I have been conducting the integrated and synthetic analyses of the cancer metabolism from the perspective of genetic and epigenetic alterations, cancer immunity, and microbiome in gastrointestinal cancer.

**Analyses on anti-tumor immunity** *T cell densities in colorectal cancer tissues, tumor CD274 (PD-L1) expression, tumor PDCD1LG2 (PD-L2) expression, and tumor SQSTM1 expression*

**Tumor SQSTM1 (p62) expression and T cells in colorectal cancer. (published)**

Evidence suggests that activation of autophagy in neoplastic cells potentiates antitumor immunity through cross-presentation of tumor-associated antigens to T cells and release of immune mediators. The SQSTM1 (sequestosome 1, p62) protein is degraded by activated autophagy, and might enhance immune response to tumor cells. We hypothesized that tumor SQSTM1 expression level might be inversely associated with T-cell densities in colorectal carcinoma tissue. We evaluated tumor SQSTM1 expression by immunohistochemistry in 601 rectal and colon cancer cases within the Nurses’ Health Study and Health Professionals Follow-up Study. Ordinal logistic regression analyses were conducted to assess the association of tumor SQSTM1 expression with CD3+, CD8+, CD45RO (CD30+), or FOXP3+ cell density in tumor tissue, controlling for potential confounders, including tumor status of microsatellite instability, CpG island methylator phenotype, long interspersed nucleotide element-1 methylation level, and KRAS, BRAF, and PIK3CA mutations. Tumor SQSTM1 expression level was inversely associated with FOXP3+ cell density (P_trend = 0.006), but not with CD3+, CD8+, or CD45RO+ cell density (with the adjusted α level of 0.01 for multiple hypothesis testing). For a unit increase in quartile categories of FOXP3+ cell density, multivariable odds ratios were 0.66 [95% confidence interval (CI), 0.45-0.98] for intermediate-level SQSTM1 expression, and 0.55 (95% CI, 0.36-0.83) for high-level SQSTM1 expression, compared with low-level SQSTM1 expression. Tumor SQSTM1 expression is inversely associated with FOXP3+ cell density in colorectal cancer tissue, suggesting a possible role of SQSTM1-expressing carcinoma cells on regulatory T cells in the tumor microenvironment.

**TIME (Tumor Immunity in the MicroEnvironment) classification based on tumor CD274 (PD-L1) expression status and tumor-infiltrating lymphocytes in colorectal carcinomas. (published)**

Inhibitors targeting the PDCD1 (programmed cell death 1, PD-1) immune checkpoint pathway have revolutionized cancer treatment strategies. The TIME (Tumor Immunity in the MicroEnvironment)
classification based on tumor CD274 (PDCD1 ligand 1, PD-L1) expression and tumor-infiltrating lymphocytes (TIL) has been proposed to predict response to immunotherapy. It remains to be determined clinical, pathological, and molecular features of TIME subtypes of colorectal cancer. Using 812 colon and rectal carcinoma cases from the Nurses’ Health Study and Health Professionals Follow-up Study, we examined the association of tumor characteristics and survival outcomes with four TIME subtypes (TIME 1, CD274low/TILabsent; TIME 2, CD274high/TILpresent; TIME 3, CD274low/TILpresent; and TIME 4, CD274high/TILabsent). In survival analyses, Cox proportional hazards models were adjusted for potential confounders, including microsatellite instability (MSI) status, CpG island methylator phenotype (CIMP) status, LINE-1 methylation level, and KRAS, BRAF, and PIK3CA mutation status. TIME subtypes 1, 2, 3 and 4 had 218 (27%), 117 (14%), 103 (13%), and 374 (46%) colorectal cancer cases, respectively. Compared with TIL-absent subtypes (TIME 1 and 4), TIL-present subtypes (TIME 2 and 3) were associated with high-level MSI, high-degree CIMP, BRAF mutation, and higher amounts of neoantigens (p < 0.001).TIME subtypes were not significantly associated with colorectal cancer-specific or overall survival. In conclusion, TIL-present TIME subtypes of colorectal cancer are associated with high levels of MSI and neoantigen load, supporting better responsiveness to cancer immunotherapy. Further studies examining tumor molecular alterations and additional factors in the tumor microenvironment may inform development of immunoprevention and immunotherapy strategies.

