSCs, while a small number of LSCs need PAR-1 for their efficient growth. Mechanistically, PAR-1 increased adherance properties of MLL-AF9 cells and promoted their engraftment to bone marrow. 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 MLL-fusion leukemia.

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Advanced Clinical Research Center

Division of Bioengineering
臓器細胞工学分野

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Assistant Professor	Marimo Sato-Matsushita, Ph.D.	助　教　学術博士	松下(佐藤)まりも

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 bed side 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 PD1 related drug. We are now analyzing the immunological parameters to further develop this powerful agent.

II. Augmentation of immune checkpoint blockade therapy with IL-18

Zhifeng Ma[1,2], Hideaki Tahara, and Haruki Okamura[1]: [1]Laboratory of Tumor Immunology and Immunotherapy, [2]Department of Orthopaedic Surgery, Hyogo College of Medicine, Nishinomiya, Hyogo, 663-8501, Japan

Immune responses that lead to anti-tumor effects require both activation of effector cells and reduction of inhibitory elements of the immune system. Although animal models and recent clinical trials demonstrated that immune checkpoint blockade enhanced effector cell responses and tumor rejection, further development and improvement of cancer immunotherapy is necessary to achieve more favorable objective responses. In this study, we examined the effect of IL-18 on the treatment of peritoneal dissemination of CT-26 mouse colon carcinoma cell line harnessing anti-programmed death ligand-1 (PD-L1) monoclonal antibody (mAb) (αPD-L1) and/or anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) mAb (αCTLA-4). The treatment of mice with αPD-L1 and/or αCTLA-4 significantly enhanced their survival. However, when combined with IL-18, αPD-L1 and/or αCTLA-4 provided

much greater therapeutic benefits. IL-18 allowed accumulation of precursor of mature natural killer (pre-mNK) cells in the peritoneal cavity, when injected with these check-point inhibitors. The results strongly suggested that IL-18 promoted therapeutic effects of immune checkpoint blockade against peritoneal dissemination of carcinoma through accumulation of pre-mNK cells, memory-type CD8+ T cells, and deletion of CD4+CD25+ T cells. Our study provides a basis for cancer immunotherapy that would involve combination of cytokines activating innate immunity and immune checkpoint blockade.

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

Marimo Sato-Matsushita, Yu Mizote, Mika Uematsu-Hamada, Hideaki Tahara

The secreted protein, milk fat globule epidermal growth 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.

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

Marimo Sato-Matsushita, Yoshihiro Hayakawa, Hideo Yagita, Hideaki Tahara

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.

V. IL-17-producing NK1.1$^-$ CD27$^-$ γδT cells promote tumor malignant progression by inducing inflammatory microenvironment.

Yoshihiro Hayakawa[3], Yoshitaka Kimura[4], Marimo Sato-Matsushita, Hideaki Tahara: [3]Institute natural Medicine, University of Toyama, [4]The University of Tokyo

Inflammatory microenvironment is an essential component of tumors and important for carcinogenesis and metastasis of tumor cells, however, the precise details of inflammatory immune responses to promote tumor malignant progression are still unclear. To characterize such tumor-promoting inflammatory immune responses, we employ a unique in vivo model in which low tumorigenic cell line QR-32 acquires high malignant phenotype after exposure to host inflammatory responses induced by an inflammation initiator. By using this model, we investigated the role of inflammatory cytokines IL-17 and IFNγ in tumor malignant progression process. We demonstrated that IL-17 and IFNγ played positive and negative roles, respectively, in the malignant progression of tumor cells and IL-17 played a predominant role in this process. Adoptive transfer of inflammatory cells from wild-type mice into IL-17-deficient mice recovered in vivo progression of QR-32 cells and the exact source of IL-17 within such inflammatory cells was determined as NK1.1$^-$ CD27$^-$ γδT cells. Furthermore, CD11b$^+$ Ly-6G$^+$ neutrophils infiltrated into the inflammatory site primed by IL-17-producing NK1.1$^-$ CD27$^-$ γδT cells in the presence of QR-32 and IL-17 played an important role for maintaining such tumor-associated inflammatory microenvironment. Collectively, our data clearly implicate that the inflammatory tumor microenvironment triggered by IL-17-producing NK1.1$^-$ CD27$^-$ γδT cells is important for tumor malignant progression. We are now further characterizing γδT cells in the inflammatory microenvironment promoting tumor malignant progression and exploring the components for downstream inflammatory immune responses triggered by IL-17.

