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 leukemia:
Leukemia-specific genetic rearrangements often result in chimeric transcription factors and tyrosine kinases, which appear to be the primary cause of those leukemias. We are studying the molecular and cellular aspects of acute and chronic leukemia as a model system.

(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) identification and characterization of somatic stem cells, b) search for molecules to affect the growth and differentiation of stem cells, and c) basic and clinical studies on bone tissue engineering.

1. *In vivo* leukemogenic potential of an interleukin 7 receptor a chain mutant in hematopoietic stem and progenitor cells.

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Somatic gain-of-function mutations in interleukin 7 receptor α chain (IL7Rα) have been described in pediatric T and B acute lymphoblastic leukemias (T/B-ALLs). Most of these mutations are in-frame insertions in the extracellular juxtamembrane- transmembrane region. By using a similar mutant, a heterozygous in-frame transmembrane insertional mutation (INS), we validated leukemogenic potential in murine hematopoietic stem/progenitor cells, using a syngeneic transplantation model. We found that ectopic expression of INS alone in hematopoietic stem/progenitor cells caused myeloproliferative disorders, whereas expression of INS in combination with a Notch1 mutant led to the development of much more aggressive T-ALL than with wild-type IL7Rα. Furthermore, forced expression of INS in common lymphoid progenitors led to the development of mature B-cell ALL/lymphoma. These results demonstrated that INS has significant in vivo leukemogenic activity and that the lineage of the resulting leukemia depends on the developmental stage in which INS occurs, and/or concurrent mutations.

2. Bcr-Abl impairs T cell development at the early stage of thymocyte differentiation.

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Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder generally believed to originate from a hematopoietic stem cell carrying the BCR-ABL fusion gene, which generally encodes 210kD and 190kD constitutively active tyrosine kinases termed as p210 and p190, respectively. In spite of the putative stem cell origin and the competence for differentiation toward mature B cells, there is a longstanding consensus that CML never involves the T cell lineage at least in chronic phase. To gain insight into this apparent conflict, we used *in vitro* T cell differentiation model from murine hematopoietic stem cells (HSCs). c-Kit$^+$Sca1$^+$Lin$^-$ (KSL) bone marrow cells were prepared by FACS from 8-weeks old C57BL/6 mice treated with 5-FU. KSL cells were similarly transduced with p190 ΔccER and were subjected to the OP9-DL1 co-culture system with or without 0.5 μM 4-HT. After 2 weeks of culture, 95% of lymphocytes from the 4-HT(−) culture revealed CD3$^+$TCRβ$^+$ phenotype, but only 30% of those were double positive in the presence of 4-HT. In addition, 90% of lymphocytes from the 4-HT(−) culture progressed to the DN2 stage with c-Kit$^-$CD44$^+$CD25$^+$ phenotype, whereas 50% of those from the 4-HT(−) culture arrested at the DN1 stage showing c-Kit$^+$CD44$^+$CD25$^-$. Since IL7 plays a central role at the stage from DN1 to DN2 of progenitor T cells, Bcr-Abl is suggested to impair T cell development possibly through interfering with the IL7 signal. The precise mechanism underlying impaired T lymphopoiesis by Bcr-Abl is under investigation.

3. Recombinant vaccinia virus therapy against hematological malignancies

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Vaccinia virus is a member of poxvirus family, and was used for global small pox prevention programs. After the eradication of small pox in 1979, usage of vaccinia virus declined significantly. However, vaccinia virus is re-evaluated due to its strong oncolytic (anti-tumor) effect. Oncolytic effects are thought to occur via infection itself and host's immune response. To determine which of the disease is ideal for vaccinia virotherapy, first, we infected different cell lines derived from acute myelogenous leukemia, chronic myeloid leukemia, acute lym-

phoblastic leukemia, malignant lymphoma, adult T-cell leukemia/lymphoma, and multiple myeloma *in vitro*. Very interestingly, myeloma showed exceptionally high susceptibility (10-100 times higher than others) to vaccinia virus, which led us to focus on myeloma. Vaccinia B5R gene is critical for viral transmission from infected cells. Although stronger infectivity is desirable for better oncolytic effect, safety is a concern. To improve its safety, we tried to make a recombinant vaccinia virus which express B5R only in normal cells but not in myeloma cells by utilizing miRNA expression pattern. We inserted a DNA sequence which is complementary to let-7a into the 3'untranslated region of viral B5R gene, because let-7a is endogenously expressed in normal tissues but not in myeloma cells. In vitro infection of a myeloma cell line (RPMI8226) showed significant infectivity even with a very low titer (MOI=0.1), while control normal cells (human skin fibroblasts) were not infected with the same titer, suggesting that let-7a in normal tissue inhibited B5 R expression of vaccinia virus, resulting in impaired transmission of vaccinia virus from infected cells to adjacent cells. As an *in vivo* infection model, 1×10^7 RPM8226-Luc cells were injected into immunodeficient mice (CB.17-SCID) subcutaneously, and 1×10^7 pfu of virus was administered via an intravenous injection 4 weeks later, then tumor volume and the amount of virus were routinely determined by *in vivo* imaging system with renilla and firefly luciferases. Without let-7a regulation, vaccinia virus infected not only myeloma cells but also normal tissues, and mice developed pock lesions in the ear, nose, mouth, foot, and tail. In contrast, infection with let-7a-regulated vaccinia virus was limited in myeloma, and the size of myeloma shrunk continuously. These data suggest that the let7a-regulated vaccinia virus will be a good candidate for future clinical application.

4. The CD7 vs CADM1 plot in FACS is useful for selection of advanced HTLV-1 carriers

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Previously, we reported the CADM1 vs CD7 plot in flow cytometry reflects multistep oncogenesis in HTLV-1 infection. In this plot in $CD4^+$ cells, CADM 1^-CD7^+(P), $CADM1^+CD7^{dim}$(D) and $CADM1^+CD7^-$ (N) subpopulations were observed and D/N subpopulations increased in advanced stages. Molecular analyses of the three subpopulations revealed that the D/N subpopulations enriched clonally expanded cells in advanced cases. In this study, we analysed HTLV-1 asymptomatic carriers (ACs, N= 29) and indolent ATL cases (smoldering- and chronic-type, N=10) to see whether the analysis is applicable for evaluation of early clinical stage in HTLV-1 infection and selection of high risk ACs for developing ATL. Using samples of ACs and indolent ATL patients, flow cytometric analysis with CADM1 vs CD7 plot in $CD4^+$ cells and inverse long PCR (clonality analysis) of FACS sorted P/D/N subpopulations were performed. ACs the CADM1 vs CD7 profile showed sample-to-sample variation, including cases with increased D/N(%) that were equivalent to indolent ATL cases. The CADM1 vs CD7 profile well correlated with HTLV-1 proviral load(PVL). All ACs with high PVL(>4copies/100 cells) had more than 10% of the D+N(%). The profile also correlated with clonality, i.e. ACs with increased D/N(%) invariably showed intense bands in these subpopulations, indicating expanded clones. In conclusion, the CADM1 vs CD7 plot is considered to be useful for evaluation of early clinical stage in HTLV-1 infection and selection of putative high-risk ACs.