**Tumor PDCD1LG2 (PD-L2) Expression and the Lymphocytic Reaction to Colorectal Cancer. (published)**

Expression of the immune checkpoint ligand CD274 (programmed cell death 1 ligand 1, PD-L1, from gene CD274) contributes to suppression of antitumor T cell-mediated immune response in various tumor types. However, the role of PDCD1LG2 (PD-L2, CD273, from gene PDCD1LG2) in the tumor microenvironment remains unclear. We hypothesized that tumor PDCD1LG2 expression might be inversely associated with lymphocytic reactions to colorectal cancer. We examined tumor PDCD1LG2 expression by IHC in 823 colon and rectal carcinoma cases within two U.S.-nationwide cohort studies and categorized tumors into quartiles according to the percentage of PDCD1LG2-expressing carcinoma cells. We conducted multivariable ordinal logistic regression analysis to assess the associations of tumor PDCD1LG2 expression with Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, or tumor-infiltrating lymphocytes, controlling for potential confounders, including microsatellite instability, CpG island methylator phenotype, long-interspersed nucleotide element-1 methylation, and KRAS, BRAF, and PIK3CA mutations. Tumor PDCD1LG2 expression was inversely associated with Crohn-like lymphoid reaction (P Rodrige = 0.0003). For a unit increase in the three-tiered ordinal categories of Crohn-like lymphoid reaction, a multivariable OR in the highest (vs. lowest) quartile of the percentage of PDCD1LG2-expressing tumor cells was 0.38 (95% confidence interval, 0.22-0.67). Tumor PDCD1LG2 expression was not associated with peritumoral lymphocytic reaction, intratumoral periglandular reaction, tumor-infiltrating lymphocytes, or patient survival (P Rodrige > 0.13). Thus, tumor PDCD1LG2 expression is inversely associated with Crohn-like lymphoid reaction to colorectal cancer, suggesting a possible role of PDCD1LG2-expressing tumor cells in inhibiting the development of tertiary lymphoid tissues during colorectal carcinogenesis.

**Association Between Inflammatory Diet Pattern and Risk of Colorectal Carcinoma Subtypes Classified by Immune Responses to Tumor. (published)**

Dietary patterns affect systemic and local intestinal inflammation, which have been linked to colorectal carcinogenesis. Chronic inflammation can interfere with the adaptive immune response. We investigated whether the association of a diet that promotes intestinal inflammation with risk of colorectal carcinoma was stronger for tumors with lower lymphocytic reactions than tumors with higher lymphocytic reactions. We collected data from the molecular pathological epidemiology databases of 2 prospective cohort studies: the Nurses’ Health Study (since 1976) and the Health Professionals Follow-Up Study (since 1986). We used duplication-method time-varying Cox proportional cause-specific hazards regression to assess the association of empirical dietary inflammatory pattern (EDIP) score (derived from food frequency questionnaire data) with colorectal carcinoma subtype. Foods that contribute to high EDIP scores include red and processed meats, refined grains, carbonated beverages, and some vegetables; foods that contribute to low EDIP scores include beer, wine, coffee, tea, yellow and leafy vegetables, and fruit juice. Colorectal tissue samples were analyzed histologically for patterns of lymphocytic reactions (Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes). During follow-up of 124,433 participants, we documented 1311 incident colon and rectal cancer cases with available tissue data. The association between the EDIP and colorectal cancer risk was significant (P Rodrige = .02), and varied with degree of peritumoral lymphocytic reaction (P heterogeneity < .001). Higher EDIP scores were associated with increased risk of colorectal cancer with an absent or low peritumoral lymphocytic reaction (highest vs lowest EDIP score quintile hazard ratio, 2.60; 95% confidence interval, 1.60-4.23; P Rodrige < .001), but not risk of tumors with intermediate or high peritumoral lymphocytic reaction (P Rodrige > .80). In 2 prospective cohort studies, we associated inflammatory diets with a higher risk of colorectal cancer subtype that contains little or no peritumoral lymphocytic reaction. These findings suggest that diet-related inflammation might contribute to development of colorectal cancer, by suppressing the
Adaptive anti-tumor immune response.

