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Table 4. Comparison of CTC and BLF methylation test

Case	Colonoscopic finding	Location	Size, mm	Histologic diagnosis	Dukes' stage	CTC diagnosis	M-score
1	CRC	Distal	30	Adenocarcinoma	B	CRC	3
2	CRC	Distal	60	Adenocarcinoma	B	CRC	2
3	CRC	Distal	60	Adenocarcinoma	B	CRC	2
4	CRC	Distal	43	Adenocarcinoma	A	CRC	2
5	CRC	Rectum	11	Adenocarcinoma in adenoma	A	Minor polyp	3
6	Minor polyp	Proximal	3	Tubular adenoma		Normal	0
7	Minor polyp	Proximal	4	Tubular adenoma		Normal	1
8	Minor polyp	Proximal	3	Tubular adenoma		Minor polyp	1
9	Normal					Normal	0

diagnostic performance of CTC. Among the subjects enrolled in this study, 9, including 5 patients with colorectal cancer, were examined using CTC (Table 4). CTC detected four colorectal cancers, while all 5 patients with colorectal cancer were positive for BLF methylation (M-score, > 2). Notably, 1 patient (case 5) developed a laterally spreading tumor (LST) that consisted of a histologically benign polypoid component and a flat adenocarcinoma component. CTC detected only the polypoid component, so the lesion was diagnosed as a minor polyp (Table 4 and Supplementary Fig. S4). Our results suggest that combining assessment of BLF methylation with CTC may improve diagnostic performance, though further study of a larger population will be necessary to confirm the clinical utility of this combination.

Discussion

Numerous studies have shown that aberrant methylation of DNA in the stool is a promising biomarker suitable for noninvasive colorectal cancer screening. For instance, *VIM*, *SFRP2*, and *TFPI2* are reported to be useful single-gene markers for a fecal DNA methylation test (9, 10, 12). In addition, other groups have shown that combinations of multiple markers improve the diagnostic efficacy of stool DNA methylation (14). In this study, we demonstrated that aberrant DNA methylation is detectable in the wash fluid of oral bowel lavage collected from the rectum of patients with colorectal cancer. Earlier studies have shown that methylation of DNA in body fluids, including pancreatic juice (23), saliva (24), and gastric juice (25), has the potential to serve as a biomarker for cancer detection and risk assessment, yet there have been no studies assessing the feasibility of using BLF for molecular screening for colorectal cancer. Importantly, we found that the utility of BLF depends on successful bowel preparation, and that residual stool may interfere with sensitive detection of tumor-derived DNA methylation. Although the total amount of extracted DNA is small, BLF specimens with sufficient bowel preparation appear to contain a greater proportion of tumor-derived DNA than those with insufficient treatment.

In this study, we tested a set of genes known to be frequently methylated in colorectal cancer, and selected the three genes with the highest sensitivities for detection of colorectal cancer (*miR-124-3*, *SFRP1*, and *LOC386758*). The *miR-124* family consists of three members (*miR-124-1*, *miR-124-2*, and *miR-124-3*), all of which are reportedly methylated in multiple types of human malignancy, including colorectal cancer and gastric cancer (26, 27). *SFRP1* encodes secreted frizzed-related protein 1, a negative regulator of Wnt signaling, and the promoter CpG island of *SFRP1* is frequently methylated in various cancers, including colorectal cancer, gastric cancer, and esophageal cancer (28–30). *LOC386758* is a frequent target of aberrant methylation newly identified in our recent epigenome analysis in colorectal cancer, though its function remains unknown (manuscript in preparation). Although BLF methylation of each of these genes could be used to detect colorectal cancer with relatively high sensitivity and specificity, we found that combining them improved diagnostic accuracy. Importantly, BLF methylation was not affected by tumor size, location, or stage, suggesting that it could potentially serve as a biomarker for both proximal and distal colon cancers.

However, the BLF methylation system failed to detect a small number of colorectal cancers as well as more than half of the precancerous lesions (minor polyps and advanced adenomas). We confirmed that the negative result was not due to the absence of methylation in the tumor tissues. In addition, we and others have previously shown that many of the 15 genes analyzed in this study are frequently methylated in colorectal premalignant lesions (31, 32). Although the true reason for the false-negative finding remains uncertain, we suspect that the presence of a too small number of exfoliated cells in the BLF is the cause. We have previously shown that DNA methylation in colonoscopically obtained mucosal wash fluids could be a predictive biomarker of tumor invasiveness (16). By performing quantitative bisulfite-pyrosequencing, we detected elevated levels of DNA methylation of tumor-related genes (*miR-34b/c*, *SFRP1*, *SFRP2*, and *DKK2*) in the mucosa of invasive tumors, though these genes were equally methylated in noninvasive and invasive tumors. Early during this study, we found that we were unable to detect BLF methylation using bisulfite-pyrosequencing, so we switched to the more sensitive MethyLight assay. We therefore suggest that the numbers

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of exfoliated cells and the amount of cell-free DNA in BLF specimens are far smaller than in the colonoscopically obtained mucosal wash fluid. Moreover, BLF specimens with high M-scores tended to show lower C_t values for Alu elements with MethyLight, which is indicative of the relative abundance of human genomic DNA (Fig. 1C). These results suggest that successful detection of BLF methylation is highly dependent on the amount of tumor-derived DNA in the BLF specimens. In addition, we speculate that differences in the sample collection steps and the methods used for methylation analysis could be major reasons why the best marker genes differed between our early studies and the present one (16).

Our findings also suggest that BLF methylation could be used to complement current colorectal cancer screening methods. FIT is one of the most commonly used and cost-effective screening tests, but its low PPV may lead to a low compliance rate among FIT-positive individuals receiving medical advice to go for secondary screening. When combined with FIT, a BLF methylation test could significantly improve PPV and more effectively select individuals who should be strongly encouraged to undergo total colonoscopy. Moreover, our data demonstrated that BLF methylation of multiple genes could be an indicator of colorectal cancer, even among FIT-negative individuals.

As compared with stool DNA tests, the biggest disadvantage of the BLF methylation test is that it requires bowel preparation. Therefore, combination with endoscopies is another feasible clinical application of BLF methylation. For instance, when combined with sigmoidoscopy, a BLF methylation test may complement the diagnostic performance for detection of proximal colon cancers. Similarly, BLF methylation could provide supportive information for patients with unsuccessful total colonoscopy. In addition, we propose that BLF methylation may improve the diagnostic performance of CTC. Although the sensitivity of CTC for detection of some colorectal cancers is equivalent to colonoscopy, its ability to detect small or flat lesions is more limited (33–35). Moreover, it is sometimes difficult to distinguish between early-stage cancers and benign adenomas using CTC. The fact that BLF methylation is highly specific for malignant tumors indicates that it could increase the ability to detect colorectal cancers using CTC. In this study, we compared BLF methylation with CTC findings in 9 individuals, including 5 patients with colorectal cancer. Using CTC, four of the colorectal cancers were successfully detected, but a flat type cancer was diagnosed as a minor polyp. In contrast, BLF methylation (M-score, > 2) was detected in all 5 patients with colorectal cancer. These results suggest that combining assessment of BLF methylation with CTC may improve the diagnostic performance, but further prospective study of a larger number of patients will be necessary to evaluate the diagnostic performance of this combination.

In sum, our results demonstrate the feasibility of using aberrant DNA methylation in BLF specimens for noninvasive colorectal cancer screening. We also found that using a panel of several marker genes further improved the sensitivity and specificity of this diagnostic system. It is noteworthy that DNA methylation was readily detectable in BLF specimens with no purification or capture of human genomic DNA. Thus, combination with other colorectal cancer detection methods that require bowel preparation, including sigmoidoscopy or CTC, would be a suitable application of the BLF methylation test. Further technical refinements, including easier bowel preparation, single-molecule detection of methylated DNA, and identification of better marker sets, would also enhance the practicality of this test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Harada, E. Yamamoto, T. Niinuma, T. Sugai, K. Imai

Development of methodology: H.-O. Yamano, T. Sugai

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Harada, E. Yamamoto, H.-O. Yamano, K. Kumegawa, K. Yoshikawa, T. Kimura, E. Harada, R. Takagi, Y. Tanaka, H. Aoki, M. Nakaoka, K. Shimoda

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Harada, E. Yamamoto, M. Nojima, R. Maruyama, A. Tuyada, M. Kai, T. Sugai

Writing, review, and/or revision of the manuscript: T. Harada, E. Yamamoto, A. Tuyada, H. Suzuki

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Study supervision: E. Yamamoto, Y. Shinomura, T. Sugai, K. Imai, H. Suzuki

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AACR American Association for Cancer Research

Cancer Prevention Research

Analysis of DNA Methylation in Bowel Lavage Fluid for Detection of Colorectal Cancer

Taku Harada, Eiichiro Yamamoto, Hiro-o Yamano, et al.

