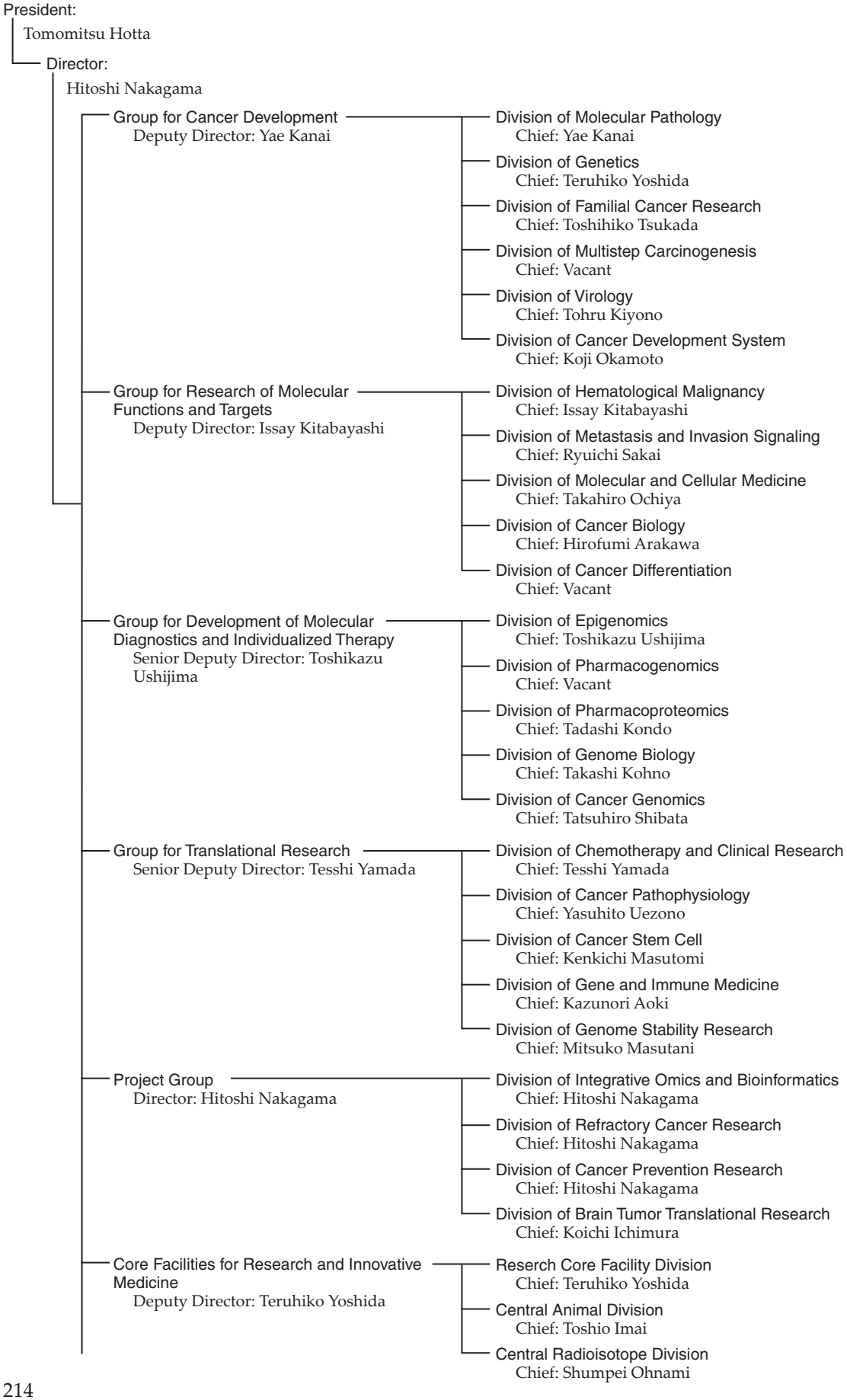
Research Institute

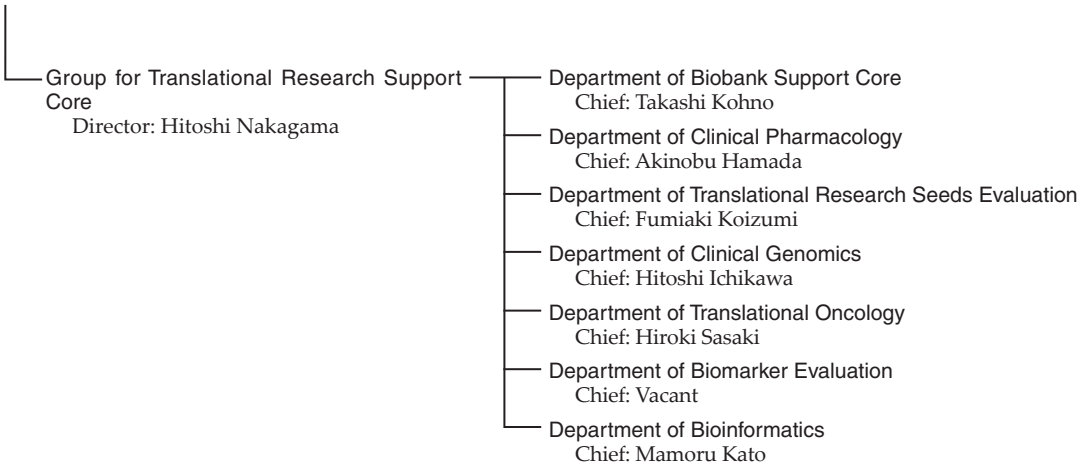
Preface

The National Cancer Center Research Institute (NCCRI) was established in 1962 as a department of the National Cancer Center (NCC), and has been the nation's leading cancer research institute for more than 50 years. The NCCRI is now internationally recognized for its major contributions to various aspects of cancer research worldwide. The mission of the NCCRI is to advance our knowledge of cancer prevention, diagnosis and therapy, toward the ultimate goal of cancer control. Collaborative research integration between other departments of the NCC, including NCC Hospitals, and the Research Institute is highly encouraged. The NCCRI is now composed of 25 divisions, and they are sub-grouped into four major Research Groups and one Project Group; namely, the Group for Cancer Development and Progression, Group for Research into Molecular Functions and Targets, Group for Development of Molecular Diagnostics and Individualized Therapy and Group for Translational Research and Project Group. Core Facilities for Research and Innovative Medicine, which consist of the Central Animal Division, Central Radioisotope Division and Core Facility Division, provide several kinds of technical support for molecular biology, high-throughput omics-type analyses, biological analysis and animal experiments for researchers in both the Research Institute and Hospitals to further encourage and facilitate the development of translational-type studies in our Institute. The NCCRI currently has approximately 90 research staff, around 30 postdoctoral fellows, and more than 180 supporting staff. Foreign scientists and research fellows are also welcomed on a regular basis. We launched the Group for Translational Research Support Core (consisting of 5 departments) to further facilitate and support the activities of TR projects, conducted between Hospital, Research Institute and other internal sections. The "Annual Report" of the NCCRI summarizes the recent research activities of each division, which cover the following areas: (i) environmental human carcinogens and cancer chemoprevention, including the use of animal models; (ii) clarification of molecular mechanisms underlying cancer development, invasion and metastasis; (iii) investigation of genetic and epigenetic alterations in a variety of cancers; (iv) clarification of the molecular bases underlying the susceptibility to cancer development; (v) exploration of novel biomarkers with diagnostic, therapeutic and prognostic value; and (vi) functional analyses of various cancer-related genes. We have also been participating in worldwide research consortia, such as the International Cancer Genome Consortium (ICGC), International Human Epigenome Consortium (IHEC), and International Cancer Biomarker Consortium (ICBC). We further encourage our members to develop international collaborative research projects in various other areas. The activities of the research institute can also be viewed on the home page: http://www.ncc.go.jp/en/nccri/index.html.

Hitoshi Nakagama, M.D., D.M.Sc.
Director, National Cancer Center Research Institute

Organization




Group for Translational Research Support Core
Director: Hitoshi Nakagama
Department of Biobank Support Core
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Department of Clinical Pharmacology
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Department of Translational Research Seeds Evaluation
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Department of Biomarker Evaluation
Chief: Vacant
Department of Bioinformatics
Chief: Mamoru Kato

Activities of the Divisions

Division of Molecular Pathology

Yae Kanai, Nobuyoshi Hiraoka, Shigeki Sekine, Masahiro Gotoh, Hidenori Ojima, Eri Arai, Taisuke Mori, Ying Tian, Masumi Tanaka, Takashi Sato, Takuya Yotani, Yuriko Yamada, Ayako Shibuya, Nanako Itoh, Michiko Suzuki

Introduction

Research in the Division of Molecular Pathology is based on a combination of clinicopathological observations and molecular pathological analyses.

Multilayer omics analysis in human cancers for personalized and preemptive medicine

We have participated in the Research Project "Comprehensive exploration of drug targets based on multilayer/integrative disease omics analyses" as a PI supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. Based on a single-CpG-resolution genome-wide DNA methylation analysis (methylome analysis) using the Infinium BeadChip system in 240 renal tissue specimens, we identified the CpG island methylator phenotype (CIMP) of clear cell renal cell carcinomas (RCCs), which is characterized by accumulation of DNA hypermethylation of CpG islands, clinicopathological aggressiveness and poorer patient outcome (Figure 1). We also have identified 17 RCC-specific CIMP marker genes. In order to establish the criteria for CIMP diagnosis, DNA methylation levels at 299 CpG sites of entire promoter CpG islands in the RCC-specific CIMP marker genes were evaluated quantitatively using a MassARRAY system. Receiver operating characteristic curve analysis showed that the area under the curve values for the 32 CpG sites were larger than 0.95. Criteria combining the 32 CpG sites discriminated CIMP-positive from CIMP-negative RCCs with 100% sensitivity and specificity in the learning cohort. Cancer-free and overall survival rates of patients with CIMP-positive RCCs were significantly lower than those of patients with CIMP-negative RCCs in the validation cohort consisting of the 100 patients. Patients with CIMP-positive RCCs in the validation cohort had a higher likelihood of both recurrence and disease-related death (hazard ratios 10.7 and 77.1, respectively). We have filed patent applications for prognostication of RCC patients using the established criteria (US61/646,044, PCT/JP2013/62650). We are now developing a new device, which is specialized to DNA methylation diagnosis and widely applicable for clinical use, in collaboration with a medical device company (Figure 1).

To reveal the molecular pathways significantly participating in CIMP-positive renal carcinogenesis, genome (whole-exome), transcriptome and proteome analyses were performed in the collaborative project study. A signaling pathway most frequently affected by multilayer omics abnormalities in CIMP-positive RCCs was identified as the potential therapeutic target (Figure 1). The effectiveness of the inhibitor of the identified pathway is now being examined in CIMP-positive RCC cell lines. Since the potential therapeutic target has been identified for clinically aggressive CIMP-positive RCCs, our criteria for CIMP diagnosis may be useful for not only prognostication but also companion diagnosis for personalized medicine.

With respect to lung carcinogenesis, a single-CpG-resolution methylome analysis in 414 lung tissue specimens reveled that DNA methylation profiles reflecting carcinogenetic factors, *i.e.*, smoking and chronic obstructive lung disease, are established even at the precancerous stage and DNA methylation alterations at the precancerous stages may determine tumor aggressiveness and patient outcome. Multilayer omics analysis identified *ADCY5 and EVX1* as potential therapeutic targets of lung adenocarcinomas. With respect to gastric carcinogenesis, a single-CpG-resolution methylome analysis in 220 gastric tissue specimens revealed that DNA methylation profiles at the precancerous stages associated with *Helicobacter pylori* infection and/or chronic atrophic gastritis were inherited by gastric cancer tissues themselves and determined tumor aggressiveness and patient outcome.

Activities in The International Human Epigenome Consortium (IHEC)

In 2010, the IHEC was established to comprehensively characterize the heterogeneity of standard epigenome profiles of multiple normal cell lineages from different human populations and to construct the epigenome database as an international research basis. Researchers and founding agencies from Canada, the EU, Italy, Germany, Japan, South Korea and the USA currently participate in the IHEC to decipher at least 1000 epigenomes (epigenome maps of 1000 cell lineage-person) within the next

7-10 years. We have participated in the IHEC as a PI supported by the Core Research for Evolutional Science and Technology (CREST) project by the Japan Science and Technology (JST) Agency. In collaboration with research groups in the National Cancer Center (NCC) and the University of Tokyo, we perform whole-genome sequencing, whole-genome bisulfite sequencing using the post-bisulfite adaptor-tagging method, chromatin immunoprecipitation-sequencing and RNA-sequencing of various cell lineages of the gastrointestinal and urinary systems.

As a contribution to the IHEC program, we first focused on epigenome mapping in hepatocytes purified from normal liver tissue and diseased liver tissue with hepatitis C virus (HCV) or hepatitis B virus (HBV)-infection. Hepatocytes were purified by collagenase perfusion via canulated hepatic veins in partial hepatectomy specimens followed by low-velocity centrifugation. More than 95% purity was immunocytochemically confirmed. Personal differentially methylated regions (pDMRs) and DMRs associated with HCV or HBV infection were identified. We are examining genome-epigenome crosstalk, *i.e.* correlations among pDMR, single-nucleotide variation and copy number variation. Under the supervision of the IHEC, we intend to disclose the data through the National Bioscience Database Center supported by the JST. Accurate standard epigenomic profiles of digestive and urogenital organ epithelial cells obtained through IHEC activities will be used to explore more useful biomarkers of and drug targets for cancers.

Clinicopathological studies of human cancers based on the practice of diagnostic pathology

Using morphological, histological, immunohistochemical and molecular pathological approaches, diagnostic and prognostic criteria which are applicable to histological specimens were explored. We collect tissue samples for the NCC Biobank and contribute to collaborative studies through providing clinicopathological information. In addition, from surgically resected materials, cancer cell lines and mouse xenograft models have been established.

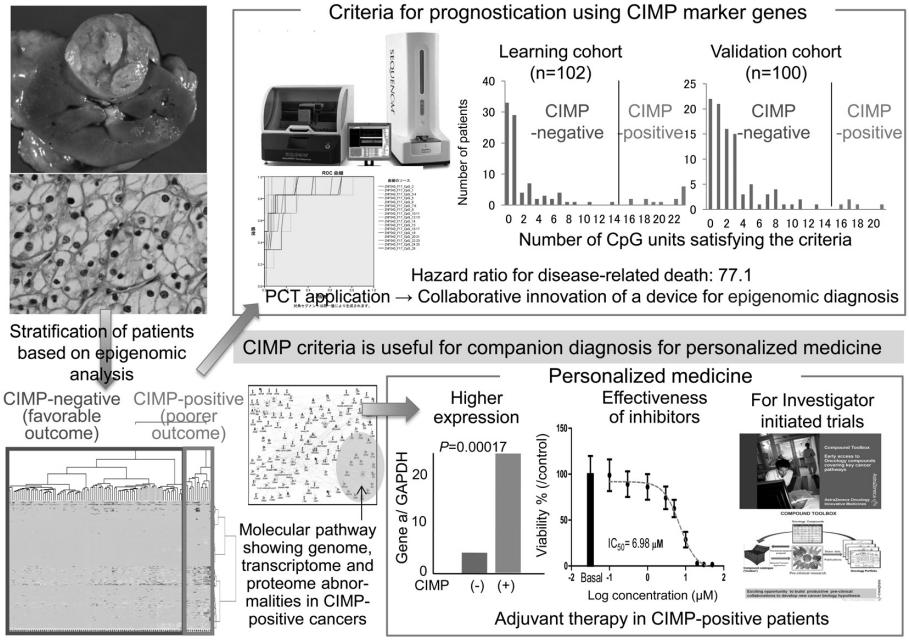

Figure 1. Personalized medicine based on epigenomic analysis of renal cell carcinomas

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Division of Genetics

Teruhiko Yoshida, Hiromi Sakamoto, Fumiaki Koizumi, Hiroki Sasaki, Hitoshi Ichikawa, Norihisa Saeki, Kazuhiko Aoyagi, Kazuyoshi Yanagihara, Sumiko Ohnami, Mineko Ushiama, Yoko Odaka, Misuzu Okuyama, Sachiyo Mitani,YousukeTomokuni, Akiko Takahashi, Masumi Shimizu, Mika Shioya, Sayaka Mito, Hiroo Takahashi, Hiroe Sakuyama, Nozomi Nakata,Noriko Koyama

Introduction

In 2013, 2 laboratory heads of the Division of Genetics, Drs. Koizumi and Sasaki were promoted to Department chiefs, and whose research activities are shown separately. The 2 major research themes of the Division were 1) molecular understanding of cancer susceptibility; and 2) development of personalized cancer diagnosis and treatment of gastric cancer.

Routine activities

We have maintained our participation in the biobanking project of the Tsukiji campus of the National Cancer Center (NCC), particularly for the peripheral blood samples.

Research activities

1) Molecular understanding of cancer susceptibility

Our previous genome-wide associated study (GWAS) identified the PSCA and MUC1 gene polymorphisms as the 2 major genetic factors for diffusetype gastric cancer. Although the associations have been successfully replicated by several laboratories, we believe that the functional understanding of the genes is important for the development of reliable preventive methods. The function of the PSCA protein has been explored by its expression pattern in other cancers and tissues and by searching the molecules associated with the PSCA protein. Whereas PSCA is up-regulated in many types of cancer, the protein was found to be down-regulated in both gallbladder and gastric cancers. In gallbladder cancer cells, the risk allele of the rs2294008 missense SNP attenuated the anti-tumor effects of the PSCA gene. However, the binding protein for PSCA has not been identified yet, and the PSCA signaling pathway still remains unknown in spite of several experimental approaches. So far, it has not been an easy task to unveil a signaling pathway and functional role of PSCA in carcinogenesis. Nonetheless, we would like to keep challenging, because the association per se appears to be solid in several ethnic groups, and the risk allele has a high frequency in the Japanese population (0.62); the combination of the PSCA and MUC1 polymorphisms would give a strong starting point to develop a combined genetic and life-style/environment profile to identify the high-risk group for diffuse-type gastric cancer, which may be less prone to decrease along with the decline of the *Helicobacter pylori* infection than the intestinal-type counterpart.

In the study on a neuroblastoma susceptibility gene, LMO1, which encodes a transcriptional regulator, several target genes of the LMO1 regulation have been identified through chromatin immunoprecipitation and DNA sequencing (ChIP-Seq).

Through collaboration with Dr. Haruhiko Sugimura at Hamamatsu University, School of Medicine, familial and/or young-onset gastric cancer cases have been analyzed with germline whole-exome sequencing (WES). We have been actively involved in the peripheral blood collection and DNA/RNA extraction in the NCC biobank project to accumulate further validation cases. Eighteen patients from 16 pedigrees with familial and/or early-onset gastric cancer were analyzed as cases with WES and SNP array analyses for structural variations. The candidate gene search from the WES data is underway based on several informatics criteria. The crucial step of the whole-exome/genome sequencing for the germline variations is an effective filtering and selection of the candidates and their validation. Two critical fundamentals for the further investigation are the large-scale Japanese reference genome sequence database and a biobank of germline DNA. We ourselves obtained the WES data of 165 control subjects from the participants of the population-based cohort study led by Dr. Shoichiro Tsugane at the NCC Research Center for Cancer Prevention and Screening. However, the reference data from several thousand members of the Japanese general population will be obtained by the Tohoku Medical Megabank project and other population-based genome cohorts, with whom we

would like to keep collaborating in the genome analyses.

2) Development of personalized cancer diagnosis and treatment of gastric cancer.

Next generation sequencer analysis has been shown to be a powerful tool to identify driver mutation candidates for the development of specific and sensitive biomarkers and therapeutics targeted to molecular aberrations. However, the proof that the observed mutation is actually a viable target of a therapeutic intervention needs a functional evaluation. Moreover, the frequency of each mutation in a particular type of cancer is often less than 5%. Therefore, establishment of cell lines from each patient with specific mutations is an important step for the functional selection of drug targets. Specifically, the Division has been analyzing clinical samples of malignant ascites from the patients with gastric cancer to identify somatic mutations and fusion genes through WES and whole transcriptome sequencing (WTS), respectively, as a part of the Integrated Disease Omics Project by NiBio. A large multi-center collaborative study on 10 malignant and non-malignant diseases has been organized, and the integrative database has been designed (Figure 1). We are in charge of the WES and SNP array analyses of 4 types of solid cancers and pediatric leukemia. The samples are being analyzed in order of the cancer types as agreed. While waiting in the queue, we successfully established 43 cancer cell lines by December 2013 from the ascites of 21diffuse-type gastric cancer patients by plate-culturing and intraperitoneal injection into SCID mice of retrieved cells (Figure 2). Our newly established cell lines can benefit the functional analyses of mutated genes and promote the preclinical study for developing new molecular target drugs.