Aspirin Use and Colorectal Cancer Survival According to Tumor CD274 (Programmed Cell Death 1 Ligand 1) Expression Status. (published)

Purpose Blockade of the programmed cell death 1 (PDCD1, PD-1) immune checkpoint pathway can improve clinical outcomes in various malignancies. Evidence suggests that aspirin (a widely used nonsteroidal anti-inflammatory drug) not only prolongs colorectal cancer survival, but can also activate T cell-mediated antitumor immunity and synergize with immunotherapy through inhibition of prostaglandin E2 production. We hypothesized that the survival benefit associated with aspirin might be stronger in colorectal carcinoma with a lower CD274 (PDCD1 ligand 1, PD-L1) expression level than in lower signaling of the immune checkpoint pathway. Patients and Methods Using data from 617 patients with rectal and colon cancer in the Nurses' Health Study and the Health Professionals Follow-Up Study, we examined the association of postdiagnosis aspirin use with patient survival in strata of tumor CD274 expression status measured by immunohistochemistry. We used multivariable Cox proportional hazards regression models to control for potential confounders, including disease stage, microsatellite instability status, CpG island methylator phenotype, long interspersed nucleotide element-1 methylation, cyclooxygenase-2 (PTGS2), and CDX2 expression, and KRAS, BRAF, and PIK3CA mutations. Results The association of postdiagnosis aspirin use with colorectal cancer-specific survival differed by CD274 expression status ($P_{interaction} < .001$); compared with aspirin nonusers; multivariable-adjusted hazard ratios for regular aspirin users were 0.16 (95% CI, 0.06 to 0.41) in patients with low CD274 and 1.01 (95% CI, 0.61 to 1.67) in patients with high CD274. This differential association seemed consistent in patients with microsatellite-stable or PIK3CA wild-type disease and in strata of PTGS2 expression, CDX2 expression, tumor-infiltrating lymphocytes, or pre-diagnosis aspirin use status. Conclusion The association of aspirin use with colorectal cancer survival is stronger in patients with CD274-low tumors than with CD274-high tumors. Our findings suggest a differential antitumor effect of aspirin according to immune checkpoint status.

Microbiome analyses (Fusobacterium nucleatum, and Bifidobacterium genus)

Diets That Promote Colon Inflammation Associate With Risk of Colorectal Carcinomas That Contain Fusobacterium nucleatum. (published)

Specific nutritional components are likely to induce intestinal inflammation, which is characterized by increased levels of interleukin 6 (IL6), C-reactive protein (CRP), and tumor necrosis factor-receptor superfamily member 1B (TNFRSF1B) in the circulation and promotes colorectal carcinogenesis. The inflammatory effects of a diet can be estimated based on an empiric dietary inflammatory pattern (EDIP) score, calculated based on intake of 18 foods associated with plasma levels of IL6, CRP, and TNFRSF1B. An inflammatory environment in the colon (based on increased levels of IL6, CRP, and TNFRSF1B in peripheral blood) contributes to impairment of the mucosal barrier and altered immune cell responses, affecting the composition of the intestinal microbiota. Colonization by Fusobacterium nucleatum has been associated with the presence and features of colorectal adenocarcinoma. We investigated the association between diets that promote inflammation (based on EDIP score) and colorectal cancer subtypes classified by level of F. nucleatum in the tumor microenvironment. We calculated EDIP scores based on answers to questionnaires collected from participants in the Nurses' Health Study (through June 1, 2012) and the Health Professionals Follow-up Study (through January 31, 2012). Participants in both cohorts reported diagnoses of rectal or colon cancer in biennial questionnaires; deaths from unreported colorectal cancer cases were identified through the National Death Index and next of kin. Colorectal tumor tissues were collected from hospitals where the patients underwent tumor resection and F. nucleatum DNA was quantified by a polymerase chain reaction assay. We used multivariable duplication-method Cox proportional hazard regression to assess the associations of EDIP scores with risks of colorectal cancer subclassified by F. nucleatum status. During 28 years of follow-up evaluation of 124,433 participants, we documented 951 incident cases of colorectal carcinoma with tissue F. nucleatum data. Higher EDIP scores were associated with increased risk of F. nucleatum-positive colorectal tumors ($P_{trend} = .03$); for subjects in the highest vs lowest EDIP score tertiles, the hazard ratio for F. nucleatum-positive colorectal tumors was 1.63 (95% CI, 1.03-2.58). EDIP scores did not associate with F. nucleatum-negative tumors ($P_{trend} = .44$). High EDIP scores associated with proximal F. nucleatum-positive colorectal tumors but not with proximal F. nucleatum-negative colorectal tumors ($P_{heterogeneity} = .003$). Diets that promote intestinal inflammation, based on EDIP score, are associated with increased risk of F. nucleatum-positive colorectal carcinomas, but not carcinomas that do not contain these bacteria. These findings indicate that diet-induced intestinal inflammation alters the gut microbiome to contribute to colorectal carcinogenesis; nutritional interventions might be used in precision medicine and cancer prevention.