5. In vitro long-term maintenance of murine iPSC-derived hematopoietic stem/progenitor cells by 4-Hydroxytamoxifen inducible HoxB4

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Hematopoietic stem/progenitor cells (HS/PCs) constitute a quite minor part of bone marrow nucleated cells (BMC) and cannot be keeping their multi-differentiation abilities of HS/PCs for a long time while expanding ex vivo. It is also difficult to efficiently induce HSCs from the pluripotent stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Recently, we generated the iPSCs from the heterozygous GATA 2-GFP knock-in mice in which GFP cDNA was inserted into exon 2 of the GATA2 gene and then we successfully established iPSCs-derived GFP+ hematopoietic progenitor cell lines that could expand in vitro for more than three months. However, these cells could repopulate in mice only for a month. In this experiment, we examined whether a consecutive expression of HoxB4 was effective in sustaining the murine iPSC-derived HS/PCs in vitro. HoxB4, a member of the Homeobox (Hox) transcriptional family, is a strong positive regulator of HSC self-renewal ex vivo when ectopically expressed in HSC. It also promotes specification of definitive HSCs from differentiating ESCs by overexpression. We transduced 4-Hydroxytamoxifen (4-HT) inducible HoxB4 gene into murine iPSCs and then induced toward hematopoietic cells. Our results showed that after culturing for two months in vitro, an iPSCs-derived cell mass including GFP+ cells kept

its multi-differentiation abilities and could expand for more than thirty weeks in vivo, by the continuous activation of 4-HT inducible HoxB4 during differentiation.

6. Maintenance of stemness of breast cancer stem-like cells by FRS2β, a feedback inhibitor for ErbB, during mammary tumorigenesis

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It is important to understand the molecular mechanisms how cancer stem cells (CSC)s are maintained. We examined how ErbB/HER signaling activity is regulated in breast CSCs. We generated knockout mice of FRS2beta adaptor protein, a feedback inhibitor for ErbB, and crossed them with MMTV-ErbB2 mice in which overexpression of ErbB2 induces breast cancer. Tumor growth in the breast tissues of wild-type mice was much faster than those of the FRS2beta mutant mice. Mammosphere forming activity of tumor cells was reduced in the FRS2beta mutant mice, suggesting that FRS2beta is important for maintenance of breast CSCs. FRS2beta was expressed in a very few luminal cells in mammary tissues. $CD24^{high}$ $CD29f^{low}$ cell population including luminal progenitor cells were reduced in the mutant mice. Moreover, mammosphere forming activity of normal mammary cells were reduced in FRS2beta mutant mice. It thus appears that FRS2beta is expressed in luminal progenitor cells that are transformed into breast CSCs and plays important roles for maintenance of not only luminal progenitor cells but also breast CSCs. Therefore, fine-tuning of ErbB by FRS2beta appears to maintain progenitor cells and CSCs.

7. Insulin-like growth factor regulates breast cancer stem cell properties

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The breast cancer stem cells (BCSCs) are thought be a source of tumor cells of breast cancer tissues. BCSCs include in $CD44^{+}/CD24^{-/low}$ $Lineage^{-}$ population and have the property of resistance against anticancer drug and radiation. We previously elucidated that heregulin (HRG), a ligand of ErbB3, plays important roles for tumor sphere formation of BCSCs through PI3K pathway (*PNAS*, 2012). By examining gene expression profiles in breast cancer cells stimulated with HRG in the presence or absence of PI3K inhibitor / NF-kB inhibitor by using DNA microarray, we obtained 217 genes as the ErbB/PI3K/NF-kB gene signature. In this study, we chose to examine the insulin-like growth factor 2 (IGF2), one of the molecules among the ErbB3/PI3K/NF-kB gene signature, because IGF2 is able to induce sphere formation of primary breast cancer cells derived from most of the patient samples. The sphere formation of human breast cancer cells induced by HRG stimulation was completely blocked by the treatment with neutralizing antibody (NAb) against IGF2. Furthermore, we found the expression levels of IGF1 receptor in BCSC population are higher than those of control cells. These findings suggest that ErbB/PI3K/NF-kB signaling maintains BCSCs through production of growth factors such as IGF2 by autocrine/paracrine mechanisms.

8. MTHFD2 is a key molecule in EGF receptor tyrosine kinase and regulates lung cancer cell growth.

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Non-small cell lung cancer (NSCLC) is a major subtype of lung cancer and is the most common and fatal cancer worldwide. Even diagnosed at early stage, 10-30% of those patients eventually relapse and die of recurrence. Previously we identified 139 genes as EGF receptor tyrosine kinase (RTK) key molecules. By using these 139 genes as a signature, we succeeded to predict the prognosis of lung adenocarcinoma patients at the early stage. In this study, we examined the role of MTHFD2 (methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase) in lung tumorigenesis which is one of the EGF RTK key molecules. MTHFD2 is localized in mitochondria and functions as an enzyme in the folate metabolism. We found that MTHFD2 was expressed at low levels in normal lung epithelial cells, while it was abundantly expressed in many lung cancer cell lines. When we knocked down the expression of *MTHFD2* by using shRNA for *MTHFD2*, the adhesion-dependent lung cancer cell growth was inhibited. Furthermore, soft agar colony forming efficiency was also inhibited. These results suggest that MTHFD2 regulates lung cancer

cell growth.

9. CD74-NRG1 is a potential oncoprotein that promotes cancer stem cell properties.

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Cancer stem cells (CSCs) are thought to be responsible for the initiation and recurrence of tumors. Therefore, targeting molecules that have a critical role in maintenance of CSCs would be a useful strategy. CD74-NRG1 fusion gene was identified in 5-15% of in invasive mucinous adenocarcinoma of the lung, a malignant type of lung adenocarcinoma. CD74-NRG1 protein contains the CD74 transmembrane domain and the EGF-like domain of the NRG1/HRG protein, suggested to mediate juxtacrine signals signaling through HER2: HER3 receptors. In this study, we expressed cDNA of CD 74-NRG1 fusion gene in BT20 human breast cancer cell line by using a lentivirus system, and investigated whether this fusion gene is involved in the promotion of CSC phenotype using a breast CSC (BCSC) assay. First, we examined the self-renewal ability of CD74-NRG1 expressing breast cancer cells by performing the sphere forming assay. CD74-NRG1 expressing cells were able to form tumor spheres without adding any growth factors, while cells infected with the lentivirus carrying control vector were not. Then, we analyzed the population of BCSCs by flow cytometry using CD44 and CD24 antibody. The percentages of $CD44^{high}/CD24^{-/low}$ BCSC-enriched population increased from 1.94% to 9.47% (variant 1) or 8.21% (variant 2). These results suggest that expression of CD74-NRG1 fusion gene promotes cancer stem cell properties and is involved in stem cell function of several types of cancers including lung and breast cancer.

10. Clinical study on bone tissue engineering

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Atrophic maxillas or mandibles 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 esthetic and prosthetic 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 and currently in progress.

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Advanced Clinical Research Center

Division of Cellular Therapy
細胞療法分野

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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 therapy targeting signal transduction pathways, (3) Characterization of a PIR (paired Ig receptors) family (LMIR/MAIR/CLM) and (4) Elucidation of molecular basis of leukemia, myelodysplastic syndromes, myeloproliferative disorders.

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

Kohtaro Nishimura, Toshihiko Oki, Toshiyuki Kawashima, Yukinori Minoshima, Ying Chun Bao, Tomonori Hatori, Yasushi Nomura, Noriko Takahashi, Takaya Satoh[1], and Toshio Kitamura: [1]Osaka City University.

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 expression of MgcRacGAP increases in the early G1 phase in parallel with Geminin, suggesting that MgcRacGAP may play some roles in G1 check point. In addition, our recent result has suggested that MgcRacGAP is subject to ubiquitin-dependent degradation in G0/G1 phase. 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.

