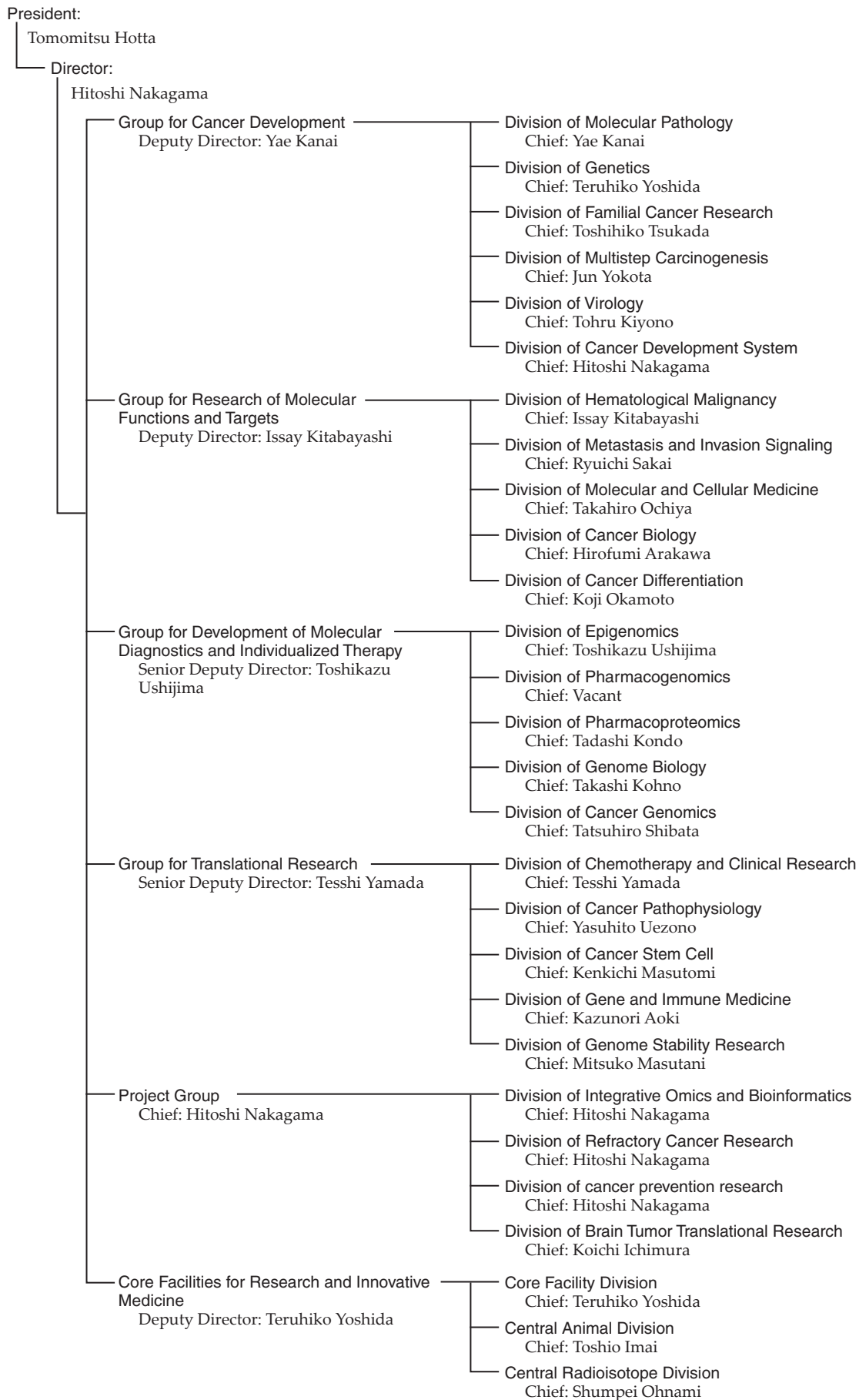# Research Institute

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 in 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 90 postdoctoral fellows, and more than 180 supporting staff. Foreign scientists and research fellows are also welcomed on a regular basis. The "Annual Report 2012" 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 interactions, such as the International Cancer Genome Consortium (ICGC), International Cancer Biomarker Consortium (ICBC), and International Human Epigenome Consortium (IHEC). We further encourage our members to develop international collaborations 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

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Tomomitsu Hotta

Director:

Hitoshi Nakagama

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  Chief: Yae Kanai
- Division of Genetics
  Chief: Teruhiko Yoshida
- Division of Familial Cancer Research
  Chief: Toshihiko Tsukada
- Division of Multistep Carcinogenesis
  Chief: Jun Yokota
- Division of Virology
  Chief: Tohru Kiyono
- Division of Cancer Development System
  Chief: Hitoshi Nakagama

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- Division of cancer prevention research
  Chief: Hitoshi Nakagama
- Division of Brain Tumor Translational Research
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  Chief: Teruhiko Yoshida
- Central Animal Division
  Chief: Toshio Imai
- Central Radioisotope Division
  Chief: Shumpei Ohnami

Activities of the Divisions

Division of Molecular Pathology

Yae Kanai, Nobuyoshi Hiraoka, Shigeki Sekine, Yoshinori Ishikawa (Ino), Masahiro Gotoh, Hidenori Ojima, Eri Arai, Taisuke Mori

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

Multilayer-omics analysis during multistage carcinogenesis

To clarify the significance of DNA methylation alterations during carcinogenesis, a methylome analysis using a single CpG resolution Infinium array was performed. Non-cancerous renal tissue samples obtained from patients with renal cell carcinomas were already at precancerous stages associated with the accumulation of DNA methylation alterations. An unsupervised hierarchical clustering analysis based on DNA methylation levels at the CpG sites, where DNA methylation alterations had occurred in precancerous stages and were inherited by and strengthened in tumorous tissue samples, identified CpG islands methylator phenotype (CIMP)-positive renal cell carcinomas (1). Clinicopathologically aggressive tumors accumulated in the CIMP-positive cluster which was characterized by the accumulation of DNA hypermethylation on CpG islands. The cancer-free and overall survival rates of patients with CIMP-positive renal cell carcinomas were significantly lower than those of patients with CIMP-negative renal cell carcinomas. DNA hypermethylation of the *FAM150A*, *GRM6*, *ZNF540*, *ZFP42*, *ZNF154*, *RIMS4*, *PCDHAC1*, *KHDRBS2*, *ASCL2*, *KCNQ1*, *PRAC*, *WNT3A*, *TRH*, *FAM78A*, *ZNF671*, *SLC13A5* and *NKX6-2* genes became hallmarks of CIMP in renal cell carcinomas (1). We have applied for patents regarding prognostication using CIMP-marker genes and are now attempting to make such prognostication techniques clinically applicable. We are now performing whole-exome, transcriptome, proteome analyses to identify molecular targets in CIMP-positive and -negative patients with renal cell carcinomas.

Whole-exome analysis of renal cell carcinomas identified frequent somatic non-synonymous mutations of *GCN1L1*, *MED12* and *CCNC*, which are members of the *CDK8* mediator complex directly regulating β-catenin-driven transcription. Mutations of *MACF1*, which functions in the Wnt/β-catenin signaling pathway, were also identified in renal cell carcinomas. A combination of methylome and transcriptome analyses further highlighted the significant role of the Wnt/β-catenin signaling pathway in renal carcinogenesis. Genetic aberrations and reduced expression of *ERC2* and *ABCA13* were frequent in renal cell carcinomas, and *MTOR* mutations were identified as one of the major disrupters of cell signaling during renal carcinogenesis. Our results confirm that a multilayer-omics analysis can be a powerful tool for revealing pathways that play a significant role in carcinogenesis.

The Infinium assay revealed that non-cancerous lung tissue obtained from patients with lung adenocarcinomas was at precancerous stages with DNA methylation alterations. The DNA methylation status of specific genes at precancerous stages significantly correlated with recurrence after establishment of lung adenocarcinomas. DNA hypermethylation of such recurrence-related genes, *ADCY5*, *EVX1*, *GFRA1*, *PDE9A* and *TBX20* genes, resulted in reduced mRNA expression in tumorous tissue samples. 5-Aza-2′-deoxycytidine treatment of lung cancer cell lines restored the mRNA expression levels of these genes. Reduced mRNA expression in tumorous tissue samples of these genes significantly correlated with tumor aggressiveness. DNA methylation alterations at precancerous stages can therefore determine tumor aggressiveness and outcome through silencing of specific genes (20).

Activiries in the international human epigenome consortium (IHEC)

The IHEC has been established by researchers and founding agencies from Canada, South Korea, the EU, Italy, Germany, Japan and the USA to comprehensively characterize the standard epigenome profiles of multiple normal cell lineages from different human populations (http://www.ihec-epigenomes.org/). We are now one of several Japanese member teams of the IHEC supported by the Core Research for Evolutional Science and Technology (CREST) division of the Japan Science and Technology (JST) Agency. We are now performing whole-genome bisulfite sequencing using the post-bisulfite adaptor-tagging method,

chromatin immunoprecipitation sequencing and RNA sequencing of purified target cells, i.e. hepatocytes, oval cells and Kupffer cells from the liver, foveolar epithelial cells, mucosal neck cells and fundic gland mucosal cells from the stomach, and foveolar epithelial cells from both the ascending and descending colon. Accurate epigenome profiling of normal cells will allow the identification of disease-specific epigenome profiles, thus facilitating a potential breakthrough in the prevention, diagnosis and therapy of diseases.

Antitumor immune responses

The host immune reaction is one of the leading players in the tumor microenvironment that is characterized by anti-tumor and pro-tumor. Tumor-infiltrating M2 macrophages, neutrophils, or the prevalence of Tregs were independent prognosticators of a worse outcome in patients with pancreatic cancers, whereas CD4$^+$ T cells, CD8$^+$ T cells, or the prevalence of HLA-DR$^+$CD68$^+$ M1 macrophages were independent prognosticators. We then connected the apparently related factors, and two variables emerged: tumor-infiltrating CD4$^+$T^{high}/CD8$^+$T^{high}/%Treg^{low} and tumor-infiltrating %M1^{high}/M2^{low}. These are independent prognosticators useful for evaluating the immune microenvironment of pancreas cancer.

Role of β-catenin in hepatocarcinogenesis

CTNNB1, encoding β-catenin, is one of the most frequently mutated oncogenes in hepatocellular carcinomas. However, it remains unclear how active β-catenin signaling confers growth advantage to hepatocytes during tumorigenesis. Our previous analyses identified several genes induced by active β-catenin signaling in hepatocellular carcinomas. To clarify the functional significance of these β-catenin-regulated genes *in vivo*, we have introduced a transposon-based model of hepatocarcinogenesis.

Now, we are analyzing the roles of hepatocellular carcinoma-related genes, including those regulated by β-catenin, as well as their functional interactions in the development of hepatocellular carcinomas.

Clinicopathological studies

From surgically resected materials of biliary tract cancers, the establishment of cancer cell lines and mouse xenograft models is routinely performed. Using originally-established cell lines and xenograft models, the efficacy of newly developed anti-cancer drugs was examined *in vitro* and *in vivo* (2). Based on such preclinical data, clinical trials of the examined drugs have recently been launched.

Widely scattered nuclear 'dot-like' focal immunoreactivity for DNA methyltransferase (DNMT) 3B in testicular seminomas reflects the potential for differentiation to embryonal carcinomas and other non-seminomatous testicular germ cell tumors: patients with stage I seminomas showing focal DNMT3B expression are at increased high risk of relapse, and should be subject to careful surveillance (3). The existence of arginase II -expressing cancer-associated fibroblasts is an indicator of a poor prognosis, as well as hypoxia, in pancreatic ductal carcinoma tissue. The histopathological examination of consecutive patients with pancreatic ductal carcinomas revealed that pancreatic intraglandular metastasis predicts a poorer outcome in postoperative patients. The presence of activating *GNAS* mutations is a characteristic genetic feature of colorectal villous adenoma (4) and pyloric gland adenomas of the stomach and duodenum. Multivariate analyses identified the independent risk factors for lymphatic and venous involvement, such as a larger tumor size, deeper invasion, and the presence of an undifferentiated-type adenocarcinoma component, in endoscopically resected gastric cancers. Other clinicopathological studies were also conducted to further the understanding of the pathogenesis and promote the diagnosis and treatment of various tumors (5-9).

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DIVISION OF GENETICS

Teruhiko Yoshida, Hiromi Sakamoto, Fumiaki Koizumi, Hiroki Sasaki, Hitoshi Ichikawa, Norihisa Saeki, Kazuhiko Aoyagi, Yasuo Kodera, Kazuyoshi Yanagihara, Takao Nishimura, Takeshi Sawada, Jun Hashimoto, Shinji Nakamichi, Sumiko Ohnami, Mineko Ushiama, Yoko Odaka, Misuzu Okuyama, Rie Komatsuzaki, Fumiko Chiwaki, Sachiyo Mitani, Akiko Takahashi, Masumi Shimizu, Mika Shioya, Sayaka Mito, Mayumi Akitaya, Yuka Kitamura, Yukiko Ito, Rumi Koyama, Hiroo Takahashi, Aya Kuchiba, Takayuki Sasaki, Akio Ashida, Hiroe Sakuyama, Nozomi Nakata, Masashi Tamaoki

Introduction

In 2012, the three major research areas of the Division of Genetics were 1) molecular understanding of cancer susceptibility; 2) transcriptome analyses of cancers and leukemia; and 3) development of personalized cancer diagnosis and treatment. We have also maintained our participation in the biobanking project of the Tsukiji campus of NCC, particularly for the peripheral blood samples.

Genetic susceptibility to cancers

We continued an investigation of prostate stem cell antigen (*PSCA*), a gene related to susceptibility to diffuse-type gastric cancer, which was identified by our previous genome-wide association study (GWAS). *PSCA* is overexpressed in many types of cancers such as prostate cancer, in which the gene has been considered to have a cancer-promoting function. However, we found that *PSCA* is down-regulated in gastric cancer and has a cell-proliferation inhibition activity in a gastric cancer cell line, suggesting its role in tumor suppression at least in the case of gastric carcinogenesis. We investigated the PSCA expression status in several cancers and found that PSCA is also down-regulated in gallbladder cancer, and functional analyses revealed a cell-proliferation inhibition activity in gallbladder cancer-derived cell lines (3). These findings indicate the *PSCA* could have a tumor-suppressive function depending on the context of the cancer type. We also reported *PSCA* expression in the islet of the normal human pancreas (4).

Clinical genetic testing on hereditary cancer syndromes has been continued as a long-standing collaborative effort with the Genetic Counseling Division of the National Cancer Center Hospital to support its genetic diagnosis.

Transcriptome analyses of cancers and leukemia

The Division has been involved in the NiBio Integrated Disease Omics Project, which is a multi-center collaborative work to identify the therapeutic molecular targets for 11 major diseases including adult solid tumors through multi-layered omics analyses. We have provided genome and transcriptome analysis core activities. This year, through an RNA sequencing analysis of 30 lung adenocarcinomas, a *KIF5B-RET* fusion gene was identified as a novel therapeutic target in a collaborative work with the researchers in the Division of Genome Biology and the National Center for Global Health and Medicine. Other collaborative research included a gene expression profiling analysis of leukemic and normal hematopoietic cells to understand the molecular pathways leading to leukemia and to develop their clinical applications (17, 18).

Development of personalized diagnosis and treatment for cancer

Five major research projects are underway in this category: First, we developed and validated mini DNA chips containing 6 marker (9) and 3 control genes for predicting gastric cancer recurrence from peritoneal washing samples with 189 first cohort and 250 second cohort samples. We have also established peritoneal metastasis model mice and 41 new gastric cancer cell lines, and identified the biological features of peritoneal metastasis-associated gastric CSCs. Second, we successfully identified 4 intrinsic subtypes (B, C1, D, F3) of ESCCs through the gene expression-based unsupervised clustering of four independent sets of 85, 72, 40, and 77 biopsy samples. The subtype D included merely 20-25% 5-year survivors treated with definitive CRT but subtype B contained 65-70%, which was clearly higher than the cases treated with neoadjuvant chemotherapy (55%, JCOG9907).