VI. 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[5], Hiroyuki Mizuguchi[6], Takafumi Nakamura[7], Hisako Katano[8], Hiroaki Uchida, Takuma Suzuki, Hideaki Tahara: [5]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan, [6]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, [7]Tottori University, [8]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 have also started to support the safety and biodistribution studies for AdSOCS3 in the context of intrapleural or intravenous administration in a mouse model. Based on these results, we are now in the phase of preparing the phase I study for the patients with malignant pleural mesothelioma using this strategy.

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

Hiroaki Uchida, Yu Okubo, Tomoko Shibata, Takuma Suzuki, Hitomi Ikeda, Tomomi Tanaka, Tomoki Shiroyama, Hideaki Tahara

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 a number of different single-chain antibodies (scFv) 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.

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

Hiroaki Uchida, Miki Yamaguchi[9], Hitomi Ikeda, Yu Okubo, Tomomi Tanaka, Hideaki Tahara: [9]Department of Molecular Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine

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 cancer, 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 with high affinity and get internalized efficiently, which has been critically required for therapeutic applications but elusive thus far.

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Advanced Clinical Research Center

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Project Senior Assistant Professor	Kiyoshi Yamaguchi Ph.D.		特任講師	薬学博士	山　口　貴世志

We have been working on the following five projects, 1) development of novel therapeutic strategies of human cancer, 2) development of anticancer drugs through a screening of chemical libraies, 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 sequence for the implementation of genomic medicine. These projects are aimed to develop strategies for better diagnosis, effective treatment, and prevention of human cancer.

1. Identification of novel molecular targets for the treatment of human cancers

Kiyoshi Yamaguchi, Yoichi Furukawa, Paul Sheridan[1], Rui Yamaguchi[1], Seiya Imoto[2], and Satoru Miyano[1]: [1]Laboratory of DNA Information Analysis, Human Genome Center, [2]Division of Health Medical Data Science, Health Intelligence Center, IMSUT

Accumulated evidence has demonstrated that aberrant epigenetic modifications are implicated in carcinogenesis. Bromodomain has been known as a protein interaction module that recognizes acetylated lysine residues within histone and non-histone proteins. Through this interaction, protein containing a bromodomain(s) conducts the assembly of nuclear factor complexes to their target sites on chromatin, resulting in the transcriptional activation. We recently found that bromodomain containing 8 (BRD8) was frequently accumulated in colorectal cancer. Although BRD8 is a component in TRRAP/TIP60-histone acetyltransferase complex, its biological role and function are largely unknown. We are investigating the biological role of BRD8 in cancer cells through integrative analysis of large-scale gene expression and ChIP-seq data. Since bromodomain-proteins have emerged as druggable targets, small molecule inhibitor targeting the bromodomain of BRD8 might be a novel therapeutic approach for the treatment of colorectal cancer.

2. Cancer drug discovery through a large chemical library screening

Kiyoshi Yamaguchi, Yoichi Furukawa

We recently developed a cell-based reporter assay system to screen effectively and specifically compounds and molecules that affect the transcriptional activity of the target molecule or signal transduction pathway. Applying this assay system, we carried out a high-throughput screening of Wnt inhibitors using a chemical library containing 20,000 compounds, and have identified several candidate small molecules. We have started the studies of their mode of action, and structure-activity relationship in collaboration with a group in the University of Tokyo. Furthermore, we are going to expand our screening of chemical libraries with the help of Drug Discovery Initiative in the University of Tokyo.

