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Genetic Alterations of Mixed Hyperplastic Adenomatous Polyps in the Colon and Rectum

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Some mixed hyperplastic adenomatous polyps (MHAPs) contain dysplastic lesions or even carcinomas. These polyps are considered to be different from ordinary hyperplastic polyps and may have a preneoplastic potential. We investigated *APC* and K-*ras* mutations in MHAPs of the colon and rectum, and also in colorectal adenomas and hyperplastic polyps to identify molecular differences between MHAPs, adenomas and hyperplastic polyps, using direct sequencing of mutation cluster regions (MCR) in *APC* and K-*ras*. No *APC* mutations were identified in 12 MHAPs and 8 hyperplastic polyps, whereas 10 of 27 (37.0%) adenomas showed somatic mutations. K-*ras* mutations were identified in one of 12 (8.3%) MHAPs, one of 8 (12.5%) hyperplastic polyps, and 10 of 27 (37.0%) adenomas. *p53* mutation was found in a carcinoma arising in an MHAP. Mutations other than *APC* mutations may play a role in the development of MHAPs.

Key words: Mixed hyperplastic adenomatous polyp — Serrated adenoma — *APC* gene — K-*ras* gene — *p53* gene

Colorectal carcinomas are considered to develop as a consequence of mutations in oncogenes and tumor suppressor genes, or DNA mismatch repair gene disruption.[1,2] Such mutations are relevant to the adenoma-carcinoma sequence, *de novo* carcinogenesis or the RER pathway.[3] In the case of the adenoma-carcinoma sequence, *APC* mutation is thought to play a key role in adenoma formation and inactivation of *APC* initiates colorectal carcinogenesis.[4] In contrast, hyperplastic polyps have no malignant potential. Hyperplastic polyps have been examined for *APC* mutations by Jen *et al*.[5] However, *APC* mutation was not identified in any hyperplastic polyps, while K-*ras* mutations were identified in both adenomas and hyperplastic polyps with almost the same frequency.[5]

Recently, the classification of MHAP was proposed by Longacre and Fenoglio-Preiser.[6] MHAP has both hyperplastic and adenomatous appearance. It has been reported that 11% of MHAPs contain areas of intramucosal carcinoma.[6] Jeevaratnam *et al*.[7] reported a case of familial hyperplastic polyposis with multiple hyperplastic polyps, adenomas and MHAPs in the colon and rectum. Colorectal cancers developed in 6 relatives in this family. Therefore, MHAP is thought to be a preneoplastic lesion. In order to examine the molecular genetics of MHAPs, we analyzed *APC* mutations and K-*ras* mutations in MHAPs.

MATERIALS AND METHODS

Tumor samples We obtained the following samples from Hamamatsu University School of Medicine, Matsuda Hospital, Seirei Sumiyoshi Hospital and Seirei Mikatabara Hospital: 12 paraffin-embedded MHAP samples (M1 to M12), 27 fresh colorectal adenoma samples (A1 to A27) including 18 tubular adenomas, 4 tubulo-villous adenomas and 5 villous adenomas, and 8 paraffin-embedded hyperplastic polyps (H1 to H8). Fresh specimens were stored at −70°C until they were used. All the specimens were reviewed by two pathologists.

In this study, the criteria for MHAPs, so-called serrated adenoma, were as follows: serrated glandular luminal appearance, increased mitotic activity in the upper zones of crypts, prominence of nucleoli, goblet cell immaturity and nuclear pseudostratification.[8] MHAP should be distinguished from a polyp with admixed hyperplastic glands

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Abbreviations: MHAP, mixed hyperplastic adenomatous polyps; APC, adenomatous polyposis coli; PCR, polymerase chain reaction; MCR, mutation cluster region; HE, hematoxylin and eosine; LOH, loss of heterozygosity; RER, replication error; HNPCC, hereditary nonpolyposis colorectal cancer.

and adenomatous glands as designated by Longacre and Fenoglio-Preiser.[6] In other words, the latter polyp is a hyperplastic polyp with adenomatous aberrant crypt foci.

