Looking at the North Building of Aichi Cancer Center Research Institute over the flower garden in bloom.
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From left to right
Ms. H. Tamaki, Dr. K. Tajima, and Mrs. J. Onishi.
Preface

First of all, let me introduce myself to you. I had been engaged in epidemiological studies at the Division of Epidemiology and Prevention during the last 30 years. I was promoted to Director of the Institute on April, 2006, as the former director, Dr. Toshitada Takahashi, M.D., D.M.Sc., became President of the Aichi Cancer Center. It is my pleasure to share with you the 20th Scientific Report (2006-2007) of the Aichi Cancer Center Research Institute. Since its establishment in 1965, Scientific Reports have been published biennially to document major research activities and highlight progress in and contributions to cancer research worldwide.

As illustrated on the following pages, the organization of the Research Institute was remodeled in 2000 to provide for 9 Divisions, consisting of three study groups: cancer prevention/epidemiology; preclinical/experimental therapy; and carcinogenesis/molecular biology. A total of 54 full-time staff members, 42 researchers and 12 research assistants, as well as 16 research residents, are now conducting a wide range of studies, together with 7 graduate school students affiliated with Nagoya University School of Medicine, Nagoya, and approximately 40 visiting research fellows. The major areas being pursued are as follows:

- descriptive and analytical epidemiology of cancers
- primary and secondary prevention of cancer
- molecular pathogenesis of gastrointestinal cancers
- molecular oncology of lung cancer
- molecular biology of translocation-junction genes of hemtopoietic tumors
- basic studies for cancer immunotherapy
- oncogenicity, molecular biology and immunology of DNA tumor viruses
- glycobiology of cancer cells in relation to metastasis
- molecular mechanisms of cell proliferation and movement
- involvement of repair mechanisms in carcinogenesis

More detailed descriptions of the research topics of each Division appear in the contents of the report. It is our sincere hope that the activities of the Institute will make a major contribution to elucidation of the mechanisms of carcinogenesis and to development of novel clinical applications in cancer diagnosis, treatment and prevention.

Finally, I would like to express my deep appreciation to the Aichi Prefectural Government for the continuous support received since this Institute was founded in 1964. Granting support from the Ministry of Education, Science, Sports, Culture and Technology, the Ministry of Health, Labor, and Welfare, the Ministry of Economy, Trade and Industry, Japan, and other related organizations, is also gratefully acknowledged.

February, 2008

Kazuo Tajima, M.D., M.P.H., D.M.Sc.
Director
Organization of the Aichi Cancer Center Research Institute

Research Institute

Director
K. Tajima
Vice Director
M. Tatematsu

Division of Epidemiology and Prevention
(K. Tajima, until Sept. 2007; H. Tanaka, as of Oct. 2007)

Division of Oncological Pathology
(M. Tatematsu)

Division of Molecular Oncology
(Y. Sekido)

Division of Molecular Medicine
(M. Seto)

Division of Immunology
(K. Kuzushima)

Division of Virology
(T. Tsurumi)

Division of Molecular Pathology
(R. Kannagi)

Division of Biochemistry
(M. Inagaki)

Central Laboratory & Radiation Biology
(K. Ishizaki)

Central Service Unit
(H. Nakamura)

Animal Facility (M. Tatematsu)

Laboratory of Translational Research

Aichi Cancer Center

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(until March, 2007)
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T. Mitsudomi

Administration Office

Chief Administrator
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(until March, 2006)
F. Ito
(as of April, 2006)
From left to right
First row: Dr. T. Kawase, Dr. K. Matsuo, Dr. H. Tanaka, Dr. A. Hiraki, Dr. T. Suzuki
Second row: Dr. J. Kanda, Dr. K. Kidokoro, Ms. S. Hiraiwa, Ms. M. Nakahama, Ms. Y. Yamauchi, Ms. T. Ito
Third row: Ms. S. Inui, Dr. S. Hosono, Ms. K. Mizutani, Ms. C. Taniguchi, Dr. T. Okasaka
Inset: Dr. K. Kuriki, Ms. M. Watanabe, Ms. S. Mano, Ms. K. Hasegawa, Ms. T. Nishiwaki, Ms. S. Irikura,
Ms. K. Suganuma,
Dr. M. Nishira, Dr. T. Paku, Ms. K. Fukaya, Ms. M. Ishida, Ms. C. Kanto, Ms. R. Saito
General Summary

The current research activities of the Division of Epidemiology and Prevention cover the following five subjects: 1) descriptive epidemiology of cancer incidence and mortality using data from the Aichi Prefectural Cancer Registry; 2) analytical epidemiology based on the hospital-based epidemiologic research program at Aichi Cancer Center (HERPACC) to determine risk and protective factors for cancer, with a particular focus on gene-environmental interactions; 3) clinical epidemiology for elucidating better cancer treatment and prevention; 4) a Korea, Japan and China (KOJACH) collaborative study focusing on those cancers which are on the increase for establishment of future prevention programs in all three countries; and 5) ethnoepidemiology of tumor viruses among Mongoloids in the Asian-Pacific area.

We are engaged in analyzing the data of the Aichi Cancer Registry to obtain estimated annual percent change of incidence rates in designated cancer sites in order to optimize cancer control programs in Aichi Prefecture. Several case-control studies using HERPACC data demonstrated that: 1) there are gene-gene interactions between polymorphisms in genes encoding alcohol-metabolizing enzymes and risk of head and neck, as well as colorectal cancers; 2) interactions exist between alcohol drinking habits and polymorphisms in the folate metabolism pathway regarding head and neck cancer; 3) there are significant associations between risk of prostate and thyroid cancer and a family history of neoplasia at the same sites; 4) intake of n-3 highly unsaturated fatty acids is associated with a lowered risk of breast cancer and gastric cancer. In collaborative studies with clinical departments, we have identified HLA allele mismatch combinations and amino acid substitution positions associated with graft-versus-leukemia (GVL) effects after unrelated hematopoietic stem cell transplantation, and different impacts of smoking on the risk of non-small cell lung cancer by types of epidermal growth factor receptor (EGFR).

In addition, international collaborative case-referent studies in Northeast Asian Countries (KOJACH Study) in Seoul, Nagoya, Nanjing, Chongqing, Benxi and Shantou are still ongoing to establish a basis for cancer prevention in the Asian Pacific region. Sero-epidemiologic investigations on HTLV-1/-2 infection among Mongoloids are also continuing in the North Pole region i.e. Greenland.
1. Epidemiological studies using data from Aichi Cancer Registry

Matsuo, K., Kawase, T., Ito, H., Tanaka, H. and Tajima, K.

Impact of habitual smoking on survival: Since 1999, information on smoking habits of cancer patients has been collected by Aichi Cancer Registry. The purpose was to examine the survival impact of habitual smoking using data registered to the Aichi Cancer Registry, Japan. The study subjects were registered cancer cases diagnosed between 1999 and 2004. The relationship between survival and smoking history (never or ever) was assessed using a Cox’s Proportional Hazard model considering age, sex, extent of disease and site.

A total of 72,789 cancer patients with smoking history and cancer stage at diagnosis were covered in the analysis. Overall, ever smokers were at higher risk of death compared with never smokers (Hazard ratio, HR: 1.28; 95% confidence interval, CI: 1.23-1.32) and this was consistently observed in both males and females. Stratified analysis also showed significant increased risk of death regardless of the disease extent. The HRs for ever smokers compared with never smokers were 1.22 (1.13-1.31), 1.29 (1.22-1.37), and 1.11 (1.05-1.17) for those whose extent of disease were local, regional and metastatic, respectively. In the cancer site specific analysis, sites in which ever smokers showed HRs greater than unity were oral, pharyngeal, esophageal, colon, gallbladder, lung, skin, uterus, breast and bladder. Lung, female breast and uterine cancers showed statistically significant increased risk of death. In contrast, only gastric cancer showed significantly lower risk of death among ever smokers compared with never smokers.

We thus found a significantly increased risk of death among ever smokers compared with never smokers, providing evidence that smoking continues to be harmful for people even after having been diagnosed with cancer.

![Figure 1](image.png)

**Fig 1. Estimated annual percent change in age-standardized incidence for designated subtype of cancer (Age 40-69).**
2. The Hospital-based epidemiologic research program at Aichi Cancer Center (HERPACC)


2.1. Gene-gene interactions between polymorphisms in alcohol-metabolizing enzyme genes regarding the risk of alcohol-related cancers

1) Head and neck cancer: Alcohol consumption is a strong risk factor for squamous cell carcinoma of the head and neck (SCCHN). The aldehyde dehydrogenase2 (ALDH2) Glu487Lys and alcohol dehydrogenase 2 (ADH2) His47Arg genetic polymorphisms, which have a strong impact on alcohol metabolism, are common in the Japanese population. To clarify the significance of these polymorphisms in SCCHN carcinogenesis, we conducted a matched case-control study with 239 incident SCCHN subjects and 716 non-cancer controls. Both ADH2 Arg/Arg and ALDH2 Glu/Lys were found to be independently associated with increased risk, with odds ratios (OR) of 2.67 (95% confidence interval [CI] 1.51-4.57) and 1.66 (1.20-2.31), respectively. Further, compared with subjects having both ADH2 His/His and ALDH2 Glu/Glu, the adjusted OR and its 95% CI for those with both ADH2 Arg/Arg and ALDH2 Glu/Lys was 5.00 (2.32-10.71) in all subjects. This combination effect was evident in heavy drinkers (11.3, 2.97-43.3) but not in moderate or non-drinkers. Statistically significant gene-environment interactions between the two polymorphisms and the drinking level were seen (ADH2, p = 0.035, ALDH2, p = 0.013). Furthermore, we also found a statistically significant gene-gene interaction between the two polymorphisms (p = 0.042). In conclusion, this case-control study showed a significantly increased risk of SCCHN in subjects with the ADH2 Arg/Arg and ALDH2 Glu/Lys polymorphisms in a Japanese population. In addition, our results demonstrated that this risk was associated with significant gene-gene interactions between ADH2 and ALDH2 polymorphisms, as well as gene-environment interactions between these polymorphisms and alcohol drinking.

2) Colorectal cancer: Alcohol consumption is recognized as a risk factor for colorectal cancer (CRC). Genetic polymorphisms, ALDH2 Glu487Lys and ADH2 His47Arg, which have a strong impact on alcohol metabolism, are common in the Japanese population but information on their significance for CRC carcinogenesis has been limited. We therefore conducted a matched case-control study with 257 incident CRC cases and 771 non-cancer controls at Aichi Cancer Center, including analysis of interactions among the polymorphisms, alcohol exposure and folate consumption. The ADH2 Arg allele was found to be associated with increased risk, the odds ratios (ORs) being 1.35 (95% confidence interval: 1.00-1.84) and 1.93 (1.06-3.53) for the His/Arg and Arg/Arg genotypes, respectively. In contrast, no apparent links were observed with the ALDH2 genotypes. Individuals having ALDH2 Glu/Glu with ADH2 Arg+, ALDH2 Lys+ with ADH2 His/His and ALDH2 Lys+ with ADH2 Arg+ showed ORs of 0.10 (0.04-0.21), 0.10 (0.06-0.19) and 1.36 (0.94-1.97), respectively, compared with ALDH2 Glu/Glu with ADH2 His/His. A statistically significant gene-gene interaction was found between the two polymorphisms for the risk of CRC (p< 0.001). The impact of ALDH2 Lys+ with ADH2 Arg+ was more evident in low folate consumers (2.32, 1.19-4.55) than those with high consumption (1.38, 0.80-2.38). In conclusion, while we failed to find any significant association with the ALDH2 polymorphism itself, significant interactions between ALDH2 and ADH2 polymorphisms were observed. Replication in future studies is warranted.

2.2. Impact of polymorphisms in alcohol-metabolizing enzyme genes on drinking behavior
1) ADH2 polymorphisms: Although functional effects of the ADH2 His(47) Arg polymorphism have been elucidated, the significance for habitual drinking has been unclear. Here, we conducted a cross-sectional study of 2,299 nonalcoholic Japanese subjects (989 men and 1,310 women). Drinking status, ethanol consumption, and physical reaction to one glass of beer were examined with regard to ADH2 and ALDH2 polymorphisms. Strength of associations was assessed with reference to age-, sex-, smoking status-, and genotype-adjusted odds ratios and their 95% confidence intervals. People with ADH2 His/Arg and Arg/Arg genotypes showed higher risk of being habitual drinkers. Among men, ALDH2 genotype- and confounder-adjusted ORs (95% CI) were 1.30 (0.89-1.89) and 3.16 (1.03-9.70), and the trend was significant (p = 0.024). A similar trend was observed among women. The combination of the two polymorphisms demonstrated a clear effect of the ADH2 Arg allele among those with ALDH2 Glu/Lys in both sexes (p(trend) = 0.007 for men and 0.024 for women). Physical reactions, such as flushing and palpitation, were significantly less common in those with Arg/Arg compared with other ADH2 genotypes, and this was more marked when combined with ALDH2 Glu/Lys. Heavy drinker status was also strongly associated with ADH2 Arg alleles. In conclusion, this study showed a significant impact of the ADH2 polymorphism on habitual drinking, regardless of the ALDH2 polymorphism.

2) Haplotypes of the ADH1B and ADH1C polymorphisms: Linkage disequilibrium (LD) between the ADH1B and ADH1C polymorphisms adds complexity to determination of their significance for drinking behavior and alcoholism. We have recently shown importance for the ADH1B polymorphism regarding habitual drinking in the Japanese population; however, the issue of LD between the ADH1B and ADH1C polymorphisms remained to be clarified. Here, we conducted a cross-sectional study in 2,299 nonalcoholic Japanese individuals. Drinking behavior was examined with regard to haplotypes of the ADH1B and ADH1C polymorphisms. Strength of association was assessed by sex and ALDH2 adjusted ORs and their 95% CIs for the haplotypes of the ADH1B and ADH1C polymorphisms.

The ORs for habitual drinking in males were significant for ADH1B*2(rapid)-ADH1C*2 (slow) (1.03; 95% CI: 1.01 -1.05), ADH1B*1 (slow) - ADH1C*1 (rapid) (1.15, 1.14-1.16), and ADH1B*1(slow)-ADH1C*2 (slow) (1.31, 1.29-1.32) compared with ADH1B*2(rapid)-ADH1C*1(rapid). Similarly, a significantly increased risk of heavy drinking was observed for each haplotype compared with ADH1B*2(rapid)-ADH1C*1(rapid). In conclusion, this study showed a significant impact of the ADH1C polymorphism on habitual drinking, regardless of the ADH1B/ALDH2 polymorphisms.

2.3. Folate and folate-related enzyme polymorphisms and cancers of the head and neck, lung and breast

A deficiency in folate is thought to increase the risk of cancer by impairing DNA repair synthesis and disrupting DNA methylation, which may lead to protooncogene activation. Polymorphisms in critical enzymes involved in the folate metabolism pathway, methylenetetrahydrofolate reductase (MTHFR C677T and A1298C), methionine synthase (MTR A2756G), methionine synthase reductase (MTRR A66G) and thymidylate synthase (TS) variable number of tandem repeat (VNTR), play important and interrelated roles in folate metabolism. We therefore evaluated associations between dietary folate intake and these polymorphisms as gene-environment interactions impacting on the risk of head and neck, lung and breast cancers.

1) Head and neck cancer

In total, 237 head and neck cancer cases and 711 age- and sex- matched non-cancer controls were included. Dietary folate intake was found to be inversely associated with head and neck cancer risk. The adjusted OR
for the top tertile intake of folate was 0.53 (95% CI, 0.32–0.89) compared with individuals in the lowest tertile. Interactions between drinking habits and MTHFR C667T (p=0.04), MTR A2756G (p=0.04) and MTRR A66G (p=0.03) polymorphisms for head and neck cancer risk were also observed.

2) Lung cancer
In total, 515 lung cancer cases and 1030 age- and sex-matched non-cancer controls were included. Folate intake was not associated with lung cancer risk, but MTHFR 677T and 1298C alleles was linked with reduced risk of squamous/small cell carcinoma (p=0.029), especially among heavy smokers (p=0.035). Furthermore, the MTHFR 677TT genotype was linked to decreased risk of squamous/small cell carcinoma development among heavy drinkers (0.17, 0.03–0.98).

3) Breast cancer
In total, 456 breast cancer cases and 912 age- and menopausal status-matched non-cancer controls were included. Breast cancer risk was inversely associated with consumption of dietary folate. The adjusted OR was 0.65 (95% CI, 0.46–0.91) for the top tertile of folate intake compared with the lowest tertile of intake (trend $P=0.010$). An increased risk of postmenopausal breast cancer with the MTHFR 677TT genotype was also observed (1.83, 1.08–3.11).

2.4. Family history and cancer risk
A family history of cancers has consistently been shown to increase the risk of neoplasia in several body sites. However, only a few studies of multiple cancer sites have been conducted. To assess familial cancer risk for common sites, we conducted a large-scale, case–control study. In total, 18,836 cancer cases (head and neck, esophagus, stomach, colorectum, liver, pancreas, lung, breast, uterus, ovary, prostate, bladder, thyroid, and lymphoma) and 28,125 age–matched and sex–matched controls, confirmed as being free of cancer, were recruited. ORs and 95% CIs for family history of cancer were determined by multiple logistic regression analysis, including stratification by family history for 14 cancer sites. The associations between family history and risk of cancer were generally stronger at the same sites than across cancer sites. Risks to first-degree relatives at the same sites were found to be significantly elevated with 8 of 14 cancer sites; especially high ORs being found for prostate (OR=5.6; 95% CI, 1.5–20.5) and thyroid cancers (13.3, 1.5–114.7). Some across-site associations were observed; esophagus–liver, stomach–bladder, colorectum–lymphoma, and pancreas–ovary. Furthermore, a significant reciprocal association between breast and prostate cancer was found.

2.5. Breast and stomach cancer risk and n-3 highly unsaturated fatty acids in erythrocytes
Breast cancer: Dietary intake of fish rich in n-3 highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been proposed to decrease cancer risk. In contrast to results from laboratory studies, however, protective effects against breast cancer have proved equivocal in epidemiological studies. In the present case-control study, we examined associations between breast cancer risk and fatty acid compositions of erythrocyte membranes as biomarkers for intake. Dietary information and blood samples were collected from 103 incident breast cancer cases

Fig.2 Breast cancer risk and erythrocyte compositions of fatty acids
and 309 non-cancer controls (matched by age and season) and erythrocyte fatty acids were measured using accelerated solvent extraction and gas-liquid chromatography. Dietary intake of n-3 HUFAs demonstrated a negative association with risk (the highest to the lowest tertile, odds ratio (OR)=0.51, 95% confidence interval (CI)=0.27–0.98; \( P_{\text{trend}} < 0.05 \)), but there was no association with those of saturated fatty acids (SFAs) and meat. Moreover, risk was inversely associated with erythrocyte compositions of EPA (0.27, 0.14–0.53; \( P_{\text{trend}} < 0.0001 \)), DHA (0.06, 0.02–0.16; \( P_{\text{trend}} < 0.0001 \)) and n-3 HUFAs (0.11, 0.05–0.24; \( P_{\text{trend}} < 0.0001 \)), and positively with that of SFAs (12.29, 4.94–30.57; \( P_{\text{trend}} < 0.0001 \)) and the ratio of SFAs/n-3 HUFAs (14.65, 5.67–37.82; \( P_{\text{trend}} < 0.0001 \)). In conclusion, we could show that erythrocyte compositions of specific fatty acids derived from fish intake, assayed as biomarkers, are associated with lower risk of breast cancer. Further studies are now needed to investigate mechanisms linked to the etiology.

**Gastric cancer:** Infection with *Helicobacter pylori* is linked to inflammation and is the main cause of peptic ulcer, gastritis, and gastric malignancies. DHA is related to anti-inflammatory and apoptosis-inducing effects. To examine associations between gastric cancer risk and the erythrocyte composition of DHA, we conducted a case-control study of 179 incident gastric cancer cases and 357 non-cancer controls. Gastric cancer risk did not seem to be directly associated with dietary intake of fish and n-3 HUFAs, including DHA. However, risk was inversely associated with erythrocyte compositions of n-3 HUFAs (0.39, 0.23–0.68; \( P_{\text{trend}} < 0.005 \)) and DHA (0.47, 0.28–0.79; \( P_{\text{trend}} < 0.01 \)). Particularly strong associations were noted for well-differentiated type lesions and n-3 HUFAs (0.10, 0.03–0.35; \( P_{\text{trend}} = 0.0005 \)) as well as DHA (0.20, 0.07–0.58; \( P_{\text{trend}} < 0.01 \)) values. In conclusion, the erythrocyte composition of DHA was found to be negatively linked to risk of gastric cancer, especially of well-differentiated adenocarcinoma. Further studies are needed to investigate mechanisms of action of DHA relevant to antitumor effects in the stomach.

