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The Use of Bone Marrow Stromal Cells (Bone Marrow-Derived Multipotent Mesenchymal Stromal Cells) for Alveolar Bone Tissue Engineering: Basic Science to Clinical Translation

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Bone tissue engineering is a promising field of regenerative medicine in which cultured cells, scaffolds, and osteogenic inductive signals are used to regenerate bone. Human bone marrow stromal cells (BMSCs) are the most commonly used cell source for bone tissue engineering. Although it is known that cell culture and induction protocols significantly affect the *in vivo* bone forming ability of BMSCs, the responsible factors of clinical outcome are poorly understood. The results from recent studies using human BMSCs have shown that factors such as passage number and length of osteogenic induction significantly affect ectopic bone formation, although such differences hardly affected the alkaline phosphatase activity or gene expression of osteogenic markers. Application of basic fibroblast growth factor helped to maintain the *in vivo* osteogenic ability of BMSCs. Importantly, responsiveness of those factors should be tested under clinical circumstances to improve the bone tissue engineering further. In this review, clinical application of bone tissue engineering was reviewed with putative underlying mechanisms.

Introduction

ATROPHIC ALVEOLAR BONE is one of the major obstacles for dental implant therapy and there are a large number of patients without sufficient bone volume. For patients with severe bone atrophy, autologous bone grafts have been performed.[1] However, even the amount of harvesting bone is small, the procedure is accompanied by swelling and pain of the donor site.[2] Although bioartificial bone substitutes have been frequently used, the ability to induce bone is limited.[3] Accordingly, less invasive and more efficient bone regeneration therapy is awaited, such as tissue engineering.

The first results of clinical bone tissue engineering were published in 2001.[4] In this study, the regeneration of long bone defects was tested using hydroxyapatite blocks together with cultured autologous bone marrow stromal cells (BMSCs). This tissue engineering-based approach proved the feasibility of this concept. The results from a preliminary clinical study of alveolar bone regeneration were published thereafter.[5] In this review, studies on clinical alveolar bone tissue engineering are summarized. Then, the problems associated with current tissue engineering were also discussed.

Bone Tissue Engineering and Stem Cells

Cells are considered as a major component of tissue engineering. Although the role of transplanted cells during bone tissue regeneration is still controversial, it has been proved that the transplanted cells could survive, proliferate, and differentiate into osteogenic phenotype.[6] There is accumulating evidence that the level and quality of regeneration is affected by the ability of transplanted cells.[7] Accordingly, it is important to establish an optimal cell culture protocol to maximize the function of osteogenic cells. Surprisingly, the BMSC ability to differentiate into osteoblast-like cells is easily diminished during passage and no bone formation was observed after several passages (Fig. 1).[7,8] Furthermore, cell seeding density and the period of induction also affect *in vivo* osteogenic ability. It has been

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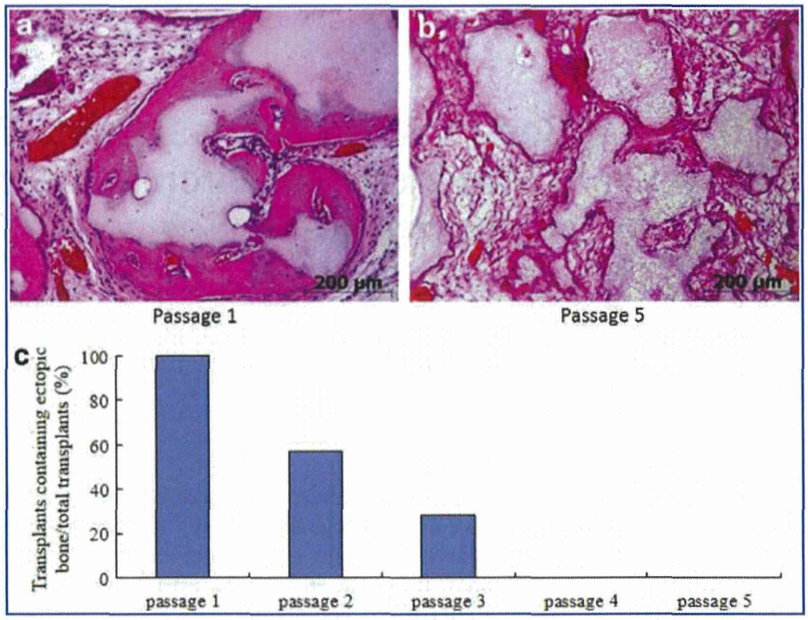

FIG. 1. Effect of passage number on ectopic *in vivo* osteogenic ability. Upper panels showing ectopic bone formation at the back of nude mice with tissue-engineered bone using passage 1 (**a**) and passage 5 (**b**) human bone marrow stromal cells (BMSCs). The success of ectopic bone formation quickly decrease after passage and no bone formation was observed after passage 4 (**c**). Note that the ability is quickly lost during passage. Modified from Agata *et al.*[7] Color images available online at www.liebertpub.com/teb

shown that basic fibroblast growth factor (bFGF) is beneficial to maintain *in vivo* osteogenic ability of BMSCs.[7]

Clinical Studies on Alveolar Bone Tissue Engineering

The results from clinical studies on alveolar bone tissue engineering using BMSCs were first reported in 2004. In this study, bone marrow-derived MSCs were mixed with platelet-rich plasma as a scaffold.[8] Bone regeneration was observed in all moderate atrophy cases. Another clinical study utilized BMSCs and hydroxyapatite granules. BMSCs were induced into osteogenic cells for 1 week and transplanted. In this study, bone formation was observed in three cases, but there was no apparent bone formation from the

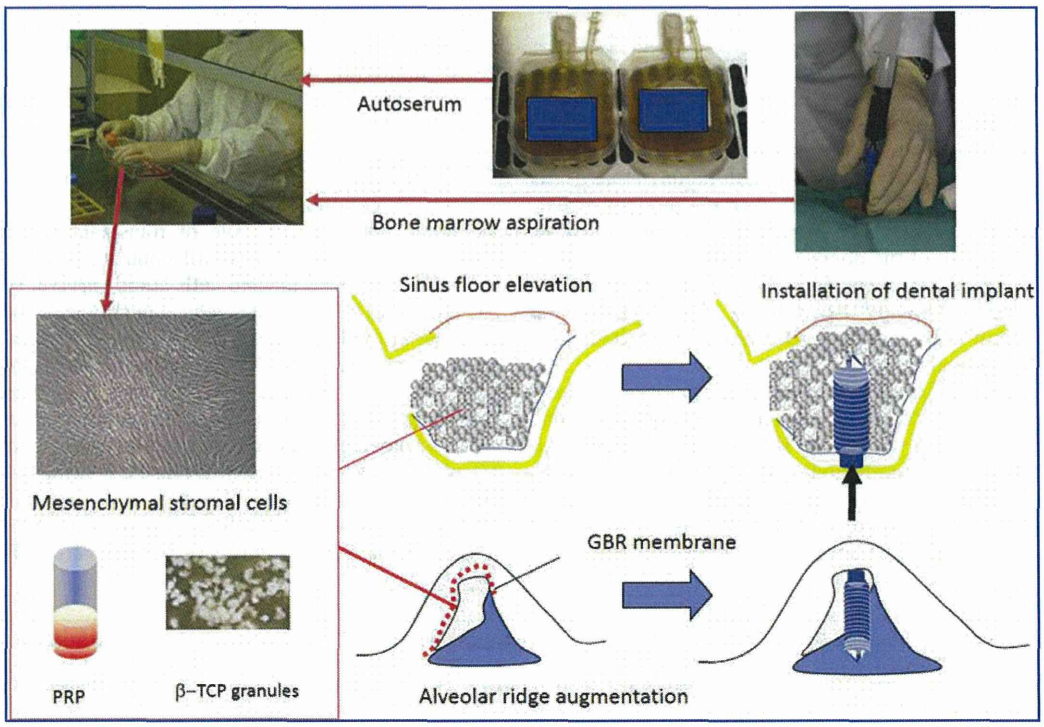

FIG. 2. The procedure for clinical study of alveolar bone regeneration at IMSUT Hospital, The Institute of Medical Science, The University of Tokyo. β-TCP, beta-tricalcium phosphate; PRP, platelet rich plasma. Color images available online at www.liebertpub.com/teb