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

Tsuneo Ikenoue and Yoichi Furukawa

Genetically engineered mice are useful tools for studying human diseases, including cancer. In this project, we have successfully established a mouse model of intrahepatic cholangiocarcinoma (ICC) by liver-specific *Kras* activation and *Pten* deletion. To investigate the origin of the tumor cells in this model, we have taken advantage of lineage tracing system using tamoxifen-inducible Cre mice and Rosa-GFP reporter mice. Furthermore, we have investigated the molecular mechanisms how *Kras* activation and *Pten* deletion induce ICC in this mouse model.

We have also generated a mouse strain carrying a conditional knockin allele of the *Fbxw7* gene, which is frequently mutated in human colon and bile duct cancer. Using this strain, we are now trying to establish novel mouse models of these tumors. Intensive investigation 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

Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa

We analyzed genetic alterations in Japanese extrahepatic biliary tract cancer (BTC) and pseudomyxoma peritonei of the colon (PMP) using multiplex PCR-based targeted enrichment and next-generation sequencing (NGS).

In the genetic analysis of BTC, we analyzed a total of 27 tumors and their matched non-cancerous tissues, and identified frequent mutations in *TP53* (14/27), *KRAS* (6/27), *PIK3CA* (6/27), and *SMAD4* (6/27). Interestingly, the frequency of the PIK3CA mutation was higher compared with Caucasian BTC cases. This result may suggest that activation of the PI3K-AKT pathway in addition to the abrogation of p53, SMAD4, and RAS-MAPK pathways may play a crucial role in the carcinogenesis of Japanese BTC.

In the PMP study, we analyzed 18 PMPs containing 10 low-grade tumors (DPAMs) and 8 high-grade tumors (PMCAs). As a result, a total of 35 somatic mutations were identified. Frequent mutations were identified in *KRAS* (14/18) and *GNAS* (8/18), but their frequencies were not significantly different between DPAMs and PMCAs. On the other hand, *TP53* mutations were found in PMCAs (3/8), but not in the DPAMs. *PIK3CA* and *AKT1* mutations were also identified in two PMCAs, but not in the DPAMs. These results suggest that *KRAS* and/or *GNAS* mutations are common genetic features of PMP, and that mutations in *TP53* and/or genes related to the PI3K-AKT pathway may render malignant properties to PMP. These data may be useful for the understanding of tumor characteristics, and facilitate the development of personalized medicine for PMP.

5. Clinical sequence for the implementation of genomic medicine

Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Mitsuhiro Komura[1], Eigo Shimizu[1], Rui Yamaguchi[1], Tetsuo Shibuya[2], Satoru Miyano[1,2], Takanori Hasegawa[3], Seiya Imoto[3], Masayuki Kobayashi[4], 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

Next-generation sequencing (NGS) has enabled 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 following projects; 1) the determination of germline mutations in patients suspected of hereditary colon tumor, 2) application of a cognitive computing system, namely IBM Watson Genomic Analytics (WGA), 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 carried out three different NGS, namely targeted sequencing, whole exome sequencing, or whole genome sequencing for ten patients with colonic polyposis. In the patients, we previously failed to identify pathological mutations within the 5′ two-thirds region of the *APC* gene by Sanger sequencing. However, NGS successfully identified pathological mutations in three of the ten patients; two were mosaic mutations in *APC*, and the other was a very rare mutation in the 3′ terminal region of *APC*. These data have corroborated the usefulness of NGS in genetic diagnosis.

In the second project, we generated a pipeline to apply genomic data to IBM WGA. After written informed consent was obtained from the patients with pseudomyxoma peritonei (PMP), they were enrolled in this study. Genetic alterations in their

tumor were determined by NGS and the data were subsequently analyzed by WGA. The results of WGA including predicted driver mutations and suggestion of actionable drugs were discussed in Tumor Board, which is composed of clinical sequencing members. Evaluation of the results is now ongoing.