In both types, the main transcriptional pathways were identified. The third project is on everolimus, an oral mTOR inhibitor, which effectively inhibited cell growth at concentrations under 100 nM (IC$_{50}$) in five of nine triple-negative breast cancer (TNBC) cell lines. All five sensitive cell lines were categorized as a basal-like subtype positive for either epidermal growth factor receptor (EGFR) or CK5/6, whereas the resistant cell lines were not of this subtype. *In vivo* assays demonstrated antitumor activity in a mouse xenograft model of basal-like breast cancer but not in the non-basal breast cancer. These results suggested that everolimus had a preferential activity against the basal-like subtypes of TNBCs (5). The forth project is the development of a novel flow-cytometry-based detection and sorting system, On-Chip Sort, for circulating tumor cells (CTCs) independent of EpCAM expression of tumor cells. The spiked tumor cells count 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 CellSearch (0%) when EpCAM-negative PC-14 cells were spiked, suggesting the superior sensitivity of our system especially in capturing the EpCAM-negative tumor cells. The fifth project has focused on the large difference in the clinical outcomes among patients with the HER2-positive type of breast cancer, which remains a key issue for trastuzumab treatment. We demonstrated that the inter-individual differences in the trastuzumab-mediated ADCC activities of the PBMC were consistent and reproducible. An *ex vivo* gene expression analysis has been developed to measure changes in the mRNA expression quantitatively after exposure to IgG. Using this technology, we found that the increased expressions of TNFSF15, IL6 and CXCL3 were significantly correlated with the ADCC activity. The association was apparently replicated in a prospective evaluation of the patients who were receiving trastuzumab-based neoadjuvant chemotherapy.

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Research Institute

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Division of Familial Cancer Research

Toshihiko Tsukada, Yuko Nagamura, Satoko Shimazu

Introduction

The Division of Familial Cancer Research is focusing 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. Drug resistance of prolactinoma and 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 previously unreported single nucleotide alteration in the *MEN1* gene, initially thought to be a missense variant, was shown to be a splicing mutation (1). Another missense menin variant identified in a patient suspected of harboring the MEN1 syndrome was determined as a causative mutation based on

the reduction of mutant menin stability. This finding encouraged us to conduct the presymptomatic genetic test for this mutation among the patient's family members, which led to the identification of asymptomatic mutation carriers. Thus, the menin stability test was proven useful for genetic diagnosis and counseling of MEN1 patients (2).

Drug resistance of prolactinoma

Dopamine (DA) agonists are used in the first-line treatment of prolactinoma, and normalize prolactin levels and reduce tumor size in most of the cases. However, some prolactinomas are resistant to DA agonists from the beginning of the treatment and need to be treated surgically. A few prolactinomas initially respond to DA agonists but become resistant after prolonged treatment with DA. Although the reduction of the dopamine D2 receptor (DRD2) expression in tumor cells may explain the resistance, the exact mechanism is not fully understood. DRD2 expression was investigated by measuring mRNAs of the short isoform (D2S) and the long isoform (D2L) of DRD2. DNA methylation patterns in the promoter region of the *DRD2* gene were also analyzed. The D2L mRNA levels were lower in the resistant tumors than in sensitive tumors. The DNA methylation patterns in the *DRD2* gene promoter region were not different between sensitive and resistant tumors. Thus, resistance of prolactinoma to dopamine agonists is correlated with a reduction in D2L expression levels. Silencing of the *DRD2* gene by methylation in the promoter region is unlikely to play a role in dopamine agonist resistance in prolactinoma (3).

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 release of several hormones. Growth hormone-producing pituitary cell GH3, ACTH-producing pituitary cell AtT-20 and catecholamine-producing adrenal chromaffin cell PC12 were treated with rikkunshito with or without forskolin, a direct adenylate cyclase activator. Intracellular cAMP levels increased in all these cell

lines following the treatment with rikkunshito and/or forskolin in a dose-dependent manner. The amounts of catecholamines released from PC12 cells increased after treatment with rikkunshito. The mRNA of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis, also increased. These findings suggest that rikkunshito acts directly on endocrine cells and enhances the biosynthesis and secretion of several hormones.

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DIVISION OF MULTISTEP CARCINOGENESIS

Jun Yokota, Naoto Tsuchiya, Reika Iwakawa-Kawabata, Hiroko Ogata-Kawata, Mariko Sasaki, Yuko Fujiwara, Masataka Takenaka, Daisuke Kurioka, Yusuke Kimura, Momoyo Nishida, Tomoyo Kobayashi, Yoshiaki Onozato

Lung cancer is the leading cause of cancer death worldwide. To develop novel ways of lung cancer prevention, diagnosis and treatment, it is important to elucidate the molecular processes of multistep lung carcinogenesis. For this reason, molecular genetic studies on lung cancer have been performed over the long term in the Division of Multistep Carcinogenesis. In 2012, the following results were obtained.

Activation of the EGFR, KRAS, and ALK oncogenes is known to define 3 different pathways of molecular pathogenesis in lung adenocarcinoma (LADC). However, many tumors lack activation of any pathway (triple-negative LDACs) thereby posing a challenge for prognosis and treatment. We reported on an extensive genome-wide expression profiling of 226 primary human stage I–II LADCs which elucidated the molecular characteristics of tumors that harbor ALK mutations or that lack EGFR, KRAS, and ALK mutations, that is, triple-negative LADCs. One hundred and seventy-four genes were selected as being upregulated specifically in 79 LADCs without EGFR and KRAS mutations. Unsupervised clustering using a 174-gene signature, including ALK itself, classified these 2 groups of tumors into ALK-positive cases and 2 distinct groups of triple-negative cases (groups A and B). Notably, group A triple-negative cases had a worse prognosis for relapse and death, compared with cases with EGFR, KRAS, or ALK mutations or group B triple-negative cases. In ALK-positive tumors, 30 genes, including ALK and GRIN2A, were commonly overexpressed, whereas in group A triple-negative cases, 9 genes were commonly overexpressed, including a candidate diagnostic/therapeutic target DEPDC1, that were determined to be critical for predicting a worse prognosis. Our findings are important because they provide a molecular basis of ALK-positive LADCs and triple-negative LADCs and further stratify more or less aggressive subgroups of triple-negative LADCs, possibly helping identify patients who may gain the most benefit from adjuvant chemotherapy after surgical resection.

Homozygous germline mutations of the PARK2 gene are responsible for the development of early-onset Parkinson's disease (PD). Homozygous PARK2 mutations have been also detected in LADCs. However, since heterozygous PARK2 germline mutations are present in a subset of non-PD individuals, the timing for the occurrence of two-hit PARK2 mutations in LADC progression is unclear. Therefore, we comprehensively analyzed mutations, expression and copy number variations of the PARK2 gene in 267 primary LADCs together with the corresponding noncancerous lung cells and 39 LADC cell lines. Heterozygous germline exonic deletions were detected in five patients with LADC, and loss of heterozygosity including the PARK2 locus was detected in 31/267 (11.6%) LADCs. However, homozygous PARK2 inactivation was not detected in any of them, including the five patients with germline mutations. Homozygous PARK2 inactivation was detected in 6/39 (15%) cell lines, two exonic deletions, one exonic duplication, and three point mutations, while heterozygous PARK2 inactivation was detected in two cell lines (both by exonic deletions). These results strongly indicate that somatic PARK2 mutations occur rarely (or do not occur) in LADC development and that germline PARK2 mutations could contribute to LADC progression but not to LADC development.

In collaboration with a research group in the Miyazaki University, the following results were also obtained. The development of oral squamous cell carcinoma (OSCC) is a multistep process that requires the accumulation of genetic alterations. To identify the genes responsible for OSCC development, we performed high-density single nucleotide polymorphism array analysis and genome-wide gene expression profiling on OSCC tumors. These analyses indicated that the absent in melanoma 2 (AIM2) gene and the interferon-inducible gene 16 (IFI16) mapped to the amplified region of chromosome 1q23 are overexpressed in OSCC. Both AIM2 and IFI16 are cytoplasmic double-stranded DNA sensors for innate immunity and act as tumor suppressors in several human cancers. Knockdown of AIM2 or IFI16 in OSCC cells resulted in the suppression of cell growth and apoptosis, accompanied by the downregulation of the nuclear factor kappa-light-chain-enhancer of activated B cells activity. Because all OSCC cell lines have reduced p53 activity, wild-type p53 was introduced in p53-deficient OSCC cells. The expression of wild-type p53 suppressed cell growth and induced apoptosis via suppression of the nuclear factor kappa-light-chain-enhancer of

activated B cells activity. Finally, the co-expression of AIM2 and IFI16 significantly enhanced cell growth in p53-deficient cells; in contrast, the expression of AIM2 and/or IFI16 in cells bearing wild-type p53 suppressed cell growth. Moreover, AIM2 and IFI16 synergistically enhanced nuclear factor kappa-light-chain-enhancer of activated B cells signaling in p53-deficient cells. Thus, expression of AIM2 and IFI16 may have oncogenic activities in the OSCC cells that have inactivated the p53 system.

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Division of Virology

Tohru Kiyono, Takashi Yugawa, Nagayasu Egawa, Tomomi Nakahara, Kenji Yamada, Satomi Kikawa, Shinichi Ohno, Takako Ishiyama, Katsuyuki Tanaka

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 the human papillomavirus (HPV). 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 critical role of MYC in cervical carcinogenesis

Recently, we have demonstrated that transduction of oncogenic HRAS, HRAS[G12V], and MYC together with HPV16 E6E7 was sufficient for tumorigenic transformation of normal human cervical keratinocytes (HCKs) (Narisawa-Saito et al., Cancer Research, 2008). Then we showed that transduction of HRAS[G12V] against the background of E6E7 expression caused accumulation of MYC protein and tumorigenic transformation of not only normal HCKs but also other normal primary human cells, including tongue keratinocytes and bronchial epithelial cells as well as hTERT-immortalized foreskin fibroblasts (1). Subcutaneous transplantation of as few as 200 HCKs expressing E6E7 and HRAS[G12V] resulted in tumor formation within 2 months. Dissecting RAS signaling pathways, constitutively active forms of AKT1 or MEK1 did not result in tumor formation with E6E7, but tumorigenic transformation was induced with the addition of MYC. Increased MYC expression endowed resistance to calcium- and serum-induced terminal differentiation and activated the mammalian target of rapamycin (mTOR) pathway. An mTOR inhibitor, Rapamycin, and MYC inhibition at a level not affecting proliferation in culture both markedly suppressed tumor formation by HCKs expressing E6E7 and HRAS[G12V]. These results suggested that a single mutation of HRAS could be oncogenic in the background of the deregulated expression of E6E7, and MYC plays a critical role in cooperation with the RAS signaling pathways in tumorigenesis. Thus inhibition of MYC and/or the downstream mTOR pathway could be a therapeutic strategy not only for the MYC-altered but also RAS-activated cancers.

HPV16 maintenance replication without E1 helicase

Papillomavirus genomes are thought to be amplified to about 100 copies per cell soon after infection, maintained constant at this level in basal cells, and amplified for viral production upon keratinocyte differentiation. Viral helicase E1 has been thought to be essential for the viral replication. By using human cervical keratinocytes harboring wild-type and an E1-deficient HPV16 genome, we demonstrate that the E1 protein is dispensable for maintenance replication but not for initial and productive replication of HPV16. Deregulated expression of E6 and E7 genes of "high risk" human papillomaviruses (HPVs) in the basal cells of stratified epithelia is a key step for malignancy, and is often caused by accidental integration of the viral genome. The E1 helicase is the only enzyme encoded by HPV and thought to be a good molecular target of anti-HPV drugs. However, our results imply that the rationale for development of E1 inhibitors as anti-HPV drugs may be more restricted than formerly envisaged (2). In collaboration with dermatologists, a novel type of HPV, HPV126, was isolated from skin warts (3).

Immortalization of normal and precancerous human cells

We have immortalized various types of normal and precancerous human cells. Among them, ovarian endometrioma cells were immortalized to analyze carcinogenesis of endometrial carcinoma (4). Immortalized skin fibroblasts from Cornelia de Lange syndrome patients were used for analyzing the abnormal cohesion acetylation cycle by HDAC8

mutations (5). From normal human tissues, corneal endothelial cells and nonluteinized granulosa cells have been newly immortalized (6, 7). Several immortalized human epithelial cells were also used for analyzing novel functions of trichoplein and CHK1 (8, 9).

A role of actin -related protein 4 in the BRG1 chromatin remodeling complex.

We found that ARP4 can form a heterocomplex with β-actin. Some mutant ARP4 which showed reduced binding to β-actin also showed reduced incorporation into BRG1 complexes. They also showed impaired interaction with Myc-associated complexes as well as TIP60 HAT complexes. Based on these findings, we proposed that β-actin-ARP4 complex formation might be a crucial feature in some chromatin-modifying enzyme complexes, such as the BRG1 complex (10).

Normal human cervical keratinocytes (HCK)

+HPV16 E6E7 → Inactivation of p53 and pRB
Telomerase activation

CIN3

+HRAS^{G12V} → MYC stabilization

Tumor formation

+c-MYC → Stem cell-like properties = "Stemness" (resistant to differentiation)

Tumor progression

Tumor initiating cell （Cancer stem-like cells）
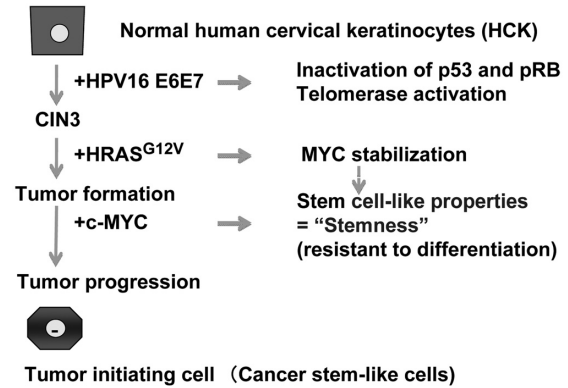
Figure 1. An *in vitro* multistep carcinogenesis model for cervical cancer

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DIVISION OF CANCER DEVELOPMENT SYSTEM

Koji Okamoto, Yoshitaka Hippou, Yukari Totsuka, Daisuke Shiokawa, Masako Ochiai, Hirokazu Ohata, Tatsuya Ishiguro, Kousuke Ishino, Masanori Gotoh, Yuki Aihara, Akihiro Sekine, Aya Sakaizawa, Aya Ohno, Emi Fukai, Waka Kato, Sachiko Dobashi, Ai Sato, Hiroaki Sakai, Emiko Yamamoto, Mayumi Mizuta, Yumi Miyamoto, Hisako Okuda

Introduction

Recent research in many laboratories has revealed the importance of cancer stem cells during the development of refractory cancer with a highly metastatic potential. In our Division, we focus on studying the dynamic regulation of stem cell-related characteristics during cancer development. The main goals of our research center around elucidation of the biological properties of cancer stem cells, and understanding the mechanisms as to how such cells develop from normal tissues. We take several experimental approaches to address these issues.