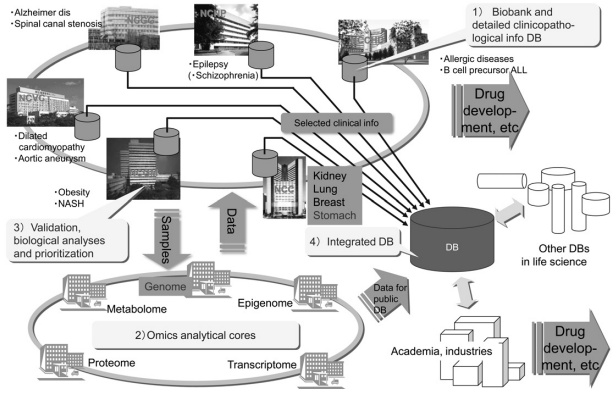

Figure 1. NiBio Integrated Disease Omics Project (FY2010-2014)

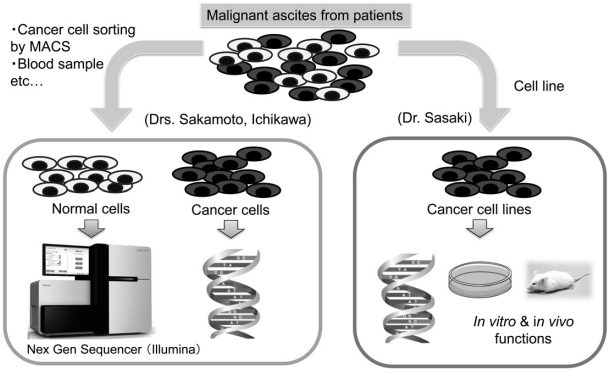

Figure 2. Functional Screening of Mutated Genes Identified by NGS Integrative Disease Omics Project Supported by NIBIO

List of papers published in 2013
Journal

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Division of Familial Cancer Research

Toshihiko Tsukada, Yuko Nagamura

Introduction

The Division of Familial Cancer Research is focusing its research activities on the development of new methods for diagnosis and treatment of familial cancer syndromes. A new diagnostic DNA test for multiple endocrine neoplasia type 1 (MEN1) was evaluated for clinical usefulness. Pharmacological actions of rikkunshito, a traditional Japanese herbal medicine, were also investigated.

Research activities

DNA diagnosis of MEN1

MEN1 is a familial cancer syndrome characterized by the multiple occurrences of endocrine tumors in the pituitary, parathyroid, and enteropancreatic endocrine tissues. MEN1 is caused by heterozygous germline mutations of the causative gene *MEN1*, which encodes a tumor suppressor protein named menin. Because the optimal therapies for MEN1-associated tumors, especially for multicentric parathyroid and pancreatic tumors, are different from those for sporadic, non-hereditary endocrine tumors, accurate differential diagnoses are mandatory before planning treatment. Germline mutation analysis of the *MEN1* gene is a powerful tool for the differential diagnosis of patients with endocrinopathy suggestive of MEN1. However, it is often difficult to distinguish a disease-causing mutation from a rare benign polymorphism especially when a novel missense mutation is identified in a patient with incomplete forms of MEN1. We previously found that mutant menin proteins associated with MEN1 were unstable and were rapidly degraded by the ubiquitin-proteasome pathway. A diagnostic test for predicting the prognosis of missense *MEN1* mutant gene carriers has been developed by exploiting this reduced stability. This method was evaluated for its clinical usefulness in collaboration with many hospitals in Japan. A missense *MEN1* mutation found in a new patient was evaluated for its pathogenicity by this method. The results indicated that the mutation will cause familial isolated primary hyperparathyroidism rather than typical MEN1 because of its residual biological activities.

The clinical characteristics and survival outcome of 32 patients with MEN1 were examined in relation to the *MEN1* gene mutation. Premature deaths related to MEN1 are suggested to be due to the development of malignant pancreatic neuroendocrine, pituitary or thymic tumors associated with mutations in exon 2, 3 and a large gene deletion (1).

Effects of rikkunshito on endocrine cells

Rikkunshito is widely used to treat appetite loss associated with various disorders, and may be a useful regimen for cancer cachexia. In order to examine possible effects of rikkunshito on hormone production in endocrine cells, we measured intracellular cAMP, which is a major regulator of biosynthesis and initiates the release of several hormones. A growth hormone-producing pituitary cell GH3 and an ACTH-producing pituitary cell AtT-20 were treated with rikkunshito with or without forskolin, a direct adenylate cyclase activator. Intracellular cAMP levels increased in both cell lines following the treatment with rikkunshito and/or forskolin in a dose-dependent manner. Release of ACTH and growth hormone from AtT-20 and GH3 cells, respectively, were suppressed by rikkunshito. These findings suggest that rikkunshito acts directly on endocrine cells and modulates secretion of hormones.

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Division of Virology

Tohru Kiyono, Takashi Yugawa, Nagayasu Egawa, Tomomi Nakahara, Kenji Yamada, Satomi Kikawa, Shin-ichi Ohno, Takako Ishiyama, Katsuyuki Tanaka, Yuki Inagawa, Kasumi Ohtsubo, Hikaru Tanaka, Kazuki Shimomura, Shotaro Tsunoda, Akiko Noguchi, Etsuko Kabasawa

Introduction

Approximately 15% of human cancers have a viral etiology, and seven viruses have been elucidated as being associated with human cancers. Among these recognized viruses, research in the Division of Virology is mainly focused on the molecular mechanisms of oncogenesis by human papillomaviruses (HPVs). A subset of HPVs including types 16 and 18 are closely associated with human cancers and have thus been called high-risk HPVs (HR-HPVs). The E6 and E7 proteins of HR-HPVs are known to inactivate the major tumour suppressors, p53 and retinoblastoma protein (pRB), respectively. By using an *in vitro* multistep carcinogenesis model for cervical cancer, we are elucidating the roles of E6, E7 and cellular oncogenes in multistep carcinogenesis (Figure 1).

A Novel function of NOTCH1 in keratinocyte differentiation

We previously elucidated that the NOTCH1 gene is transcriptionally activated by p53 and repressed by ΔNp63α. Since NOTCH1 is a key inducer of keratinocyte differentiation, ΔNp63α maintains the self-renewing capacity of normal human keratinocytes and cervical cancer cells partly through transcriptional repression of the Notch1 gene and imply a novel pathogenetic significance of frequently observed overexpression of ΔNp63α together with p53 inactivation in SCCs. ROCK, an effector of the small GTPase RHO, is also implicated in keratinocyte differentiation, and its inhibition efficiently potentiates immortalization of human keratinocytes and greatly improves the survival of dissociated human pluripotent stem cells. However, the molecular basis for ROCK activation is not fully established in these contexts. We elucidated that the intracellular forms of NOTCH1 trigger the immediate activation of ROCK1 independent of its transcriptional activity, promoting differentiation and resulting in decreased clonogenicity of normal human keratinocytes (Figure 2). Knock-down of NOTCH1 abrogated ROCK1 activation and conferred sustained clonogenicity upon differentiation stimuli. Treatment with a ROCK inhibitor, Y-27632, or ROCK1 silencing substantially rescued the growth defect induced by activated NOTCH1. Furthermore, we revealed that impaired self-renewal of human induced pluripotent stem cells upon dissociation is, at least in part, attributable to NOTCH-dependent ROCK activation. Thus, the present study unveils a novel NOTCH-ROCK pathway critical for cellular differentiation and loss of self-renewal capacity in a subset of immature cells (1).

Isolation of a novel type of HPV

E7 proteins of most HPVs as well as animal papillomaviruses conserve the Leu-X-Cys-X-Glu pRB-binding motif. However, some of the HPVs causing flat warts do not. In collaboration with dermatologists, a novel type of HPV, HPV160, belonging to the Alpha-PV species 2 was isolated from a flat wart of an immunocompetent patient (2).

Immortalization of Normal and Precancerous Human cells

We have immortalized various types of normal and precancerous human cells. Among them, immortalized ovarian endometrioma cells were used for analyzing the molecular mechanisms of progestin to inhibit their growth (5). A novel cell line, AM-3, was established from an ameloblastoma patient to analyze a pathway to induce osteoclastogenesis (4). Immortalized skin fibroblasts from severe combined immunodeficiency (SCID) patients with mutations in the Artemis gene were used for identifying a novel function of Artemis as a molecular switch that converts stalled replication forks harboring single-stranded gap DNA lesions into DSBs, thereby activating the ATM signaling pathway following prolonged replication fork stalling (8). From normal human amniotic tissues, epithelial cells and mesenchymal stem cells were newly immortalized (6, 9). By using several immortalized human cells as tools for cellular biology, Ser99 on Plk1 was identified as a novel mitosis-specific phosphorylation site, which operates

independently of Plk1-Thr210 phosphorylation (3) and beta1,4-galactosyltransferase 6 (B4galt6) in addition to B4galt5 was confirmed to play a role as a lactosylceramide synthase (7).

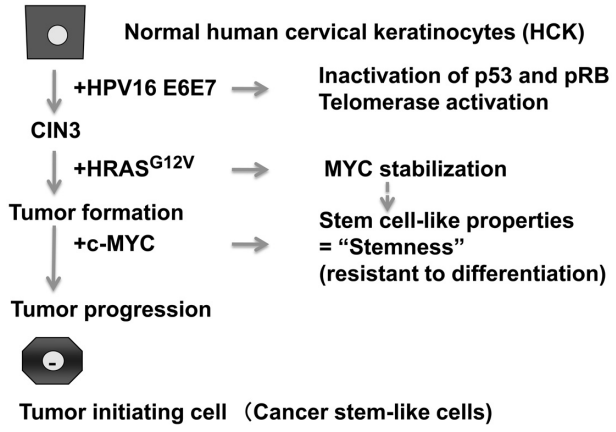

Figure 1. An *in vitro* multistep carcinogenesis model for cervical cancer

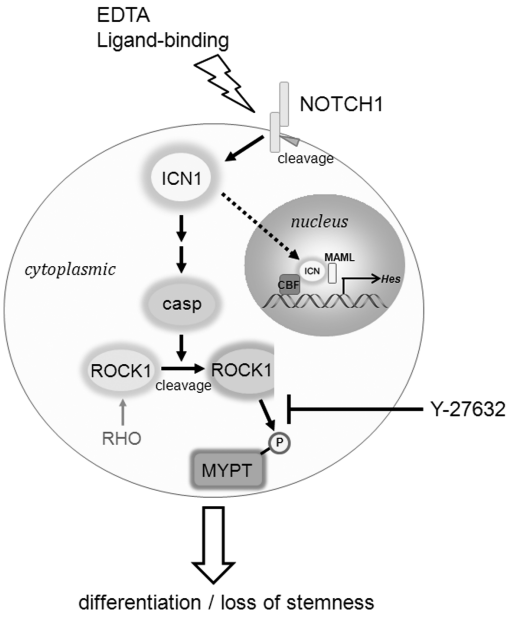

Figure 2. Proposed model for the NOTCH-ROCK pathway and its biological signif

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Division of Cancer Development System

Koji Okamoto, Yoshitaka Hippou, Yukari Totsuka, Daisuke Shiokawa, Masako Ochiai, Mitsuyoshi Yoshimoto, Hirokazu Ohata, Masanori Gotoh, Kazuhiro Shiizaki, Noriyuki Yokomichi, Tetsuya Matsuura, Emi Fukai, Akira Baba, Toshihiro Tsujita, Waka Kato, Yuki Yokoi, Naoko Osada, Sachiko Dobashi, Ai Sato, Hiroaki Sakai, Emiko Yamamoto, Mayumi Mizuta, Yumi Miyamoto, Kaoru Orihashi, Yukie Matumura

Introduction

Cancer stem cells (CSCs) play important roles during the development of refractory cancer with highly metastatic potential. Our group mainly focuses on studying CSCs from various refractory tumors. In particular, we cultivate CSCs *in vitro* from various clinical specimens, and attempt to understand how CSCs contribute to metastatic capability and chemo-resistance of cancer through our research on the biological properties of CSCs.

Routine activities

A weekly conference is held with members of Division of Cancer Development System.

Research activities

In vitro cultivation and characterization of cancer stem cells from human refractory cancer

Accumulating reports indicate that CSCs are responsible for metastatic processes as well as the tumorigenicity and chemoresistance of cancer. In our previous studies, we isolated cancer stem cells from human colon cancer, and established the conditions that allow stable *in vitro* propagation of colon CSCs in a spheroid form. We expanded our studies to cultivate CSCs not only from the primary tumor but also from metastatic foci in the liver. The CSCs from metastatic foci are likely to show higher metastatic capability. We are comparing the metastatic and non-metastatic CSCs through a series of 'Omics' data analyses, in order to elucidate the biological feature that is linked to the capability of CSCs to metastasize. Similar studies on CSCs from ovarian cancer and glioma are in progress.

In addition to the systematic Omics studies, we continued to investigate the regulation of CD44, whose induction after suppression of Rho-associated protein kinase is linked to the stemness (stem-like qualities) of colon CSCs. We demonstrated that mTOR is responsible for the induction of CD44, and in fact the upregulation of mTOR is indispensable for the stemness of colon CSCs.

Functional identification and characterization of a regulatory factor of cancer metastasis

In our previous studies, miR-493 was functionally isolated as an inhibitor of liver metastasis of colon cancer cells, and the following studies indicated that up-regulation of miR-493 during carcinogenesis prevents liver metastasis via the induction of cell death of metastasized cells. We demonstrated that MKK7, a MAP-related kinase, was identified as a direct target of miR-493, and its inhibition partially phenocopied the anti-metastatic effects. Thus, in combination with IGF-1R, a previously identified target of miR-493, MKK7 functions to promote liver metastasis of colon cancer, presumably in response to surviving signals from the liver microenvironment, and the inhibition of such signals by miR-493 blocks liver metastasis.

Recapitulation of multi-step adenocarcinogenesis for diverse organs through an *in vitro* approach

Whereas both genetic and environmental factors cooperate for tumorigenesis *in vivo*, we demonstrated that the lentivirus-mediated introduction of genetic alterations in murine primary epithelial cells could lead to development of adenocarcinoma in the dorsal skin of immune-deficient mice. Notably, tumor initiation and subsequent step-wise progression from normal cells via pre-cancerous lesions to carcinoma could be accurately recapitulated for various vital organs in a cell-autonomous manner. By taking this approach, genetic and/or environmental interactions toward tumorigenesis could be conveniently investigated *in vitro*, which would likely accelerate elucidation of the molecular mechanisms underlying carcinogenesis.

Identification of Novel Mutagens/Carcinogens

Nanomaterials are commonly used in various industrial fields. Because the genotoxicity and safety of nanomaterials are of serious concern, we examined the genotoxicity of magnetite nanoparticles (MGT) on human A549 and Chinese hamster ovary (CHO) AA8 cells. Treatment with MGT increased the frequency of micronuclei (MN), DNA double strand

breaks (DSB), sister chromatid exchange (SCE), and production of reactive oxygen species (ROS) in *in vitro* systems. These findings suggested that MGT, through induction of ROS, induces DSB, which is followed by clastogenic events including MN and SCE. Reports related to other environmental mutagens/carcinogens are listed in the attached references.

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Division of Hematological Malignancy

Issay Kitabayashi, Kazutsune Yamagata, Takuo Katsumoto, Yutaka Shima, Yoko Ogawara, Emi Takamatsu, Yukiko Aikawa, Mika Shino, Akiko Kittaka, Rieko Furuya, Miu Adachi, Mariko Saito

Introduction

Acute myeloid leukemia (AML) is the most common leukemia in Japan and the U.S. With current standard chemotherapy, Approximately 70% of adults with AML can be expected to attain complete remission status following appropriate induction therapy. However, many of the AML patients relapse and only 25-30% of young adults and fewer than 10% of older patients survive longer than 5 years, suggesting the presence of AML stem cells that are resistant to chemotherapy. AML stem cell eradication is therefore thought to be crucial to effect a cure for AML. Chromosome abnormalities, which results in a generation of specific fusion genes, are observed in ~50% of AML patients. In cases of AML associated with fusion genes involving *MLL, MOZ, CALM* or *NUP98* the outcome is extremely poor. Normal cytogenetics portend average-risk AML. Recent genome analyses revealed that mutations in NPM, IDH1/IDH2/TET2, DNMT3a and FLT3 genes are often simultaneously observed in patients with normal cytogenetics. The purpose of our research is to establish new therapeutic methods by identifying molecular targets that are essential for the maintenance of AML cells, especially AML stem cells.

Research activities

Chromosomal translocations that involve the monocytic leukemia zinc finger (MOZ) gene are typically associated with human AML and often predict a poor prognosis. Overexpression of HOXA9, HOXA10, and MEIS1 were observed in AML patients with MOZ fusions. To assess the functional role of HOX upregulation in leukemogenesis by MOZ-TIF2, we focused on bromodomain-PHD finger protein 1 (BRPF1), a component of the MOZ complex that carries out histone acetylation for generating and maintaining proper epigenetic programs in hematopoietic cells. An immunoprecipitation analysis showed that MOZ-TIF2 forms a stable complex with BRPF1, and a chromatin immunoprecipitation analysis showed that MOZ-TIF2 and BRPF1 interact with HOX genes in MOZ-TIF2-induced AML cells. Depletion of BRPF1 decreased the MOZ localization on HOX genes, resulting in loss of transformation ability induced by MOZ-TIF2. Furthermore, mutant MOZ-TIF2 engineered to lack histone acetyltransferase activity, was incapable of deregulating HOX genes as well as initiating leukemia. These data indicate that the MOZ-TIF2/BRPF1 complex upregulates HOX genes mediated by MOZ-dependent histone acetylation, leading to the development of leukemia. We suggest that activation of the BRPF1/HOX pathway through MOZ HAT activity is critical for MOZ-TIF2 to induce AML.

AML1/RUNX1 is a frequent target of chromosome translocations and mutations in myeloid and B-cell leukemias, and upregulation of AML1 is also observed in some cases of T-cell leukemias and lymphomas. Our study showed that the incidence of thymic lymphoma in p53-null mice is less frequent in the Aml1+/- than in the Aml1+/+ background. AML1 is upregulated in p53-null mouse bone-marrow cells and embryonic fibroblasts. In the steady state, p53 binds to and inhibits the distal AML1 promoter. When the cells are exposed to stressors, p53 is released from the distal AML1 promoter, resulting in upregulation of AML1. Overexpression of AML1 stimulates T-lymphocyte proliferation. These results suggest that upregulation of AML1 induced by loss of p53 promotes lymphoid-cell proliferation, thereby inducing lymphoma development.

The PML gene is frequently fused to the retinoic acid receptor α (RARα) gene in acute promyelocytic leukemia (APL), generating a characteristic PML-RARα oncogenic chimera. PML-RARα disrupts the discrete nuclear speckles termed nuclear bodies (NBs) which are formed in PML, suggesting that NB disruption is involved in leukemogenesis. NB formation that relies upon PML oligomerization and its stabilization of the hypoxia-inducible protein kinase HIPK2 is disrupted by expression of the PML-RARα chimera. We reported that disruption of NBs is also mediated by PML-RARα inhibition of PML oligomerization. The PKA-mediated phosphorylation of PML-RARα blocked its ability to inhibit PML oligomerization and destabilize HIPK2. Our results established that both PML oligomerization and HIPK2 stabilization at NBs are important for APL cell differentiation, offering insights into the basis for the most common pro-

differentiation therapies of APL used clinically.

Monocytic leukemia zinc finger (MOZ)/KAT6A is a MOZ, Ybf2/Sas3, Sas2, Tip60 (MYST)-type histone acetyltransferase that functions as a coactivator for acute myeloid leukemia 1 protein (AML1)- and Ets family transcription factor PU.1-dependent transcription. We previously reported that MOZ directly interacts with p53 and is essential for p53-dependent selective regulation of p21 expression. We showed in a subsequent study that MOZ is an acetyltransferase of p53 at K120 and K382 and colocalizes with p53 in promyelocytic leukemia (PML) nuclear bodies following cellular stress. The MOZ–PML–p53 interaction enhances MOZ-mediated acetylation of p53, and this ternary complex enhances p53-dependent p21 expression. Moreover, we identified an Akt/protein kinase B recognition sequence in the PML-binding domain of MOZ protein. Akt-mediated phosphorylation of MOZ at T369 has a negative effect on complex formation between PML and MOZ. As a result of PML-mediated suppression of Akt, the increased PML–MOZ interaction enhances p21 expression and induces p53-dependent premature senescence upon forced PML expression. Our study demonstrated that MOZ controls p53 acetylation and transcriptional activity via association with PML.

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Division of Metastasis and Invasion Signaling

Ryuichi Sakai, Hitoyasu Futami, Hideki Yamaguchi, Takamasa Uekita, Takuya Shirakihara, Katsuhiko Nakashima

Introduction

The invasive and metastatic nature of cancers is the major threat for cancer patients and is a serious problem during cancer treatment. It has also been recently highlighted that unique interactions of cancer cells with neighboring cells such as stromal fibroblasts have critical roles in the invasion and metastasis of cancers. Numerous types of genetic and epigenetic alterations found in cancers might utilize several common pathways responsible for these malignant characteristics. An understanding of the signal pathway regulating these cancer-specific properties could lead to the development of new therapeutic targets specific to cancers. One of our approaches to identify these cancer-specific pathways is to investigate aberrant phosphotyrosine-containing proteins during cancer development and progression. As substrates of tyrosine kinases, these protein might include the mediators of cancer-specific signals and potential targets of therapeutics. The main object of our research is to establish models of the novel therapy for progressive cancer by regulating phosphotyrosine-dependent signals in cancer cells and their microenvironments.