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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, colon cancer, lung cancer, breast cancer, nasopharyngeal cancer, cholangiocarcinoma, malignant melanoma, and malignant lymphoma have also been used for testing efficacy and safety.

Studies using glioma-derived cancer stem cells

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-derived 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.

Publications

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Advanced Clinical Research Center

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

Professor Fumitaka Nagamura, M.D., D.M.Sc

教 授 医学博士 長 村 文 孝

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 Research Hospital

Minako Kouno, Riyo Owada, Makiko Karasawa, Masanori Nojima, Fumitaka Nagamura

In Research 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 2014, we supported 4 investigator-initiated investigational new drug application (IND) clinical trials and 2 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 Institutional Review Board 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.

Makiko Karasawa, Hiroshi Yasui, Fumitaka Naga-

mura

Ministry of Education, Culture, Sports, Science and Technology launched "Translational Research Network Program" to promote translational researches based on the results of basic science at academia. This grogram was transferred to Japan Agency for Medical Research and Development in 2015 and has been expected to support TRs from basic science to seek obtaining intellectual property to early stage of clinical trial. In 2015, we supported 17 basic researches, 15 preclinical studies, and 6 clinical studies. The number of studies we assist has been increasing year by year. Organization reinforcement is the urgent problem.

Publications

1. 長村文孝　ウイルス療法のガイドライン・ガイダンスについて 実験医学 印刷中
2. Nagamura F. Collaboration between academia for the development of translational research. Nihon Yakugaku Zasshi. 145: 211-15, 2015

Advanced Clinical Research Center

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

Associate Professor Naoya Kato, M.D., Ph.D.
Project Assistant Professor Ryosuke Muroyama, M.D., Ph.D.

准教授 医学博士 加藤直也
特任助教 医学博士 室山良介

Our major goal is to establish personalized medicine for patients with infectious diseases or cancers, especially those of gastrointestinal and hepatic fields, based on human or pathogenic microbe genome information.

1. Fusion HBx form HBV integrant might affect ER stress response and be associated with HCC

Ryosuke Muroyama, Kaku Goto, Yasuo Matsubara, Ryo Nakagawa, Sayuri Morimoto, Sayaka Ito, Naoya Kato

Backgrounds/Objectives: Hepatitis B virus (HBV) is a major risks factor associated with hepatocellular carcinoma (HCC), and HBV integration has been suggested to be associated with hepatocarcinogenesis. However, its molecular mechanisms remains unclear. In this study, we identified fusion HBx from HBV integrant in human hepatoma cell line, and investigated its role in hepatocarcinogenesis.

Methods: 1) We identified fusion HBx translated from HBV integrant in Hep3B cells, which consisted of 3′-truncated HBx following 61 amino acids from human sequences, and established stably knocked-down (KD) cells against fusion HBx by siRNA. 2) Using KD cells, we examined the effect of fusion HBx on cell growth, invasion ability and tumorigenicity in vivo. 3) We examined the expression change of mRNAs in KD cells using microarray, and gene set enrichment analysis (GSEA) was performed to investigate the signature of fusion HBx.

Results: 1) We established KD cells in which fusion HBx was disappeared by immunofluorescence. 2) In KD cells, cell proliferation and invasion ability was reduced. In addition, KD cells could not develop any visible tumor in nude mice when we injected KD cells subcutaneously into nude mice. 3) We identified 305 up-regulated and 115 down-regulated genes in KD cells by more than two folds. In GSEA, up-regulated genes in KD cells were enriched in endoplasmic reticulum (ER) stress response.