Routine activities

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

Research activities

In vitro cultivation and characterization of cancer stem cells from human colon and ovarian cancer

Accumulating reports indicate that "cancer stem cells" exist in various types of cancer, and that they are responsible for metastatic processes as well as the tumorigenicity and chemoresistance of cancer. In order to examine the role of cancer stem cells in metastasis, we isolated cancer stem cells from human colon cancer, and established the condition that allows stable *in vitro* propagation of colon cancer stem cells in a spheroid form. We found that inhibition of Rho kinase greatly facilitated the establishment of spheroids from primary colon cancer. Under such conditions, the spheroid cells expressed cancer stem cell markers, showed the ability to differentiate, and induced tumors in mice. The spheroids were composed of cells that expressed various levels of CD44, and that CD44^{high} cells exhibited characteristics associated with cancer stem cells. As expected from the predicted hierarchy, CD44^{high} cells differentiated into CD44$^{-/low}$ cells. Unexpectedly, a fraction of CD44$^{-/low}$ cells generated CD44^{high} cells, and we hypothesize a model in which the transition from the CD44$^{-/low}$ to CD44^{high} state enhances tumorigenicity by maintaining a CD44^{high} fraction in colon cancer.

We also found that inhibition of Rho kinase also promoted the establishment of spheroids from primary ovarian cancer. Biochemical and biological evaluation of the established spheroid cells is in progress.

Functional identification and characterization of regulatory factors of cancer metastasis

We developed an experimental model in which liver metastasis of colon cancer was generated with high efficiency in highly immunocompromised NOG mice. This metastasis model was used to functionally isolate regulatory factors involved in the metastasis to the liver of colon cancer cells. First we looked for miRNAs that could inhibit liver metastasis of colon cancer cells by applying a systematic screening approach (dropout screening). Through the dropout screening of a miRNA library after the introduction of HCT116 colon cancer cells, miR-493 was isolated that reproducibly inhibited metastasis of colon cancer cells to the liver. Subsequently IGF1R was identified as a direct target of miR-493, and its inhibition partially phenocopied the anti-metastatic effects. High levels of miR-493 in primary colon cancer were inversely related to the presence of liver metastasis, and attributed to an increase of miR-493 expression during carcinogenesis. Therefore, our data indicated that, in a subset of colon cancer, up-regulation of miR-493 during carcinogenesis may prevent liver metastasis via the induction of cell death of the metastasized cells.

We also attempted to isolate genes that regulate liver metastasis of colon cancer by using a functional screening method that is conceptually similar to the dropout screening for miRNA. Screening of the shRNA library identified several candidate genes, and individual evaluation of these genes is in progress.

Establishment of *in vitro* model of the early steps of colon carcinogenesis

We established an *in vitro* model of colon epithelium based on a recent report (Sato et al,

Nature 2009). These experiments resulted in the formation of a caricature of the colon / intestine epithelium or "organoid" *in vitro* in the presence of defined growth factors. Such organoids will develop from normal epithelium as well as from mouse colon cancerous tissue, and could be used to examine the dynamic alteration of the colon epithelium during early colon carcinogenesis. In addition, we showed that xenograft tumors could be generated from the organoids after lentivirus-mediated knockdown of APC and p53 in the presence of oncogenic K-ras, potentially establishing a novel model for CRC *in vitro*. Similar approaches will be taken to study human colon carcinogenesis using surgery specimens.

Identification of Novel Mutagens/Carcinogens

Nanomaterials are useful for their characteristic properties, and are commonly used in various fields. The assessment of the genotoxicity and safety of nanomaterials is therefore of serious concern. So far, we have examined the genotoxic effects of multi-walled carbon nanotubes (MWCNTs) using *in vitro* micronuclei, sister chromatid exchange, *in vivo* DNA damage and mutation assays. Overall, MWCNTs were shown to be genotoxic both in *in vitro* and *in vivo*; the mechanisms probably involve oxidative stress and inflammatory responses. Reports related to other environmental mutagens/carcinogens can be found in the attached list of references.

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Division of Hematological Malignancy

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

Introduction

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 chemotherapy-resistant AML stem cells. Thus, AML stem cell eradication is thought to be crucial to offer a complete cure for AML. Chromosome abnormalities, which result in the generation of specific fusion genes, are observed in ~50% of AML patients. Cases with AML who are associated with fusion genes involving *MLL, MOZ, CALM* or *NUP98* have an extremely poor outcome. Normal cytogenetics portend average-risk AML. Recent genome analysis has 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 was to establish new therapeutic methods by identifying the molecular targets that are essential for the maintenance of AML cells, especially AML stem cells.

Research activities

To investigate the molecular mechanism of AML, we established mice models. AML models with fusion genes, MOZ-TIF2, MLL-AF10, NUP98-HOXA9 or CALM-AF10, were generated by introducing the respective genes to bone marrow cells. We have found that the expression of M-CSFR was specifically high in stem cells of mice AML models and human AML patients. Using transgenic mice expressing a drug-inducible suicide gene controlled by the M-CSFR promoter, we demonstrated that AML was cured by eradication of M-CSFR-high cells. Administration of an mM-CSFR-specific ADCC-antibody reduced the numbers of leukemia cells and slowed the progression of AML in the mouse model. To develop antibody medicine, we obtained hM-CSFR-specific antibodies in collaboration with the RIKEN institute (adopted by the Program for Dug Discovery and Medical Technology Platforms).

We found that the expression of M-CSFR, HOXA9 and c-KIT was high in AML stem cells, and that MOZ- and MLL-fusions induced the expression of m-csfr, c-kit and hoxa9 by interacting with PU.1, c-MYB and BRPF1, respectively. Analysis using mice deficient for these factors demonstrated that PU.1, c-MYB, BRPF1 and M-CSFR were essential for MOZ-TIF2 to induce and maintain AML. These results indicated that the PU.1/M-CSFR, c-MYB/cKIT and BRPF1/HOX pathways were critical for maintenance of AML stem cells. A dual kinase inhibitor for M-CSFR and cKIT (PLX3397) slowed the progression of AML in mice.

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

Figure 1
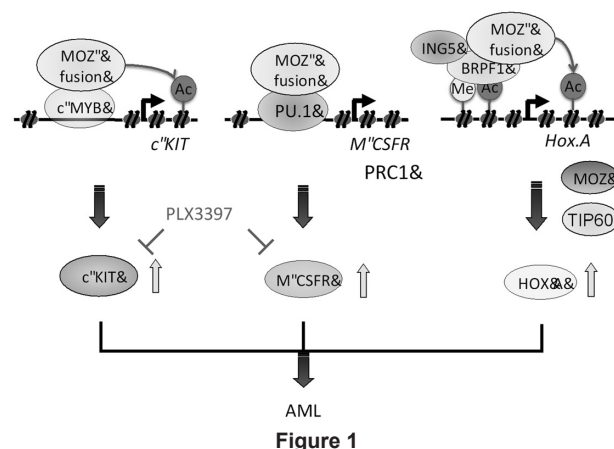
DIVISION OF METASTASIS AND INVASION SIGNALING

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

Introduction

The malignant characteristics of cancers causing the invasion into surrounding tissue and metastasis to distant organs are serious threats to the clinical treatment of cancer. Interaction of cancer cells with neighboring cells such as cancer associated fibroblasts (CAFs) has recently been shown to have critical roles in this procedure. It is also suggested that numbers of receptor and non-receptor tyrosine kinases are involved in the multiple steps of cancer progression. Signals from activated tyrosine kinases are mediated through phosphorylation of substrate molecules to modulate cell characteristics during tumor proliferation and metastasis. The main object of our Division is to elucidate the roles of signaling molecules during cancer metastasis and invasion. One of the goals of our research is to establish models of the novel therapy of progressed cancer by regulating phosphotyrosine-dependent signals in cancer cells and their microenvironments.

Models of cancer invasion and metastasis

Scirrhous gastric carcinoma (SGC) has the worst prognosis among the various types of gastric cancer, owing to its rapid expansion through progressive invasion, peritoneal dissemination and frequent metastasis to lymph nodes. Because massive proliferation of stromal fibroblasts occurs within SGC lesions, CAFs have been proposed to support the progression of SGC. However, the biological and molecular basis of the interaction between SGC cells and CAFs remains largely unknown. We investigated the role of CAFs in invasion and extracellular matrix (ECM) remodeling by SGC cells. When SGC cells were cocultured with CAFs on three-dimensional (3D) Matrigel, they were attracted together to form large cellular aggregates that invaded the Matrigel. Time-lapse imaging of SGC and CAFs along with fluorescent microspheres embedded in the Matrigel revealed that this process was associated with extensive contraction and remodeling of the ECM. Phosphorylation of the myosin light chain significantly increased in CAFs when they were cocultured with SGC cells and blebbistatin, a myosin II inhibitor, blocked the 3D invasion and ECM remodeling by SGC cells and CAFs. These results indicated that SGC cells promote the actomyosin-mediated contractility of CAFs to remodel ECM during invasion.

CDCP1 (CUB-domain-containing protein 1) is a transmembrane protein that regulates anchorage-independent growth and cancer cell migration and invasion. Expression of CDCP1 is detected in a number of cancer cell lines and tissues and is closely correlated with a poor prognosis. 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). MT1-MMP accumulates in the invadopodia through targeted delivery via membrane trafficking. We have revealed that CDCP1 is required for ECM degradation by invadopodia in human breast cancer and melanoma cells. CDCP1 localized to caveolin-1-containing vesicular structures and lipid rafts and was detected in close proximity to invadopodia. Further biochemical analysis revealed that CDCP1 was an essential regulator of the trafficking and function of MT1-MMP- and invadopodia-mediated invasion of cancer cells.

We demonstrated that CDCP1 was required for the 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 RNAs (siRNAs) 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 the loss of CDCP1 could induce caspase-independent cell death. Instead, the loss of CDCP1 induced 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 inhibition of autophagy which contributes to the anoikis resistance of lung cancer cells.

Oncogenic signals in neuroblastomas

Recently, activation of anaplastic lymphoma kinase (ALK), either by mutation or overexpression, has been indicated as a significant oncogenic factor in neuroblastoma formation. To investigate the role of ALK receptor tyrosine kinase in neuroblastoma oncogenesis, we investigated phosphotyrosine-containing proteins associated with ALK in neuroblastomas using mass-spectrometry analysis. Various types of phosphoproteins were identified as binding partner of ALK in neuroblastoma tumors. Flotillin-1 (FLOT1), a plasma membrane protein known to be involved in endocytosis, was found among those binding partners of ALK. It was suggested that FLOT1 controls the amount of ALK protein at the cell surface through the regulation of receptor endocytosis. Decreased binding affinity of oncogenic ALK mutants to FLOT1 may cause the activation of ALK signaling which leads to the poor prognosis of neuroblastoma cases harboring these mutations. Further studies on the tumor suppressive function of FLOT1 in neuroblastomas are currently in progress.

On the other hand, it was observed that expression of Ret, a receptor tyrosine kinase which is highly expressed in some of the neuroblastoma cell lines, was suppressed by knockdown of ALK or by the ALK inhibitor in the neuroblastoma cell lines. Since the activation of Ret kinase by its ligands such as GDNF was shown to contribute anchorage independent growth of neuroblastoma cells, the indirect effect of ALK activation through Ret kinase might affect the oncogenic aspects of neuroblastomas. The combinatory effect of inhibiting both ALK and Ret kinases is being analyzed for evaluation of its clinical significance.

DIVISION OF MOLECULAR AND CELLULAR MEDICINE

Takahiro Ochiya, Fumitaka Takeshita, Masaki Kawamata, Nobuyoshi Kosaka, Ryou-U Takahashi, Ayako Inoue, Wakako Kobayashi, Maki Abe, Makiko Ono, Yu Fujita, Takeshi Katsuda, Luc Gailhouste, Muriel Thirion, Satoshi Seino, Hiroaki Miyazaki, Yusuke Yoshioka, Keitaro Hagiwara, Naoomi Tominaga, Keita Uchino, Shingo Ikeda

Introduction

The main focus of the Division of Molecular and Cellular Medicine is the development of novel strategies to study tumorigenesis, cancer metastasis, and drug resistance. The specific activities in 2012 were as follows: 1) Studies on microRNA regulation in cancer cells and development of RNAi-based therapeutics; 2) An exosome-carrying microRNAs as a novel diagnosis and therapeutic tool against cancer; 3) Generation of genetically modified rats from embryonic stem cells for development of novel animal models for cancer research; and 4) Hepatic differentiation of mesenchymal stem cells and its therapeutic application.

1) Studies on microRNA Regulation in Cancer Cells and Development of RNAi-based Therapeutics

Since small interfering RNA (siRNA) and microRNA (miRNA) are silencing small RNAs that can modulate tumor-related genes and pathways, siRNA and miRNA are expected to be an attractive new class of anticancer drugs (1, 19). The novel RNAi agents have been developed that are single stranded RNAs with high stability (2).

We previously identified Ribophorin-2 (RPN2) as a novel regulator for drug resistance and maintenance of cancer stem cells (CSCs) in breast cancer. For the clinical application of siRNA targeting RPN2, pre-clinical trials with naturally-occurring breast cancer in dogs have been performed (20). For analysis of CSCs in colon cancer, stable cell lines having CSC properties from colon cancer patients were established (3). In these cell lines, irinotecan could induce the transition from LGR5(+) to LGR5(-) drug-resistant state. These results provide new biological insights into drug resistance of CSCs.

The alteration of methylation in promoter regions coding miRNA is linked to transcriptional change in cancers. The analyses of methylations and expression of miRNAs in the drug resistant cell lines derived from human breast cancer revealed the epigenetic similarities and differences between miRNA and protein-coding genes (4).

We also have focused on the elucidation of regulation for iron homeostasis in cancer. The miR-210 suppresses two molecules for iron homeostasis, transferrin receptor 1 (TfR) and ISCU (5). Our study reveals that miR-210 works as an iron sensor and is involved in the maintenance of iron homeostasis by sustaining the TfR expression to affect the cell proliferation and survival in the hypoxic region within tumors.

The safe dietary intake of natural products could reduce the risk of a wide range of human cancers. We reported that resveratrol promotes the expression and activity of Ago2, thereby inhibiting breast cancer stem-like cell characteristics by increasing the expression of tumour-suppressive miRNAs, including miR-16, -141, -143, and -200c (6). Our study suggested that the dietary intake of natural products contributed to the prevention and treatment of cancer by regulating the RNAi pathway.

2) An exosome-carrying microRNAs as a Novel Diagnosis and Therapeutic Tool against Cancer

Circulating miRNAs could be found in variety of body fluids including serum, plasma, urine, saliva and breast milk (7). The existence of circulating miRNAs in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker (8). For this reason, a new method for the highly sensitive detection of circulating miRNAs has been developed (9). The circulating miRNAs are secreted from variety types of cell via the extracellular vesicles called exosomes. We provided a method to directly detect and quantify exosomes in human serum from prostate cancer patients using anti-CD63 and anti-CD9 antibodies. These results suggested that the ExoScreen system enabled us to detect circulating exosomes, and thus provided a novel biomarker. Normal epithelial cells regulate the secretion of humoral factors that prevent aberrant growth of neighboring cells. In this homeostatic regulation, exosomal tumor-suppressive miRNAs secreted by normal cells acted as anti-proliferative signal entities (10, 11). In addition, the application of exosomal tumor-suppressive miRNAs has been proposed as a novel nucleic acid therapy against cancer development.

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3) Generation of Genetically Modified Rats from Embryonic Stem Cells for Development of Novel Animal Models for Cancer Research

For cancer studies, development of suitable animal models for human cancer is essential (12). Rats have important advantages as an experimental system for pharmacological investigations. We have established rat embryonic stem cells (ESCs) and generated Oct4-Venus transgenic rats. Using this transgenic ESC line, we have succeeded in generating p53 knockout rats. Homozygous KO males developed normally, whereas females rarely survived due to neural tube defects. The homozygous male died within 4 months due to tumor development. In contrast to this phenotype, knockout chimeras generated via blastocyst injection with p53-null ESCs exhibited high rates of embryonic lethality in both sexes. These results demonstrate that p53 functions as a guardian of embryogenesis as well as a tumor suppressor in rats (13).