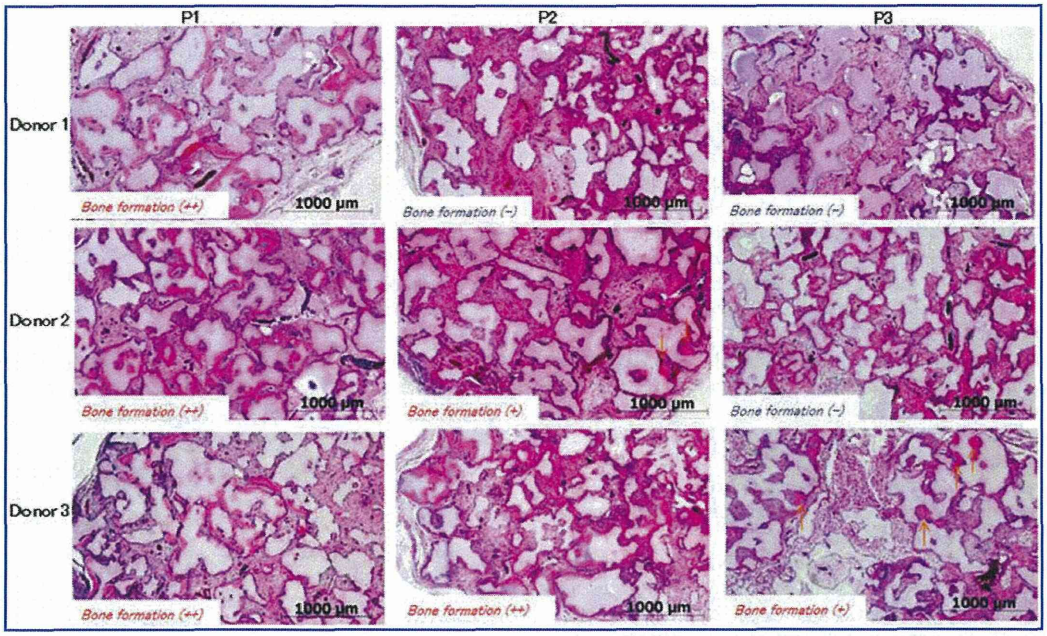

FIG. 3. Individual (donor) variations of *in vivo* osteogenic ability and their changes during passage. Note that the effect of passage differed between individuals. Modified from Agata.[9] Color images available online at www.liebertpub.com/teb

transplanted cells in cases where the atrophy was severe. Thus, the efficacy of clinical alveolar bone tissue engineering for severe atrophy cases remains controversial.

We have conducted a clinical study of bone tissue engineering for severe atrophy of alveolar bone. In this study, autologous BMSCs were transplanted together with platelet-rich plasma gel and beta-tricalcium phosphate (β-TCP) granules as scaffolds (Fig. 2). The results from a 2-year observation showed that bone regeneration was observed in all patients, although significant individual variations in cell growth, differentiation, and levels of bone regeneration were observed (Asahina *et al.*, manuscript in preparation). This type of study, focused on severe atrophy cases, may prove the usefulness of alveolar bone tissue engineering. In terms of safety, no side effects or related complications have been reported, which may imply the relatively safety nature of alveolar bone tissue engineering using BMSCs.

Toward the Establishment of Reliable Alveolar Bone Tissue Engineering Using BMSCs

Although clinical studies have confirmed the feasibility and safety of alveolar bone tissue engineering using BMSCs, one of the important clinical benchmarks is the efficacy for severe atrophy cases. The results from focused studies with selected cases will provide the evidence. Another important problem is the individual variation as shown by basic and preliminary clinical studies. Since the shape and the size of bone defect vary among individuals, it might be impossible to completely eliminate such variations. Accordingly, it should be important to minimize the variation in other factors, such as cells. In terms for BMSCs, there was no significant difference in the expression of mesenchymal stem cell markers during passage.[7] In contrast, a large variation was observed in the *in vivo* bone forming ability among donors and during passage (Fig. 3).[7,8,9] We believe the usage of early passage cells as well as growth factors (bFGF) may minimize the variation, which should be tested under clinical settings.

In spite of the number of studies and the clinical efficacy of bone tissue engineering, it is not a standard treatment at present. It is necessary to show the superiority of clinical outcome compared with standard autologous bone transplantation and allogenic (or xenogenic) transplantation. Furthermore, tissue engineering requires special facility for cell culture and there is a requirement for many safety examinations, which may also increase the cost for treatment. Those technologies, which may support the widespread use of bone tissue engineering, should be investigated.

Tissue engineering is one of the most rapidly progressing fields and alveolar bone is still an attractive target for tissue engineering.[10] The application of bone tissue engineering is not limited for dental implants and is successfully applied for other diseases such as nonunion fractures[11] and alveolar clefts.[12,13]

Disclosure Statement

No competing financial interests exist.

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ORIGINAL ARTICLE – COLORECTAL CANCER

Association of MicroRNA-31-5p with Clinical Efficacy of Anti-EGFR Therapy in Patients with Metastatic Colorectal Cancer

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ABSTRACT

Background. Gene mutations in the pathway downstream of epidermal growth factor receptor (EGFR) are considered to induce resistance to anti-EGFR therapy in colorectal cancer (CRC). We recently reported that microRNA-31 (miR-31)-5p may regulate *BRAF* activation and play a role in the signaling pathway downstream of EGFR in CRC. Therefore, we hypothesized that miR-31-5p can be a useful biomarker for anti-EGFR therapy in CRC.

Methods. We evaluated miR-31-5p expression and gene mutations [*KRAS* (codon 61 or 146), *NRAS* (codon 12, 13, or 61), and *BRAF* (*V600E*)] in the EGFR downstream pathway in 102 CRC patients harboring *KRAS* (codon 12 or 13) wild-type who were treated with anti-EGFR therapeutics. Progression-free survival (PFS) and overall survival (OS) were evaluated.

Results. *KRAS* (codon 61 or 146), *NRAS*, and *BRAF* mutations were detected in 6.9, 6.9, and 5.9 % patients, respectively. Compared with CRCs with at least one mutation ($n = 20$), significantly better PFS ($P = 0.0003$) but insignificantly better OS were observed in CRCs harboring all wild-type genes (*KRAS*, *NRAS*, and *BRAF*). High miR-31-5p expression was identified in 11 % ($n = 11$) patients and was significantly associated with shorter PFS ($P = 0.003$). In CRCs carrying all wild-type genes, high miR-31-5p was associated with shorter PFS ($P = 0.027$).

Conclusions. High miR-31-5p expression was associated with shorter PFS in patients with CRC treated with anti-EGFR therapeutics. Moreover, in CRCs carrying all wild-type genes, high miR-31-5p was associated with shorter

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PFS, suggesting that it may be a useful and additional prognostic biomarker for anti-EGFR therapy.

In the era of targeted therapies, monoclonal antibodies against the epidermal growth factor receptor (EGFR) have expanded the spectrum of therapeutic options and have improved the clinical outcome of patients with metastatic colorectal cancer (CRC).[1–20] Deregulation of the signaling pathways downstream of EGFR, including the RAS/RAF/MEK/ERK pathway, is believed to induce resistance to anti-EGFR therapy.

Mutations in *KRAS* codon 12 or 13 are widely recognized as a major cause of deregulation of the RAS/RAF/MEK/ERK pathway and are negative predictors for EGFR-targeted therapies in metastatic CRC.[4–6,20–22] The *KRAS* codon 12 or 13 mutations are found in approximately 30–40 % of metastatic CRCs; despite the prediction, more than half of the patients without a mutation in *KRAS* codon 12 or 13 do not benefit from anti-EGFR therapy.[2–6,23]

With respect to resistance to anti-EGFR therapeutics, a mutation in *KRAS* codon 61 or 146 (which is rare but activates the RAS signaling pathway as well as mutation in codon 12 or 13) is actively studied as a possible additional predictive biomarker for anti-EGFR therapy.[7,20] Moreover, several studies have demonstrated that mutations in gene such as *NRAS* and *BRAF* may account for the resistance, because these genes are downstream effectors of the EGFR signaling pathway, similar to the *KRAS* gene.[8–20] Therefore, there is a need to identify additional biomarkers for more accurate selection of patients for anti-EGFR therapy.