Conclusions: Fusion HBx translated from HBV integrant might affect ER stress response and play an important role in hepatocarcinogenesis

2. Small molecules for MICA regulation

Kaku Goto[1], Masahisa Jinushi[2], Ryosuke Muroyama[1], Wenwen Li[1], Ryo Nakagawa[1], Sayaka Ito[1], Yasuo Matsubara[1], Yasushi Tanoue[1,3], Toshio Fujisawa[1,4], Naoya Kato: [1]Division of Advanced Genome Medicine, IMSUT; [2]Institute for Advanced Medical Research, Keio University Graduate School of Medicine; [3]Department of Gastroenterology and Hepatology, JCHO Tokyo Takanawa Hospital; [4]Department of Gastroenterology, NTT Medical Center Tokyo

A natural killer (NK) group 2D (NKG2D) ligand MHC class I polypeptide-related sequence A (MICA) was identified to be a genetic susceptibility factor for hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) in our genome-wide association study (Kumar V *et al.,* Nat Genet 2011). Lower levels of MICA expression were associated with the elevated risk of HCC development in patients, and preventive effects of MICA expression on hepatocarcinogenesis were suggested. We therefore aimed to find drugs for regulation of MICA expression. Our new MICA promoter reporter system detected an anti-cancer agent as the top hit in a screen for an FDA-approved drug library. The drug treatment indeed upregulated the expression of MICA in HCC cells, boosting NK cell-mediated cytotoxicity in coculture and inhibiting tumor growth via NK cells in vivo. The mode of MICA expression induction and further possibilities of pharmacological modulation of MICA are currently investigated. Findings are expected to lead to novel immunotherapies for prevention and elimination of HCC (Goto K *et al.,* J Gastroenterol 2015; Goto K *et al.,* Nippon Rinsho 2015).

3. The AMPK-related kinase SNARK in viral hepatitis and hepatocellular carcinoma

Kaku Goto[1], Raymond T. Chung[2], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]GI Unit, Massachusetts General Hospital, Harvard Medical School

Our genome-wide RNAi screen for host cellular cofactors for hepatitis C virus (HCV) replication (Tai AW *et al.,* Cell Host Microbe 2009) identified sucrose, non-fermenting 1/AMP-activated protein kinase-related kinase (SNARK) to be a proviral gene. We then revealed that SNARK supported HCV replication by its phosphorylation and phosphotransferase activity and conversely SNARK expression level was upregulated by HCV infection in patients and cell culture, interfering with intracellular signalings. These effects were abrogated by SNARK kinase inhibitor, raising SNARK as an effective target of therapies against the virus and pathogenesis (Goto K *et al.,* J Hepatol 2013). Our investigation into substrates, interactors, and signalings targeted by the kinase is underway, clarifying involvement of SNARK in hepatitis virus pathogenesis and hepatocellular carcinoma development with pharmacological regulation of the kinase activity examined further.

4. Decreased miR-425 induced inflammatory cytokine production in CD4+ T cells of primary biliary cirrhosis via upregulating N-Ras expression

Ryo Nakagawa[1,2], Ryosuke Muroyama[1], Kazuhiko Koike[2], Sayaka Ito[1], Keiko Takano[2], Kaku Goto[1], Masahiro Nakano[2], Chisato Saeki[2], Yasuo Matsubara[1], Naoya Kato[1], Mikio Zeniya[2]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Department of Gastroenterology and Hepatology, The Jikei University School of Medicine

Background: Primary biliary cirrhosis (PBC) is an autoimmune liver disease of unknown pathogenesis. Moreover, therapeutic targets for the autoimmunity of PBC are not yet found. Since $CD4^+$ T cells are known to play a pivotal role in the immunological disorder of PBC, we analyzed microRNA (miRNA) and mRNA of $CD4^+$ T cells integratedly to reveal its pathogenesis.

Methods: Clinically and pathologically diagnosed 7 PBC patients and 7 healthy controls, who agreed to provide samples with written informed consent, were enrolled in this study. Then, we analyzed the expression profile of miRNA and mRNA in $CD4^+$ T cells of PBC patients and controls by microarray and qRT-PCR using a gene set enrichment analysis (GSEA). The biological function of differentially expressed and GSEA-enriched miRNAs was evaluated by miRNA overexpression assay, reporter assay, and CD3 stimulation assay using cultured cells.

