Yonago Acta medica 2006;49:1–7

HRAS1 Variable Number of Tandem Repeats Polymorphism in Japanese Patients with Colorectal Adenoma and Cancer

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The highly polymorphic *HRAS1* variable number of tandem repeats (VNTR) has been described as an inherited predisposing factor in various human cancers. The aim of the present study was to evaluate the association between the presence of rare *HRAS1* VNTR alleles and colorectal adenoma and cancer. A total of 165 Japanese patients underwent total colonoscopy with informed consent, and were divided into 2 groups: colorectal neoplastic and non-neoplastic patients. Two hundred and sixteen *HRAS1* VNTR alleles from 108 colorectal neoplastic patients (67 adenomas and 41 cancers) and 114 alleles from 57 non-neoplastic patients were genotyped using PCR-based long-agarose gel electrophoresis assay of peripheral blood leukocyte DNA. Rare alleles were differentiated from 4 types of common allele (a1, a2, a3 and a4) by shifts in electrophoretic mobility. The prevalence of rare *HRAS1* VNTR alleles was higher in colorectal neoplastic patients than in non-neoplastic patients (25.4% and 34.1% versus 8.8%). The adjusted odds ratio with at least one rare allele was 8.65 (95% confidence interval = 2.93–25.53, $P < 0.0001$) in colorectal neoplastic patients. The presence of rare *HRAS1* VNTR alleles could be a genetic predisposing factor for risk of colorectal neoplasm in Japanese people.

Key words: colorectal neoplasm; *HRAS1* variable number of tandem repeats; polymorphism

Recently, the rate of colorectal cancer has been rapidly increasing in Japan, and colorectal cancer is now the main cause of death from malignant disease as in many other countries (Yoshimi and Sobue, 2004). In fact, age-standardized incident rates are similar to rates in US populations of Caucasians (Yiu et al., 2004). The reason has generally been ascribed to the westernized diet, characterized by a high intake of fat and meat especially after World War II (Kono, 2004), whereas the relevance of genetic predispositions has not been sufficiently analyzed.

Colorectal cancer is a multifactorial disease, with dietary factors, lifestyle habits and genetic predispositions contributing to its development. The multifarious molecular changes in cancer development frequently involve alterations on minisatellites or variable number of tandem repeats (VNTR) as well as allelic deletions or loss of heterozygosity. Since the *HRAS1* VNTR allelic polymorphism in normal individuals and cancer patients was surveyed by Krontiris et al. in 1985, this polymorphism has been reported to be an inherited predisposing factor in various cancers, including colorectal, ovarian and lung cancers, so far (Krontiris et al., 1993; Lindstedt et al., 1999; Weitzel et al., 2000). The proto-oncogene *HRAS1*, located on the short arm of chromosome 11, encompasses 4 exons flanked by a VNTR region at the 3' end which consists of a basic 28 bp

Abbreviations: CI, confidence interval; OR, odds ratio; PCR, polymerase chain reaction; VNTR, variable number of tandem repeats

consensus sequence. The *HRAS1* VNTR consists of four common progenitor alleles, which were previously identified as a1, a2, a3 and a4 having repeats of 30, 46, 68 and 84 units, respectively. In addition, several rare variants are thought to derive from germ-line mutations of the nearest common alleles (Kasperczyk et al., 1990). These common alleles represent 94% in Caucasian populations (Krontiris et al., 1985). Minisatellite alleles frequently vary not only in the number of repeat copies but also in the interspersion pattern of the repeat sequence along the VNTR (Conway et al., 1996; Ding et al., 1999). Although the actual role of specific *HRAS1* VNTR alleles in cancer susceptibility is not yet known, it has been shown that the *HRAS1* VNTR binds at least 4 members of the *rel* NF-κB family of transcriptional regulatory factors, suggesting that the VNTR could affect cancer susceptibility by transcriptional modulation of *HRAS1* or other nearby genes (Trepicchio and Krontiris, 1992; Green and Krontiris, 1993; Mukhopadhyay et al., 1995). The aim of the present study is to examine whether Japanese individuals with rare *HRAS1* VNTR alleles are at an increased risk for developing colorectal neoplasm.

Materials and Methods

Subjects

A total of 165 Japanese subjects (78 women and 87 men; age range, 19–90 years; median, 67 years) were studied between August 2003 and March 2005. All of the subjects had undergone total colonoscopy just before the enrollment. For extraction of DNA, 5 mL of peripheral blood was obtained from each of the 108 patients with histologically confirmed colorectal neoplasm (41 cancers and 67 adenomas) and 57 non-neoplasm (neoplasm-free) subjects. Subjects were interviewed for medical history, family history of neoplastic disease and health habits such as alcohol intake and cigarette smoking. Before enrollment and blood sampling, written informed consent was obtained from all patients and non-neoplasm subjects. This study was approved by the Institutional Review Boards of Tottori University Faculty of Medicine (approval number: G 16).