2. Molecular therapy targeting signal transduction pathways using small molecule compounds

Toshiyuki Kawashima, Akiho Tsuchiya, Yukinori Minoshima, 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 collaborate with a US biotech venture company in modification of RSJI-1 for optimization to develop anti-cancer drugs, and have developed JP1156 which kill the tumor cells with much lower IC50. JP1156 is effective in inhibiting growth of various tumor cell lines in mouse tumor-burden models, and we are now searching for a partner company to support phase studies of this compound.

3. Identification and characterization of a novel family of paired Ig (immunoglobulin-like) receptors LMIRs.

Kumi Izawa, Masahiro Sugiuchi, Ayako Kaitani, Mariko Takahashi, Akie Maehara, Yoshinori Yamanishi, Toshihiko Oki, Fumi Shibata, Kaori Tamitsu, Si-Zhou Feng, Hideaki Nakajima[2], Jiro Kitaura, and Toshio Kitamura: [2]Keio University School of Medicine

We originally identified and characterized two mouse cDNAs from a mouse bone marrow-derived mast cell cDNA library. They encoded type I transmembrane proteins including a single variable immunoglobulin (Ig) motif in the extracellular domain with about 90% identity of amino acids. LMIR1 contains immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, while LMIR2 harbors a short cytoplasmic tail associating with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as DAP12. In addition to LMIR1/2, related genes were identified by homology search in the close proximity on the same chromosome 11: LMIR3 is an inhibitory type receptor like LMIR1, and LMIR4-8 are activation type receptors like LMIR2. It is of note that LMIR3 has a unique property to associate with FcRγ and thereby functions as an activating receptor in concert with TLR4 stimulation. LMIRs are also called CLMs or MAIRs. Those receptors are mainly expressed in cells involved in innate immunity including mast cells, neutrophils, monocytes, and dendritic cells. In addition, some of the receptors are also expressed in some cells in colon, trachea, and lung, indicating that these receptors play important roles in innate immunity.

LMIR5 is a DAP12-coupled activating receptor predominantly expressed in myeloid cells. We have identified T cell Ig mucin 1 (TIM1) as a possible ligand for LMIR5 by retrovirus-mediated expression cloning. TIM1 interacted only with LMIR5 among the LMIR family, whereas LMIR5 interacted with TIM4 as well as TIM1. Stimulation with TIM1 or TIM4 induced LMIR5-mediated activation of mast cells. Notably, LMIR5 deficiency suppressed TIM1-Fc-induced recruitment of neutrophils in the dorsal air pouch, and LMIR5 deficiency attenuated neutrophil accumulation in a model of ischemia/reperfusion injury in the kidneys in which TIM1 expression is up-regulated. In that model, LMIR5 deficiency resulted in ameliorated tubular necrosis and cast formation in the acute phase. Collectively, our results indicate that TIM1 is an endogenous ligand for LMIR5 and that the TIM1-LMIR5 interaction plays a physiological role in immune regulation by myeloid cells.

We have also identified ceramides as ligands for LMIR3, and demonstrated that LMIR3 plays critical roles in inhibiting allergic response caused by mast cells using LMIR3 knockout mice. Our results suggest that ceramides present in the skin attenuate the activation of mast cells when they are activated by IgE and antigens.

4. Molecular basis of acute leukemia, myelodysplastic syndromes (MDS), MDS overt leukemia, and myeloproliferative disorder (MPD).

Daichi Inoue, Kojin C Kawabata, Reina Nagase, Sayuri Horikawa, Naoko Watanabe, Yukiko Komeno, Naoko Kato, Yutaka Enomoto, Toshihiko Oki, Hideaki Nakajima[2], Yuka Harada[3], Hironori Harada[3], Tetsuya Nosaka[4], Jiro Kitaura, and Toshio Kitamura: [3]Department of Hematology/Oncology, Juntendo University, and [4]Mie University School of Medicine.

To elucidate the molecular mechanisms of leukemia, MDS, and MPD, we established mouse model using bone marrow transplant (BMT); we transduced mouse bone marrow cells with genes of leukemogenic mutations derived from patients including MLL-fusions and BCR-Abl, and mutant forms of AML1 and C/EBPa using retroviruses. The bone marrow cells transduced with these mutant genes derived from patients were transplanted to irradi-

ated mice. Using this mouse BMT model, we have shown several interesting things; 1) Combination of class I (MLL-Sept6) and class II mutations lead to development of acute leukemia; 2) A class II mutation (AML1 mutations) induced MDS-like disease, and some of the mice progressed to acute leukemia with additional mutations such as overexpression of Evi1; 3) Combination of BCR-Abl and Hes1 expression induced CML blast crisis (BC) like disease. In fact, overexpression of Hes1 was demonstrated in 8 of 20 patients with CML-BC but not in patients with CML-chronic phase; 4) Two classes of C/EBPa mutations (N-terminal and C-terminal mutations) collaborate with each other in inducing acute leukemia in mouse BMT models, probably working as class I and class II mutations.

Recent progress using high-speed sequencing has identified mutations in genes that are not categorized to class I and class II mutations. These include mutations of epigenetic factors, splicing factors, and molecules of the Cohesin complex. Experiments are now under way to clarify the molecular mechanisms by which mutations of epigenetic factors including TET2, EZH2 and ASXL1 induce hemopoietic malignancies using mouse BMT models and transgenic mice.

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Advanced Clinical Research Center

Division of Infectious Diseases
感染症分野

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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 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.

1. Structure of TCR and antigen complexes at an immunodominant CTL epitope in HIV-1 infection

Akihisa Shimizu, Ai Kawana-Tachikawa, Atsushi Yamagata[1,2], Chung Yong Han, Dayong Zhu, Hitomi Nakamura[3], Michiko Koga, Tomohiko Koibuchi[4], Jonathan Carlson[5], Eric Martin[6], Chanson J. Brumme[7], Shi Yi[8], George F. Gao[8], Zabrina L. Brumme[6,7], Shuya Fukai[1,2], Aikichi Iwamoto; [1]Structural Biology Laboratory, Life Science Division, Synchrotron Radiation Research Organization and Institute of Molecular and Cellular Biosciences, The University of Tokyo, [2]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, [3]Department of Infectious Diseases Control, International Research Center for Infectious Diseases, IMSUT, [4]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT, [5]Microsoft Research, Los Angeles, CA, USA [6]Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada. [7]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada. [8]CAS Key Laboratory for Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Science, Beijing, China.

We investigated the crystal structure of an HLA-A*2402-restricted CTL epitope in the HIV-1 nef gene (Nef134-10) before (pHLA) or after TCR docking. The wild type epitope and two escape mutants were included in the study. Y135F was an early-appearing major mutation, while F139L was a late-appearing mutation which was selected in the patients without Y135F. F139 was an eminent feature of the Nef134-10 epitope. Wild type-specific TCR was less fit to F139L mutant suggesting that F139L is an escape from the CTL against the wild type epitope. Although Y135F mutation disrupted the hydrogen bond to HLA-A*2402 His70, newly formed hydrogen bond between T138 and His70 kept the conformation of the epitope in the reconstituted pMHC. TCR from Y135F- or dually-specific CTL had unique mode of binding to the mutant epitope. Y135F has been reported as a processing mutant but CTL carrying structurally adequate TCR can be found in the patients.