Mediators of cancer invasion and metastasis signaling

Resistance to cell death caused by detachment (so-called anoikis) is one of the properties that cancer cells need to acquire for metastasis to distant organs. We have identified a transmembrane protein CDCP1 (CUB-domain-containing protein 1) from metastatic lung cancer cells as a key molecule responsible for resistance to anoikis. Expression of CDCP1 is detected in a number of cancer cell lines and tissues and is closely correlated with a poor prognosis. We demonstrated that CDCP1 is induced by activation of the Ras-Erk pathway and phosphorylated at tyrosines by activated Src family kinases (SFKs) in cancer cells. In other words, CDCP1 is a functional link between Ras and Src signaling during the multistage progression of human malignant tumors, highlighting CDCP1 as a potent target for treatment in the broad spectrum of human cancers associated with activation of the Ras pathway. Inhibition of CDCP1 expression using small interfering RNA (siRNA) induced cell death of suspended cancer cells without generating cleaved caspase-3, a marker of apoptosis, and the cell death was not inhibited by a general caspase inhibitor, suggesting that loss of CDCP1 induced a caspase-independent cell death. Instead, the loss of CDCP1 induced the LC3-II protein and the formation of autophagosomes. Moreover, the cell death of suspended lung cancer cells induced by the CDCP1 siRNA was reduced by an autophagy inhibitor 3-Methyladenine. These results indicated that CDCP1 signaling plays a critical role in the inhibition of autophagy which contributes to the anoikis resistance of lung cancer cells.

We have revealed that CDCP1 is required for ECM degradation by invadopodia in human breast cancer and melanoma cells. CDCP1 was localized to caveolin-1-containing vesicular structures and lipid rafts and was detected in close proximity to invadopodia. Invadopodia are actin-based protrusions on the surface of invasive cancer cells that promote the degradation of the extracellular matrix (ECM) via localized proteolysis, which is mainly mediated by membrane-type 1 matrix metalloproteinase (MT1-MMP). Further biochemical analysis revealed that CDCP1 is an essential regulator of the trafficking and function of MT1-MMP- and invadopodia-mediated invasion of cancer cells.

Investigation of molecular targets for scirrhous gastric carcinoma

Scirrhous gastric carcinoma (SGC) has the worst prognosis among various types of gastric cancers, owing to its rapid expansion through progressive invasion, peritoneal dissemination and frequent metastasis to lymph nodes. Because massive proliferation of stromal fibroblasts (SF) occurs within SGC lesions, interaction between SGCs and SF cells might play a role in the invasive properties of SGC cells. When SGC cells were cocultured with SF cells on three-dimensional Matrigel, they were attracted together to form large cellular aggregates that invaded the Matrigel. A myosin II inhibitor, blebbistatin, blocked the invasion and ECM remodeling by SGC and SF cells. These results indicated that SGC cells promote actomyosin-mediated contractility of SF cells to remodel the ECM during invasion. The

formation of these invasive foci was monitored by fluorescent imaging, and utilized for screening of inhibitor libraries. Several regents such as Rho inhibitors and Src inhibitors were picked up as candidate drugs targeting cancer-stromal interaction in SGCs.

Oncogenic Signals in Neuroblastomas

Activation of anaplastic lymphoma kinase (ALK) either by mutation or overexpression, has been indicated as a significant oncogenic factor in neuroblastoma formation. Investigation of phosphotyrosine-containing proteins associated with ALK in neuroblastomas was performed using mass-spectrometry analysis to elucidate the unique signals associated with neuroblastomas. Among various types of novel and known binding partners of ALK, Flotillin-1 (FLOT1), a plasma membrane protein known to be involved in endocytosis, was identified by mass-spectrometry analysis. Knockdown of FLOT1 in neuroblastoma cells caused dissociation of ALK from endosomes along with membrane accumulation of ALK, which resulted in activation of ALK and downstream signals. Suppression of FLOT1 expression also enhanced the oncogenic properties of neuroblastoma cells both *in vitro* and *in vivo*. On the other hand, oncogenic ALK mutants showed less binding affinity to FLOT1 than wild-type ALK. Lower expression levels of FLOT1 were observed in highly malignant subgroups of human neuroblastoma tissues. Taking these findings together, the results suggested that decreased levels of FLOT1 or defects in the binding affinity between ALK mutants and FLOT1 may cause malignant phenotypes of neuroblastoma through the activation of ALK signaling.

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Division of Cancer Biology

Hirofumi Arakawa, Yasuyuki Nakamura, Hiroki Kamino, Hitoya Sano, Yuri Saito, Ryuya Murai, Izumi Hyo

Introduction

The scope of the research at the Division of Cancer Biology is broad, covering numerous areas including the cloning of genes involved in carcinogenesis, biological and structural analyses of proteins, analyses of animal models, and the development of new strategies for cancer therapy. In particular, the tumor suppressor p53 and the genes that are directly regulated by p53 have been studied to uncover the mechanism of p53-mediated tumor suppression, based on which new cancer preventive, diagnostic, and therapeutic strategies could be developed.

Research activities

Identification and characterization of p53-target genes

Using a combination of a microarray analysis and a chromatin immunoprecipitation assay, identification of p53-target genes in the human genome has been conducted. Thus far, a number of p53-target genes including *DFNA5*, *SEMA3F*, *BLNK*, *UNC5A*, *NEEP21*, and *TMPS* have been identified and characterized at the Division. Along the line, a new p53-target gene was identified, and designated *Mieap* for mitochondria-eating protein, reflecting its unusual function of the protein. Surprisingly, the function of Mieap is involved in mitochondrial quality control (MQC).

Mieap-induced accumulation of lysosome-like organelles within mitochondria

Mieap controls mitochondrial quality via two distinct novel mechanisms. One of the mechanisms has been designated MALM for Mieap-induced accumulation of lysosome-like organelles within mitochondria (*PLoS ONE* 6: e16054, 2011). In this mechanism, Mieap induces the accumulation of intramitochondrial lysosomal proteins in order to eliminate oxidized mitochondrial proteins in response to mitochondrial damage. This leads to a decrease in reactive oxygen species generation and an increase in mitochondrial ATP synthesis activity, implying MALM plays a role in repairing unhealthy mitochondria.

BNIP3 and NIX, mitochondrial outer membrane proteins, two Mieap-interacting proteins, mediate the translocation of lysosomal proteins from the cytosol into the mitochondria during MALM by forming an unknown pore in the mitochondrial double membrane (*PLoS ONE* 7: e30767, 2012). 14-3-3γ mediates the degradation of oxidized mitochondrial proteins within mitochondria during MALM (*Scientific Reports* 2: 379, 2012).

Mieap-induced vacuole

Alternatively, the other mechanism has been designated MIV for Mieap-induced vacuole (*PLoS ONE* 6: e16060, 2011). When MALM is inhibited, Mieap induces a vacuole-like structure, MIV. The MIV engulfs the damaged mitochondria and accumulates lysosomes, leading to the degradation of unhealthy mitochondria. MIV likely represents a novel mechanism for mitochondrial autophagy, also called "mitophagy". Therefore, Mieap controls mitochondrial quality by repairing or eliminating unhealthy mitochondria via MALM or MIV generation, respectively (Figure 1).

Mitochondrial quality control and cancer

The accumulation of unhealthy mitochondria results in mitochondrial dysfunction, which has been implicated in aging, degenerative diseases and cancer. The Mieap-regulated MQC is frequently inactivated by p53 mutations or Mieap-methylation or BNIP3 methylation in more than 80% primary colorectal cancer tissues. In order to further evaluate the clinical significance of the Mieap-regulated MQC, the status of p53 (gene mutation), Mieap (methylation), and BNIP3/NIX (methylation) are being examined in many primary cancer tissues including pancreatic and gastric cancer patients.

Aerobic glycolysis is a common feature of human cancers, which is also known as the Warburg effect. Although the nature of cancer cells has been applied to the development of positron emission tomography (PET) for the whole body screening of human cancers, the mechanism for the phenomenon remains to be elucidated. The p53-Mieap pathway is frequently inactivated in human cancers because of p53 mutations and/or Mieap methylation. This leads to the accumulation of unhealthy mitochondria and consequently the Warburg effect (Figure 2). The accumulated unhealthy mitochondria in cancer cells also produce high levels of reactive oxygen

species (ROS). The increased mitochondrial ROS dramatically enhance cancer migration and invasion (Figure 2).

New therapeutic strategies for cancer therapy

Adenovirus-mediated gene transfer of *Mieap* has been found to strongly suppress tumor growth, suggesting that normalization of unhealthy mitochondria could be a novel strategy to suppress cancers *in vivo*. Toward the development of new strategies for cancer therapy, the *in vitro* and *in vivo* antitumor effects of these genes are being examined in this Division.

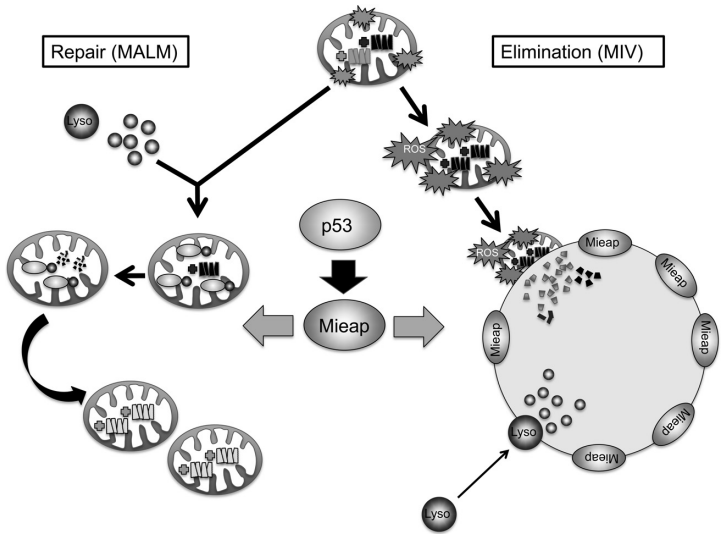

Figure 1

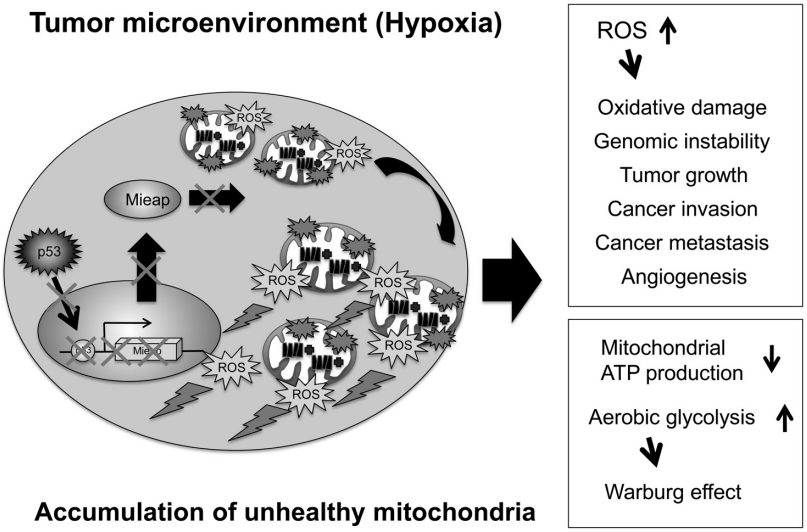

Figure 2

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1. Zhu Y, Li Y, Haraguchi S, Yu M, Ohira M, Ozaki T, Nakagawa A, Ushijima T, Isogai E, Koseki H, Nakamura Y, Kong C, Mehlen P, Arakawa H, Nakagawara A. Dependence receptor UNC5D mediates nerve growth factor depletion-induced neuroblastoma regression. J Clin Invest, 123:2935-2947, 2013
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Division of Molecular and Cellular Medicine

Takahiro Ochiya, Fumitaka Takeshita, Masaki Kawamata, Nobuyoshi Kosaka, Ryou-u Takahashi, Ayako Inoue, Wakako Kobayashi, Maki Abe, Yu Fujita, Hiroaki Miyazaki, Takeshi Katsuda, Makiko Ono, Yusuke Yoshioka, Luc Gailhouste, Muriel Thirion, Yuki Konishi, Kurataka Ootsuka, Yutaka Nezu, Keitaro Hagiwara, Naoomi Tominaga

Introduction

The focus of the Division of Molecular and Cellular Medicine lies in the development of novel treatments and diagnosis to advance cancer therapy. The specific activities were as follows: 1) Studies on microRNA (miRNA) regulation in cancer cells and development of RNA interference (RNAi) -based therapeutics; 2) An exosome as a novel diagnosis and therapeutic tool against cancer; 3) Study of stem cells and their therapeutic applications.

1) Studies on miRNA regulation in cancer cells and development of RNAi-based therapeutics.

RNAi-based therapeutics is a promising approach as novel and potentially more effective treatments for cancer and miRNA are one of the targets involved in the regulation of tumor-related genes (1-5, 31). We show miRNA (miR)-582-5p and -3p, which are strongly decreased in high-grade bladder cancer clinical samples (6). The overexpression of miR-582-5p or -3p reduced the proliferation and invasion of human bladder cancer cells. Furthermore, transurethral injections of miR-582 suppressed tumor growth and metastasis in an animal model of bladder cancer. Thus, local treatment with RNAi is anticipated to become available for clinical use. We have developed novel RNAi agents (PnkRNA™, nkRNA®) that show knockdown of the target gene in tumor cells on lung tissues through inhalation without any sophisticated delivery technology in mice (7).

Our group also identified miR-148a as a liver-specific miRNA highly expressed in the adult liver and found that miR-148a was critical for hepatic differentiation through the direct targeting of DNA methyltransferase (DNMT) 1, a major enzyme responsible for epigenetic silencing (8, 32). We also found that miR-148a directly targeted the c-Met oncogene in human liver cancer. Therefore, our study suggests that miR-148a plays an important dual role in hepatic maturation and liver tumor suppression.

We previously identified Ribophorin-2 (RPN2) as a novel regulator for drug resistance. We also found that RPN2 played an important role in the regulation of breast cancer stem cell (CSC) properties such as tumorigenic and metastatic activities via stabilizing p53 mutants (9). These findings reveal a previously undescribed molecular mechanism for mtp53 stabilization in breast cancer and suggest that the RPN2/mtp53 regulatory network could be a promising target for anti-CSC therapy. For clinical application of siRNA targeting RPN2, pre-clinical trials with naturally occurring breast cancer in dogs have been performed. The first clinical trials of siRNA in Japan will be started next year at the National Cancer Center Hospital (NCCH).

2) An exosome as a novel diagnostic and therapeutic tool against cancer

Circulating exosomes have been found in a variety of body fluids including serum, plasma, urine, saliva, and breast milk (10). The existence of circulating exosomes in the blood of cancer patients has raised the possibility that exosomes may serve as a novel diagnostic marker (11-12, 33). For this reason, a new method for a highly sensitive method of identifying circulating exosomes has been developed (13,14). Cell-cell communication of cancer cells and microenvironmental cells is critical for the acquisition of malignancy in human cancer, however, the precise molecular mechanisms of this cell-cell communication remain unclear (15,16). We have demonstrated that a tumor-suppressive miRNA secreted from non-cancerous cells via the neutral sphingomyelinase 2 (nSMase2)-mediated exosomal pathway could be transported between cells and exert gene silencing in the recipient cancer cells, thereby leading to an inhibition of cancer cell growth. We recently showed the contribution of nSMase2-mediated exosomes from cancer cells to the cancer cell metastasis *in vivo* via the induction of angiogenesis in the tumor (17). These findings prompted us to consider the idea for the application of the exosome in the diagnosis of and therapy against cancer development (18, 19).

3) Study of stem cells and their therapeutic applications

We are interested in the therapeutic potential of stem cells, including induced pluripotent stem

(iPS) cells, embryonic stem (ES) cells (20-24), and mesenchymal stem cells (MSCs) (25-28). Our main focus is particularly on the realization of the clinical application of adipose tissue derived-mesenchymal stem cells (ADSC) in liver diseases (25, 26). We have also reported that a stem or progenitor cell-based approach is beneficial in the regeneration of functional liver tissue (23, 24). Recently, MSCs are attracting much attention not only for their own potential as cells, but also for their secretory capacity of exosomes that can have therapeutic benefits (27). We recently reported the novel therapeutic potential of exosomes secreted from human ADSCs against Alzheimer's disease (AD) (28). We found that hADSCs secrete exosomes carrying enzymatically active neprilysin, the most important β-amyloid peptide (Aβ)-degrading enzyme in the brain. We have also studied the relationships between infection with the hepatitis viruses and cancer pathogenesis (29, 30).

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Division of Epigenomics

Toshikazu Ushijima, Eriko Okochi-Takada, Satoshi Yamashita, Kiyoshi Asada, Tohru Niwa, Hideyuki Takeshima, Naoko Hattori, Yukie Yoda, Takamasa Takahashi, Yasuyuki Shigematsu, Satoshi Yoshida, Emil Rehnberg, Akiko Mori, Naoko Kobayashi, Yuko Miyaji, Aya Nakajima, Zong Liang

This Division has been focusing on the epigenetic mechanisms of carcinogenesis, and has identified many aberrantly methylated CpG islands (CGIs) in various cancers, *i.e.*, gastric cancers, breast cancers, pancreatic cancers, lung cancers, ovarian cancers, neuroblastomas, and melanomas. This has led to the identification of novel tumor-suppressor genes (TSGs) in gastric cancers, development of a powerful prognostic marker in neuroblastomas, and establishment of the concept of an "epigenetic field for cancerization". This Division continues its activity through revealing epigenetic alterations in various cancers, and by developing clinically useful biomarkers, a novel approach of cancer prevention, and epigenetic therapy.

Identification of novel epigenetic alterations

Identification of TSGs inactivated by aberrant DNA methylation is important. During 2013, by focusing on genes on chromosome X that can be inactivated only by a single hit, *FHL1* was identified as a TSG inactivated in gastrointestinal cancers by either aberrant DNA methylation or somatic mutation. Inactivation of *FHL1* in normal-appearing tissues was suggested to be involved in the formation of an epigenetic field for cancerization (1).

The recent development of personal sequencers and bead array technology has made it possible to obtain integrated pictures of genetic and epigenetic alterations on the same set of cancer samples. This Division showed that the number of aberrantly methylated genes was highly variable among individual gastric cancers, and that the CpG island methylator phenotype (CIMP) was associated with mutations of oncogenes, such as *ERBB2* and *PIK3CA* (2).

Development of biomarkers

This Division previously revealed that neuroblastomas with CIMP have a worse prognosis than those without (3). At the same time, aberrant DNA methylation of individual TSGs has also been reported to be associated with a poor prognosis. During 2013 it was revealed that CIMP had a stronger prognostic power than methylation of individual genes (Figure 1) (4). The clinical usefulness of CIMP in neuroblastomas is currently being analyzed using materials collected in a prospective manner. In gastric cancers, levels of accumulated methylation in normal-appearing tissues are expected to be useful as a cancer risk marker, and a prospective study is being conducted to bring this concept into clinical practice. The degree of aberrant methylation in gastric mucosae was correlated with the severity of *Helicobacter pylori (H. pylori)*-related gastritis (5).

Contamination of normal cells is almost always present in tumor samples and affects their molecular analyses, and development of a biomarker that can estimate the fraction of cancer cells in a tumor DNA sample is important. Using esophageal squamous cell carcinoma (ESCC) as an example, this Division isolated three genomic regions, *TFAP2B*, *ARHGEF4*, and *RAPGEFL1*, whose DNA methylation levels reflected the fraction of cancer cells in a tumor DNA sample (6).

Development of cancer prevention

Suppression of aberrant DNA methylation is a novel approach to cancer prevention that has the potential to reverse risk once accumulated. This Division revealed that suppression of aberrant DNA methylation by a DNA demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), can prevent *H. pylori*-induced gastric cancers using a Mongolian gerbil model (7). It was also shown that *Il1b* induced by *H. pylori* infection enhanced mouse gastric carcinogenesis (8). It was suggested that removal of aberrant DNA methylation and/or suppression of DNA methylation induction could become a novel target for cancer prevention.

Development of a novel technique to detect a combination of epigenetic modifications

A combination of epigenetic modifications specifically present in cancer cells is a possible target in developing cancer cell-specific epigenetic therapy.

Regardless of the importance of combinations of epigenetic modifications, techniques to detect combinations are limited. This Division developed a novel technique to visualize a combination of epigenetic modifications (designated as imaging of a combination of histone modifications, iChmo) (9). This technique was able to visualize a combination of epigenetic modifications not only in cultured cells but also in tissue samples, and offered advantages in the detection of combinations in samples with heterogeneous cell population and also in tissue samples.

Other activities

This Division assisted other research groups with the epigenetic analysis in *UNC5D* in neuroblastoma (10), that of *PTEN* in colorectal cancer (11), and those in various animal models (12-14).