HRAS1 VNTR genotyping

Genomic DNA was extracted from peripheral blood samples using DnaQuick II DNA extraction kits (Dainippon Pharmaceutical, Osaka, Japan) as recommended by the supplier. Using the extracted DNA as a template, the *HRAS1* VNTR region was amplified by polymerase chain reaction (PCR) using a commercially available kit, Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The sequences of the primers were: the forward primer, 5'-ATC TAC AGT CCC CCT TGC CG -3'; the reverse primer, 5'-GCA ACT GAC CGT GCA AGT CA -3'. Reactions were carried out using the following conditions: 2 min at 95°C and then amplified for 10 cycles consisting of 15 s at 94°C, 30 s at 62°C and 2 min at 72°C, followed by 20 cycles of 15 s at 94°C, 30 s at 62°C and 2 min plus cycle elongation of 5 s for each cycle at 72°C. A final extension step at 72° C for 7 min was added to terminate the amplification.

Subsequently, the PCR product was electrophoresed on a 1% agarose gel 30 cm in length using TBE buffer (45 mM Tris-borate, pH 8.3 and 2 mM EDTA) to confirm the size. Molecular size marker IV (Nippon Gene, Tokyo, Japan) and a 100 bp ladder marker (Bio-Rad Lab., Hercules, CA) were loaded in approximately every 7th and 8th lane on all gels to minimize artifacts due to gel distortion. Gels were stained with ethidium bromide and destained in water to allow direct visualization of the alleles (Fig. 1). The 4 most common *HRAS1* VNTR alleles were labeled a1, a2, a3 and a4. The other alleles were labeled in terms of the difference in number of 28 bp repeat units from that of "the common allele" closest in size. For example, "a1+" refers to the allele with 28 bp repeat unit more than a1, "a1-" refers to the

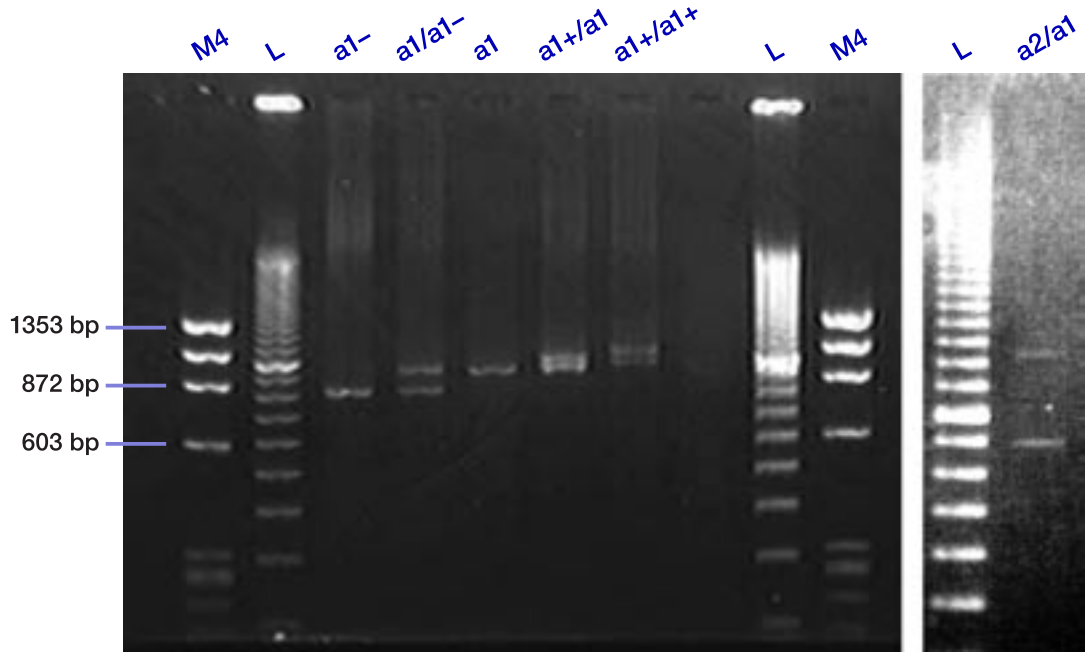

Fig. 1. Representative examples of the *HRAS1* VNTR allele. Rare alleles are indicated by the addition (+) or deletion (–) of 28 bp motifs from common alleles. Lanes M4 and L: molecular weight standards.

allele with 28 bp repeat unit less than a1; these are referred to as "rare alleles".