4) Hepatic Differentiation of Mesenchymal Stem Cells and Its Therapeutic Application

Adipose-derived mesenchymal stem cells (ADSCs) are attractive in the context of future clinical applications (14). It was found that ADSCs and induced pluripotent stem cells could differentiate into hepatocytes and demonstrate the functional properties of primary human hepatocytes (15-18).

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Division of Cancer Biology

Hirofumi Arakawa, Yasuyuki Nakamura, Hiroki Kamino, Masaki Yoshida, Ryuya Murai, Yuri Saito, Hitoya Sano, 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 way, a new p53-target gene was identified, and designated *Mieap* for mitochondria-eating protein, reflecting the unusual function of the protein. Surprisingly, the function of Mieap is involved in mitochondrial quality control (MQC).

Mieap-induced accumulation of lysosome-like organella 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 that MALM plays a role in repairing unhealthy mitochondria.

BNIP3 and NIX, mitochondrial outer membrane proteins, were identified as Mieap-interacting proteins (1), and were shown to mediate the translocation of lysosomal proteins from the cytosol into mitochondria during MALM by forming an unknown pore in the mitochondrial double membrane (1). 14-3-3γ was also identified as a Mieap-interacting protein to mediate the degradation of oxidized mitochondrial proteins within mitochondria during MALM (2).

Mieap-inudced 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. In cancer cell lines, we found that the Mieap-regulated MQC is frequently inactivated by p53 mutations or Mieap-methylation or BNIP3 methylation. In order to 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 primary cancer tissues of colorectal and pancreatic 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
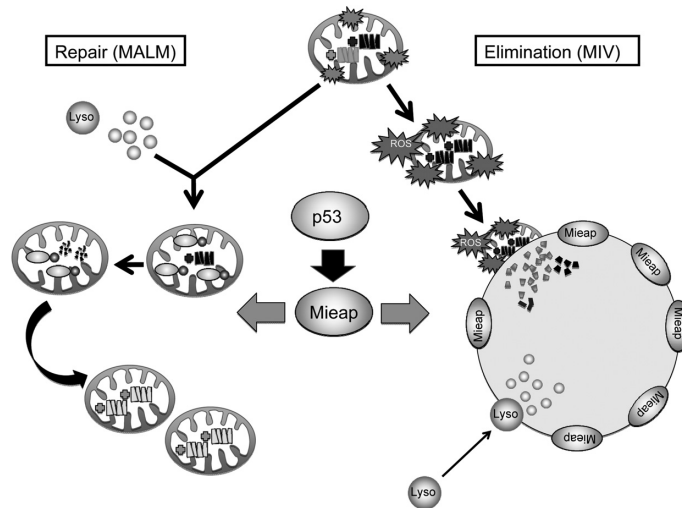
Figure 1.

Tumor microenvironment (Hypoxia)

ROS ↑
↓
Oxidative damage
Genomic instability
Tumor growth
Cancer invasion
Cancer metastasis
Angiogenesis

Mitochondrial ATP production ↓

Aerobic glycolysis ↑
↓
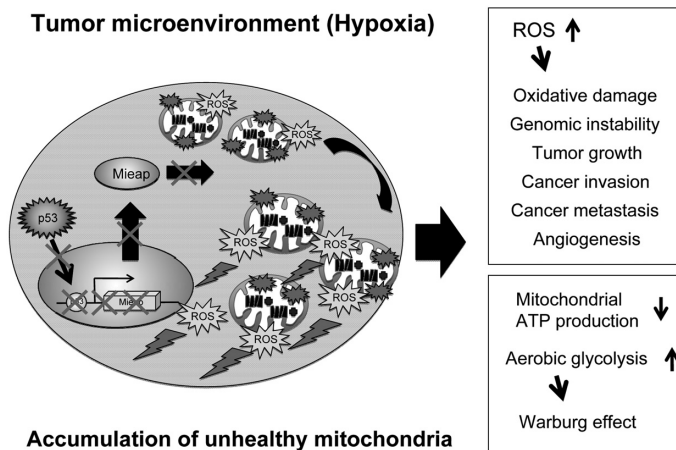Warburg effect

Accumulation of unhealthy mitochondria

Figure 2.

and consequently the Warburg effect (Figure 2). This finding could explain the reason why cancer cells preferentially utilize aerobic glycolysis, as observed by Warburg. Therefore, the mechanisms of maintenance of healthy mitochondria are currently being investigated in this Division.

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.

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Journal

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Division of Epigenomics

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

This Division has been focusing on the epigenetic mechanisms of carcinogenesis, mainly DNA methylation, 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 identification of a novel tumor-suppressor gene (TSG) 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 in identifying novel epigenetic alterations in various cancers and normal tissues, and is applying its past discoveries to the development of clinically useful biomarkers. It is also interested in the development of epigenetic therapy and clarification of mechanisms of how epigenetic alterations are induced.

Identification of novel epigenetic alterations

Identification of TSGs silenced by aberrant methylation is important, but has been hampered by a large number of genes methylated as passengers of carcinogenesis. To overcome this issue, this Division took advantage of the fact that the majority of genes methylated in cancers lack, in normal cells, RNA polymerase II (Pol II) and have trimethylation of histone H3 lysine 27 (H3K27me3) in their promoter CGIs. It was shown that some TSGs had Pol II and lacked H3K27me3 in normal cells, being outliers to the general rule, and that novel TSGs could be identified by searching for such outliers (1).

Aberrant hypermethylation is known to be present in predisposed epithelial cells. In a study conducted this year, hypomethylation of repetitive elements was shown to be present in the background mucosae of esophageal squamous cell carcinoma (2). State-of-the-art technologies are constantly employed for genome-wide methylation analyses, and bead array technology and high-throughput sequencing technologies are now being adopted.

Development of biomarkers

This Division previously revealed that accumulation of aberrant methylation induced by *Helicobacter pylori* infection was deeply involved in predisposition to gastric cancers (epigenetic field for cancerization). Based on the fact, accumulation levels of aberrant methylation are expected to become a useful gastric cancer risk marker. This year, novel risk markers, highly informative among individuals with past *H. pylori* infection, were developed (Figure 1) (3). A prospective clinical study is being conducted to bring these risk markers into practical application.

Detection of lymph node metastasis is critically important for determination of the treatment strategy for gastric cancers, and this Division identified CGIs whose methylation levels were associated with the presence of lymph node metastasis in gastric cancers (4). In neuroblastomas, the clinical usefulness of the prognostic marker mentioned above is being analyzed using materials prospectively collected.

Development of epigenetic therapy

Epigenetic therapy is expected to be a next-generation strategy in cancer chemotherapy. Since many genes are known to be silenced in a single cancer, simultaneous reversal of silencing of multiple genes is expected to be an effective treatment. This Division is working on this strategy as a novel therapeutic concept using neuroblastomas as a model. At the same time, a screening system for novel epigenetic drugs is also being developed.

Induction mechanisms of epigenetic alterations

Clarification of the induction mechanisms of epigenetic alterations is critically important for public health, including cancer prevention. This Division showed that chronic inflammation is critically important for induction of aberrant methylation (5). Regarding components of inflammation, it
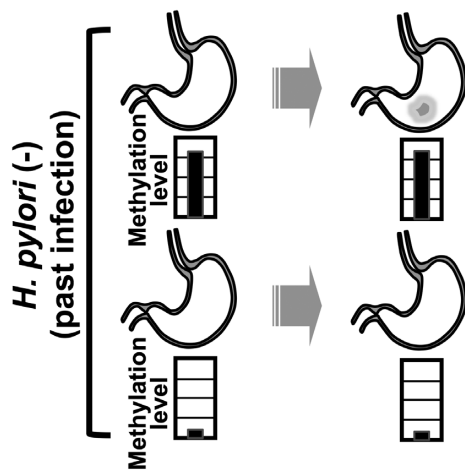
Figure 1. Novel gastric cancer risk marker, informative among individuals with past *H. pylori* infection

was shown that functional T and B cells are non-essential for the epigenetic field for cancerization by analysis of aberrant methylation in severe combined immunodeficiency mice in a mouse colitis model (6).

Although aberrant DNA methylation is involved in the field for cancerization, it had been unclear for H3K27me3. This Division demonstrated that aberrant H3K27me3 could be induced by exposure to a specific environment, such as colitis, and suggested that aberrant histone modification, in addition to aberrant DNA methylation, was involved in the formation of a field for cancerization (7).

Other activities

This Division assisted in the epigenetic analysis of *miR-7-1* in gastric cancers (8), that of *Mat1A* and *Mat2A* in liver cancers (9), and research on gastric cancer risk (10). This Division also contributed to communicating recent findings in inflammation-associated cancer development and epigenetic changes (11).

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DIVISION OF PHARMACOPROTEOMICS

Tadashi Kondo, Daisuke Kubota, Hiroshi Ichikawa, Noriyuki Hosoya, Takashi Tajima, Kenta Mukaihara, Kazutaka Kikuta, Yoko Takai, Ruriko Sakamoto, Kazuya Arai, Mayo Kikuchi, Yukiko Nakamura, Fusako Kito, Marimu Sakumoto, Yutaka Sugihara, Hirotaka Yonemori, Ayako Haga, Ryosuke Yamaka

Introduction

The aim of this Division is to create clinically useful tools through cancer research and to contribute to the better clinical outcome of cancer patients (Figure 1). For this aim, we challenge biomarker research to optimize therapeutic strategies, and also attempt to elucidate drug targets. The Division is characterized by the applications based on an original proteomics approach, and the use of clinical materials/clinical-pathological information on the basis of collaborations with clinicians and pathologists in the National Cancer Center Hospital and domestic/overseas hospitals (Table 1). Development of novel proteomics modalities is one of the major themes of this Division, and interdisciplinary collaborations have been established with universities and companies. Among many malignancies investigated in the Division, sarcomas are presently the most focused on, based on the long time collaboration with orthopedicians.

Research activities

1. Biomarker research for personalized medicine

Biomarkers to optimize the therapeutic strategy were challenged using the proteomics modality. Proteomic biomarker research for sarcomas generated many promising biomarkers, and external validation and *in vitro* functional studies were undertaken (1-3).

In gastrointestinal stromal tumors (GISTs), DDX-39 was identified as a biomarker to predict metastasis after surgery, and its clinically significant characteristics were immunohistochemically validated (4). The immunohistochemical validation of a novel prognostic biomarker, pfetin, which was previously discovered as a prognostic biomarker for GISTs in this Division, was conducted in combination with DDX-39 (5). The immunohistochemical validation study on the prognostic values of pfetin was performed in approximately 500 GIST cases from six domestic hospitals.

In hepatocellular carcinomas (HCCs), the decreased expression of selenium-binding protein 1 (SBP-1) promoted tumor invasiveness by increasing the activity of glutathione peroxidase 1 and diminishing HIF-1α (6). Immunohistochemistry revealed that SBP-1 was a novel prognostic biomarker for HCCs. A novel serum biomarker for microvascular invasion was discovered with mass spectrometry, and its clinical utilities were confirmed with an ELISA. APC-binding protein EB1 (EB1), which was previously discovered as a prognostic biomarker for HCC in this Division, was identified as a potential prognostic biomarker in colorectal cancer (7). A proteomic study was performed on cholangiocarcinomas, using tumor tissues, a xenograft model, and primary culture cells from the patients with different response to gemcitabine treatments, and macrophage-capping protein (CapG) was identified as a novel predictive biomarker. The prognostic values of CapG were immunohistochemically confirmed (8).

Other biomarkers were also discovered by comprehensive profiling of proteins and microRNA in osteosarcoma, gastric cancer, and renal cell carcinoma. An external immunohistochemical validation study and functional assessment of the identified biomarkers were undertaken, and promising candidates were discovered. The clinical applications of the identified proteins will be our next big challenge.

2. Discovery of therapeutic targets

Identification of targets was performed with the focused proteomics approach for druggable proteins. The comprehensive expression profiling of tyrosine kinases identified many promising drug candidates in sarcomas. Unique enzymes in the protein complex of biomarker candidates were identified by the interactome approach using gel electrophoresis and mass spectrometry. *In vitro* inhibition assays were performed to evaluate the possibility for further investigation.

3. Development of proteomics modalities and their application in cancer research

Proteomics modalities to investigate the heterogenous tumor tissues were developed and used to identify the proteins associated with malignant cancer features. Using laser microdissection and a large format two-dimensional difference gel electrophoresis (2D-DIGE) technology, we identified cathepsin D as a unique protein to lung adenocarcinoma (10), and validated its clinical utilities with a tissue microarray. A novel application of the technology associated with Laser-assisted in Situ Keratomileusis (LASIK) was developed for proteomic studies, and applied to invadopodia of breast cancer cells, resulting in the identification of novel metastasis-associated proteins. *In vitro* functional validation confirmed the biological significances (11).

The combination of gel electrophoresis and mass spectrometry was employed to perform a comprehensive expression study on intact proteins to enable further understanding of the molecular backgrounds behind the clinical utility of biomarker proteins such as nucleophosmin (NPM), which was identified as a prognostic biomarker for Ewing sarcoma in this Division. The clinically significant characteristics of NPM-binding proteins were confirmed with a meta-analysis of mRNA data.

An automatic protein sample processor is under development for comprehensive and quantitative protein expression study in collaboration with commercial manufacturer.

The application of antibody libraries is one of the challenging approaches for cancer research. Using monoclonal antibodies for approximately 600 nuclear factors, the proteins for early recurrence were identified in HCC. The *in vitro* and *in vivo* functional assessments and immunohistochemical validation using the tissue microarray technique were performed to assess the clinical usefulness of the identified nuclear factors. Posttranslational modifications of the proteins from chromosome 21 were examined using antibodies from the Human Protein Atlas and a unique Western blotting method (12). Detailed and comprehensive studies on posttranslational modifications of proteins will be challenged using antibodies.

4. Contribution to research community

The Division contributed to the guidelines in the worldwide public antibody databases such as the Human Proteome Project (Human Proteome Organization) and the Antibodypedia (Nature Publishing Group). The division also contributed to the Chromosome-centric Human Proteome Project (Human Proteome Organization)(12).

Table 1. Cases examined for biomarker research and target discovery in 2012

	Malignancies	Research theme	No. of cases examined*
1	Osteosarcoma	Biomarker to predict response to pre-operative chemotherapy	37
2	Rhabdomyosarcoma	Biomarker for differential diagnosis	23
3	Myxoid liposarcoma	Biomarker to predict prognosis after surgery	29
4	Mixofibrosarcoma	Biomarker for differential diagnosis	27
5	Alveolar soft part sarcoma	Discovery of drug target	13
6	Epithelioid sarcoma	Discovery of drug target	12
7	Gastrointestinal stromal tumor	Biomarker to predict metastasis after surgery	371
8	Lung cancer	Discovery of drug target	40
9	Gastric cancer	Biomarker to predict lymph node metastasis before surgery	217
10	Colorectal cancer	Biomarker to predict prognosis after surgery	200
11	Hepatocellular carcinoma	Serum biomarker for microvascular invasion	727
12	Renal cell carcinoma	Biomarker to predict prognosis after surgery	89
13	Metastatic bone tumor	Discovery of drug target	11
Total			1796

*Cases include those from the National Cancer Center Hospital and the other domestic and overseas hospitals

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Division of Genome Biology

Takashi Kohno, Hideaki Ogiwara, Kouya Shiraishi, Yoko Shimada, Tatsuji Mizukami, Teruhide Ishigama, Takashi Mitachi, Norihide Yoshikawa, Olivia Schreiber, Hideyuki Hayashi

Introduction

Somatic mutations in the cancer genome and inter-individual variations in the human genome are critical keys to improving the efficacy of treatment in 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 (Figure 1). We are working together with 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

Novel genes rearranged in lung cancer were searched for by conducting whole RNA sequencing of lung adenocarcinoma tissues supplied from the National Cancer Center Biobank using high-speed DNA sequencers. We identified in-frame fusion transcripts of KIF5B (the kinesin family 5B gene) and the RET oncogene, which are present in 1–2% of lung adenocarcinomas (LADCs) from patients in Japan and the United States (1). The KIF5B-RET fusion leads to aberrant activation of RET kinase and is considered to be a new driver mutation of LADC because it segregates from mutations or fusions in EGFR, KRAS, HER2 and ALK, and a RET tyrosine kinase inhibitor, vandetanib, suppresses the fusion-induced anchorage-independent growth activity of NIH3T3 cells. Kinase inhibitors are now standard treatment for patients with lung cancer whose tumors harbor specific mutant kinases, and the RET fusion protein was considered potentially to be responsive to existing targeted therapies using RET kinase inhibitors. The development of assays to assess RET fusions and other driver mutations in each patient will offer the potential to routinely parse lung cancer into multiple different clinically relevant molecular disease types in the near future. A project focusing on this issue has started in collaboration with NCC staff from hospitals. An investigator-initiated clinical trial to address the therapeutic efficacy of vandetanib will start in 2013.