For the evaluation of the mitotic activity, Ki67 immunostaining was performed, because Ki67 is thought to be a marker of proliferating cells.[9] Colorectal adenomas show a uniform distribution of Ki67-positive cells throughout the sections, including the cells of the adenoma surface, while Ki67 staining in the normal mucosa is confined to the middle third and lower third of the crypts.[10] As MHAPs, we used samples which met the criteria of MHAP and were stained by anti-Ki67 antibody (Dako) not only on the bottom, but also sparsely in the upper zones of the crypts, using a modified peroxidase-antiperoxidase technique.[11] Hyperplastic polyps and adenomas were also stained by anti-Ki67 antibody. All the hyperplastic polyps were stained only in the lower half of the crypts, and all the adenomas were stained profusely from the bottom to the upper zone of the entire glandular lesion.

The characteristics of the samples are summarized in Table I. Carcinomas were observed in two MHAP cases: one was a carcinoma in an MHAP and the other was an advanced carcinoma synchronized with multiple adenomas, MHAPs and hyperplastic polyps. The microscopic appearance of MHAP with focal cancer is shown in Fig. 1.

DNA extraction DNA was extracted from fresh frozen tissues, as described elsewhere.[12]

Six 10 μm sections were cut from each paraffin-embedded tissue block, and one section was stained with HE. Using the HE-stained section as a guide, adenoma, hyperplastic polyp, MHAP, carcinoma and normal tissues were microdissected, and DNA was extracted as described in a previous article.[13]

Analysis of tumors for *APC* mutations MCR (codons 1286 to 1513) of *APC* [4] was analyzed by direct sequencing. In brief, two overlapping fragments of *APC* covering codons 1260 to 1410 and 1389 to 1547 were amplified by PCR using 0.1 μg of genomic DNA, 0.25 μl of 0.35 μg/μl of each of the appropriate primers (upstream fragment, 5′-AGACTTATTGTGTAGAAGATAC-3′ and 5′-ATGGTTCACTCTGAACGGA-3′; downstream fragment, 5′-TCTGTCAGTTCACTTGATAG-3′ and 5′-CATTTGATTCTTTAGGCTGC-3′), 1.5 μl of 78 m*M* $MgCl_2$, 1.5 μl of 25 m*M* dNTPs, 2.5 μl of dimethyl sulfate, 2.5 μl of 10× PCR buffer (16.6 m*M* NH_4SO_4, 67 m*M* Tris pH 8.8, 2.5 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol and 6.7 μ*M* EDTA), and 0.25 μl of 5 u/μl Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) in 25 μl. Amplifications were performed for 37 cycles of denaturation (30 s at 95°C), annealing (2 min at 50°C) and extension (5 min at 70°C).

All of the PCR products were purified using C3TTK filtration tubes (Millipore, Tokyo). Purified PCR products were sequenced using internal primers as follows: forward sequencing primer for the upstream fragment 5′-CATTATCATCTTTGTCATCAGC-3′ and reverse sequencing primer 5′-TTCACTCTGAACGGAGCTGG-3′; forward sequencing primer for the downstream fragment 5′-ACTTGATAGTTTTGAGAGTCG-3′ and reverse sequencing primer 5′-GGAGGCATTATTCTTAATTCC-3′; each primer was labeled at the 5′ end with rhodamine with a Thermo Sequenase cycle sequencing kit (Amersham, Tokyo) according to the manufacturer's protocol. Detection was carried out by using an FMBIO 100 (Takara, Kyoto).

Analysis of LOH of *APC* In three cases (M1, M7 and M12), of which two were associated with cancer, LOH of the *APC* locus was analyzed. A CA repeat marker is located 30–70 kb downstream from *APC*, and can be amplified by PCR.[14] Another CA repeat marker is located upstream from *APC*, and can also be amplified by PCR.[15] In the three cases, DNAs of normal mucosa tissue, MHAP tissue and cancer tissue were amplified by PCR at the 2 CA repeat markers, and the PCR products were separated by denaturing polyacrylamide gel electrophoresis.

K-*ras* mutation analysis Exon 1 of the K-*ras* was amplified for all the samples using forward primer 5′-ACCTTATGTGTGACATGTTCT-3′ and reverse primer 5′-AGAGAAACCTTTATCTGTATC-3′. Direct sequencing of the PCR products was carried out to identify K-*ras* mutations, using an FMBIO 100 (Takara).