MicroRNAs (miRNA) constitute a class of small non-coding RNA molecules (21–25 nucleotides) that function as posttranscriptional gene regulators. miRNAs can function as oncogenes or tumor suppressors. Therefore, they have been increasingly recognized as useful biomarkers of various human cancers.[24–44] In CRC, several miRNAs are known to be deregulated and to target genes in the downstream part of the EGFR pathway, for example, miR-143 and miR-145 for *KRAS* and *BRAF*, respectively.[27,28]

Recently, we reported that high microRNA-31 (miR-31)-5p expression was strongly associated with *BRAF* mutation and poor prognosis in a large statistical sample of CRCs.[45] In addition, we found downregulation of *BRAF* target proteins after transfection of a miR-31-5p inhibitor into the cells. These findings suggest that miR-31-5p may regulate activation of the *BRAF* gene in CRC and may play an important role in the signaling pathway downstream of EGFR.

Therefore, we hypothesized that miR-31-5p can be a useful biomarker for anti-EGFR therapy in patients with CRC. To test this hypothesis, we assessed the association of miR-31-5p expression or gene mutations [*KRAS* (codon 61 and 146), *NRAS* (codon 12, 13, and 61), and *BRAF* (*V600E*)] with clinical outcomes in patients with metastatic CRC treated with anti-EGFR therapy.

MATERIALS AND METHODS

Patients and Tissue Specimens

We collected formalin-fixed, paraffin-embedded (FFPE) tissues of 102 primary tumors of CRCs of patients who underwent surgical treatment and chemotherapy with anti-EGFR antibodies at Sapporo Medical University Hospital or Keiyukai Sapporo Hospital between 1997 and 2013. All patients underwent surgical resection of primary tumor of CRC before receiving anti-EGFR therapy. None of the patients received neoadjuvant chemotherapy or radiation therapy. To clarify the association between microRNA-31-5p expression and survival in patients with metastatic CRC, we limited the patients who received 5-FU-based adjuvant chemotherapy before receiving anti-EGFR therapy. Moreover, patients with metastatic CRC who were treated with other targeted therapies [i.e., antivascular endothelial growth factor (VEGF) therapy] or radiation therapy before or after the surgical resection were excluded. The primary cancer tissues of all patients were confirmed to be both histologically EGFR-positive and without mutations in *KRAS* codons 12 and 13.

Patients' response to anti-EGFR therapy was evaluated every 8 weeks during the treatment. The therapeutic response was assessed using the response evaluation criteria in solid tumors (RECIST).[46] Progression-free survival (PFS) was defined as the time from the beginning of anti-EGFR therapy to progression or death by any cause. Patients who did not meet these criteria were censored at the date of last administration. Overall survival (OS) was defined as the time from the diagnosis of CRC to death by any cause or last follow-up. The patients were followed until death or December 2013, whichever came first. Informed consent was obtained from all patients before specimen collection. This study was approved by the institutional review boards of the participating institutions.

DNA or RNA Extraction and Molecular Analysis

DNA or RNA was extracted from FFPE tissues or colon cancer cell lines. miR-31-5p or -3p expression were analyzed by quantitative reverse transcription-PCR. For gene mutation analysis of *KRAS* (codons 61 and 146), *NRAS* (codons 12, 13, and 61), and *BRAF* (*V600E*), PCR and targeted pyrosequencing were performed. The details of these methods are provided in Supplementary Methods.

Statistical Analysis

JMP (version 10) software was used for statistical analysis (SAS Institute, Cary, NC, USA). All *P* values were two-sided. Univariate analysis was performed to assess clinicopathological and molecular characteristics according to the miRNA expression level; the Chi-square test or Fisher's exact test was used for categorical data, whereas analysis of variance was used to compare the mean patient age. To account for multiple hypothesis testing in associations between miRNA expression and the other ten covariates, the *P* value for significance was adjusted using the Bonferroni correction to $P = 0.005$ (=0.05/10).

In survival analysis, the Kaplan–Meier method and log-rank test were used to assess survival time distribution. Cox proportional hazards regression models were used to compute mortality hazard ratio (HR) according to the molecular status.

RESULTS

MicroRNA-31 Expression in CRCs

The study included 102 patients with metastatic CRC who were treated with anti-EGFR therapy. All patients were tested for miR-31-5p or -3p expression. Eleven patients were in miR-31-5p high-expression group and 91 were in miR-31-5p low-expression group. By contrast, 25 patients were in miR-31-3p high-expression group and 77 were in miR-31-3p low-expression group (details provided in Supplementary Methods). The relative expression levels of both miRNAs were significantly correlated ($r = 0.82$; $P < 0.0001$, Supplementary Fig. 1).

Clinicopathological and Molecular Features of Patients with CRC

Table 1 shows the clinicopathological and molecular features according to miR-31-5p expression level. There was no significant difference between the miR-31-5p high-expression group and low-expression group in all clinicopathological and molecular features. Of the 102 patients with CRC treated with anti-EGFR therapy, 44 (43 %) received cetuximab and 58 (57 %) received panitumumab. *KRAS* mutations (codon 61 or 146), *NRAS* mutations (codon 12, 13, or 61), and *BRAF* mutations (*V600E*) were detected in 7 (6.9 %) patients, 7 (6.9 %) patients, and 6 (5.9 %) patients, respectively. No patient had simultaneous mutations in these three genes. Eighty-two (80 %) patients had all wild-type *KRAS*, *NRAS*, and *BRAF*.

Mutational Status and Efficacy of Anti-EGFR Therapy

During the follow-up of the 102 patients with CRC treated with anti-EGFR therapy who were eligible for survival analysis, 61 patients died (all deaths were confirmed to be attributable to CRC). The median follow-up periods for PFS and OS were 6.0 and 48 months, respectively.

In Kaplan–Meier analysis, a significant difference in PFS ($P = 0.004$) was observed between the mutated *RAS* (at least one mutation in *KRAS* or *NRAS*) group ($n = 14$) and the *RAS* wild-type group ($n = 88$; Fig. 1a). There was no significant difference in OS ($P = 0.53$) between them (Supplementary Fig. 2a). With regard to *BRAF*, no significant difference in PFS ($P = 0.055$; Fig. 1b) and significant difference in OS ($P = 0.029$; Supplementary Fig. 2b) were observed between the patients with CRC with mutation ($n = 6$) and those without mutation ($n = 96$).

In addition, compared with CRCs with at least one mutation in *KRAS*, *NRAS*, or *BRAF* ($n = 20$), significantly better PFS ($P = 0.0003$) was observed in CRCs possessing all wild-type copies of *KRAS*, *NRAS*, and *BRAF* ($n = 82$; Fig. 1c), whereas no significant difference was detected in OS ($P = 0.13$; Supplementary Fig. 2c) between them. In univariate Cox regression analysis, similar results were observed with PFS [HR 3.37; 95 % confidence interval (CI) 1.61–6.67; $P = 0.0018$; Table 2].

MicroRNA-31 Expression and Efficacy of Anti-EGFR Therapy

Median PFS and OS in the miR-31-5p high-expression group were 2.8 and 32 months, respectively; those in the miR-31-5p low-expression group were 6.1 and 51 months, respectively. In Kaplan–Meier analysis, a high miR-31-5p expression level was significantly associated with shorter PFS ($P = 0.003$; Fig. 2a). On the other hand, there was no significant difference in OS ($P = 0.86$) between them (Supplementary Fig. 3). In univariate Cox regression analysis, similar results were observed with PFS (HR 2.88; 95 % CI 1.30–5.73; $P = 0.012$; Table 2).