Association of Dietary Patterns With Risk of Colorectal Cancer Subtypes Classified by Fusobacterium nucleatum in Tumor Tissue. (published)

Fusobacterium nucleatum appears to play a role in colorectal carcinogenesis through suppression of the hosts' immune response to tumor. Evidence also suggests that diet influences intestinal F. nucleatum. However, the role of F. nucleatum in mediating the relationship between diet and the risk of colorectal cancer is unknown. To test the hypothesis that the associations of prudent diets (rich in whole grains and
dietary fiber) and Western diets (rich in red and processed meat, refined grains, and desserts) with colorectal cancer risk may differ according to the presence of F. nucleatum in tumor tissue. A prospective cohort study was conducted using data from the Nurses’ Health Study (June 1, 1980, to June 1, 2012) and the Health Professionals Follow-up Study (June 1, 1986, to June 1, 2012) on a total of 121700 US female nurses and 51529 US male health professionals aged 30 to 55 years and 40 to 75 years, respectively (both predominantly white individuals), at enrollment. Data analysis was performed from March 15, 2015, to August 10, 2016. Exposures are prudent and Western diets. Incidence of colorectal carcinoma subclassified by F. nucleatum status in tumor tissue, determined by quantitative polymerase chain reaction. Of the 173229 individuals considered for the study, 137217 were included in the analysis, 47449 were male (34.6%), and mean (SD) baseline age for men was 54.0 (9.8) years and for women, 46.3 (7.2) years. A total of 1019 incident colon and rectal cancer cases with available F. nucleatum data were documented over 26 to 32 years of follow-up, encompassing 3643562 person-years. The association of prudent diet with colorectal cancer significantly differed by tissue F. nucleatum status (P = .01 for heterogeneity); prudent diet score was associated with a lower risk of F. nucleatum-positive cancers (P = .003 for trend; multivariable hazard ratio of 0.43; 95% CI, 0.25-0.72, for the highest vs the lowest prudent score quartile) but not with F. nucleatum-negative cancers (P = .47 for trend, the corresponding multivariable hazard ratio of 0.95; 95% CI, 0.77-1.17). There was no significant heterogeneity between the subgroups in relation to Western dietary pattern scores. Prudent diets rich in whole grains and dietary fiber are associated with a lower risk for F. nucleatum-positive colorectal cancer but not F. nucleatum-negative cancer, supporting a potential role for intestinal microbiota in mediating the association between diet and colorectal neoplasms.