***p53* mutation analysis** Direct sequencing of exon 5 to exon 8 of the *p53* gene was performed for two cases: a focal carcinoma in MHAP (M1) and a synchronous advanced carcinoma associated with multiple MHAPs (M7). Each exon of the *p53* gene was amplified by PCR as described above, with the following primers: (exon 5, 5′-ATGTTTGTTTCTTTGCTGCCGT-3′ and 5′-TCCAAATACTCCACACGCAAAT-3′; exon 6, 5′-CTCAGATAGCGATGGTGAG-3′ and 5′-GGAGAAAGCCCCCCTACT-3′; exon 7, 5′-CTCCCCAAGGCGCACTG-3′ and 5′-TGGGAGCAGTAAGGAGATTC-3′; exon 8, 5′-AACCTGTGGCTTCTCCTCC-3′ and 5′-ACCGCTTCTTGTCCTGCTTG-3′). After purification of the PCR products, direct sequencing of the PCR products was performed, as described for *APC* direct sequencing, with 5′ biotinylated inner primers: (exon 5, forward primer 5′-CAACTCTGTCTCCTTCCTCTTCCT-3′; exon 6, reverse primer 5′-CAGAGACCCCAGTTGCAAACCAGA-3′; exon 7, forward primer 5′-CCTCATCTTGGGCCTGTGTTATCT-3′; exon 8 forward primer 5′-TTACTGCCTCTTGCTTCTCTTTTCC-3′).

Analysis of LOH of the *p53* LOH of *p53* was also analyzed for 3 cases (M1, M7 and M12). A CA repeat marker has been detected at the *TP53* locus by Jones *et al.*[16] DNAs of normal mucosa tissues, MHAP tissues and cancer tissues were amplified by PCR, using published

Table I. Characteristics of MHAPs, Adenomas and Hyperplastic Polyps

Patients	Age	Sex	Location	Diameter (cm)	Shape	Other condition	
M1	77	F	T	3.0×2.5	FL	cancer in MHAP	
M2	47	M	S	1.5	P		
M3	49	F	R	1.5×1.0	P		
M4	54	M	T	0.6	SP		
M5	65	M	S	1.2	P		
M6	54	M	S	1.5	P		
M7	65	M	R	2.0×1.5	FL	diffuse hyperplastic polyposis with synchronous advanced cancer	
M8	53	M	T	1.3	P		
M9	50	F	D	0.4	SP		
M10	61	M	R	0.6	SP		
M11	50	M	D	1.0	SP		
M12	58	M	R	0.9	SP	multiple MHAPs	
A1	64	M	S	0.7	P	T	
A2	55	M	R	6.0×5.0	FL	V	cancer in adenoma
A3	57	F	S	2.5	P	TV	
A4	66	F	S	4.0×2.2	FL	TV	cancer in adenoma
A5	71	F	S	6.0×3.5	FL	V	
A6	63	M	S	1.5	P	T	
A7	73	F	A	3.0	FL	TV	
A8	72	F	C	2.0×2.0	FL	T	
A9	63	M	R	2.0×3.0	FL	T	
A10	71	F	T	3.0×3.0	FL	V	
A11	29	F	S	0.8	P	T	
A12	56	M	A	0.7	P	T	
A13	29	F	A	0.7	P	T	
A14	40	M	S	1.0	P	T	
A15	75	M	A	1.2×0.8	P	T	
A16	70	F	R	1.5	P	T	
A17	66	F	S	1.0	SP	T	
A18	69	M	R	1.5	P	T	
A19	67	F	S	0.5	P	T	
A20	57	F	S	2.0×1.3	P	T	
A21	48	M	R	1.2	P	T	
A22	55	M	R	2.1×1.5	SP	T	
A23	59	M	S	1.0×1.5	P	T	
A24	54	M	S	1.2	P	T	
A25	89	M	S	5.0×6.0	FL	V	cancer in adenoma
A26	65	M	R	3.0×1.5	SP	TV	
A27	47	F	R	2.5	FL	TV	
H1	52	M	S	0.6	SP		
H2	52	F	S	3.0	SP		
H3	64	F	A	2.0	SP		
H4	69	M	R	0.5	SP		
H5	24	M	R	0.5	SP		
H6	58	M	D	0.4	S		
H7	50	M	A	0.4	S		
H8	45	M	S	0.6	S		

Sex: M, male; F, female. Location: C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum. Shape: P, pedunculated type; SP, semi-pedunculated type; FL, flat type. Pathological type: T, tubular adenoma; TV, tubulo-villous adenoma; V, villous adenoma.