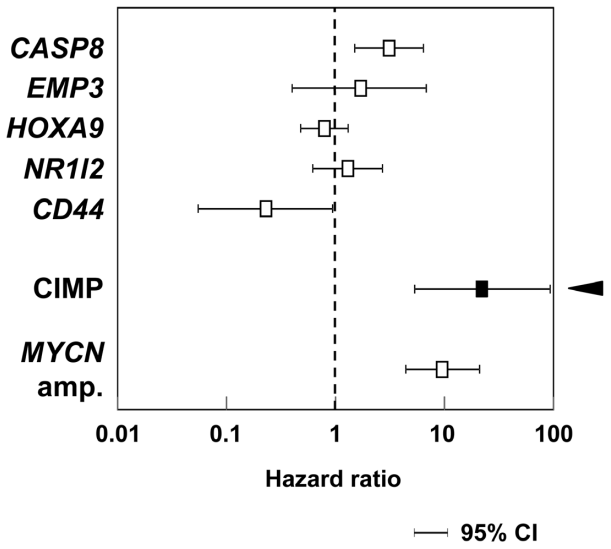

Figure 1. Stronger prognostic power of the CpG island methylator phenotype (CIMP)

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Division of Pharmacoproteomics

Tadashi Kondo, Takashi Tajima, Fusako Kito, Marimu Sakumoto, Kazutaka Kikuta, Kenta Mukaihara, Naofumi Asano, Yuta Kurota, Daisuke Kubota, Hiroshi Ichikawa, Yutaka Sugihara, Yuki Tani

Introduction

Our research goal is to contribute to the development of effective anti-cancer therapy. To achieve this goal, we have developed our current research plan, which is aimed at the discovery of biomarkers and drug targets, on the basis of interdisciplinary collaborative projects. All malignancies are the research subjects, and recently we focused more on sarcomas.

Research activities

In sarcomas, we investigated biomarker candidates for personalized medicine and therapeutic targets for additional indications. In osteosarcomas, we discovered novel protein and miRNA biomarkers to predict the response to neo-adjuvant treatments, and launched a collaboration with diagnostic companies. We aim to commercialize our discovery over the next few years. One protein was considered as a candidate for a drug target, and we confirmed the inhibitory effects of the small molecule approach on osteosarcoma cells. In gastrointestinal stromal tumors, we discovered pfetin as a novel prognostic biomarker, achieved multi-institutional validations, and commercialized our original antibody as a research tool in partnership with a domestic company. We plan to reveal the clinical usefulness of measuring pfetin expression in a prospective clinical study. In alveolar soft tissue sarcomas, we surveyed all tyrosine kinases using specific antibodies, identified over-expressed kinases in tumor tissues, and started to find specific inhibitors for additional indications. In mixoid liposarcomas, we examined the protein complex of the fusion gene products of sarcomas with a proteomic approach, and revealed the functional properties of complex components. In epithelioid sarcomas and rhabdomyosarcomas, we identified proteins with specific expression in tumor tissues, and started molecular characterization of their properties. We also applied our approach to other malignancies. In gastric cancer, we identified proteins associated with lymph node metastasis. In renal cancer, we found prognostic biomarkers. In metastatic bone tumors, we identified proteins and miRNAs with unique expression in metastatic tumors, and characterized their functional properties *in vitro*. A novel protein fractionation system for proteomic analysis has been under development in collaboration with an industrial company. To reveal the molecular backgrounds of biomarkers, *in vitro* experiments were extensively performed.

Education

Four young doctors and two students had training in translational research. The doctors who stayed in our laboratory were supervised regarding their presentations, papers, and grant applications. One student started his professional career in the field of translational research. The Division Chief has given lectures in various universities as a visiting professor or lecturer.

Perspectives

Using clinical materials and comprehensive analysis, we are identifying various promising biomarkers and drug targets. Through functional experiments and independent validations, we are selecting candidates for further research. In each stage we have several candidates, and examine more malignancies using the same approach. For pfetin and the several other biomarkers, we have reached the stage of commercialization. We will soon clarify the clinical utility of our biomarkers and their targets in future clinical studies.

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Division of Genome Biology

Takashi Kohno, Naoto Tsuchiya, Hideaki Ogiwara, Kouya Shiraishi, Motonobu Saito, Yoko Shimada, Mariko Sasaki, Ayaka Otsuka, Yuko Fujiwara, Tatsuji Mizukami, Reika Iwakawa-Kawabata, Masaki Takenaka, Takashi Nakaoku, Hideyuki Hayashi, Takashi Mitachi, Yujin Ishihara, Shino Kasuga, Daisuke Kurioka, Momoyo Nishida, Tomoyo Kobayashi, Yoshiaki Onozato, Mei Tanabe

Introduction

Somatic mutations in the cancer genome and inter-individual variations in the human genome are critical keys to improving the efficacy rate of cancer clinics. The aim of our division is to find "seeds" that improve the treatment and prevention of cancer by identifying and elucidating the biological significance of somatic mutations in cancer genomes and genetic polymorphisms of cancer patients. We are working together with National Cancer Center (NCC) staff from hospitals, the Research Center for Cancer Prevention and Screening, and the Center for Cancer Control and Information Service to fight lung cancer, the most common cause of cancer-related deaths in Japan and worldwide.

Research activities

1. Genes for personalized therapy

Whole RNA sequencing of 30 lung adenocarcinomas (LADCs) led us to identify the RET fusion gene as a new druggable driver oncogene present in 2% of LADCs. A phase II clinical trial, which is investigating the therapeutic effect of a RET-tyrosine kinase inhibitor, vandetanib, has been started as described below (Figure 1). Future personalized therapies including all druggable oncogene targets will cover >60% and >30% of Japanese and US/European LADC patients, respectively.

SMARCA4/BRG1, encoding an ATPase functioning as a catalytic subunit of the SWI/SNF chromatin remodeling complex, is deficient by genetic and epigenetic alterations in 10% of lung cancers, preferentially in those without driver oncogene aberrations. Our functional studies, including that on DNA double strand break repair, revealed that ablation of the ATPase activity of SMARCA2/BRM, another SWI/SNF catalytic subunit, specifically causes senescence of BRG1-deficient cancer cells based on synthetic lethality. BRM-ATPase inhibitory therapy is a promising approach for the treatment of BRG1-deficient cancers, including lung cancers without mutations in known therapeutic target genes (Figure 1). Specific inhibitors against BRM-ATPase are searched for by conducting a collaborative study with a pharmaceutical company. Patients with BRG1-deficient cancers will benefit from the BRM-ATPase inhibitory therapy.

2. Genes for personalized prevention

Our genome-wide association study (GWAS) verified two previously reported loci, TERT and TP63, and identified two new susceptibility loci, BPTF (Bromodomain PHD finger transcription factor), encoding a chromatin remodeling protein, and BTNL2 (Butyrophilin-like 2), encoding an immune-modulating protein. All the defined SNPs were common ones with minor allele frequencies >0.25 and per allele odds ratios of 1.1-1.4. Associations of these four loci were validated in an independent sample set. International and pan-Japan collaborative GWASs are underway to further identify genetic factors involved not only in the susceptibility to but also in the prognosis of lung cancer.

3. Biological roles of microRNAs in cancer development

A tumor-suppressive microRNA, miR-22, is a regulator for intrinsic tumor-suppressor networks organized by p53. Through the miR-22 target screening, we recently identified NIMA-related kinase 9, NEK9, as a novel factor required for cell cycle progression in p53-mutated cancer cells. NEK9 depletion repressed cell proliferation selectively in p53 deficient cancer cells *in vitro* and *in vivo*, suggesting its inhibition could be a novel strategy for the development of cancer therapy. Furthermore, based on the detailed analysis of exosomal miRNAs that were circulating in the serum, we have successfully identified several miRNAs as promising diagnostic biomarkers for the detection of colon cancer patients.

Clinical trials

A phase II clinical trial, which investigates the therapeutic effect of a RET-tyrosine kinase inhibitor, vandetanib, has been started by us in Japan in Q1 of 2013, and positive therapeutic responses have been

observed. For the purpose, >300 nonsmall cell lung carcinoma cases have been screened by an all-Japan consortium consisting of >125 hospitals, LC-SCRUM-Japan (Lung Cancer Genomic Screening Project for Individualized Medicine in Japan), using RT-PCR and FISH assays developed by us. LC-SCRUM-Japan will shortly include the screening of two more driver oncogenic aberrations, ROS1 fusion and BRAF mutation, for clinical trials of corresponding kinase inhibitors.

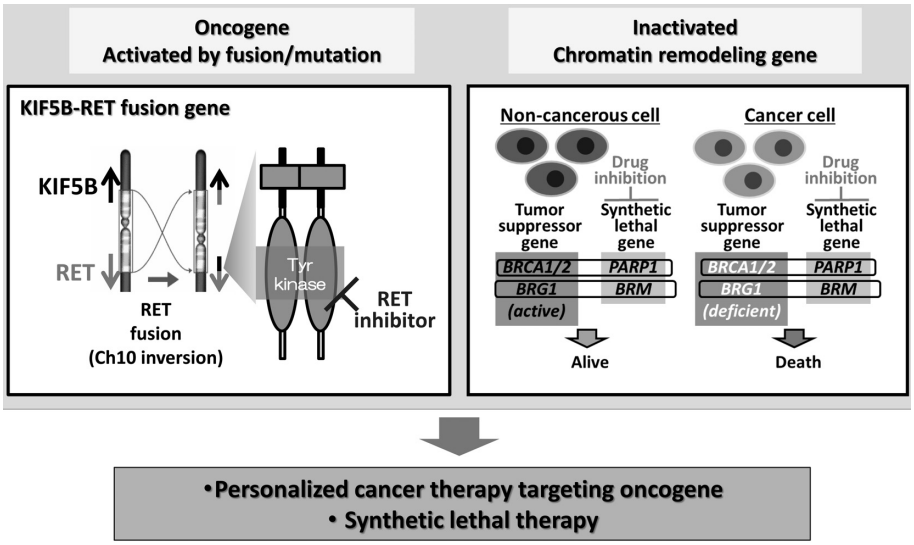

Figure 1. Personalized cancer therapy based on genetic aberrations

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Division of Cancer Genomics

Tatsuhiro Shibata, Fumie Hosoda, Yasushi Totoki, Yasuhito Arai, Hiromi Nakamura, Tomoki Shirota, Hirofumi Rokutan, Naoko Okada, Tomoko Urushidate, Hiroko Shimizu, Shoko Ohashi, Wakako Mukai, Isao Kurosaka, Arisa Hara, Yasuko Konagai, Momoko Nagai, Asmaa M. Elzawahry

Introduction

The Division of Cancer Genomics focuses on comprehensive characterization of the cancer genome on the basis of tumor pathology and aims to make a "breakthrough" by identifying novel cancer-related genes, including potential therapeutic targets and biomarkers, and to understand the cancer genome as representing heterogeneous and interconnected "biological systems" that contribute to the pathogenesis of cancer. This Division has also organized the facility and developed new informatics methodologies for the analysis of a next-generation high-performance sequencer.

Research activities

Whole genome/exome sequencing of liver cancer and the International Cancer Genome Project

We have participated in the International Cancer Genome Consortium to generate a comprehensive high-resolution catalog of genomic changes for major cancer types (1-3). We performed whole genome sequencing of 31 Japanese liver cancers and identified high accurate sets of somatic mutations. We provided this dataset for the analysis of mutational signatures between various cancer types (1). We also performed whole exome sequencing of 213 Japanese liver cancers and executed the joint collaboration of two large-scale cancer genome projects (ICGC and TCGA) and comparatively analyzed the trans-ethnic liver cancer genome data of 608 cases.

Novel druggable fusion genes in cholangiocarcinoma

Cholangiocarcinoma is an intractable cancer, with limited therapeutic options, in which the molecular mechanisms underlying tumor development remain poorly understood. We performed whole transcriptome sequencing (WTS) analysis in eight specimens from cholangiocarcinoma patients without KRAS/BRAF/ROS1 alterations, and identified two fusion kinase genes, FGFR2-AHCYL1 and FGFR2-BICC1. In reverse transcriptase polymerase chain reaction (RT-PCR) screening, the FGFR2 fusions were detected in 9 patients with cholangiocarcinoma, exclusively in the intrahepatic subtype (9/66, 13.6%). The rearrangements were mutually exclusive with KRAS/BRAF mutations. Expression of the fusion kinases in NIH3T3 cells activated MAPK, and conferred anchorage-independent growth and in vivo tumorigenesis of subcutaneous transplanted cells in immune-compromised mice. Treatment with FGFR kinase inhibitors, BGJ398 and PD173074, effectively suppressed transformation. The expression pattern of these fusions in association with sensitivity to FGFR inhibitors warrant a new molecular classification of cholangiocarcinoma and suggest a new therapeutic approach to the disease.

Oncogenic ROS1 rearrangement in lung cancer

Non-small-cell lung cancer (NSCLC) has recently been undergoing extensive molecular subclassification. We performed a histological analysis, WTS and RT-PCR analysis of 799 surgically resected NSCLC samples and identified 15 cases of ROS1-rearranged cancer. Half of the cases harbored mucinous cribriform or signet ring cell features similar to the ALK-rearranged type (4). We established a transgenic mouse model for ROS1-rearranged lung cancer, and revealed a pivotal role of the ROS1-fusion gene in lung carcinogenesis (5).

Transcriptome sequencing analysis for other tumor types

To understand the genetic basis and to identify new drug targets in sarcomas and other cancer types, a transcriptome sequencing approach has been undertaken (6-8). WTS and a verification experiment with the RT-PCR method identified more than a hundred in-frame fusion genes in 68 gastric cancers examined. Those fusion genes include good candidates for therapeutic targets: two oncogenic protein kinase gene fusions, two oncogenic pathway-activating gene fusions, and four recurrent promoter-swapping gene fusions. Functional analyses of these fusion genes are in progress.

Metabolomic analysis of the GLO1 oncogene in gastric cancer

We have identified a frequent amplification of chromosome 6p21 in gastric cancer and six potential

cell growth-activating genes in this region. Among them, GLO1 that encodes glyoxalase I, a detoxifying enzyme of a cytotoxic methylglyoxal, exhibited the strongest oncogenic activity. Downregulation of GLO1 resulted in growth inhibition of gastric cancer cells. A metabolomic analysis of GLO1-downregulated gastric cancer cells revealed that GLO1 functions in the activation of the central carbon metabolism: glycolysis, the pentose phosphate pathway, and TCA cycle. These data indicated that GLO1 is a multifunctional metabolic oncogene and could be an important therapeutic target for gastric cancer.

Bioinformatics platform and support for other cancer research

We developed highly efficient and accurate in-house algorithms to detect and analyze somatic mutations, structural alterations including fusion genes, virus integrations, transcriptome and small RNAs. Using them, we supported a bioinformatics analysis for non-coding RNAs and gene expression (9-12). As an expert pathological function, further collaborative clinical research with the Division and hospital groups has been performed (13). We also developed a somatic mutation caller for low tumor purity samples and subclonal mutations.

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Division of Chemotherapy and Clinical Research

Tesshi Yamada, Masaya Ono, Kazufumi Honda, Mari Masuda, Nami Miura, Ayako Mimata, Masahiro Kamita, Tomoko Umaki, Yuko Miyamoto, Hiroko Ito, Haruyo Tozaki, Akihiko Miyanaga, Takafumi Watanabe, Yukio Watabe, Nobuhiko Nishijima

Introduction

Even for cancers having the same origin and histology, their clinical courses may vary among individuals. Accurate prediction of disease progression and therapeutic efficacy is therefore essential for optimization of therapy in individual patients. The Division has shifted its main focus from basic research to the identification and clinical application/translation of biomarkers applicable for therapy personalization. We reported 2 prognostic and 1 predictive biomarkers during 2013.

Distinct outcome of stage I lung adenocarcinoma with ACTN4 cell motility gene amplification

Even if detected at an early stage, a substantial number of lung cancers relapse after surgery. Patients with such tumors are likely to benefit from adjuvant therapy, but no method for discriminating among them has been established. We identified that amplification of a single metastasis-related gene was able to recognize a small (8-16%) but substantial subset of stage I lung adenocarcinomas with a markedly unfavorable outcome. Actinin-4 is an actin-binding protein that we originally identified as being associated with enhanced cell motility and cancer invasion. Overall survival was significantly worse for patients with stage I lung adenocarcinoma harboring actinin-4 (ACTN4) gene amplification than for those whose tumors showed no such gene amplification (P<0.001, log-rank test). Multivariate analysis revealed that ACTN4 gene amplification in stage I lung adenocarcinoma was an independent factor associated with a higher risk of death (hazard ratio, 6.78; P<0.001, Cox regression analysis) (Table 1). The result was first astonishing to us, because to the best of our knowledge no single biomarker has yet been identified as having such a high prognostic significance for early-stage lung cancer. We carefully confirmed its reproducibility in multiple cohorts totaling 1774 patients. We may say that ACTN4 gene amplification in lung adenocarcinoma is equivalent to HER2/CERBB2 gene amplification in breast cancer.

Diagnostic and prognostic significance of the alternatively spliced ACTN4 variant in high-grade neuroendocrine pulmonary tumors

High-grade neuroendocrine tumors (HGNT) of the lung manifest a wide spectrum of clinical behavior, and patients whose prognosis is predicted to be unfavorable might benefit from intense adjuvant chemotherapy. So far, however, no prognostic biomarker for HGNT has been established.

In a study, we demonstrated that the variant form of actinin-4 is a novel biomarker predictive of an unfavorable postsurgical outcome in HGNT patients. We established a monoclonal antibody specifically recognizing the product of the alternatively spliced *ACTN4* transcript, and used it to examine the expression of variant actinin-4 immunohistochemically in a total of 609 surgical specimens of various histological subtypes of lung cancer. Variant actinin-4 was expressed in 55% (96/176) of HGNTs. Expression of variant actinin-4 was significantly associated with poorer overall survival in HGNT patients ($P < 0.001$, log-rank test). Multivariate analysis using the Cox proportional hazards model showed that expression of variant actinin-4 was the most significant independent negative predictor of survival in HGNT patients (hazard ratio, 2.18; $P < 0.001$) after the presence of lymph node metastasis.

Soluble Interleukin-6 receptor is a serum biomarker that predicts the response of esophageal squamous cell carcinoma to neoadjuvant chemoradiotherapy

Preoperative chemoradiotherapy (PCRT) has been shown to improve the outcome of patients with esophageal caner, but response to the therapy varies among patients. Esophageal squamous cell carcinoma (ESCC) patients with high levels of serum vascular endothelial growth factor and C-reactive protein have been reported to respond poorly to PCRT, but no reliable biomarker that can predict the efficacy of PCRT has yet been established.

We performed comprehensive profiling of 84 serum cytokines in 37 ESCC patients who were able to receive neoadjuvant PCRT. We found that the baseline level of soluble interleukin-6 receptor (sIL6R) was significantly higher in 30 patients who failed to achieve a histological complete response to PCRT ($P = 0.005$). Multivariate analysis revealed that the increased level of sIL6R was one of several

significant independent predictors of an unfavourable outcome (hazard ratio, 2.87; $P = 0.017$). The increased level of sIL6R in patients who did not obtain a complete response was reproducibly observed in an independent cohort of 34 patients. These findings were further subjected to independent validation using retrospective and prospective cohorts.

Table 1. Hazard Ratios for Death in 284 Stage I Cases of TMA, According to Prognostic Factors

Characteristics		Univariate analysis			Multivariate analysis		
		Hazard Ratio	[95% CI¶]	*P* Value*	Hazard Ratio	[95% CI¶]	*P* Value*
Sex	(Female vs. Male)	2.98	[1.06-8.36]	**.04**	1.73	[0.40-7.47]	.16
Age	(<65 yr vs. ≥65 yr)	3.24	[1.28-8.23]	**.01**	3.61	[1.31-9.94]	**.01**
Smoking history	(Never smoked vs. Current and former smokers)	3.23	[1.25-8.34]	**.02**	1.31	[0.32-5.26]	.71
Pathological stage†	(IA vs. IB)	6.88	[2.26-21.0]	**< .001**	3.53	[1.11-11.2]	**.03**
Histological differentiation	(Well differentiated§ vs. Moderately and poorly differentiated)	4.83	[1.72-13.6]	**.003**	2.55	[0.86-7.59]	.09
EGFR mutation	(Absent vs. Present)	0.20	[0.07-0.62]	**.005**	0.43	[0.12-1.49]	.18
KRAS mutation‡	(Absent vs. Present)	1.57	[0.45-5.41]	.48			
ACTN4 gene amplification	(Negative vs. Positive)	10.53	[4.15-26.7]	**< .001**	6.78	[2.59-17.7]	**< .001**

¶ Abbreviation: CI, confidence interval.

* Cox regression analysis. *P* values of < .05 are shown in bold

† According to the International Union Against Cancer (UICC) TNM Classification of Malignant Tumours, 6th edition (2002).

§ Includes bronchioloalveolar carcinoma [World Health Organization (WHO) Histological Typing of Lung and Pleural Tumours, 3rd edition (1995)].

‡ Six cases for whom *KRAS* mutaion data were not avilable were excluded.