We also analyzed the association of miR-31-5p expression with the efficacy of anti-EGFR therapy in patients with CRC with no mutations in *KRAS*, *NRAS*, or *BRAF*. In the *RAS* wild-type group ($n = 88$), significantly shorter PFS ($P = 0.035$; Fig. 2b) was observed in the high-expression group compared with low-expression group in Kaplan–Meier analysis. Similarly, significant shorter PFS ($P = 0.0014$; Fig. 2c) was observed in the high-expression groups than low-expression group in patients with *BRAF* wild-type CRC ($n = 96$). Furthermore, a significant difference was detected in PFS ($P = 0.027$; Fig. 2d) between

TABLE 1 Clinicopathological and molecular features of 102 CRC patients who received anti-EGFR therapy

Clinicopathological or molecular feature	Total	MicroRNA-31-5p expression		*P*
		Low-expression	High-expression	
All cases	102	91	11	
Gender				
Male	72 (71 %)	62 (68 %)	10 (91 %)	0.37
Female	30 (29 %)	29 (32 %)	1 (9.1 %)	
Age (mean ± SD)	60.4 ± 11.8	60.2 ± 11.7	60.6 ± 12.1	0.77
Tumor location				
Rectum	36 (35 %)	32 (35 %)	4 (36 %)	0.83
Distal colon (splenic flexure to sigmoid colon)	42 (41 %)	37 (41 %)	5 (45 %)	
Proximal colon (cecum to transverse colon)	24 (24 %)	22 (24 %)	2 (18 %)	
Stage at resection of primary tumor				
III	44 (43 %)	40 (44 %)	4 (36 %)	0.20
IV	58 (57 %)	51 (56 %)	7 (64 %)	
Anti-EGFR agents				
Cetuximab	44 (43 %)	40 (44 %)	4 (36 %)	0.63
Panitumumab	58 (57 %)	51 (56 %)	7 (64 %)	
Line of anti-EGFR therapy				
First line	13 (13 %)	12(13 %)	1 (9.1 %)	0.92
Second line	17 (16 %)	15 (16 %)	2 (18 %)	
Third line and beyond	72 (71 %)	64 (70 %)	8 (73 %)	
KRAS mutation				
Wild-type	95 (93 %)	86 (95 %)	9 (82 %)	0.11
Codon 61 mutated	3 (2.9 %)	2 (2.2 %)	1 (9.1 %)	
Codon 146 mutated	4 (3.9 %)	3 (3.3 %)	1 (9.1 %)	
NRAS mutation				
Wild-type	95 (93 %)	85 (93 %)	10 (91 %)	0.77
Codon 12 mutated	3 (2.9 %)	3 (3.3 %)	0 (0 %)	
Codon 13 mutated	2 (2.0 %)	2 (2.2 %)	0 (0 %)	
Codon 61 mutated	2 (2.0 %)	1 (1.1 %)	1 (9.1 %)	
BRAF mutation (*V600E*)				
Wild-type	96 (94 %)	87 (96 %)	9 (82 %)	0.12
Mutated	6 (5.9 %)	4 (4.4 %)	2 (18 %)	
KRAS (codon 61 or 146) or *NRAS* or *BRAF* mutation				
All wild-type	82 (80 %)	76 (84 %)	6 (55 %)	0.037
At least one mutation	20 (20 %)	15 (16 %)	5 (45 %)	

Percentage (%) indicates the proportion of cases with a specific clinicopathological or molecular feature within a given dichotomous category of microRNA-31-5p expression by qRT-PCR. *P* values were calculated by analysis of variance for age and by a Chi-square test or Fisher's exact test for all other variables. To account for multiple hypothesis testing in associations between microRNA-31-5p expression and other 10 covariates, the *P* value for significance was adjusted by Bonferroni correction to $P = 0.005$ (=0.05/10)

CRC colorectal cancer, *EGFR* epidermal growth factor receptor, *SD* standard deviation

the high- and low-expression groups in patients with CRC with all wild-type copies of *KRAS*, *NRAS*, and *BRAF* ($n = 82$).

By contrast, there was no significant difference in PFS ($P = 0.13$) and OS ($P = 0.49$) between the miR-31-3p high- and low-expression groups (Supplementary Fig. 4).

MicroRNA-31 Expression in Colon Cancer Cell Lines

Supplementary Fig. 5 shows relative miR-31-5p or -3p expression levels of eight cell lines. Relative expression levels of miR-31-5p were more than ten times compared with those of miR-31-3p.

TABLE 2 Cox regression analysis of CRC patients treated with anti-EGFR therapy according to gene mutational status or microRNA-31-5p expression

	Progression-free survival			
	Median (months)	Hazard ratio	95 % confidence interval	*P*
KRAS (codon 61 or 146) (mutation vs. wild-type)	2.5 versus 6.1	3.16	0.75–9.15	0.11
NRAS (codon 12, 13, or 61) (mutation vs. wild-type)	2.6 versus 6.1	2.61	0.89–6.16	0.077
BRAF (*V600E*) (mutation vs. wild-type)	2.0 versus 6.1	2.65	0.79–6.71	0.11
KRAS (codon 61 or 146) or *NRAS* or *BRAF* (at least one mutation vs. all wild-type)	2.1 versus 6.4	3.37	1.61–6.67	0.0018
MicroRNA-31-5p expression (high expression vs. low expression)	2.8 versus 6.1	2.88	1.30–5.73	0.012

CRC colorectal cancer, *EGFR* epidermal growth factor receptor

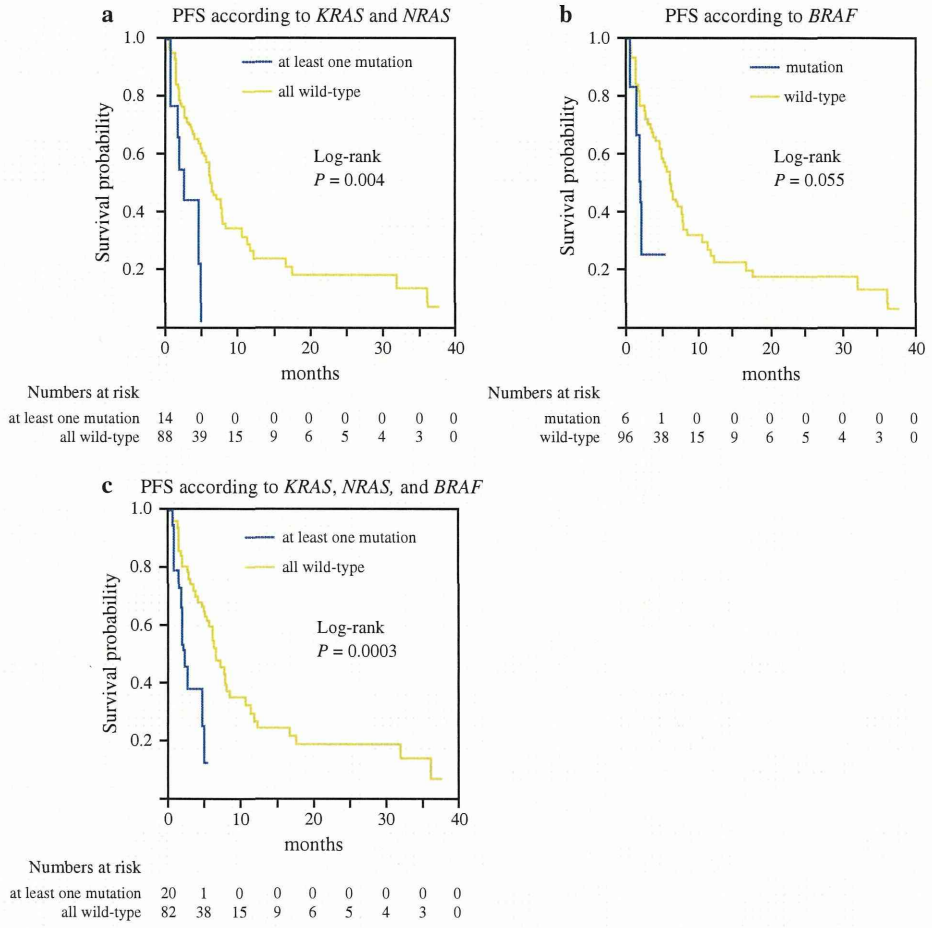

FIG. 1 Kaplan–Meier survival curves of patients treated with anti-EGFR therapy according to the mutational status in *KRAS*, *NRAS*, and *BRAF* genes. **a** Progression-free survival (PFS) of patients with at least one mutation in *KRAS* (codon 61 or 146) or *NRAS* (codon 12, 13, or 61) versus patients with all wild-type copies of the 2 genes. **b** PFS of patients with mutation in *BRAF* versus patients with wild-type copies of *BRAF*. **c** PFS of patients with at least one mutation in *KRAS*, *NRAS*, and *BRAF* versus all wild-type copies of the three genes