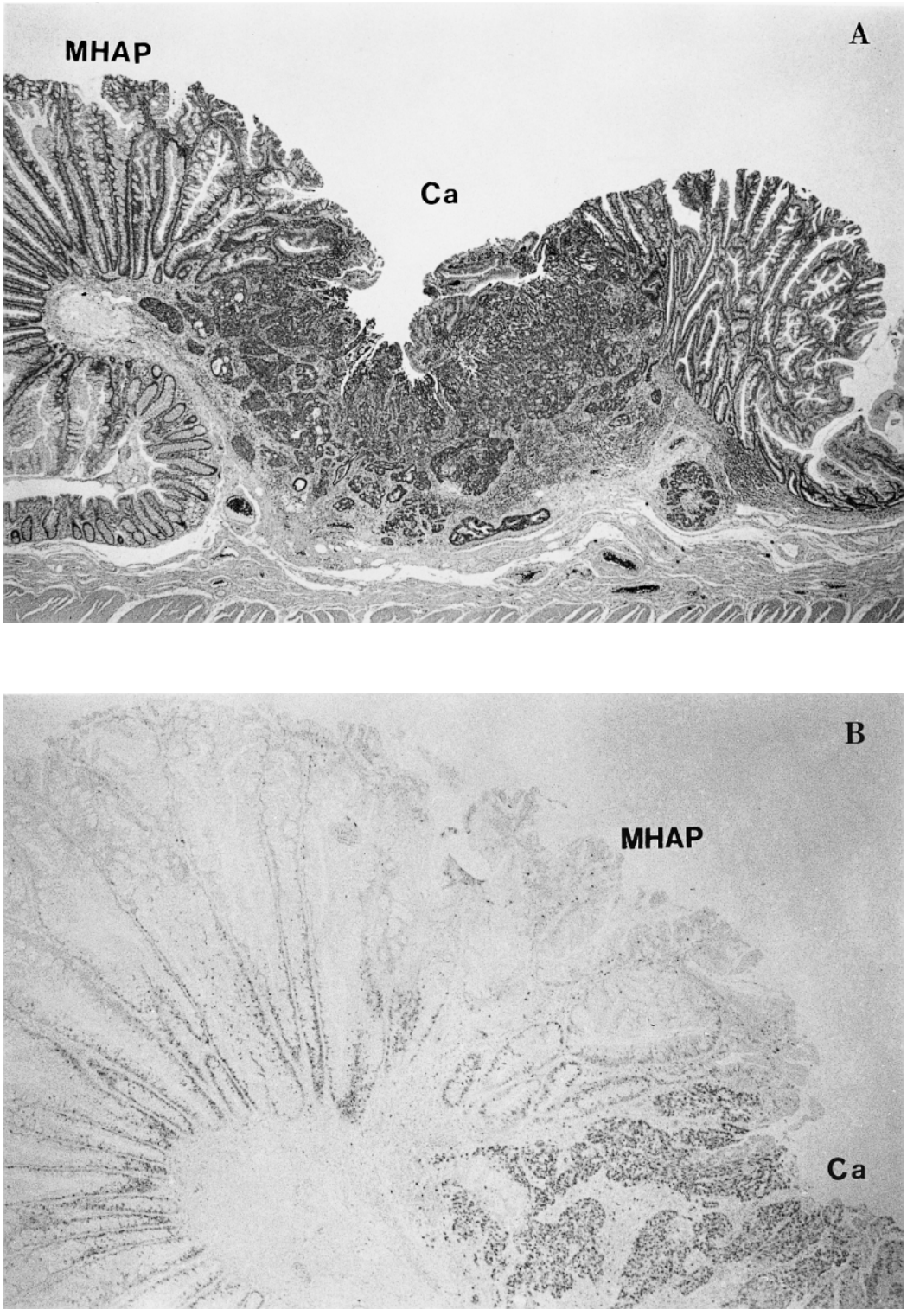

Fig. 1. A mixed hyperplastic adenomatous polyp (M1). A, Focal cancer in MHAP. HE ×25. B, Marked staining on the bottom of crypts, sparse staining in the middle and upper zone of crypts for Ki67 in MHAP, and diffuse and strong staining for Ki67 in the focal cancer.