Statistical analysis

As in other published studies, alleles were categorized into two sets: common and rare. The association between rare *HRAS1* VNTR alleles and colorectal neoplasm was assessed by comparing the proportions of patients and non-neoplasm subjects with one or more rare alleles. The statistical significance of the different means or proportions was measured using analysis of variance or chi-square test. To evaluate increased risk of colorectal neoplasm associated with the presence of rare *HRAS1* alleles, odds ratios (ORs) and 95% confidence intervals (CIs) were computed and adjusted by logistic regression for several covariates potentially associated with neoplastic risk such as age, sex, family history of neoplasm, alcohol intake and smoking status. The statistical analyses were performed using the software package SPSS II for Windows (version 11.0 J, SPSS Japan, Tokyo).

Results

Characteristics of research subjects

The characteristics of participants are summarized in Table 1. Adenoma cases were more likely than non-neoplasm cases to be male and to have a family history (all of malignancy), and cancer cases were more likely to be older and to smoke habitually. No differences between neoplastic and non-neoplastic cases were observed in other clinicopathological variables.

HRAS1 VNTR allelic patterns

The *HRAS1* VNTR allelic patterns are shown in Table 2. We genotyped 216 *HRAS1* VNTR alleles from 108 patients with colorectal neoplasm (41 cancers and 67 adenomas) and 114 alleles from 57 non-neoplasm subjects. Regarding the distribution of common alleles in our study, most subjects had a1 alleles, and none had a3 or a4 alleles.

Table 1. Characteristics of research subjects

Variables	Non-neoplasm [57]		Colorectal neoplasm					
			Adenoma [67]		*P*†	Cancer [41]		*P*†
	Number	(%)	Number	(%)		Number	(%)	
Age (year)	60.6 ± 16.6		65.4 ± 9.4		0.11	68.4 ± 10.9		0.01
Sex (male/female)	23/34		41/26		0.02	23/18		0.12
Family history								
All of malignancies	18/50	(36.0)	32/53	(60.4)	0.01	19/41	(46.3)	0.32
Only colorectal cancer	7/50	(14.0)	3/53	(5.7)	0.15	8/41	(19.5)	0.48
Alcohol*(60 mg/day)	9/53	(17.0)	12/53	(22.6)	0.46	7/41	(17.1)	0.99
Smoking*(habitually)	15/52	(28.8)	22/53	(41.5)	0.17	21/41	(51.2)	0.03

Continuous variables expressed as mean ± SD.
[ ], number of subjects.
* Assessed only in available patients.
† *P* values were calculated versus non-neoplasm. Statistical significance was defined as $P < 0.05$.

Association between HRAS1 VNTR and colorectal neoplasm

To evaluate increased risk of colorectal neoplasm associated with the presence of rare *HRAS1* alleles, odds ratios (ORs) and 95% CIs were computed and adjusted by logistic regression for several covariates potentially associated with neoplastic risk such as age, sex, family history of neoplasm, alcohol intake and smoking status (Table 3). The adjusted OR for colorectal neoplasm was 8.65 (95% CI = 2.93–25.53, $P < 0.0001$). Repeating the analyses among cancer cases only, the OR was 13.16 (95% CI = 3.69–47.00, $P < 0.0001$). The OR in adenoma was calculated at 6.59 (95% CI = 2.07–21.02, $P < 0.001$).

Discussion

In addition to many environmental factors, genetic polymorphisms are thought to influence the carcinogenesis of colorectal cancer. Although there have been many candidate genes associated with colorectal carcinogenesis, only 2 genes, *HRAS1* VNTR and *APC-1* 1307K, were found

Table 2. *HRAS1* VNTR allelic patterns

		Non-neoplasm		Colorectal neoplasm			
				Adenoma		Cancer	
		Number	(%)	Number	(%)	Number	(%)
Common alleles	a1	103	(90.3)	100	(74.6)	54	(65.9)
	a2	1	(0.9)	0	(0)	0	(0)
	a3	0	(0)	0	(0)	0	(0)
	a4	0	(0)	0	(0)	0	(0)
	Subtotal	104	(91.2)	100	(74.6)	54	(65.9)
Rare alleles	~a1-	9	(7.9)	19	(14.2)	17	(20.7)
	a1+~a2-	1	(0.9)	15	(11.2)	11	(13.4)
	a2+~a3-	0	(0)	0	(0)	0	(0)
	a3+~a4-	0	(0)	0	(0)	0	(0)
	a4+~	0	(0)	0	(0)	0	(0)
	Subtotal	10	(8.8)	34	(25.4)	28	(34.1)
Total		114		134		82	
		[57 individuals]		[67 individuals]		[41 individuals]	