DISCUSSION

In the current study on patients with CRC who underwent surgical treatment, we elucidated the association of miR-31-5p expression or gene mutations in the pathway downstream of EGFR with the efficacy of anti-EGFR therapy. High miR-31-5p expression was strongly associated with shorter PFS in patients with CRC treated with anti-EGFR therapeutics. Moreover, in CRCs carrying all wild-type genes, high miR-31-5p expression was associated with shorter PFS. Thus, our data support the hypothesis that miR-31-5p can be a useful biomarker for anti-EGFR therapy.

It is well established that mutations in *KRAS* codon 12 or 13 predict the lack of response to anti-EGFR therapeutics.[4–6] Recent studies have demonstrated a correlation

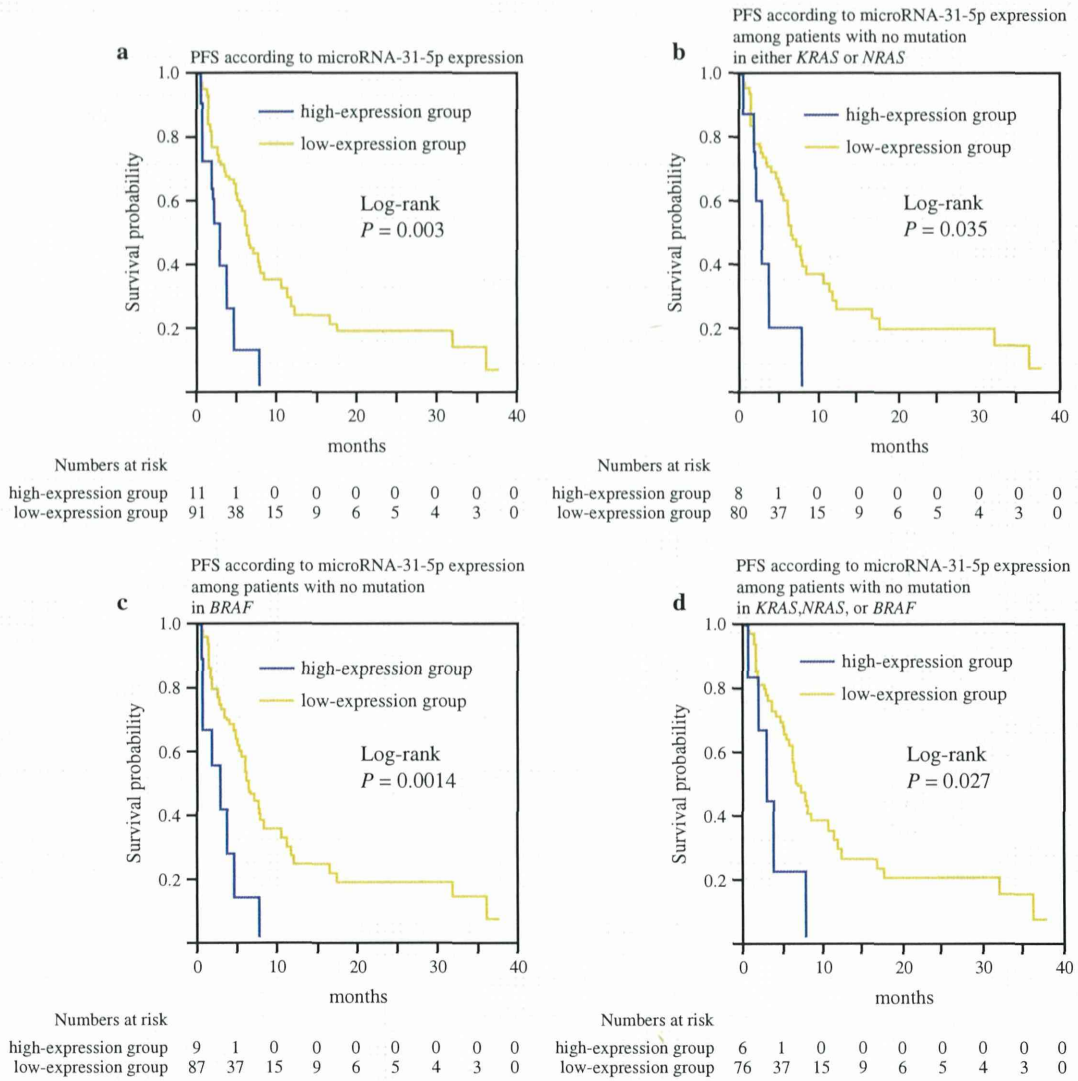

FIG. 2 Kaplan–Meier survival curves of patients treated with anti-EGFR therapy according to microRNA-31-5p expression. **a** Progression-free survival (PFS) of the high-expression group versus the low-expression group. **b** PFS of the high-expression group versus the low-expression group among patients with no mutations in either *KRAS* or *NRAS*. **c** PFS of the high-expression group versus the low-expression group among patients with no mutations in *BRAF*. **d** PFS of the high-expression group versus the low-expression group among patients with no mutations in *KRAS*, *NRAS*, or *BRAF*

between mutation in *KRAS* codons 61 or 146 and resistance to anti-EGFR therapeutics.[7,8,20] Moreover, as additional RAS-activating mutations, *NRAS* mutations have been reported to predict the response in patients with CRC treated with anti-EGFR therapy.[8,20,47–49] *BRAF* also has been reported to demonstrate a correlation between mutation and resistance to treatment with anti-EGFR therapeutics.[7,11,13,14,16,50]

In the current study, mutations in *KRAS* (codon 61 and 146), *NRAS* (codon 12, 13, and 61), and *BRAF* (*V600E*) were detected in 7, 7, and 6 patients, respectively, among 102 patients treated with anti-EGFR therapy. We observed significantly better PFS in CRCs possessing all wild-type copies of *KRAS*, *NRAS*, and *BRAF* compared with CRCs with at least one mutation of *KRAS*, *NRAS*, and *BRAF*, although no significant differences in OS was observed between them. These findings are almost consistent with those of previous studies and support the validity of our pyrosequencing assay for examining the gene mutations in the pathway downstream of EGFR.[8,14,16,19,48]

Several miRNAs (i.e., miR-143 or let-7) have been reported to be associated with the efficacy of anti-EGFR therapy in metastatic CRC.[39,40] Nevertheless, mutational analysis in these studies was performed only on *KRAS*

codons 12 and 13. In the present study, we found that high miR-31-5p expression is associated with resistance to anti-EGFR therapeutics in patients with metastatic CRC. The strength of our current study is that we conducted mutational analysis for *KRAS*, *NRAS*, and *BRAF* in those patients. With regard to miR-31, a recent study reported that upregulation of miR-31-3p is associated with poor prognosis in patients with CRC who received anti-EGFR therapy.[51] In the present study, no significant association was observed between miR-31-3p expression and prognosis of patients with CRC who received anti-EGFR therapy. miR-31-3p originates from the opposite arm of the same pre-miRNA of miR-31-5p. In our data, the relative expression levels of both miRNAs were strongly correlated and the relative expression level of miR-31-3p was extremely low compared with that of miR-31-5p in colon cancer cell lines or FFPE tissues. Previous studies, which reported upregulation of miR-31 in CRCs, support our data; this is because in all of those studies, the upregulated miR-31 was not miR-31-3p but miR-31-5p.[33–38] Thus, the deregulation of miR-31-3p in patients with CRC may be represented by a secondary change in the deregulation of miR-31-5p. These results indicate that miR-31-5p is more adequate as a biomarker than miR-31-3p.