Whole gene expression profiling data of human primary lung epithelial cells stimulated with epidermal growth factor (EGF) in the presence or absence of a clinically used EGF receptor tyrosine kinase (RTK)-specific inhibitor, gefitinib, were subjected to a mathematical simulation using the State Space Model. A risk scoring model was constructed to classify high- or low-risk patients based on the expression signatures of 139 gefitinib-sensitive genes in surgical specimens of lung adenocarcinomas (2). This system will be useful to identify early stage lung adenocarcinoma patients with a poor prognosis who will benefit from adjuvant therapy after a surgical operation.

Genes involved in DNA repair and/or chromatin remodeling are being analyzed to improve the efficiency of existing therapeutic methods. Non-homologous end joining (NHEJ) and homologous recombination (HR) are major repair pathways for DNA double strand breaks (DSBs) generated by ionizing radiation and anti-cancer drugs. We revealed that CBP/p300 histone acetyltransferases (HATs) promote DSB repair by facilitating NHEJ and HR (3), and are therefore possible target for sensitization of tumors to radio- and chemotherapies. In fact, garcinol, a natural compound with an inhibitory activity against CBP/p300 HATs, was identified as a promising radiosensitizer (4).

2. Genes for personalized prevention

Genetic factors underlying the specific risk of lung adenocarcinoma in Asians are being searched for to comprehensively understand the molecular mechanism of lung carcinogenesis (5-6). A genome-wide association study comprising a total of 6,029 individuals with lung adenocarcinoma (cases) and 13,535 controls confirmed two previously reported risk loci, 5p15.33 (rs2853677, $P = 2.8 \times 10^{-40}$, odds ratio (OR) = 1.41) and 3q28 (rs10937405, $P = 6.9 \times 10^{-17}$, OR = 1.25), and identified two new susceptibility loci, 17q24.3 (rs7216064, $P = 7.4 \times 10^{-11}$, OR = 1.20) and 6p21.3 (rs3817963, $P = 2.7 \times 10^{-10}$, OR = 1.18) (7). Another genome-wide association study of 5,510 never-smoking female lung cancer cases and 4,544 controls, which were drawn from 14 studies from mainland China, South Korea, Japan, Singapore, Taiwan and

Hong Kong, identified three new susceptibility loci at 10q25.2 (rs7086803, P = 3.54 × 10^{-18}), 6q22.2 (rs9387478, P = 4.14 × 10^{-10}) and 6p21.32 (rs2395185, P = 9.51 × 10^{-9}) (8). These data provide evidence supporting a role for genetic susceptibility in the development of lung adenocarcinoma in Asians. These results represent basic information to improve the prevention of lung adenocarcinoma through identification of high-risk individuals for development.
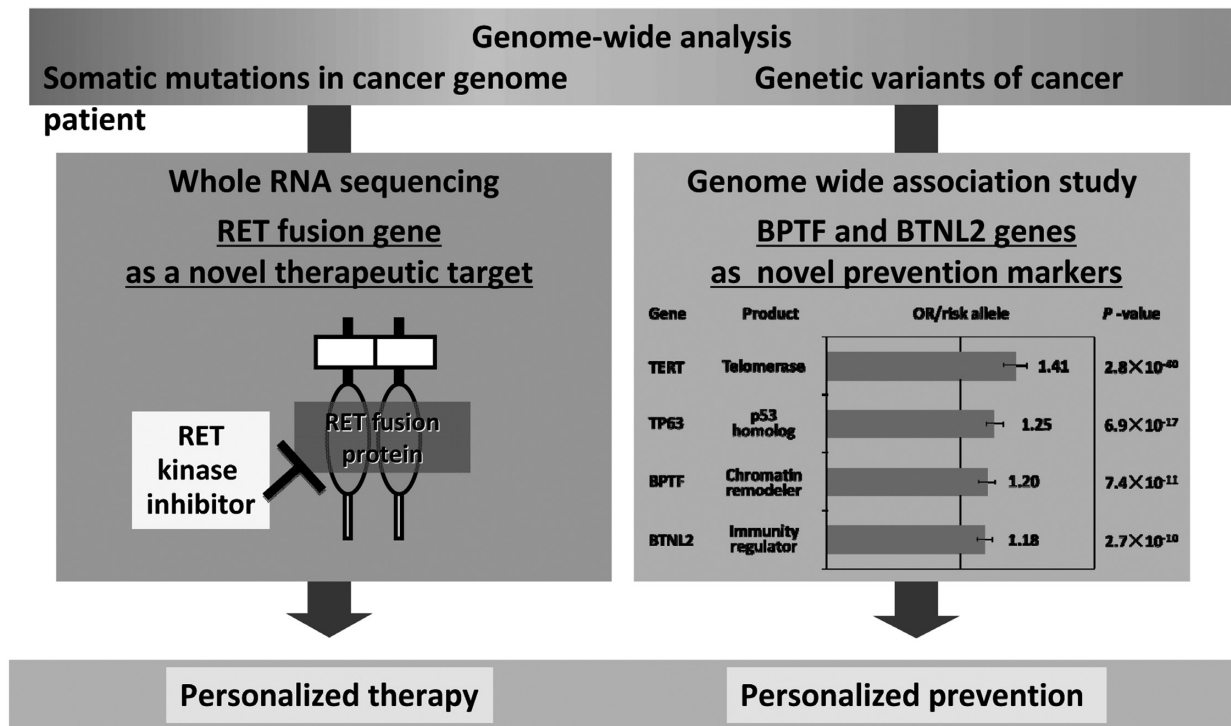

Figure 1

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Journal

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DIVISION OF CANCER GENOMICS

Tatsuhiro Shibata, Fumie Hosoda, Yasushi Totoki, Mamoru Kato, Yasuhito Arai, Hiromi Nakamura, Natsuko Hama, Wataru Munakata, Tomoki Shirota, Naoko Okada, Tomoko Urushidate, Hiroko Shimizu, Shoko Ohashi, Wakako Mukai, Isao Kurosaka

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 global 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 sequencing analysis of liver cancer and the International Cancer Genome project

Thirteen countries including Japan participated in the International Cancer Genome Consortium to generate a comprehensive, high-resolution catalog of genomic changes for major cancer types worldwide. The National Cancer Center has joined this consortium and the Division of Cancer Genomics has taken the initiative in the execution of this international project as a representative research group to analyze virus-associated liver cancer.

Whole genome sequencing of 27 hepatocellular carcinoma (HCC) cases including 14 hepatitis B and 11 C virus-associated HCCs revealed the significant influences of diverse environmental and genetic backgrounds on the somatic mutation patterns and an important role of epigenetic remodeling by genetic alterations in liver carcinogenesis (1, 2).

Whole genome sequencing and genetic analysis of sarcomas

Chondrosarcoma accounts for more than 20% of primary bone sarcomas, with an overall incidence estimated at approximately one in 200,000. However, the etiological background of chondrosarcomagenesis remains largely unknown, along with detailed information on molecular alterations, including potential therapeutic targets. We performed massive parallel sequencing of 10 chondrosarcoma genomes along with the matched normal genomes to identify somatic mutations, structural alterations including fusion genes, and mutation signatures that may help to comprehensively characterize the molecular features of this tumor. Frequent co-amplification and co-expression of CDK4 and MDM2 genes was elucidated in high-grade osteosarcoma specimens (3).

Whole exome sequencing analysis of breast cancer

Metastasis is the main cause of therapeutic failure and death in cancer patients. To understand the genetic basis underlying metastatic progression of breast cancer, a whole exome sequencing (WES) analysis of 16 trios of primary breast cancer, lymph node metastatic tumor and their matched noncancerous tissue has been done. A preliminary result of the WES analysis identified some nonsynonymous mutations which were commonly observed in primary and metastatic tumors and a large number of mutations specific to primary or metastatic tumors, respectively. The result suggests that heterogeneous genetic alterations occur during the tumor progression and the metastatic process in individual breast cancers. Pathological analysis of chemosensitivity in triple-negative breast cancers was performed (4).

Genome-wide genetic analyses of childhood cancer and other tumors

Comprehensive analysis of the five key genes, *WT1*, *CTNNB1*, *WTX*, *IGF2* and *RASSF1*, from Japanese Wilms tumor patients revealed that methylation of the RASSF1 promoter was a prognostic biomarker (5), and that loss of IGF2 imprinting was specifically low in the Japanese cohort (6). In germ cell tumors, meiosis error and subsequent genetic and epigenetic alterations may have caused malignant transformation (7). Frequent deletion of the TNFAIP3/A20 gene was identified in classical Hodgkin lymphoma (8).

Oncogenic fusion genes in lung cancer

To explore the molecular genetics of, and identify new molecular targets in lung cancer, whole transcriptome analysis was performed in non-small cell lung cancer (NSCLC) tissues. We identified novel in-frame fusion kinase genes, EZR-ROS1 and others,

and showed their transforming activities in colony formation were suppressed by the corresponding kinase inhibitors. Established transgenic mouse lines specifically expressing EZR-ROS1 in lung alveolar epithelial cells developed multiple adenocarcinoma nodules in both lungs at an early age.

Transcriptome sequencing analysis of gastric cancer

To understand the genetic basis underlying the development of gastric cancer and to identify new drug targets in the diffused type of gastric cancer, a transcriptome sequencing approach has been undertaken. RNA sequence analysis predicted 43 fusion gene candidates in 27 out of 37 tumors examined. Twenty-four in-frame gene fusions have been identified among the candidates with the RT-PCR method. Those fusion genes including two oncogenic protein kinase-fusions could be good candidates for therapeutic targets.

Metabolome analysis of the NRF2 oncogene

NRF2, a key regulator for the maintenance of redox homeostasis, has been shown to contribute to malignant phenotypes of cancers including aggressive proliferation. However, the mechanisms with which NRF2 accelerates proliferation are not fully understood. We showed that NRF2 redirects glucose and glutamine into anabolic pathways, especially under the sustained activation of PI3K-Akt signaling (9).

Bioinformatics platform and support for clinical sequencing and other cancer research

As a part of a Phase I Center, we developed a computer system for clinical sequencing which utilizes the latest DNA sequencing technology to identify DNA aberrations in clinical samples to achieve personalized medicine. We also supported a bioinformatics analysis to classify DNA adducts in mass-spec data, to identify micro-RNAs related to carcinogens, and to characterize genes related to cancer stem cells. In addition, we collaborated with research groups outside NCC on identification of a new gene in neuroblastoma and analysis of small RNA (10).

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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, Naoko Yasuno, Yuko Miyamoto, Hiroko Ito, Haruyo Tozaki, Akihiko Miyanaga, Takafumi Watanabe, Yukio Watabe

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 so-called "-omics" technologies have made rapid advances in recent years. It is anticipated that application of these technologies will greatly facilitate the discovery of molecules reflecting the diverse clinical behaviors of cancers. As cancer development occurs in parallel with protein dysfunction, comprehensive analyses of cancer proteomes would seem to be a more practical approach for the development of diagnostics and therapeutics.

Novel post-translational regulation of the PML tumor suppressor function

Using a comprehensive shotgun mass spectrometry approach, we had previously identified PML as one of 70 proteins commonly co-immunoprecipitated with the anti-T-cell factor-4 (TCF4) antibody from two colorectal cancer cell lines. PML is a tumor suppressor involved in the pathogenesis of acute promyelocytic leukemia (APL). Loss of PML-NBs has been reported in many different human neoplasms, including colorectal carcinoma, but the mechanisms involved have not been fully elucidated. We show for the first time that β-catenin interacts with PML isoform IV and disrupts PML-IV function and PML-NB formation by inhibiting RanBP2-mediated SUMOylation of PML-IV.

Plasma and serum biomarker discovery using 2DICAL

2DICAL (2-dimensional image converted analysis of liquid chromatography and mass spectrometry) is a proteomics analysis system originally developed at the NCCRI and applied for medical and biological proteomics. With regard to the medical application of 2DICAL, biomarkers have been discovered for two urological cancers. Carbonic anhydrase I (CAI) was detected by 2DICAL as a novel plasma biomarker of prostate cancer. The 2DICAL result was validated with an enzyme-linked immunosorbent assay (ELISA) of 185 plasma samples, and this confirmed that the plasma CAI concentration significantly increased in prostate cancer patients. Especially in the PSA "gray zone" of 4 -10 ng/ml, determination of the plasma CAI concentration increased the prostate cancer discrimination rate when combined with the PSA test. In clear cell renal cell carcinoma, 2DICAL identified fibronectin 1 as a novel plasma biomarker. Using Amplified Luminescent Proximity Homogeneous Assay technology (AlphaLISA), the identified biomarker candidate was validated in a cohort of 77 patients with clear cell renal cell carcinoma and 130 healthy controls.

Reviewing the 2DICAL approach for biomarker discovery, we have adopted a pathway of sample recruitment, sample preparation, biomarker discovery, and validation. 2DICAL has played an important part in the discovery of new biomarkers. Using this approach, we have succeeded in finding plasma or serum biomarkers for pancreatic cancer and colorectal cancer, and have become better able to predict both the adverse effects of chemotherapy for pancreatic cancer and the survival of patients.

Proteomics approach for biological experiments

The interactome is a comprehensive proteomics approach for discovery of proteins that bind specifically to others. 2DICAL interactome analysis (IP-2DICAL) has identified NPM1 (nucleophosmin), which binds to DDX31 (DEAD box polypeptide 31) shown to be upregulated exclusively in clear cell renal cell carcinoma with a genome-wide gene expression profiling analysis. The interaction between DDX31 and NPM1 plays a critical role in carcinogenesis through interruption of the p53–HDM2 pathway of apoptosis by blocking the interaction between HDM2 and NPM1 in the nucleoplasm or cytoplasm.

Mieap is an important P53-related protein that controls the quality of mitochondria. 2DICAL

has identified 14-3-3γ as a novel Mieap-interacting protein. Biological investigations have revealed a critical role of 14-3-3γ in eliminating oxidized mitochondrial proteins during the MALM (<u>M</u>ieap-induced <u>a</u>ccumulation of <u>l</u>ysosome-like organelles within <u>m</u>itochondria) process by interacting with Mieap within mitochondria. Thus, interactome analysis is a very useful tool for revealing novel interactions of molecules that are of specific interest to researchers.

Multi-institutional validation study

Among patients with the more common human malignancies, those with invasive ductal carcinoma of the pancreas have the worst prognosis. The poor outcome seems to be at least partly attributable to difficulty in early detection. In fact, over 95% of patients with pancreatic cancer are not diagnosed until the disease has progressed to stage III or IV. We have performed a comprehensive comparative plasma proteomic analysis of pancreatic cancer patients and healthy controls using a newly developed quantitative mass spectrometry system. We reported that two modified forms of plasma/serum apolipoproteins were reduced in patients with pancreatic diseases. Although their reduction is not specific to pancreatic cancer, it has considerable potential for early detection of the disease. The significance of this discovery was further validated using a total of 1099 plasma/serum samples, consisting of 3 cohorts collected from 8 medical institutions in two countries.