Results: An integral miRNA-mRNA analysis revealed 4 decreased miRNAs (miR-181a, -181b, -374 b, -425) coordinately dysregulated T cell receptor (TCR) signaling pathway in $CD4^+$ T cells of PBC. Especially, N-Ras in the upper stream of TCR signaling pathway was targeted by 4 decreased miRNAs. *In vitro* assays revealed miR-425 downregulated N-Ras expression and therefore suppressed production of inflammatory cytokines (IL-2, IL-10, and IFN-γ).

Conclusion: Decreased miR-425 in $CD4^+$ T cells upregulates N-Ras and induces the inflammation through dysregulation of TCR signaling pathway in PBC. The restoration of decreased miR-425 and/or downregulation of N-Ras expression in $CD4^+$ T cells could be novel therapeutic options for PBC.

5. Novel zinc finger protein in gastrointestinal tract

Yasuo Matsubara[1,2], Kazuaki Takahashi[2], Masahiro Arai[2], Jun Miwa[2], Shunji Mishiro[2], Ryosuke Muroyama[1], Kaku Goto[1], Ryo Nakagawa[1], Sayaka Ito[1], Sayuri Morimoto[1], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Department of Medical Science, Toshiba General Hospital

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 ZNF-114-like hypothetical protein in the gastric biopsy specimen by mass spectrometry. Its mRNA sequence and other mRNAs with similar sequences were determined by RACE. The expression vector was constructed and transfection was performed. Apparent phenotype change was not revealed in transfected cultured cells of upper gastrointestinal tract. Knock-down studies will be executed for further functional analysis.

6. *H. pylori* in HIV-infected patients.

Yasuo Matsubara[1,2], Ryosuke Muroyama[1,2], Kaku Goto[1], Ryo Nakagawa[1], Sayaka Ito[1], Sayuri Morimoto[1], Naoya Kato[1,2]: [1]Division of Advanced Genome Medicine, IMSUT: [2]Department of Advanced Medical Science, IMSUT Hospital

The prevalence of *H. pylori* infection in the HIV-infected patients is to be elucidated. Some studies showed higher rates of infection in HIV-negative patients than HIV-positive. Ones One of hypotheses is an appropriate amount of CD4+ cells is needed for colonization of *H. pylori*. We investigated *H. pylori* in HIV-infected patients using biopsy specimens taken by upper gastrointestinal endoscopy. Rate of *H. pylori* infection diagnosed by light microscopy was lower in HIV-positive subjects. There was not significant relation between blood CD4 count and *H. pylori* infection.

7. HBx-binding compounds are potential target for treatment of HBV-induced HCC

Sayuri Morimoto, Sayaka Ito, Ryosuke Muroyama, Ryo Nakagawa, Kaku Goto, Yasuo Matsubara, Naoya Kato

Chronic Hepatitis B virus (HBV) infection is a risk factor for developing hepatocellular carcinoma (HCC). Hepatitis B virus X protein (HBx) plays an important role in the development of HCC in HBV-infected patients. HBx has a variety of functions such as control of HBV replication, transcriptional trans-activating ability on cellular genes, and ability to induce apoptosis of cells. a compound that binds to HBx and modifies its ability to perform these functions could be a potential drug for treatment of HBV-induced HCC. Therefore, we constructed glutathione S-transferase (GST)-tagged HBx proteins for screening chemical compounds. To confirm that the presence of GST does not affect the function of HBx, GST-tagged HBx's trans-activating ability was compared to that of HBx by luciferase assay in HeLa cells transfected with HBx or GST-HBx harboring plasmids. GST-HBx showed a comparable level of trans-activation to that of HBx, so the fusion protein was amplified and purified for use in screening. Compounds that bind to GST-HBx will be further analyzed.