In the present study, we also compared the utility of miR-31-5p expression with the utility of gene mutations (*KRAS*, *NRAS*, and *BRAF*) for identifying patients with metastatic CRC to be treated with anti-EGFR therapeutics. In Cox regression analysis, significantly shorter PFS was observed in miR-31-5p high-expression group than in the low-expression group. With regard to gene mutational analysis, no significant differences in PFS were detected according to single gene analysis, whereas there was significant difference according to combination analysis. The HR according to combination analysis of gene mutation was higher than that according to miR-31-5p expression in PFS. These results suggest that miR-31-5p may be inferior to combination analysis but better than mutational analysis of a single gene in the pathway downstream of EGFR as prognostic biomarker for anti-EGFR therapy.

Our study has some limitations, including the cross-sectional and observational design and the relatively small sample size of prognostic analysis. In addition, all patients underwent resection of the primary lesion of CRC before chemotherapy in the present study. These limitations may have affected prognostic analysis; OS of patients with CRC in the present study was relatively longer compared with that in large clinical trials. No significant association was detected between OS and molecular status except for *BRAF*. Future large and independent studies are necessary to confirm the correlation between miR-31-5p and unfavorable prognosis in patients with metastatic CRC who received anti-EGFR therapy.

In conclusion, we found that high miR-31-5p expression was associated with survival in patients with metastatic CRC who underwent surgical treatment and chemotherapy with anti-EGFR antibodies. Moreover, high miR-31-5p expression was associated with shorter PFS in CRC patients without gene mutations in the downstream part of the EGFR pathway, suggesting that miR-31-5p may be a useful and additional prognostic biomarker for anti-EGFR therapy.

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DISCLOSURE None.

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Clinicopathological and Molecular Characteristics of Serrated Lesions in Japanese Elderly Patients

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Key Words
Aging · Colonoscopy · Colorectal cancer · Hyperplastic polyp

Abstract
Background: The population in Japan is aging more rapidly than in any other country. However, no studies have determined the characteristics of the large population of elderly patients with colorectal tumors. Therefore, we examined the clinicopathological and molecular features of these tumors in elderly patients. ***Methods:*** In total, 1,627 colorectal tumors (393 serrated lesions, 277 non-serrated adenomas and 957 colorectal cancers) were acquired from patients. Tumor specimens were analyzed for *BRAF* and *KRAS* mutations, CpG island methylator phenotype-specific promoters *(CACNA1G, CDKN2A, IGF2* and *RUNX3), IGFBP7, MGMT, MLH1* and *RASSF2* methylation, microsatellite instability (MSI) and microRNA-31 (miR-31). ***Results:*** The frequency of elderly patients (aged ≥75 years) with sessile serrated adenomas (SSAs) with cytological dysplasia was higher than that of those with other serrated lesions and non-serrated adenomas ($p < 0.0001$). In elderly patients, all SSAs were located in the proximal colon (particularly the cecum to ascending colon). High miR-31 expression, *MLH1* methylation and MSI-high status were more frequently detected in SSAs from elderly patients than in those from non-elderly patients. In contrast, no significant differences were found between older age of onset and high-grade dysplasia for traditional serrated adenomas or non-serrated adenomas in any of these molecular alterations. ***Conclusion:*** In elderly patients, all SSAs were located in the proximal colon. Furthermore, cytological dysplasia and molecular alterations were more frequently detected in elderly patients with SSAs than in non-elderly patients. Thus, careful colonoscopic examinations of the proximal colon are necessary for elderly patients because SSAs in those patients may exhibit malignant potential.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide [1–7]. The serrated neoplasia pathway has attracted considerable attention as an alternative pathway for CRC development, and serrated le-

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Table 1. Clinical and pathological features of patients with serrated lesions and non-serrated adenomas

Clinical and pathological features	Histopathology							p
	HPs	SSAs without cytological dysplasia	SSAs with cytological dysplasia	TSAs without HGD	TSAs with HGD	non-serrated adenomas without HGD	non-serrated adenomas with HGD	
All cases	142	122	10	103	16	137	140	
Male	93 (65)	72 (59)	5 (50)	61 (59)	10 (63)	89 (65)	74 (53)	
Female	49 (35)	50 (41)	5 (50)	42 (41)	6 (37)	48 (35)	66 (47)	0.40
Age, years	58.5±12.0	57.2±11.5	74.1±4.7	61.5±13.1	62.4±13.2	66.5±10.1	65.7±10.0	<0.0001
Tumor size, mm	9.3±3.6	12.0±7.3	12.3±6.4	9.6±4.5	13.3±4.5	12.6±7.4	26.9±16.4	<0.0001
Tumor location								
Rectum	15 (11)	0	0	17 (17)	5 (31%)	15 (11)	29 (21)	
Distal colon[1]	52 (37)	17 (14)	1 (10)	46 (45)	7 (44%)	45 (34)	36 (26)	
Proximal colon[2]	73 (52)	105 (86)	9 (90)	39 (38)	4 (25%)	73 (55)	74 (53)	<0.0001

Data are expressed as mean ± SD, or n with percentage in parentheses. Percentages indicate the proportion of patients of each histological type who met the criteria for a specific clinical feature. p values were calculated by ANOVA for age and tumor size, and by χ^2 or Fisher's exact test for tumor location.
[1] Sigmoid colon to splenic flexure.
[2] Transverse colon to cecum.

sions exhibit unique clinical, pathological or molecular features [1, 8–19]. Sessile serrated adenomas (SSAs) and traditional serrated adenomas (TSAs) are premalignant lesions, although an SSA is the principal serrated precursor of CRC [18].

The CpG island methylator phenotype (CIMP) is a distinct form of epigenomic instability [2, 17, 20–23], which causes most sporadic microsatellite instability (MSI)-high CRCs by epigenetically inactivating *MLH1* [24]. Independent of MSI, CIMP-high CRCs are associated with proximal tumor location, older age of onset, female gender and *BRAF* mutation [7, 22, 23]. In particular, there are many clinical and molecular similarities between SSA and CIMP-high CRC; for example, their proximal tumor location, *BRAF* mutation status and comparable *MLH1* methylation [10, 12, 16–18, 25, 26]. Therefore, SSAs have been hypothesized to be precursor lesions that develop into CIMP-high CRCs with *BRAF* mutations in the proximal colon [1, 10, 12, 16–18, 25, 26].

The number of elderly CRC patients is expected to increase because of the aging populations in many countries. Previous studies conducted in the USA and European countries have shown that the clinical and molecular features of elderly CRC patients include being female, proximal tumor location, *BRAF* mutation, *MLH1* methylation, CIMP-high and MSI-high status [22, 27–29]. The population in Japan is aging more rapidly than in any other country. However, no studies have determined the characteristics of CRCs and premalignant lesions, including serrated lesions, in this large population of elderly Japanese patients. Therefore, we examined the clinical, pathological and molecular features of colorectal tumors in elderly Japanese patients.

Materials and Methods

Histopathological Evaluations of Colorectal Serrated Lesion Tissue Specimens

We collected and analyzed 1,627 tissue specimens of colorectal serrated lesions (n = 393), non-serrated adenomas (tubular or tubulovillous adenomas, n = 277) and CRCs (n = 957) from patients who underwent endoscopic resection or surgical treatment at Sapporo Medical University Hospital, Keiyukai Sapporo Hospital or JR Sapporo Hospital between 2001 and 2013. The patient demographic and clinical information is presented in table 1.

Histological findings relating to all colorectal serrated lesion specimens were evaluated by pathologists who were blinded to the clinical and molecular information. Serrated lesions, including hyperplastic polyps (HPs), SSAs and TSAs, were classified on the basis of the current World Health Organization (WHO) criteria [18]. HPs were further subdivided into microvesicular HPs and goblet cell HPs, with all being found to be microvesicular.