*1 Department of Head and Neck Surgery, Aichi Cancer Center Hospital
*2 Department of Gastroenterological Surgery, Aichi Cancer Center Hospital
*3 Department of Clinical Laboratory, Aichi Cancer Center Hospital

### 3. Clinical applications of epidemiology

#### 3.1. Identification of HLA allele mismatch combinations and amino acid substitution positions associated with GVL effects after unrelated HSCT: Analysis from the Japan Marrow Donor Program


Graft-versus-leukemia (GVL) effects are considered to reduce relapse rates due to eradication of residual leukemia cells after allogeneic hematopoietic stem cell transplantation (HSCT). Segregation from graft-versus-host disease (GVHD) has been a major issue clinically. We recently clarified 16 high-risk HLA mismatch combinations and eight high-risk specific amino acid substitution positions for severe acute GVHD in six HLA loci. In the current study, we clarified...
HLA allele mismatch combinations and amino acid substitution positions associated with GVL effects. A total of 4,643 patients consecutive transplanted for hematological malignancy (ALL, AML, CML, MDS, MM and ML) with T cell replete marrow from serologically HLA-A, -B and -DR antigen-matched donors through the Japan Marrow Donor Program were registered in this cohort study. All HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 alleles were retrospectively typed. The effect of HLA locus mismatch in allele level, the HLA allele mismatch combinations in HLA six loci and amino acid substitution positions on reduced relapse rates was analyzed using a multivariable competing risk regression model. As results: (1) mismatches of HLA-C (p<0.0001) and HLA-DPB1 (p<0.0001) were strongly reduced leukemia relapse, and HLA-A (p=0.9), HLA-B (p=0.91), HLA-DRB1 (p=0.54) and HLA-DQB1 (p=0.54) were not; (2) a total of 10 HLA mismatch combinations were significantly associated with GVL effects, four in HLA-C alleles (donor Cw*0303-patientCw*1502 (n=25), Cw*0102-Cw*1402 (n=16), Cw*0801-Cw*0102 (n=10) and Cw*1402-Cw*0304 (n=23)), and six in HLA-DPB1 alleles (DP*0402-DP*0201 (n=66), DP*0501-DP*0201 (n=351), DP*0501-DP*0401 (n=53), DP*0501-DP*0402 (n=121), DP*0901-DP*0201 (n=50) and DP*1301-DP*0201 (n=21)), but none in HLA-A, -B, -DRB1 and -DQB1 alleles (with the exception of two combinations in HLA-C, all were different from high-risk mismatches for severe acute GVHD); (3) specific amino acid substitutions at positions 9, 99, 156 in HLA-C molecule was elucidated to be significant factors responsible for GVL effects, and one of three was different from substitutions responsible for severe acute GVHD. As for HLA-DPB1, no significant association between the positions of specific amino acid substitutions and GVL were found. In conclusion, our large scale comprehensive analysis made it possible to identify 4 HLA-C and 6 HLA-DPB1 mismatch combinations responsible for GVL effects, some of which differed from those responsible for acute GVHD. Responsible amino acid substitutions on specific position were also elucidated in HLA-C, but not in HLA-DPB1. These findings suggest that donor selection according to these results could segregate GVL from acute GVHD. In addition, we speculate that the molecular basis of GVL caused by HLA-DPB1 mismatch might differ from that in HLA-C.

*1Japanese Red Cross Tokyo Metropolitan Blood Center
*2Division of Molecular Science, Tokai University
*3HLA Laboratory
*4Department of Cell Transplantation & Regenerative Medicine, Tokai University
*5International Medical Center of Japan
*6Department of Hematology, Japanese Red Cross Nagoya First Hospital
*7Department of Hematology and Cell therapy, Aichi Cancer Center

3.2. Exploration of risk factors for non-small-cell lung cancer (NSCLC) with or without epidermal growth factor receptor (EGFR) mutations

Matsuo, K., Ito, H., Suzuki, T., Hiraki, A., Yatabe, Y. and Mitsudomi, T.

Recently, the epidermal growth factor (EGFR), which is a receptor tyrosine kinase (TK) activating several signaling pathways resulting in cell proliferation, escape from apoptosis, invasion or metastasis, all of which are associated with cancer phenotypes, has been identified. Elevated levels of EGFR are frequently seen in a variety of epithelial tumours, including NSCLCs. Activating mutations of EGFR have been reported in a subset of NSCLC usually highly sensitive to kinase inhibitors. This implies that NSCLCs can be classified into two distinct groups, namely EGFR-mutated (EGFRmut) and EGFR-wild-type (EGFRwt) as with the estrogen/progesterone receptor status in determining breast cancer treatment. Recent cross-sectional studies among NSCLC cases so far have revealed that the
frequency in non-smokers is significantly different between \( EGFR^{\text{mut}} \) and \( EGFR^{\text{wt}} \) NSCLCs.

The present study aimed to assess the impact of smoking and sex on the risk of non-small-cell lung cancer (NSCLC) with or without \( EGFR \) mutations. We conducted a case-control study using 152 patients with \( EGFR \)-mutated (\( EGFR^{\text{mut}} \)) NSCLCs, 283 with \( EGFR \)-wild-type (\( EGFR^{\text{wt}} \)) NSCLCs and 2,175 age- and sex-frequency-matched controls. Smoking was a significant risk factor for \( EGFR^{\text{wt}} \) NSCLCs [odds ratio (OR) for ever-smokers: 4.05; 95% confidence interval: 2.79-5.88] but not for \( EGFR^{\text{mut}} \) NSCLCs (OR=0.73, 0.46-1.14). Sex did not affect this association, which was consistently observed across other smoking-related parameters including pack-years. Sex was the sole risk factor for \( EGFR^{\text{mut}} \) NSCLCs (OR for women relative to men: 2.19, 1.41-3.39) and there was no significant interaction between being female and smoking. In contrast, sex, smoking and their interaction were significant in \( EGFR^{\text{wt}} \) NSCLCs. The impact of sex on \( EGFR \) mutation status was assessed with reference to several indicators of reproductive history among women. Total fertile years showed a significant positive association with \( EGFR^{\text{mut}} \) NSCLCs but not with \( EGFR^{\text{wt}} \) NSCLCs. Other indicators showed similar trends and this result may partly explain the sex difference in the acquisition of \( EGFR \) mutations. In conclusion, our case-control study clearly demonstrated that impacts of smoking and sex on the risk of \( EGFR^{\text{mut}} \) NSCLC are different from those for \( EGFR^{\text{wt}} \) NSCLCs. Our findings provide clear evidence that NSCLCs can be divided on the basis of their \( EGFR \) mutation status.

4. Comparative epidemiological study of cancers on the increase, focusing on Korea, Japan and China (KOJACH study)


To establish a basis for cancer prevention in the Asian Pacific region, we started a case-referent study on colorectal cancers in 2000, based on a standardized epidemiological approach, in Korea (Seoul), Japan (Nagoya) and China (Nanjing, Chongqing, Benxi and Shantou), the so called KOJACH Study (Fig.4). In Korea they developed their own SQFFQ and we established semi-quantitative food frequency questionnaires (SQFFQs) in the four cities in China. The validity and reproducibility of all SQFFQs established in the four cities in China were evaluated for.
further epidemiologic studies. We have collected lifestyle data by standardized SQFFQs and blood samples for plasma and DNA after obtaining informed consent from 400 colorectal cancer cases in Seoul, Nagoya, Nanjing and Chongqing, and in total more than 1,600 cases in the three countries by 2005. The same number of referents matched by age and sex were recruited from hospital patients in Korea and Japan and from the general population in China. A detailed analysis on risk and protective factors for colorectal cancer in each country was conducted and main results published in the several international journals. Furthermore, detailed analysis of combined risk impact of lifestyles and genetic polymorphisms are ongoing in the three countries. Furthermore, since 2005 we have focused on breast cancer which is also rapidly increasing in all three countries. We are collecting a total of 1,800 breast cancer cases with the same number of controls in Seoul, Nagoya and Nanjing by the same methods as conducted for colorectal cancer. We had collected more than 70% of cases and controls by the end of 2007 and should finish completely by the end of 2008 to allow analysis of risk impact for breast cancer in the three countries according to the same methods conducted for colorectal cancer.

*1 Department of Preventive Medicine, Nagoya University of Medical School, Nagoya, Japan
*2 Department of Planning and Information, Aichi Prefectural Institute of Public Health, Nagoya, Japan
*3 Division of Epidemiology, Cancer Institute of Jiangsu Province, Nanjing, China
*4 National Cancer Center, Korea
*5 Department of Preventive Medicine, College of Medicine, Seoul National University, Seoul, Korea
*6 Laboratory of Molecular Toxicology, Third Military Medical University, Chongqing, China

5. Ethnoepidemiologic studies on virus-related cancer

Tajima, K., Sonoda, S.*1, Chiba, H.*2, Senoo, H.*3

Human T-cell leukemia virus type 1 (HTLV-1), the main cause of adult T-cell leukemia/lymphoma, is found throughout the world but with microgeographical clusters of hyperendemicity. Epidemiologic studies among Mongoloids showed that HTLV-1 is highly endemic in South Japan (one million carriers) and in the Andes district of South America. In contrast, HTLV-II (also a risk factor for adult T-cell leukemia/lymphoma) is broadly distributed in all of South America, except the Andes line. After sero-epidemiologic studies on HTLV-I antibodies among Tibetan people in China, Sahme people in North Norway and Nenets people in Northwest Russia in 2001, 2003 and 2005, respectively, we confirmed the lack of HTLV-I/II clusters among Mongoloids in the Central Asia and North Scandinavian and Russian polar areas. In 2006 we tried to conduct field work in Northern Canada to study HTLV-I/II epidemic patterns among Inuit people, but finally our study plan was not accepted by the Canadian government. Now we are planning a new challenge to clarify the HTLV-I/II distribution among Inuit people in Greenland who have migrated to the most Northeastern polar areas in the world and finally we hope to complete our worldwide sero-epidemiologic survey of HTLV-I/II among Mongoloid people in the world.

*1 Southern Region Hospital, Kagoshima, Japan
*2 Department of Laboratory Medicine, Hokkaido University School of Medicine, Sapporo, Japan
*3 Department of Anatomy, Akita University School of Medicine, Akita, Japan
From left to right
First row: Dr. T. Toyoda, Dr. T. Tsukamoto, Dr. M. Tatematsu, Dr. H. Nakanishi, and Dr. R. Fukuyama
Second row: Mr. S. Takasu, Ms. K. Inoue, Dr. M. Yamamoto, Ms. H. Miyamoto, Ms. I. Hanaoka, and Dr. X. Zhang
Third row: Dr. H. Suzuki, Dr. S. Kondo, Dr. M. Matsui, and Dr. D. Takagi
Inset: Dr. M. Ota, Ms. T. Hagiwara, Dr. Y. Ito, Mr. H. Tanaka, Ms. M. Yoshimura, and Dr. A. Hirata
General Summary

The responsibility of the Division of Oncological Pathology includes autopsy and research activities. From the establishment of this laboratory in 1965 to the end of 2007, the number of autopsy cases amounted to 2605. Postmortem examinations are a source of valuable information on the behavior of neoplasms and their response to therapy. Autopsy findings also supply the basis for total evaluation of the course of disease, including the accuracy of clinical diagnosis, effectiveness or failure of drugs, irradiation and surgery, and the appearance of complications such as opportunistic infection and hemorrhage during treatment. The main research theme of the laboratory is carcinogenesis and progression of gastrointestinal malignancies. During 2006-2007, the research activities were divided into the following three main areas. The first deals with links between clinicopathological findings and phenotypes using several gastric and intestinal phenotypic markers. Reduction of MUC2 expression may be associated with occurrence and progression of colorectal carcinomas arising though both the adenoma-carcinoma sequence pathway and de novo carcinogenesis. The second area concerns gastric cancers and their prevention. Nordihydroguaiaretic acid (NDGA) and 3, 4-vinyl-2,6-dimethoxyphenol (canolol) suppress gastric carcinogenesis in Helicobacter pylori (H. pylori) in-
fected Mongolian gerbils (Mgs) while a high-salt diet dose-dependently enhance *H. pylori*-associated gastritis and stomach carcinogenesis. A high-salt diet works synergistically with *H. pylori* infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mgs, and our results support the hypothesis that excessive salt intake may be associated with progression of *H. pylori*-induced gastritis. The third research area involves basic research on tumor progression and metastasis, especially micrometastasis and related clinical applications. Selection of the patients with high-risk for peritoneal relapse detected by genetic diagnosis and subsequent intraperitoneal chemotherapy targeting peritoneal micrometastasis is now under clinical trial as a potential new therapeutic strategy for gastric cancer patients.

1. **Loss of MUC2 expression correlates with progression along the adenoma-carcinoma sequence pathway as well as de novo carcinogenesis in the colon**

Mizoshita, T., Tsukamoto, T., Inada, K., Hirano, N., Tajika, M.*, Nakamura, T.*, Ban, H. and Tatematsu, M.

We have previously demonstrated links between clinicopathological findings and phenotypes using several gastric and intestinal phenotypic markers in stomach and pancreatic cancers. However, the clinicopathological significance of the phenotype and Cdx2 expression has hitherto remained unclear in colorectal carcinogenesis. We examined the correlation between gastric and intestinal phenotypic expression in 91 primary early carcinomas of the colon. MUC2 expression demonstrated a significant decrease from tubular/tubulovillous adenomas with moderate atypia, through intramucosal carcinomas, to cancers with submucosal invasion (*P* < 0.0001). Intramucosal de novo carcinomas (flat type carcinomas without adenomatous components) exhibited a greater decrease of MUC2 than intramucosal lesions with adenomatous components. Expression of MUC5AC also decreased significantly with progression according to the tubular/tubulovillous adenoma-carcinoma sequence, carcinomas with villous adenomatous components having a higher level compared with their tubular adenomatous counterparts, suggesting differences in the pathway of malignant transformation. Cdx2 nuclear expression was maintained in all of the adenomas and early carcinomas examined. Our data suggest that reduction of MUC2 expression may be associated with the occurrence and progression of colorectal carcinomas in both the adenoma-carcinoma sequence pathway and de novo carcinogenesis. Tumor-suppressive effects of Cdx2 may be preserved during early stages of colorectal carcinogenesis.

*1 Department of Gastroenterology, Aichi Cancer Center Hospital, Chikusa-ku, Nagoya 464-8681, Japan

2. **Inhibitory effects of nordihydroguaiaretic acid, a plant lignan, on Helicobacter pylori-associated gastric carcinogenesis in Mongolian gerbils**


Recent epidemiological studies have demonstrated that consumption of certain natural products can lower cancer risk in humans. For example, plant-derived lignans have been shown to exert chemopreventive effects against cancer in vitro and in vivo. In the present study, the effects of three such lignans, termed arctiin, arctigenin, and nordihydroguaiaretic acid (NDGA), on the proliferation of *Helicobacter pylori* and *H. pylori*-associated gastric cancer development were investigated in Mongolian gerbils. To examine the effects of arctigenin and NDGA on stomach carcinogenesis, specific pathogen-free male, 5-week-old gerbils were infected with *H. pylori*, administered 10 p.p.m. *N*-methyl-*N*-nitrosourea in their drinking water and fed diets containing various concentrations of lignans until they were killed after 52 weeks. At a dietary level of 0.25%, NDGA significantly decreased the incidence of gastric adenocarcinomas. Arctigenin, in contrast, failed to attenuate neoplasia at a level of 0.1%. Both NDGA and arctigenin significantly reduced serum 8-hydroxy-2'-deoxyguanosine levels at doses of 0.25 and 0.05% (NDGA), and 0.1% (arctigenin). Administration of 0.25% NDGA significantly suppressed the formation of intestinal metaplasia both in the antrum and the corpus. Although all three lignans dose-dependently inhibited the *in vitro* proliferation of *H. pylori*, there were no differences in the titers of anti-*H. pylori* antibodies or the amount of the *H. pylori*-specific urease A gene among all *H. pylori*-infected groups. These results suggest that NDGA might be effective for prevention of gastric carcinogenesis. The possible mechanisms appear to be related to inhibitory ef-
fects on progression of gastritis and antioxidative activity rather than direct antimicrobial influence.

Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0212, Japan

Central Research Laboratories, Yomeishu Seizo Co., Ltd, Kamiina-Minowa, Nagano 399-4601, Japan

Central Clinical Laboratories, Shinshu University Hospital, Matsumoto, Nagano 390-8621, Japan

Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan

Department of Pathology, Wakayama Medical University, Kimiidera, Wakayama 641-0012, Japan

3. 4-Vinyl-2,6-dimethoxyphenol (canolol) suppresses oxidative stress and gastric carcinogenesis in Helicobacter pylori-infected carcinogen-treated Mongolian gerbils

Tsukamoto, T., Cao, X., Seki, T. \textsuperscript{1}, Tanaka, H., Morimura, S. \textsuperscript{2}, Cao, L., Mizoshita, T., Ban, H., Toyoda, T., Maeda, H. \textsuperscript{2} and Tatematsu, M.

Oxidative stress is linked to gastric carcinogenesis because of its ability to damage DNA. Here we examined antioxidative and anti-inflammatory effects of 4-vinyl-2,6-dimethoxyphenol (canolol), a recently identified potent antioxidative compound obtained from crude canola oil, on Helicobacter (H.) pylori-induced gastritis and gastric carcinogenesis using a Mongolian gerbil model. The animals were allocated to H. pylori-infection alone (12 weeks) or H. pylori + N-methyl-N-nitrosourea (MNU) administration (52 weeks). After oral inoculation of H. pylori, they were fed for 10 and 44 weeks with or without 0.1% canolol. H. pylori-induced gastritis, 5′-bromo-2′-deoxyuridine (BrdU) labeling and scores for cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) immunohistochemistry were attenuated in the canolol-treated groups. Expression of interleukin-1beta (IL-1beta), tumor necrosis factor-alpha (TNF-alpha), COX-2 and iNOS mRNA in the gastric mucosa were significantly increased in H. pylori-infected groups (P < 0.01 and P < 0.05, respectively), while no significant effects were noted in non-infected animals. There was significant synergistic interaction between H. pylori infection and 10% NaCl diet on the expression of iNOS (P < 0.05) and also a tendency for enhanced COX-2 expression (P = 0.0599). The present results suggest that a high-salt diet works synergistically with H. pylori infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils, and support the hypothesis that excessive salt intake.

4. Synergistic upregulation of inducible nitric oxide synthase and cyclooxygenase-2 in gastric mucosa of Mongolian gerbils by a high-salt diet and Helicobacter pylori infection

Toyoda, T., Tsukamoto, T., Hirano, N., Mizoshita, T., Kato, S., Takasu, S., Ban, H. and Tatematsu, M.

Intake of salt and salty food is known as a risk factor for gastric cancer. We have previously demonstrated that a high-salt diet dose-dependently enhances Helicobacter pylori (H. pylori)-associated gastritis and stomach carcinogenesis in Mongolian gerbils. In this study, we focused on the influence of excessive salt intake on the expression of inflammatory mediators involved in progression of H. pylori-induced chronic gastritis.

A total of 45 stomach samples from Mongolian gerbils were evaluated by immunohistochemistry. The animals were infected with H. pylori and fed basal (0.32%) or a high-salt (10%) diet, and sacrificed after 40 weeks. Proliferative activity and expression of cyclooxygenase-2 (COX-2) in gastric mucosa were significantly increased in H. pylori-infected gerbils. The additional high-salt diet significantly up-regulated the expression of inducible nitric oxide synthase (iNOS) and COX-2 in H. pylori-infected groups (P < 0.01 and P < 0.05, respectively), while no significant effects were noted in non-infected animals. There was significant synergistic interaction between H. pylori infection and 10% NaCl diet on the expression of iNOS (P < 0.05) and also a tendency for enhanced COX-2 expression (P = 0.0599). The present results suggest that a high-salt diet works synergistically with H. pylori infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils, and support the hypothesis that excessive salt intake.
may be associated with progression of *H. pylori*-induced gastritis.

5. Role of IKK and oscillatory NFkappaB kinetics in MMP-9 gene expression and chemoresistance to 5-fluorouracil in RKO colorectal cancer cells

Fukuyama, R.*1, Ng, K.P.*1, Cicek, M.*1, Kelleher, C.*1, Niculaite, R.*1, Casey, G.*1, Sizemore, N.*1

Nuclear factor kappa B (NFkappaB) is a central participant in the metastasis and chemoresistance of colorectal cancer (CRC). However, it is not fully understood to what extent NFkappaB contributes to induction of the metastasis-associated matrix metalloprotease-9 (MMP-9) gene and sensitivity to the commonly used chemotherapeutic 5-fluorouracil (5-Fu) in CRC. Using the RKO human CRC cell line and two NFkappaB signaling deficient RKO mutants, we investigated NFkappaB’s role in the induction of MMP-9 and 5-Fu sensitivity in RKO CRC cells. Tumor necrosis factor alpha (TNFalpha) failed to induce MMP-9 in either of the NFkappaB signaling mutants. RKO cells exhibited a robust, oscillatory NFkappaB activity in response to TNFalpha not seen in either of the NFkappaB mutant cell lines, which instead demonstrated diminished, nonoscillatory NFkappaB activation. Analysis of TNFalpha-induced phosphorylation and MMP-9 promoter recruitment of the p65 NFkappaB subunit revealed a significant reduction in p65 phosphorylation as well as reduced and altered recruitment of p65 to the MMP-9 gene promoter in either of the NFkappaB signaling mutants. RKO cells exhibited a robust, oscillatory NFkappaB activity in response to TNFalpha not seen in either of the NFkappaB mutant cell lines, which instead demonstrated diminished, nonoscillatory NFkappaB activation. Analysis of TNFalpha-induced phosphorylation and MMP-9 promoter recruitment of the p65 NFkappaB subunit revealed a significant reduction in p65 phosphorylation as well as reduced and altered recruitment of p65 to the MMP-9 gene promoter in the mutants compared to the parental RKO cell line. 5-Fu only activated NFkappaB in the parental RKO cells through induction of IkappaB-kinase (IKK) activity and increased sensitivity to 5-Fu was observed in both NFkappaB mutant lines. Our results suggest that TNFalpha-dependent induction of MMP-9 gene expression is tightly regulated by oscillatory/cumulative activation of NFkappaB and that 5-Fu stimulates NFkappaB and RKO CRC cell survival through induction of IKK activity.

6. A new diagnostic and therapeutic strategy targeting micrometastasis for prevention of peritoneal recurrence in gastric cancer patients

Nakanishi, H., Ito, S., *1 Kodera, Y., *2 and Tatematsu, M.