**Bifidobacterium genus in colorectal carcinoma tissue in relation to tumor characteristics and patient survival. (accepted)**

Evidence indicates a complex link between gut microbiome, tumor characteristics, and host immune system in the tumor microenvironment. In experimental studies, *Bifidobacterium* genus appears to regulate intestinal epithelial cell differentiation factors. Accumulating evidence suggests that bifidobacteria may enhance the anti-tumor immune response and efficacy of immunotherapy. We hypothesized that the amount of bifidobacteria in colorectal carcinoma tissue might be associated with tumor differentiation, and higher immune response to colorectal cancer. Using a molecular pathological epidemiology database of 1,313 rectal and colon cancers, we measured the amount of *Bifidobacterium* DNA in carcinoma tissue by a quantitative PCR assay, which was not significantly different from that in normal colon tissue. The multivariable ordinal regression model was used to adjust for potential confounders, including microsatellite instability status, CpG island methylator phenotype, long-interpersed nucleotide element-1 methylation, KRAS, BRAF and PIK3CA mutations. Intratumor bifidobacteria were detected in 393 (30%) cases. The amount of bifidobacteria was associated with the extent of signet ring cells (P = 0.002). Compared with *Bifidobacterium*-negative cases, multivariable odd ratios for the extent of signet ring cells were 1.29 (95% confidence interval, 0.74-2.24) for *Bifidobacterium*-low cases and 1.87 (95% confidence interval, 1.16-3.02) for *Bifidobacterium*-high cases (P \_trend = 0.010). The association between intratumor *Bifidobacterium* and signet ring cells suggests a possible role of *Bifidobacterium* in determining distinct tumor characteristics in colorectal cancer.

**Genetic and epigenetic changes in colorectal cancer**

**Aspirin exerts high anti-cancer activity in PIK3CA-mutant colon cancer cells. (published)**

Evidence suggests that nonsteroidal anti-inflammatory drug aspirin (acetylsalicylic acid) may improve patient survival in PIK3CA-mutant colorectal carcinoma, but not in PIK3CA-wild-type carcinoma. However, whether aspirin directly influences the viability of PIK3CA-mutant colon cancer cells is poorly understood. We conducted in vitro experiments to test our hypothesis that the anti-proliferative activity of aspirin might be stronger for PIK3CA-mutant colon cancer cells than for PIK3CA-wild-type colon cancer cells. We measured the anti-proliferative effect of aspirin at physiologic concentrations in seven PIK3CA-mutant and six PIK3CA-wild-type human colon cancer cell lines. After exposure to aspirin, the apoptotic index and cell cycle phase of colon cancer cells were assessed. In addition, the effect of aspirin was examined in parental SW48 cells and SW48 cell clones with individual knock-in PIK3CA mutations of either c.3140A>G (p.H1047R) or c.1633G>A (p.E545K). Aspirin induced greater dose-dependent loss of cell viability in PIK3CA-mutant cells than in PIK3CA-wild-type cells after treatment for 48 and 72 hours. Aspirin treatment also led to higher proportions of apoptotic cells and G0/G1 phase arrest in PIK3CA-mutant cells than in PIK3CA-wild-type cells. Aspirin treatment of isogenic SW48 cells carrying a PIK3CA mutation, either c.3140A>G (p.H1047R) or c.1633G>A (p. E545K), resulted in a more significant loss of cell viability compared to wild-type controls. Our findings indicate that aspirin causes cell cycle arrest, induces apoptosis, and leads to loss of cell viability more profoundly in PIK3CA-mutated colon cancer cells than in PIK3CA-wild-type colon cancer cells. These findings support the use of aspirin to treat patients with PIK3CA-mutant colon cancer.

**LINE-1 methylation level and prognosis in pancreas cancer: pyrosequencing technology and literature review. (published)**
Global DNA hypomethylation plays an important role in genomic instability and carcinogenesis. The long interspersed nucleotide element-1 (LINE-1) methylation level is a good surrogate marker of the global DNA methylation level. Previously, we demonstrated a strong relationship between LINE-1 hypomethylation and poor prognosis in certain cancers. However, the relationship between the LINE-1 methylation level and the clinical outcome of pancreatic cancer (PC) remains unclear. We used a pyrosequencing assay to measure LINE-1 methylation levels in 126 samples of resected PC and evaluated the prognostic value of the LINE-1 methylation level. LINE-1 methylation levels were significantly lower in PC tissues than in matched noncancerous pancreatic tissues (p = 0.039, n = 36). The tumoral LINE-1 methylation range was 41.3-92.8 (n = 126, mean 77.7, median 78.5, standard deviation 5.7). The LINE-1 methylation level was unrelated to clinical and pathological features. Moreover, LINE-1 hypomethylation was not significantly associated with overall survival, cancer specific survival, or disease-free survival (log-rank p = 0.30, p = 0.18 and p = 0.50, respectively). The LINE-1 methylation level appears not to be associated with poor prognosis in PC. The effect of the LINE-1 methylation level on the survival of PC patients needs to be confirmed in a larger-cohort study.