2. Switching and emergence of CTL epitopes in HIV-1 infection

Chungyong Han, Ai Kawana-Tachikawa, Akihisa Shimizu, Dayong Zhu, Hitomi Nakamura, Eisuke Adachi[1], Tadashi Kikuchi[1], Michiko Koga, Tomohiko Koibuchi[1], George F. Gao, Yusuke Sato, Atsushi Yamagata, Eric Martin, Shuya Fukai, Zabrina L. Brumme, Aikichi Iwamoto; [1]Department of Infectious Diseases and Applied Immunology, IMSUT hospital, IMSUT.

Human Leukocyte Antigen (HLA) class I restricted Cytotoxic T Lymphocytes (CTLs) exert substantial evolutionary pressure on HIV-1, as evidenced by the reproducible selection of HLA-restricted immune escape mutations in the viral genome. An escape mutation from tyrosine to phenylalanine at the 135th amino acid (Y135F) of the HIV-1 *nef* gene is frequently observed in patients with HLA-A*24:02, an HLA Class I allele expressed in ～70% of Japanese persons. The selection of CTL escape mutations could theoretically result in the *de novo* creation of novel epitopes, however, the extent to which such dynamic "CTL epitope switching" occurs in HIV-1 remains incompletely known. Two overlapping epitopes in HIV-1 *nef*, Nef126-10 and Nef134-10, elicit the most frequent CTL responses restricted by HLA-A*24:02. Thirty-five of 46 (76%) HLA-A*24:02-positive patients harbored Y135F mutation in their plasma HIV-1 RNA. Nef codon 135 plays a crucial role in both epitopes, as it represents the C-terminal anchor for Nef126-10 and the N-terminal anchor for Nef134-10. While the majority of patients with 135F exhibited CTL responses to Nef126-10, none harboring the wild-type Y135 did so, suggesting that Nef 126-10 is not efficiently presented in the latter patients. Consistent with this, peptide binding and limiting dilution experiments confirmed F, but not Y, as a suitable C-terminal anchor for HLA-A*24:02. Moreover, experiments utilizing antigen specific CTL clones to recognize endogenously-expressed peptides with or without Y135F indicated that this mutation disrupted the antigen expression of Nef 134-10. Critically, the selection of Y135F also launched the expression of Nef126-10, indicating that the latter epitope is created as a result of escape within the former. Our data represent the first example of the *de novo* creation of a novel overlapping CTL epitope as a direct result of HLA-driven immune escape in a neighboring epitope. The robust targeting of Nef126-10 following transmission (or *in vivo* selection) of HIV-1 containing Y135F may explain in part the previously reported stable plasma viral loads over time in the Japanese population, despite the high prevalence of both HLA-A* 2402 and Nef-Y135F in circulating HIV-1 sequences.

3. Effect of Maraviroc intensification on HIV-1-specific T cell immunity in recently HIV-1-infected individuals

Ai Kawana-Tachikawa, Josep M Llibre[1], Isabel Bravo[1], Roser Escrig[1], Beatriz Mothe[1,2], Jordi Puig[1], Maria C. Puertas[2], Javier Martinez-Picado[2,3], Julia Blanco[2], Christian Manzardo[4], Jose Maria Miro[4], Aikichi Iwamoto, Anton L. Pozniak[5], Jose M. Gatell[4], Bonaventura Clotet[1,2], Christian Brander[2,3], and the MARAVIBOOST investigators; [1]Lluita contra la SIDA Foundation. HIV Unit, University Hospital Germans TriasiPujol, [2]Irsicaixa AIDS Research Institute－HIVACAT, Autonomous University of Barcelona, [3]Institució Catalana de Recerca i Estudis Avancats (ICREA), [4]Hospital Clínic-IDIBAPS, University of Barcelona, [5]HIV/GUM Department, Chelsea and Westminster Hospital.

The effect of maraviroc on the maintenance and the function of HIV-1-specific T cell responses remains unknown. Subjects recently infected with HIV-1 were randomized to receive anti-retroviral treatment with or without maraviroc intensification for 48 weeks, and were monitored up to week 60. PBMC and *in vitro*-expanded T cells were tested for responses to the entire HIV proteome by ELISpot analyses. Intracellular cytokine staining assays were conducted to monitor the (poly)-functionality of HIV-1-specific T cells. Analyses were performed at baseline and week 24 after treatment start, and at week 60 (3 months after maravirocdiscontinuation). Maraviroc intensification was associated with a slower decay of virus-specific T cell responses over time compared to the non-intensified regimen in both, direct *ex-vivo* as well as *in in-vitro* expanded cells. The effector function profiles of virus-specific $CD8^+$ T cells were indistinguishable between the two arms and did not change over time between the groups. Maraviroc did not negatively impact any of the measured parameters, but was rather associated with a prolonged maintenance of HIV-1-specific T cell responses. Maraviroc, in addition to its original effect as viral entry inhibitor, may provide an additional benefit on the maintenance of virus-specific T cells which may be especially important for future viral eradication strategies.

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Advanced Clinical Research Center

Division of Bioengineering
臓器細胞工学分野

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Our division has been conducting basic research projects related to the cancer and transplantation immunology. The reagents, modalities, and concepts developed in this division have been clinically applied as translational research projects by the clinicians involved in related clinical trials 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.

Development of innovative cancer therapy using immunologic approaches

I. The practical application for cancer immunotherapy through the investigation chronic inflammation in IL-23/Th17 axis

Marimo Sato-Matsushita and Hideaki Tahara

In this study, we investigated whether bone marrow-derived dendritic cells (BM-DCs) adenovirally transduced with genes encoding murine IL-23 have therapeutic benefits for antitumor immunotherapy. We made RGD fiber-mutant adenovirus (Ad) vectors encoding IL-23 or EGFP. The MCA205 fibrosarcoma was intradermally inoculated to C57BL/6 on day 8, the mice were injected intratumorally with BM-DCs transduced with Ax3CAmIL23/RGD (Ad-IL-23-DCs). The tumors of mice treated with AD-IL-23-DCs resulted in significant growth suppression when compared to that with BM-DCs transduced Ad-EGFP-F/RGD. Ad-IL-23-DCs treatment induced MCA-205-specific and potent CTL responses. In addition, the significant induction of IFN-γ and IL-17 and decrease of T-regs in TIL were strongly suggested in the mice injected with Ad-IL-23-DCs.

This strategy designed to deliver genetically modified DCs to tumor sites is associated with systemic and therapeutic antitumor immunity and could be an alternative approach to those using delivery of DCs loaded with defined tumor antigens. The evaluation of chronic inflammation in Ad-IL-23-DCs treatment using immunological analyses and immunohistochemical methods is currently on going.

II. Analysis of immunotherapy markers in oncology

Marimo Sato - Matsushita, Hideaki Tahara, Francesco M Marincola[#] ([#]Disease and Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, Associate Director National Institute of Health)

We focused on novel cutting-edge strategies suitable for high-throughput screening of clinical samples for the identification. Such biomarkers will be more likely identified by paired comparison of pre- and post-treatment samples, and selection and validation of biomarkers relevant to disease outcome and/or serve as surrogate equivalents to clinical outcome. The critical factor in identification of predictive markers for treatment is the availability of samples from patients homogenously treated within the treatment arm from clinical trials. Thus, collec-

tion of tissue samples needs to be mandated in each clinical trial. Paired pre-treatment and post-treatment samples collected at various time points need to be considered for these studies to identify optimal collection/measurement time points. Currently available high-throughput genomic/epigenetic/proteomic approaches for profiling of small amount of tissues should facilitate progress in this area. In the current study, we evaluated molecular profile of peripheral cells from healthy donors, chronically viraemic HCV-treatment-naïve patients and patients who spontaneously achieved virus eradication by whole genome gene expression analysis (GeneChipR Human Gene 1.0 ST Array - Affymetrix).