Peritoneal metastasis is a major cause of gastric cancer death. Development of new diagnostic and therapeutic methods for prevention of peritoneal recurrence is therefore an extremely urgent issue to be addressed in gastric cancer research. Genetic diagnosis to detect micrometastasis in the peritoneal cavity with CEA qRT-PCR developed in our laboratory in 1999 is now widely recognized to be a highly sensitive method for free cancer cells and clinical utility for prediction of peritoneal relapse after curative surgery was validated in a prospective study. A phase II clinical trial of adjuvant chemotherapy for qRT-PCR positive patients showed reduction of peritoneal recurrence after curative surgery with marginal significance. New genetic diagnostics such as a custom DNA microarray with an algorithm (SVM) using 59 genes for peritoneal washes has been developed for prediction of peritoneal metastasis. Furthermore, 6 new genetic markers including a stem cell marker, CD133, were also found by genome-wide microarray analysis and the resultant multiple qRT-PCR method exhibited even more diagnostic accuracy. Preclinical study using a GFP-tagged peritoneal micrometastasis model showed that micrometastases in the peritoneal cavity are highly chemo-sensitive, especially to intraperitoneal chemotherapy (IP) with paclitaxel. A phase I clinical trial of IP for far-advanced gastric cancer patients was initiated. High intraperitoneal paclitaxel concentrations were maintained following IP administration and ascites diminished during the course of the treatment. These results suggest that intraperitoneal chemotherapy tailored to patients at high-risk for peritoneal relapse would be a new potential therapeutic modality for advanced gastric cancer patients.

*1 Department of Gastroenterological Surgery, Aichi Cancer Center Central Hospital, Nagoya, Japan
*2 Department of Surgery II, Nagoya University School of Medicine, Nagoya, Japan
From left to right
First row: Ms. Ikuko Tomimatsu, Dr. Keiko Shinjo, Ms. Mari Kizuki, and Ms. Eri Nishikawa
Second row: Dr. Yutaro Suzuki, Dr. Taijiro Ozawa, Dr. Yutaka Kondo, Dr. Byonggu An, Dr. Koji Kawaguchi, Dr. Yoshitaka Sekido, Dr. Hideki Murakami, Dr. Hirotaka Osada, Dr. Makiko Fujii, Mr. Yoshio Tatematsu and Dr. Motokazu Ito.
Division of Molecular Oncology

Yoshitaka Sekido M.D., Ph.D., Chief
Hirotaka Osada, M.D., Ph.D., Section Head
Yutaka Kondo, M.D., Ph.D., Section Head (as of April 2007)
Hideki Murkami, M.D., Ph.D., Section Head (as of April 2006)
Makiko Fuji, D.D.S., Ph.D., Senior Researcher (as of July 2007)
Yoshio Tatematsu, B.S., Research Assistant
Ikuko Tomimatsu, Semi-regular Employee (as of April 2006)
Mari Kizuki, Semi-regular Employee (as of June 2007)

Research Resident
Hideo Sakamoto, M.D., Nagoya City University School of Medicine (until March 2007)
Byonggu An, M.D., Shiga University of Medical Science (as of April 2007)

Visiting Trainees
Wentao Gao, M.D., Research fellow of the 29th Japan-China Sasagawa (until March 2007)
Toshikiko Yokoyama, M.D., Nagoya University School of Medicine (until March 2007)
Naohiro Sato, M.D., Nagoya University School of Medicine (until March 2006)
Tetsuo Taniguchi, M.D., Nagoya University School of Medicine (until September 2007)
Yasuhiro Goto, M.D., Nagoya University School of Medicine (until September 2007)
Koji Kawaguchi, M.D., Nagoya University School of Medicine (as of April 2006)
Yutaro Suzuki, M.D., Nagoya University School of Medicine (as of July 2006)
Motokazu Ita, M.D., Nagoya University School of Medicine (as of October 2006)
Keiko Shinjo, M.D., Nagoya University School of Medicine (as of August 2007)
Taijiro Ozawa, M.D., Nagoya City University School of Medicine (as of August 2007)
Eri Nishikawa, Nagoya University School of Medicine (as of August 2007)

General Summary
Our goal is to determine genetic lesions giving rise to human solid cancers and use this information for prevention, diagnosis, and treatment of disease. Currently, we are focusing on lung cancer, malignant mesothelioma, colon cancer, hepatoma, and head and neck cancer. Our studies also provide opportunities to dissect biochemical and pathological pathways of malignant phenotypes including dysregulated cell growth, differentiation, invasion and metastasis. Human cancers arise because of genetic mutations in oncogenes and tumor suppressor genes and our approach is to study candidate genes, with a systematic molecular analysis of biochemical pathways and global profiling of gene expression by microarrays, along with comparative genomic hybridization to detect chromosomal abnormalities. Epigenetic changes with DNA methylation and histone modification may also be identified as important mechanisms of inactivation of tumor suppressor genes. We also functionally analyze candidate genes by transfecting wild type copies into human cancer cells and testing for their ability to suppress malignancy in vitro and in vivo as well as characterizing their protein products biochemically. Alternatively, we inactivate expression using RNA interference (RNAi) in either tumor or normal cells and then study the resulting phenotype. Understanding the functions of mutated genes and disrupted signaling pathways should provide a foundation for translational research for human malignancies, from bench to bedside.

1. Genomic profiling of malignant pleural mesotheliomas with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including JUN amplification on 1p32
Taniguchi, T., Karnan, S.*1, Fukui, T.*2, Yokoyama, T., Tagawa, H.*1, Yokoi, K.*3, Ueda, Y.*3, Mitsudomi,
Genome-wide array-based comparative genomic hybridization analysis of malignant pleural mesotheliomas (MPMs) was carried out to identify regions that display DNA copy number alterations. Seventeen primary tumors and nine cell lines derived from 22 individuals were studied, some of them originating from the same patients. Regions of genomic aberrations observed in >20% of individuals were 1q, 5p, 7p, 8q24 and 20p with gains, and 1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 9p21.3, 10p, 13q33.2, 14q32.13, 18q and 22q with losses. Two regions at 1p32.1 and 11q22 showed a high copy gain. The 1p32.1 region contains a protooncogene, JUN, and we could further demonstrate overexpression of JUN with real-time polymerase chain reaction analysis. As MPM cell lines did not overexpress JUN, our findings suggested that induction of JUN expression might be involved in the development of MPMs in vivo, with gene amplification in a subset of tumors. However, the most frequent alteration was 9p21.3 deletion, which includes the p16(INK4a)/p14(ARF) locus. With polymerase chain reaction analysis, we determined the extent of the homozygous deletion regions of the p16(INK4a)/p14(ARF) locus in MPM cell lines and found the deletion regions to vary with the line. Our results with array comparative genomic hybridization analysis have provided new insights into the genetic background of MPM, and hopefully will give clues to development of new molecular target therapies for this neoplasm.

3. Variable DNA Methylation Patterns Associated with Progression of Disease in Hepatocellular Carcinomas

Hepatocellular carcinoma (HCC) most commonly arises with a background of chronic inflammation due to viral infection, and features both genetic and epigenetic abnormalities. However, a global picture of epigenetic changes in HCC has hitherto been lacking. To address this question, we conducted genome-wide screening for aberrantly methylated CpG islands in cancerous tissue and corresponding adjacent non-cancerous tissue (chronic hepatitis, CH, or liver cirrhosis, LC) from

2. CLCP1 interacts with semaphorin 4B and regulates motility of lung cancer cells

Osada, H., Nagai, H.1, Sugito, N.1, Matsubara H1, Tatematsu Y, Hida T2, Sekido Y, Nagino M2, Nimura Y2, Takahashi T.a

We previously established a highly metastatic subline, LNM35, from the NCI-H460 lung cancer line, and demonstrated up-regulation of a novel gene, CLCP1 (CUB, LCCL-homology, coagulation factor V/VIII homology domains protein), also called ESDN/DCBL2, in LNM35 and lung cancer specimens. We have subsequently focused on the potential roles of the gene in cancer metastasis. First, we established stable LNM35 RNAi clones, in which CLCP1 expression was suppressed by RNAi, and found that their motility was significantly reduced, though growth rates were not changed. Next, in vitro selection of a phage display library demonstrated that a phage clone displaying a peptide similar to a sequence within the Sem domain of semaphorin 4B (SEMA4B) interacted with LNM35. Immunoprecipitation experiments confirmed interaction of CLCP1 with SEMA4B. Finally, SEMA4B down-regulated the expression level of CLCP1 protein through induction of ubiquitination and proteasome degradation. These results are the first to indicate that CLCP1 plays a role in cell motility, while also showing that at least one of the ligands is SEMA4B and that their interaction mediates proteasome degradation by CLCP1. Although the physiological role of the interaction between CLCP1 and SEMA4B remains to be investigated, this novel gene may become a target of therapy to inhibit metastasis of lung cancers.

1Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine
2Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine
3Department of Pulmonary Medicine, Aichi Cancer Center Hospital

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HCC patients using the methylated CpG island amplification-microarray (MCAM) technique with 6,458 CpG islands. MCAM data were confirmed by bisulfite pyrosequencing for 56 genes. MCAM identified 719 prominent targets of hypermethylation in HCCs, which represent 11% of all CpG islands. HCCs arising from LC had very much more methylation than those arising from CH (1,249 genes or 19% vs. 444 genes or 7%). There were four patterns of aberrant methylation: Type I (4%, e.g. MMP14) represents methylation that develops substantially at the transition between normal tissue and adjacent tissue and does not increase further in cancer. Type II (55%, e.g. RASSF1A) shows progressively increasing methylation from adjacent tissue to HCC. Type III (4%, e.g. GNA14) shows decreased methylation in adjacent tissue but either similar or increased methylation in HCC. Type IV (37%, e.g. CDKN2A) shows low levels of methylation in normal tissue and adjacent tissue but high levels in HCC. Methylation in the type IV genes is characteristic of moderately/poorly differentiated cancer. Our extensive analyses revealed that CpG island promoters become methylated in different patterns during progression of the disease, suggesting different mechanisms for acquisition of epigenetic changes. These data support the notion that molecular pathways linked to aberrant DNA methylation dictate the clinical features of HCCs in multiple steps of tumorigenesis. In addition we provide hundreds of markers that could be of diagnostic utility in HCCs and pre-neoplastic lesions.

1Department of Gastroenterological Surgery, Aichi Cancer Center Hospital
2Department of Gastroenterology, Aichi Cancer Center Hospital
First row (from left to right): Dr. T. Miyata, Dr. M. Seto, Dr. S. Tsuzuki, Ms. Y. Komai.
Second row (from left to right): Dr. K. Karube, Ms. Sato, Dr. K. Honma, Mr. S. Karnan, Dr. M. Nakagawa.
Research in this laboratory is aimed at generating a better understanding of the genetic and molecular bases of human cancer, with eventual application of the acquired knowledge in the field of medical oncology. Our work has been mainly focused on hematologic malignancies, in cooperation with physicians of the Department of Hematology and Cell Therapy (Chief, Dr. Yasuo Morishima) and the Department of Endoscopy (Chief, Dr. Tsuneya Nakamura), Aichi Cancer Center Hospital. Research on hematologic malignancies offers several advantages for exploration of the molecular bases of neoplasia. Chromosomal abnormalities have been analyzed by a large number of researchers and the observed strong association between specific chromosome changes and specific hematopoietic tumors provides direct evidence that the resultant gene alterations play a pivotal role in disease development or clinicopathological manifestation. During the last two years, we have studied the issues detailed below.

The function of API2-MALT1 associated with mucosa-associated lymphoid tissue lymphomas is now being studied to identify target genes. An array-CGH (comparative genomic hybridization) that contains 2300 BAC clones on a slide glass and can scan the entire genome in 1.4 MB segments on average was applied for various hematopoietic malignancies. TEL-AML1 leukemia frequently found in childhood was thereby found to have a characteristic genome profile and oncogenic function of the TEL-AML1 chimeric gene was analyzed in a bone marrow transplantation system. The significance of the AML1 isoform was also analyzed. Mucosa-associated lymphoid tissue (MALT) lymphoma is unique in its clinicopathologic features. A single institution analysis on reactivity to anti-\textit{H. pylori} therapy was conducted and it was found that a half of non-reactive group (15% of total cases) had \textit{API2-MALT} chimeric gene. The remaining half of the cases with gastric MALT lymphomas were found to have copy number changes. Specific deletion at 6q23.3 for ocular adnexal MALT lymphoma was analyzed in detail with contig array CGH and it was found that the target gene was TNFAIP3, indicating that NF-kB plays a pivotal role in development of the MALT lymphoma. Peripheral T-cell lymphomas, unspecified (PTCL-U), were also analyzed by array CGH and examples with genomic alterations showed similarity to lymphoma type adult T-cell leukemias/lymphomas (ATLLs).
1. Genetic abnormalities involved in t(12;21) TEL-AML1 acute lymphoblastic leukemia: analysis by means of array-based comparative genomic hybridization

Tsuzuki, S., Karnan S. and Seto, M.

The TEL (ETV6)-AML1 (RUNX1) chimeric gene fusion is the most common genetic abnormality in childhood acute lymphoblastic leukemias. Evidence suggests that this chimeric gene fusion constitutes an initiating mutation that is necessary but insufficient for the development of leukemia. In a search for additional genetic events that could play a role, we applied a genome-wide array-comparative genomic hybridization technique to 24 TEL-AML1 leukemia samples and two cell lines. It was found that at least two chromosomal imbalances were involved in all samples. Recurrent regions of chromosomal imbalance (>10% of cases) and representative involved genes were gain of chromosomes 10 (17%) and 21q (25%; RUNX1) and loss of 12p13.2 (87%; TEL), 9p21.3 (29%; p16INK4a/ARF), 9p13.2 (25%; PAX5), 12q21.3 (25%; BTG1), 3p21 (21%; LIMD1), 6q21 (17%; AIM1 and BLIMP1), 4q31.23 (17%; NR3C2), 11q22-q23 (13%; ATM) and 19q13.11-q13.12 (13%; PDCD5). Enforced expression of TEL and to a lesser extent BTG1, both single genes known to be located in their respective minimum common region of loss, inhibited proliferation of the TEL-AML1 cell line Reh. Together, these findings suggest that some of the genes identified as lost by array-comparative genomic hybridization may partly account for the development of leukemia.

2. Isoform-specific potentiation of stem and progenitor cell engraftment by AML1/RUNX1

Tsuzuki, S., Matsuo K. and Seto, M.

BACKGROUND: AML1/RUNX1, the most frequently mutated gene in leukemia, is central to the normal biology of hematopoietic stem and progenitor cells. However, the role of different AML1 isoforms within these primitive compartments is unclear. We therefore investigated whether altering relative expression of AML1 isoforms might impact on the balance between cell self-renewal and differentiation in vitro and in vivo.

METHODS AND FINDINGS: The human AML1a isoform encodes a truncated molecule with DNA-binding but no transactivation capacity. We used a retrovirus-based approach to transduce AML1a into primitive hematopoietic cells isolated from a mouse and observed that enforced AML1a expression increased the competitive engraftment potential of murine long-term reconstituting stem cells. Thus the proportion of AML1a-expressing cells became elevated over time in both primary and secondary recipients. Furthermore, AML1a expression dramatically increased primitive and committed progenitor activity in engrafted animals as assessed by long-term culture, cobblestone formation, and colony assays. In contrast, expression of the full-length isoform AML1b abrogated engraftment potential. In vitro, AML1b promoted differentiation while AML1a enhanced proliferation of progenitors capable of short-term lymphomyeloid engraftment. Consistent with these findings, the relative abundance of AML1a was highest in the primitive stem/progenitor compartment of human cord blood, and forced expression of AML1a in these cells elevated maintenance of primitive potential both in vitro and in vivo. CONCLUSIONS: Our data demonstrate that the "a" isoform of AML1 has the capacity to potentiate stem and progenitor cell engraftment, required for successful clinical transplantation. This activity is consistent with its expression pattern in both normal and leukemic cells. Manipulating the balance of AML1 isoform expression may offer novel therapeutic strategies, exploitable in the contexts of leukaemia and also cord blood transplantation in adults, in whom stem and progenitor cell numbers are often limiting.

*1 Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Japan

3. Chromosomal imbalances are associated with outcome of Helicobacter pylori eradication in t(11;18)(q21;q21) negative gastric MALT lymphomas

Fukuhara, N. *1,2, Nakamura, T. *3, Nakagawa, M., Tagawa, H., Takeuchi, I. *4, Yatabe, Y. *5, Morishima, Y. *6, Nakamura, S. *7 and Seto, M.

Approximately 70% of gastric mucosa-associated lymphoid tissue (MALT) lymphomas can be successfully treated with H. pylori eradication. The translocation t(11;18)(q21;q21) characteristic of MALT lymphomas is recognized as a marker for H. pylori independence, but this marker is found in only half of the cases resistant to eradication of the bacterium. To address this anomaly, we performed array-based comparative ge-
nomic hybridization (array-CGH) for 29 gastric MALT lymphomas treated with H. pylori eradication. These comprised ten cases of t(11;18) positive MALT, nine cases of t(11;18) negative MALT with H. pylori dependence and ten cases of t(11;18) negative MALT with H. pylori independence. Array-CGH analysis demonstrated no significant genetic alterations in t(11;18) positive MALT lymphomas, but numerous genomic alterations were detected in t(11;18) negative cases. This indicated that genomic imbalance is associated with H. pylori independence in t(11;18) negative gastric MALT lymphomas. Gene alterations may thus play an important role in the development of H. pylori independence.

*1 Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan
*2 Department of Rheumatology and Hematology, Tohoku University Graduate School of Medicine, Sendai, Japan
*3 Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Japan
*4 Division of Computer Science, Mie University Graduate School of Engineering, Tsu, Japan
*5 Department of Pathology and Molecular Diagnostics, *6 Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Japan
*7 Pathology/Clinical Laboratories, Nagoya University Hospital, Nagoya; All the above institutions are located in Japan.

4. Identification of the target gene at chromosome deletion 6q23.3-24.1 in ocular adnexal mucosa associated lymphoid tissue lymphomas

Honma, K., Kim, W-S. *1, Ko, Y-H.*2 and Seto, M.

Our recent study showed that ocular adnexal MALT lymphomas feature recurrent genomic deletion including homozygous loss in the 2.9 Mb region at chromosome band 6q23.3-24.1. As deletion 6q23.3-24.1 is the most common genomic alteration, we assumed that this is a crucial genetic alteration for lymphomagenesis of this specific type of MALT lymphoma. In an attempt to identify the target gene in this region, we applied contig array CGH analysis to nine ocular MALT lymphoma samples and three lymphoma cell lines with 6q23.3-24.1 loss. We narrowed the minimal common region down to a length of 586kb region where two genes and four expression sequence tags (ESTs) exist. All of these genes and ESTs were subjected to RT-PCR and real-time quantitative RT-PCR. A correlation between genomic loss and expression level was found only with TNFAIP3 and we thus concluded that this is the target gene for the deleted region. TNFAIP3 is an inhibitor of NF-kB signaling so that its loss may result constitutive activation of NF-kB, which has also been implicated in generation of MALT lymphomas with t(11;18)(q21;q21) or t(14;18)(q32;q21). Thus, TNFAIP3 may act as a tumor suppressor gene for ocular adnexal marginal zone B cell lymphoma. The function of TNFAIP3 in oncogenesis remains unclear so studies aimed at its elucidation are now underway.

*1 Department of Internal Medicine, Samsung Medical Center, Seoul, Korea
*2 Department of Pathology, Samsung Medical Center, Seoul, Korea

5. Array CGH analysis of PTCL-U for identification of a distinct subgroup

Nakagawa, M., Nakagawa-Oshiro, A., Karnan, S., Nakamura, S.*1, Takeuchi, I.*2, Ohshima, K.*3 and Seto, M.

Peripheral T-cell lymphoma unspecified (PTCL-U) comprises histopathologically and clinically heterogeneous groups. Although clarification of the subgroups in PTCL-U is therefore of major importance, the molecular basis of the clinical heterogeneity is still poorly understood. In this study, we used array CGH for high-resolution analysis of 51 PTCL-U patients and identified 32 regions with frequent genomic imbalance, one region with high-copy number gain at 14q32.2 and one with homozygous loss at 9p21.3. Gains of 7p and 7q, and loss of 9p21.3 showed significant associations with a poor prognosis. A detailed analysis disclosed that PTCL-U cases with genomic alteration show distinct histopathological and prognostic features. Interestingly, they manifest remarkable genetic, histopathologic and prognostic resemblance to lymphoma-type adult T-cell leukemia/lymphoma (ATLL), supporting the notion that PTCL-U cases featuring genomic alteration might comprise a distinct subgroup.