This study was approved by the institutional review boards of the participating institutions. Informed consent was obtained from all patients prior to specimen collection.

DNA Extraction and Pyrosequencing for KRAS and BRAF Mutations and MSI Analysis

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, Calif., USA) [1]. Extracted genomic DNA was used for PCR and targeted pyrosequencing for *KRAS* (codons 12 and 13) and *BRAF (V600E)* [6]. MSI analysis was performed as previously described [1].

Sodium Bisulfite Treatment and Real-Time PCR (MethyLight) to Determine CACNA1G, CDKN2A (p16), IGFBP7, IGF2, MGMT, MLH1, RASSF2 and RUNX3 Promoter Methylation

Bisulfite modification of genomic DNA was conducted using the BisulFlash™ DNA Modification Kit (Epigentek, Brooklyn, N.Y., USA) [17]. DNA methylation was quantified for four CIMP-specific promoters *[CACNA1G, CDKN2A (p16), IGF2* and *RUNX3]* and *IGFBP7, MGMT, MLH1* and *RASSF2* using Real-Time PCR (MethyLight) [17, 30, 31]. CIMP-high status was defined as the presence of 3 of 4 or more methylated promoters and CIMP-low/zero as the presence of 0–2 of 4 methylated promoters [17].

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) for MicroRNA-31

Total RNA was extracted from FFPE tissues using the miRNeasy FFPE Kit (Qiagen) [1]. MicroRNA-31 (miR-31)-5p expression was analyzed by qRT-PCR using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif., USA) and TaqMan microRNA Assays (Applied Biosystems), as previously described [1].

Statistical Analysis

JMP (version 10) software was used for all statistical analyses (SAS Institute, Cary, N.C., USA). Univariate analysis was used to determine the clinical, pathological and molecular characteristics associated with serrated lesions and non-serrated adenomas for elderly (≥75 years) and non-elderly (<75 years) patients. Group results for tumor sizes were compared by ANOVA, and results for other variables were compared by χ^2 or Fisher's exact test. A p value <0.05 was considered significant, and all p values were two-sided. 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 correlations between miR-31 expression level and tumor size.

Results

Clinical and Pathological Features of Elderly Patients with Serrated Lesions and Non-Serrated Adenomas

As shown in table 1, patients with SSAs with cytological dysplasia were significantly older (mean ± SD 74.1 ± 4.7 years) than those with HPs (58.5 ± 12.0 years), those with SSAs without cytological dysplasia (57.2 ± 11.5 years), those with TSAs without high-grade dysplasia (HGD; 61.5 ± 13.1 years), those with TSAs with HGD (62.4 ± 13.2 years), those with non-serrated adenomas without HGD (66.5 ± 10.1 years) and those with non-serrated adenomas with HGD (65.7 ± 10.0 years, all p < 0.0001).

Elderly patients (aged ≥75 years) with serrated lesions (n = 39) accounted for 9.6% of the 403 patients with serrated lesions. As shown in figure 1, the frequency of elderly patients with SSA with cytological dysplasia (6/10, 60%) was significantly higher than that of those with HP (10/142, 7.0%), those with SSA without cytological dysplasia (7/122, 5.7%), those with TSA without HGD (13/103, 13%) and those with TSA with HGD (3/16, 19%, all p < 0.0001).

Among the elderly patients, all 13 SSAs (cecum, n = 5; ascending colon, n = 6; transverse colon, n = 2) were located in the proximal colon. No significant difference was found in the size of SSAs between the elderly (10.7 ± 5.4 mm) and non-elderly (12.2 ± 7.3 mm, p = 0.49). With regard to HPs and TSAs, no significant differences were found between elderly and non-elderly patients in any of these clinical features.

In contrast, the frequency of elderly patients with non-serrated adenoma with HGD (26/140, 19%) was nearly the same as that of those with non-serrated adenoma without HGD (28/137, 20%). No significant differences were found between elderly patients with non-serrated adenomas and non-elderly patients for other clinical features (gender, tumor size or tumor location).

Molecular Features of Serrated Lesions and Non-Serrated Adenomas in Elderly Patients

Table 2 shows the molecular features of HPs, SSAs, TSAs and non-serrated adenomas. *MLH1* methylation (46%, p = 0.027), MSI-high status (23%, p = 0.0042), and high miR-31 expression (69%, p = 0.022) were more frequently detected in SSAs from elderly patients (aged ≥75 years) than in those from non-elderly patients (18, 1.7 and 36%, respectively, aged <75 years). No significant differences were found between elderly patients with SSAs and non-elderly patients for other molecular features.

Figure 2 represents the correlations between miR-31 expression and tumor size according to age (aged ≥75 or <75 years) in SSAs. No significant correlations were found between miR-31 expression and tumor size in elderly patients (r = 0.52, p = 0.068) or non-elderly patients (r = 0.037, p = 0.69). With regard to HPs, TSAs and non-serrated adenomas, no significant differences were found between elderly and non-elderly patients in any of these molecular features.

Clinicopathological and Molecular Features of Elderly Patients with CRC

Elderly CRC patients (aged ≥75 years, n = 264) accounted for 28% of the 957 patients with CRCs. The proportion of females (55%) and the frequencies of proximal colon location (43%), *BRAF* mutation (8.3%) and CIMP-high status (36%) were significantly higher for elderly CRC patients (aged ≥75 years) than for non-elderly pa-

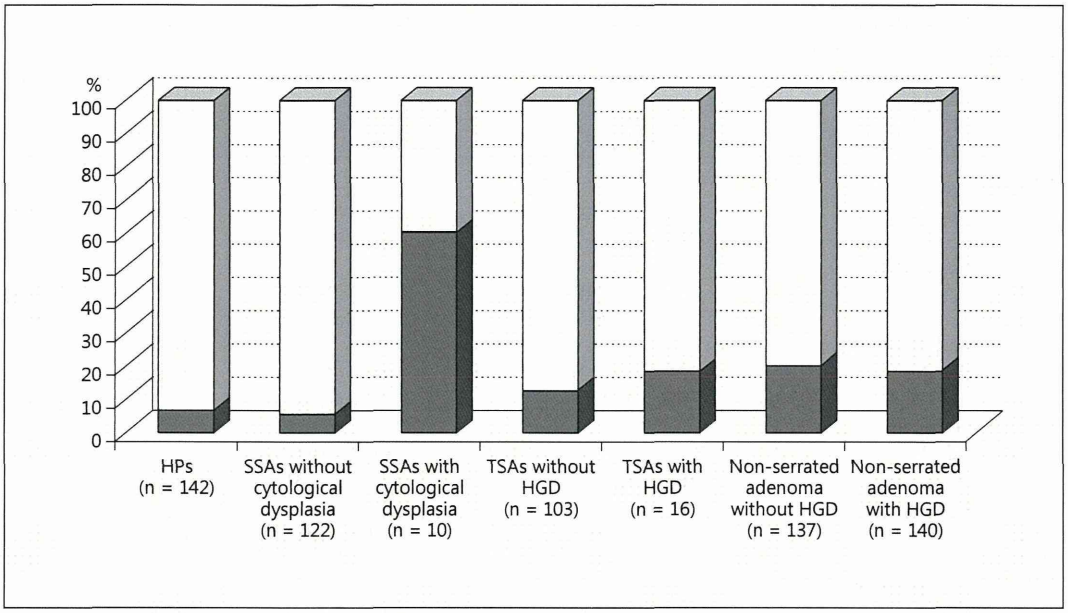
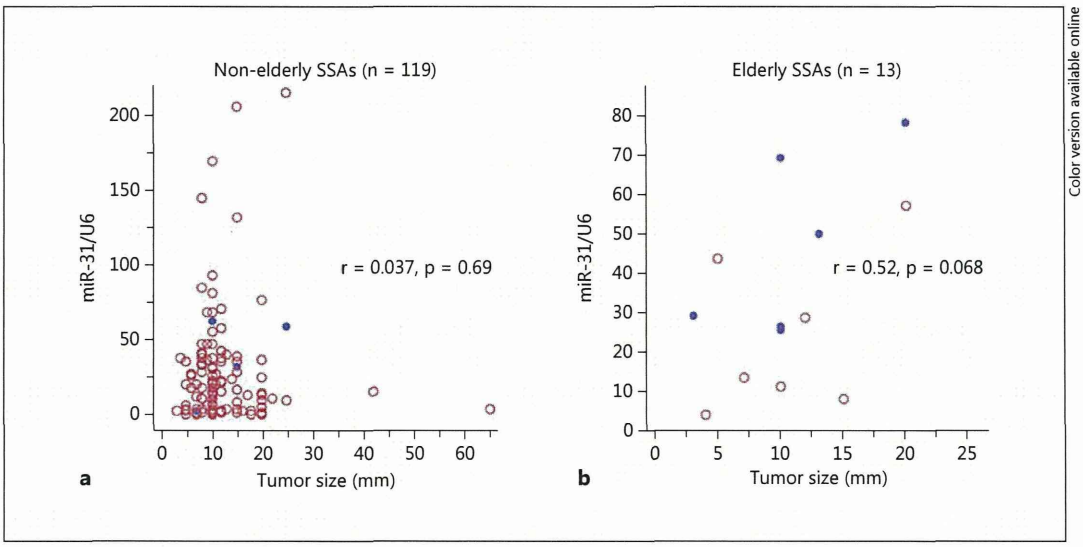