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DIVISION OF CANCER PATHOPHYSIOLOGY

Yasuhito Uezono, Seiji Shiraishi, Masami Suzuki, Kanako Miyano, Yuka Sudo, Yumi Sawada, Junko Ezuka, Yukiko Araki, Kiyoshi Terawaki, Katsuya Morita, Katsuya Ohbuchi, Junichi Ogata, Koichiro Minami, Shun Muramatsu, Naoyo Motoyama, Tohru Yokoyama, Maho Ashikawa, Miki Inoue, Naofumi Oyanagi, Yohei Kashiwase, Yoshihiko Tasaki, Atsumi Nagasawa, Akinobu Yokoyama

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) studies on the prevention and development of novel treatment for cancer cachexia. In particular, basic to clinical, and also clinical to basic translational collaborative research with the divisions of Palliative Care and Psychooncology in the National Cancer Center Hospital comprises our main research protocols and is now ongoing.

Improvement of pain treatment for patients with severe and intolerable cancer pain

In the treatment of pain in cancer patients, opioids and related analgesics are mainly and routinely used. However, the opioids currently available can prove ineffective in not a few patients. For such patients, development of clinically available novel opioid analgesics is indispensable and attractive. We are studying distinct pharmacological properties among each of the opioid analgesics to finally develop and then introduce novel opioids in the clinical field; such a trial is one of our main research themes. In addition, several adjuvant analgesics such as anti-convulsants, anti-depressants, anesthetics, anti-arrhythmias and the $GABA_B$ receptor against baclofen are used for pain control; they are chosen based mainly on the history of their clinical experience. In order to clarify the mechanisms by which adjuvant analgesics have analgesic effects in some particular types of pain, basic research analyses with molecular and cellular biological approaches are conducted in this Division (1, 2, 3, 4, 5, 6). For instance, voltage-dependent Na^+ channels (Nav) in the peripheral neurons could be involved in certain types of intolerable pain. Accordingly, one of our ongoing studies involves elucidating the mechanisms as to how Nav is modulated by several drugs or endogenous active agents (1, 3, 4). In addition, the transient receptor potential (TRP) channel family, especially the TRP Vanilloid channels 1 (TRPV1) and TRP ankyrin 1 (TRPA1) are reported to transduce a large group of signals such as pain. We are trying to investigate the mechanisms of the TRP family functions (2).

Study on the prevention and effective treatment of cancer cachexia

Cancer cachexia is often observed in patients with advanced cancer, and is characterized by anorexia and weight loss associated with reduced muscle mass and adipose tissue. The prevention and effective treatment of cachexia are important in the management of patients with cancer because cachexia induces increased morbidity and mortality, and impinges on the patients' quality of life. There is also a trend towards lower response rates with the use of chemotherapy in patients with cancer cachexia. The study of cancer cachexia is indispensable to improve the quality of life in cancer patients and is being conducted in this Division. With support from a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, we demonstrated that a Japanese *kampo* (traditional Oriental medicine) medication *rikkunshito* and other compounds improved cancer anorexia-cachexia symptoms in cancer cachexia (7, 8). The roles of neuropeptides and/or neurotransmitters in the central nervous system on the cause and emergence of cancer cachexia are also investigated in our Division (9, 10). Additional interests in our Division are the roles of neurotransmitters/neuropeptides in the brain in other pathophysiological states (10, 11).

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Division of Cancer Stem Cell

Kenkichi Masutomi, Satoko Yamaguchi, Mami Yasukawa, Keita Kinoshita, Naoko Okamoto

Introduction

Research in the Division of Cancer Stem Cells is focused on deciphering the mechanisms that establish and maintain cancer stem cells and to develop a novel approach 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

It is widely known that the telomerase is a ribonucleoprotein complex that elongates telomeres. Human telomerase (hTERT) acts as an RNA dependent DNA polymerase (RdDP) and synthesizes telomere DNA from a non-coding RNA (ncRNA) template *human TERC (hTERC)*. We analyzed in detail the mechanisms of telomere elongation at single seeded telomeres in human cells, and we reported that telomere elongation was strictly regulated both temporally and spatially in the S phase of the cell cycle (1). We also found that in addition to *hTERC*, hTERT binds a second non-coding RNA, *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). Moreover, we confirmed that the phenotypes of *RMRP* null mice are embryonicaly lethal. From these observations, we considered the possibilities that the hTERT-*RMRP* complex might be essential for ontogeny and biological functions.

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 BRG1 and the nucleolar GTP-binding proteins, nucleostemin (NS) or GNL3L, and the complex composed of hTERT, BRG1 and NS or GNL3L 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/GNL3L 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/GNL3L exhibit increased CD133 and CD44 expression and enhanced tumorigenicity at limiting cell numbers. These observations indicate that the TERT–BRG1–NS/GNL3L complex is essential for the maintenance of TICs. Because NS contributes to the maintenance of TICs, we hypothesized that NS may act as a predictive marker for recurrence after neoadjuvant chemotherapy. We examined the expression of CD133, CD44, NS, GNL3L, and TWIST with immunohistochemistry in a series of 54 surgically-resected specimens of esophageal squamous cell carcinomas after neoadjuvant chemotherapy. We identified that a high NS proportion, TWIST intensity, and an advanced pathological N (lymph nodes) stage significantly correlated with poor relapse-free survival (2). Moreover, we confirmed that a high NS proportion, strong TWIST intensity, and an advanced pathological N stage significantly correlated with poor recurrence-free survival in a multivariate analysis adjusted for pathological T (tumor) and N stages. In addition, we examined the correlation between NS and TWIST using several human esophageal cancer cell lines (2). We confirmed that the ectopic expression of NS induced the upregulation of TWIST expression, and we also found that the endogenous NS expression level correlated with the TWIST expression (2). These observations implicated NS and TWIST as the predictive markers for postoperative recurrence, and suggest that the expression level of NS was correlated with the clinical prognosis in esophageal cancer patients. Moreover, these represented the first practical attempt to examine the clinical impact of the cancer stem cell factor(s) of NS in esophageal cancer.
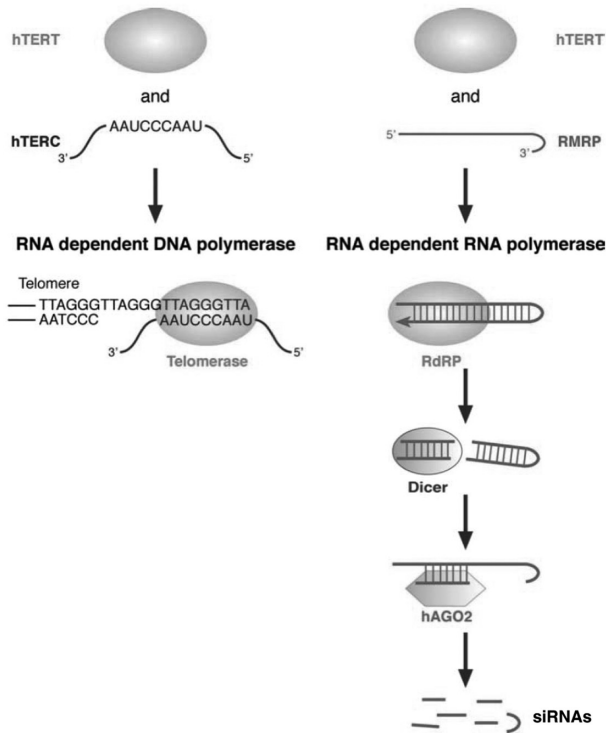
Figure 1

**Increased fraction of
tumor initiating cells**

Figure 2

Telomerase and epigenetics

Previously reports have shown that functional non-coding RNA is widely involved in the physiology of organisms through its epigenetic regulation. We therefore focused on studying the molecular basis of maintenance of the heterochromatin formation by RNAs, especially by non-coding RNAs such as siRNAs, miRNAs and snoRNAs. 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. Since previous studies have shown that hTERT expression reduces the frequency of dicentric chromosomes and suppresses aneuploidy, we speculated that hTERT would monitor genome stability during centromeric heterochromatin maintenance as well as telomere maintenance. Moreover, we have identified that hTERT acts as RdRP, and produces a double-stranded, endogenous siRNA (Figure 1). These observations indicated that the mammalian homologue of RdRP (TERT) may regulate heterochromatin formation through its epigenetic regulation, and we are analyzing the link between telomerase and epigenetics.
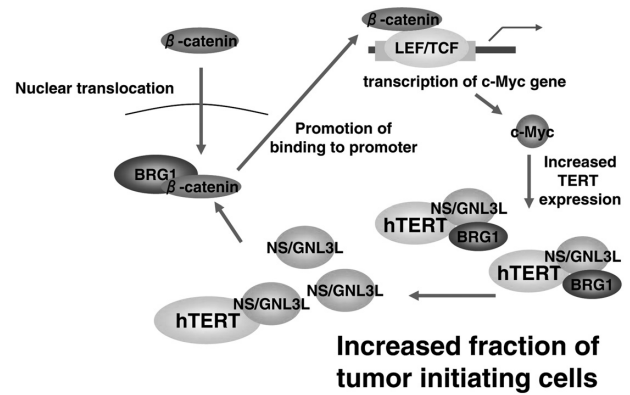
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Division of Gene and Immune Medicine

Kazunori Aoki, Kenta Narumi, Naoko Goto, Yoko Kobayashi, Kouichirou Aida, Takeshi Udagawa, Koji Suzuki, Reina Miyakawa, Yuki Yamamoto, Naoto Shimokawatoko, Kazuki Miura, Keito Taniwaka

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 library approach. The specific activities in 2012 were as follows: 1) Preclinical study of intratumoral injection of IFN-β plasmid/liposome complex for sarcomas; 2) Mechanism of antitumor immunity induced by the combination of hematopoietic stem cell transplantation and immune gene therapy; 3) Development of a peritoneal dissemination-targeting adenovirus vector.

Research activities

Type I IFN gene therapy against sarcomas

Sarcomas at advanced stages remain a clinically challenging disease. Interferons (IFN) can target cancer cells by multiple antitumor activities including the induction of cancer cell death and enhancement of the innate and adaptive immune response. The Division examined whether a type I IFN gene transfer could induce an effective antitumor effect against sarcomas. First, the Division found that a type I IFN gene transfer significantly suppressed the cell growth of various sarcoma cell lines. Then, to examine the antitumor effect *in vivo*, the legs of BALB/c nude mice were inoculated with 143B human osteosarcoma or SK-UT-1B leiomyosarcoma cells, and an IFN-β-liposome complex was then injected directly into the tumors 3 or 6 times. The IFN-β gene transfer showed a significant suppressive effect against the 143B tumors in a dose-dependent manner, while a three-time injection of IFN-β plasmid-liposome was sufficient to eradicate the SK-UT-1B tumors. No adverse effect was recognized in the treated mice. The results showed that an intratumoral IFN gene transfer could be a promising therapeutic strategy for sarcomas. To translate the basic research to a clinical setting, the Division is collaborating with the Central Hospital, and is planning a Phase I clinical trial on intratumoral injection of an IFN-β plasmid/liposome complex in patients with sarcomas at advanced stages. At present, the protocol is under review in the Gene Therapy Ethics Committee of the National Cancer Center.

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

T cells recognize tumor-associated antigens under the condition of lymphopenia-induced homeostatic proliferation (HP), however, HP-driven antitumor responses gradually decay in association with tumor growth. The Division examined whether a tumor-specific immune response induced by IFN could enhance and sustain HP-induced antitumor immunity in CT26 murine colon cancer models. An intratumoral IFN gene transfer resulted in marked tumor suppression when administered in the early period of syngeneic hematopoietic stem cell transplantation, and was evident even in distant tumors that were not transduced with the IFN vector (1). IFN gene transfer was then combined with syngeneic HSCT in murine osteosarcoma 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 (2). The treated mice showed no significant adverse events. To clarify the mechanism of antitumor immunity induced by the combination therapy, CD11c+ cells were isolated from the regional lymph nodes of treated tumors. Flow-cytometry showed that an intratumoral delivery of the IFN gene promoted the maturation of CD11c+ cells in the tumors and effectively augmented the antigen-presentation capacity of the cells. An analysis of the cytokine profile showed that the CD11c+ cells in the treated tumors secreted a large amount of immune-stimulatory cytokines including IL-6. The CD11c+ cells rescued effector T-cell proliferation from regulatory T cell-mediated suppression, and IL-6 played a dominant role in this phenomenon (1)(Fig. 1). The intratumoral IFN gene transfer created an environment strongly supporting the enhancement of antitumor immunity in reconstituted lymphopenic recipients through the induction of tumor-specific immunity and suppression of immunotolerance.
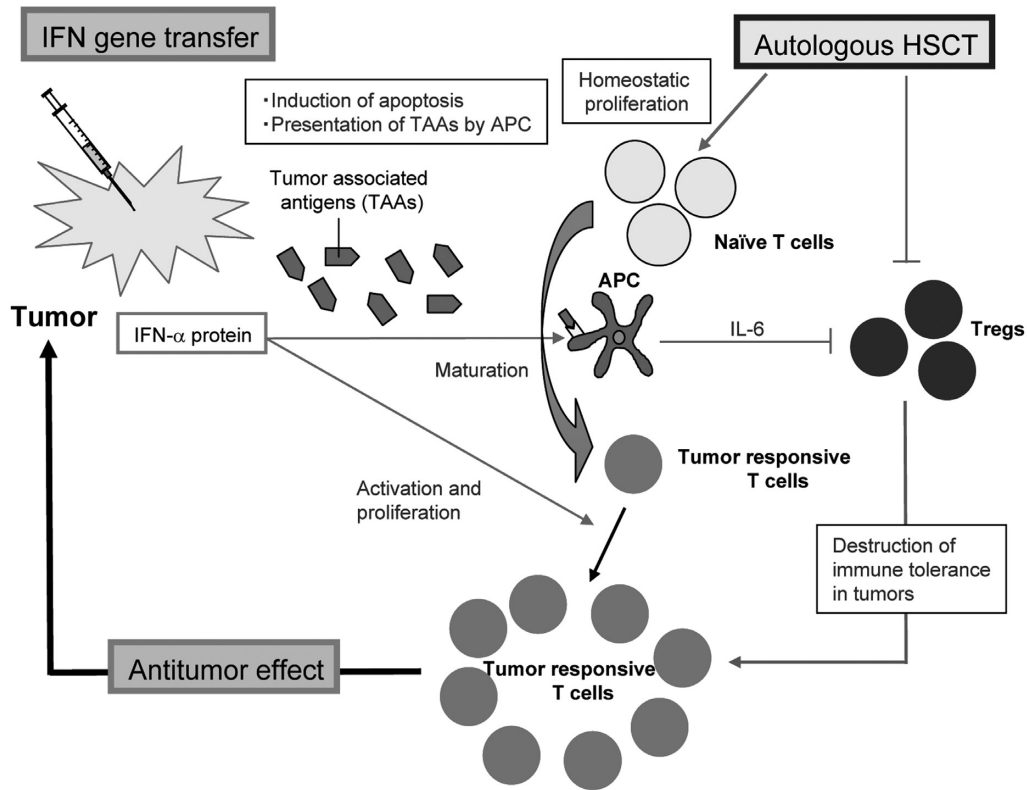
Figure 1

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

The targeting of gene transfer at the cell-entry level is one of the most attractive challenges in vector development. To develop cancer-targeting adenovirus vectors, the Division has constructed a random peptide library displayed on the adenoviral fiber knob, and has successfully selected targeted vectors by screening the library on cancer cell lines *in vitro*. The infection of targeted vectors was considered to be mediated by specific receptors on target cells. However, the expression levels and kinds of cell surface receptors may be substantially different between an *in vitro* culture and *in vivo* tumor tissue. Therefore, the Division screened the peptide display-adenovirus library in the peritoneal dissemination model of AsPC-1 pancreatic cancer cells. The vector displaying a selected peptide (PFWSGAV) showed higher infectivity in the AsPC-1 peritoneal tumors but not in organs and other peritoneal tumors as compared with a non-targeted vector (3). Furthermore, the infectivity of the PFWSGAV-displaying vector for AsPC-1 peritoneal tumors was significantly higher than that of a vector displaying a peptide selected by *in vitro* screening, indicating the usefulness of *in vivo* screening in exploring the targeting vectors.