*1 Pathology/Clinical Laboratories, Nagoya University Hospital, Japan
*2 Division of Information Engineering, Graduate School of Engineering, Mie University, Japan
*3 Department of Pathology, School of Medicine, Kurume University, Japan
From left to right

First row: Dr. H. Torikai, Dr. Y. Akatsuka, Dr. K. Kuzushima, Dr. Y. Watanabe
Second row: Ms. K. Shiraishi, Ms. T. Tsuboi, Dr. S. Morishima, Ms. K. Nishida
Insets: Dr. M. Kamei, Dr. A. Demachi-Okamura, Ms. M. Tanimoto, Mr. T. Nagao, Dr. K. Tsujimura
Division of Immunology

Kiyotaka Kuzushima, M.D.  Chief
Yoshiki Akatsuka, M.D.  Section Head
Kunio Tsujimura, M.D.  Section Head (until March 2007)
Yoshinori Ito, M.D.  Senior Researcher (until March 2006)
Yukiko Watanabe, M.D.  Senior Researcher (as of April 2007)
Hiroki Torikai, M.D.  Senior Researcher (as of July 2007)
Ayako Demachi-Okanuma, Ph.D.  Research Resident (until March 2007)
Michi Kamei, M.D.  Research Resident (as of April 2007)
Satoko Morishima, M.D.  Research Resident (as of April 2007)
Yasue Matsudaira, B.S.  Senior Research Assistant (until March 2006)
Keiko Nishida, B.P.  Senior Research Assistant
Fumiyo Ando, Semi-regular Employee (until October 2006)
Michiyo Nakayama, Semi-regular Employee (until January 2006)
Yumi Nakao-Ohashi, Semi-regular Employee (until March 2007)
Keiko Shirai, Semi-regular Employee
Miyoko Tanimoto, Semi-regular Employee
Tomiko Tsunoi, Semi-regular Employee

Visiting Trainees
Ayako Demachi-Okanuma, Ph.D.  Research Resident of Foundation for Promotion of Cancer Research (Japan) (as of April 2007)
Takakazu Kawase, M.D.  Cancer Genetics, Nagoya University Graduate School of Medicine (until March 2007)
Satoko Morishima, M.D.  Cancer Genetics, Nagoya University Graduate School of Medicine (until March 2007)
Hiroki Torikai, M.D.  Third Department of Internal Medicine, National Defense Medical College (until June 2007)
Masataka Haneda, M.D.  Department of Immunology, Nagoya University Graduate School of Medicine (as of May 2007, until September 2007)
Takashi Nagao, M.D.  Department of Pediatrics, Okayama University School of Medicine (as of May 2007, until January 2008)
Mayumi Narita, Okayama University School of Medicine (as of September 2007, until November 2007)
Kazue Watanabe, M.S.  Medical & Biological Laboratories Co., Ltd. (until January 2006)

General Summary
The object of our research is to characterize and understand T lymphocyte responses to antigens expressed on cancers and virus-infected cells. The major projects undertaken over the past two years are summarized below.

In the field of transplantation immunology, three projects are in progress focusing on epitope identification and fine characterization of the epitope processing, to be recognized by minor histocompatibility (H) antigen-specific cytotoxic T lymphocytes (CTLs). Firstly, HLA-A*3101 and -A*3303-restricted CTL epitopes were determined looking at the Cathepsin H molecule. This was found to be relatively ubiquitously expressed at the protein level, but CTL clones predominantly lysed targets of hematopoietic cell origin. Although the mechanisms involved in the differential susceptibility remain to be determined, these data suggest that the minor H antigens could be targets for graft-versus-leukemia effects. Secondly, a novel HLA-B44–restricted minor H antigen was identified by means of cDNA expression cloning studies. The CTL epitope was encoded by a novel allelic splice variant of the HMSD (Histocompatibility Minor, Serpin Domain containing) gene. Interestingly, the immunogenicity is generated by a novel mechanism, namely differential protein ex-
pression due to alternative splicing controlled by an intronic single-nucleotide polymorphism located in the consensus 5' splice site adjacent to an exon. Thirdly, we have determined that the nonamer peptide VLHDDLLEA, the HLA-A*0201-restricted HA-1 H, could be also presented by HLA-A*0206. The finding points to expansion of the patient population who can benefit from HA-11-based immunotherapy.

Another field of our research is to characterize the T cell responses to EBV-related malignancies. To this end, several CD4+ T cell clones specific to Epstein-Barr virus (EBV) nuclear antigen (EBNA) 1 have been established and well characterized. Most importantly, one example proved capable of killing EBV-carrying NK and T cell lines derived from patients with EBV-associated disease, suggesting that EBNA1-specific CD4+ T cells may be useful for immunotherapy targeting EBV carrying NK and T cell malignancies. The clones were used as probes to verify the structure for targeting EBNA1 to the lysosomal/endosomal pathway in mRNA-transfected antigen presenting cells. Among those tested, constructs consisting of the signal sequence of heat shock protein gp96, EBNA1 and lysosome-associated membrane protein 1 proved most effective.

1. The human cathepsin H gene encodes two novel minor histocompatibility antigen epitopes restricted by HLA-A*3101 and -A*3303

Torikai, H., Akatsuka, Y., Miyazaki, M.*1, Tsujimura, A.2, Yatabe, Y.3, Kawase, T., Nakao, Y., Tsujimura, K.4, Motoyoshi, K.5, Morishima Y.6, Koder Y.7, Kuzushima, K., and Takahashi, To.8

Minor histocompatibility (H) antigens play crucial roles in the induction of graft-versus-host disease (GVHD) and/or graft-versus-leukemia (GVL) effects following HLA-identical hematopoietic stem cell transplantation (HSCT). We identified a novel minor H antigen epitope using two HLA-A*3101 and -A*3303-restricted cytotoxic T lymphocyte (CTL) clones derived from different HSCT recipients with high-risk acute leukemia who both maintained durable remission more than 2 years after well-controlled acute/chronic GVHD. The epitopes identified by cDNA expression cloning were encoded by the same nonsynonymous single nucleotide polymorphism (SNP) in the leader sequence of the Cathepsin H gene. The polymorphic amino acid residue controlling the antigenicity was located at the C terminus of identified epitopes which is the major anchor motif (e.g. Arg or Lys) for both HLA-A*3101 and -A*3303. Fine epitope mapping revealed that the nonamer sequence ATLPLL-LCAR was defined as an HLA-A*3101-restricted epitope (CTSHR/A31), while a decameric peptide featuring a one N-terminal amino acid extension, WATLPLLCAR, was presented by HLA-A*3303 (CTSHR/A33). HLA peptide binding assays clearly revealed that the immunogenicity of both epitopes was totally dependent on the differential HLA binding capacity. CTSH is relatively ubiquitously expressed at the protein level and thus may be involved in GVHD and anti-leukemic/tumour responses. Interestingly, however, CTL clones predominantly lysed targets of hematopoietic cell origin, which could not be explained in terms of the immunoproteasome system. Although the unique mechanisms involved in the differential susceptibility remain to be determined, these data suggest that CTSH-encoded minor H antigens could be targets for GVL effects.

*1Department of Internal Medicine & Molecular Science, Nagoya City University Graduate School of Medical Sciences
*2Department of Hematology, Japanese Red Cross Nagoya First Hospital
*3Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Central Hospital
*4Department of Infectious Diseases, Hamamatsu University School of Medicine
*5Third Department of Internal Medicine, National Defense Medical College
*6Department of Hematology and Cell Therapy, Aichi Cancer Center Central Hospital
*7Aichi Health Plaza

2. Identification of a novel minor histocompatibility antigen generated by alternative splicing due to an intronic SNP in HMSD

Kawase, T., Akatsuka, Y., Torikai, H., Morishima, S., Oka, A.1, Tsujimura, A.2, Miyazaki, M.3, Tsujimura, K.4, Miyamura, K.5, Ogawa, S.6, Inoko, H.7, Morishima, Y.6, Koder, Y.7, Kuzushima, K., Takahashi, To.8

Minor histocompatibility (H) antigens are MHC (human leukocyte antigen, HLA in human)-associated peptides originating mainly from
polymorphisms in the genome that trigger T cell responses between MHC identical allogeneic individuals. Graft-versus-host disease and graft-versus-leukemia/lymphoma (GVL) effects in hematopoietic stem cell transplant recipients are initiated by donor T cell recognition of minor H antigens on recipient cells. Identification of suitable target antigens for selective GVL effects is warranted.

We have identified a novel HLA-B44–restricted minor H antigen (designated as ACC-6) with expression limited to hematopoietic cells using a cytotoxic T lymphocyte (CTL) clone isolated from a post-transplant patient with acute myeloid leukemia. cDNA expression cloning studies demonstrated that the CTL epitope of interest was encoded by a novel allelic splice variant of the HLA-B44 allele (designated as HMSD). The immunogenicity of the epitope was generated by differential protein expression due to alternative splicing, which was completely controlled by 1 intronic single-nucleotide polymorphism located in the consensus 5' splice site adjacent to an exon. Both HMSD and HMSD transcripts were selectively expressed at higher levels in mature dendritic cells, primary leukemia cells, especially those of myeloid lineage, and primary myeloma cells. Engraftment of myeloid leukemia stem cells that were positive for the ACC-6 minor H antigen into nonobese diabetic/severe combined immunodeficient (NOD/SCID)/γnull mice was completely inhibited by in vitro preincubation with an ACC-6-specific CTL clone, suggesting that this minor H antigen epitope is expressed on leukemic stem cells. The patient from whom the CTL clone was isolated demonstrated a significant increase of ACC-6-specific T cells in post-transplant peripheral blood, whereas ACC-6-specific T cells were undetectable in peripheral blood from his donor. These findings suggest that the ACC-6 minor H antigen, designated ACC-6, could serve as a target antigen for immunotherapy against hematologic malignancies. The clinical significance of the ACC-6 minor H antigen will be evaluated in a vaccination study in post-transplant patients with high risk of malignancies.

*1Department of Genetic Information, Division of Molecular Life Science, Tokai University School of Medicine
*2Department of Hematology, Japanese Red Cross Nagoya First Hospital
*3Department of Internal Medicine & Molecular Science, Nagoya City University Graduate School of Medical Sciences
*4Department of Infectious Diseases, Hamamatsu University School of Medicine
*5Department of Regeneration Medicine for Hematopoiesis, Graduate School of Medicine, University of Tokyo
*6Core Research for Evolutional Science and Technology (CREST) of Japan, Science and Technology Corporation (JST)
*7Department of Hematology and Cell Therapy, Aichi Cancer Center Central Hospital

3. The HLA-A*0201-restricted minor histocompatibility antigen HA-1H peptide can also be presented by another HLA-A2 subtype, A*0206

Torikai H., Akatsuka, Y., Miyauchi, H., Terakura, S.1, Onizuka, M.1, Tsujimura, K.2, Miyamura K.1, Morishima Y.3, Kodera Y.1, Kuzushima, K., and Takahashi, To4.

HA-1H is one of the most attractive minor histocompatibility (H) antigens as a target for immunotherapy of hematopoietic malignancies, but HLA-A*0201 and -B60 molecules capable of presenting HA-1H-derived peptides are less common in eastern Asian when compared with Caucasian populations. Therefore, an attempt was made to search for novel epitopes presented by HLA alleles other than those previously reported by generating CTL lines from patients undergoing HLA-identical, HA-1 disparate hematopoietic stem cell transplantation (HSCT) by stimulation with a 29-mer HA-1H peptide spanning a central polymorphic histidine (His). Two CTL clones established were found to be restricted by HLA-A*0206, which is the second or third most common HLA-A2 subtype worldwide. Epitope mapping revealed that the clones recognized the same nonamer peptide as A*0201-restricted HA-1H, VLHDDLLEA. This epitope was unexpected, since it does not contain any preferred anchor motifs for HLA-A*0206. However, HLA peptide binding assays revealed stronger binding of this peptide to A*0206 than to A*0201. We next examined the in vivo immunogenicity of HLA-A*0206-restricted, HA-1H-specific T cells by means of tetramers. Peripheral blood mononuclear cells (PBMCs) were obtained after HSCT from a patient who was positive for both HLA-A*0201 and -A*0206 and received an HA-1 disparate marrow transplant. After HA-1H peptide
pulsed antigen presenting cell stimulation, individual tetramers detected tetramer+ CD8+ cells. Thus, A*0201 and A*0206 restricted HA-1H might even be immunogenic. This finding points to expansion of the patient population who might benefit from HA-1H-based immunotherapy.

*1Department of Hematology, Japanese Red Cross Nagoya First Hospital  
*2Department of Infectious Diseases, Hamamatsu University School of Medicine  
*3Department of Hematology and Cell Therapy, Aichi Cancer Center Central Hospital  
*4Aichi Health Plaza

4. Epstein-Barr virus (EBV) nuclear antigen 1-specific CD4+ T cells directly kill EBV-carrying NK and T cells


Epstein-Barr virus (EBV) nuclear antigen (EBNA) 1 is expressed in every EBV-infected cell, regardless of the state of EBV infection. Although EBNA1 is thought to be a promising antigen for immunotherapy of all EBV-associated malignancies, it is less clear whether EBNA1-specific CD4+ T cells can act as direct effectors. Here, we investigated the ability of CD4+ T cell clones induced with overlapping peptides covering the C-terminal region of EBNA1, and identified minimal epitopes and their restricted MHC class II molecules. Of these, a novel epitope, EYHQEGGPD, was found to be presented by DRB1*0401, 0403, and 0406. Five CD4+ T cell clones produced IFN-γ upon stimulation with autologous EBV-transformed lymphoblastoid cell lines (LCLs) recognizing epitopes produced through autophagic pathways, as confirmed by RNA interference (RNAi) of an autophagy-related gene. One example proved capable of killing EBV-carrying NK and T cell lines derived from patients with chronic active EBV infection (CAEBV), presenting an epitope through autophagic pathways, suggesting that EBNA1-specific CD4+ T cells may be helpful for immunotherapy targeting EBV carrying NK and T cell malignancies.

5. Effective antigen presentation to EBNA1-specific CD4+ T lymphocytes

Watanabe, Y., Demachi-Okamura, A., and Kuzushima, K.

Induction of potent and sustained antitumor immunity is dependent on efficient and longitudinal activation of cytotoxic T lymphocytes (CTLS) and CD4+ T lymphocytes. Antigens expressed in mRNA-transfected antigen presentation cells (APCs) are mainly processed in the MHC class I pathway. Targeting antigens to the lysosomal/endoosomal pathway in APCs can enhance presentation of MHC class II-restricted epitopes derived from tumor and viral antigens. We therefore explored structures for effective antigen presentation to CD4+ T lymphocytes. Essentially three constructs, lysosome-associated membrane protein 1 (LAMP1), invariant chain and autophagy-related light chain 3, were compared for their ability to introduce EBNA1 to the class II pathway. As signal sequences, we used both of LAMP1 and the heat shock protein gp96. Autologous CD40-activated B cells transfected with mRNA encoding EBNA1 with the targeting signals to class II were used to stimulate an EBNA1-specific CD4+ T clone. IFN-γ production from the clone was evaluated by enzyme-linked immunospot assays. As a result, gene products from gp96-EBNA1-LAMP1 were most effectively presented among those tested. CD40-activated B cells transfected with the same structural mRNA were able to stimulate an EBNA1-specific CTL clone, indicating that the class I pathway is also enhanced by the modification. Currently, simultaneous induction of EBNA1-specific CTL and CD4+ T lymphocytes using the APCs is ongoing. Our findings should prove useful not only for identification of novel epitopes but also for monitoring T-cell responses during immunotherapy.
From left to right
First row: Mr. S. Toyama, Dr. S. Iwahori, Dr. T. Tsurumi, Dr. S. Nakayama, Dr. A. Kudoh, and Mr. Y. Nishikawa.
Second row: Dr. S. Nakasu, Dr. T. Murata, Dr. H. Isomura, and Mr. Y. Sato.
General Summary

Approximately 15% of all human cancers have a viral etiology, but only six viruses have actually been implicated in their development. Among these the Epstein-Barr virus (EBV) is the object of our own studies. EBV is a ubiquitous gamma herpesvirus associated with several malignant diseases, including Burkitt’s lymphoma, nasopharyngeal lymphoma, a subset of Hodgkin’s lymphomas, some gastric cancers, and B cell lymphomas in immunosuppressed patients. Our research aims are to elucidate the molecular mechanisms of viral proliferation and oncogenesis of EBV as part of the world-wide effort to combat virus-infected cancers. During the period 2006-2007, our research interest was concentrated on the following issues: 1) phosphorylation of MCM4 at sites inactivating DNA helicase activity of the MCM4-6-7 complex during Epstein-Barr Virus productive replication; 2) expression of Epstein-Barr Virus BZLF1 immediate-early protein induces p53 degradation independent of MDM2, leading to repression of p53-mediated transcription; 3) enhanced phosphorylation of transcription factor Sp1 in response to herpes simplex virus type 1 infection is dependent on the ataxia telangiectasia-mutated protein; 4) purification of the product of the Epstein-Barr virus BZLF1 gene; 5) a cis-element between the TATA Box and the transcription start site of the Major Immediate-Early (MIE) promoter of human cytomegalovirus determines efficiency of viral replication.

1. Phosphorylation of MCM4 at sites inactivating DNA helicase activity of the MCM4-6-7 complex during Epstein-Barr virus productive replication

Kudoh, A. and Tsurumi, T.

Induction of Epstein-Barr virus (EBV) lytic replication blocks chromosomal DNA replication notwithstanding an S-phase like cellular environment with high CDK2 activity. We report here that the phosphorylated form of MCM4, a subunit of the MCM complex essential for chromosomal DNA replication, increases with progression of lytic replication, Thr-19 and Thr-110 being CDK2 targets whose phosphorylation inactivates MCM4-6-7 complex-associated DNA helicase. Expression of EBV-encoded protein kinase (PK) in HeLa cells causes phosphorylation of these sites on MCM4. In vitro, the sites of MCM4 in the MCM4-6-7 hexamer were confirmed to be phosphorylated by EBV-PK with the same loss of helicase activity as with CDK2/cyclin A. Introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, while EBV-PK inhibited the helicase activity of both the wild type and the mutant MCM4-6-7 hexamer. Therefore, phosphorylation of MCM4 by the consecutive actions of CDK2 kinase and EBV-PK during lytic replication might provide one mechanism to block chromosomal DNA replication in infected cells, through inactivation of DNA unwinding by the MCM4-6-7 complex.

2. Expression of Epstein-Barr virus BZLF1 immediate-early protein induces p53 degradation independent of MDM2, leading to repression of p53-mediated transcription

Sato, Y. and Tsurumi, T.
Induction of the Epstein-Barr virus (EBV) lytic program elicits ATM-dependent DNA damage responses, with activation of the ATM-Chk2-p53 signal transduction pathway, resulting in phosphorylation of p53 at Ser15. This prevents interaction with its negative regulator MDM2. However, p53-downstream signaling is blocked and the molecular mechanism involved in this process remains to be clarified. In this study, we found that during lytic infection p53 is actively degraded in a proteasome-dependent manner even with a reduced level of MDM2. Turnover of p53 is stimulated in the lytic phase compared with that in the latent phase, suggesting that EBV regulates p53 protein-levels also after induction of lytic replication. Co-immunoprecipitation analysis revealed that the EBV BZLF1 immediate-early protein interacts with the DNA-binding domain near the N-terminus of p53. It was confirmed that BZLF1 protein elicited proteasome-dependent degradation of p53 and repressed the p53-mediated transactivation in SaOS-2 cells. Degradation of p53 was observed even in the presence of Nutlin-3, an inhibitor of p53-MDM2 interaction, and also in mouse embryo fibroblasts lacking the mdm2 gene, indicating the BZLF1 protein-induced degradation of p53 to be independent of MDM2. Furthermore, Nutlin-3 increased the level of p53 in the latent phase of EBV infection but not in the lytic phase. Thus, although the p53 level is regulated by MDM2 in the latent phase, it might be mediated by the BZLF1 protein-associated E3 ubiquitin ligase in the lytic phase. These findings provide insight into how EBV regulates the cellular environment advantageous for lytic replication through degradation of p53, leading to inhibition of p53 downstream signaling.

3. Enhanced phosphorylation of transcription factor Sp1 in response to herpes simplex virus type 1 Infection is dependent on the ataxia telangiectasia-mutated protein

Iwahori, S. and Tsurumi, T.

The ATM protein, a member of the related PI-3-like kinase family encoded by a gene responsible for the human genetic disorder ataxia telangiectasia, regulates cellular responses to DNA damage and viral infection. Transcription factor Sp1 functions as a trans-activator of gene expression and its recognition elements are distributed widely in various promoters of cellular and viral genes. As with many other transcription factors, the transcription activity of Sp1 is regulated in part by post-translational modifications, which include phosphorylation, glycosylation, acetylation and sumoylation. It has been previously reported that herpes simplex virus type 1 (HSV-1) infection induces activation of protein kinase activity of ATM and hyper-phosphorylation of transcription factor, Sp1.

In the present study, we showed that ATM is intimately involved in Sp1 hyper-phosphorylation during HSV-1 infection, rather than individual HSV-1 encoded protein kinases. In ATM-deficient cells or cells silenced for ATM expression by shRNA targeting, hyper-phosphorylation of Sp1 was prevented even as HSV-1 infection progressed. Mutational analysis of putative ATM phosphorylation sites on Sp1 and immunoblot analysis with phosphopeptide-specific Sp1 antibodies clarified that at least Ser-56 and Ser-101 residues on Sp1 become phosphorylated upon HSV-1 infection. Serine to alanine mutations at both sites on Sp1 considerably abolished hyper-phosphorylation of Sp1 upon infection. Although ATM phosphorylated Ser-101 but not Ser-56 on Sp1 in vitro, phosphorylation of Sp1 at both sites was not detected at all upon infection in ATM-deficient cells, suggesting that some cellular kinase(s) activated by ATM could be involved in phosphorylation at Ser-56. Upon viral infection Sp1-dependent transcription in ATM expression-silenced cells was almost the same as that in ATM-intact cells, suggesting that ATM-dependent phosphorylation of Sp1 might have only limited effects on transcriptional activity during HSV-1 infection. ATM-dependent Sp1 phosphorylation appears to be a global response to various DNA damage stresses including viral DNA replication.

4. Purification of the product of Epstein-Barr virus BZLF1 Gene

Nakasu, S. and Tsurumi, T.

The product of the BZLF1 gene (pBZLF1) of the Epstein-Barr virus (EBV) is a nuclear protein which activates the lytic cycle in cells latently infected with EBV. pBZLF1 has been suggested to activate genes required for the lytic cycle and to induce viral DNA replication as a DNA binding protein specific for the viral lytic origin of DNA replication (ori lyt).