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

Marimo Sato-Matsushita, Hideaki Tahara

The secreted protein, milk fat globule epidermal growth factor-8 (MFG-E8), stimulates disease progression through coordinated vβ3 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 finding 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.

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

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

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, CD 11b$^+$ Ly-6G$^+$ neutrophils infiltrated into the inflammatory site primed by IL-17-producing NK1.1$^-$ CD 27$^-$ 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.

V. Development of robotized cell culture system

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In collaboration with Kawasaki Heavy Industries, Inc., we are developing robotized cell culture system which could be applied to a variety of procedures including virus production as a funded project by NEDO. In order to obtain information to develop this culture system, Dr. Wakitani is now developing clinical trials for regeneration of articular cartilage using manually cultured autologous bone marrow mesenchymal cell transplantation following the guideline for the clinical experiment using human stem cells.

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)

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In collaboration with the research team, we have prepared the replication-defective recombinant adenoviral vector expressing the suppressor of cytokine signaling 3 (SOCS3) 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.

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Advanced Clinical Research Center

Division of Clinical Genome Research
臨床ゲノム腫瘍学分野

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Research Projects
We have been working on the following four projects, 1) development of novel therapeutic strategies of human cancer, 2) functional analysis of molecules associated with human cancer, 3) establishment and investigation of mouse models of human cancer, and 4) development of novel diagnostic strategies for hereditary tumors. 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, Rui Yamaguchi[1], Seiya Imoto[2], and Satoru Miyano[1,2]: [1]Laboratory of Sequence Analysis, [2]Laboratory of DNA Information Analysis, Human Genome Center, IMSUT

It has become increasingly recognized that aberrant epigenetic modifications play an important role in carcinogenesis. Bromodomain containing protein BRD8, a component in TRRAP/TIP60-histone acetyltransferase (human NuA4) complex, has been reported to be implicated in human carcinogenesis. Previously, we found that BRD8 was accumulated in colorectal cancer, and that BRD8 interacted with proto-oncogene MRGBP (MRG-binding protein). We additionally found that BRD8 was a relatively short-lived protein and degraded through ubiquitin-proteasome pathway. Overexpression of wild-type MRGBP suppressed ubiquitination of BRD8, but that of mutant MRGBP lacking the binding region failed the suppression. Immunoprecipitation experiments revealed that the bromodomain of BRD8 was responsible for the interaction and ubiquitination. These findings suggest that degradation of BRD8 is inhibited by the suppression of ubiquitination through the interaction with MRGBP. Further analysis of BRD8 will contribute to a better understanding of colorectal carcinogenesis and the development of novel therapeutic strategies.

We have also identified SET and MYND domain containing 3 (SMYD3) as a novel therapeutic target for colorectal cancer. Accumulating evidence suggests that SMYD3 catalyzes methylation of histone lysines, with implications for human carcinogenesis. However, the molecular mechanism by which SMYD3 promotes cancer progression remains largely unknown. Since SMYD3 methylates histone tails and interacts with RNA polymerase II, we hypothesized that SMYD3 may regulate downstream genes through the simultaneous modulation of chromatin structure and the recruitment of transcription factors. To identify the downstream target gene(s), we recently explored gene expression profile of colorectal cancer cells treated with or without SMYD3 siRNAs using DNA microarray, and investigated SMYD3-associated regions by chromatin immunoprecipitation and sequencing (ChIP-seq) method. The analyses revealed a set of genes whose expression might be directly regulated by SMYD3. Further investigations through gene ontology and

pathway analyses using these data should be helpful for the better understanding of human carcinogenesis involving SMYD3 overexpression.

2. Functional analysis of Smyd3 *in vivo*

Kiyoshi Yamaguchi, Tsuneo Ikenoue, and Yoichi Furukawa

SMYD3 is a histone methyltransferase whose expression levels are enhanced in human colon, liver, and breast cancer. We have revealed that zebrafish Smyd3 plays a crucial role in morphogenesis of heart and skeletal muscle. To clarify the physiological function of Smyd3 in mammal, we have established Smyd3 knockout mice. Now, the phenotypes of their heart, muscle, as well as intestine are under investigation. We also analyze methylation status of histone tails in Smyd3 knockout mice. To clarify the roles of Smyd3 in intestinal tumorigenesis, further investigation will be performed by crossing the smyd3 knockout mice with Apc+/- mice, which are widely used as a model recapitulating human intestinal tumor.

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

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Genetically engineered mice are useful tools for studying human diseases, including cancer. In this project, we have successfully established mouse model of intrahepatic cholangiocarcinoma 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.

We have also generated a mouse strain carrying a conditional knockin allele of the *Fbxw7* gene, which is frequently mutated in human colon and liver 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. Genetic diagnosis using next generation sequencer

Kiyoshi Yamaguchi, Tsuneo Ikenoue, Yoichi Furukawa, Seiya Imoto[1], Mitsuhiro Komura[1], Yuichi Shiraishi[1], Teppei Shimamura[1], Rui Yamaguchi[2], Tetsuo Shibuya[2], and Satoru Miyano[1,2]: [1]Laboratory of DNA Information Analysis, [2]Laboratory of Sequence Analysis, Human Genome Center

Improved sequencing technologies have enabled us to identify genetic variations of human genome in individuals and neoplasms. Biliary tract cancer is one of the most frequent cancers in Japan. Although genetic alterations in this type of cancer have been studied for various genes, the profile of mutations in Japanese patients have not been fully understood. We recently searched for genetic alterations in Japanese biliary tract cancer tissues by multiplex PCR-based targeted enrichment and next generation sequencing (NGS). Our findings may be useful for developing a personalized approach to cancer treatments.

In collaboration with Human Genome Center, we started two studies using NGS last year; 1) the determination of germ-line mutations in patients suspected for hereditary colorectal cancer, and 2) identification of somatic mutations in hematopoietic malignancies and solid tumors. Using NGS and a highly secure supercomputer system, we performed whole genome sequencing to identify pathogenic mutation in two patients with colonic polyposis without family history. As a result, we identified more than 4.6 million germ line variants in one of the two patients. Among them, approximately thirty thousands were located in exons or splicing sites. Interpretation of the variants is now ongoing. Additionally somatic mutations in their polyps are also under investigation. These studies are aimed to return the data of personal genome and/or cancer genome to patients in IMSUT Hospital, and apply them to their diagnosis and treatment.

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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 therapy 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 a 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, and malignant melanoma 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 HSVs-1 that util-

ize tumor/tissue-specific promoters have been created that replicates efficiently in CSCs as well as slow growing tumor cells.