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Division of Cancer Pathophysiology

Yasuhito Uezono, Seiji Shiraishi, Masami Suzuki, Kanako Miyano, Yumi Sawada, Yuka Sudo, Junko Ezuka, Yukiko Araki, Katsuya Morita, Kiyoshi Terawaki, Katsuya Ohbuchi, Koichiro Minami, Tohru Yokoyama, Naofumi Oyanagi, Yohei Kashiwase, Akinobu Yokoyama, Hitomi Nishimura

Introduction

Since its establishment in January 2009, the Division of Cancer Pathophysiology has focused on two major research issues regarding 1) improvement of the quality of life of patients with cancer suffering from severe or intolerable pain, and 2) prevention and development of novel treatments for cancer cachexia symptoms. Based on the 2nd Basic Plan to Promote Cancer Control Programs established in Japan in 2012, basic to clinical, and also clinical to basic translational collaborative research with the clinical laboratory groups comprises our main research protocols and has been ongoing.

Research activities

Translational research to innovate and develop new strategies to improve pain analgesia in cancer patients

The purpose of our studies is to develop new therapies for both refractory cancer pain and chemotherapy-induced peripheral neuropathy, which make the quality of life of cancer patients even worse. One of the targets is severe pain with bone-metastasized patients and patients undergoing cancer chemotherapy. A second target is stomatitis induced by chemotherapy and/or radiotherapy.

We previously showed that a platelet-activating factor (PAF) antagonist produced profound and long lasting anti-allodynia effects in several different neuropathic pain models in mice including a partial sciatic nerve ligation injury model and streptozotocin-induced diabetes model (5). Also we have found that the PAF antagonist showed extremely excellent analgesic effects on both the bone-metastasized cancer pain model mice and the chemotherapy-induced peripheral neuropathy model mice (Morita and Shiraishi et al., PLOS ONE (2014), in press). The pain-relieving action of PAF antagonists were found to be effective in neuropathic pain animal models (5), and patent was issued covering the compounds; "COMPOSITION FOR TREATMENT OF CANCER PAIN AND USE THEREOF WO/2012/077775 PCT/JP2011/078508".

The cancer patients who undergo chemotherapy, radiotherapy and terminal paliative care often have a wide range of stomatitis, which induces severe pain and limits the fundamental basics of life, namely “eating, drinking and talking”. In clinical sides, lidocaine is normally used for cancer patients with stomatitis to relieve oral pain. However, lidocaine removes not only the pain but also the ability to discriminate taste and texture, since it nonselectively suppresses the activation of all neurons by blocking the voltage-gated Na^+ channels. Therefore, a novel analgesic drug, which blocks selectively the pain-related neuron alone, is required to allow patients to eat without losing or changing the taste and texture. We have focused on a "compound X" as the novel analgesic drug, and have elucidated the effects of the compound X on oral pain indueced by stomatitis. We estabilised the method to evaluate the intensity of oral pain using stomatitis model animals. With the model, lidocaine inhibited not only the pain but also caused numbness in normal oral mucosa. On the other hand, the compound X suppressed the pain in the ulcer, but had no effects on normal tissues. Further, the analgesic effect of the compound X was longer than that of lidocaine, indicating that the compound X is a more superior analgesic drug than lidocaine.

In a basic study with cultured cell models, we have been elucidating the pharmacological actions of the compound X (*e.g.*, how does it block only the pain-related neurons?). By connecting such a basic study to a clinical study, we want to develop "the new pain-killer the compound X, which can remove the oral pain without changing the texture and taste of food" for the cancer patients with severe painful stomatitis.

Prevention, and decrease the cachexic symptoms that make the quality of life of caner patients even worse

We established novel cancer cachexia animal models (2). We then undertook molecular and cellular analyses to identify the mechanisms of action of the expected compounds to improve the quality of life of patients suffering from cancer cachexia with biological, biochemical and electrophysiological approaches (1, 2, 4). We then established a novel cancer cachexic model by inoculating human gastric

cancer cells (85As2) (2), and found that a Japanese Kampo (traditional Oriental) medicine "rikkunshito" usually administered for the prevention of gastritis, nausea and vomiting since the 17th century in Japan, improved the symptoms of cancer cachexia (Terawaki K et al., Am J Physiol Endocrinol Metab, 306:E373-E387, 2014). We summarize the mechanisms of traditional Japanese Kampo medicines and their potential use for improvement of the symptoms of cancer cachexic patients and the side effects in cancer patients who take anticancer agents. We further try to elucidate novel methods to overcome cancer cachexic symptoms and make them clinically available, in additional to rikkunshito, in collaboration with several clinical groups in Japan.

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Division of Cancer Stem Cell

Kenkichi Masutomi, Yoshiko Maida, Satoko Yamaguchi, Mami Yasukawa, Yosuke Satomura

Introduction

Research in the Division of Cancer Stem Cell is focused on deciphering the mechanisms that establish and maintain cancer stem cells and to develop novel therapeutic approaches to treating cancer through targeting cancer stem cells. In particular, the Division studies the molecular links between a) telomerase and RNA dependent RNA polymerase; b) telomerase and cancer stem cells; and c) telomerase and epigenetics.

Telomerase and RNA dependent RNA polymerase

Telomerase is a ribonucleoprotein complex that elongates telomeres. Human TERT (hTERT) is known as the catalytic subunit of the enzyme. TERT acts as an RNA dependent DNA polymerase (RdDP) and synthesizes telomere DNA from a non-coding RNA (ncRNA) template, human *TERC* (*hTERC*). In addition to *hTERC*, we found that hTERT binds a second ncRNA, *RMRP*, the RNA component of RNase MRP, and TERT and *RMRP* act as an RNA-dependent RNA polymerase (RdRP) and produce double-stranded *RMRP* that can be processed into an endogenous small interfering RNA (siRNA) to regulate *RMRP* expression levels (Figure 1). To further investigate the biological functions of hTERT RdRP, we generated a new anti-hTERT monoclonal antibody and established a RdRP assay using hTERT immune complexes isolated from cell lysate (IP-RdRP assay). We confirmed that both hTERT levels and hTERT-associated RdRP activity are increased during mitosis while telomerase activity is upregulated in S phase. These observations indicate a non-telomere directed function of hTERT during mitosis.

Telomerase and cancer stem cells

Previous studies indicated that hTERT has activities beyond telomere maintenance, and it is speculated that the constitutive expression of hTERT not only stabilizes telomere length and facilitates cell immortalization but also contributes to tumor susceptibility and alters stem cell cycling *in vivo* even when telomere lengths are not limited. We showed that hTERT forms a protein complex with the SWI/SNF component, Brahma-related gene 1 (BRG1) and the nucleolar GTP-binding proteins, nucleostemin (NS), and the complex composed of hTERT, BRG1 and NS (TBN complex) participates in the regulation of tumor initiating cells (TICs) phenotypes through telomere-independent mechanisms (Figure 2). We also confirmed that the cells that constitutively express NS exhibited increased beta-catenin signaling and elevated MYC, OCT3/4, KLF4 and TWIST (master regulator of epithelial mesenchymal transition [EMT]) expression. Moreover, cells that constitutively express elevated levels of hTERT, BRG1 and NS exhibit increased CD133 and CD44 expression and enhanced tumorigenicity at limiting cell numbers. These observations indicate that the TBN complex is essential for the maintenance of TICs.

TERT, BRG1 and NS are upregulated in malignant cells. We found that the expression levels of BRG1 and NS as well as hTERT are increased in the cells arrested in M phase. We further confirmed that the hTERT, BRG1 and NS complex assembles specifically in mitosis. Because hTERT exerts RdRP activity in mitotic cells, we hypothesized that the TBN complex may function as a RdRP complex. To investigate the hypothesis, we performed an IP-RdRP assay using either BRG1 immune complexes or NS immune complexes and found RdRP activity in both of the immune complexes as observed with the hTERT immune complexes. The results indicate a novel function of the TBN complex as a RdRP.

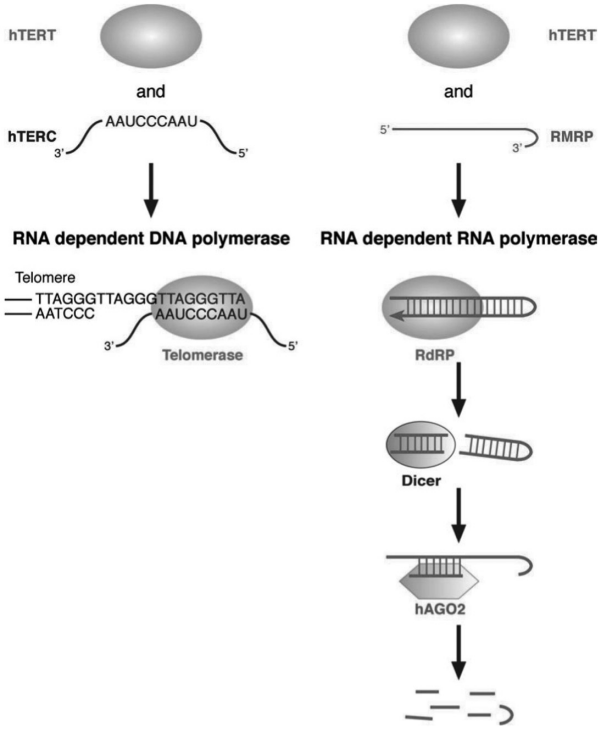

Figure 1. hTERT exerts RdRP activity

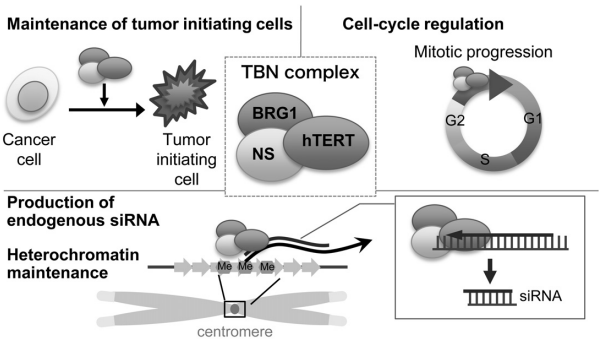

Figure 2. Various functions of the TBN complex

Telomerase and epigenetics

Previously reports have shown that functional non-coding RNA synthesized via RdRP is involved in the physiology of model organisms through its epigenetic regulation. RdRP regulates centromeric heterochromatin formation and it is required for proper chromosome segregation in mitosis. The RNA-directed RNA polymerase complex (RDRC) and the RDRC-like complex maintain heterochromatin in yeast and worms, respectively, and both complexes contain RdRP and RNA helicase. Because hTERT has RdRP activity, and BRG1 has helicase activity, we speculated that the TBN complex might have similar functions with the RDRC and the RDRC-like complex. We therefore focused on studying the molecular basis of maintenance of the heterochromatin formation by RNAs, especially by non-coding RNAs such as siRNAs, and RdRP in human cells. It is widely known that epigenetic abnormalities contribute to tumor progression, but the detailed mechanisms are unclear. It is thus important to understand the detailed mechanisms of epigenetics regulation. We found that hTERT localizes not only at centromeres but also on mitotic spindles in M phase. We further found that the TBN complex contributes to heterochromatin maintenance at centromeres and transposons. Acting as a RdRP, the TBN complex produced double stranded RNAs homologous to centromeric repeat elements and transposons that were processed into small interfering RNAs targeted heterochromatin regions. These small interfering RNAs promoted heterochromatin assembly in a manner dependent on the RNA interference pathway. These observations indicated that the mammalian homologue of RdRP (TERT) regulates heterochromatin formation through its epigenetic regulation. We further confirmed that suppression of hTERT, BRG1 or NS increases the cells arrested in mitosis, suggesting a regulatory effect of the TBN complex on mitotic progression (Figure 2). Our findings suggest that inhibitors for the novel functions of hTERT may prove useful in targeting cancer stem cells.

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Division of Gene and Immune Medicine

Kazunori Aoki, Kenta Narumi, Naoko Goto, Yoko Kobayashi, Kouichirou Aida, Yuki Yamamoto, Reina Miyakawa, Yosei Rin, Tsukasa Shinohara, Kei Nakano

Introduction

Research programs in the Division of Gene and Immune Medicine consist of the development of gene and cell therapies for solid cancers based on the analysis of host-immune response against cancer, and the development of novel cancer-targeting vectors by the peptide-displaying viral library approach. The specific activities in 2013 were as follows: 1) Combination of hematopoietic stem cell transplantation and immune gene therapy against solid cancers; 2) Cancer-targeting vectors using the peptide-display adenovirus library.

Research Activities

Combination of hematopoietic stem cell transplantation and immune gene therapy against solid cancers

Lymphopenia-induced homeostatic proliferation (HP) of T cells following autologous hematopoietic stem cell transplantation (HSCT) skews the T-cell repertoire by engaging tumor-associated antigens (TAAs), leading to an induction of antitumor immunity. Since the tumor-reactive lymphocytes preferentially proliferate under the condition of HP, the Division examined whether the priming of donor lymphocytes to TAAs could enhance HP-induced antitumor immunity in autologous HSCT recipients. Since type I interferon (IFN) maturates the dendritic cells and promotes the priming of T cells, the Division investigated whether the further priming of donor cells by IFN-α could strengthen the antitumor effect of HSCT. The intratumoral IFN-α gene transfer significantly increased the number of IFN-γ-positive lymphocytes in response to CT26 cells but not the syngeneic lymphocytes in donor mice. The infusion of primed donor lymphocytes markedly suppressed the tumor growth in recipient mice, and cured 64% of the treated mice (Figure 1) (1). Autologous HSCT with the infusion of primed donor lymphocytes is a promising strategy to induce an effective antitumor immunity for solid cancers.

On the other hand, how HSCT alters the immunosuppressive microenvironment in the tumors is unknown. The Division analyzed the kinetics of regulatory T cells (Tregs) in the tumors after syngeneic HSCT. Unexpectedly, the frequency of $CD4^+$ cells expressing Foxp3 was increased in the spleen, whereas the frequency was clearly decreased in the tumors after HSCT. Next, to examine the mechanism of Treg suppression after HSCT, dendritic cells were isolated from tumors. A large amount of the Treg-inhibitory cytokine IL-6 was secreted from the dendritic cells in the tumors but not in the spleens in the recipient mice. Furthermore, to understand what factor affected the activity of dendritic cells in the tumors after HSCT, we analyzed the expression of various cytokines/chemokines with mouse cytokine antibody arrays, and noticed that VEGF-D concentration was increased in the tumors in the early period after HSCT. The dendritic cells produced IL-6 in response to VEGF-D stimulation, and an administration of VEGFR-3 neutralizing antibody significantly suppressed the production of IL-6 from dendritic cells accompanied with the increase of Tregs in the tumors of HSCT recipients (Figure 2)(2). Autologous HSCT creates an environment strongly supporting the enhancement of antitumor immunity in reconstituted lymphopenic recipients through the suppression of Tregs. In fact, the Division previously reported that in osteosarcoma mouse models, intratumoral IFN gene transfer markedly suppressed the growth of vector-injected tumors and inhibited formation of spontaneous lung and liver metastases in syngeneic HSCT mice, and an infiltration of many immune cells was recognized in metastatic tumors of the treated mice. To translate the basic research to a clinical setting, the Division collaborates with the Central Hospital, and is planning a Phase I clinical trial on intratumoral injection of IFN-β plasmid/liposome complex in patients with sarcoma at advanced stages.

Development of cancer-targeting vectors using the peptide-display adenovirus library

Recently, adenovirus vectors have been applied for oncolytic virotherapy and in vivo imaging technology (3). Although a conditionally replicative adenovirus is an efficient anticancer agent designed to replicate selectively in tumor cells, the addition of a targeting strategy is necessary to enhance oncolysis and secure safety. However, redirection of the adenovirus vectors by engineering the capsid-

coding region has shown limited success because proper targeting ligands are generally unknown. To overcome this limitation, the Division constructed an adenovirus library displaying random peptides on the fiber knob, and its screening led to successful selections of several particular targeted vectors (4). In the previous library construction method, the procedures were complicated and time-consuming, and some of the vectors in the library were defective with no displaying peptide. To resolve these problems, the Division developed a novel method employing an in-cell Cre recombination and fiberless adenovirus, which greatly simplified the library-making steps. The high quality live adenovirus library may be able to facilitate the development of targeted adenovirus vectors for a variety of applications in medicine.

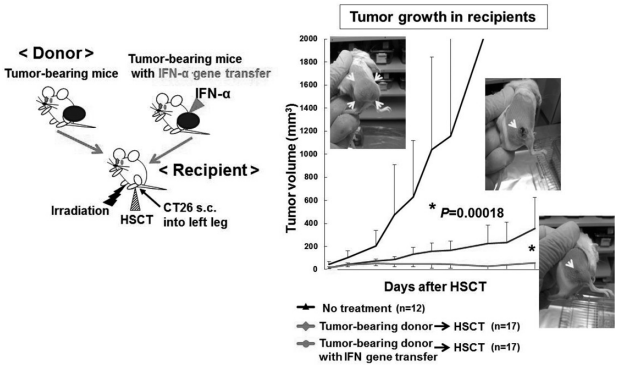

Figure 1. Donor priming enhances the antitumor immunity of HSCT

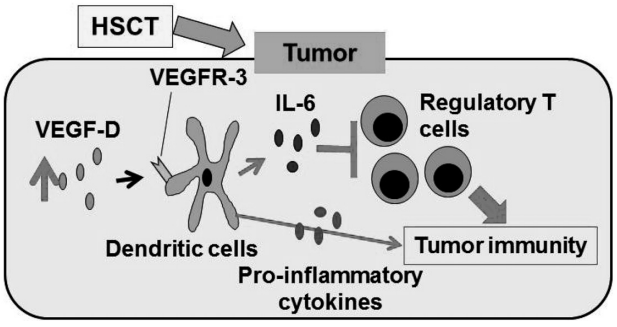

Figure 2. Breakup of the immunotolerant microenvironment by HSCT

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Division of Genome Stability Research

Mitsuko Masutani, Ken-ichi Yoshioka, Hiroaki Fujimori-Sakuma, Akira Asto, Kengo Inoue, Takahisa Hirai, Hiromi Harada, Junhui Wang, Soichiro Saito, Yuko Atsumi, Yuko Kudo, Tomoyuki Osawa, Hiroaki Mukai, Tasuku Itoh, Miyuki Hozumi, Sota Kikuhara, Motoharu Kohata, Masako Yamazaki, Tsubasa Sekiguchi

Introduction

This Division has been focusing on the biology of genome stability and trying to apply the evidence towards the development of strategies in cancer therapy and prevention. One of the pathways we focus on is the poly (ADP-ribosylation) pathway. Poly (ADP-ribose) polymerases (PARPs) and poly (ADP-ribose) glycohydrolase (PARG) are the major enzymes of poly (ADP-ribosylation) and are involved in DNA damage response and chromatin regulation. This pathway is being studied at molecular/cellular levels, and furthermore in animal models to pursue the development and evaluation of the inhibitors of poly (ADP-ribosylation) for cancer treatment. Radiation biology is also being studied to establish biological radiosensitization strategies in tumor radiotherapy and to elucidate the mechanisms of carcinogenesis.

Involvement of Parp-1 in germ cell tumor development

Parp-1 deficient embryonic stem (ES) cells showed preferential differentiation into a trophoblast lineage during tumorigenesis when grafted subcutaneously. This was demonstrated to associate the overexpression of *H19*, a non-coding gene, under *Parp-1* deficiency. The forced expression of the *H19* triggered the differentiation of ES cells into trophoblasts. The *H19* overexpression was able to promote trophoblast lineage commitment even under the suppressive pressure by the transcription factor Oct3/4. It is necessary to clarify whether aberrant *H19* overexpression induced during human carcinogenesis is related to PARP-1 dysfunction. When *Parp-1* deficient ES cells were grafted into uteri, trophoblasts were only present in the induced *Parp-1* deficient tumors and these tumors were associated with higher frequencies of invasive and metastatic lesions.

Intervention of the poly (ADP-ribosylation) pathway in cancer treatment

PARP inhibitors are reported to be effective against cancer cells, which are defective in homologous recombination pathways. Clinical trials suggested that further studies on factors that affect the effectiveness of inhibitors should be carried out. By screening with a shRNA library, candidate genes that affect the lethal effects of PARP inhibitors were identified and are being evaluated.

PARG inhibition in cancer cells induced S-phase arrest and PAR accumulation, inhibited DNA repair after alkylation damage and induced cell death. Therefore, PARG has also been suggested as a potential anti-cancer target. However, useful and specific PARG inhibitors are not currently available. A collaboration study with other institutions aimed at developing PARG inhibitors has therefore been conducted. From random and focused library screening, PARG inhibitors were identified and structural optimization is ongoing.

We also studied PAR metabolism *in vivo* using mouse models. PAR is rapidly converted into metabolites in the bloodstream. Dynamic states of these metabolites are currently being analyzed.

Radiation damage response and radiosensitization

For optimization of radiation therapy from basic biology, strategies for radiosensitization of various forms of radiation, including low and high linear-transfer (LET) radiation should be developed and evaluated. PARG functional inhibition augmented the level of PAR, blocked repair of DNA double-strand breaks (DSBs) and enhanced cell death caused by γ- and carbon-ion irradiation. Accumulated levels of PAR, which has been indicated to cause cell death, were particularly higher after carbon-ion irradiation compared with γ-irradiation, suggesting that PARG inhibition may be beneficial, especially for sensitization to particle-ion radiation.