In order to understand the role of pBZLF1 in induction of the lytic cycle, we have focused on purification and characterization of its biochemical
features. We first tried to purify pBZLF1 from insect cells infected with baculoviruses which overproduced pBZLF1. However, partially purified pBZLF1 from insect cells tended to form aggregates and was eluted with a wide range of salt concentrations from ion exchange chromatography columns. The results suggested an altered conformation of the pBZLF1 produced in the insect cells. Therefore we next tried to purify pBZLF1 from B95-8 cells, a cell line latently infected with EBV.

The lytic cycle was induced by chemicals and pBZLF1 could be extracted from the induced cells with high salt buffer (0.6 to 1 M NaCl) and purified more than 100 fold with hydrophobic chromatography, DEAE Sephacel chromatography, and phospho (P) cellulose chromatography. Judging from sucrose gradient centrifugation, most pBZLF1 was present in complexes with other proteins in low salt buffer (< 0.12 M NaCl) during these steps and behaved as a monomer protein in high salt buffer (e.g. 1.5 M NaCl). Thus the steps were necessary to purify pBZLF1 under high salt conditions. We found pBZLF1 to flow through hydroxyapatite in the presence of phosphate and become absorbed to hydroxyapatite in the absence of phosphate under high salt conditions. After hydroxyapatite chromatography, a sample was absorbed to heparin sepharose in 0.3 M NaCl buffer and pBZLF1 was eluted with 0.7 M NaCl buffer. Starting from 3 l culture, pBZLF1 and several other proteins were visible on silver staining of the SDS page of the final fraction (0.4 ml) but were only faint with CBB staining. Therefore a larger scale purification procedure is now required for further investigation.

### 5. A cis-element between the TATA Box and the transcription start site of the major immediate-early (MIE) promoter of human cytomegalovirus determines efficiency of viral replication

Isomura, H. and Tsurumi, T.

The promoter of the major immediate-early (MIE) genes of human cytomegalovirus (HCMV), also referred to as the CMV promoter, possesses a cis-acting element positioned downstream of the TATA box between -14 and -1 relative to the transcription start site (+1). We determined the role of the cis-acting element in viral replication by comparing recombinant viruses with the cis-acting element replaced with other sequences. Like the wild-type virus, recombinant virus with the simian CMV counterpart replicated efficiently in human foreskin fibroblasts. In contrast, replacement with the murine CMV counterpart caused inefficient MIE gene transcription, RNA splicing, MIE and early viral gene expression, and viral DNA replication. To determine which nucleotides in the cis-acting element are required for efficient MIE gene transcription and splicing, we constructed mutations within the cis-acting element in the context of a recombinant virus. While mutations in the cis-acting element had only a minor effect on in vitro transcription, effects on viral replication were major. The nucleotides at -10 and -9 in the cis-acting element relative to the transcription start site (+1) affected efficient MIE gene transcription and splicing at early times after infection. The cis-acting element also acts as a cis-repression sequence when the viral IE86 protein accumulates in infected cells. We were thus able to demonstrate that the cis-acting element has an essential role in viral replication.
From left to right
First row: Dr. Zenta Yasukawa, Dr. Osamu Taguchi, Dr. Reiji Kannagi, Dr. Mamoru Kyogashima and Dr. Akiko Kanamori.
Second row: Ms. Keiko Miyazaki, Ms. Akiko Nishioka, Ms. Mineko Izawa, Dr. Akiko Yusa, Dr. Keiko Tamiya-Koizumi, Dr. Lim Khe-Ti, Ms. Yoshiko Goto, Ms. Sachiko Kondo, and Mr. Kouji Tanaka.
Third row: Dr. Jun Yin, Dr. Hirokazu Yagi, Dr. Masahiro Fujii, Dr. Keiichiro Sakuma
Division of Molecular Pathology

Reiji Kannagi, M.D., D.M.Sc., Chief
Osamu Taguchi, D.M.Sc., Section Head
Mamoru Kyogashima, M.D., D.M.Sc., Senior Researcher
Akiko Kanamori, Ph.D., Senior Researcher
Keiichiro Sakuma, M.D., D.M.Sc., Senior Researcher (as of April, 2007)
Zenta Yasukawa, Ph.D., Research Resident
Akiko Yusa, Ph.D., Research Resident (as of April 2007)
Mineko Izawa, B.A., Research Assistant (until March, 2007)
Yoshiko Goto, D.V.M.S., Research Assistant
Sasako Eguchi, Semi-regular Employee
Akiko Nishioka, M.T., Semi-regular Employee
(March 2007)

Visiting Scientists
Hiroshi Ikeda, M.D., D.M.Sc., Aichi Medical University
Jun Yin, Ph.D. Foundation for Promotion of Cancer Research (as of April, 2006)
Khe-Ti Lim, Ph.D., National Institute of Biomedical Innovation
Guo-Yun Chen, M.D., D.M.Sc., Japan Science and Technology Agency (until March, 2007)
Keiko Miyazaki, M.T., Japan Science and Technology Agency
Mineko Izawa, B.A., Japan Science and Technology Agency (as of April, 2007)
Takashi Murate, M.D., D.M.Sc., Nagoya University School of Health Sciences
Keiko Tamiya-Koizumi, Ph.D., Nagoya University School of Health Sciences

Visiting Trainees
Naoko Kimura, B.Ph., Nagoya City University
Masahiro Fujii, M.D. Nagoya University School of Medicine (as of June, 2006)
Kazumi Hagiwara, M.T., Nagoya University School of Health Sciences
Sayaka Sobue, M.T., Nagoya University School of Health Sciences
Hirokazu Yagi, M.P. Nagoya City University
Sachiko Kondo, M.E. Nagoya City University
Akiko Yusa, Ph.D., Aichi University of Education (until March, 2007)
Kouji Tanaka, B.Ph., Shinshu University School of Medicine
Risa Umeyama, Sugiyama Jogakuen University (until December, 2006)
Sayaka Miyagi, Sugiyama Jogakuen University (as of February, 2007)
Kumiko Oseto, Sugiyama Jogakuen University (as of February, 2007)
Tetsufumi Koike, M.D., Fukushima University School of Medicine (until March, 2006)
Atsushi Akutagawa, M.D., Nagoya University School of Medicine (until March, 2006)

General Summary

Cell adhesion mediated by selectins and their carbohydrate ligands, sialyl Lewis X and sialyl Lewis A, plays an important role in cancer metastasis and tumor angiogenesis. Expression of these carbohydrate determinants is markedly enhanced in cancer cells compared to non-malignant epithelial cells. Our recent studies indicated the presence of further-modified complex forms of sialyl Lewis X/A in non-malignant epithelial cells, which have additional sulfation or sialylation. Such complex determinants serve as specific ligands for another family of carbohydrate-recognizing molecules, siglec s, and maintain immunological homeostasis in normal mucous membranes. The impairment of these additional modifications in cancer cells, which we term "incomplete synthesis," leads to considerable accumulation of sialyl Lewis X/A in cancer cells at early stages of malignant transformation. Epigenetic gene silencing through DNA methylation and/or histone deacetylation is proposed to confer the incomplete synthesis.
In later stages of cancer progression, increased transcription of several glycogenes responsible for the synthesis of sialyl Lewis X/A determinants by hypoxia-inducible factors further accelerates expression of these determinants on cancer cells. Hematogenous metastasis mediated by sialyl Lewis X/A-selectin interactions determines the prognostic outcome of patients with advanced cancers. Cell adhesion molecules equipped with specific carbohydrate-recognition domains figure prominently in the biological behavior of cancer cells as well as in cell-cell interactions in normal mucosal membranes. During the period 2006-2007, we have made progress in elucidating the mechanisms involved in induction of cancer-associated carbohydrate determinants including sialyl Lewis X, sialyl Lewis A and \( N \)-glycolyl GM2 in human cancers. At the same time, we have also studied the role of CD44 in cancer progression, and identified several important molecules involved in immune recognition.

1. Significance of interconversion between carbohydrate ligands for selectins and siglecs expressed on normal colonic epithelium and cancer cells

Miyazaki, K. Izawa, M., Ohmori, K.*1, Hashimoto, Y.*2 and Kannagi, R.

Cancer-associated carbohydrate determinants, such as sialyl Lewis A and sialyl Lewis X, are known to serve as ligands for selectins, and mediate cancer cell adhesion to vascular beds in the course of hematogenous metastasis. Expression of these determinants is markedly increased in cancer cells compared to normal epithelial cells. We have recently shown that non-malignant epithelial cells express carbohydrate determinants, such as disialyl Lewis A or sialyl 6-sulfo Lewis X, having more complex structures than sialyl Lewis A/X. Their expression is lost at an early stage of colon carcinogenesis due to the epigenetic silencing of glycogenes involved in their synthesis. Loss of these normal carbohydrate determinants then leads to accumulation in cancers of less-complex determinants such as sialyl Lewis A and sialyl Lewis X. Disialyl Lewis A on normal epithelial cells serves as a ligand for siglec-7 and siglec-9, but sialyl Lewis A on cancer cells has no binding activity. Binding assays at the cellular level indicated that sialyl 6-sulfo Lewis X also serves as a ligand for siglec-7, but sialyl Lewis X does not. It is noteworthy that only the carbohydrate determinants on normal epithelial cells serve as ligands for siglecS, while cancer-associated determinants do not. Siglec-7/-9 are known to have ITIM-motifs which inhibit signal transduction in immune cells by recruiting tyrosine phosphatases SHP-1 and SHP-2. On transfection into human macrophage cell lines, Siglec-7/-9 suppressed LPS-induced COX2 and IL-12 production. These results imply that normal glycans of colonic epithelial cells exert a suppressive effect on tissue macrophage COX2 expression in colonic mucosa, thus maintaining immunological homeostasis in normal mucosal membranes. The results also suggest that abnormal glycosylation during early carcinogenesis abrogates this suppressive effect.

*1 Department of Laboratory Medicine, Kyoto University, School of Medicine.
*2 Glyco-chain Functions Laboratory, Frontier Research System, RIKEN Institute.

2. Tumor hypoxia induces expression of gangliosides containing abnormal sialic acid on cancer cells

Yin, J., Izawa, M., Miyazaki, K., Yasukawa, Z. Kitajima, K.*1 and Kannagi, R.

Tumor hypoxia plays important roles in malignant progression by altering intracellular glucose metabolism and inducing angiogenic factor production, thus selecting and expanding more aggressive hypoxia-resistant cancer cell clones. Little is known, however, regarding hypoxia-induced changes in cell surface molecules, which can serve as markers of hypoxia-resistant cancer cells.

We therefore have investigated expression of NeuGc-GM2, a cancer-associated ganglioside GM2 containing a non-human sialic acid, \( N \)-glycolyl sialic acid (NeuGc), in human cancers. Tissues prepared from breast
and colon cancers frequently expressed NeuGc-GM2, while it was virtually absent in non-malignant mammary and colonic epithelia. Studies on cultured cancer cells have indicated that non-human sialic acid can be incorporated from culture medium. Hypoxic culture markedly induced mRNA for a sialic acid transporter, sialin, and this was accompanied by enhanced incorporation of NeuGc. Transfection of cells with the Sialin gene conferred accelerated sialic acid transport and induced cell surface expression of NeuGc-GM2.

From these results we propose that the preferential expression of NeuGc-GM2 in cancers is closely associated with tumor hypoxia. This latter induces expression of the sialic acid transporter, and enhances incorporation of non-human sialic acid from the external milieu. A consequence of this is acquisition of cancer-associated cell-surface gangliosides, typically GM2, containing abnormal non-human sialic acid (NeuGc), which is not endogenously synthesized through CMP-NeuAc hydroxylase, since humans lack a gene for the synthetic enzyme. As hypoxia is associated with diminished response to radio- and chemotherapy, NeuGc-GM2 is a potential therapeutic target for hypoxic cancer cells.

Expression of many other carbohydrate determinants is also induced by tumor hypoxia. One must bear in mind here that hypoxia-induced change is not limited to malignant cells, and that normal cells respond to hypoxia as well. If cell surface determinants induced simply by hypoxia are chosen as a target of therapeutic attack, such determinants can be induced on the surfaces of hypoxic normal cells, which will also be open to attack. In this context, it is interesting to note that the GM2 ganglioside, even that with normal sialic acid, is known to be preferentially expressed on cancer cells, and is regarded as a good target for immune therapy of cancers. NeuGc-GM2 is therefore expected to be a better target for therapy of hypoxia-resistant highly-aggressive cancers than GM2 having normal sialic acid.

3. Studies on cell adhesion activity of CD44

3.1. Roles of carbohydrate side chains of the CD44 molecule

Lim, K.T., Miyazaki, K., Kimura, N., Murakami, Y.*1 and Kannagi, R.

Cell adhesion molecules such as CD44 are modified by a variety of carbohydrate chains including N-glycans, O-glycans and glycosaminoglycans. Using human cancer cells we have studied functional roles of the carbohydrate side chains of CD44, known to specifically bind to a carbohydrate ligand, hyaluronate. Sialylation of CD44 is known to suppress, while 6-sulfation facilitates this binding to hyaluronate. Western blotting analyses of CD44 in cultured human colon cancer cells indicated that it carried the sialyl Lewis X determinant, which is known as a ligand for E-selectin. CD44 in colon cancer cells were also found to express sialyl Lewis A and sialyl 6-sulfo Lewis X determinants.
We previously showed that the sialyl Lewis A determinant serves as a ligand for E-selectin as well as sialyl Lewis X, and that the sialyl 6-sulfo Lewis X determinant is a specific ligand for L-selectin. These carbohydrate determinants are carried mainly by the splicing variants of CD44 (CD44v) in cancer cells. Introduction of a gene for the sialyltransferase that transfers sialic acid to N-acetylglucosamine residues induced expression of the disialyl Lewis A determinant, which we recently showed to serve as a ligand for siglec-7, a member of the immunosuppressive receptor family recognizing sialic acid. These results suggest that carbohydrate side chains of CD44v molecules in colon cancers not only regulate the binding to hyaluronate, but also function as ligands for multiple cell adhesion molecules, and that expression of functional carbohydrate side chains is highly dependent on presence of the splicing variants.

*1 Institute of Medical Science, Tokyo University

3.2. Potentiation of CD44-mediated leukocyte adhesion to the hyaluronan substratum by SHAP

Zhuo, L.S.*1, Kanamori, A., Kannagi, R. Itano, N.*1, Wu, J.W.*1, Hamaguchi, M.*2, Ishiguro, N.*2, and Kimata, K.*1

CD44-hyaluronan (HA) interaction is involved in diverse physiological and pathological processes. Regulation of interaction avidity is well studied for CD44 but less is known regarding HA. We discovered a unique covalent modification of HA by a protein, SHAP, that corresponds to the heavy chains of inter-α-trypsin inhibitor family molecules circulating in blood. Formation of the SHAP-HA complex is often associated with inflammation, a process well known to involve CD44-HA interaction. We therefore examined the effect of SHAP on the CD44-HA interaction-mediated lymphocyte adhesion. Under both static and flowing conditions, Hut78 cells (CD44-positive) and CD44-transfected Jurkat cells (originally CD44-negative) adhered preferentially to immobilized SHAP-HA complexes rather than to HA. The enhanced adhesion was exclusively mediated by the CD44-HA interaction, because it was inhibited by HA, but not Ixzl, and was completely abolished by pretreating the cells with anti-CD44 antibodies. SHAP appears to potentiate the interaction by increasing the avidity of HA for CD44 and altering their distribution on cell surfaces. Large amounts of the SHAP-HA complex accumulate in the hyperplastic synovium of rheumatoid arthritis patients. Infiltrating leukocytes are strongly positive for HA, SHAP, and CD44 on their surfaces, suggesting a role for the adhesion-enhancing effect of SHAP in pathogenesis.

*1 Institute for Molecular Science of Medicine, Aichi Medical University.
*2 Nagoya University, School of Medicine.

4. Diversity of sulfatide molecular species from biological materials by MALDI-TOF MS

Kyogashima, M., Tadano-Aritomi, K.*1, Murate, T., Tamiya-Koizumi, K., Hara, A.*2, Aoyama, T.*2 and Kannagi, R.

By combining the partition method for enrichment of sulfatides without any chromatographic procedures and a preparation method for lysosulfatides, we have succeeded in analyzing sulfated glycosphingolipids from biological materials by MALDI-TOF MS within a single day. We found SM4s (galactosylsulfatide) to be composed of different species. While the exact composition depended on the source material, SM4s always contained hydroxy fatty acids to various degrees. In addition to the common sphingoid 4-sphingenine (d18:1), uncommon/unusual sphingoids phytosphingosine (t18:0), 4-eicosasphinganine (d20:0), 4-eicosasphingenine (d20:1), and sphingadiene (d18:2) were readily detected. Finally, in addition to SM4s, sulfatide SM3 (sulfated lactosylceramide) and SM2 (sulfated gangliotriaosylceramide) were clearly present in renal tubule cells. The major SM4s were found to comprise ceramides possessing d18:1 with C22 hydroxy fatty acids (C22:0h), C23:0h, and C24:0h, whereas the major SM3/SMD forms were composed of ceramides possessing t18:0 with C22 normal fatty acids (C22:0, C23:0, C24:0). Namely, in
these two series of sulfatides, either fatty acids or sphingoids were hydroxylated, and chain lengths of these components were exactly the same, consequently resulting in a similar polarity of ceramide moieties. These results demonstrate diversity of sulfatide molecular species, not only with respect to sugar- but also to ceramide moieties, which is probably important for specific effective functions in particular microenvironments, such as lipid membrane microdomains.

*1 Department of Biochemistry, Teikyo University School of Medicine.  
*2 Department of Metabolic Regulation, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine.

5. Molecular profiling of heparan sulfate glycosaminoglycans in human cancers using monoclonal antibodies

Yusa, A., Fujii, M., Goto, Y., Suzuki, K. *1, Ishimaru, T. *1, Yamamoto, K. *1, Kim, Y. *2, Kimata, K. *3, Iwata, H. *4, Kyogashima, M., and Kannagi, R.

Cancer cell surface heparan sulfate proteoglycans (HSPGs) play important roles in cell proliferation, invasion and metastasis. Information, however, on sugar chain structures of the glycosaminoglycans (GAGs) on malignant cells has been more limited, as compared to the core proteins, because of their extreme complexity. In order to address this issue, we have profiled cancer HSPGs by flow-cytometry and immunohistochemistry using various monoclonal anti-HS antibodies, including 10E4 recognizing GlcNS and GlcNAc, JM403 recognizing GlcNH2, and newly generated antibodies, NAH46, AS22 and ACH55. NAH46 was generated by immunizing mice with KLH-coupled bacterial polysaccharide K5 heparosan. AS22 and ACH55 were generated with KLH-coupled acharan sulfate prepared from the giant African snail, and its desulfated form acharan, respectively. Specificities of these antibodies were tested using a panel of GAGs, including chondroitin sulfate, keratan sulfate, hyaluronic acid, heparin and HS. NAH46, AS22 and ACH55 were confirmed to specifically recognize GlcA-GlcNAc, IdoA2S-GlcNAc, and IdoA-GlcNAc, respectively. Several human colon, squamous cell, lung, and breast cancer cell lines as well as breast cancer tissues were then examined by flow-cytometry and immunohistochemistry using these antibodies. While NAH46 intensively stained all cell lines, staining patterns with 10E4 and JM403 were variable, suggesting that the degree of HSPG sulfation differed depending on the cancer cell. AS22 and ACH55 stained some colon cancer cell lines and breast cancer tissues, indicating that HSPGs having the IdoA2S-GlcNAc and/or IdoA-GlcNAc structures may also be expressed in human cancers.

*1 Central Research Laboratories, Seikagaku Corporation.  
*2 Natural Products Research Institute, College of Pharmacy, Seoul National University.  
*3 Institute for Molecular Science of Medicine, Aichi Medical University  
*4 Department of Breast Oncology, Aichi Cancer Center Hospital.

6. Studies on molecules involved in immune regulation


Chen, G.Y., Osada, H. *1, Santamaria-Babi, L.F.*,2 and Kannagi, R.

Selectin-mediated cell adhesion is involved in hematogenous metastasis of cancer cells and tissue infiltration by leukemic cells. It is also implicated in extravasation of leukocytes in inflammatory diseases as well as in routine homing of lymphocytes. Selectin-mediated cell adhesion is regulated by levels of selectins and their carbohydrate ligand, sialyl Lewis X, whose expression on cell surfaces depends on transcriptional regulation of the fucosyltransferase VII gene ($FUT7$), encoding the rate-limiting enzyme for sialyl Lewis X synthesis. We have previously shown that enhanced sialyl Lewis X expression on adult T cell leukemic cells is due to induction of $FUT7$ transcription by the Tax protein, an oncogenic transcription activator encoded by the etiological virus HTLV-1. The induction was mediated by
phosphorylation-independent interaction of CREB/ATF-family transcription factors with the Tax protein.

We have now further investigated transcription factors involved in regulation of the FUT7 gene. The 5′-regulatory region of FUT7 turns out to be equipped with binding sites for multiple transcription factors, including Sp1, MZF-1, CREB/ATF, GATA-3 and T-bet. Among these factors, only GATA-3 repressed, while the other promoted, FUT7 transcription. All formed transcription factor complexes on the FUT7 promoter; GATA-3 interacted with HDAC-3/-5, T-bet and CREB/ATF, and T-bet interacted with GATA-3 CBP/P300 and Sp1. The GATA-3/ T-bet interaction most dynamically regulated composition of the transcriptional complex on the FUT7 promoter. GATA-3 repressed FUT7 transcription through phosphorylation-dependent interaction with HDAC-3 and -5, while T-bet promoted its transcription through recruiting CBP/P-300.