8. Detection of HCV NS5A L31/Y93 mutations conferring treatment resistance

Sayaka Ito[1], Kaku Goto[1], Yasushi Tanoue[1,2], Ryosuke Muroyama[1], Yasuo Matsubara[1], Wenwen Li[1], Ryo Nakagawa[1], Toshio Fujisawa[1,3], Shinsho Yoshiba[2], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, The Institute of Medical Science, The University of Tokyo; [2]Department of Gastroenterology and Hepatology, JCHO Tokyo Takanawa Hospital; [3]Department of Gastroenterology, NTT Medical Center Tokyo

Background and aims: Recently interferon-free regimen for HCV infection was attained by the combination treatment with NS5A inhibitor and protease inhibitor. However, amino acid mutations conferring resistance to NS5A inhibitor were identified in the N-terminal region of NS5A protein. We here focused on the resistance mutations at L31 and Y93 of NS5A reported in genotype 1b HCV infection, and examined their emergence in patients using our newly established detection system. Methods: Following the isolation of viral RNA from patient serum and its reverse transcription, the region covering the mutations was amplified by nested PCR. Subsequently L31/Y93 mutations were investigated by direct sequencing. Zero Blunt TOPO PCR Cloning Kit was used for the construction of plasmids encoding NS5A fragments with L31F or Y93 H. Results: Reverse transcribed viral RNA from serum of HCV-infected patient was used for the nested PCR with primers designed based on HCV genotype 1b genome sequences. Then we identified the primer set with the highest efficiency and specificity in amplification and subsequent direct sequencing. The established method was capable of sensing each mutation in the mixture of the plasmids encoding L31F or Y93H, with the individual mutation content of 25% or more and in a mixing ratio-dependent manner. So far L31F/M and Y93H were observed in 5 (5%) and 12 (12%) out of 102 cases, respectively, and both emerged in the two breakthrough cases without L31/Y93 mutations before the treatment with daclatasvir and asnaprevir. Conclusions: We newly generated a detection system of NS5A inhibitor resistance mutations in NS5 A of HCV from patient serum. Our system is expected to be conducive for designing treatment

strategies to evade emergence of resistant viruses and achieve SVR.

9. Induction of MICA expression by anti-cancer drugs for novel HCC immunotherapy

Yasushi Tanoue[1,2], Kaku Goto[1], Toshio Fujisawa[1,3], Sayaka Ito[1], Ryosuke Muroyama[1], Ryo Nakagawa[1], Yasuo Matsubara[1], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, The Institute of Medical Science, The University of Tokyo; [2]Department of Gastroenterology and Hepatology, JCHO Tokyo Takanawa Hospital; [3]Department of Gastroenterology, NTT Medical Center Tokyo

Identification of an anti-tumor ligand MHC class I polypeptide-related sequence A (MICA) as a genetic susceptibility factor for HCV-induced hepatocellular carcinoma (HCC) in our genome-wide association study (Kumar V et al., Nat Genet 2011) indicated the hepatocarcinogenesis-preventive potential of MICA expression. In our screen for an FDA-approved drug library using a luciferase reporter system, an anti-cancer agent was found to strongly upregulate MICA promoter activity, suggesting further potencies of related drugs. Currently their effects on MICA biogenesis and anti-HCC activities of NK cell are examined, with novel HCC immunotherapies in view.

10. IL-13Rα2 as a novel prognostic biomarker for human pancreatic cancer

Toshio Fujisawa[1,2] Yasushi Tanoue[1,3], Kaku Goto[1], Sayaka Ito[1], Ryosuke Muroyama[1], Ryo Nakagawa[1], Yasuo Matsubara[1], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Department of Gastroenterology, NTT Medical Center Tokyo; [3]Department of Gastroenterology and Hepatology, JCHO Tokyo Takanawa Hospital

Interleukin-13 Receptor α2 (IL-13Rα2) is known as a cancer testis antigen and a potential target for cancer immunotherapy. IL-13Rα2 is overexpressed in about 70% pancreatic cancer samples and involved in cancer invasion and metastasis. We investigated a possible correlation between IL-13Rα2 expression in pancreatic cancer and patient prognosis.