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

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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. Identification of functional SNPs in the promoter region of MICA which alter transcriptional activity

Ryosuke Muroyama, Kaku Goto, Wenwen Li, Ryo Nakagawa, Norie Kowatari, Chisiro Wakabayashi, Yasuo Matsubara, Naoya Kato

Hepatocellular carcinoma (HCC) reveals a very high mortality rate in the world, and hepatitis B virus (HBV) or hepatitis C virus (HCV) is one of the major etiological factors for developing HCC. Previously, we reported SNP (rs2596542) located in the promoter region of MHC class I polypeptide-related chain A (MICA) was significantly associated with the risk of HBV/HCV-induced HCC and also with serum levels of soluble MICA. However, functional SNPs were not fully elucidated so far because SNPs in the MICA locus show strong linkage disequilibrium. In this study, we tried to identify functional SNPs which alter transcriptional activity of MICA. We constructed MICA promoter-reporter plasmids using the sequence from HLE cells (G allele of rs2596542) or Huh7 cells (A allele of rs 2596542), and compared the transcriptional activity between them by luciferase assay. The result showed that the promoter sequence which had G allele of rs2596542 exhibited 3-4 folds higher transcriptional activity than that which had A allele of rs2596542. We further analyzed the difference of transcriptional activity between them by reporter plasmids which containing serial truncated promoter region of MICA, and found that two functional SNPs (rs6906175 and 2301750) in the promoter region of MICA altered transcriptional activity. Therefore, these functional SNPs might be associated with the expression of MICA and the risk of HBV/HCV-induced HCC.

2. Small molecules for MICA expression regulation

Kaku Goto[1], Masahisa Jinushi[2], Ryosuke Muroyama[1], Wenwen Li[1], Ryo Nakagawa[1], Norie Kowatari[1], Chisiro Wakabayashi[1], Yasuo Matsubara[1], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Research Center for Infection-Associated Cancer, Institute for Genetic Medicine, Hokkaido University

A genome-wide association study (GWAS) identified an anti-tumor ligand MHC class I polypeptide-related sequence A (MICA) to be a genetic susceptibility factor for HCV-induced hepatocellular carcinoma (HCC). Lower levels of MICA expression were associated with the elevated risk of HCC de-

velopment in patients, implying preventive effects of MICA expression induction on hepatocarcinogenesis. Hence we sought to find small molecules for regulation of MICA expression. Sodium butyrate (NaB), a well-known HDAC inhibitor and a reported MICA inducer in multiple cancer cell lines, significantly upregualted the MICA mRNA levels in hepatoma cells. After the construction of active luciferase reporters encoding MICA promoter sequences, stable hepatoma cell transformants harboring the reporters were established via antibiotics selection, which responded to the NaB treatment in a dose-dependent fashion likewise. Using the luciferase reporter cell system, a primary screen for an FDA-approved drug library discovered that multiple drugs including an anti-cancer agent induced the MICA expression significantly at mRNA and protein levels, and the mode and impact of the ligand modulation are currently investigated. Findings in this study would expectedly serve to develop anti-tumor immunotherapies in virus-induced HCC.

3. Identification of a Functional Variant in the MICA Promoter Which Regulates MICA Expression and Increases HCV-Related Hepatocellular Carcinoma Risk

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Hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma (HCC) in Japan. We previously identified the association of SNP rs 2596542 in the 5' flanking region of the MHC class I polypeptide-related sequence A (MICA) gene with the risk of HCV-induced HCC. In the current study, we performed detailed functional analysis of 12 candidate SNPs in the promoter region and found that a SNP rs2596538 located at 2.8 kb upstream of the MICA gene affected the binding of a nuclear protein(s) to the genomic segment including this SNP. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, we identified that transcription factor Specificity Protein 1 (SP1) can bind to the protective G allele, but not to the risk A allele. In addition, reporter construct containing the G allele was found to exhibit higher transcriptional activity than that containing the A allele. Moreover, SNP rs2596538 showed stronger association with HCV-induced HCC ($P = 1.82 \times 10 - 5$ and $OR = 1.34$) than the previously identified SNP rs2596542. We also found significantly higher serum level of soluble MICA (sMICA) in HCV-induced HCC patients carrying the G allele than those carrying the A allele ($P = 0.00616$). In summary, we have identified a functional SNP that is associated with the expression of MICA and the risk for HCV-induced HCC.

4. A genome-wide association study of HCV induced liver cirrhosis in the Japanese population identifies novel susceptibility loci at MHC region

Yuji Urabe[1,2], Naoya Kato[3], Vinod Kumar[1], Ryosuke Muroyama[3], Motoyuki Otsuka[4], Ryosuke Tateishi[4], Paulisally Hau Yi Lo[1], Chizu Tanikawa[1], Masao Omata[4], Kazuhiko Koike[4], Michiaki Kubo[5], Kazuaki Chayama[2], Yusuke Nakamura[1], Koichi Matsuda[1]: [1]Laboratory of Molecular Medicine, Human Genome Center, IMSUT; [2]Departments of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University; [3]Unit of Disease Control Genome Medicine, IMSUT; [4]Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo; [5]Center for Genomic Medicine, RIKEN

We performed a genome-wide association study (GWAS) of hepatitis C virus (HCV)-induced liver cirrhosis (LC) to identify predictive biomarkers for the risk of LC in patients with chronic hepatitis C (CHC). A total of 682 HCV-induced LC cases and 1,045 CHC patients of Japanese origin were genotyped by Illumina Human Hap 610-Quad bead Chip. Eight SNPs which showed possible associations ($P<1.0 \times 10(-5)$) in the GWAS stage were further genotyped using 936 LC cases and 3,809 CHC patients. We found that two SNPs within the major histocompatibility complex (MHC) region on chromosome 6p21, rs910049 and rs3135363, were significantly associated with the progression from CHC to LC ($P(\text{combined}) = 9.15 \times 10(-11)$ and $1.45 \times 10(-10)$, odds ratio $(OR) = 1.46$ and 1.37, respectively). We also found that HLA-DQA1*0601 and HLA-DRB1*0405 were associated with progression from CHC to LC ($P = 4.53 \times 10^{-4}$ and 1.54×10^{-4} with $OR = 2.80$ and 1.45, respectively). Multiple logistic regression analysis revealed that rs3135363, rs 910049, and HLA-DQA1*0601 were independently associated with the risk of HCV-induced LC. In addition, individuals with four or more risk alleles for these three loci have a 2.83-fold higher risk for LC than those with no risk allele, indicating the cumu-

lative effects of these variations. Our findings elucidated the crucial roles of multiple genetic variations within the MHC region as prognostic/predictive biomarkers for CHC patients.

5. AMPK-related kinase SNARK in chronic HCV infection

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Host cellular cofactors for hepatitis C virus (HCV) infection are recognized as attractive antiviral targets due to their independence from viral sequence. Our genome-wide RNAi screen for host cellular cofactors (Tai AW *et al*., Cell Host Microbe 2009) identified that sucrose, non-fermenting 1/AMP-activated protein kinase-related kinase (SNARK) positively regulated HCV replication. We therefore sought to clarify the mechanisms of reciprocal regulation between SNARK and HCV. Knockdown of SNARK decreased levels of HCV replication in both OR6 replicon and JFH1 infection systems. Overexpressed siRNA-resistant wild type SNARK rescued the suppressed viral replication, which was abrogated by either a kinase deficiency or phospho-deficient mutation. Conversely, SNARK mRNA level was upregulated by HCV infection in patients and cell culture, deranging cellular signalings. These SNARK-mediated effects on both virus and host were cancelled by a SNARK kinase inhibitor. Hence viral induction of the proviral kinase was speculated to promote HCV pathogenesis. We are presently investigating substrates and signalings targeted by the kinase and their pharmacological regulation.