T-bet interfered with binding of GATA-3, which in turn interfered with binding of T-bet to the FUT7 promoter as evidenced by chromatin immunoprecipitation assays. Since GATA-3 and T-bet are two opposing factors in T-helper-1 (Th1) and T-helper-2 (Th2) development, this well explains the preferential expression of sialyl Lewis X in Th1 compared to Th2 cells. In addition, we have generated evidence implying that HIF (hypoxia inducible factor) also binds to the FUT7 promoter. This would explain the hypoxia-induced expression of sialyl Lewis X in cancer cells, as described in our previous report.

*1 Division of Molecular Oncology, Aichi Cancer Center.

*2 Department of Dermatology, Hospital del Mar, IMAS, Barcelona, Spain.

6.2. Identification of cutaneous lymphocyte antigen as sialyl 6-sulfo-Lewis X, a selectin ligand expressed on a subset of skin-homing helper memory T cells

Ohmori, K.*1, Sakuma, K., Kiso, M.*2, Imai, T.*3, Yoshie, O.*4, Hasegawa, H.*5, Matsushima, K.*6 and Kannagi, R.

We previously identified the carbohydrate determinant sialyl 6-sulfo Lewis X as the major L-selectin ligand on high endothelial venules of peripheral lymph nodes. In recent studies, we examined the distribution of the sialyl 6-sulfo Lewis X determinant among peripheral lymphocytes. It was thereby found to be expressed on a subset of helper memory T and NK cells. Helper memory T cells expressing sialyl 6-sulfo Lewis X were CD45RObright+PSGL-1high+CCR4+L-selectin+CCR7+ but lacked α4β7 integrin and CCR9, indicating that they were the skin-homing population of central memory T cells. The T-cell subset significantly expressed mRNAs for 6-sulfotransferase HECGlcNAc6ST and fucosyltransferase Fuc-T VII, responsible for the synthesis of sialyl 6-sulfo Lewis X. Characteristics of the T-cell population were similar to those previously described for cutaneous lymphocyte-associated antigen (CLA)–positive T cells defined by the HECA-452 or 2F3 antibodies. Binding of the T-cell subset with the specific anti–sialyl 6-sulfo Lewis X antibody G152 was almost completely abrogated by HECA-452 or 2F3. Binding of recombinant E-, P-, and L-selectins to the T-cell subset was also sig-
nificantly inhibited by G152 and by HECA-452 antibodies. We propose that CLA, which is expressed without any activation stimuli on peripheral skin-homing helper memory T cells in healthy persons, is at least partly the sialyl 6-sulfo Lewis X determinant.

*1 Kyoto University, School of Medicine.
*2 Gifu University, School of Agriculture.
*3 Kan Research Institute.
*4 Kinki University, School of Medicine.
*5 Ehime University, School of Medicine.
*6 Tokyo University, School of Medicine.

6.3. Identification of α2-6 sialylated 6-sulfo-LacNAc as a preferred ligand for CD22/siglec-2 on human B-lymphocytes

Kimura, N., Ohmori, K. *1, Miyazaki, K., Izawa, M., Matsuzaki, Y. *2, Yasuda, Y. *2, Takematsu, H. *3, Kozutsumi, Y. *3, Moriyama, A. *4, and Kanagi, R.

CD22/Siglec-2, an important inhibitory co-receptor on B-lymphocytes, is known to recognize α2-6-sialylated glycans as a specific ligand. Here we propose that the α2-6-sialylated 6-GlcNAc-sulfated determinant serves as a preferred ligand for CD22, because binding of a human B-cell line to CD22 was almost completely abrogated after incubation with NaClO₃, an inhibitor of cellular sulfate metabolism, and also significantly inhibited with a newly generated monoclonal antibody specific to the α2-6-sialylated 6-sulfo-LacNAc determinant (KN343, murine IgM). The α2-6-sialylated 6-sulfo-LacNAc determinant defined by the antibody was significantly expressed on a majority of normal human peripheral B-lymphocytes as well as follicular B-lymphocytes in peripheral lymph nodes. The determinant was also expressed by endothelial cells of high-endothelial venules of secondary lymphoid tissues including lymph nodes, tonsils and gut-associated lymphoid tissues, more strongly than on B-lymphocytes, suggesting a role for CD22 in B cell interaction with blood vessels and trafficking. These results indicate that the α2-6-sialylated 6-sulfo-LacNAc determinant serves as an endogenous ligand for human CD22, and provide evidence that 6-GlcNAc sulfation as well as α2-6 sialylation may regulate CD22/Siglec-2 functions in humans.

*1 Kyoto University, School of Medicine.
*2 Central Research Laboratories, Seikagaku Corporation.
*3 Kyoto University, School of Pharmaceutical Sciences.
*4 Nagoya City University, School of Natural Science.

6.4. Alpha-internexin as a corneal autoantigen in a spontaneous auto-immune keratitis mouse model

Hattori, T. *1, Takeuchi, M. *1, Usui, M. *1 and Taguchi, O.

The purpose of this study was to identify target antigens of autoimmune keratitis in a disease-prone mouse model. BALB/c nude mice grafted with embryonic rat thymi (TG nude mice) develop various organ-localized autoimmune lesions, including keratitis. A hybridoma producing a monoclonal antibody...
(OT-20), specific for corneal epithelium was established using spleen cells from this mouse model of keratitis, and the target of OT-20 was identified by immunoblot analysis. T-cell proliferation and cytokine production by TG nude mice with keratitis were then examined. Immunoblot analysis revealed alpha-internexin to be the target antigen of OT-20 that specifically recognizes corneal epithelium. Sera from TG nude mice with keratitis reacted with alpha-internexin on Western blot analysis, and the T cells of these mice on stimulation with alpha-internexin exhibited proliferation responses and produced IL-2, IFN-gamma and TNF-alpha, but not IL-4 or IL-5. These results suggest that alpha-internexin is one of the corneal antigens associated with keratitis developing spontaneously in TG-nude mice, with a probable pathogenic role.

*1 Department of Ophthalmology, Tokyo Medical University, Tokyo.
From left to right
First row: Dr. M. Ibi, Ms. Y. Takada, Dr. Y. Ohmuro, Ms. T. Yuhara
Second row: Dr. T. Shiromizu, Dr. Y. Ikegami, Dr. Y. Hayashi, Dr. H. Goto
Third row: Dr. M. Inagaki, Dr. K. Kasahara, Mr. M. Enomoto, Dr. I. Izawa, Dr. A. Inoko
Inset: Dr. A. Kawajiri, Dr. P. Zou, Dr. T. Yamaguchi, Dr. T. Oguri
Division of Biochemistry

Masaki Inagaki, M.D. Chief
Ichiro Izawa, M.D. Section Head
Hidemasa Goto, M.D. Section Head
Akihito Inoko, M.D. Senior Researcher
Kousuke Kasahara, Ph.D. Researcher (as of April 2007)
Yuko Hayashi, Ph.D. Research Assistant
Peng Zou, M.D. Research Resident (until March 2007)
Takashi Siromizu, Ph.D. Research Resident (as of April 2006)
Miho Ibi, D.D.S. Research Resident (as of April 2007)

Visiting Trainees
Yuki Ohmuro, Ph.D. Research Resident of Foundation for Promotion of Cancer Research (as of April 2007)
Masato Enomoto, M.S. Department of Cellular Oncology, Graduate School of Medicine, Nagoya University (as of April 2006)
Yousuke Ikegami, M.D. Department of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences (as of April 2006)
Aie Kawajiri, Ph.D. Department of Pathology, Nagoya University School of Medicine (until March 2006)
Takashi Oguri, M.S. Department of Cancer Genetics, Nagoya University School of Medicine (until March 2006)
Takashi Shiromizu, M.S. Department of Cancer Genetics, Nagoya University School of Medicine (until March 2006)
Tomoya Yamaguchi, M.S. Department of Cancer Genetics, Nagoya University School of Medicine (until March 2007)

General Summary
Cells need to respond to environmental signals to proliferate in a coordinated fashion during development and differentiation. In epithelia, the architecture is also intimately involved in control of cell cycle machinery. Mutations in genes functioning in cell cycle control and the maintenance of tissue architecture lead to uncontrolled proliferation, genetic instability, and cell invasion (metastasis) in cancer cells. However, the precise mechanisms of carcinogenesis remain largely unknown.

Our research aim is to elucidate mechanisms by which the cell cycle (including cell cycle checkpoints) and tissue architecture (including the intracellular cytoskeletal network) are controlled. Our attention is focused on 2 specific areas: (1) Identification and functional analysis of protein kinases involved in cell cycle checkpoints; (2) Regulation of cytoskeletal proteins (especially intermediate filaments) and associated elements active in cell adhesion and determination of cell polarity.

1. Chk1 phosphorylation by cyclin-dependent kinase 1 controls mitotic entry through cytoplasmic sequestration of Chk1

Enomoto, M., Goto, H., Kasahara, K., Ikegami, Y., Yamaguchi, T., Shiromizu, T., Tomono, Y. ’1, Tsujimura, K. ’2, Kiyono, T. ’3 and Inagaki, M.

Chk1 is one of the most important players in genetic stability, disorders of which are often observed in cancer cells. In response to stalled replication and genotoxic stresses, Chk1 is phosphorylated at Ser317 and Ser345 by ataxia-telangiectasia mutated- and Rad3-related (ATR) and thereby activated. This prevents premature entry into mitosis. However, Chk1 dynamics in mitosis remain to a great extent unknown.

We recently reported Chk1 to be phosphorylated at Ser286 and Ser301 by cyclin-dependent kinase (Cdk) 1 during mitosis. In order to elucidate functional consequences, we produced a rat monoclonal antibody which specifically recognizes Chk1 phosphorylated at Ser301. Immunocytochemical analyses using this antibody revealed Chk1-Ser301 phosphorylation from entry into mitosis (prophase). The level of phosphorylation appeared to peak at
metaphase and to gradually decrease after anaphase. In prophase when chromosome condensation occurs without nuclear envelope breakdown, we observed two phosphorylation patterns. At earlier stages, the phosphorylation tended to be observed mainly in the nucleus. However, at later stages of prophase, Chk1 phosphorylated at Ser301 appeared to exist mainly in the cytoplasm. This translocation was inhibited by treatment with leptomycin B, which blocks Crm1-mediated nuclear export. To further address the role of mitotic Chk1 phosphorylation, we established HeLa cells in which Chk1 wild type (WT) or Chk1 mutated to Ala at Ser286 and Ser301 (S286A/S301A) were expressed in a tetracycline-dependent manner. Introduction of S286A/S301A caused significant delay in mitotic entry, compared with the WT case. In S286A/S301A mutant prophase cells, staining was observed mainly in the nucleus although in WT cells location was also found in the cytoplasm. GST-pull down assays revealed that Chk1 could bind to 14-3-3 proteins in a Ser301- phosphorylation-dependent manner. These results suggested that Cdk1 controls the cytoplasmic sequestration of Chk1 likely through binding to 14-3-3 proteins. We propose that this pathway is a key control mechanism for maintaining the G2/M transition.

2. Spatial regulation of Chk1 phosphorylation in checkpoint response


Precise chromosome duplication is critical to cellular functions, and errors in the process can result in aneuploidy, which is often observed in cancer cells. Cells respond to DNA damage and replication blocks by activating a checkpoint network, a system which arrests the cell cycle and facilitates DNA repair. Chk1, one of the key players in the checkpoint system, is controlled by several protein kinases such as ATR and Cdk1. However, the roles of each phosphorylation site on Chk1 are not fully understood.

For analysis, we produced site- and phosphorylation state-specific antibodies for Ser296, Ser301 (a Cdk1 site), or Ser345 (an ATR site) on Chk1. Hydroxyurea (HU) treatment or ultraviolet (UV) irradiation were confirmed to induce Ser296 and Ser301 phosphorylation, together with Ser345 phosphorylation, by ATR. Treatment with UCN-01, a Chk1-specific inhibitor, reduced the phosphorylation at Ser296 but not at Ser345, suggesting that Ser296 may be an autophosphorylation site on Chk1. Interestingly, in response to HU treatment or UV irradiation, Chk1 phosphorylated at Ser296 or Ser345 formed foci in the nucleus, but the foci didn’t completely overlap with each other. These results imply that Chk1 function may be spatially regulated by phosphorylation at different sites in the checkpoint response. Now, we are further analyzing the specific roles of each site phosphorylation in checkpoint responses.

*1 Division of Molecular and Cell Biology, Shigei Medical Research Institute
*2 Department of Infectious Diseases, Hamamatsu University School of Medicine
*3 Virology Division, National Cancer Center Research Institute

3. Palmitoylation of ERBIN is required for its plasma membrane localization

Izawa, I., Nishizawa, M., Hayashi, Y. and Inagaki, M.

LAP (leucine-rich repeats (LRR) and PSD-95/Dlg/ZO-1 (PDZ)) family proteins, including Scribble, LET-413, ERBIN, Densin-180, and Lano, are involved in the regulation of cell polarity. Drosophila Scribble is a cell-junction localized protein implicated in control of epithelial cell polarity and growth. In addition, it has been shown that Scribble mutants cooperate with oncogenic Ras, Raf, or Notch to cause invasive and metastatic neoplasia in Drosophila larvae. The LRR domains of LAP proteins are reported to mediate basolateral membrane localization and to be essential for their function. To further dissect the mechanisms of plasma membrane localization of ERBIN, we introduced various mutants of ERBIN into cultured cells and observed the intracellular localization. When a LRR domain mutant lacking amino acid residues 1-32 at the amino (N) terminal region was overexpressed in cells, localization was in the cytoplasm, rather than at the plasma membrane. We found that cysteines 14 and 16 in the N-terminal region of ERBIN are palmitoylated and that this palmitoylation is required for its plasma membrane localization.
localization. The overexpressed 1-196 amino acids of ERBIN, without the latter half of LRR, were palmitoylated but did not localize at the plasma membrane, suggesting that both palmitoylation and LRR are required for the localization. Overexpression of wild-type ERBIN in Neuro2a cells resulted in formation of cellular processes, lacking in the mutant in which cysteines 14 and 16 were changed to serines. These results indicate that the palmitoylation of ERBIN is critical for its biological function.

4. Trichoplein and Albatross: unexpected passengers among keratin filaments, centrioles and cell-cell contacts

Inoko, A., Zou, P., Ohmuro-Matsuyama, Y., Shiromizu, T., Ibi, M., Hayashi, Y., Yonemura, S.*1, Nakayama, M.*2, Kaibuchi, K.*2, Kiyono, T.*3, Izawa, I. and Inagaki, M.

To reveal the function of cytoskeleton elements particular to epithelial cells, we have searched for binding partners with keratin 8/18 filaments and recently identified two novel keratin filament-binding proteins: trichoplein and Albatross. Trichoplein was found to localize not only on keratin filaments but also on mother centrioles. Knockdown in HeLa cells revealed that trichoplein is essential for anchoring of microtubules and ninein on appendages of mother centrioles. In addition, overexpression and knockdown of keratin in SW13 of keratin-deficient cells and T24 bladder carcinoma cells, respectively, revealed that keratins promote the accumulation of associated trichoplein on themselves. Furthermore, we found that trichoplein localizes even on desmosomes in the small intestine and T84, colon cancer, cells. Experiments are now ongoing to clarify the function of trichoplein at cell-cell borders with trichoplein stable knockdown T84 cells.

With Albatross, localization is not only on keratin filaments but also in the vicinity of the apical junctional complex (AJC), a cell-cell adhesive apparatus composed of tight junctions, adherens junctions and desmosomes. Knockdown in A549, lung adenocarcinoma, cells revealed that Albatross regulates the formation of AJC and lateral domains in cells. In addition, we found that keratin promotes this function, using keratin-rescued SW13 cells.

Furthermore, we found that Albatross also localizes even on centrioles and clarification of functions in this site is now being targeted with knockdown experiments.

Finally, we found that both trichoplein and Albatross have a similar domain designated as the trichohyalin/plectin homology domain (TPHD), which binds to keratins. Screening is ongoing to find other TPHD proteins.

*1 RIKEN Center for Developmental Biology
*2 Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University
*3 Virology Division, National Cancer Center Research Institute
From left to right
First row: Ms. S. Matsumoto, Ms. N. Saito and S. Kitajima, D.D.S.
Second row: Dr. K. Ishizaki, Dr. Hid. Nakamura, Dr. Y. Yasui and Dr. H. Kumi moto
Insets: H. Furue, D.D.S. and Dr. K. Ijichi
General Summary

One of our main research projects is molecular genetic analysis of human esophageal and oral tumors. An especial focus is on mutations in mitochondrial DNA and we have found an increased mutation frequency in Japanese esophageal tumors. To ascertain the mechanisms responsible for mitochondrial mutations in tumor cells, we are also studying X-ray induced mutations. So far we have obtained results suggesting that tumor cells with p53 mutations exhibit high frequencies of induced mutations in mitochondrial DNA.

Another research project is the study of genetic effects of low-dose-rate radiation (LDR) on human cells. For this purpose we have established immortal cell lines derived from normal individuals and patients with ataxia telangiectasia (AT), NBS, or Artemis deficiency. The Artemis and NBS genes are known to function downstream of the AT gene. Using these cell lines we have revealed that cytotoxic effects and mutation induction by LDR in normal cells is much lower than with high-dose-rate radiation (HDR) but that AT cell lines exhibit similar radiation sensitivity independent of the dose-rate. In contrast, NBS and Artemis deficient cells show higher resistance to LDR than to HDR while exhibiting the same sensitivity to HDR as AT cells. Using phosphorylated H2AX foci as indicators of DNA double strand breaks (DSBs), we have demonstrated that AT cells exhibit a partial but significant defect in the repair of DSBs, while NBS and Artemis deficient cells feature only a minor repair deficiency after LDR.

1. Mitochondria DNA instability due to ionizing radiation
Kumimoto, H., Saito, N. and Ishizaki, K.

Previously we analyzed mutations in the D-loop region of mitochondrial DNA (mtDNA) in esophageal tumors and found frequent somatic mutations (in 34% of tumors) (Kumimoto et al. Int J Cancer, 108, 228-231, 2004). We also determined nuclear genomic instability, but did not find any correlation between somatic mtDNA mutations and nuclear genomic instability, suggesting that instability of mtDNA in esophageal cancer might be independent of nuclear genomic instability. To know whether mtDNA mutations could be induced by mutagenic agents such as X-ray, we screened for mtDNA mutations in 4 esophageal cancer cell lines, KYSE-30, 110, 150 and 410, and a normal cell line, SuSa/T-n, after X-ray irradiation. These cell lines were irradiated with X-ray at the doses giving survival rate of 0.01 in each cell line. After single-cell colonies were picked up, DNA was extracted. Since there are up to $10^3$ copies of mtDNA in each cell and the same mutations may not occur in all mtDNA, evaluation was conducted by assessing the ratio of mutated mtDNA to the total mtDNA. Since the D310 region which has a 7 continuous C stretch in mtDNA frequently showed 1 base insertion or deletion mutations, we analyzed this region by GeneScan. Colonies with more than
0.3 for the proportion of mutated mtDNA were defined as mutant colonies (Fig. 1). In SuSa/T-n, no change was observed in D310 length after X-ray irradiation, and two esophageal cancer cell lines, KYSE-150 and 410, also did not exhibit change alteration. On the other hand, 11.8% and 31.8% of colonies from irradiated cells of the esophageal cancer cell lines, KYSE-30 and 110, exhibited length changes in the D310 region. Since the p53 gene was mutated in these two mtDNA-mutation positive cell lines but not in the mtDNA-mutation negative cell lines, the p53 gene might play an important role in mtDNA stability.

2. Role of ATM in repair of the double-strand break induced by low-dose-rate radiation

Nakamura, Hid., Yasui, Y., Kitajima S., Saito, N. and Ishizaki, K.

Effects of low-dose-rate radiation (LDR) on cells derived from normal humans (Normal) and patients with ataxia-telangiectasia (AT), NBS (NBS1, NBS2 and NBS3) and Artemis (Artemis1 and Artemis2) deficiency were investigated. Non-proliferating cells were irradiated with LDR (0.3 mGy/min) continuously for up to 2 weeks or were exposed to high-
dose-rate radiation (HDR; 1 Gy/min). AT cells showed hypersensitivity to HDR radiation compared with normal cells, a property shared by NBS and Artemis cells. While the survival of normal cells after LDR radiation was significantly higher than that after HDR radiation, AT cells showed essentially similar survival in both cases. In contrast, survival of Artemis deficient cells after LDR radiation was more resistant (Fig. 2). Surprisingly, the survival of NBS cells after LDR radiation was to that of normal cells (Fig. 3). Although only a few γH2AX foci were observed in LDR-irradiated normal cells, significant numbers remained in AT cells. In Artemis deficient cells, numbers of γH2AX foci after LDR irradiation were about half those in AT cells. Only a few foci were observed in the case of LDR irradiated NBS cells. These results suggest that ATM plays an important role in repairing the double-strand breaks (DSBs) induced by LDR radiation. Moreover, they indicate that ATM might activate not only Artemis but also other factors in the DSB-repair pathway. On the other hand, functions of NBS in cells irradiated with LDR might differ from those in HDR-irradiated cells.

3. Lethal effects of a heavy-ion beam on AT-heterozygote cells

Kitajima, S., Nakamura, Hid., Yasui, Y. and Ishizaki, K.