Table 3. Association between *HRAS1* VNTR and colorectal neoplasm

Genotypes	Non-neoplasm [57]	Neoplasm [108]	Type of tumor	
			Adenoma [67]	Cancer [41]
Common/common	50	60	42	18
Common/rare or rare/rare	7	48	25	23
Odds ratio	1.00	8.65	6.59	13.16
95% confidence interval	2.93–25.53	2.07–21.02	3.69–47.00	
P	Not significant	< 0.0001	< 0.001	< 0.0001

[ ], number of subjects.
P: less than 0.05 is significant.

to have a polymorphism that increases colorectal carcinogenesis in meta-analysis reported by Houlston and Tomlinson (2001). De Jong et al. (2002) reviewed 20 genes (30 gene polymorphisms) discussed in several reports, and speculated that *HRAS1* VNTR, *GSTT1*, *NAT2* (phenotype), *ALDH2* and *TNF*-α (α2 allele) increase carcinogenetic risk. They summarized that *HRAS1* VNTR was involved in colon carcinogenesis with an OR of 2.50 to 2.67. However, these studies of the association between *HRAS1* VNTR polymorphisms and colorectal cancer were performed on Caucasians, while there has been no study performed on Japanese. Racial differences in the *HRAS1* VNTR allele distribution have been reported (Weston et al., 1991). In our subjects, the most common alleles were a1, and no larger alleles (a3 and a4 alleles) were noted. In a previously reported *HRAS1* VNTR allele distribution in Japanese people (Ishikawa et al., 1987; Honda et al., 1988; Pierce et al., 2000), a1 accounted for a high ratio, and the frequencies of a2, a3 and a4 alleles were less than 10% in each allele. The absence of a3 and a4 in our study suggests the presence of regional differences, in addition to racial differences, but the details are not clear because of the small number of patients.

In this study, in agreement with previous reports on Caucasians (Klingel et al., 1991; Krontiris et al., 1993; Gosse-Brun et al., 1998; Vega et al., 2001), the frequency of the rare alleles was significantly higher in the neoplasm group and the adjusted OR of colorectal neoplasm with at least one rare allele was 8.65 ($P < 0.0001$), showing the close association of *HRAS1* VNTR polymorphisms with colorectal neoplasms in Japanese. Furthermore, the adjusted OR of colorectal cancer was markedly higher. The recent increase in colorectal cancer patients in Japanese may be due to additional external factors such as post-war dietary changes in individuals with genetic predispositions.

This is the first report to clarify the significant relationship of *HRAS1* VNTR and colorectal adenoma. Previous studies reported only some comparison with colorectal cancer, and did not discuss adenoma. Generally, neoplasms develop in the adenoma-carcinoma sequence (Vogelstein et al., 1988) in most colorectal cancers. It has been suggested that *HRAS1* VNTR polymorphisms are involved in the early stage of tumorigenesis as a genetic predisposing factor.

We also found the difference in OR between adenoma (OR; 6.59) and cancer (OR; 13.16) patients. Although the reason for the increase in OR in colorectal cancer patients is not clear, many hereditary predispositions may be involved in the carcinogenesis in a complex manner.

Since Krontiris et al. (1985) reported on the association of *HRAS1* VNTR with carcinogenesis, other associations with various other cancers besides colorectal cancer have been reported mainly in Europe and America. However, some reports presented negative views of a causal relationship in recent cases of breast cancer and urinary bladder cancer (Firgaira et al., 1999; van Gils et al.,

2002; Tamimi et al., 2003). This may have been due to advances in molecular-biological techniques that have allowed the resolution of single-repeat differences (particularly in larger sizes), leading to a more precise classification of common and rare alleles than classification by Southern blot. In recent reports of colorectal cancer using the PCR method (Vogelstein et al., 1988; Vega et al., 2001), a significant correlation was noted between *HRAS1* VNTR and colorectal cancer. In this study, we also used the PCR method, and found significant differences between non-neoplasm and neoplasm patients.

In conclusion, there is a strong association between rare alleles of the *HRAS1* VNTR and colorectal neoplasm in Japan. If these results are confirmed, then from the standpoint of preventive medicine, *HRAS1* VNTR polymorphisms may be useful in identifying risk groups for colorectal neoplasms. Further investigation of differences in *HRAS1* VNTR polymorphisms in development sites, histologic types, and prognosis of colon cancer may be necessary.

Ackowledgments: The authors are indebted to Prof. Eiji Nanba and Mrs. Kaori Adachi of the Gene Research Center, Tottori University Faculty of Medicine, for technical support.

This study has been awarded prizes at United European Gastroenterology Week 2004 in Prague, the World Conference of Gastroenterology 2005 in Montreal, and Digestive Disease Week-Japan 2005 in Kobe.

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Received December 9, 2005; accepted December 21, 2005

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