Using shRNA libraries, around 100 candidate target genes of various categories for radiosensitization were selected and are being validated using siRNA. The radiosensitization effect for several cell lines was confirmed by knocking down gene A, resulting in increased S-phase arrest.

Boron-captured neutron therapy

The project of introduction of accelerator-based BNCT (boron-captured neutron therapy) system in NCC is ongoing. To support the biological evaluation of this new system, a collaborative study of BNCT with other institutes was initiated in 2012. Along

with a supporting study for biological evaluation, the study to understand the mechanisms of tumor cell death induced by BNCT and biomarkers for damage response is being pursued. For this purpose, transcriptome and proteome analyses after boron-captured neutron reaction in human cancer cell lines have been conducted. Transcriptome analysis after BNCT in cancer cells revealed that several particular transcription factors and immune-response-related genes were upregulated. Proteome analysis also revealed augmented levels of several proteins specifically after BNCT irradiation. The biological significance of these genes in monitoring of BNCT effectiveness will be evaluated in cellular or animal models.

Role of Arf/p53, and H2AX in the maintenance of genomic stability

Aging is a risk factor for cancers that develop with genomic instability and mutations, such as in the ARF/p53 module. Immortality is developed after abrogation of the H2AX-diminished state, which is associated with genomic instability (often with tetraploidy) and the induction of mutations in either the Arf or p53 gene. Although the involvement of p53 for the protection from immortalization is firmly established, the dependence of ARF has remained unclear. Both Arf and p53 were shown to be required for the down-regulation of H2AX and formation of the growth-arrested state. This observation is consistent with the previous reports that show the immortality development with the mutations in either ARF or p53 and the resulting recoveries of H2AX and cell growth. Notably, whereas tetraploidization was essential for immortalization of wild-type MEFs, this event was not required for immortalization of MEFs containing mutations in Arf/p53 and these cells still underwent mitotic catastrophe-associated cell death to get rid of their octaploid population. The Arf/p53-dependent down-regulation of H2AX was also shown to contribute to the selective survival of normal cells against treatment with anticancer drugs.

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Division of Integrative Omics and Bioinformatics

Hitoshi Nakagama, Tsutomu Ohta, Akinobu Hamada, Masaru Katoh, Mamiko Miyamoto, Yuuki Yamamoto, Teruaki Tsuji, Shuichi Shimma, Yuki Takashima

Introduction

This division consists of Ohta's Unit, Hamada's Unit and Katoh's Unit. Our goal is the development of innovative diagnostics and therapeutics for cancer based on an integrative omics approach.

Ohta's Unit

Oxidative and electrophilic stresses are sensed by Keap1, which activates transcription factor Nrf2 to achieve cytoprotection by regulating the expression of drug-metabolizing and anti-oxidative stress enzymes/proteins. The constitutive activation of Nrf2 leads to resistance against anti-cancer drugs and growth stimulation in lung cancer. This suggests that inhibition of *NRF2* may provide a new direction for therapeutic approaches in lung cancers with activation of Nrf2. The inhibitors for NRF2 are investigated and identified using *in vitro* and *in vivo* analyses.

Failure to expeditiously repair DNA at sites of double-strand breaks (DSB) ultimately leads to human disorders including cancer. NBS1 plays an important role in the cellular response to DSB damage. A rare polymorphic variant of *NBS1* that resulted in an isoleucine to valine substitution at amino acid position 171 (I171V) was first identified in childhood acute lymphoblastic leukemia. The *NBS1* polymorphic variant locates in the N-terminal region that is a protein interaction region with DNA repair factors. An aplastic anemia (AA) patient (a Japanese child) with a homozygous polymorphic variant of *NBS1*-I171V was previously described. The chromosomes of lymphoblastic cell lines derived from this patient contained a remarkable number of structural chromosomal aberrations. However, it was unclear whether the *NBS1*-I171V polymorphic variant affected DSB repair activity and chromosomal instability. The reduced DSB repair activity of *NBS1*-I171V polymorphic variant was detected.

Hamada's Unit

The activity of Hamada's Unit is described in the report from the Department of Clinical Pharmacology.

Katoh's Unit

Omics medicine, producing large amounts of omics data on genetics, genomics, epigenomics, transcriptomics, proteomics and metabolomics, consists of three layers (Figure 1). The first layer corresponds to clinical medicine that is involved with clinical sampling of blood and tissues or bio-banking. The second layer corresponds to basic medicine that produces large amounts of omics data from clinical samples and generates curated databases by using algorithms. The third layer corresponds to translational medicine that develops novel diagnostics and therapeutics and generates a knowledgebase from manuscripts and curated databases (Reference 1). Katoh's Unit has been involved in translational medicine since 2002.

WNT, FGF, Hedgehog, Notch and TGFβ signaling cascades are the major themes of Katoh's Unit (Reference 1), while human genes that had been first discovered in the Katoh's Unit are sub-themes of Katoh's Unit (References 2-4). Forkhead-box (FOX) family members are DNA-binding proteins with a FOX domain. Germ-line mutations in the *FOX* family of genes cause hereditary diseases, because FOX proteins are involved in transcriptional regulation and DNA repair. Somatic mutations in the *FOX* family of genes, including gene amplifications, point mutations, and translocations, occur in a variety of human cancers (Reference 2). The *Drosophila Asx* gene encodes an epigenetic regulator that is associated with the Polycomb-group repressor complex and the trithorax-group activator complex. ASXL1, ASXL2 and ASXL3 are human homologs of the *Drosophila* Asx. ASXL family members are epigenetic

scaffolding proteins that assemble epigenetic regulators and transcription factors to specific genomic loci with histone modifications. Germline mutations in *ASXL1* occur in Bohring-Opitz syndrome, while somatic mutations in *ASXL1* occur in colorectal cancer with microsatellite instability, hematological malignancies and castration-resistant prostate cancer (Reference 3). GIPC1, GIPC2 and GIPC3 are GIPC family members that consist of GH1, PDZ and GH2 domains. GIPC1 is an adaptor protein with a dimerizing ability that loads PDZ ligands as cargoes for MYO6-dependent endosomal trafficking. GIPC1 is required for the cell-surface expression of IGF1R and TGFβR3. GIPC1 is also required for integrin recycling during cell migration, angiogenesis and cytokinesis. GIPC1 upregulation in breast, ovarian and pancreatic cancers promotes tumor proliferation and invasion, whereas GIPC1 downregulation in cervical cancer with human papillomavirus type 18 infection leads to resistance to cytostatic TGFβ signaling. GIPC2 is downregulated in acute lymphocytic leukemia owing to epigenetic silencing, while Gipc2 is upregulated in estrogen-induced mammary tumors. Somatic mutations of GIPC2 occur in malignant melanoma, and colorectal and ovarian cancers (Reference 4).

Extracellular DNA and circulating miRNAs are key topics in translational medicine, and epigenetics play a key role in cancerous and non-cancerous diseases. Katoh underlined diagnostic techniques utilizing circulating miRNAs in exosomes and microvesicles, therapeutics utilizing siRNAs in polymer-based hydrogel nanoparticles and therapeutics targeted to a field of epigenetic alterations (Reference 5).

Masaru Katoh has been contributing to the global scientific community based on manuscript publication, reviewer activity and editor activity. Katoh carried out peer review of grant proposals or journal manuscripts written in English 82 times in 2013. Katoh is an Academic Editor of *PLoS ONE*, and has carried out editorial decision 160 times in 2013 that resulted in 51 final decisions for manuscripts submitted to PLoS ONE. Masaru Katoh was inaugurated as the Chief Editor of *Frontiers in Molecular Medicine* that aims to address the gap between cell and developmental biology and clinical medicine and to promote development of novel diagnostics and therapeutics for various types of cancers and non-cancerous diseases (http://www.frontiersin.org/Molecular_Medicine).

Manuscript citation count in the Web of Science Database (Thomson Reuters) is a surrogate marker of contribution to the global science community. Katoh's manuscripts were cited 548 times by others in 2013.

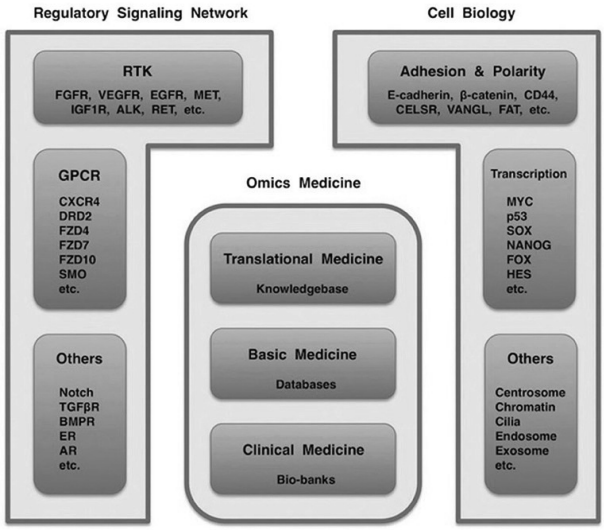

Figure 1. Three layer structure of omics medicine (http://www.frontiersin.org/Journal/10.3389/fcell.2013.00001/full)

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Journal

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3. Katoh M. Functional and cancer genomics of *ASXL* family members. Br J Cancer, 109:299-306, 2013
4. Katoh M. Functional proteomics, human genetics and cancer biology of GIPC family members. Exp Mol Med, 45:e26, 2013
5. Katoh M. Therapeutics targeting angiogenesis: Genetics and epigenetics, extracellular miRNAs and signaling networks. Int J Mol Med, 32:763-767, 2013

Division of Refractory Cancer Research

Hitoshi Nakagama, Masato Enari, Shinichi Yachida, Rieko Ohki, Erina Takai, Yuko Hibiya, Yukie Aita, Chika Shima, Keiko Igarashi, Ryo Otomo, Makoto Miyazaki, Yoshinori Asano, Issei Ezawa, Kozue Saito, Miku Shimizu, Shiori Suzuki, Chen Yu, Yuhei Takano, Junko Ohtsuka, Raira Saigawa, Maiko Minegishi, Shu Matsushita, Haruka Takigawa

Introduction

Our main focus is to clarify the molecular mechanisms of tumor progression in refractory cancers including lung cancers, pancreatic cancers and brain tumors, and to develop various novel therapeutic strategies for cancer prevention. In particular, the Division studies how cancer cells acquire invasiveness, metastatic activity and drug resistance, which are characteristics of refractory cancers. The specific activities in 2013 were as follows: 1) Requirement of NuMA for the selective induction of p53-target genes; 2) PHLDA3 is a p53-regurated repressor of Akt and a novel suppressor of neuroendocrine tumors; 3) New Approaches of Early Detection of Pancreatic Cancers; and 4) Metagenomics: Role of the Human Gut Microbiome in Colorectal Carcinogenesis.

Routine activities

A weekly conference is held with the members of the Division of Refractory Cancer Research, and a biweekly conference is held with the members of the Division of Cancer Development System. In addition, a monthly progress report is held with the members of the research institute.

Research activities

1) Requirement of NuMA for the selective induction of p53-target genes

The p53 tumor suppressor protein is a transcription factor controlling various outcomes, such as growth arrest and apoptosis, through the regulation of a different set of target genes. The Mitotic Apparatus protein (NuMA) plays important roles in spindle-pole organization during mitosis and in chromatin regulation in the nucleus during interphase. Although NuMA has been shown to co-localize with several nuclear proteins including high-mobility group proteins I/Y and GAS41, a role for NuMA during the interphase remains unclear. We have reported that NuMA binds to p53 to modulate p53-mediated transcription. The acute and partial ablation of NuMA attenuates induction of the pro-arrested p21 gene following DNA damage, subsequently causing impaired cell-cycle arrest. Interestingly, the NuMA knockdown had little effect on induction of the p53-dependent pro-apoptotic PUMA gene. Furthermore, NuMA is required for the recruitment of Cdk8, a component of the Mediator complex and promoter of the p53-mediated p21 gene function. These data demonstrate that NuMA is critical for the target selectivity of p53-mediated transcription (Ohata H. et al. *Molecular and Cellular Biology* 2013).

2) PHLDA3 is a p53-regulated repressor of Akt and a novel suppressor of neuroendocrine tumors

p53 and Akt are critical players regulating tumorigenesis with opposite effects: whereas p53 transactivates target genes to exert its function as a tumor suppressor, Akt phosphorylates its substrates and transduces downstream oncogenic signals. In addition, p53 and Akt negatively regulate each other to balance oncogenic and tumor-suppressive signals within a cell. We have identified PHLDA3 as a p53 target gene, which encodes a PH domain-only protein. We found that PHLDA3 competes with the PH domain of Akt for binding of membrane lipids, thereby inhibiting Akt translocation to the cellular membrane and activation (Kawase T. et al. *Cell* 2009). We demonstrated the suppression of anchorage-independent cell growth by PHLDA3, and furthermore, frequent loss of the PHLDA3 genomic locus in primary endocrine tumors. In addition, we demonstrated hyperactivation of Akt and hyperplasia in endocrine tissues in PHLDA3 deficient mice. These results collectively indicate that PHLDA3 is a novel tumor suppressor of endocrine tumors. Our results reveal a new mode of coordination between the p53 and Akt pathways, and show that PHLDA3 is an important downstream mediator of p53 to regulate Akt activity.

3) New Approaches of Early Detection of Pancreatic Cancers

We previously estimated that the time from tumor initiation to metastatic dissemination is at

least a decade in pancreatic ductal adenocarcinomas (Yachida S. et al. *Nature* 2010; Yachida S. et al. *Oncogene* 2013). This finding indicates that there is a large window of opportunity for medical intervention before the cancer spreads to distant organs. The goal of this project is to establish novel methods to detect the early stages of pancreatic ductal adenocarcinomas, for which we use two approaches: 1. The Genomic approach: *KRAS* and other mutations in plasma samples using next-generation sequencing technology; and 2. The Metabolomic approach: Applications of the metabolism characteristic to pancreatic ductal adenocarcinoma to develop a new tracer on PET.

4) Metagenomics: Role of the Human Gut Microbiome in Colorectal Carcinogenesis

Recent evidence based on metagenomics has highlighted the potential contribution of the microbiome in the maintenance of host homeostasis. In this project, we will clarify the relationships between the luminal microbiota and colorectal cancers and the mechanisms of potential contribution of the microbiome towards colorectal cancer development. The study is conducted with patients who undergo total colonoscopy in the National Cancer Center Hospital. Their lifestyle, such as dietary habits, is obtained from detailed questionnaires. Fecal samples are collected before colonoscopy, frozen immediately and DNA is purified by standard methods. Sequencing is carried out by whole-genome shotgun using standard protocols.

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8. Okano K, Oshima M, Kakinoki K, Yamamoto N, Akamoto S, Yachida S, Hagiike M, Kamada H, Masaki T, Suzuki Y. Pancreatic thickness as a predictive factor for postoperative pancreatic fistula after distal pancreatectomy using an endopath stapler. Surg Today, 43:141-147, 2013
9. Takai E, Tsukimoto M, Kojima S. TGF-β1 downregulates COX-2 expression leading to decrease of PGE2 production in human lung cancer A549 cells, which is involved in fibrotic response to TGF-β1. PLoS One, 8:e76346, 2013.

Division of Cancer Prevention Research

Hitoshi Nakagama, Michihiro Mutoh, Gen Fujii, Rikako Ishigamori, Masami Komiya, Ruri Nakanishi

Introduction

We are investigating mechanisms of carcinogenesis, searching for early diagnostic markers for cancer, and developing practicable cancer chemopreventive agents. Abnormal activation of β-catenin signaling, including TCF/LEF transcription factor activation, is a well-known cause of many carcinogeneses. On the other hand, dyslipidemia, alterations of adipocytokine balance and pro-inflammatory status have been suggested to be involved in the development of many cancers. In animal studies, improvement of dyslipidemia, adipocytokine imbalance and inflammation status suppressed carcinogenesis. However, underlying suppressive mechanisms are not known in detail, such as lipid metabolism changes in the cancer cells and cross-talk changes between the epithelial cells, adipocytes and macrophages. Investigations clarifying the mechanisms of dyslipidemia-, obesity- and inflammation-related carcinogenesis may lead us to develop early diagnostic markers for cancer. Based on the molecular findings, cancer chemopreventive agents were selected from clinically used medicines. Drug repositioning will be a clue to the development of effective approaches for human cancer prevention.

Research activities

Apc-deficient mouse model: P-glycoprotein (P-gp; encoded by *Mdr1a* gene) has been shown to be associated with intestinal tumorigenesis through activation of the TCF/LEF transcription factor. We examined inhibition of the P-gp function with verapamil (a clinically used antihypertensive drug), and found that verapamil could decrease the number of intestinal polyps which developed in the mice with loss of function of the *Apc* gene product.

Mouse lung carcinogenesis model: We have shown that *in vivo* SPECT imaging with ^{111}In-DOTA-c(RGDfK) is a useful method to detect early pancreatic cancer in a hamster pancreatic carcinogenesis model. Thus, we aimed to demonstrate the great potential for detecting urethane-induced A/J mice lung cancer by a combination of SPECT/CT imaging. ^{111}In-DOTA-c(RGDfK) was clearly visualized in small lung nodules, suggesting that SPECT/CT is a useful non-invasive imaging approach for evaluating the characteristics of lung tumors in mice, linking to a human imaging approach.

Hp-induced gastritis model: *Helicobacter pylori* (*Hp*) infection causes gastritis and is considered a gastric cancer risk. We have reported that codfish meal markedly enhanced *Hp*-induced gastritis in Mongolian gerbils. Thus, we examined the effects of components in codfish meal in the model, and found that intake of calcium compounds, such as hydroxyapatite and calcium carbonate, may contribute to enhance *Hp*-induced gastritis. These findings of novel risk factors may link to the development of new chemopreventive agents

Clinical trials: A double-blind, randomized, placebo-controlled clinical study was performed to evaluate the influence of low-dose aspirin enteric-coated tablets (100 mg/day for 6-10 months) in 34 familial adenomatous polyposis subjects (17 each in the aspirin and placebo groups). The sizes of colorectal polyps tended to be greater in the placebo as compared to the aspirin group, and subgroup analysis revealed that subjects with a mean baseline polyp diameter of ≤ 2 mm showed a significant reduction of mean polyp sizes in the aspirin group.

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2. Fujimoto K, Fujii G, Mutoh M, Yasunaga M, Tanaka H, Wada M. Suppression of intestinal polyp development in *Apc*$^{Min/+}$ mice through inhibition of P-glycoprotein using verapamil. Eur J Cancer Prev, 22:8-10, 2013
3. Iimuro M, Nakamura S, Arakawa T, Wakabayashi K, Mutoh M. Effects of dietary calcium on *Helicobacter pylori*-induced gastritis in Mongolian gerbils. Anticancer Res, 33:3667-3674, 2013
4. Hayakawa T, Mutoh M, Imai T, Tsuta K, Yanaka A, Fujii H, Yoshimoto M. SPECT/CT of lung nodules using ^{111}In-DOTA-c(RGDfK) in a mouse lung carcinogenesis model. Ann Nucl Med, 27:640-647, 2013
5. Komiya M, Fujii G, Takahashi M, Iigo M, Mutoh M. Prevention and intervention trials for colorectal cancer. Jpn J Clin Oncol, 43:685-694, 2013

Division of Brain Tumor Translational Research

Koichi Ichimura, Shintaro Fukushima, Taishi Nakamura, Hirokazu Takami, Emiko Yamamoto, Hideyuki Arita, Yuko Matsushita

Introduction

Our laboratory focuses on translational research into various types of malignant brain tumor. Brain tumors are a rare form of cancer, however some of them remain as one of the most difficult cancers to cure in humans. There are more than 130 different types of brain tumors, each developing through a distinct molecular pathogenesis. To facilitate an effective personalized therapy, elucidating the molecular basis of each individual tumor is essential. We therefore set our aim as follows: 1) Conduct comprehensive investigational research into the molecular basis of key malignant brain tumors such as gliomas, primary central nervous system lymphomas (PCNSL) and intracranial germ cell tumors (iGCT) to identify novel tumor markers for making a more accurate diagnosis or predicting the outcome of the patients as well as therapeutic targets; 2) Establish an optimal assay for molecular marker testing to utilize in clinical trials and routine clinical practice ; and 3) Organize and/or participate in a multicenter study to collect a large number of tumor materials to facilitate the above research and to validate the findings.