It is well recognized the heavy-ion radiotherapy is effective for cancer. However, it is not clear what effects beam exposure might have on AT-heterozygotes, which are thought to account for about 1% of the population. In this study, we analyzed effects of carbon and iron-ion beams with various energy levels (expressed as LET) on AT-heterozygote cells. LETs of the heavy-ion beam used were 24, 40, 50, 60 and 200 keV/µm. Survival was determined by colony formation assay, and relative biological effectiveness (RBE) was obtained (Fig.4). To calculate the RBE, we used the radiation dose which gave a survival rate of 0.1 (D_{10}) after X-rays and heavy ions, and the D_{10} for X-rays was divided by that for the heavy-ion beams with various LETs. In a normal cell line, RBE was flat from 24-40 keV/µm but increased LET-dependently above 40 keV/µm. In contrast, although RBEs of AT-heterozygote cell lines at 24 keV/µm were similar to that of normal cells, increase LET-dependently was noted above 24 keV/µm. In an AT cell line, the RBE increased from 24-40 keV/µm. However, above 40 keV/µm, a plateau was evident. These results suggest that ATM heterozygosity could influence cellular survival after heavy-ion beam therapy. We now need to determine the mechanisms underlying the heavy-ion sensitivity of AT-heterozygotes.
From left to right,
First row: Dr. M. Yamamoto and Ms. M. Nishizawa
Second row: Dr. Hir. Nakamura and Mr. Y. Minoura
Inset: Mr. Har. Tanaka
The Central Service Unit fulfills many functions in assisting the investigations performed by the Institute and has responsibilities for the maintenance and operation of various instruments for biotechnology research. These include a DNA sequencer (ABI 3100), two flow-cytometers (Becton-Dickinson FACS Calibur), imaging analyzers (Fujix BAS-2500Mac, Amersham-Pharmacia ImageMaster-CL and FluorImager-595), two X-ray machines (Hitachi MBR-1520R3), a scanning electron microscope (Hitachi SEM), two real-time PCR set-ups (ABI 7500 Fast Real-Time PCR System and Roche Light Cycler), seven ultracentrifuges (Beckman-Coulter and Hitachi), a microplate reader (TECAN GENios) and computer systems for image analysis (Windows and Macintosh systems).

Furthermore, we maintain and manage the radioisotope experimental facilities, SPF and conventional animal rooms, laboratories for translational research, a hazardous chemical storehouse, ultra-low temperature freezers, cold rooms, a liquid nitrogen storage room, security systems, air-conditioning, and water purifying and waste water treatment systems, as well as a carbon dioxide gas supply, thereby contributing to many other of the Institute’s functions. During the last two years we replaced several instruments such as a confocal laser microscope (Carl-Zeiss LSM510 META), and an X-ray machine for SPF animals (Hitachi MBR-1520R3) and have held technical seminars on advanced biotechnology. Our activities thus provide essential background support for all of the research carried out by the Research Institute.
From Left to right
First row: Ms. T. Yasuda, Ms. T. Shibata
1. Roles of mitotic kinases in carcinogenesis


Appropriate chromosome duplication cycles are critical to cellular function, and mitosis is the final step in which duplicated chromosomes are divided into two daughter cells correctly. Errors in mitosis can result in aneuploidy, which is often observed in cancer cells. Mitotic chromosomal dynamics are regulated by the coordinated activities of many mitotic kinases, such as cyclin-dependent kinase 1 (Cdk1), Aurora-B or Polo-like kinase 1 (Plk1), but mechanisms of coordination remain largely unknown. We have found two novel examples of signaling crosstalk among mitotic kinases.

(1) Complex Formation of Plk1 and INCENP Required for Metaphase-Anaphase Transition

We have established that Cdk1 phosphorylates Thr59 and Thr388 on the inner centromere protein (INCENP), which regulates localization and kinase activity of Aurora-B in the transition from prophase to metaphase. INCENP depletion disrupts Plk1 localization specifically at the kinetochore. This phenotype is rescued by the exogenous expression of INCENP wild type (WT) and INCENP mutated at Thr59 to Ala (T59A), but not at Thr388 to Ala (T388A). Replacement of endogenous INCENP with T388A results in delay of progression from metaphase to anaphase. We propose that INCENP phosphorylation by Cdk1 is necessary for recruitment of Plk1 to the kinetochore, and that complex formation of Plk1 and Aurora-B on INCENP may play critical roles in the regulation of chromosomal dynamics.

(2) Chk1 phosphorylation by cyclin-dependent kinase 1 controls mitotic entry through cytoplasmic sequestration of Chk1

We recently reported Chk1 to be phosphorylated at Ser286 and Ser301 by cyclin-dependent kinase (Cdk) 1 during mitosis. In order to elucidate functional consequences, we produced a rat monoclonal antibody which specifically recognizes Chk1 phosphorylated at Ser301. Immunocytochemical analyses using this antibody revealed Chk1-Ser301 phosphorylation from entry into mitosis (prophase). The level of phosphorylation appeared to peak at metaphase and to gradually decrease after anaphase. In prophase when chromosome condensation occurs without nuclear envelope breakdown, we observed two phosphorylation patterns. At earlier stages, the phosphorylation tended to be observed mainly in the nucleus. However, at later stages of prophase, Chk1 phosphorylated at Ser301 appeared to exist mainly in the cytoplasm. This translocation was inhibited by treatment with leptomycin B, which blocks Crm1-mediated nuclear export. To further address the role of mitotic Chk1 phosphorylation, we established HeLa cells in which Chk1 wild type (WT) or Chk1 mutated to Ala at Ser286 and Ser301 (S286A/S301A) were expressed in a tetracycline-dependent manner. Introduction of S286A/S301A caused significant delay in mitotic entry, compared with the WT case. In S286A/S301A mutant prophase cells, staining was observed mainly in the nucleus although in WT cells location was also found in the cytoplasm. GST-pull down assays revealed that Chk1 could bind to 14-3-3 proteins in a Ser301-phosphorylation-dependent manner. These results suggested that Cdk1 controls the cytoplasmic sequestration of Chk1 likely through binding to 14-3-3 proteins. We propose that this pathway is a key control mechanism for maintaining the G2/M transition.
2. Array CGH analysis of malignant lymphomas and its application for molecular diagnosis

Tagawa, H.

Array comparative genomic hybridization (array CGH) analysis has identified frequent 13q31 amplification in diffuse large B-cell lymphomas (DLBCLs) and Burkitt's lymphomas. We found the C13orf25/miR-17 cluster to include the responsible gene in this region and could show that C13orf25 contains the microRNA-17-18-20-92 polycistron. We further demonstrated that MYC and the miR-17 cluster synergistically contribute to cancer development from rat fibroblast (Rat-1) transfectants through agarose colony formation assays as well as in nude mice. In addition, we identified TGFβRII as the true target for the miR-17 cluster. In an attempt to apply array CGH data for molecular diagnosis, we examined whether genomic copy number gains and losses detected in this way could be used to differentiate lymphoma diseases or subtypes. Copy number gains and losses in 46 DLBCLs and 29 mantle cell lymphoma (MCL) were assessed by array CGH and gene expression in the DLBCL cases was profiled. Hierarchical clustering revealed 28 to be of activated B-cell (ABC) type while 18 were of germinal center-B-cell (GCB) type. A computer algorithm was developed to classify lymphoma diseases or subtypes on the basis of copy number gains and losses whose application allowed correct classification of the DLBCL and MCL subtypes in 89% of cases, and of ABC and GCB subtypes in 83%. These results demonstrate that copy number gains and losses detected by array CGH can be used for classifying lymphomas into biologically and clinically distinct diseases or subtypes.
Journals


J115. Matsubara, H., Takeuchi, T., Nishikawa, E., Yanagisawa, K., Hayashita, Y., Ebi, H.,


J157. Oshima, H., Matsunaga, A., Fujimura, T.,


Ozaki, Y., Sawai, S., Tezuka, N., Fujino, S., Itoh, Y., Taguchi, O., Kannagi, R. and Ogasawara, K.: Successful tumor eradication was achieved by collaboration of augmented cytotoxic activity and anti-angiogenic effects following therapeutic vaccines containing helper-activating analog-loaded dendritic cells and tumor antigen DNA. Cancer vaccines containing helper-activating analog-loaded anti-angiogenic effects following therapeutic collaboration of augmented cytotoxic activity and


K., Tokunaga, K., Inoko, H., Saji, H., Ogawa, S., Juji, T., Sasazuki, T., Kodera, Y., and Morishima, Y.: Donor activating killer immunoglobulin-like receptor (KIR) genotype and the prophylactic ATG pre-administration are the critical factors for the adverse effect of the HLA-C-KIR ligand mismatch on acute graft-versus-host disease (aGVHD) in unrelated T cell-replete HLA-A,-B,-DR-matched bone marrow transplantation. Biol Blood Marrow Transplant, 14: 75-87, 2007. (PMID: 18158964)


Reviews and Books


Abstracts for international conferences


A052. Nakagawa, M., Nakagawa-Oshiro, A,


Record of Seminars

Invited Speakers

2006


Jun. 30 Urano, T. (Dept. of Molecular Biochemistry, Nagoya University Graduate School of Medicine): Ca2+/calmodulin regulates Aurora-A kinase activity at anaphase.

July 24 Oshima, M. (Division of Genetics, Cancer Research Institute, Kanazawa University): Gastric tumorigenesis through cooperation of Wnt signaling and COX-2/PGE2 pathway.


Oct. 04 Shen, L. (Department of Leukemia, MD Anderson Cancer Center): CpG island methylation is a poor prognostic factor in myelodysplastic syndrome patients and is reversed by Decitabine therapy-Results of a Phase III randomized study.

Oct. 26 Hirano, N. (Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School): Long-lived CTL generated using artificial antigen-presenting cells: Translational cancer research from bench to bedside.

Oct. 31 Riddell, S. R. (Fred Hutchinson Cancer Research Center, University of Washington): Establishing T cell memory by adoptive transfer of T cell clones.

Nov. 28 Rickinson, A. (Cancer Research UK Institute for Cancer Studies, University of Birmingham): T cell responses to Epstein-Barr virus infection.

Dec. 06 Takenawa, T. (Department of Biochemistry, The Institute of Medical Science, The University of Tokyo): Breakdown of regulation of cell motility and cancer invasion/metastasis.

2007

Mar. 05 Hoffman, R. M. (Department of Surgery, University of California): The use of fluorescent protein imaging to visualize new cellular and subcellular targets for cancer chemotherapy in vivo.

May 09 Ohta, K. (Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo): ADLib system: rapid and flexible design of monoclonal antibodies by the chicken DT40 cell line.

May 17 Tanaka, H. (Department of Cancer Control and Statistics, Osaka Medical Center for Cancer and Cardiovascular Diseases): Epidemiology of liver cancer and national policy for liver cancer control.
May 25 Eriksson, J. E. (Dept. of Biology, Abo Akademi University, Turku, Finland and Turku Center for Biotechnology, Univ. of Turku and Abo Akademi University): Intermediate filaments as signaling organizers.

Magin, T. M. (Institute for Physiological Chemistry, Division of Cell Biochemistry and LIMES, University of Bonn): Characterization of a novel multidomain protein involved in cell polarity and tight junction formation.

May 29 Yamaguchi, T. (Center for Experimental Medicine, Institute of Medical Science, University of Tokyo/ Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science And Technology): Establishment of transgenic mice that express SV40 large T antigen by Cre/loxP recombination and applications for immortalized blood/lymphatic endothelial cells.

Jun. 28 Matsuyama, M. (Center for Developmental Biology, RIKEN Kobe): The role of secreted frizzled-related protein (Sfrp) in the Wnt signaling pathway.

Aug. 08 Minami, M. (Division of Hematology-Oncology, Department of Medicine and Moores - UCSD Cancer Center, University of California San Diego School of Medicine, USA): BCR-ABL-transformed leukemia stem cells display innate resistance to imatinib.

Sep. 10 Cavalli, F. (Oncology Institute of Southern Switzerland, and President of UICC): Extranodal lymphomas in Europe/Lugano lymphoma conference; history and future.

Institute Speakers

2006


Feb. 16 Yamaguchi, T. (Biochemistry): Phosphorylation by Cdk1 induces Plk1-mediated vimentin phosphorylation during mitosis.


Mar. 23 Nakamura, Hir. (Central Laboratory & Radiation Biology): Summary of my research work.


July 31 Cao, X. (Oncological Pathology): Chemoprevention of N-methyl-N-nitrosourea induced gastric cancer using Helicobacter pylori infected Mongolian gerbils.


2007

Jan. 31  Chen, G.-Y. (Molecular Pathology): Transcriptional regulation of sialyl Lewis X expression.

Feb. 15  Peng, Z. and Inoko, A. (Biochemistry): Unexpected correlations among keratins, centrosomes and apical junctional complex - A novel mechanism for cancer growth which is different from EMT?-

Mar. 05  Akatsuka, Y. (Immunology): From bed to bench to cell processing facility, and finally to bed.


Aug. 08  Nakanishi, H. (Oncological Pathology): Basic research and clinical application of micrometastasis of gastrointestinal malignancy.

Sep. 13  Fujii, M. (Molecular Oncology): Enhancement of c-Myc transcriptional activity by SNIP1 (Smad Nuclear Interacting Protein 1).

Oct. 30  Nakagawa, M. (Molecular Medicine): Subtyping of peripheral T-cell lymphoma, unspecified, by array CGH.

Record of Symposia

The 12th Aichi Cancer Center International Symposium
“Perspective of Oncological Strategy for Gastrointestinal Cancer”

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Program of symposium

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Integrated genetic and epigenetic analysis identifies colon cancer as three different diseases

Jean - Pierre Issa
M. D. Anderson Cancer Center, Houston, Texas, USA

Background: Colon cancer affects 6 percent of the population in the US, and is one of the most common malignances. Colon cancer has been viewed as the result of progressive accumulation of genetic and epigenetic abnormalities. However, this view does not fully reflect the molecular heterogeneity of the disease.

Methods: We have analyzed both genetic (mutations of BRAF, KRAS and p53, as well as microsatellite instability) and epigenetic alterations (DNA methylation of 27 CpG island promoter regions) in a group of 97 primary colorectal cancer patients. Two clustering analyses on the basis of either epigenetic profiling or a combination of genetic and epigenetic profiling were performed to identify subgroups with distinct molecular signatures.

Results: Unsupervised hierarchical clustering of the DNA methylation data identified 3 distinct subgroups of colon cancers named CpG island methylator phenotype (CIMP) 1, CIMP2 and CIMP negative. Genetically, these three clusters correspond to very distinct profiles; CIMP1 cases are characterized by MSI (80%) and BRAF mutations (53%) but rare KRAS and p53 mutations (16% and 11%, respectively). CIMP2 is associated with 92% KRAS mutations but rare MSI, BRAF or p53 mutations (0%, 4% and 31% respectively). CIMP negative cases have a high rate of p53 mutations (71%) and lower rates of MSI (12%) or mutations of BRAF (2%) or KRAS (33%). Clustering based on both genetic and epigenetic parameters also identifies three distinct (and homogeneous) groups that largely overlap with the previous classification. The three groups are independent of age, gender or stage, but CIMP 1 and 2 are more common in proximal tumors.

Conclusion: Integrated genetic and epigenetic analysis reveals that colon cancers correspond to three molecularly distinct diseases. These diseases also differ in terms of histology, precursor lesion and clinical course, suggesting truly distinct pathogenesis.

DNA methylation as a marker for the past and future

Toshikazu Ushijima
Carcinogenesis Division, National Cancer Center Research Institute Tokyo, Japan

DNA methylation, a representative epigenetic modification, is stably inherited upon cell replication, and can ‘permanently’ repress gene transcription. In spite of the deep involvement of aberrant methylation in gastric cancers, its induction mechanisms have been unclear.

We quantified methylation levels of eight CpG islands (CGIs) of seven genes in the gastric mucosae of healthy individuals with and without Helicobacter pylori (HP) infection, a potent gastric carcinogen. HP infection was detected by the serum antibody test and/or the culture method, reflecting the current or recent infection status. It was found that methylation levels were 5- to 50-fold higher in individuals with current HP infection than those without. We further isolated 48 promoter CGIs that can be methylated in gastric cancers, and found that specific genes were methylated in individuals with HP. It was suggested that a field defect with inactivation of multiple genes was induced by HP infection and that this can be detected by methylation of specific genes.

Methylation levels of the initial eight CGIs were also analyzed in non-cancerous gastric mucosae of cases with a gastric cancer. Individuals with current HP infection showed high methylation levels regardless of the presence or absence of gastric cancers. In contrast, among individuals without current HP infection, cases with a gastric cancer had 2- to 20- fold higher methylation levels than healthy individuals. We further showed that the level of FLNc methylation had an increasing trend in the order of: healthy individuals, cases with a single gastric cancer, and cases with multiple gastric cancers. The risk given by high methylation levels was independent from that given by the extent of endoscopic atrophy. It was indicated that methylation levels in gastric mucosae of individuals without current HP infection can be used to estimate a future risk of gastric cancers.

It is generally known that, for specific cancers, DNA methylation of some genes in cancer tissues is associated with responses to chemotherapies and that of other genes is with patient prognosis. Our
study here showed DNA methylation in non-cancerous tissues is also useful, and might be related to methylation patterns in cancer tissues.

**HER family as potential molecular targets for anticancer therapy against gastrointestinal malignancies**

Hayao Nakanishi

*Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan*

HER family, a receptor tyrosine kinase including HER1 (EGFR), HER2 (neu/erbB2), HER3 (erbB3) and HER4 (erbB4), regulates essential cellular functions, such as growth, differentiation and apoptosis in epithelial cells, and their activation is associated with carcinogenesis and progression in various cancers including gastrointestinal malignancies. The binding of ligands to the extracellular region of HER family induces receptor homo- or hetero-dimerization and activation of cytoplasmic tyrosine kinase which in turn leads to autophosphorylation and initiation of downstream signaling. Recently, drugs targeting EGFR and HER2 is clinically used for lung and breast cancer patients, and their clinical efficacy has been proved. In gastrointestinal malignancies, a chimeric EGFR monoclonal antibody (mAb) (cetuximab) is approved for clinical application in metastatic colorectal cancer, but molecular targeting therapy for gastric cancer is still unavailable. We found that gastric cancer metastasized to the liver overexpresses HER2 at a significantly higher incidence than primary gastric cancers. We thus developed three new HER2 overexpressing gastric cancer cell lines (GLM-1, GLM-2, GLM-4) without EGFR mutations from such liver metastasis, two of which had HER2 gene amplifications. Interestingly, all these GLM series of cell lines were highly sensitive to gefitinib (Iressa) in vitro, a specific inhibitor of EGFR tyrosine kinase, whereas most of the HER2 low expressing counterparts were not. In these HER2 overexpressing GLM series, Akt, but not ERK1/2 was constitutively phosphorylated, and gefitinib efficiently inhibited this Akt phosphorylation, induced strong apoptosis in vitro and exhibited antitumor activity in subcutaneous (sc) tumor xenografts in nude mice. On the other hand, a humanized HER2 mAb (trastuzumab) only weakly inhibited growth of sc tumor xenografts, but showed a marked anti-tumor effect against peritoneal metastasis of HER2 overexpressing GLM-1 cells after intraperitoneal (ip) administration. In these GLM cell lines, no significant inhibition of Akt phosphorylation and induction of apoptosis were observed by trastuzumab treatment in vitro, whereas significant antibody-dependent cellmediated cytotoxicity (ADCC) was observed by trastuzumab treatment. These results suggest that the anti-tumor effects of gefitinib and the anti-metastatic (peritoneal) effects of trastuzumab against GLM cell lines are mainly due to the effective inhibition of HER2- driven constitutive activation of phosphatidylinositol-3-kinase (PI3K)/Akt pathway and induction of ADCC, respectively. In colorectal cancers, we found that intact EGFR expressing COLM-5 cells, a poorlydifferentiated colorectal cancer cell line with high metastatic potential established in our laboratory, showed super-sensitivity to both gefitinib and cetuximab in vivo. Primary sc tumor growth, lymph node metastasis and peritoneal metastasis were markedly inhibited by gefitinib and cetuximab monotherapy in some of the treated nude mice cured. However, no apparent apoptosis induction and inhibition of Akt and ERK1/2 phosphorylation were observed in vitro. Although the mechanism of the anti-tumor effect of gefitinib and cetuximab on COLM-5 cells remains unclear, these results suggest the possible presence of a subset of aggressive colorectal cancers with high sensitivity for EGFR targeting drugs. Gastric and colorectal cancers with HER family overexpression would therefore be potential targets for molecular therapy with gefitinib, cetuximab, trastuzumab and possibly other agents.

**Perspectives in pancreatic cancer chemotherapy**

Malcolm J. Moore

*Department of Medical Oncology & Hematology, Princess Margaret Hospital, Toronto, Canada*

Gemcitabine has represented the gold standard for palliative chemotherapy in advanced pancreatic cancer for over a decade. This benchmark was established by a phase III trial which randomized 126 symptomatic patients with advanced pancreatic cancer to either gemcitabine or 5-fluorouracil. Treatment with gemcitabine was associated with improvements in one-year survival (18% versus 2%) and clinical benefit response (24% versus 5%) over 5-FU. Since this trial was published in 1997, many large international phase III trials involving thousands of patients have been conducted comparing gemcitabine alone (as the reference
standard) to a gemcitabine combination with either a cytotoxic or molecularly targeted agent. Almost all have failed to meet the standard of improving overall survival.