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DIVISION OF GENOME STABILITY RESEARCH

Mitsuko Masutani, Ken-ichi Yoshioka, Hiroaki Fujimori-Sakuma, Kengo Inoue, Takahisa Hirai, Anna-Margareta Rydén, Yasuhisa Okajima, Hiromi Harada, Junhui Wang, Soichiro Saito, Yuko Atsumi, Yuko Kudo, Tomoyuki Osawa, Hiroaki Mukai, Shuhei Yoshida, Tasuku Itoh, Miyuki Hozumi, Masako Yamazaki, Tsubasa Sekiguchi

Introduction

This Division pursues mechanisms underlying the genomic stability against diverse direct and indirect DNA damage caused by radiation, chemotherapeutic agents and various forms of cellular stress. In parallel with these studies, research projects into the development of novel strategies for chemotherapy and radiation therapy have been conducted in collaboration with institutions and clinical researchers inside and outside of the NCC. The 31st meeting of Molecular Pathology was organized in Ena by this Division.

Research activities

Radiation damage response and radiosensitization

For the studies on radiosensitization, evaluated irradiation systems delivering a variety of radiation types are necessary. An X-ray irradiation system in the Research Institute has been set up and physical and biological evaluation were carried out in collaboration with the Departments of Radiation Oncology of the NCC. A model for metastasized tumors in the mouse brain and a treatment model with local X-ray irradiation were optimized.

Radiosensitization by a PARP inhibitor for low and high LET (linear energy transfer) radiation was observed and the involvement of a possible increase of DSB (double strand break)-like lethal DNA lesions (Figure 1) has been investigated (2). Using an shRNA library, target genes for radiosensitization were widely screened and more than 100 candidate genes of various categories, including cell cytoskeleton, DNA damage response, and transcription, were picked up and are being validated using siRNA.

Basic research on BNCT

Boron neutron capture therapy (BNCT) is a unique cancer cell-targeted therapeutic strategy, for which clinical trials are now ongoing. To understand the mechanism of tumor cell death induced by BNCT and to optimize BNCT condition, we used rat tumor graft models with boronophenylalanine and histological and biochemical analyses was carried out focusing on DNA damage response in collaboration with other institutions. The persistent staining of γH2AX and poly(ADP-ribose) (PAR) suggested accumulated double-strand breaks after BNCT. The γH2AX and PAR were found to be the markers for monitoring DNA damage induced by BNCT. Collaboration studies for the biomarkers of BNCT have also been started with Kyoto University Research Reactor Institute and several other institutions.

Epigenetic dysregulation caused by PARP inhibitor

Clinical trials have demonstrated the significance of PARP inhibitors in the treatment of tumors showing *BRCA* dysfunction. Utilizing mouse embryonic stem cells (ESCs) as a model, the effect on epigenetic regulation was investigated. Dysregulation of DNA methyltransferases and DNA hypomethylation were induced in ESCs by a PARP inhibitor, which accompanied up-regulation of particular genes and a differentiation disorder. This study suggested that a PARP inhibitor might cause epigenetic dysregulation possibly affecting therapeutic efficacy and may also cause potential side-effects to normal cells, especially to stem cells (4, 5).

Functional studies of PARG and development of PARG inhibitors for cancer therapy

PARG is involved in DNA repair and its inhibition has been shown to cause accumulation of PAR synthesis. PAR accumulation also triggers cell death. PARG siRNA knockdown suggests that PARG dysfunction enhances the cell death caused by γ- and carbon-ion irradiation as well as alkylating agents. PARG inhibitors may enhance the effect of radiation therapy and chemotherapeutic agents. Specific and potent PARG inhibitors are not known. Screening of PARG inhibitors from chemical libraries and an optimization study have been conducted as a collaborative study with other institutions. Several PARG inhibitors have been developed and structural optimization is being investigated.
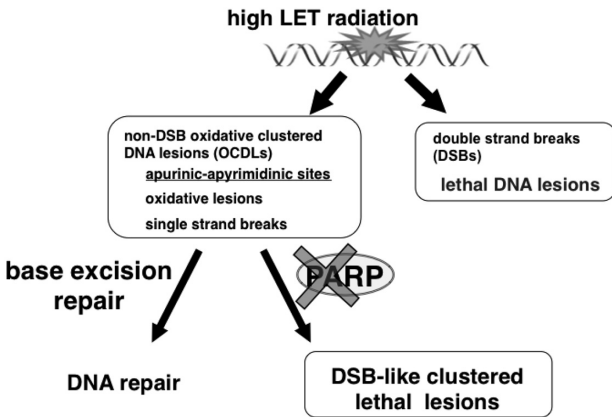
Figure 1. A model for increase of DSB-like clustered lethal DNA lesions by PARP inhibitor after exposure to high LET radiation
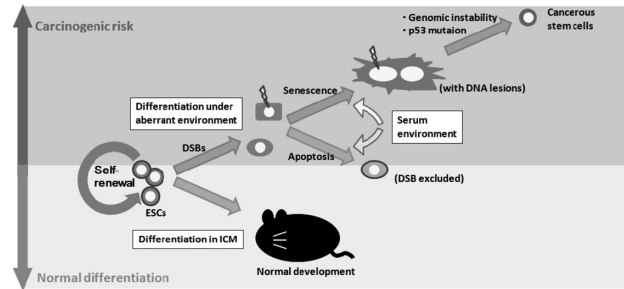
Figure 2. Transformation of ESCs under aberrant niche or environment

Induction of genomic instability in stem cells

Although stem-cell maintenance depends on their microenvironment, it remains to be elucidated whether an environmental aberrancy can act as a carcinogenic stressor for cellular transformation of differentiating stem cells into cancer stem cells. Utilizing mouse ESCs as a model, environmental aberrancy during differentiation was demonstrated to lead to the emergence of pluripotent cells showing cancerous characteristics (Figure 2). This suggests that stem cells differentiating in an aberrant environment are at a risk of cellular transformation into malignant counterparts (1).

Arf/p53-dependent downregulation of H2AX and sensitivity to anti-cancer drugs

Cancer cells are generally more sensitive to anticancer drugs than normal somatic cells. However, the factors that determine this differential sensitivity are poorly understood. It was observed that Arf/p53-dependent downregulation of H2AX induced the selective survival of normal cells, resulting in the preferential targeting of cancer cells (3). Treatment with camptothecin, a topoisomerase I inhibitor, caused normal cells to downregulate H2AX while inducing a quiescent cellular state, a process which required both Arf and p53. In contrast, transformed cells are generally mutated in either Arf or p53, thereby not down-regulating H2AX and sensitively responding to the drug, unless they have developed drug resistance. This effect of discrimination between normal and cancer cells is much larger than that of p53-mediated apoptosis induction. Therefore both the H2AX expression level and γH2AX level are critical factors that determine drug sensitivity, which should be considered when administering chemotherapeutic agents.

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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, consisting of Ohta's Unit, Hamada's Unit and Katoh's Unit, is focused on development of innovative cancer diagnosis and treatment as well as analyses of pharmaco-dynamics 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. 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 have been searched for using *in vitro* and *in vivo* analyses.

The t(X;18)(p11.2;q11.2) translocation found in synovial sarcomas results in a fusion between the SYT gene on chromosome 18 and an SSX gene on the X chromosome. Although SYT-SSX fusion proteins appear to trigger synovial sarcoma development, little is known about the functions of SYT-SSX. The SYT-SSX fusion protein produces a dominant-negative function for the SYT, which is a transcriptional co-activator. The SYT-SSX fusion protein complex is purified from cells and the association proteins are analyzed using mass spectrometry.

Hamada's unit

Research in clinical pharmacology and pharmaco-imaging is focused on the PK/PD analysis of anticancer agents in clinical trial and the development of an integrated pharmacokinetics system. This novel system provides drug exposure levels in the blood and tissue using a high-sensitivity triple-quadrupole mass spectrometer and non-label pharmaco-imagining (*i.e.*, imaging mass spectrometry in a mass microscope). Our aim and research goal are to provide a revolutionary new analyzing system for clinical pharmacology and drug development for use in clinical trials.

Imaging mass spectrometry (IMS) is now widely used in several research fields for pharmacology in particular, IMS can provide novel visualization information that differs from conventional pharmaco-imaging technologies such as autoradiography and positron emission tomography, due to its non-labeled feature. The parent drug and its metabolites can be individually visualized using IMS. The basic of IMS is tissue surface analysis using mass spectrometry. The workflow of IMS is shown in Fig.1. Tissue sections are prepared in a cryo-microtome, after which a thin matrix layer is formed on the tissue surface to absorb UV laser for ionization using a spray or chemical vapor deposition. In IMS, matrix-assisted laser desorption/ionization (MALDI) is used as an ionization method. During IMS measurement, all mass spectra obtained directly from the tissue surface are stored with ionization position information. A peak intensity map of interested m/z is reconstructed using the spectra. Here, m/z means the mass-to-charge ratio which depends on molecular weight. Therefore, if we can confirm m/z peaks correspond to the target drug compounds, tissue distribution is available without labeling.

Katoh's unit

Atypical Cadherin Fat, involved in tumor suppression and planar cell polarity (PCP), is a *Drosophila* homolog of human FAT1, FAT2, FAT3 and FAT4. FAT1 and FAT4 undergo the first proteolytic cleavage by Furin and are predicted to undergo the second cleavage by γ-secretase to be released into the intracellular domain. Ena/VAPS-binding to FAT1 induces actin polymerization at lamellipodia and filopodia to promote cell migration, while Scribble-binding to FAT1 induces phosphorylation and functional inhibition of YAP1 to suppress cell growth. *FAT1* is preferentially downregulated in invasive breast cancer and is repressed in oral cancer due to homozygous deletion or epigenetic silencing. On the other hand, *FAT1* is upregulated in leukemia. Prognosis of preB-acute lymphocytic leukemia (ALL) patients with *FAT1* upregulation is poor. FAT4 directly interacts with MPDZ/MUPP1

to recruit membrane-associated guanylate kinase MPP5/PALS1. FAT4 is involved in the maintenance of PCP and inhibition of cell proliferation. *FAT4* mRNA is repressed in breast cancer and lung cancer due to promoter hypermethylation. The *FAT4* gene is recurrently mutated in several types of human cancers, such as melanomas, pancreatic cancer, gastric cancer and hepatocellular carcinomas. FAT1 and FAT4 suppress tumor growth via activation of Hippo signaling, whereas FAT1 promotes tumor migration via induction of actin polymerization. FAT1 is tumor suppressive or oncogenic in a context-dependent manner, whereas FAT4 is tumor suppressive. The copy number aberration, translocation and point mutation of the *FAT1*, *FAT2*, *FAT3*, *FAT4*, *FRMD1*, *FRMD6*, *NF2*, *WWC1*, *WWC2*, *SAV1*, *STK3*, *STK4*, *MOB1A, MOB1B, LATS1, LATS2, YAP1* and *WWTR1/ TAZ* genes should be comprehensively investigated in various types of human cancers to elucidate the mutation landscape of the FAT Hippo signaling cascades. Because YAP1 and WWTR1 are located at the crossroads of adhesion, the G-protein-coupled receptor (GPCR), receptor-type tyrosine kinase (RTK) and the stem cell signaling network, cancer genomics of the FAT signaling cascades could be applied for diagnostics, prognostics and therapeutics in the era of personalized medicine.

Katoh contributes to the global science community based on manuscript publication, reviewer activity and editor activity. Katoh carried out peer reviews of grant proposals or journal manuscripts written in English 70 times in 2012. Katoh is an editorial board member of several scientific journals, such as *PLoS ONE*, the *Asia-Pacific Journal of Clinical Oncology*, and t*he International Journal of Oncology*. Katoh made editorial decisions regarding 135 manuscripts submitted to *PLoS ONE* in 2012.

The 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 530 times by others in 2012.
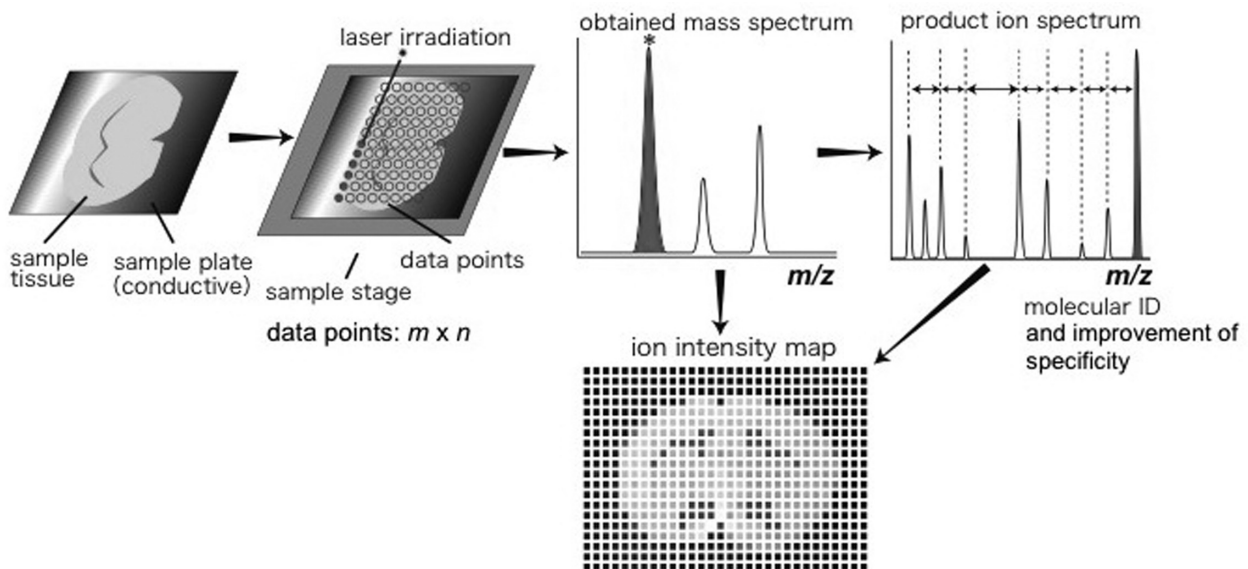

Figure 1. Workflow of imaging mass spectrometry

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7. Katoh M. Function and cancer genomics of *FAT* family genes. Int J Oncol, 41:1913-1918, 2012

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Division of Refractory Cancer Research

Hitoshi Nakagama, Masato Enari, Shinichi Yachida, Rieko Ohki, Yuko Hibiya, Yukie Aita, Ryo Otomo, Makoto Miyazaki, Yoshinori Asano, Issei Ezawa, Kozue Saito, Shoko Ohde, Miku Shimizu, Shiori Suzuki, Chen Yu, Yuhei Takano

Introduction

The Division's 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 2012 were as follows: 1) p53 inactivation by anaplastic lymphoma kinase through the direct tyrosine phosphorylation of p53; 2) PH domain-only protein PHLDA3, a p53-regurated repressor of Akt and a novel suppressor of endocrine tumors; 3) clinicopathologic and genetic analysis of pancreatic cancers.

p53 inactivation by anaplastic lymphoma kinase through the direct tyrosine phosphorylation of p53

The tumour suppressor protein p53 is a transcription factor that activates various genes which are responsible for cell growth arrest and apoptosis in response to cellular stress. The inactivation of the p53 pathway is crucial for tumour formation. We have previously found that the nuclear clathrin heavy chain (CLTC) protein, which has been identified as a cytosolic protein functioning that functions in endocytosis and protein sorting, binds to p53 and is required for p53-mediated transcription (Genes Dev. 20,1087-1099, 2006; Oncogene 27, 2215-2227, 2008; J. Mol. Biol. 394, 460-471, 2009). To investigate the role of CLTC in tumours, we first examined the instances of CLTC in various tumors which had fused to anaplastic lymphoma kinase (CLTC-ALK) generated by chromosomal translocation, and investigated the function of the CLTC-ALK fusion on p53-mediated pathways. Unexpectedly, we found that CLTC-ALK inhibited the p53 pathway through ALK-mediated tyrosine kinase activity. Other ALK-fusion proteins including NPM-ALK and EML4-ALK also inhibited the p53 pathway, suggesting that tyrosine phosphorylation was required for the inhibition of the p53 pathway. We performed an immunoprecipitation assay to explore the ALK-phosphorylated proteins that interact with p53 and strikingly found that p53 is directly phosphorylated by ALK-fusion proteins. Mutational analyses revealed that three tyrosine residues in the DNA-binding and C-terminal domains of p53 were phosphorylated to inhibit the p53 pathway. These phosphorylation events enhance Mdm2 binding, leading to decreased p53 stability and the retention of p53 in the cytoplasm. The relationship between ALK and p53 has so far been unclear and we proposed that oncogenic ALK-fusion proteins inhibit p53 function through the direct tyrosine phosphorylation of p53.