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Current Topics
Paradigm Shift in Cancer
Research, Treatment
and Education

Juntendo Medical Journal
2014. 60 (3), 234~237

Overview and Future Prospects of "Promotion Plan for the Platform of Human Resource Development for Cancer"

KOHZOH IMAI

Current Topics
Paradigm Shift in Cancer Research, Treatment and Education
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2014. 60 (3), 234~237

Overview and Future Prospects of "Promotion Plan for the Platform of Human Resource Development for Cancer"

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Promotion Plan for the Platform of Human Resource Development for Cancer (CancerHR Phase Two) had been launched in FY 2012. The newly selected five-year-project consists of 43 chairs in faculty of medicine: 9 chairs specialized in radiation treatment, 7 chairs specialized in chemotherapy, 10 chairs specialized in palliative care, and 17 other chairs. The above plans are expected to improve the platform of cancer education and research.

Key words: Promotion Plan for the Platform of Human Resource Development for Cancer, radiation treatment, palliative care, patient-oriented medical treatment

Human Resource Development Plan for Cancer (hereinafter referred to as CancerHR) was one of the educational projects established by Ministry of Education, Culture, Sports, Science and Technology in Japan (MEXT) in fiscal year 2007. The program had been highly evaluated so far.

Increasing the number of death from cancer having reached 350,000 a year, and increasing the awareness of cancer patients and their family which is represented by numerous Cancer Patient Meetings eventually brought it to enforce the Cancer Control Act. Based on such a background, CancerHR had been launched. I would like to describe my expectation for "Promotion Plan for the Platform of Human Resource Development for Cancer" (CancerHR Phase Two) started in FY 2012 since I have an opportunity to oversee the entire project as a Chairman of the Committee of Promotion of Human Resource Development for Cancer (Table-1).

CancerHR is a program for graduate student who specialized in clinical cancer, which has several features. First feature is that it aims to establish nationwide cancer treatment in cooperation with cancer medical treatment cooperation base hospitals. Second is that it aims to foster specialists, not only medical doctors but also nurses, pharmacists, and radiological medical physicist etc. In 4 years, the program achieved to foster more than 2,000 candidates (about 1,200 medical doctors) specialized in cancer. Third feature is that it placed great importance on radiation treatment, palliative care, and chemotherapy.

The research group of Ministry of Health, Labour and Welfare in Japan estimated that 2.25 million patients (diagnosed within 5 years) need cancer treatment in 2015. Although it seems like the actual number of cancer patients in need of treatment will go up to 4-5 million. Currently 15,000 "General Clinical Oncologists" who have the latest and comprehensive knowledge are certified (as of April 2014) despite 50,000 specialists are necessary. Further, only 514 Certified Nurses Specialist in cancer nursing and 2,684 Certified Nurses in cancer nursing are certified as of January 2014. These numbers are far from sufficient.

Having been launched recently, CancerHR started to achieve fostering cancer specialists. At

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Table-1 List of members of the Committee of Promotion of Human Resource Development for Cancer

	Shinsuke Amano	Executive Director of Group Nexus Japan, a nonprofit organization
Chairman	Kohzoh Imai	Director of Research Hospital, The institute of Medical Science, The University of Tokyo
	Hiroko Komatsu	Professor at Faculty of Nursing and Medical Care, Keio University
	Hiroshi Suzuki	Professor at The University Hospital, The University of Tokyo
	Nagara Tamaki	Director of Graduate School of Medicine, Hokkaido University
	Kazuo Tamura	Professor at Faculty of Medicine, Fukuoka University
	Satoru Tsuneto	Professor at a Corporate Sponsored Research Program in Graduate School of Medicine, Osaka University
	Keiichi Nakagawa	Associate Professor at The University of Tokyo Hospital
Vice chairman	Masahiko Nishiyama	Professor at Research Institute for Development Therapeutics, Saitama Medical University
	Okio Hino	Professor at Faculty of Medicine, Juntendo University
	Shigeri Hosaka	Executive Board Member of Japan Medical Association
	Mayumi Honda	Staff writer, Social Security News Department, The Yomiuri Shimbun

the same time it just started to collaborate between the specialists to make use of their professional skills, and universities and university hospitals had not been collaborated enough.

In view of the above, Promotion Plan for the Platform of Human Resource Development for Cancer (CancerHR Phase Two) had been launched in FY 2012 (Table-2). Based on the results and problems of Phase One, the following three results or effects are mainly picked up to make clear of target needs to proceed in Phase Two.

Construction of the cancer education research platform

Cancer education, research and medical treatment in Japan have been fallen far behind the international medical platform due to the separated operation in each of the internal organ specialties or medical specialties. This is because of insufficient cooperation between the internal organ specialties and medical specialties. Also the number of cross-disciplinary internal organ specialty chairs such as radiation treatment (separated from radiation diagnosis), chemotherapy (pharmacotherapy), and palliative care etc. are inadequate in number. Nevertheless, the newly selected five-year-project consists of 43 chairs: 9 chairs specialized in radiation treatment, 7 chairs specialized in chemotherapy, 10 chairs specialized in palliative care, and 17 other chairs. The above plans are expected to improve the platform of cancer education and research.

Promotion of cancer education reform

The project will accept 1,800 medical doctors specializing cancer treatment, and 1,200 medical personnel such as nurses and pharmacists in graduate school for 5 years. Other programs or education reforms are planned as follows:

1) In addition to educating cancer professionals in radiation treatment, chemotherapy, and palliative care, this project further set up a training course for surgical treatment, cancer treatment at home, pediatric cancer, gynecologic cancer, cancer rehabilitation, medical professionals, researcher and instructors who are involved in the latest and next-generation cancer research with the aim of extension of cancer professional education.
2) This project schedule to construct a new educational model to take advantage of characteristics of each university including a construction of e-Learning system, continuous interchange of personnel between local universities and universities in metropolitan area, programs applied to local networks having rooted in the area, programs principally aiming to team treatment, and education of researchers who are involved in the latest and next-generation research with the resource preserved in each university.

Table-2 List of Universities selected for Promotion Plan for the Platform of Human Resource Development for Cancer

Selected University	Name of Program	Number of Universities
Hokkaido University Asahikawa Medical University *Sapporo Medical University Health Sciences University of Hokkaido	Program of Human Resource Development for Leading Cancer Treatment in Hokkaido	4
*Tohoku University Yamagata University Fukushima Medical University Niigata University	Promotion Plan of Human Resource Development for Cancer in Tohoku	4
*University of Tsukuba Ibaraki Prefectural University of Health Sciences Dokkyo Medical University Gunma University Gunma Prefectural College of Health Sciences Saitama Medical University Chiba University Nippon Medical School	International Training Program for Co-operative Experts in Clinical Oncology	8
Jichi Medical University *The University of Tokyo Toho University Yokohama City University	Education Program for the Breakthrough in Cancer Treatment	4
Hirosaki University Akita University *Tokyo Medical and Dental University Tokyo Medical University Tokyo University Institute of Technology Tokyo University of Pharmacy and Life Sciences	Training Program for Next Generation Specialists to Promote Cancer Therapy	6
International University of Health and Welfare *Keio University Tokai University Tokyo Dental College Tokyo Metropolitan University St. Luke's International University Kitasato University St. Marianna University School of Medicine University of Yamanashi Shinshu University	Education Program for Professional Leader for Development in High-level Cancer Treatment	10
Iwate Medical University *Juntendo University Tokyo University of Science Meiji Pharmaceutical University Rikkyo University Tottori University Shimane University	Restoration Plan for Cancer Treatment through ICT and Human Resources	7
Kyorin University Teikyo University *Tokyo Women's Medical University Komazawa University	Tokyo Oncology Professional	4
University of Toyama *Kanazawa University Kanazawa Medical University Ishikawa Prefectural Nursing University University of Fukui	Hokuriku Training Program of Oncology Specialist	5
Gifu University Hamamatsu University School of Medicine *Nagoya University Nagoya City University Aichi Medical University Fujita Health University Meijo University	Education Program for Medical Professionals for Cancer Treatment between organizations	7
Mie University Shiga University of Medical Science *Kyoto University Kyoto Pharmaceutical University Osaka Medical College	Training Program for Next Generation Researchers and Medical Professionals Specialized in Cancer	5
Kyoto Prefectural University of Medicine *Osaka University Osaka University of Pharmaceutical Sciences University of Hyogo Kobe Pharmaceutical University Nara Medical University Wakayama Medical University	Human Resource Development Program for Cancer Coordinating Between Regions and Professions	7
Osaka City University Osaka Prefecture University Kansai Medical University *Kinki University Kobe University Hyogo College of Medicine Kobe City College of Nursing	7-University Joint Project: Advanced Creative Plan for Cancer Education Base	7
*Okayama University Kawasaki Medical School Hiroshima University Yamaguchi University The University of Tokushima Tokushima Bunri University Kagawa University Ehime University Kochi University University of Kochi	Mid-West Japan Cancer Professional Education Consortium	10
*Kyushu University Kurume University University of Occupational and Environmental Health, Japan Fukuoka University Fukuoka Prefectural University Saga University Nagasaki University Kumamoto University Oita University University of Miyazaki Kagoshima University University of the Ryukyus	Kyushu Promotion Plan for the Platform of Human Resource Development for Cancer	12

* University in charge of application
15 programs in total (100 universities)

Equal Accessibility to cancer treatment

Regardless of the residential area, patients should be able to access to cancer treatment of high quality based on a scientific knowledge (Equal Accessibility to cancer treatment). This project will foster cancer specialized professionals to work in those areas. The Following cases illustrate in detail.