Fig. 1. Frequencies of different lesions in patients classed as elderly (≥75 years old, ■) and non-elderly (<75 years old, □). Based on the histopathology of resected specimens, serrated lesions were classified as HP, SSA without cytological dysplasia, SSA with cytological dysplasia, TSA without HGD, or TSA with HGD. The frequency of elderly patients with SSA with cytological dysplasia (6/10, 60%) was significantly higher than that of those with HP (10/142, 7.0%), those with SSA without cytological dysplasia (7/122, 5.7%), those with TSA without HGD (13/103, 13%) and those with TSA with HGD (3/16, 19%, all $p < 0.0001$).



Color version available online

Fig. 2. Correlations between miR-31 expression and tumor size in SSAs (SSAs without cytological dysplasia, ○; SSAs with cytological dysplasia, ●) according to age: non-elderly (<75 years old, n = 119; **a**) and elderly (≥75 years old, n = 13; **b**).

Table 2. Associations between patient age and epigenetic features, CIMP and MSI status, and miR-31 expression in serrated lesions according to histopathology

	HPs (n = 142)	p	SSAs (n = 132)	p	TSAs (n = 119)	p
BRAF mutated cases						
Non-elderly	47% (62/132)	0.67	86% (102/119)	0.43	66% (68/103)	0.83
Elderly	40% (4/10)		77% (10/13)		69% (11/16)	
KRAS mutated cases						
Non-elderly	20% (26/130)	0.47	3.4% (4/119)	0.36	20% (20/102)	0.63
Elderly	30% (3/10)		0% (0/13)		25% (4/16)	
IGFBP7 methylated cases						
Non-elderly	13% (17/132)	0.10	38% (45/119)	0.61	17% (17/103)	0.83
Elderly	0% (0/10)		31% (4/13)		19% (3/16)	
MGMT methylated cases						
Non-elderly	3.9% (5/129)	0.42	8.4% (10/119)	0.14	8.8% (9/102)	0.098
Elderly	10% (1/10)		23% (3/13)		0% (0/16)	
MLH1 methylated cases						
Non-elderly	3.1% (4/129)	0.34	18% (21/119)	0.027	3% (3/101)	0.34
Elderly	10% (1/10)		46% (6/13)		0% (0/16)	
RASSF2 methylated cases						
Non-elderly	3.8% (5/132)	0.39	18% (21/119)	0.28	14% (14/103)	0.60
Elderly	0% (0/10)		31% (4/13)		19% (3/16)	
CIMP-high cases						
Non-elderly	7.8% (10/129)	0.24	41% (49/119)	0.38	19% (19/102)	0.27
Elderly	20% (2/10)		54% (7/13)		31% (5/16)	
MSI-high cases						
Non-elderly	1.5% (2/132)	0.59	1.7% (2/119)	0.0042	2% (2/102)	0.38
Elderly	0% (0/10)		23% (3/13)		6.3% (1/16)	
High miR-31 cases						
Non-elderly	16% (21/132)	0.74	36% (43/119)	0.022	43% (43/100)	0.37
Elderly	20% (2/10)		69% (9/13)		31% (5/16)	

Percentages indicate the proportion of patients based on age (non-elderly, aged <75 years; elderly, aged ≥75 years) according to histopathology.

tients (36, 33, 4.3 and 9.2%, respectively, aged <75 years, all $p < 0.01$). No significant differences were found between elderly CRC patients and younger patients for other clinical and molecular features. Based on Kaplan-Meier analysis, older age (elderly) was not significantly associated with patient survival for CRC-specific mortality (data not shown).

Discussion

We conducted this study to identify the clinical, pathological and molecular features of colorectal tumors found in elderly Japanese patients. The frequency of elderly patients with SSAs with cytological dysplasia was significantly higher than that of those with other serrated lesions and non-serrated adenomas. In elderly patients, all SSAs were located in the proximal colon. With regard to molecular alterations, high miR-31 expression, *MLH1* methylation and MSI-high status were more frequently detected in SSAs from elderly patients than in those from non-elderly patients. In contrast, no significant differences were found between older age of onset and HGD for TSAs or non-serrated adenomas in any of these molecular alterations.

A number of investigators have reported that SSAs with cytological dysplasia have genetic and epigenetic abnormalities and are at a high risk of progression to CRCs [10, 12, 16–18, 26, 32]. *MLH1* methylation leads to MSI-high CRC, and repeat tract mutations in genes such as *TGFβRII* are restricted to those lesions with cytological dysplasia in SSAs [26, 33–35]. We recently reported that

the frequency of CIMP-high status was much higher in SSAs with cytological dysplasia than in those without dysplasia [17]. Moreover, our current data showed that *MLH1* methylation and MSI-high status were more frequently detected in SSAs from elderly patients than in those from non-elderly patients. These results suggest that *MLH1* methylation that leads to MSI-high CRC may accumulate with aging and play an important role in SSA progression.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that act as posttranscriptional gene regulators and have been increasingly recognized as useful biomarkers for CRC [1, 17]. Using microRNA array analysis, we recently discovered that miR-31 expression was significantly upregulated in *BRAF*-mutated CRCs compared with that in wild-type CRCs [1]. Moreover, associations were found between miR-31 expression, proximal tumor location and poor CRC prognosis. We also reported that the frequency of high miR-31 expression was higher in SSAs with cytological dysplasia than that in SSAs without dysplasia [17]. In the current study, our data showed that the frequency of high miR-31 expression was significantly higher in SSAs from elderly patients than in those from non-elderly patients. Because no previous study has reported on an association between the age of onset and miRNA expression in colorectal serrated lesions, this is the first report to show that miR-31 may be an age-dependent factor for SSA.

Previous studies conducted in the USA and European countries reported that the frequencies of being female, proximal colon location, *BRAF* mutations and CIMP-high status were significantly higher for elderly CRC patients than for non-elderly patients [22, 27–29]. Similar results were observed in the current study using a large number of samples from Japanese CRC patients. This suggests that there are no differences in the clinical and molecular features between elderly Japanese and Caucasian patients with CRC.

In conclusion, in elderly patients, all SSAs were located in the proximal colon. Furthermore, cytological dysplasia was more frequently detected in elderly patients with SSAs than in non-elderly patients. High miR-31 expression, *MLH1* methylation and MSI-high status were more frequently detected in SSAs from elderly patients than in those from non-elderly patients, suggesting that in SSAs these molecular alterations may accumulate with aging. Therefore, these findings indicate that a careful colonoscopic examination of the proximal colon (particularly the cecum to ascending colon) is necessary for elderly patients because SSAs in these patients may exhibit a malignant potential.

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Disclosure Statement

No conflict of interest.

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