Research activities

1. Investigation for novel biomarkers in adult gliomas

Through a genetic analysis of 546 gliomas, we identified that somatic mutations at two hotspots (C228T, C250T) in the promoter region of *TERT*, the reverse-transcriptase subunit of telomerase, occur very frequently among adult gliomas. We found that *TERT* promoter mutations were particularly common among primary glioblastomas (70%), making it the most frequently mutated gene in glioblastomas. We showed that *TERT* promoter mutations invariably upregulate *TERT* expression while *TERT* promoter methylation alone did not. Thus, we showed for the first time that *TERT* promoter mutations are the main driver of telomerase upregulation, the phenomenon which has been widely known over the decades while its mechanism remained unknown up until now. *TERT* mutations are also common among oligodendroglial tumors and strongly associated with the presence of 1p19q loss and *IDH1/2* mutations, however they are uncommon in astrocytic tumors with concurrent *IDH1/2* and *TP53* mutations. These findings make *TERT* promoter mutations an ideal diagnostic marker. We are currently validating the efficacy of *TERT* promoter mutations as a biomarker in a large independent cohort of adult gliomas. We have also established a novel pyrosequencing-based assay for *MGMT* methylation testing. The presence of *MGMT* methylation has been established as a prognostic marker in glioblastomas, as well as a predictive marker for the response to temozolomide in elderly glioblastoma patients. We are currently utilizing the assay for a clinical trial to validate whether the postoperative treatment of the patients could be determined based on the *MGMT* methylation status (see below).

2. A comprehensive genome analysis on intracranial germ cell tumors

Intracranial germ cell tumors (iGCT) are the second most common brain tumors among the patients under the age of 14, however they are rare in the Western population. iGCTs are one of the few pediatric brain tumors that remain largely unexplored. We have established an Intracranial Germ Cell Tumor Genome Analysis Consortium to centrally collect surgical specimens of iGCTs nationwide and conduct a comprehensive genome analysis on them. Our current iGCT sample cohort consists of 125 iGCTs, 65 testicular germ cell tumors and 8 metastatic GCTs, which is by far the largest iGCT series in the world. We analyzed 41 iGCTs with exome sequencing and identified about 40 candidate genes, for which all the remaining tumors are being investigated using the IonTorrent system.

3. Establishment of the Japanese Pediatric Molecular Neuro-oncology group (JPMNG)

Numerous large-scale genome analyses have uncovered the molecular pathogenesis of various types of pediatric brain tumor in the last few years. A novel molecular classification which can better predict the outcome of the patients has already been developed in several tumor types such as medulloblastomas and ependymomas. To establish a standardized molecular classification system in Japan based on the international consensus and to

utilize it for better prognostication of the patients or stratification for personalized target therapy, a Japanese Molecular Neuro-Oncology Group (JPMNG) has been established as a joint project between the Japan Society for Neuro-Oncology and the Japanese Society for Pediatric Neurosurgery. As one of the core members of JPMNG, we are jointly in charge of performing molecular tests for medulloblastomas and ependymomas that are currently being collected nationwide.

Clinical trials

We are in charge of the methylation testing in the EGGTRIAL on newly diagnosed elderly glioblastomas. This clinical trial has been set to validate the earlier reports that elderly glioblastoma patients with *MGMT*-methylated tumors respond to temozolomide better than those with *MGMT*-unmethylated tumors. Patients at the age of 70 or more with newly diagnosed glioblastoma are eligible for enrollment in the EGGTRIRAL. We perform and report the results of the *MGMT* methylation test within three weeks after the operation. The patient will be treated with temozolomide alone when *MGMT* is methylated while they will receive radiation therapy alone when *MGMT* is unmethylated. The registration to the trial started in May 2013 and is scheduled to continue until 2015.

List of papers published in 2013
Journal

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Research Core Facility Division

Teruhiko Yoshida, Yasuhito Arai, Toshio Imai, Yoshinori Ikarashi, Hitoshi Ichikawa, Tetsuya Ishikawa, Shumpei Ohnami, Masaya Ono, Takahiro Ochiya, Koji Okamoto, Yae Kanai, Takuo Katsumoto, Issay Kitabayashi, Tadashi Kondo, Hiromi Sakamoto, Hiroki Sasaki, Tatsuhiro Shibata, Fumie Hosoda, Tesshi Yamada

Introduction

The Research Core Facility (CF) originated from the 1st lecture given by Dr. Hitoshi Nakagama on May 9, 2011 after his appointment as the Director of the National Cancer Center (NCC) Research Institute (RI). Along with the biobank, the CF has been positioned between the NCCRI and the NCC hospital thus establishing a bidirectional translational bridge (Figure 1). Ample collection of high quality clinical samples combined with advanced and reliable analytical power should be a crucial asset of our Institute, especially in light of international collaboration and competition. However, the latest genome and other omics technologies demand heavy investments from our researchers both in hardware, its maintenance and human expertise, which are increasingly difficult if not impossible to afford by individual laboratories, especially those staffed by young PIs and physician scientists. Animal histopathology, reproductive engineering and advanced cell biology are other areas where the availability of highly skillful CF support would make a huge difference in the progress and outcome of the research. As a consequence, the CF has become an essential component integrated in many leading biomedical research institutes in the world.

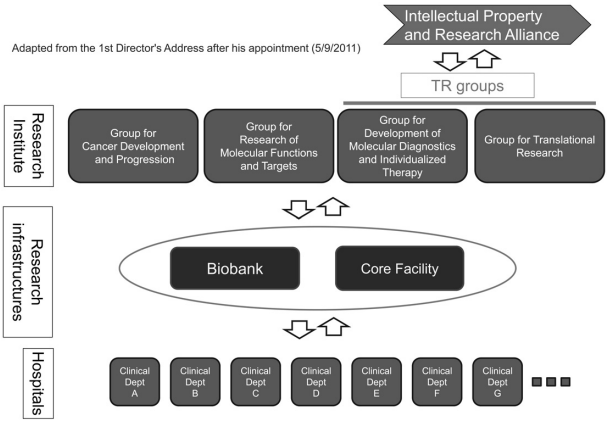


Figure 1. Concept of CF: Director's Initiative

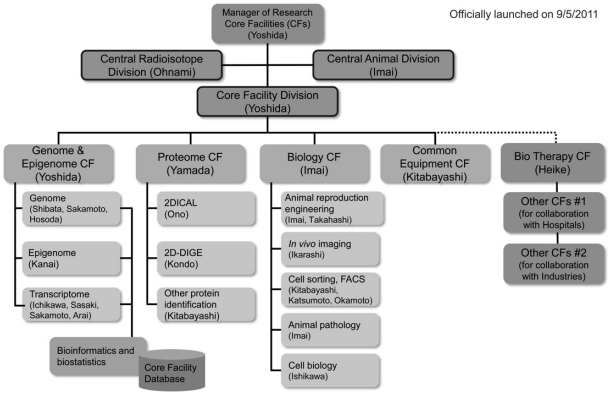


Figure 2. CF Organization

Routine activities

Following the Director's lecture, the CF was officially started on September 5, 2011 with the organization shown in Figure 2. The important point is that the CF is a virtual facility, putting together and further facilitating the existing collaborative effort by the individual research scientists and laboratories, each engaging in their own competitive research. The CF has 4 major arms to share technical expertise, *i.e.*, Genome & Epigenome, Proteome, Biology, and Common Equipment for self-service use of shared resource-demanding machines in terms of cost, space and other installation specifications.

The mission of the CF is not restricted to the mutual support and collaboration inside the NCCRI, but extends to other sectors of the NCC as a whole. For instance, the CF offers genotyping service for population-based cohort studies in the Research Center for Cancer Prevention and Screening (RCCPS), and helping observation studies in the framework of clinical trials in the hospital. Some CF staff are also involved in the NCC EPOC (Exploratory Oncology Research & Clinical Trial Center) and its translational research. The CF is also supporting a transitional zone between research and clinical practice, such as genetic diagnosis of hereditary cancer syndromes. One of the major uses of the CF is the large-scale analysis of clinical samples, and thus, the CF is closely related with several Biobank-based projects (Figure 3).

Research activities

Because the CF covers such diverse activities, its performance is difficult to quantify, but just as a simplified example, the numbers of the individual research projects and samples submitted to the CF are summarized in Figure 4. As shown on the rightmost columns, some of the projects supported by CF were directed by PIs outside of the NCCRI. Although not apparent in the table, one of the most important contributions of the CF may be the discussion and consultation BEFORE offering the actual CF service.

The activities of the Biology arm of the CF can be found in the report of the Central Animal Division.

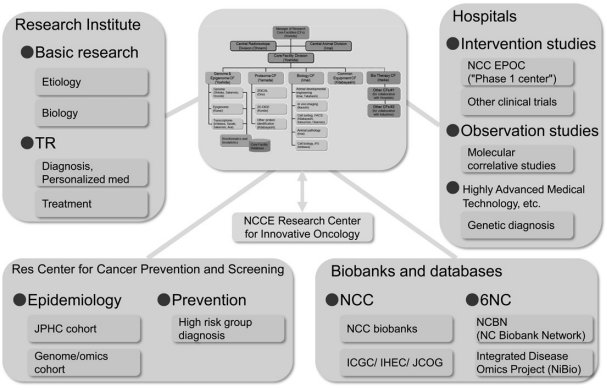


Figure 3. CF Interactions and Participations

Table 1. CF Activities in FY 2011-2013 (excluding the self-service type)

CF Area	Applications	# projects			# samples			PIs outside RI	
		FY 2011	FY 2012	FY 2013	FY 2011	FY 2012	FY 2013	Hosp	RCCPS
Genome	Next Generation Sequencer	18	19	11	739	995	346	○	○
	SNP array/TagMan assay	10	9	9	1993	1574	1777	○	○
	Agilent array and others	5	9	4	366	652	123		○
Epigenome	NGS	2	2	1	102	14	8		
	Infinium array	7	6	9	1646	569	801		○
Transcriptome	NGS	5	9	1	148	169	8		
	Affymetrix GeneChip	5	4	2	97	76	110	○	
	Agilent array	5	3	4	58	56	68		
Proteome	2DICAL	7	2	2	524	112	54	○	
	2D-DIGE	0	7	4	0	308	83		
Animal reproduction engineering	Embryo/ sperm freezing stock	2	5	5	9	36	17		
	Microbiological cleaning	1	1	5	0	1	5		
In vivo imaging	IVIS	10	2	4	-	-	-		
	OV110	2	2	0	-	-	-		
Animal histopathology	FFPE, frozen sections	12	12	11	1743	2974	1778		
	Examination and diagnosis	4	4	6	-	-	-		
Cell biology	Cell line establishment	1	1	0	5	2	0		
Total		96	97	78	7,430	7,538	5,178		

Central Animal / Radioisotope Divisions

Toshio Imai, Mami Takahashi, Tetsuya Ishikawa, Yoshinori Ikarashi, Kotomi Otsubo, Naoaki Uchiya, Momoko Kobayashi, Teruo Komatsu, Masashi Yasuda, Manabu Tsuchida, Ayami Kawashima, Satoshi Ikeda, Junichi Zukeyama, Fukase Masashi, Seki Yudai, Takuya Matsuyama, Junya Asahira, Shumpei Ohnami

Introduction

The Central Animal Division belongs to the Core Facilities for Research and Innovative Medicine, and a pivotal role of this division is supportive actions for basic/clinical/public health researchers on the basis of biological resources in the National Cancer Center.

The Central Radioisotope Division provides advanced technical training and education for researchers in the fields of molecular genetics and radiology. This division is equipped with separate laboratories where registered users can conduct experiments safely with various types of radioisotopes.

Routine activities

The important role of the Central Animal Division is health management of the experimental animals and maintenance of the animal experimentation facility in the National Cancer Center Research Institute. Some researchers and technical staff act also in the Core Facilities for Research and Innovative Medicine, and several support services are provided based on their biological skills, such as reproductive technologies for animal cleaning/embryo-sperm preservation, histopathological techniques for animal tissues and establishment of expandable cells/xenograft transplantable models from clinical cancer tissues.

Research activities

Research activities of the Central Animal Division have focused on studies of chemical carcinogenesis using laboratory animals and genetically modified cancer developing animal models, the process of graft-versus-host disease using *in vivo* imaging technologies and human induced hepatic stem cells for anti-cancer drug screening. Research activities of the Central Radioisotope Division have been performed in collaboration with the Division of Genetics and the Division of Gene and Immune Medicine.

1) Promotion of colorectal and pancreatic carcinogenesis by A^y allele

Epidemiologically, obesity has been associated with colorectal and pancreatic cancer risks, but the underlying mechanisms are not clearly understood. The A^y mice possessing the agouti yellow allele expresses ectopically the agouti gene product, which regulates energy metabolism, and become obese. We reported that A^y allele promotes colorectal tumor development in diabetic KK mice. We also found that the A^y allele promoted pancreatic carcinogenesis in *K-ras* mutant mice. These results suggest involvement of the agouti/melanocortin receptor pathway in obesity-associated cancer.

2) Pancreatic Ductal Carcinogenesis and Epithelial Mesenchymal Transition in Hamsters

Elevations in mucin 1 (MUC1) mRNA levels were found to be prominent among the up-regulated genes in atypical hyperplasias in a hamster model. Immunohistochemistry for the MUC1 cytoplasmic domain (MUC1-CD), which was not detected in normal-like pancreatic ducts, was positive in apical surfaces of the epithelia of atypical hyperplasias and invasive ductal carcinoma (IDC) areas with distinct tubular patterns. In contrast, its translocation to cytoplasmic/nuclear positivity was observed in the invasive front of IDCs. The co-expression of epithelial-mesenchymal transition (EMT)-related proteins, such as slug and vimentin, with cytoplasmic/nuclear MUC1-CD was also found. These results indicate that the alteration in the expression level and subcellular localization of MUC1 is a biphasic phenomenon and the latter may be associated with EMT in pancreatic carcinogenesis in hamsters.

3) Mechanisms of Promotion/progression of Mammary Carcinogenesis associated with a High-fat Diet

The effects of a high-fat diet (HFD) during prepubertal and pubertal stages were investigated in 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in female F344 rats. The results obtained indicated that HFD promoted carcinogenesis, and, in addition, affected aggressive phenotypes of the induced carcinomas. The molecular mechanisms of the promotion/progression as assessed with DNA microarray analysis for the carcinoma tissues were speculated to be associated

with increased expression of a couple of cell cycle/differentiation-related genes, which were reported to be up-regulated in human breast carcinoma cell lines.

4) *In Vivo* Fluorescence Imaging of Donor Cells after Allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

Visualizing the *in vivo* dynamics of donor cells after allogeneic HSCT could be useful for an understanding of the process of graft-versus-host disease (GVHD) and donor cell engraftment. The *in vivo* fluorescence imaging technique can visualize GFP donor cells in small animals in a whole-body manner. Furthermore, the combination with *in vivo* imaging and immunohistochemistry simultaneously visualize both proliferating and apoptotic cells and their origin, donor or host. These techniques enable a great deal of understanding regarding the mechanism of GVHD.

5) Human Induced Hepatic lineage-oriented Stem Cells for Drug Discovery and Regenerative Medicine in Cancer Therapy

We generated human induced hepatic lineage-oriented stem (iHS) cells from fibroblasts by gene transfer (OCT3/4, SOX2, and KLF4). The human iHS cells self-renewed during more than 80 passages. They were differentiated in a chemically defined medium without any differentiation growth factors through 12-days-culture. The almost all *in vitro*-differentiated cells were strongly positive for albumin, and negative for neuroectodermal and mesodermal marker proteins. The cells expressed hepatic genes such as *ALB*, *AFP*, *AAT*, *TTR*, *FABP1*, cytochrome P450 enzymes, and conjugating enzymes. The hepatocyte-like cells were functional for the uptake of indocyanine green, storage of glycogen, and production of urea. A protocol for fully functional hepatic differentiation of iHS cells is under investigation.

List of papers published in 2013

Journal

1. Imai T, Cho YM, Takahashi M, Kitahashi T, Takami S, Nishikawa A, Ogawa K. High susceptibility of heterozygous (+/*fa*) lean Zucker rats to 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis. Oncol Rep, 29:1914-1922, 2013
2. Ito K, Ishigamori R, Mutoh M, Ohta T, Imai T, Takahashi M. A^y allele promotes azoxymethane-induced colorectal carcinogenesis by macrophage migration in hyperlipidemic/diabetic KK mice. Cancer Sci, 104:835-843, 2013
3. Takahashi M, Mutoh M, Ishigamori R, Fujii G, Imai T. Involvement of inflammatory factors in pancreatic carcinogenesis and preventive effects of anti-inflammatory agents. Semin Immunopathol, 35:203-227, 2013

Department of Biobank Support Core

Takashi Kohno, Izumi Kobayashi, Mari Tomoda

Introduction

The Department of Biobank Support Core was established in May 2013 to support the management of the National Cancer Center (NCC) Biobank. This Department supports committees and working groups of this Biobank and the National Center Biobank Network (NCBN).

Routine activities

1. Support of the NCC Biobank

This Department functions as a secretariat for the NCC Biobank Coordination Committee and has held coordination committee conferences eight times. The consent ratio of NCC patients for cooperation in Biobanking in 2013 was 86.4%. Information on the NCC Biobank is being published through the periodical updating of the NCC-internal website and also by tours (16 including the ones for the Ministries, University Hospitals and National Institutes abroad), interviews and answering queries. The Department also functions as a contact window to researchers who need NCC Biobank samples.

2. Support of NCBN

This Department supported several conferences of NCBN committees. The topics discussed included how we should disclose research findings, including incidental ones, to patients, and how banked samples and clinical information should be provided to researchers who need them. The Department also supported the construction and publication of a catalog database on the NCBN webpage.

3. Support for translational research in the NCC

The Department supported 10 monthly Research Conferences (Table 1) inviting 28 researchers as presenters. In total, 1,694 discussants participated in the conferences. The Department also held 3 conferences for discussion on TR (Table 2) inviting three researchers as presenters. A conference is being held to promote "seeds push" and "needs pull" TRs through discussion among Group for Translational Research Support Core staff, NCC hospitals staff and EPOC staff (Figure 1).

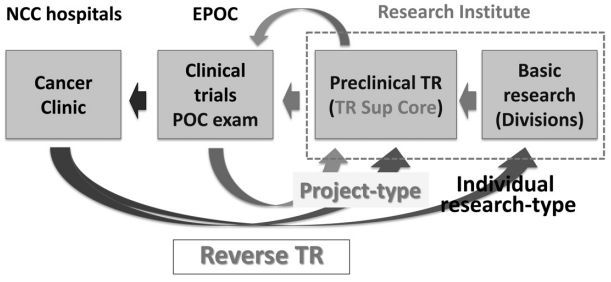


Figure 1. "Seeds Push" & "Needs-Pull" translational research approaches in the NCC

Table 1. Research conferences in 2013

Date	Topic	Presenter	No.of participant
Jan8,2013	Molecular imaging	Kenji Tamura, Hirofumi Fujii, Akinobu Hamada	215
Feb12,2013	Biobank	Yae Kanai, Izumi Kobayashi, Kazufumi Honda, Teruhiko Yoshida	173
Mar12,2013	Surgery & Medical device	Masaaki Ito, Kiyokazu Nakajima, Ichiro Sakuma	134
Apr16,2013	Clinical sequencing	Takayuki Yoshino, Takashi Kohno	231
May14,2013	Adverse event	Yoshiro Saito, Ken Kato, Teruhiko Yoshida	157
Jun11,2013	Liquid biopsy	Takahiro Ochiya, Shintaro Kanda	200
Jul16,2013	Biomarker	Shoichiro Tsugane, Motoki Iwasaki, Atsushi Goto	136
Sep10,2013	Liquid biopsy	Makiko Ono, Fumiaki Koizumi, Tsuchiya Naoto	172
Oct8,2013	Sarcoma	Akira Kawai,Tadashi Kondo	125
Dec10,2013	Familial cancer	Noriyuki Katsumata, Teruhiko Yoshida, Yoichi Naito	153

Table 2. Monthly discussion on translational research (TR) in 2013

Date	Topic	Presenter
25-Sep	Clinical sequencing in TOPICS-1	Kenji Tamura
23-Oct	TR of novel FGFR fusion in cholangiocarcinoma	Tatsuhiro Shibata
27-Nov	Reverse TR & Clinical benefit	Yasuhide Yamada

Department of Clinical Pharmacology

Akinobu Hamada, Shuichi Shimma, Yoichi Kurata, Satoko Osawa, Yu Yamanoi, Yuki Takashima, Masanobu Nishidate, Toshiyuki Hata

Introduction

The Clinical Pharmacology Group is focused on the development of pharmacokinetic analyzing system. This system provides the drug exposure in blood and tissues using a high-sensitivity triple-quadrupole mass spectrometer and mass microscope for non-label imagining mass spectrometry. Imaging mass spectrometry (IMS) is now widely used in several research fields. For pharmacokinetic studies in particular, IMS can provide novel visualization information that differs from conventional imaging technologies such as autoradiography and positron emission tomography due to its non-labeled feature.

Research activities

Our aim is to provide evaluation methods for novel drugs in clinical trial. In 2013, we started to evaluate IMS using animal models and clinical samples obtained from surgical operations and biopsies. During this evaluation, we have developed standard operational procedures from tissue sampling to obtained ion imaging analysis. The most important breakthrough was the development of a new matrix application method to provide higher ionization efficiency and tissue surface protection. Using this method, the ionization efficiency was improved by 40 times compared with previous techniques. We found this new sample preparation method was essential for IMS. In addition to the matrix application method, a tissue sectioning procedure for small specimens such as biopsies also had to be developed. In general, small tissue specimens are embedded in a polymer-based compound for sectioning. However, the compound is a well-known contaminant in IMS. The tissue surface is directly analyzed in IMS, hence the residual polymers on the tissue surface are preferentially ionized. The cluster of polymer peaks is usually detected at a high intensity compared to the target molecules. We developed a sectioning procedure without embedding, and we confirmed the feasibility of IMS for small specimens. Due to our developments in sample preparations, we started to apply IMS for a few clinical trials of molecular targeting drugs (Figure 1).