1) This project is scheduled to open 83 courses to foster cancer professionals who will contribute the cancer treatment in local area, in which it will accept about 900 professionals in 5 years.
2) This project set up about 4 chairs specialized in local cancer medical cooperation such as local cancer treatment cooperation chair and local cancer treatment promotion chairs etc., in which it will promote to foster local oncologist, to construct local network, and to send oncologist to the local areas.
3) This project will open about 133 short-term learning courses (intensive courses) in graduate schools nationwide to accept about 3,000 professionals a year. This program enables the local medical professionals to learn the latest knowledge or treatment method of cancer preserved in universities.

Request from Committee of Promotion

Committee of Promotion requested the participating universities for the project as follows.

1) Conduct the plan accordingly as well as review to improve programs continuously to implement PDCA cycle with reference to an annual external evaluation conducted by external experts, comments from Committee of Promotion and social needs etc.
2) Return the result or effects to a society as much as possible and contribute the development of cancer treatment in Japan through educating excellent oncologists, and performing cutting-edge research and medical treatment for cancer.
3) Visualize the effort and effect as clear as possible to announce the public to recognize.

Committee of Promotion will continue to assess the progress of the project at interim evaluations and to support to enrich and develop the project.

Cancer control has been a critical issue for life and health of citizen in Japan thus Committee of Promotion requested the government to continue its financial support in which clearly stated on MEXT website.

As CancerHR Phase Two started, "National CancerHR Assembly" has been formally established by the universities selected for this program. The assembly is currently a private organization established with the aim to support programs at universities fostering cancer professionals involved in cancer treatment. Another objective of its establishment is to provide an occasion to exchange opinions of all universities belonged to the 15 main consortiums. Professor Nariaki Matsuura of Osaka University took the post as a Director. The Assembly held annual sectional meetings on palliative care in the first year (FY 2012), on radiation treatment in the second year (FY 2013), and will hold meeting on chemotherapy in the third year (FY 2014). Those meetings are available to exchange opinions of each university and to improve the program. Also some programs are open to general public to expand a patient-oriented medical treatment. All the above activities are in progress every year.

Japan has one of the highest life expectancy rates in the world, together with high incidence of cancer and death caused by cancer. Longer life results in increasing cancer for which the current situation would be regretful. Yet improvement of cancer treatment will improve the Quality of Life of patients and further bring vitality to a family, an office, and a community. The achievement of our country will greatly contribute many other countries with aging population around the world. This could be the grounds to launch "CancerHR Phase Three". Such successive CancerHR program will show a great value for cancer treatment and will foster personnel for contributing to cancer treatment worldwide in the future.

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ORIGINAL ARTICLE

Everyday clinical practice in IgG4-related dacryoadenitis and/or sialadenitis: Results from the SMART database

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Abstract

Objective. Immunoglobulin (Ig)G4-related disease (IgG4-RD) is a new disease entity that has only been identified this century. Clinical information is thus lacking. We established the Sapporo Medical University and Related Institutes Database for Investigation and Best Treatments of IgG4-related Disease (SMART) to clarify the clinical features of IgG4-RD and provide useful information for clinicians.

Methods. Participants comprised 122 patients with IgG4-related dacryoadenitis and/or sialadenitis (IgG4-DS), representing lacrimal and/or salivary lesions of IgG4-RD, followed-up in December 2013. We analyzed the sex ratio, mean age at onset, organ dysfunction, history or complications of malignancy, treatments, rate of clinical remission, and relapse.

Results. The sex ratio was roughly equal. Mean age at diagnosis was 59.0 years. Positron emission tomography revealed that the ratio of other organ involvements was 61.4%. Complications of malignancy were observed in 7.4% of cases. Glucocorticoid was used to treat 92.1% of cases, and the mean maintenance dose of prednisolone was 4.8 mg/day. Rituximab was added in three cases, and showed good steroid-sparing effect. The clinical remission rate was 73.8%, and the annual relapse rate was 11.5%. Half of the cases experienced relapses within 7 years of initial treatment.

Conclusion. We analyzed the clinical features and treatments of IgG4-DS using SMART, providing useful information for everyday clinical practice.

Keywords

Autoimmune pancreatitis, Cancer, IgG4-related disease, Mikulicz's disease, rituximab

History

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Immunoglobulin (Ig)G4-related disease (IgG4-RD) (see Supplementary Table 1 available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2014.950036) has recently attracted attention in many medical areas. IgG4-RD is characterized as a condition involving marked infiltration of IgG4-bearing plasmacytes and fibrosis in enlarged organs and elevated serum levels of IgG4 [1]. Organs that can be affected include the lacrimal and salivary glands, pancreas, bile duct, kidneys, lungs, pituitary gland, mammary glands, and prostate [2]. Until recently, lacrimal and salivary lesions in IgG4-RD have been called Mikulicz's disease (MD), and are now called "IgG4-related dacryoadenitis and sialadenitis" (IgG4-DS) [3]. In rheumatology and clinical immunology, IgG4-DS had been considered identical to Sjögren's syndrome (SS) [4], but has now been recognized as a separate pathological entity and differences from SS have been discussed [5].

A nationwide survey in Japan estimated that there were 8000 patients with IgG4-RD and 4300 patients with IgG4-DS [6]. With the growing awareness of this disease, rheumatologists have been identifying patients with IgG4-RD more often in daily practice, but information about the disorder remains unclear. We established the Sapporo Medical University and Related Institutes Database for Investigation and Best Treatments of IgG4-related Disease (SMART) to clarify the features of IgG4-DS and provide feedback on important and useful information for clinicians. We describe the results herein.

Materials and methods

Subjects were patients with IgG4-RDS diagnosed between April 1997 and December 2013 and currently under follow-up

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in our hospital or related facilities. All patients provided written informed consent and were registered to SMART. Patients who declined cohort participation or were lost to follow-up were excluded. Participants comprised all 122 patients with IgG4-DS who were registered to SMART as of December 2013. IgG4-DS was diagnosed according to the comprehensive diagnostic criteria for IgG4-RD [7] or the diagnostic criteria for IgG4-related MD [8], and we confirmed no Ig-heavy chain gene rearrangement. When Ig-heavy chain gene rearrangement was detected, we diagnosed B cell lymphoma. Patients encountered prior to the development of these criteria were diagnosed based on physical, serological, and pathological findings. The diagnoses of these patients were not changed during follow-up in any cases.

We analyzed the sex ratio, mean age at onset, distributions of age of onset, current age, involvement of organs other than the lacrimal and salivary glands as confirmed by positron emission tomography (PET) or computed tomography (CT), serum levels of IgG and IgG4 in patients with and without other organ involvement (OOI) as items prior to treatment, and treatments for IgG4-DS and adverse events, rate of clinical remission; MD assessment questionnaire (MAQ) scores (see Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2014.950036) [9], relapse-free survival rate, and complications of malignancies as items during follow-up.

Systemic evaluation for complications of IgG4-RD and malignancies was performed using enhanced CT every year and ^{18}F-fluorodeoxyglucose (FDG)-PET at the diagnosis of IgG4-RD. If necessary, gastroenteroscopy or gynecological examination was added. Histological examination was performed to diagnose complications where possible. Physical and serological examinations were checked every 1–3 months. The MAQ comprises four questions to assess the degree to which lacrimal and salivary glands were enlarged and the occurrence and severity of sicca symptoms. Patients checked the boxes that best corresponded with current symptoms: disappearance of symptoms (0 points); slight improvement of symptoms (1 point); unchanged symptoms (2 points); or worsening of symptoms (3 points). The mean of these four questions was then used as the MAQ score, allowing comparison of patient condition compared with the first visit. Scores were checked at each visit. Clinical remission was defined as the disappearance of swollen lacrimal and/or salivary glands and no OOI by medical examination and imaging. Relapse was defined as re-enlargement of lacrimal and/or salivary glands or new/re-appearance of OOI after clinical remission.

In our treatment protocol, starting prednisolone at a dose of 0.6 mg/kg/day is considered appropriate for single-organ failure, increasing to 1.0 mg/kg/day with multiple-organ failure. However, the attending physician is permitted to decrease the initial dose due to age or physical complications. The initial dose of prednisolone is continued for 2–4 weeks, tapering the dose by 10% every 2 weeks. The attending physician can set a dose less than 10 mg/day. For relapsed or steroid-dependent cases, the dose of prednisolone is increased or concomitant use of immunosuppressants is selected (see Supplementary Figure 2 http://informahealthcare.com/doi/abs/10.3109/14397595.2014.950036).