**Epidemiological analyses**

**Body mass index and risk of colorectal carcinoma subtypes classified by tumor differentiation status.** (published)

Previous studies suggest that abnormal energy balance status may dysregulate intestinal epithelial homeostasis and promote colorectal carcinogenesis, yet little is known about how host energy balance and obesity influence enterocyte differentiation during carcinogenesis. We hypothesized that the association between high body mass index (BMI) and colorectal carcinoma incidence might differ according to tumor histopathologic differentiation status. Using databases of the Nurses' Health Study and Health Professionals Follow-up Study, and duplication-method Cox proportional hazards models, we prospectively examined an association between BMI and the incidence of colorectal carcinoma subtypes classified by differentiation features. 120,813 participants were followed for 26 or 32 years and 1528 rectal and colon cancer cases with available tumor pathological data were documented. The association between BMI and colorectal cancer risk significantly differed depending on the presence or absence of poorly-differentiated foci ($P_{\text{heterogeneity}} = 0.006$). Higher BMI was associated with a higher risk of colorectal carcinoma without poorly-differentiated foci ($\geq 30.0$ vs. 18.5-22.4 kg/m2: multivariable-adjusted hazard ratio, 1.87; 95% confidence interval, 1.49-2.34; $P_{\text{trend}} < 0.001$), but not with risk of carcinoma with poorly-differentiated foci ($P_{\text{trend}} = 0.56$). This differential association appeared to be consistent in strata of tumor microsatellite instability or FASN expression status, although the statistical power was limited. The association between BMI and colorectal carcinoma risk did not significantly differ by overall tumor differentiation, mucinous differentiation, or signet ring cell component ($P_{\text{heterogeneity}} > 0.03$, with the adjusted $\alpha$ of 0.01). High BMI was associated with risk of colorectal cancer subtype containing no poorly-differentiated focus. Our findings suggest that carcinogenic influence of excess energy balance might be stronger for tumors that retain better intestinal differentiation throughout the tumor areas.

**Dietary glycemic and insulin scores and colorectal cancer survival by tumor molecular biomarkers.** (published)

Accumulating evidence suggests that post-diagnostic insulin levels may influence colorectal cancer (CRC) survival. Yet, no previous study has examined CRC survival in relation to a post-diagnostic diet rich in foods that increase post-prandial insulin levels. We hypothesized that glycemic and insulin scores (index or load; derived from food frequency questionnaire data) may be associated with survival from specific CRC subtypes sensitive to the insulin signaling pathway. We prospectively followed 1,160 CRC patients from the Nurses' Health Study (1980-2012) and Health Professionals Follow-Up Study (1986-2012), resulting in 266 CRC deaths in 10,235 person-years. CRC subtypes were defined by seven tumor biomarkers (KRAS, BRAF, PIK3CA mutations, and IRS1, IRS2, FASN and CTNNB1 expression) implicated in the insulin signaling pathway. For overall CRC and each subtype, hazard ratio (HR) and 95% confidence interval (95% CI) for an increase of one standard deviation in each of glycemic and insulin scores were estimated using time-dependent Cox proportional hazards model. We found that insulin scores, but not glycemic scores, were positively associated with CRC mortality (HR = 1.19, 95% CI = 1.02-1.38 for index; HR = 1.23, 95% CI = 1.04-1.47 for load). The significant positive associations appeared more pronounced among PIK3CA wild-type cases and FASN-negative cases, with HR ranging from 1.36 to 1.60 across insulin scores. However, we did not observe statistically significant interactions of insulin scores with PIK3CA, FASN, or any other tumor marker ($P_{\text{interaction}} > 0.12$). While additional studies are needed for definitive evidence, a high-insulinogenic diet after CRC diagnosis may contribute to worse CRC survival.