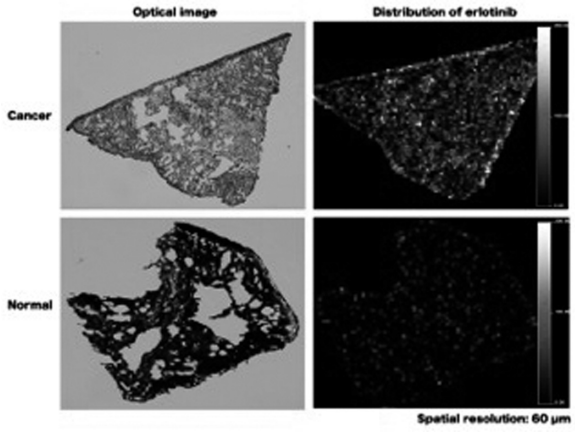


Figure 1.

List of papers published in 2013
Journal

1. Shimma S, Takashima Y, Hashimoto J, Yonemori K, Tamura K, Hamada A. Alternative two-step matrix application method for imaging mass spectrometry to avoid tissue shrinkage and improve ionization efficiency. J Mass Spectrom, 48:1285-1290, 2013
2. Hashiguchi Y, Hamada A, Shinohara T, Tsuchiya K, Jono H, Saito H. Role of P-glycoprotein in the efflux of raltegravir from human intestinal cells and CD4+ T-cells as an interaction target for anti-HIV agents. Biochem Biophys Res Commun, 439:221-227, 2013
3. Tomita Y, Yuno A, Tsukamoto H, Senju S, Kuroda Y, Hirayama M, Irie A, Kawahara K, Yatsuda J, Hamada A, Jono H, Yoshida K, Tsunoda T, Kohrogi H, Yoshitake Y, Nakamura Y, Shinohara M, Nishimura Y. Identification of promiscuous KIF20A long peptides bearing both CD4+ and CD8+ T-cell epitopes: KIF20A-specific CD4+ T-cell immunity in patients with malignant tumor. Clin Cancer Res, 19:4508-4520, 2013
4. Tsubata Y, Okimoto T, Miura K, Karino F, Iwamoto S, Tada M, Honda T, Hamaguchi S, Ohe M, Sutani A, Kuraki T, Hamada A, Isobe T. Phase I clinical and pharmacokinetic study of bi-weekly carboplatin/paclitaxel chemotherapy in elderly patients with advanced non-small cell lung cancer. Anticancer Res, 33:261-266, 2013

Department of Translational Research Seeds Evaluation

Fumiaki Koizumi, Takeshi Sawada, Shigehiro Yagishita, Yoshitaka Seki, Satoshi Kitazono, Jun Hashimoto, Takayuki Sasaki, Yuka Kitamura, Misaki Ono, Mayumi Akitaya, Rumi Koyama, Yukiko Ito

Introduction

The Department of Translational Research Seeds Evaluation is a newly established section of the Translational Research Support Group. The main goal of our team is to develop safe and effective cancer biomarkers for molecular targeted cancer drugs. We have two main projects ongoing to achieve this goal: the "ADCC project" and the "CTC project".

Research activities

Objective

1. CTC project (Figure 1.)

The aim of this project is to develop a novel flow-cytometry-based circulating tumor cell (CTC) detection and sorting system (On-Chip Sort) independent of the tumor cell EpCAM expression. The system is expected to provide useful clinical information on prognosis, cancer staging, drug choice, and therapeutic efficacy.

2. ADCC project (Figure 2.)

This project's goal is to develop a new assay system for predicting the antibody-dependent cellular cytotoxicity (ADCC) activity and the clinical outcome in patients receiving antibody therapy.

Approach

1. CTC project

In our assay, 4 mL of samples is hemolyzed and CD45+ leucocytes are eliminated using magnetic beads coated with an anti-CD45 antibody. After negative CTC enrichment, samples are fixed and labeled with FITC-cytokeratin, PE-EpCAM and Alexa700-CD45 antibodies, and analyzed with our flow-cytometry-based CTC detection and sorting system. In a preclinical study, various kinds of cell line were spiked into human blood. The sensitivity in detection and the possibility of mutation analysis from captured cells were evaluated. In a clinical study, 40 advanced lung cancer patients, 40 advanced breast cancer patients and 5 healthy donors were recruited at the National Cancer Center Hospital (NCCH). The results of the CTC enumeration were compared with those from CellSearch®.

2. ADCC project

We used the peripheral blood mononuclear cells (PBMCs) of eight healthy volunteers (HVs) to examine the degree of ADCC with the calcein assay. To identify molecular markers that might be correlated with ADCC activity, we adopted an *ex vivo* gene expression analysis in which changes in the mRNA expression after exposure to IgG can be measured quantitatively.

Current Research Outcomes

1. CTC project

Enumeration of the spiked tumor cells was linear over a range of 10 to 1000 cells, with a recovery rate of ≥ 90%. A significantly higher recovery rate was observed with our system (90 - 102%) than with CellSearch® (0%) when EpCAM-negative PC-14 cells were spiked, suggesting a superior sensitivity of our system in capturing EpCAM-negative tumor cells. A mutation analysis of captured cells from spiked cultured cells in peripheral blood was successfully performed. In 22 blood samples from lung cancer patients, CTCs detected by our system ranged from 0 - 18 CTCs (median, 6.5), and 77.3% (17/22) of the samples were above the threshold level (≥ 4 / 4 mL). On the other hand, with CellSearch®, only 27% of the samples had a ≥ 2 / 7.5 mL threshold level. In 2 blood samples from healthy donors, our system detected 0 and 3 CTCs, and CellSearch® detected 0 and 1 CTC. In 24 blood samples from breast cancer patients, CTCs detected by FISHMAN-R ranged from 0 - 829 CTCs (median, 4.5), and 86.3% (19/22) of the samples were above the threshold level (≥ 2 / 4 mL). On the other hand, CellSearch® detected CTCs in only 22.7% (5/22) of the samples which had a CTC threshold level (≥ 2 CTCs / 7.5 ml PB). In 2 blood samples from healthy donors, 1 CTC was detected by On-Chip Sort and the CellSearch detected none. These results of a clinical feasibility study showed our system to be significantly more sensitive for CTC enumeration in lung and breast cancer. Isolation of CTCs is achieved in lung cancer and breast cancer patients by our novel CTC system. We also demonstrated mutation detection (EGFR and PIK3CA mutations) from isolated CTCs.

2. ADCC project

We demonstrated that the inter-individual differences in trastuzumab-mediated ADCC activities of the PBMCs were consistent and reproducible. Using this technology, we tested 14 candidate genes

and found that the increase (expressed as a fold increase (FI)) in the expressions of several cytokines were significantly correlated with the ADCC activity. Next, we conducted a prospective evaluation in 18 patients who were receiving trastuzumab-based neoadjuvant chemotherapy, to determine whether the FIs in the expressions of these 14 genes were associated with a pathological complete response (pCR). Patients who showed a pCR showed higher FIs in the expressions of four genes (among the four selected genes, two were also selected in an *in vitro* ADCC association experiment). than those who did not show a pCR ($p = 0.004$, $p = 0.015$, $p = 0.0495$, and $p = 0.014$, respectively).

Future Perspectives

1. CTC project

We plan to apply this approach in the assay of clinical samples from patients with a variety of cancer types and investigate the correlation among gene mutations in sorted CTCs, circulating free DNA and primary lesions.

2. ADCC project

A prospective clinical trial to validate our results and use this system in the clinical setting is now in the planning stage. In the near future, we will also propose conducting clinical trials for rituximab and cetuximab.

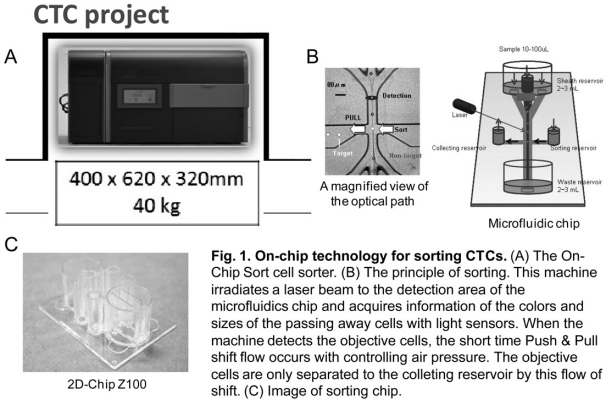
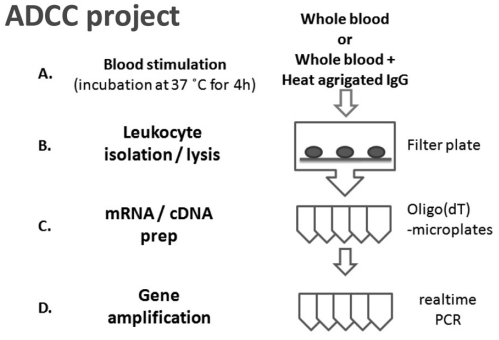


Fig. 1. On-chip technology for sorting CTCs. (A) The On-Chip Sort cell sorter. (B) The principle of sorting. This machine irradiates a laser beam to the detection area of the microfluidics chip and acquires information of the colors and sizes of the passing away cells with light sensors. When the machine detects the objective cells, the short time Push & Pull shift flow occurs with controlling air pressure. The objective cells are only separated to the colleting reservoir by this flow of shift. (C) Image of sorting chip.

Figure1.



Assay principle and procedure

A blood stimulation.
B Thawed sample is transferred to filter plate to trap leukocytes on membrane, then lysis buffer is added into a well.
C Lysate is transferred to oligo(dT)-immobilized microplate for poly(A)$^+$ mRNA isolation, followed by cDNA synthesis on the same plate.
D The cDNA solution is transferred to 384-well plate for real time PCR.

Figure2.

List of papers published in 2013
Journal

1. Katanasaka Y, Kodera Y, Kitamura Y, Morimoto T, Tamura T, Koizumi F. Epidermal growth factor receptor variant type III markedly accelerates angiogenesis and tumor growth via inducing c-myc mediated angiopoietin-like 4 expression in malignant glioma. Mol Cancer, 12:31, 2013
2. Kondo S, Ueno H, Hosoi H, Hashimoto J, Morizane C, Koizumi F, Tamura K, Okusaka T. Clinical impact of pentraxin family expression on prognosis of pancreatic carcinoma. Br J Cancer, 109:739-746, 2013
3. Nishio M, Horai T, Horiike A, Nokihara H, Yamamoto N, Takahashi T, Murakami H, Koizumi F, Nishio K, Yusa W, Koyama N, Tamura T. Phase 1 study of lenvatinib combined with carboplatin and paclitaxel in patients with non-small-cell lung cancer. Br J Cancer, 109:538-544, 2013
4. Nakadate Y, Kodera Y, Kitamura Y, Tachibana T, Tamura T, Koizumi F. Silencing of poly(ADP-ribose) glycohydrolase sensitizes lung cancer cells to radiation through the abrogation of DNA damage checkpoint. Biochem Biophys Res Commun, 441:793-798, 2013

Department of Clinical Genomics

Hitoshi Ichikawa, Fumie Hosoda, Sachiyo Mitani, Shizuka Shinohara

Introduction

The Department of Clinical Genomics was organized in 2013 in order to support genome, epigenome and transcriptome analyses of clinical samples in translational research with the aim of realizing personalized cancer treatment based on those omics data. For this purpose, small-scale next generation sequencers, Illumina MiSeq and Ion Proton, have been set up in this department, and sequencing services are provided which can detect base-substitution and insertion/deletion mutations, gene amplifications, and gene fusions from cancer tissues including formalin-fixed paraffin-embedded (FFPE) samples.

Research activities

Target sequencing analysis from FFPE cancer tissues

Next generation sequencing technology has enabled us to unravel most of the genetic alterations generated in cancer genomes. However, in actual research projects, available cancer tissue samples are often limited and not enough for typical next generation sequencing analysis. This Department accepts FFPE tissue samples which are easier to access than frozen tissue samples, and supports target sequencing of tens to hundreds of cancer genes on commercial or in-house cancer panels using the small-scale next generation sequencers. This year thymic cancer and pancreatic cancer were analyzed upon request from researchers in Hospital and Research Institute.

Development of clinical sequencing system

Individual cancers harbor a set of genetic aberrations such as mutations, gene amplifications and gene fusions, and some of them are expected to become informative biomarkers to predict the therapeutic response in molecular targeted therapies. Next generation sequencers have the potential to become an important tool in diagnosis and therapeutic decision-making in cancer treatment because of their ability to sequence a large number of clinically relevant cancer genes in a single test and to detect mutations with high sensitivity. As the basis of realizing omics data-driven personalized cancer treatment in the National Cancer Center (NCC), we have developed a clinical sequencing system which can identify genetic alterations of targetable or actionable cancer genes with next generation sequencers. In the present version of this system, 90 potentially targetable or actionable genes were selected as an in-house cancer panel (NCC oncopanel v2), and all exons of these 90 genes and introns of 10 genes among them were captured and sequenced for detection of mutations/gene amplifications and gene fusions, respectively. By using a novel algorithm developed by researchers of the Department of Bioinformatics, mutations, gene amplifications and gene fusions could be called out. This sequencing system was adopted in the TOPICS-1 (Trial of Onco-Panel for Introduction into Clinical Study-Phase 1) study, a clinical study to investigate the feasibility and utility of clinical sequencing in early phase clinical trials, and the performance of this system in the clinical setting is now under investigation.

Department of Translational Oncology

Hiroki Sasaki, Kazuhiko Aoyagi, Masashi Tamaoki, Rie Komatsuzaki, Fumiko Chiwaki, Shinzo Mayuzumi, Akio Ashida

Introduction

In 2013, the two major research areas of the Department of Translational Oncology were 1) preclinical studies using newly established gastric cancer cell lines for derivation of industrial and academia seeds/drugs to EPOC, and 2) development of personalized cancer diagnosis and treatment.

Preclinical Studies Using Newly Established Gastric Cancer Cell Lines

Genome-wide genetic information in 770 cancer cell lines is available on COSMIC DB (Sanger Center, UK); however, among them, only 21 cell lines are derived from gastric cancer (GC). Almost all of the 21 GC cell lines were established many years ago. Only insufficient clinical and pathological information is attached. The establishment of new GC cell lines, especially from metastatic sites after therapy, has been urgently required. Peritoneal metastasis is most frequent in GCs, especially diffuse-type GCs. In collaboration with the Division of Genetics, we have newly established 43 diffuse-type GC derived cell lines from the cancer ascites of 21 patients. Now we possess 74 GC cell lines including 66 diffuse-type (new 43 and existing 23) and 8 intestinal-type. We have also established peritoneal metastasis model mice (1). In collaboration with two pharmaceutical companies, *in vitro* and *in vivo* preclinical studies of 4 molecular-targeted drugs using 39 representative cell lines (33 diffuse- and 6 intestinal-type) expressing luciferase and GFP were conducted to derivate them to the Exploratory Oncology Research & Clinical Trial Center (EPOC).

Development of Personalized Diagnosis and Treatment for Cancer

Two major research projects are underway in this category: First, we developed mini DNA chips containing 6 marker and 3 control genes for predicting gastric cancer recurrence from peritoneal washings. Peritoneal cytology (CY) offers important prognostic information for gastric cancer after surgery; however, CY provides only a limited sensitivity and the task requires great skill. Our goal is to develop a sensitive tool that could be used in a clinical laboratory agency as a substitute for skilled cytology. We recently developed a mini DNA chip assay by using 98 retrospectively-collected peritoneal washings (Ann Surg Oncol 14:1694-1702, 2007). Next, we conducted a prospective study on 189 advanced cancers with more than 4 years of follow up. Prognoses of 36 CY0/DNA chip+ cases were found to be as poor as those of 34 CY1 cases. In 140 P0CY0 patients, LN+/DNA chip+ cases showed the highest recurrence (83%, 19/23), while LN-/DNA chip- cases showed the lowest (4%, 2/45). Our collaborating company prepared many supporting data for submitting the mini DNA chip to the Pharmaceuticals and Medical Devices Agency (PMDA) for marketing approval as an IVD. Second, we successfully identified 5 intrinsic subtypes of esophageal squamous cell carcinomas by gene expression-based unsupervised clustering of 274 biopsy samples obtained before treatment. The 274 profiles were divided into a test set (107 cases containing 35 and 72 cases received chemoradiotherapy (CRT) or surgery) and a validation set (167 cases containing 90 and 77 cases, respectively). Five intrinsic subtypes including 2 new subtypes were identified in the test set, and these were reproducibly found in the validation set. The two new subtypes were very similar to the previously-identified subtypes, D and B. Subtype-D included 26% 5-year survivors with CRT, whereas subtype-B contained 72%, which was clearly higher than the cases treated by neoadjuvant chemotherapy (55%, JCOG9907). Furthermore, we could find certain molecular pathways acting in each subtype that lead to not only elucidation of responsiveness to CRT but also the future treatment.

List of papers published in 2013
Journal

1. Fujita T, Yanagihara K, Takeshita F, Aoyagi K, Nishimura T, Takigahira M, Chiwaki F, Fukagawa T, Katai H, Ochiya T, Sakamoto H, Konno H, Yoshida T, Sasaki H. Intraperitoneal delivery of a small interfering RNA targeting *NEDD*1 prolongs the survival of scirrhous gastric cancer model mice. Cancer Sci, 104:214-222, 2013
2. Aoyagi K, Tamaoki M, Nishumura T, Sasaki H. Technical considerations for analyzing EMT-MET data from surgical samples. Cancer Lett, 341:105-110, 2013
3. Ono H, Chihara D, Chiwaki F, Yanagihara K, Sasaki H, Sakamoto H, Tanaka H, Yoshida T, Saeki N, Matsuo K. Missense allele of a single nucleotide polymorphism rs2294008 attenuated antitumor effects of prostate stem cell antigen in gallbladder cancer cells. J Carcinog, 12:4, 2013
4. Ishii H, Sasaki H, Aoyagi K, Yamazaki T. Classification of gastric cancer subtypes using ICA, MLR and Bayesian network. Stud Health Technol Inform, 192:1014, 2013

Department of Biomarker Evaluation

Takashi Kohno, Yoshinori Ishikawa

Introduction

The Department of Biomarker Evaluation was established in May 2013 to conduct evaluation of candidate biomarkers developed for personalized cancer therapy.

Research activities

By participating in conferences for discussion on translational research (TR, see the report of The Department of Biobank Support Core), TRs that enable us to identify and develop novel biomarkers were considered. Candidates for biomarkers will be obtained both by "seeds push" and "needs pull" TR projects in the near future, therefore, the Department will support the development of biomarkers applicable in cancer clinics through immunohistochemical and other analyses.

Department of Bioinformatics

Mamoru Kato

Introduction

Department of Bioinformatics launches officially on November of this year. Missions of our department are 1) bioinformatics analysis support for experimental departments in Group for Translational Research Support Core, 2) bioinformatics analysis support for other groups in the National Cancer Center (NCC), and 3) to develop new bioinformatics and data-analysis methods necessary for emerging genomics technologies.

Research activities

- We took charge of the bioinformatics part in the clinical sequencing project in the NCC. We developed a new software system that processes a large amount of data produced by the next generation sequencer (NGS) to detect SNVs/indels/fusion genes and to check data quality. The sensitivity and the specificity of the SNV detection system were nearly 100% even for a simulated tumor purity of 20% when we compared with SNP array data. When we used NGS data to compare our SNV detection tool with other computational tools, we confirmed that ours showed a much higher performance than the other tools, in particular, for data from the Ion sequencer. Also, when we made a comparison in the detection of fusion genes, ours was far superior to another commonly-used tool.

- We setup a database that stores clinical sequencing data to be made use of for new findings.

- We provided bioinformatics analysis support for studies on liver cancer as a part of ICGC, metastasis of breast cancer, bile duct cancer in the Division of Cancer Genomics, for studies on DNA adductomics, gene expression of cancer stem cells, miRNAs in the Division of Cancer Development System, and for a study on germinoma in the Division of Brain Tumor Translational Research.

- We started a project on single-cell sequencing to reveal intra-tumor heterogeneity and related cancer-cell evolution, collaborating with the Division of Caner Genomics and the Division of Cancer Development System. We succeeded in sequencing with the new technology for some single cells.

List of papers published in 2013
Journal

1. Rajaram M, Zhang J, Wang T, Li J, Kuscu C, Qi H, Kato M, Grubor V, Weil RJ, Helland A, Borrenson-Dale AL, Cho KR, Levine DA, Houghton AN, Wolchok JD, Myeroff L, Markowitz SD, Lowe SW, Zhang M, Krasnitz A, Lucito R, Mu D, Powers RS. Two Distinct Categories of Focal Deletions in Cancer Genomes. PLoS One, 8:e66264, 2013