Ethical considerations

Written consent to use case information was obtained from all patients prior to enrolment, in accordance with the Declaration of Helsinki. This study was conducted with the approval of Sapporo Medical University Hospital Institutional Review Board (SMU 22–57, 24-155).

Results

Smart

As of the end of December 2013, 122 patients with IgG4-DS had been enrolled. The mean follow-up period was 4.33 years, and SMART covered 528.81 person-years. Diagnoses for 76 patients were made according to the diagnostic criteria for IgG4-related MD, and all other patients met the comprehensive diagnostic criteria. Patients comprised 62 men and 60 women, representing a sex ratio of 1.03:1. Mean age at onset was 58.7 ± 12.6 years (range, 23–81 years), and the distributions of ages of onset are shown in Figure 1. The largest age stratum comprised patients in their 60s, and patients >60 years old comprised 56.6%. Mean (± standard deviation) current age was 64.5 ± 11.9 years.

OOI detected by PET

PET was performed in 70 patients before treatment. Involvement of organs other than the lacrimal and salivary glands was detected in 43 cases (61.4%). Organ failure excluding IgG4-DS was seen with one involvement in 27 cases (38.6%), two involvements in 8 cases (11.4%), and three or more lesions in 8 cases (11.4%) (Figure 2). Of these, 11 cases (15.7%) showed complications of type I autoimmune pancreatitis. Retroperitoneal fibrosis was seen in 14 cases (20.0%). Six of those 14 cases (42.9% of patients with retroperitoneal fibrosis) showed involvement of the renal hilum, and 3 of those 6 cases (50.0% of patients with lesions of the renal hilum) developed hydronephrosis. Soft tissues in the perivertebral space were observed in five cases (35.7% of patients with retroperitoneal fibrosis). Only two cases showed lesions around the ureter. Periaortitis was revealed in six cases. Lung involvement was detected in six cases (8.6%). Other lesions were detected in the prostate gland (nine cases, 12.9%), pericardium (three cases, 4.3%), thyroid gland (two cases, 2.9%), liver (two cases, 2.9%), and mammary glands (two cases, 2.9%). All cases of OOI described above were also confirmed with PET. In these 70 patients, OOI detected by CT alone were as follows: IgG4-related kidney disease in 16 cases (22.9%); and tracheal and bronchial lesions in 3 cases (4.3%). Involvement of the pituitary gland (one case, 1.4%) and seminal vesicles (one case, 1.4%) was detected only on magnetic resonance imaging (MRI).

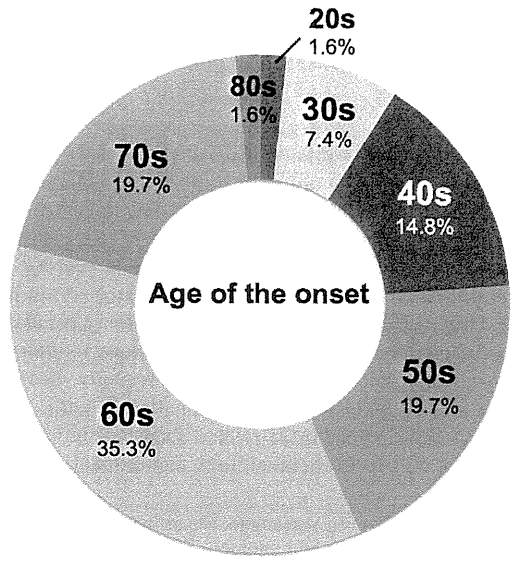

Figure 1. Distribution of age at onset in SMART. Patients over 60 years old comprised >55% of the database.

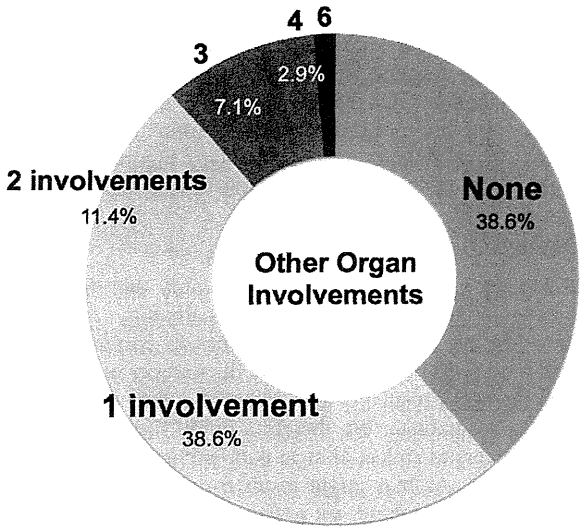

Figure 2. OOI by PET and CT evaluation. Involvement of organs other than the lacrimal and salivary glands was seen in 61.4% of IgG4-DS cases. More than three lesions were observed in about 11% of cases.

Serological evaluation at diagnosis

Sixty-nine patients (56.6%) with IgG4-DS showed hypergammaglobulinemia. Elevated levels of serum IgG4 and IgE were observed in 116 (95.1%) and 37 patients (30.3%), respectively. Hypocomplementemia was detected in 41 patients (33.6%). We divided patients into groups according to the presence or absence of OOI, and compared levels of these serological markers. The OOI-positive group comprised 75 patients (61.5%), and the OOI-negative group comprised 47 patients (38.5%). Mean levels of serum IgG were 2482 ± 1,351.1 mg/dL in the OOI-positive and 1872.5 ± 854.2 mg/dL in the OOI-negative group ($p < 0.01$). Mean serum IgG4 levels were 862.1 ± 714.8 mg/dL and 478.1 ± 363.3 mg/dL, respectively ($p < 0.001$). Levels of these markers differed significantly between groups (Figure 3); however, no significant difference was seen between groups in the proportions of patients presenting with abnormally elevated IgE levels or hypocomplementemia.

Maintenance treatment for IgG4-DS

Subjects in this analysis comprised 89 patients who presented with relapse in 2013, after excluding untreated patients and patients with tapering of glucocorticoid. Drug-free patients with clinical remission were included. The rate of glucocorticoid prescription was 92.1%, and the mean steroid dose for maintenance was 4.8 mg/day. Figure 4 shows the dose distribution. Patients treated with less than 5 mg/day of prednisolone comprised 52.8%, but some patients needed > 10 mg/day for the maintenance of clinical remission.

Immunosuppressants were used in combination with steroid for 9.0% of cases. The oral immunosuppressants used were azathioprine, and cyclosporine A. Rituximab was prescribed in three cases, each of which presented with several relapses (see Supplementary Table 2 http://informahealthcare.com/doi/abs/10.3109/14397595.2014.950036). The regimen comprised rituximab at only 500 mg/body every 6 months, with gradual extension of the intervals. The glucocorticoid-sparing effect seen as a reduction from 13.0 ± 2.7 mg/day to 3.7 ± 0.6 mg/day (-71.8%) using rituximab.

Clinical remission, relapse, and adverse events

An MAQ score of 0 was seen in 44.9% of patients (Figure 5) according to the analysis of patients treated with maintenance therapy. In all 122 patients, clinical remission had been reached and maintained in 73.8% of cases as of 2013. The rate of steroid discontinuation was 5.7%. Levels of serum IgG4 were still high (> 135 mg/dL) in some patients who achieved clinical remission, but levels of all serological markers (e.g., IgG, IgG4, IgE, and complements) were within normal ranges in all patients who achieved drug-free remission. The annual relapse rate was 11.5%. New OOI was seen in 28.6% of cases with recurrence. The mean dose of prednisolone at relapse was 3.2 ± 3.7 mg/day. A Kaplan–Meier relapse-free survival curve estimated that relapse occurred in half of patients within 7 years (Figure 6) using the data collected from April 1997 to December 2013.

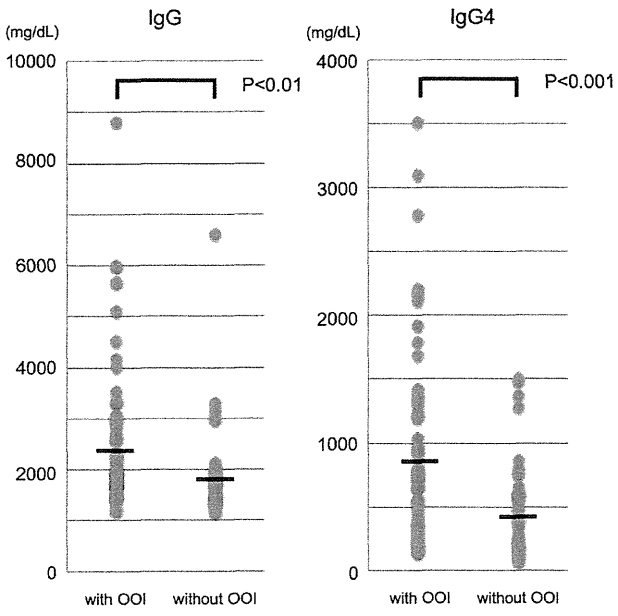

Figure 3. Levels of serum IgG and IgG4 in OOI-positive and -negative groups. The OOI-positive group showed significantly elevated levels of serum IgG and IgG4 compared to the OOI-negative group.