6. Mutations in hepatitis B Virus (HBV) X region are hepatocellular carcinoma risk factors for HBV genotype C infected patients

Wenwen Li[1], Ryosuke Muroyama[1], Kaku Goto[1], Ryo Nakagawa[1], Norie Kowatari[1], Chishiro Wakabayashi[1], Yasuo Matsubara[1], Qiang Li[2], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Jinan Infectious Disease Hospital, Shandong University, Jinan, China

Chronic hepatitis B virus (HBV) infection accounts for approximately 50% of the hepatocellular carcinoma (HCC) cases worldwide and even 80-90 % in the areas where HBV is highly prevalent. The role of virus mutation in the interaction between virus and host during carcinogenesis is still unclear, especially in patients infected with specific genotypes considered to be more oncogenic than other ones.

Aim: We sought to clarify potential HCC characteristic mutations in HBV genotype C infected patients. HBV genotype C sequences were downloaded from online global databases. Sequences were then screened based upon the criteria: 1. Full length HBV X; 2. genotype C; 3. human sera origin; 4. with diagnosis information and thus classified into Non-HCC or HCC group. Continuous data were expressed as Mean ± SD and were compared by t-test. Categorical data were analyzed by Fisher's exact test (SPSS 16.0). $P<0.05$ was considered to be significant difference. Logistic regression was performed to evaluate the effects of mutations on HCC risk. 1) Four hundred and ninety-five HBV genotype C sequences (HCC: 153; Non-HCC: 342) were finally extracted out of the downloaded 5380 HBV X sequences. 2) Twenty nucleotide positions showed significantly different distribution between HCC and Non-HCC groups. Six of them were also located in overlapped Enhancer 2 (Enh2) region and 14 in overlapped core promoter (CP) region. 3) Logistic regression showed that mutations A1383C (OR: 2.00, 95% CI: 1.08-3.71), A1479C/G/T (OR: 2.93, 95% CI: 1.49-5.79; OR: 2.79, 95% CI: 1.32-5.90; OR: 6.70, 95% CI: 2.81-15.99), C1485T (OR: 2.63, 95 % CI: 1.50-4.60), C1653T (OR: 1.95, 95% CI: 1.15-3.31), and A1762T (OR: 1.85, 95% CI: 1.01-3.40) were independent risk factors for genotype C HBV-related HCC. Five point mutations in genotype C HBV X region were risk factors of HCC though these mutations seemed to be more associated with the disease progression than HCC. Further longitudinal studies are needed to verify the roles of these mutations in earlier disease stages and the procession of oncogenesis.

7. Novel drug screening system based on the essential role of HBx trans-activation activity in HBV replication

Wenwen Li[1], Kaku Goto[1], Ryosuke Muroyama[1], Ryo Nakagawa[1], Chishiro Wakabayashi[1], Norie Kowatari[1], Yasuo Matsubara[1], Qiang Li[2], Naoya Kato[1]: [1]Division of Advanced Genome Medicine, IMSUT; [2]Jinan Infectious Disease Hospital, Shandong University, Jinan, China

Current efficacy of anti-hepatitis B virus (HBV) drugs is limited in eradicating nuclear covalently closed circular DNA (cccDNA), a vital factor helping the establishment of persistent HBV infection, which necessitates the development of novel anti-HBV drugs for eliminating cccDNA. One of the HBV proteins, HBx, has been proved to be essential for HBV replication through the interaction with cccDNA and the transactivation function. Therefore, we hypothesized that suppressing HBV cccDNA via inhibition of HBx transcriptional transactivity should be a promising therapeutic strategy against

HBV. Hence, we constructed genotype C HBx encoding plasmids and then examined the effects of HBx on major signaling pathways reported ever (i. e., NF-kappaB, AP-1, SRE, etc.) by luciferase assay, out of which two most stimulated signalings were selected. Subsequently, those luciferase reporters were stably transfected in a hepatoma cell line, and clones with lower backgrounds and high sensitivity were isolated. Meanwhile a Tet-on system controlled HBx-encoding plasmid is also under construction. We will establish a dual stable transfectant hepatoma cell line with two types of plasmid, a signaling pathway luciferase reporter and the HBx Tet-on system vector, using multiple antibiotics selection. Finally, drug libraries, an FDA-approved drug library and the one from the open innovation center for drug discovery in the University of Tokyo, will be used for new drug screenings. Potential molecular mechanism of the interaction between viral onco-protein and host signaling pathways will be further analyzed.

8. Specifically expressed miRNA in CD4$^+$ T cells participates in the pathogenesis of primary biliary cirrhosis

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Primary biliary cirrhosis (PBC) is a chronic inflammatory autoimmune liver disease. Although detailed mechanisms of the pathogenesis of PBC remain unknown, CD4$^+$ T cells are suggested to play an important role. Recently microRNA (miRNA) was reported to be involved in the pathogenesis of PBC.

Aim: We analyzed the expression profile of miRNA and their target genes in CD4$^+$ T cells of PBC patients to reveal their participation in pathogenesis of PBC. 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. Total RNA, including miRNA, was extracted from CD4$^+$ T cells purified from peripheral blood. The comprehensive analysis of miRNA was conducted using microarray and quantitative real-time PCR (qRT-PCR). We predicted the target genes of miRNA, which was expressed specifically in PBC, using bioinformatics. The dynamics of predicted target genes were analyzed by microarray and qRT-PCR. Then, luciferase assay and miRNA mimic assay were performed to examine the binding of the specific miRNA to 3′untranslated region (3′UTR) of target genes. Finally, we tested the potential role of specifically expressed miRNA against target genes by overexpressing miRNA in cultured cells. Microarray miRNA study showed 2 increased and 13 decreased miRNAs in PBC ($p<0.05$). Among them, 5 miRNAs were validated to be down-regulated in PBC ($p<0.05$) by qRT-PCR. A total of 4,855 target genes were predicted from 5 miRNAs by bioinformatics. In the mRNA microarray analysis, the expression of 2,565 genes was significantly different between PBC and control. Comparison of the target prediction and gene expression microarray study revealed 238 target genes were specifically expressed in PBC. Among 238 target genes, we analyzed 10 genes that were the target for more than 3 specifically expressed miRNAs to reveal the regulation of the target genes by miRNAs. The expression of 3 target genes, which were reported to be associated with T cell development and function, were validated by qRT-PCR. Luciferase assay and miRNA overexpression assay demonstrated that the specific miRNAs regulate these target genes by binding to their 3′ UTR. We have identified PBC specific expression of miRNA and their target genes. These miRNA may participate in the immunological pathogenesis of PBC through the regulation of the target genes in CD4$^+$T cells.

9. Novel zinc finger protein in gastrointestinal tract

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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. The aim of this study is to find another factor involved in segmental formation of gastrointestinal tract. Crude extracts from gastrointestinal tract biopsy specimens: esophagus, gastric fundus, duodenum and colon were subjected to PAGE. Specific band (less than 40kDa) in the gastric fundus sample was analyzed by mass spectrometry, and ZNF-114-like hypothetical protein deduced from genome sequence was identified. Its mRNA sequence was determined by RACE. Functional

analysis will be performed using expression vectors.