Gemcitabine and 5-fluorouracil have been the most widely used chemotherapy agents in pancreatic cancer. It is rational, therefore, to combine these agents in the hopes of achieving benefit over either drug alone. SAKK/CECOG compared GemCap, the combination of gemcitabine and capecitabine, an oral prodrug of 5-FU, to gemcitabine alone in patients with locally advanced or metastatic cancer. Consistent with the majority of phase III trials in pancreatic cancer before it, this trial by Herrmann et al. failed to reach their primary endpoint; in this case, a 2-month improvement in median overall survival. ECOG 2297, enrolled 327 patients to standard doses of gemcitabine, given weekly 3 weeks out of 4, with or without 5-FU by bolus infusion at a dose of 600 mg/ m2 weekly in the same schedule. The median survival for gemcitabine alone was 5.4 months, compared to 6.7 months with gemcitabine and 5-FU (p=0.09). Modulating the effect of 5-FU, by the use of 24-hour infusion and leucovorin were not successful in improving efficacy when combined with gemcitabine. A phase III trial of 533 patients conducted in the UK comparing GemCap to gemcitabine alone has been reported in abstract form, suggesting a significant survival advantage [HR=0.80] to the combination therapy. Similarly studies of gemcitabine with oxaliplatin, irinotecan and pemetrexed have not shown benefit. After ten years of exhaustive clinical investigations with gemcitabine doublet regimens in advanced pancreatic cancers, the arguments in favor of combination chemotherapy are modest. The only positive study with gemcitabinecapecitabine is tempered by a similar study that was negative and several other negative gemcitabine-fluoropyrimidine studies.

A wave of new molecularly targeted agents has been emerging into the clinic and many more agents are in preclinical development. The combination of gemcitabine with molecularly targeted agents is a potentially more fruitful avenue of exploration. Encouraging results in phase II trials have been documented with EGFR antagonists, such as erlotinib and cetuximab, as well as antiangiogenic agents, such as bevacizumab. Other agents on the horizon include multitargeted tyrosine kinase inhibitors, such as sorafenib and sunitinib, inhibitors of the mammalian target of rapamycin (mTOR), such as RAD001 and temsirolimus, and Src kinase inhibitors. Gemcitabine and erlotinib, an orally available antagonist of the epidermal growth factor receptor (EGFR), was the first combination regimen to demonstrate statistically significant superiority in terms of overall survival over gemcitabine alone. The hazard ratio was 0.81 which translates to an overall 23% improvement in survival; in absolute terms this translated to an average improvement in survival of 5 weeks. Further analyses suggest there is a subset of patients with pancreatic cancer who receive a greater benefit although at this time we cannot prospectively identify these patients.

Phase II trials of novel agents need to continue and more resources need to be devoted to the development of new agents. These trials also provide the platform for necessary translational research to identify predictive biomarkers for these new agents. By returning to the benchtop from the bedside, we might be able to establish an enriched subset of patients who might benefit from any of a number of available combination therapies. To date, such predictive biomarkers have been elusive for EGFR antagonists and antiangiogenic agents. However, with the use of innovative strategies, such as functional imaging, genomics and other related technologies, we may finally move toward truly individualized and targeted treatment for patients with advanced pancreatic cancer.

Takuji Okusaka
Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, Tokyo, Japan

Despite recent advances in imaging modalities, most patients with pancreatic cancer are surgically unresectable at the time of their diagnosis and have an extremely poor prognosis. Even for those who undergo resections, the risk of recurrence is exceedingly high, and patient outcome remains unsatisfactory. Therefore, to improve the prognosis of pancreatic cancer patients, the development of effective non-surgical treatments for this disease is essential.

Gemcitabine, a deoxycytidine analogue, showed a significant impact on survival and clinical benefit response, such as pain alleviation and improved performance status, in a randomized trial comparing it to 5-FU. However, there is still substantial room for improvement in chemotherapy for pancreatic
cancer, because gemcitabine can only confer a limited survival benefit. S-1, an oral fluoropyrimidine-based antineoplastic agent consisting of the fluorouracil (5-FU) prodrug tegafur combined with two modulators, gimeracil and potassium oxonate, showed a promising result in our early and late phase II studies. Furthermore, S-1 may be useful in combination with gemcitabine, because the toxicity of S-1 was generally mild and its profile was distinct from that of gemcitabine. Currently, we are conducting a multi-institutional phase II study of this combination treatment for metastatic pancreatic cancer.

A pharmacokinetics study for gemcitabine in Japanese cancer patients showed that carriers of the cytidine deaminase *3 allele had a reduced gemcitabine clearance and a corresponding increase in the neutrophil reduction ratio. Evolving understanding of molecular and genetic biology should facilitate research to establish individualized therapy regimens and to develop novel non-surgical treatment for this disease.

The future direction of chemotherapy for pancreatic cancer

Akira Sawaki

Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Japan

Pancreatic cancer is a devastating illness that has the briefest survival of any solid tumor, accounting for approximately 20,000 deaths per year in Japan. The efficacy of gemcitabine was reported by significantly prolonged overall survival demonstrating the existence of a subpopulation of pancreatic cancer patients who benefit from chemotherapy. Prediction of treatment outcome helps tailor more effective treatment strategies and is important to avoid over-treatment. We mention the possibility of tailor made treatment according to the readily available clinical data and molecular processes associated with the development and progression of the disease.

We investigated pre-treatment characteristics and computerized tomography findings in patients, in order to identify the most effective readily available prognostic factors in predicting survival for metastatic pancreatic cancer patients. Multivariate analysis identified performance status, primary tumor location, and C-reactive protein as important independent predictive factors. Patients were divided into three groups according to the score based on coefficients of the Cox model. The internally validated cindex (receiver operating characteristics area under the curve) of this model was 0.711. Applied to another data set, the externally validated c-index was 0.692. This index improved predictive ability in patients with metastatic pancreatic cancer treated with gemcitabine.

We describe the preliminary results of expression of multiple genes in pancreatic cancer with DNA microarray systems. EUS-FNA, which is established as the preferred method to confirm a diagnosis of pancreatic cancer, can also be used to provide pancreatic cancer tissue for microarray analysis. Total mRNA was harvested from each sample which was amplified to provide adequate mRNA for the analysis. The patient with the highest dCMP deaminase or RRM1 resulted in progressive disease (PD) for gemcitabine. DNA microarray-based gene expression profiling combined with EUSFNA may be a promising tool to predict chemoresistance in advanced pancreatic cancer. The rapidly evolving understanding of the molecular biology of pancreatic cancer may contribute to the development and use of targeted therapies with novel agents for even more effective treatments in the near future.

Current thoughts on the role of chemotherapy and radiation in advanced head and neck cancer

Carol R. Bradford

University of Michigan Medical School and Comprehensive Cancer Center, Ann Arbor, Michigan, USA

The paradigm for the management of advanced squamous cell carcinoma of the upper aerodigestive tract has shifted from a predominantly surgically-based treatment approach to an organ preservation approach that utilizes combinations of chemotherapy and radiation. This approach has been shown to be a valid approach in which organ preservation is feasible and can be attached without sacrificing the potential for meaningful survival. Careful pretreatment clinical, radiographic, and endoscopic assessments of these patients are essential for accurate tumor staging. Treatment strategies focusing on the integration of chemotherapy and radiation will be discussed including the role of induction chemotherapy and tumor response assessment. One of the critical issues is the post-treatment surveillance of these patients. The management of advanced nodal
disease in this patient population will be described including the indications and timing of salvage neck dissection. The high incidence of postoperative complications with salvage surgery for local-regional recurrence is mitigated by the use of free tissue transfer. Careful attention should be paid to quality of life and functional outcomes following surgical versus nonsurgical approaches. Targeted treatment using intensity modulated radiation therapy (IMRT) to spare saliva (parotid) function and the pharyngeal constrictors has resulted in improved swallowing results. Our present approach for advanced cancers of the oropharynx is concomitant carboplatin, docetaxel, and IMRT. Our approach for advanced laryngeal cancers not amenable to surgical organ preservation is induction chemotherapy (cisplatin/5-fluorouracil) followed by concomitant cisplatin and radiation therapy in responders. Biomarkers such as p53 and Bcl-xL have proven important for predicting response to organ preservation approaches. In oropharynx cancers, presence of human papillomavirus portends an excellent prognosis whereas overexpression of epidermal growth factor receptor portends a poor prognosis. Patients who continue to smoke ("current smokers") have a poorer outcome than patients who have quit and never smokers.

**Prediction of chemosensitivity for head and neck cancer**

Tetsuya Ogawa

*Department of Head and Neck Surgery, Aichi Cancer Center Hospital, Nagoya, Japan*

**Introduction:** Head and neck squamous cell carcinoma (HNSCC) is classified as a tumor with high sensitivity to anti-cancer agents. From the viewpoint of clinical efficacy, however, chemotherapy for head and neck tumors remains challenging. Because conventional clinical and pathologic parameters cannot be used to accurately predict the response to chemotherapy or disease outcome for patients with HNSCC, there exists a great need to identify new markers with which to define the subset of patients who will respond to chemotherapy. To help establish order-made cancer chemotherapy, we performed multigene analysis to identify predictive markers for response to chemotherapy in patients with HNSCC.

**Method:** Patients undergoing radical treatment for HNSCC at the Department of Head and Neck Surgery, Aichi Cancer Center Hospital were included in the present study, and tumor specimens were collected from surgery or biopsy. 1: The chemosensitivity of surgically respected specimens was investigated in vitro using histoculture drug response assay (HDRA) for 5-fluorouracil (5-FU) and cisplatin, respectively. TS and DPD activities were also measured. 2: Biopsy specimens were taken from patients before administration of induction chemotherapy with 5-FU and cisplatin, and their clinical responses were estimated. 3: Using specimens collected from both surgery and biopsy, we subsequently analyzed the mRNA expression levels of 13 markers that we thought were likely predictors of response to anti-cancer agents. These mRNA expressions were quantified by real-time reverse transcription polymerase chain reaction (real-time RT-PCR) assay, after which we investigated the associations of these mRNA expression levels with chemosensitivity in the HDRA, TS and DPD activities, and clinical response, respectively.

**Results:** We found that HER2 mRNA expression level was inversely correlated with 5-FU and cisplatin sensitivity in the HDRA, respectively. An inverse correlation was also found between beta-tubulin expression level and cisplatin sensitivity in the HDRA. Moreover, associations of cisplatin sensitivity in the HDRA with MRP1 and Rb1 expression levels were also demonstrated, respectively, albeit just above the level of statistical significance. There was a positive correlation between TS and DPD activity, and an inverse correlation was detected between TS activity and 5-FU sensitivity in the HDRA. However, associations of TS and DPD activity with their mRNA expression levels were not detected. In the patients who were subjected to induction chemotherapy, the overall response rate was 73.5% (complete response, 38.2%; partial response, 35.3%). However, no significant correlation was observed between any of the investigated mRNA expressions and clinical response to chemotherapy.

**Conclusion:** Further studies are required to determine whether HER2 expression will be a useful predictive maker for chemosensitivity in patients with HNSCC. A study with additional patients to provide more data on the response to chemotherapy is currently underway.

**Radiotherapy for esophageal cancer -Current status and future directions-**

Satoshi Ishikura

*Clinical Trials and Practice Support Division, Center for...*
Carcinoma of the esophagus has been a challenging disease. In contrast to Western countries where the number of patients with adenocarcinoma has been increasing, most patients still have squamous cell carcinoma (SCC) in Japan. Recently, the number of patients with stage I disease has been increasing, although most patients are still diagnosed with advanced disease and dismal prognosis. The standard therapy for patients with resectable disease has been surgery with / without adjuvant therapy in Japan. In 80’s, radiotherapy alone had been indicated in unresectable or medically inoperable patients as a definitive or palliative treatment. In 90’s, chemoradiotherapy (CRT) became a standard for patients who received non-surgical treatment. Recent data suggested that patients who achieved complete response (CR) after CRT had a substantial risk of late toxicity such as pericarditis, pleural effusion, heart failure, and radiation pneumonitis, especially when treated with traditional 2-dimensional radiotherapy. The intergroup randomized study RTOG 9405 / INT 0123, which compared standard dose (50.4 Gy) vs. high dose (64 Gy) radiotherapy with concurrent chemotherapy, also showed that local failure was still dominant even with high dose radiotherapy, and suggested that the tumor control probability of current CRT approaches reached a plateau.

In 2003, we changed the treatment scheme which intended to prevent late toxicity by reducing the dose to normal tissues without compromising the efficacy. This included 3-dimensional conformal radiotherapy, radiotherapy dose reduction from 60 Gy to 50.4 Gy, and selected salvage surgery. So far, the treatment outcome seems good, although it is preliminary and long-term follow up is necessary. We also expect that newer cytotoxic drugs and / or molecular targeted therapy in combination with radiotherapy may improve local control and overall survival in the near future.

Definitive chemoradiotherapy for stage I-III esophageal squamous cell carcinoma: Current results in Japan.

Kei Muro

Department of Clinical Oncology, Aichi Cancer Center Hospital, Nagoya, Japan

Background: In Japan, extended radical esophagectomy is thought to be the only way to obtain the cure of EC. Purpose: The purpose is to evaluate the efficacy and toxicities of CRT for clinical stage I-III (T1-3N0-1 and M0) esophageal squamous cell carcinoma (ESCC). Methods: For stage I, treatment consisted of two 4-week courses of cisplatin 70 mg/m2 (day 1) and 5FU 700 mg/m2/day (days 1-4) combined with concurrent radiotherapy of 60 Gy in 30 fractions over 7 weeks with one week break. For stage II, III, consisted of two 5-week courses of cisplatin 40 mg/m2 (day 1, 8) and 5FU 400 mg/m2/day (days 1-5, 8-12) combined with concurrent radiotherapy of 60 Gy in 30 fractions over 8 weeks with 2-week break. For responders, two 4-week courses of chemotherapy of cisplatin 80 mg/m2 (day 1) and 5FU 800 mg/m2/day (days 1-5) were added. Results: From June 1997 to September 2003, consecutive 158 patients were retrospectively analyzed. Patient characteristics were as follows: median age of 63 (range 34-78), male/female; 132/26, PS 0/1/2; 84/70/4, stage I/IIA/IIB/III; 70/25/18/4, scc/others with scc component; 154/4. Of 70 patients (pts) with stage I, there were 64 complete responses (CRs) for CR rate of 91.4% (95% confidence interval (CI); 85-98%). Regarding stage I, 6 pts with residual tumor successfully underwent endoscopic mucosal resection (EMR) or esophagectomy, and local recurrence or new lesion occurred in 11 pts (17%) of 64 CRs, who successfully underwent EMR in 8 and esophagectomy in 3 as salvage treatment, respectively. Of 88 pts with stage II, III, there were 58 CRs for CR rate of 66% (95%CI; 56-75%). Regarding stage II, III, 28 pts (48%) of 58 CRs showed recurrent disease, and 22 pts (79%) of 28 recurrences underwent EMR or esophagectomy as salvage. On the other hand, of 30 non-CRs, there were 10 pts (33%) who underwent EMR or esophagectomy as salvage. With a median follow-up duration of 4.5 years, the 1, 3, 5-year survival rates in pts with stage I were 96%, 79%, and 74%, respectively. With a median follow-up duration of 3.4 years, the 1, 2, 3-year survival rates in pts with stage II, III were 76%, 56%, and 46%, respectively. These survival data are comparable with those obtained by ordinary surgery in Japan. Acute toxicities and late radiation morbidities increased dependent on the steps of stages, but they were tolerable and manageable. There was one treatment related death due to severe diarrhea in stage III EC pt. Conclusions: Definitive CRT for stage I-III ESCC is effective in CR rate and
short-term survival with acceptable toxicities, but salvage treatment for locoregional failure after CRT is necessary to improve the prognosis.

**Systemic chemotherapy for colorectal cancer**

Claus-Henning Köhne

*Clinic of Oncology and Haematology, Klinikum Oldenburg, Oldenburg, Germany*

Colorectal cancer is a curative disease. About 50% of patients will be cured by the surgeon, however the other half of patients will experience a relapse. Adjuvant chemotherapy has been established as an important treatment option to increase the cure rate. Twelve cycles of FOLFOX are the most efficacious therapy. Long lasting peripheral neuropathy are however potential sides effects that may not be tolerable for everyone. Oral fluoropyrimidienes either with capecitabine or with UFT are potential less toxic options. Adjuvant therapy is established for node positive disease (UICC stage III), but controversial for stage II patients. A group of high risk stage II patients has been defined; the value of systemic chemotherapy is however less well established. The liver is the main site of metastases in colorectal cancer. If resectable cure is possible in about 30% - 50% of patients. In patients in which liver metastases have been resected the use of adjuvant chemotherapy is again controversial. Unfortunately, only 15-20% of patients may have respectable disease and treatment of theses patients is mostly palliative. Systemic chemotherapy has become more efficacious with the use of regimens including infusional 5-FU modulated with folinic acid and combined with either irinotecan and or oxalaplatin. Response rate of 50-60% may be expected especially in patients with metastases confined to the liver. Phase II studies have been performed with carefully selected patients that were considered non-resectable by their surgeon but probably resectable after systemic chemotherapy. Although criteria of nonresectability may differ between surgeons about 50% of these previously nonresectable cases became resectable. In other phase II studies not selecting patients as carefully the resection rate was 20%. Secondary resection of metastases is reported in phase III studies mainly as an incidental event in below 10%. The rate of secondary resection depends on the selection of patients and on the efficacy of the used preoperative chemotherapy. The new targeted agents such as bevacizumab and cetuximab are candidates to further increase the response rate and potential resection rate. Currently available data suggest that cetuximab may be a good candidate as it has single agent efficacy and high response rates have been reported in combination with chemotherapy. Bevacizumab may cause peri-operative bleeding complications and thus has to be used with caution in this setting. Future trials will be necessary to better define the relative value to these new agents.

Secondary resection may be considered as a paradigmatic shift from palliation to cure in patients with metastatic disease and should be considered in any patient with a good response following chemotherapy.

Nowadays, we have to decide whether a curative approach might be possible in order to initiate the most efficacious regimen that may result in resection of metastases. Hereby, a higher degree of toxicity may be acceptable to achieve this goal. In a more palliative approach, a low degree of toxicity with a maximum effect of tumor control is the aim and the sequential use of chemotherapy agents probably with a stop and go strategy to maximise quality of life in patients is a potential strategy.

**Systemic chemotherapy for colorectal cancer - Current status and future perspectives in Japan**

Atsushi Ohtsu

*Division of Gastrointestinal Oncology/Digestive Endoscopy, National Cancer Center Hospital East, Kashiwa, Japan*

Chemotherapy for colorectal cancer is a rapidly evolving field. This is largely due to the development of various novel agents, such as irinotecan, oxaliplatin, capecitabine, bevacizumab, cetuximab, and panitumumab, over the past decade. These agents have provided not only survival prolongation in patients with metastatic disease but also additional cure rate after curative resection in stage III disease. Although the approval status of these agents had been far behind from the Western countries, the delay is now being improved and these advantage will be available very soon for the Japanese patients.

There are few ethnic differences in PK / PD of these new agents between the West and Japanese population, except for oral fluorouracils where Japanese patients have lower incidence of diarrhea
as compared to Caucasians. Nowadays, oxaliplatin or irinotecan in combination with infusional fluorouracil (FOLFOX / FOLFIRI) are becoming popular in most of the Japanese institutions where medical oncologists lead the chemotherapy. The registration trials of molecular targeting agents such as bevacizumab, cetuximab, and panitumumab have already completed and these agents will be commercially available soon. During these developments, there have been no obvious differences in safety and efficacy results as compared with those in overseas and we are ready to accept global results. According to the recent change of regulatory guidelines in Japan which facilitate to enter into global studies, we are also planning to participate global IND studies with newly developing (next generation) agent. In case of adjuvant trials, several Japanese institutions have participated in a global IND trial comparing FOLFOX4 with FOLFOX4 + bevacizumab or XELOX + bevacizumab (AVANT study). The number of post-marketing studies is also increasing and various combination regimens including oral fluorouracil which seems favorable for Japanese population, and some of them are being investigated in randomized trials. These studies, either global or domestic, will resolve the issues whether there are true ethnic differences in terms of efficacy and safety between Japanese and non-Japanese populations.

Mortality rate from colorectal cancer in Japan has already become similar to those in the West and it should be an urgent issue for Japanese health. We have just entered into the global development line of new agents for colorectal cancer and will play an important role in this field, which would provide more advantage for the patients with colorectal cancer over the world.

Hepatic arterial infusion chemotherapy for liver metastasis from colorectal cancer
Yoshitaka Inaba
Department of Diagnostic and Interventional Radiology, Aichi Cancer Center Hospital, Nagoya, Japan

Some RCTs comparing 5-FU based systemic chemotherapy and hepatic arterial infusion chemotherapy (HAIC) have not shown the impact of HAIC contributes to improving on the survival prolongation in patients with liver metastasis from colorectal cancer in the last 20 years. In these studies, although HAIC was superior to systemic chemotherapy on the local response, HAIC had low feasibility and high incidence of extra-hepatic foci. However, there is a great difference in techniques for HAIC between western countries and Japan. The indwelling catheter for HAIC is placed under laparotomy in western countries, while it is performed with percutaneous radiological procedures in Japan. HAIC in Japan actually has high feasibility and low invasion. In Japanese phase II studies, the median survival time (MST) of HAIC with 5-FU only showed over 20 months. At that time, there were not new agents such as irinotecan and oxaliplatin. But since HAIC was the regional therapy, it could not prevent the extra-hepatic disease from spreading anyway.

Now systemic chemotherapy for advanced or recurrent colorectal cancer has approximately 50% of the response rate and over 20 months of the MST, but liver metastasis is still one of the most important survival limiting factors. In US, combination therapy with hepatic arterial infusion and systemic irinotecan or oxaliplatin might be more useful recently. HAIC may have a potential role in pushing up the MST of patients with liver metastasis even after the failure of systemic chemotherapy.

HAIC supported by the adequate techniques is safely administered. HAIC with 5-FU only is reasonable, while new agents are costly. In considering the disease condition as well as quality of life and medical expenses of patients, we are groping the new strategy in which HAIC is performed only in the time when liver metastasis is dominant and it is converted into systemic chemotherapy in the time when extrahepatic lesions are growing.
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Aichi Cancer Center Research Institute
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