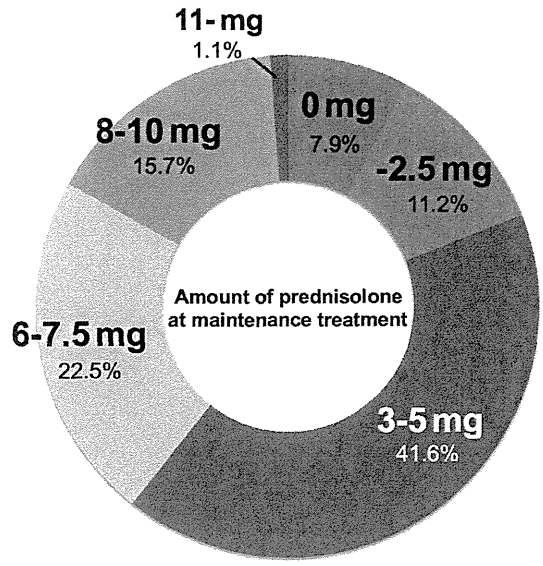

Figure 4. Dose of glucocorticoid for maintenance therapy. Mean steroid dose was about 5 mg/day. Control could be achieved on < 5 mg/day of prednisolone in about half of cases, but some required > 11 mg/day of steroids.

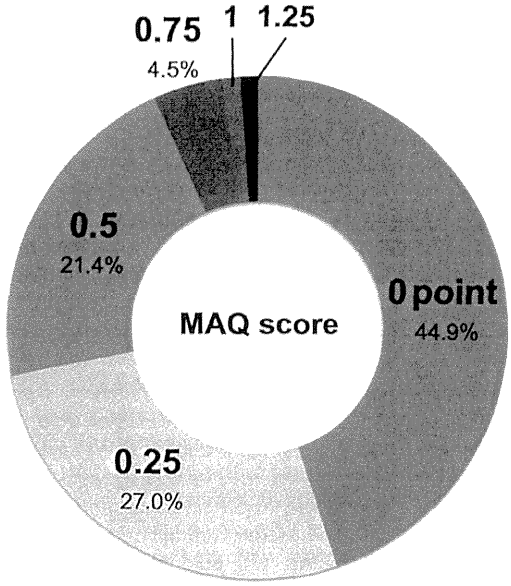

Figure 5. Distribution of MAQ scores. Only 44.9% of cases satisfied the criterion for improvement of clinical symptoms.

Severe adverse events were encountered in nine cases, comprising one case each of lymphoma, acute myeloid leukemia, sebaceous carcinoma, sepsis, pneumonia, and Mallory–Weiss syndrome, and three cases of osteonecrosis. Adverse events other than the above were observed in 13 cases, comprising 1 case each of suppurative osteomyelitis, scrotal edema, depression, angina pectoris, and umbilical hernia, and 4 cases each of herpes zoster and osteoporosis. The rate of adverse events was no higher in cases treated with immunosuppressants (including rituximab) than in cases treated using steroid monotherapy.

Complications of malignancy

On the other hand, the frequency of a history or complications of malignancy as of the end of 2013 was 7.4%, with lymphoma and breast cancer in two cases each, and acute promyelocytic leukemia, skin cancer, colon cancer, tongue cancer, and gastrointestinal stromal tumor (GIST) in one case each. Lymphoma comprised mucosa-associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma. PET was useful for detecting the cases with lymphoma, colon cancer, and GIST. Malignancy and IgG4-DS were diagnosed simultaneously in the patients with MALT lymphoma, colon cancer, and GIST. Six patients (66.7%) showed complications of malignancy within 3 years after the diagnosis of IgG4-DS (see Supplementary Figure 3 http://informahealthcare.com/doi/abs/10.3109/14397595.2014.950036).

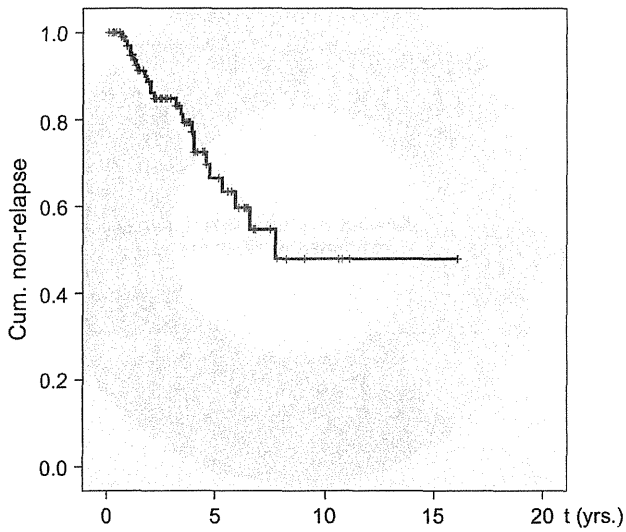

Figure 6. Kaplan–Meier relapse-free survival curve in IgG4-DS. The Kaplan–Meier survival curve showed that relapse occurred in about half of IgG4-DS patients within 7 years.

Discussion

IgG4-RD is a newly established disease entity, and the frequency of rheumatologists encountering this pathology is increasing in daily practice. However, the information currently available regarding IgG4-RD is insufficient, as the number of cases seen at a single facility is relatively low, and useful clinical data are thus difficult to accumulate. We therefore established SMART using multiple centers to collect clinical data, including patient profiles and treatments. As bias might result from us selecting cases to introduce to this database, we registered all cases from multiple facilities to eliminate the problem. This project will be continued into the future, and 122 patients had been registered to SMART and followed-up as of the end of 2013.

We have previously reported that patients with IgG4-DS, previously known as MD, were predominantly female [8]. The sex ratio recently seems to have shifted to either no bias or toward males with the gradual accumulation of cases, and is now close to the tendency seen with type I autoimmune pancreatitis [10]. The reason for this change remains unclear, but we believe that the data are getting closer to the correct sex ratio with the accumulation of IgG4-DS cases. The distribution of age at onset remains skewed toward patients over 60 years old, occupying about 60%. Considering IgG4-RD as a whole as showing a predilection toward older individuals might be appropriate. On the other hand, performing differential diagnosis for IgG4-DS using only the age at onset is not feasible, because the SMART showed some patients with IgG4-DS arising in their 20s.

Our study revealed that about 60% of cases with IgG4-DS showed the involvement of organs other than the lacrimal or salivary glands. This is a higher rate than expected, and involvement of more than two other organs comprised nearly 25%. IgG4-RD could be considered as a systemic disorder, and complications need to be evaluated systemically and carefully. We should recognize that these lesions are not necessarily single.

Next, we analyzed differences in serological markers compared to the first visit to identify factors predictive of OOI. Serum IgG and IgG4 levels were significantly higher in patients with OOI. No conclusion has been reached concerning whether these markers reflect the disease activity of IgG4-RD, but careful examination of whether high levels are present is important on the first visit. In daily practice, complications have to be checked for by routine use of contrast-enhanced CT and FDG-PET at diagnosis. Almost all patients in whom FDG-PET was not performed were experiencing economic hardships. Detection of organ involvement appears fairly straightforward. Several recent reports have described the utility of FDG-PET [11,12], but some organs are unsuited to the detection of lesions using FDG-PET, namely the kidneys and pituitary gland. Contrast-enhanced CT may overcome this weakness of FDG-PET if renal function is unhindered, but hypophysitis is difficult to detect on either FDG-PET or CT. We do not yet know the frequency of pituitary IgG4-RD. Whether MRI of the pituitary gland is needed as a routine screening examination on the first visit must be discussed. At present, we consider MRI as warranted when symptoms of pituitary hormone deficiency or pituitary gland compression are seen [13]. As described below, latent malignancies are an important problem in IgG4-RD. Only FDG-PET cannot differentiate between inflammation of IgG4-RD and malignancy,

but FDG-PET remains useful for the detection of lesions requiring careful examination.

With regard to the treatment of IgG4-DS, glucocorticoids are the first-line agents in clinical practice. This is because glucocorticoids can induce clinical remission and offer good efficacy for the improvement of clinical symptoms [14]. With regard to the initial dose of prednisolone for induction therapy, no consensus has yet been reached and hard evidence remains lacking. We analyzed the cases that initial dose had to be decreased because of older age or other complication, comparing to the cases treated with our protocol, and found that cases treated using a lower dose of glucocorticoid tended to relapse more easily [15]. Our data reveal that most patients with IgG4-DS continue glucocorticoid treatment as maintenance therapy. Until now, data on maintenance doses for clinical remission have been unavailable. The present analysis revealed that half of cases presented with good control on less than 5 mg/day of prednisolone, but several populations could not be regulated on over 10 mg/day. This difference in response might directly reflect disease activity, but the differences in pathogenesis involved remain unclear. Devising treatment strategies for cases requiring high doses of steroid for maintenance treatment may be necessary. The present analysis revealed that oral immunosuppressants are prescribed for cases presenting with frequent relapse, or cases needing over 10 mg/day of prednisolone to achieve clinical remission. Khosroshahi et al. recently reported the efficacy of rituximab for IgG4-RD [16]. We also treated three cases using rituximab, and found that the steroid-sparing effect was higher than that with traditional oral immunosuppressants. The safety of rituximab is also not inferior to those agents. We might therefore consider the option of rituximab for younger patients who have experienced several relapses; however, the mode of action for rituximab is as yet unknown, and clarification of the target of rituximab in IgG4-RD is needed.