10. IL28B minor allele is associated with a younger age of onset of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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IL28B polymorphisms were shown to be associated with response to peg-interferon based treatment in chronic hepatitis C (CHC) and spontaneous clearance. However, little is known about how this polymorphism affects the course of CHC, including the development of hepatocellular carcinoma (HCC). The aim of this study is to evaluate the influence of IL28B polymorphisms on hepatocarcinogenesis in CHC patients. We genotyped the rs8099917 single-nucleotide polymorphism in 351 hepatitis C-associated HCC patients without history of IFN-based treatment, and correlated the age at onset of HCC in patients with each genotype. The mean ages at onset of HCC for TT, TG, and GG genotypes were 69.9, 67.5 and 66.8, respectively. In multivariate analysis, IL28B minor allele (TG and GG genotypes) was an independent risk factor for younger age at onset of HCC ($p = 0.02$) in males ($p < 0.001$) with higher body mass index (BMI; $p = 0.009$). IL28B minor allele was also associated with a lower probability of having aspartate aminotransferase-to-platelet ratio index (APRI)>1.5 (minor vs. major, 46.7% vs. 58.6%; $p = 0.01$), lower AST (69.1 vs. 77.7 IU/l, $p = 0.02$), lower ALT (67.8 vs. 80.9 IU/l, $P = 0.002$), higher platelet count (12.8 vs. 11.2×104/μl, $p = 0.002$), and higher prothrombin time (79.3% vs. 75.4%, $p = 0.002$). In conclusion, IL28B minor allele was associated with lower inflammatory activity and less progressed fibrosis of liver, however, constituted a risk factor for younger-age onset of HCC in CHC patients.

11. Impact of IL28B genetic variation on HCV-induced liver fibrosis, inflammation, and steatosis: a meta-analysis

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IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression. A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses. We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003-1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988-1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050-1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110-1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351-0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415-2.717; P = 0.901). IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs 8099917 TT genotypes.

12. Patatin-like phospholipase-3 (rs738409 C>G) polymorphism is associated with the development of hepatocellular carcinoma in patients with chronic hepatitis C infection

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An isoleucine to methionine substitution at position 148 in the PNPLA3 gene (p.I148M, rs738409) has recently been identified as a susceptibility factor for liver damage in steatohepatitis. However, little is known about the influence of this polymorphism on hepatocarcinogenesis. The aim of this study is to assess the impact of PNPLA3 polymorphism on the development of hepatocellular carci-

noma (HCC) which is thought to be one of the major steatosis-related complications in patients with chronic hepatitis C. We genotyped the rs738409 single-nucleotide polymorphism (SNP) in 358 hepatitis C-related HCC patients, and correlated the age at onset of HCC and the duration between the hepatitis C virus (HCV) infection and the development of HCC. The median age at onset of HCC for the GG genotype was significantly younger compared to for non-GG genotypes (67.81 *vs.* 69.87 years, $P < 0.001$), and the median interval between HCV infection and the development of HCC was significantly shorter in patients with the GG genotype (39.96 *vs.* 40.85 years, $P = 0.008$). PNPLA3 GG genotype was also associated with a higher AST level (69.5 *vs.* 59.0 IU/l, $P = 0.02$), lower prothrombin time (73.0% *vs.* 78.0%, $P = 0.008$), and a higher prevalence of histological steatosis (40.0% *vs.* 22.2%, $P = 0.01$) at the time of HCC onset. In conclusion, the PNPLA3 genotype GG may be associated with accelerated hepatocarcinogenesis in CHC patients through increased steatosis in the liver.

13. SOCS1 abrogates IFN's antiviral effect on hepatitis C virus replication

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Suppressor of cytokine signaling 1 (SOCS1) and suppressor of cytokine signaling 3 (SOCS3) have been thought to block type I interferon (IFN) signaling. We have previously reported that SOCS3 suppresses HCV replication in an mTOR-dependent manner. However, the relationship between SOCS1 and HCV replication remains unclear. Here, we found that overexpression of SOCS1 alone did not have an effect on HCV RNA replication. However, suppression of HCV replication by IFN-α was rescued by SOCS1 overexpression. The upregulation of HCV replication by SOCS1 overexpression in the presence of IFN is likely a result of the impairment of IFN signaling by SOCS1 and subsequent induction of ISGs. Knockdown of SOCS1 alone with specific shRNA enhanced the antiviral effect of IFN compared with negative control. Thus, SOCS1 acts as a suppressor of type I IFN function against HCV.

14. Kinetic differences in the induction of interferon stimulated genes by interferon-α and interleukin 28B are altered by infection with hepatitis C virus

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Several genome-wide association studies (GWAS) have identified a genetic polymorphism associated with the gene locus for interleukin 28B (IL28B), a type III interferon (IFN), as a major predictor of clinical outcome in hepatitis C. Antiviral effects of the type III IFN family have previously been shown against several viruses, including hepatitis C virus (HCV), and resemble the function of type I IFN including utilization of the intracellular Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway. Effects unique to IL28B that would distinguish it from IFN-α are not well defined. By analyzing the transcriptomes of primary human hepatocytes (PHH) treated with IFN-α or IL28B, we sought to identify functional differences between IFN-α and IL28B to better understand the roles of these cytokines in the innate immune response. Although our data did not reveal distinct gene signatures, we detected striking kinetic differences between IFN-α and IL28B stimulation for interferon stimulated genes (ISGs). While gene induction was rapid and peaked at 8 hours of stimulation with IFN-α in PHH, IL28B produced a slower, but more sustained increase in gene expression. We confirmed these findings in the human hepatoma cell line Huh7.5.1. Interestingly, in HCV-infected cells the rapid response after stimulation with IFN-α was blunted, and the induction pattern resembled that caused by IL28B. Conclusion: The kinetics of gene induction are fundamentally different for stimulations with either IFN-α or IL28B in hepatocytes, suggesting distinct roles of these cytokines within the immune response. Furthermore, the observed differences are substantially altered by infection with HCV.

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
Senior Assistant Professor (Project)	Masanori Nojima, M.D., PhD, M.P.H.		特任講師	医学博士	野　島　正　寛

Division of Advanced Medicine Promotion was established in December 2011. Our mission is to assist the development 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 utilization. In this sense, our role is the translation from the results of basic science at our Institute to the conduct of clinical trials at the Research Hospital. At Research 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

Noriko Fujiwara, Makiko Karasawa, Masanori Nojima, Fumitaka Nagamura

In Research Hospital, we work together with staffs of Department of Clinical Trial Safety Management. 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 Department of Clinical Trial Safety Management.

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

Noriko Fujiwara, Makiko Tajima, 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 planed 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. Statistical consulting.

Masanori Nojima, Fumitaka Nagamura

We have started statistical consulting in April, 2013. This consulting is for study design and statistical analysis in any research including clinical re-

search (confirmatory and exploratory), basic medical/biological research. We have collaborated with other 12 departments in IMSUT this year through the consulting.

4. Statistical education program

We also held statistical education program using EZR (Bone Marrow Transplantation 2013: 48, 452-458) from September to December in 2013. EZR is a graphical user interface based on R, a free programming language for statistical analysis. The program consisted of 6 sections (listed below), and total 38 researchers were attended.

1. Data Handling/Two-group Comparison
2. Confounding and Interaction—Stratified Analysis and Multivariate Analysis—
3. Diagnosis, Multiple Comparisons, Analysis for Paired Data, Nonparametric Method
4. Survival Analysis
5. Practice of Multivariate Analysis—Variable Selection—
6. Confirmation of Hypothesis and Sample Size

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長村文孝　米国FDAにおける抗がん剤の審査　医薬品・医療機器承認取得のためのデータ・情報の取得とまとめ方　技術情報協会　印刷中

長村文孝　絶対に必要な医学の基礎知識&その他のがん　がん患者のこころに寄り添うために　実践的サイコオンコロジー　真興社　印刷中