Totally, 236 samples of surgically resected pancreatic cancer tissue (adenocarcinoma) were obtained from two different institutions. Samples were immunohistochemically stained for IL-13Rα2 expression and its intensity was evaluated by total 4 investigators. The expression of IL-13Rα2 was correlated with patient characteristics including prognosis and clinicopathological parameters.

As a result, 63%-68% of pancreatic cancer samples overexpressed IL-13Rα2. Kaplan-Meier survival analysis revealed that patients with IL-13Rα2 positive pancreatic cancer had significantly lower survival compared to patients with IL-13Rα2 negative cancer ($P < 0.0057$ and 0.0002). On multivariate analysis, only IL-13Rα2 expression and UICC-stages were identified as common prognostic factor at both institutions. Interestingly, tumors that had significant nerve invasion frequently correlated with high IL-13Rα2 expression. IL-13Rα2 could be a prognostic biomarker of surgically resected pancreatic cancer patients. Because IL-13Rα2 expression is related with invasion to nerve, immunotherapy targeting IL-13Rα2 might not only prolong patient survival but also suppress symptoms by nerve invasion including cancer pain.

Samples used in this study were all Japanese. Therefore, we will perform additional analysis using TCGA data from US national cancer institute, and confirm the interracial universality of the results in the next year.

11. Development of anti-metastasis treatment targeting IL-13Rα2 for pancreatic cancer

Toshio Fujisawa[1,2], Kaku Goto[1], Ryosuke Mukoyama[1], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Department of Gastroenterology, NTT Medical Center Tokyo

Pancreatic cancer is an aggressive disease with only limited therapeutic options available. Interleukin-13 receptor α2 chain (IL-13Rα2), which is a high-affinity receptor for IL-13, is overexpressed in a variety of human solid cancers including pancreatic cancer. We have previously reported that histone acetylation was a key mechanism to control IL-13Rα2 expression in pancreatic cancer cells, and histone deacetylation (HDAC) inhibitors upregulated IL-13Rα2 expression and enhanced the efficacy of immunotoxin targeting IL-13Rα2. On the other hand, IL-13Rα2 helps invasion and metastasis of pancreatic cancer through activation of extracellular signal-regulated kinase 1/2 and activator protein-1 (AP-1) nuclear factors. We, thereupon, hypothesized that histone acetyltransferase (HAT) inhibitors, which oppositely works from HDAC inhibitors, and AP-1 inhibitors decrease IL-13Rα2 expression and metastasis of pancreatic cancer. Last year, we examined IL-13Rα2 expression in pancreatic cancer cell lines and picked up two cell lines, HS766T and MIA-PaCa2, because of their aggressive metastasis. And we took notice of three HAT inhibitors for inhibiting IL-13Rα2 expression. This year, we examined the efficacy of these drugs for inhibiting IL-13Rα2 expression in the pancreatic cancer cells. Epigallocatechin gallate showed the strongest effect for IL-13Rα2 inhibition and it was promising to prevent pancreatic cancer metastasis. Continuously, the possibility of the drugs for inhibition of cancer invasion and metastasis was exam-

ined. All three drugs showed a certain measure of inhibiting effects for cancer invasion *in vitro*. As well as IL-13Rα2 inhibition, Epigallocatechin gallate most strongly prevent pancreatic cancer invasion and metastasis. For confirming these effects, we are now planning animal study of pancreatic cancer.

Publications

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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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Project Professor	Shinichi Muramatsu, M.D., Ph.D.		特任教授(兼務，非常勤)	医学博士	村　松　慎　一
Senior Assistant Professor	Sumimasa Nagai, M.D., Ph.D.		講　師	医学博士	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 are currently preparing Japanese multicenter clinical trial of CD19-CAR-T cell therapy for adult patients with relapsed and refractory B-ALL. CD19-CAR-T gene therapy for malignant B-cell lymphoma was conducted in one patient at Jichi Medical University Hospital as Phase I/II clinical research.

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