While the rate of clinical remission as judged by physicians was 73.8%, the frequency of an MAQ score of 0, which reflects patient satisfaction, was 44.9%. A gap of about 30% thus exists between the judgment of doctors and patients. This was somewhat surprising. Methods combining the subjective and objective evaluation of IgG4-RD are lacking at present, and need to be established. Although the data are not shown, the rate of clinical remission has recently tended to increase, and the rate of annual relapse has tended to decrease, presumably because we try to follow the cases with IgG4-DS according to both clinical symptoms and levels of serum biomarkers, such as serum levels of IgG, IgG4, IgE, and complement. Importantly, use of serological markers alone cannot completely predict relapse [17]. Levels of serum IgG4 are insufficient as a disease activity marker in IgG4-RD. On the other hand, patients who achieved discontinuation of steroid showed normalization of immunological marker levels. This suggests that immunological remission is necessary to achieve cure of IgG4-RD, and this might be impossible using glucocorticoid monotherapy alone in most cases. No data have previously been available regarding doses of steroid at relapse, but SMART disclosed a dose of 3.2 mg/day. This supports the notion that 40% of cases are treated with 3–5 mg/day as maintenance. This study also provided an understanding of the long-term prognosis. Half of the cases presented with recurrence within 7 years from initial treatment. The annual relapse rate tended to remain relatively steady. We have already reported that half of the cases presented with new OOI at relapse [18]. There are two possibilities in the interpretation. The first is a case that the lesions have already formed at first, but they are difficult to detect by PET. The second is a case that new lesions are formed in the process of tapering glucocorticoid. We don't know yet whether these possibilities are correct. Consideration in conjunction with this fact suggests the necessity of following patients with IgG4-DS systemically for several years from initial treatment. Analysis of differences in cases presenting with and without relapse is needed in the future. In terms of adverse events, we should note that the incidence of osteonecrosis is very high in IgG4-DS. With regard to autoimmune disorders, the high incidence of femoral head avascular necrosis in systemic lupus erythematosus has already been reported [19]. The rate in IgG4-DS is still unknown, but a previous survey showed the frequency was again high [20]. SMART revealed a similar tendency. The risk of osteonecrosis should be recognized at induction therapy for IgG4-DS.

As described above, CT and FDG-PET are also useful for detecting malignancies. We found that 10% of cases with IgG4-DS in SMART had some history or complications of malignancy. We have previously reported that the standardized incidence of malignancy in IgG4-RD within 3 years from diagnosis was 3.5-fold higher than in the general population [21]. The risk of latent malignancy has been pointed out for autoimmune pancreatitis [22]. The relationship between IgG4-RD and malignancy needs further discussion [23], but analysis of the time at which malignancies were diagnosed from SMART revealed that two-thirds of the nine patients showed malignancies within 3 years of the diagnosis of IgG4-DS, and the remaining three patients received simultaneous diagnoses. No tendencies toward specific kinds of malignancy were apparent. These findings suggest that screening for malignancy is also important at the time of diagnosing IgG4-RD.

We will keep trying to unearth useful information for clinicians from this database in order to aid in the development of new therapeutic strategies to completely regulate IgG4-DS.

Conclusion

We established SMART through multiple institutions, and have described the clinical features and treatment of IgG4-DS in daily clinical practice. Systemic screening for complications and malignancies at the diagnosis of IgG4-DS is important. Glucocorticoid can easily achieve clinical remission, but most cases require maintenance therapy with glucocorticoid. The efficacy of rituximab for cases with frequent relapse needs to be validated as soon as possible.

Conflict of interest

None.

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Supplementary Figures 1–3, Tables 1 and 2.

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ORIGINAL ARTICLE

IGF2 differentially methylated region hypomethylation in relation to pathological and molecular features of serrated lesions

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Abstract

AIM: To investigate *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation in relation to clinicopathological and molecular features in colorectal serrated lesions.

METHODS: To accurately analyze the association between the histological types and molecular features of each type of serrated lesion, we consecutively collected 1386 formalin-fixed paraffin-embedded tissue specimens that comprised all histological types [hyperplastic polyps (HPs, *n* = 121), sessile serrated adenomas (SSAs, *n* = 132), traditional serrated adenomas (TSAs, *n* = 111), non-serrated adenomas (*n* = 195), and colorectal cancers (CRCs, *n* = 827)]. We evaluated the methylation levels of *IGF2* DMR0 and long interspersed nucleotide element-1 (LINE-1) in HPs (*n* = 115), SSAs (*n* = 120), SSAs with cytological dysplasia (*n* = 10), TSAs (*n* = 91), TSAs with high-grade dysplasia (HGD) (*n* = 15), non-serrated adenomas (*n* = 80), non-serrated adeno-

mas with HGD (n = 105), and CRCs (n = 794). For the accurate quantification of the relative methylation levels (scale 0%-100%) of *IGF2* DMR0 and LINE-1, we used bisulfite pyrosequencing method. Tumor specimens were analyzed for microsatellite instability, *KRAS* (codons 12 and 13), *BRAF* (*V600E*), and *PIK3CA* (exons 9 and 20) mutations; *MLH1* and *MGMT* methylation; and IGF2 expression by immunohistochemistry.

RESULTS: The distribution of the *IGF2* DMR0 methylation level in 351 serrated lesions and 185 non-serrated adenomas (with or without HGD) was as follows: mean 61.7, median 62.5, SD 18.0, range 5.0-99.0, interquartile range 49.5-74.4. The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geqslant$ 74.5, Q2 62.6-74.4, Q3 49.6-62.5, Q4 $\leqslant$ 49.5) for further analysis. With regard to the histological type, the *IGF2* DMR0 methylation levels of SSAs (mean ± SD, 73.1 ± 12.3) were significantly higher than those of HPs (61.9 ± 20.5), TSAs (61.6 ± 19.6), and non-serrated adenomas (59.0 ± 15.8) (P < 0.0001). The *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level (r = -0.21, P = 0.0051). *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas (P < 0.0001). Multivariate logistic regression analysis also showed that *IGF2* DMR0 hypomethylation was inversely associated with SSAs (P < 0.0001). The methylation levels of *IGF2* DMR0 and LINE-1 in TSAs with HGD (50.2 ± 18.7 and 55.7 ± 5.4, respectively) were significantly lower than those in TSAs (61.6 ± 19.6 and 58.8 ± 4.7, respectively) (*IGF2* DMR0, P = 0.038; LINE-1, P = 0.024).

CONCLUSION: *IGF2* DMR0 hypomethylation may be an infrequent epigenetic alteration in the SSA pathway. Hypomethylation of *IGF2* DMR0 and LINE-1 may play a role in TSA pathway progression.

Key words: *BRAF*; Colon polyp; Colorectal neoplasia; Colorectum; Genome; Insulin-like growth factor; Long interspersed nucleotide element-1; Microsatellite instability; Serrated pathway

Core tip: The serrated pathway attracts considerable attention as an alternative colorectal cancer (CRC) pathway. We previously reported the association of *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation with prognosis and its link to LINE-1 hypomethylation in CRC; however, there have been no studies describing its role in the serrated pathway. Therefore, we evaluated the methylation levels of *IGF2* DMR0 and long interspersed nucleotide element-1 (LINE-1) in 351 serrated lesions and 185 non-serrated adenomas. Our results suggest that the *IGF2* DMR0 may be an infrequent epigenetic alteration in the sessile serrated adenoma pathway. Moreover, we found that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of traditional serrated adenoma.

Naito T, Nosho K, Ito M, Igarashi H, Mitsuhashi K, Yoshii S, Aoki H, Nomura M, Sukawa Y, Yamamoto E, Adachi Y, Takahashi H, Hosokawa M, Fujita M, Takenouchi T, Maruyama R, Suzuki H, Baba Y, Imai K, Yamamoto H, Ogino S, Shinomura Y. *IGF2* differentially methylated region hypomethylation in relation to pathological and molecular features of serrated lesions. *World J Gastroenterol* 2014; 20(29): 10050-10061 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i29/10050.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i29.10050

INTRODUCTION

The serrated neoplasia pathway has attracted considerable attention as an alternative pathway of colorectal cancer (CRC) development, and serrated lesions exhibit unique clinicopathological or molecular features[1-23]. According to the World Health Organization (WHO) classification[24], colorectal premalignant (or non-malignant) neoplastic lesions with serrated morphology currently encompass three major categories: hyperplastic polyp (HP), sessile serrated adenoma (SSA), and traditional serrated adenoma (TSA).