PH domain-only protein PHLDA3; a p53-regulated repressor of Akt and a novel tumor suppressor of endocrine tumors

p53 and Akt are critical players regulating tumorigenesis with opposite effects: while 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 (Cell 136, 535-550, 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.

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Clinicopathologic and genetic analysis of pancreatic cancers

Pancreatic ductal adenocarcinoma is an aggressive malignancy, usually with widespread metastatic disease. Genetic alterations of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* are the most frequent events in pancreatic ductal adenocarcinoma. We determined the extent to which these 4 alterations were coexistent in the same carcinoma, and their impact on patient outcome. Pancreatic cancer patients who underwent an autopsy were studied (n = 79). The number of genetically altered driver genes in the adenocarcinoma tissues was variable, with identification of an alteration in all 4 genes being seen in only 29 patients (37%). The number of altered driver genes was significantly correlated with disease free survival, overall survival and metastatic burden at autopsy. On multivariate analysis, the number of driver gene alterations in pancreatic ductal adenocarcinoma remained independently associated with overall survival. Carcinomas with only 1 to 2 driver alterations were enriched for those patients with the longest survival (median 23 months, range 1 to 53). Determinations of the status of the 4 major driver genes in pancreatic cancer, and specifically the extent to which they are coexistent in an individual patient's cancer, provides distinct information regarding disease progression and survival that is independent of the clinical stage and treatment status. We also confirmed the clinical significance of the number of driver gene alterations as a predictive marker of postoperative survival outcome in a population of Japanese patients (Ann Surg 2013, *in press*).

Pancreatic neuroendocrine neoplasms are the second most common tumor among pancreatic tumors. They include well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs). Furthermore, NECs are divided into large cell NEC and small cell NEC. We reported that small cell NECs are genetically similar to large cell NECs, and these genetic changes are distinct from those reported in NETs.

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3. Yachida S. Novel therapeutic approaches in pancreatic cancer based on genomic alterations. Curr Pharm Des, 18:2452-2463, 2012

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5. Fu B, Yachida S, Morgan R, Zhong Y, Montgomery EA, Iacobuzio-Donahue CA. Clinicopathologic and genetic characterization of traditional serrated adenomas of the colon. Am J Clin Pathol, 138:356-366, 2012

Division of Cancer Prevention Research

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

Introduction

Obesity and abnormal lipid metabolism are associated with the development of many cancers, including colon and pancreas cancer. Dyslipidemia, alterations of adipocytokine balance and pro-inflammatory status have been suggested to be involved in the development of colon and pancreatic cancer (1). In animal studies, improvement of dyslipidemia and an abnormal adipocytokine balance suppressed both colon and pancreas carcinogenesis (2, 3). However, the 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. Thus, we are investigating the mechanisms of obesity- and dyslipidemia-related carcinogenesis in the colon and pancreas to develop effective approaches for human cancer prevention.

Research activities

Molecular Targets for Cancer Prevention and the Search for a Chemopreventive Agent against Colon Cancer

Obesity is a risk factor for human colorectal cancer development. Last year we clearly showed that obese KK-A^y mice are highly susceptible to azoxymethane (AOM)-induced colorectal aberrant crypt foci (ACF) and tumor development. KK-A^y mice showed high serum triglyceride, Pai-1, leptin and IL-6 levels and low adiponectin levels. Thus, we examined the effects of pioglitazone on AOM-induced colorectal ACF development in KK-A^y mice. Pioglitazone is a peroxisome proliferator-activated receptorγ (PPARγ) agonist, which used as an anti-diabetic drug with the potential to improve dyslipidemia. Administration of pioglitazone reduced the number of KK-A^y colon ACF / mouse with significant reduction of serum triglyceride and insulin levels (4). Moreover, mRNA levels of Pai-1 and leptin in the visceral fat also decreased. Along the lines of developing novel cancer chemopreventive agents, we demonstrated that a novel chemically synthesized compound SK-1009 suppressed IL-6 mRNA levels in human colon cancer cells through blocking NF-kappaB pathways. As IL-6 is an important biological mediator playing an important role in inflammation and cancer, but few inhibitors and suppressors are known, it is possible that our data may provide important information to enable further development of chemopreventive agents (5). Moreover, one of the promising colorectal cancer chemopreventive agents, indomethacin, also suppresses lung carcinogenesis in urethane treated male A/J mice. This examination also indicated that respiration-gated X-ray micro-computed tomography (micro-CT) was a useful non-invasive imaging approach for evaluating the characteristics and suppression of lung tumors in mice treated with cancer chemopreventive agents (6). In addition, it has been shown that *in vivo* SPECT imaging with ^{111}In-DOTA-c(RGDfK) was a useful method to detect early pancreatic cancer in a hamster pancreatic carcinogenesis model (7).

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Journal

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Division of Brain Tumor Translational Research

Koichi Ichimura, Shintaro Fukushima, Kensuke Tateishi, Ayaka Otsuka, Emiko Yamomoto, Hideyuki Arita, Yuko Matsushita

Introduction

Our laboratory focuses on translational research on brain tumors. The prognosis of malignant brain tumors, which make up approximately one third of all primary brain tumors, is dismal. In adults, the great majority of primary malignant brain tumors are gliomas. Glioblastomas (WHO grade IV) are the most malignant and fatal brain tumor, the patients' median survival being only about 14 months after surgical removal. Glioblastomas with MGMT methylation respond to temozolomide better than those without, especially among elderly patients. The presence of IDH1/2 mutations is associated with longer survival and defines a biologically distinct subtype among adult gliomas. Thus, accurately assessing those molecular markers in gliomas is critical in interpreting the outcome of clinical trials in these tumors, as well as in routine clinics. The on-going genome-wide studies using high throughput sequencing technologies will undoubtedly identify novel molecular markers and possibly a therapeutic target. However, to fully evaluate the significance of newly identified glioma-associated genes, an extensive validation using a large cohort of tumors as well as functional analysis are necessary using a suitable model system such as cultured cells.

In contrast to adults, brain tumors are very common among children, being the most frequent solid malignancy in pediatric patients. The spectrum of brain tumors in children differs from that of adults. Recent extensive genetic studies in medulloblastomas, pilocytic astrocytomas, pediatric glioblastomas and ependymomas have identified a number of novel molecular features in these tumors. Based on those findings, medulloblastomas are now subdivided into four groups that have distinctly different molecular profiles and clinical courses. The results are now being translated into molecular classifications and may possibly lead to the development of individualized treatment. Intracranial germ cell tumors (iGCTs) are one of the few pediatric brain tumors that are yet to be explored. They are the second most common brain tumor in children under the age of 14 in Japan. iGCTs are histopathologically divided into 5 subtypes, *i.e.*, germinomas, teratomas, embryonal carcinomas, choriocarcinomas and yolk sac tumors.

Mature teratomas may be surgically removed, and germinomas generally respond to combined radio-chemotherapeutic regimens. However a subset of germinomas and other iGCTs may be resistant to therapy and the patients' prognosis may be poor. Their molecular pathogenesis is largely unknown. These tumors require more attention and full molecular investigations.

Study of MGMT methylation and clinical trials

As outlined above, MGMT methylation is one of the most important molecular markers in glioblastomas as well as other brain tumors such as anaplastic astrocytomas and primary central nervous system lymphomas (PCNSL). Methylation testing of MGMT is employed in most major clinical trials involving gliomas using various methods such as methylation-specific PCR. However an optimal method is yet to be agreed upon. The quantitative MSP-based method used in the Phase III trials in Europe and USA is not available in Japan. We have developed a robust pyrosequencing-based MGMT methylation assay. To validate the efficacy of the assay for predicting the prognosis of the patients, the assay is being compared with the outcome of approximately 140 primary glioblastomas which were treated with radiation and temozolomide. Brain tumor specimens from all clinical trials that take place in Japan will then be collected and analyzed in our laboratory. The optimized assay will also be made available for routine neurosurgical clinics through a diagnostic company with whom we have formed a collaborative partnership.

Molecular profiling of gliomas and PCNSL

To validate the significance of novel candidate oncogenes/tumor suppressor genes (TSG) for brain tumors, we are currently setting up a large series of tumor samples for gliomas and PCNSL. So far, through a collaborative effort, over 200 tumor samples have been collected from the Department of Neurosurgery and Neuro-Oncology, National Cancer Center Hospital, as well as other neurosurgical centers. Genetic profiling of known genes, such as

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IDH1, as well as chromosomal abnormalities (e.g., total 1p/19q loss) has been performed to stratify the sample cohort. Target genes will be selected based on the results of the on-going genome analysis of gliomas and PCNSL. We are also generating a cellular model of gliomas through serial introduction of genetic alterations into human neural stem cells. A neuronal stem cell line has been established in the lab and the recombinant adeno-associated vectors have been provided from Horizon Discovery.

Genome analysis of intracranial germ cell tumors

To comprehensively study the biology of iGCTs, we have organized and established the Intracranial Germ Cell Tumors Genome Analysis Consortium of Japan, a nation-wide collaborative initiative to centrally collect patients' materials and clinical information. Over 40 centers in Japan and 3 in Korea have joined the Consortium, through which nearly a hundred iGCT cases of various histological subtypes have been obtained so far. DNA and RNA have been extracted from all tumors. A set of genes involved in the MAPK pathway, including c-kit and RAS, have been examined for the presence of mutations. mRNA /protein expression of c-kit and copy number abnormalities have also been studied. The results showed that mutations of c-kit are very common and associated with its elevated expression in germinomas but not in other types of iGCTs. A whole exome sequencing for 40 iGCTs is being carried out. The results will be validated in an independent cohort of tumors of up to 100.

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CENTRAL ANIMAL / RADIOISOTOPE DIVISIONS

Toshio Imai, Mami Takahashi, Tetsuya Ishikawa, Yoshinori Ikarashi, Kotomi Otsubo, Naoaki Uchiya, Momoko Kobayashi, Natsumi Suda, Teruo Komatsu, Masashi Yasuda, Manabu Tsuchida, Masahiro Nakashima, Ayami Kawashima, Daiju Mutoh, Satoshi Ikeda, Kouki Tamasaka, Junichi Zukeyama, Takuya Matsuyama, 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 from clinical cancer tissues.

Research activities

Research activities of the Central Animal Division have focused on studies of chemical carcinogenesis using laboratory animals, 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) Fatty infiltration in the pancreas in association with invasive ductal carcinogenesis in hamsters and man

The influence of obesity and pancreatic fatty infiltration (FI) in pancreatic carcinogenesis is being investigated in human and animal models. Syrian golden hamsters, which are susceptible to chemical carcinogenesis in the pancreatic ducts, are in a hyperlipidemic state and suffer severe FI of the pancreas. The association between the degree of pancreatic FI and pancreatic cancer was investigated in a case-control study in humans. The degree of FI in non-cancerous parts of pancreatic sections was significantly higher in pancreatic cancer patient cases than in the controls, and was positively associated with pancreatic cancer development. In addition, there was a case in which severe fatty pancreas had been observed on CT-scan imaging 5 years before detection of pancreatic adenocarcinoma. These data suggest that severe pancreatic FI could be a risk factor for pancreatic cancer.

2) Pancreatic ductal carcinogenesis and epithelial mesenchymal transition in hamsters

The poor prognosis of pancreatic cancer has been attributed to the difficulty in detection of this cancer in its early operable stages, resulting from its aggressive invasive and distant metastatic activities. To clarify the mechanisms of increased motility and invasiveness of pancreatic carcinoma cells, in the context of epithelial to mesenchymal transition (EMT), the expression of Slug was evaluated in early and advanced stage lesions in a BOP-treated hamster model. Immunohistochemical analysis revealed Slug accumulation, which was associated with cytoplasmic/nuclear localization of a membrane-bound mucin MUC1, in invasive areas of carcinoma but not in early stage lesions and glandular areas of carcinoma. The results might suggest that the activation of MUC1 plays a role in EMT via Slug accumulation in pancreatic carcinoma cells.

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

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in 7, 12-dimethyl -benz(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. 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-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 and is a very useful tool for exploring immunomodulatory reagents for GVHD and understanding the action and mechanism of the reagents.

5) Human induced malignant stem cells and human induced hepatic stem cells for compound and/or target screening in anti-cancer drug discovery

Gene transfer of OCT3/4, SOX2, KLF4, and (c-Myc) was able to establish human induced malignant stem (iMS) cells and human hepatic stem (iHS) cells from normal or cancer tissues. The expandable iMS cells and iHS cells are similar to human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells in morphology. Human iHS cells markedly expressed hepatocyte-specific genes and human iMS cells have aberrations such as mutations in endogenous tumor suppressor genes and/or endogenous cancer-related genes. In addition, these cells express ES/iPS cell-specific genes at an equivalent level. Such iMS cells and iHS cells would be useful for compound and/or target screening in anti-cancer drug discovery.

6) Functional analysis of genetic polymorphisms associated with folate metabolism

Epidemiological and clinical studies have suggested an inverse association between folate intake and risk for cancer, in particular pancreatic, colon and esophagus cancer, whereas folate deficiency is associated with DNA strand breaks, impaired DNA repair, increased mutations and aberrant DNA methylation. Some genetic polymorphisms involved in folate metabolism such as methylenetetrahydrofolate reductase (*MTHFR C677T* and *A1298C*) and methionine synthase reductase (*MTRR G66A* and *C1862T*), may modulate the effect of dietary folate on DNA methylation and cancer susceptibility.

To elucidate the functional significance of the *MTHFR* or *MTRR* variants, we transfected the plasmid vector expressing the *MTHFR* or *MTRR* cDNA to human embryonic kidney 293 cells, which express an undetectable level of endogenous MTHFR or MTRR protein. We found that the exogenous MTHFR protein was produced at lower levels in the *MTHFR* transfectants harboring the *MTHFR 677TT* or *1298CC* cDNA, as compared with the *677CC* or *1298AA* transfectants harboring the wild-type. On the other hand, the exogenous MTRR protein showed no obvious differences between the *MTRR 66AA* or *1862TT* variants and wild-type. All *MTHFR* transfectants showed dramatically decreased homocysteine levels in culture mediums and a lower level of the total methyl-CpG content than those of Lac-Z control transfectants, but not in *MTRR* transfectants.

These preliminary results may facilitate our understanding of the functional genetic polymorphisms associated with folate metabolism that affect susceptibility to cancer through individual variation.

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