SSA and TSA are premalignant lesions, but SSA is the principal serrated precursor of CRCs[15]. In particular, there are many clinicopathological and molecular similarities between SSA and microsatellite instability (MSI)-high CRC, for example, right-sided predilection, *MLH1* hypermethylation, and frequent *BRAF* mutation[7,15,17-19,25-28]. Therefore, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon[7,15,17,25,26,28,29].

In contrast, TSAs are much less common than SSAs, and thus, there are fewer data on their molecular profile[15,25]. TSAs typically do not show *MLH1* hypermethylation or develop to MSI-high CRCs, but they do commonly have *MGMT* hypermethylation[15,25,26]. With regard to the *PIK3CA* gene, a previous study reported that no mutation was found in serrated lesions, and that mutations were uncommonly, but exclusively, observed in non-serrated adenomas (1.4%)[30]. Because some HPs do share molecular features with TSAs (*e.g.*, *KRAS* mutation)[3,25,26,31], it has been suggested that the TSA pathway (HP-TSA-carcinoma sequence) may diverge from the SSA pathway (HP-SSA-SSA with cytological dysplasia-carcinoma sequence) on the basis of *KRAS vs BRAF* mutations and/or *MLH1 vs MGMT* hypermethylation within subsets of HPs[15]. However, a definite precursor of TSA has not been established. In addition, the key carcinogenic mechanism involved in this TSA pathway remains largely unknown.

Loss of imprinting (LOI) of *insulin-like growth factor 2* (*IGF2*) has been shown to be associated with an increased risk of CRC[32,33], suggesting that it may play a role in colorectal carcinogenesis. The imprinting and expression of *IGF2* are controlled by CpG-rich regions known as differentially methylated regions (DMRs)[34-37]. In particular, *IGF2* DMR0 hypomethylation has been

suggested as a surrogate-biomarker for *IGF2* LOI[38]. Previously, we reported that *IGF2* DMR0 hypomethylation in CRC was associated with poor prognosis and might be linked to global DNA hypomethylation [long interspersed nucleotide element-1 (LINE-1) hypomethylation][38]. However, to date, there have been no studies describing the role of *IGF2* DMR0 hypomethylation in the early stage of colorectal carcinogenesis.

To investigate the role of *IGF2* DMR0 hypomethylation in serrated lesions we examined *IGF2* DMR0 and LINE-1 methylation levels as well as other molecular alterations using a large sample of 1330 colorectal tumors (351 serrated lesions, 185 non-serrated adenomas, and 794 CRCs).

MATERIALS AND METHODS

Histopathological evaluation of tissue specimens of colorectal serrated lesions

Histological findings related to all colorectal serrated lesion specimens were evaluated by a pathologist (Fujita M) who was blinded to the clinical and molecular information. Serrated lesions (HPs, SSAs, and TSAs) were classified on the basis of the current WHO criteria[24]. HPs were further subdivided into microvesicular HPs and goblet cell HPs.

SSAs are characterized by the presence of a disorganized and distorted crypt growth pattern that is usually easily identifiable upon low-power microscopic examination. Crypts, particularly at the basal portion of the polyp, may appear architecturally distorted, dilated, and/or branched, particularly in the horizontal plane, which leads to the formation of boot, L, or anchor-shaped crypts. The cytology is typically quite bland, but a minor degree of nuclear atypia is allowable, particularly in the crypt bases[15,25,26].

To accurately analyze the association between the histological types and molecular features of each type of serrated lesion we consecutively collected more than 100 formalin-fixed paraffin-embedded (FFPE) tissue specimens of each histological type (HP, SSA, and TSA). In total, 364 tissue specimens of serrated lesions [121 HPs, 122 SSAs, 10 SSAs with cytological dysplasia, 96 TSAs, and 15 TSAs with high-grade dysplasia (HGD)] from patients who underwent endoscopic resection or other surgical treatment at Sapporo Medical University Hospital, Keiyukai Sapporo Hospital or Teine-Keijinkai Hospital between 2001 and 2012 were available for assessment. All of HPs were microvesicular HPs.

The serrated lesions were classified by location: the proximal colon (cecum, ascending and transverse colon), distal colon (splenic flexure, descending, sigmoid colon) and rectum. Informed consent was obtained from all the patients before specimen collection. This study was approved by the institutional review boards of the participating institutions. The term "prognostic marker" is used throughout this article according to the REMARK Guidelines[39].

Tissue specimens of CRC and non-serrated adenomas

FFPE tissue specimens of 827 CRCs (stages I -IV), 85 non-serrated adenomas (*i.e.*, tubular or tubulovillous adenomas), and 110 non-serrated adenomas with HGD from patients who underwent surgical treatment or endoscopic resection at the above hospitals were also collected. The criterion for diagnosing cancer was invasion of malignant cells beyond the muscularis mucosa.

DNA extraction and pyrosequencing for KRAS, BRAF, and PIK3CA and MSI analysis

Genomic DNA was extracted from the FFPE tissue specimens of the colorectal tumors using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, United States). PCR and targeted pyrosequencing were then performed using the extracted genomic DNA to determine the presence of *KRAS* (codons 12 and 13), *BRAF* (*V600E*) and *PIK3CA* (exons 9 and 20) mutations[40,41]. MSI analysis was performed as previously described using 10 microsatellite markers[14]. MSI-high was defined as instability in $\geqslant$ 30% of the markers and MSI-low/microsatellite stable (MSS) as instability in $<$ 30% of the markers[14].

Sodium bisulfite treatment and pyrosequencing to measure IGF2 DMR0 and LINE-1 methylation levels

Bisulfite modification of genomic DNA was performed using a BisulFlash™ DNA Modification Kit (Epigentek, Brooklyn, NY, United States).

We measured the relative methylation level at the *IGF2* DMR0 using a bisulfite-pyrosequencing assay as previously described[38]. The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated as percentage (scale 0%-100%). We calculated the average of the first and second CpG sites in the *IGF2* DMR0 as the *IGF2* DMR0 methylation level. Likewise, to accurately quantify the LINE-1 methylation levels we utilized a pyrosequencing assay, as previously described[42].

Pyrosequencing to measure MGMT and MLH1 promoter methylation

Pyrosequencing for *MGMT* and *MLH1* methylation was performed using the PyroMark kit (Qiagen). We used a previously defined cut-off of $\geqslant$ 8% methylated alleles for *MGMT* and *MLH1* hypermethylated tumors[43].

Immunohistochemistry for IGF2 expression

For IGF2 staining, we used anti-IGF2 antibody (Rabbit polyclonal to IGF2; Abcam, Cambridge, MA, United States) with a subsequent reaction performed using Target Retrieval Solution, Citrate pH 6 (Dako Cytomation, Carpinteria, CA, United States). In each case, we recorded cytoplasmic IGF2 expression as no expression, weak expression, moderate expression, or strong expression relative to normal colorectal epithelial cells. IGF2 expression was visually interpreted by Nosho K, who was unaware of the other data. For the agreement study of IGF2 expression, 128 randomly selected cases were examined by a second pathologist (by Naito T), who was also unaware

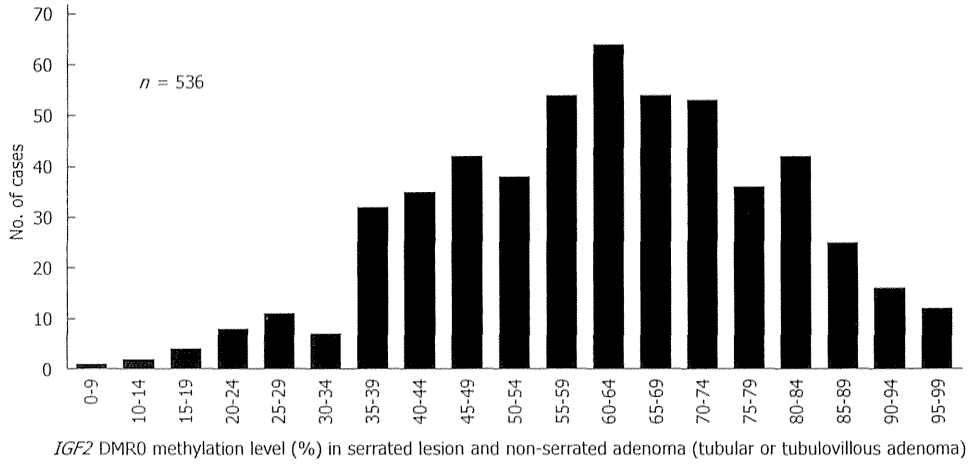

Figure 1 Distribution of *IGF2* differentially methylated region 0 methylation levels in 351 serrated lesions. Hyperplastic polyp, sessile serrated adenoma (SSA), SSA with cytological dysplasia, traditional serrated adenoma (TSA) and TSA with high-grade dysplasia (HGD) and 185 non-serrated adenomas (tubular adenoma, tubular adenoma with HGD, tubulovillous adenoma and tubulovillous adenoma with HGD). DMR: Differentially methylated region; *IGF2*: *Insulin-like growth factor 2*.

of the other data. The concordance between the two pathologists ($P < 0.0001$) was 0.84 ($\kappa = 0.69$), indicating substantial agreement.

Statistical analysis

JMP (version 10) software was used for all statistical analyses (SAS Institute, Cary, NC, United States). All P values were two-sided. Univariate analyses were performed to investigate the clinicopathological and molecular characteristics including *IGF2* DMR0 and LINE-1 hypomethylation, according to histological type, classified as serrated lesion, non-serrated adenoma, and CRC. P values were calculated by analysis of variance for age, tumor size, and the methylation levels of *IGF2* DMR0 and LINE-1 and by χ^2 or Fisher's exact test for all other variables. A multivariate logistic regression analysis was employed to examine associations with *IGF2* DMR0 hypomethylation (as an outcome variable), adjusting for potential confounders. The model initially included sex, age, tumor size, tumor location, histological type, and the LINE-1 methylation level, and MSI, *BRAF*, *KRAS*, and *PIK3CA* mutations. In the CRC-specific survival analysis, the Kaplan-Meier method and log-rank test were used to assess the survival time distribution. The Spearman correlation coefficient was used to assess the correlation of the *IGF2* DMR0 methylation level and IGF2 expression.

RESULTS

The IGF2 DMR0 methylation level in serrated lesion and non-serrated adenomas

We assessed 559 FFPE tissue specimens of serrated lesions and non-serrated adenomas in the *IGF2* DMR0 methylation assay and obtained 536 (96%) valid results. The distribution of the *IGF2* DMR0 methylation level in 351 serrated lesions and 185 non-serrated adenomas (with or without HGD) was as follows: mean 61.7, median 62.5, SD 18.0, range 5.0-99.0, interquartile range 49.5-74.4 (all on a 0-100 scale) (Figure 1). The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geqslant$ 74.5, Q2 62.6-74.4, Q3 49.6-62.5, Q4 $\leqslant$ 49.5) for further analysis.

We evaluated the *IGF2* DMR0 methylation level in serrated lesions (HP, SSA, and TSA) and non-serrated adenomas according to their histological type. The *IGF2* DMR0 methylation levels of SSAs ($n = 120$, mean $\pm$ SD, 73.1 $\pm$ 12.3) were significantly higher than those of HPs ($n = 115$, 61.9 $\pm$ 20.5, $P < 0.0001$), TSAs ($n = 91$, 61.6 $\pm$ 19.6, $P < 0.0001$), and non-serrated adenomas ($n = 80$, 59.0 $\pm$ 15.8, $P < 0.0001$) (Figure 2).

IGF2 DMR0 hypomethylation was associated with larger tumor size in serrated lesions and non-serrated adenomas (Table 1). With regard to the histological type, *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in HPs, TSAs, and non-serrated adenomas ($P < 0.0001$) (Table 1). Multivariate logistic regression analysis also showed the *IGF2* DMR0 hypomethylation was inversely associated with SSAs ($P < 0.0001$).

Association of IGF2 expression and IGF2 DMR0 methylation level in serrated lesions and non-serrated adenomas

We examined IGF2 overexpression in 168 colorectal serrated lesions and non-serrated adenomas. The *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level ($r = -0.21$, $P = 0.0051$).

IGF2 DMR0 methylation level in colorectal cancer

A total of 827 paraffin-embedded CRCs (stages I -IV) were subjected to an *IGF2* DMR0 methylation assay with 794 (96%) valid results. The distribution of the *IGF2* DMR0 methylation level in these 794 CRCs was as follows: mean 54.7, median 55.0, SD 13.7, range 7.5-98.0, interquartile range 46.1-63.0 (all on a 0-100 scale). The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geqslant$ 63.0, Q2 55.0-62.9, Q3 46.1-54.9, Q4 $\leqslant$ 46.0) for

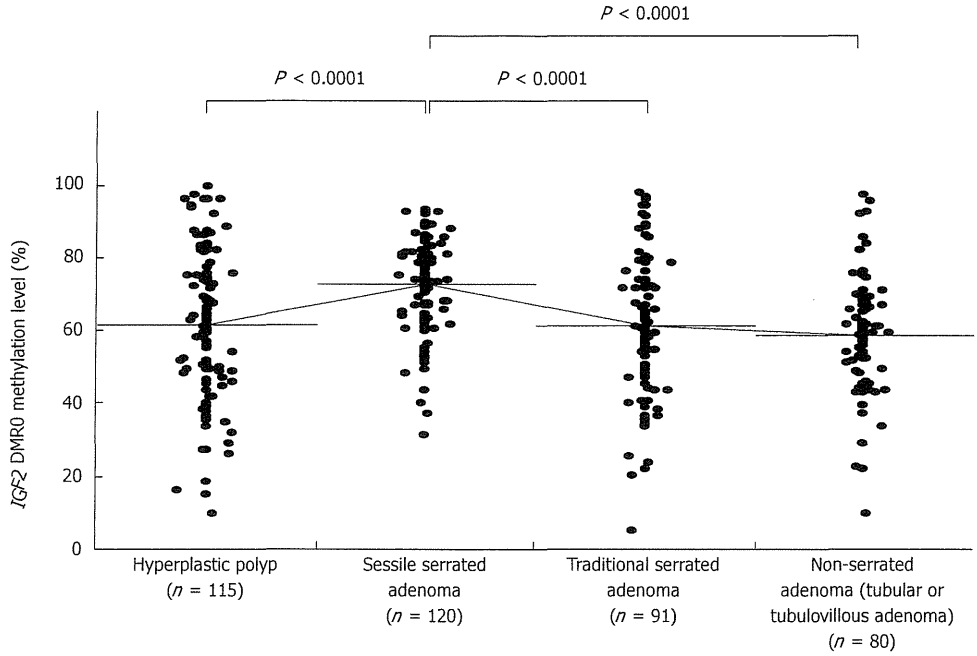

Figure 2 *IGF2* differentially methylated region 0 methylation level according to histological type. *Insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 methylation levels of sessile serrated adenoma (mean ± SD; 73.1 ± 12.3) were significantly higher compared with those of hyperplastic polyp (61.9 ± 20.5, $P < 0.0001$), traditional serrated adenoma (61.6 ± 19.6, $P < 0.0001$), and non-serrated adenoma (59.0 ± 15.8, $P < 0.0001$). *P*-values were calculated by analysis of variance.

Table 1 *IGF2* differentially methylated region 0 hypomethylation in serrated lesions and non-serrated adenomas *n* (%)

Clinicopathological feature	Total *n*	*IGF2* DMR0 methylation (quartile)				*P* value
		Q1 (≥ 74.5)	Q2 (62.6-74.4)	Q3 (49.6-62.5)	Q4 (≤ 49.5)	
All cases	536	134	130	131	141	
Sex						
Male	326 (61)	78 (58)	80 (62)	92 (70)	76 (54)	0.041
Female	210 (39)	56 (42)	50 (38)	39 (30)	65 (46)	
Age (mean ± SD)	61.5 ± 12.2	59.9 ± 12.3	60.8 ± 12.0	63.1 ± 11.6	62.3 ± 13.0	0.150
Tumor size (mm) (mean ± SD)	14.3 ± 11.4	9.9 ± 4.0	13.4 ± 7.4	14.7 ± 11.1	19.1 ± 17.6	< 0.0001
Tumor location						
Rectum	70 (13)	11 (8.5)	14 (11)	18 (14)	27 (20)	0.061
Distal colon	161 (31)	35 (27)	43 (33)	37 (29)	46 (33)	
Proximal colon	296 (56)	84 (65)	72 (56)	75 (58)	65 (47)	
Histological type						
Hyperplastic polyp (HP)	115 (21)	33 (25)	25 (19)	23 (18)	34 (24)	< 0.0001
Sessile serrated adenoma (SSA) without cytological dysplasia	120 (22)	60 (45)	39 (30)	15 (11)	6 (4.3)	
SSA with cytological dysplasia	10 (1.9)	1 (0.8)	3 (2.3)	6 (4.6)	0 (0)	
Traditional serrated adenoma (TSA) without high-grade dysplasia (HGD)	91 (17)	22 (16)	21 (16)	23 (18)	25 (18)	
TSA with HGD	15 (2.8)	2 (1.5)	2 (1.5)	2 (1.5)	9 (6.4)	
Non-serrated adenoma (tubular or tubulovillous adenoma) without HGD	80 (15)	11 (8.2)	17 (13)	32 (24)	20 (14)	
Non-serrated adenoma with HGD	105 (20)	5 (3.7)	23 (18)	30 (23)	47 (33)	

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age and tumor size and by χ^2 or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.010 (= 0.05/5).

further analysis.

Colorectal cancer patient survival and IGF2 DMR0 methylation level

The influence of the *IGF2* DMR0 methylation level on clinical outcome was assessed in CRC patients. During the follow-up of 398 patients with metastatic CRC (stages Ⅲ-Ⅳ) who were eligible for survival analysis, mortality occurred in 134, including 118 deaths confirmed to be attributable to CRC. The median follow-up period for