Table II. Mutation Analysis of K-*ras*, *APC*, *p53* for MHAPs, Adenomas and Hyperplastic Polyps

Case no.	Examined lesion	K-*ras*		*APC*		*p53*	
		codon	mutation	codon	mutation	codon	mutation
M1	MHAP	ND		ND			ND
	focal ca	ND		ND		174	CGC–CAC
M2	MHAP	ND		ND			
M3	MHAP	ND		ND			
M4	MHAP	ND		ND			
M5	MHAP	ND		ND			
M6	MHAP	ND		ND			
M7	MHAP	ND		ND			ND
	syn ca	ND		ND			ND
M8	MHAP	12	GGT–TGT	ND			
M9	MHAP	ND		ND			
M10	MHAP	ND		ND			
M11	MHAP	ND		ND			
M12	MHAP	ND		ND			
A1	ad	ND		1393	CTT–CTTT		
A2	ad	12	GGT–GAT	ND			
A3	ad	ND		ND			
A4	ad	12	GGT–TGT	ND			
A5	ad	12	GGT–GTT	ND			
A6	ad	ND		1411	AGTG–AGG		
A7	ad	ND		ND			
A8	ad	12	GGT–GAT	ND			
A9	ad	ND		1309	GAAAAGAT–GAT		
A10	ad	12	GGT–GAT	1370	AAA–TAA		
A11	ad	ND		ND			
A12	ad	ND		ND			
A13	ad	ND		1309	GAA–TAA		
A14	ad	12	GGT–GTT	ND			
A15	ad	ND		1438	AACA–ACA		
A16	ad	ND		ND			
A17	ad	ND		ND			
A18	ad	ND		ND			
A19	ad	12	GGT–GAT	ND			
A20	ad	ND		1438	AACAC–AACC		
A21	ad	12	GGT–GAT	ND			
A22	ad	ND		1376	TATGT–TGT		
A23	ad	ND		1363	AAA–TAA		
A24	ad	12	GGT–GTT	1450	CGA–TGA		
A25	ad	12	GGT–GAT	ND			
A26	ad	ND		ND			
A27	ad	ND		ND			
H1	HP	ND		ND			
H2	HP	ND		ND			
H3	HP	ND		ND			
H4	HP	ND		ND			
H5	HP	ND		ND			
H6	HP	ND		ND			
H7	HP	ND		ND			
H8	HP	12	GGT–GCT	ND			

ad, adenoma; MHAP, mixed hyperplastic adenomatous polyp; HP, hyperplastic polyp; focal ca, focal cancer in MHAP; syn ca, synchronous cancer; ND, not detected; blank, not examined.

Table III. Genetic Alterations of Cancerous and Benign Portion of Three MHAPs

Case no.	Lesion	K-*ras*	*APC* mutation	LOH	*p53* mutation	LOH	RER
M1	MHAP	ND	ND	ND	ND	ND	ND
	focal ca	ND	ND	ND	+	+	ND
M7	MHAP	ND	ND	ND	ND	ND	ND
	syn ca	ND	ND	ND	ND	ND	ND
M12	MHAP	ND	ND	ND	ND	ND	ND

MHAP, benign portion of mixed hyperplastic adenomatous polyp; focal ca, focal cancer in MHAP; syn ca, synchronous cancer; ND, not detected; +, detected.

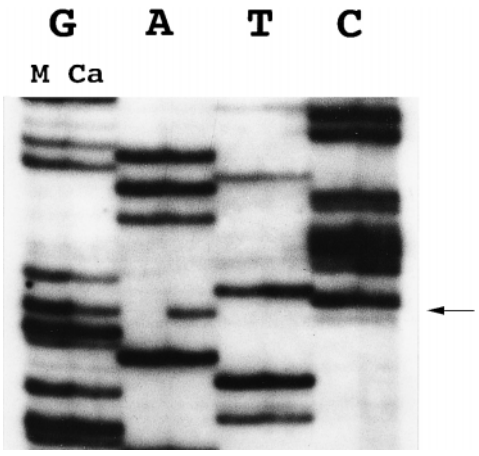

Fig. 2. PCR direct sequencing of *p53* of M1. M, MHAP; Ca, focal cancer. The arrow indicates nucleic acid substitution; codon 174 CGC (Arg) to CAC (His).

primers.[16] The PCR products were separated by denaturing polyacrylamide gel electrophoresis.

RESULTS

Analysis of *APC* mutations and LOH The results of *APC* analysis in colorectal tumors are shown in Tables II and III. Ten of 27 (37.0%) adenomas showed somatic mutations but no mutation was identified in 12 MHAPs and 8 hyperplastic polyps.

No LOH of the *APC* locus was identified in any of three cases (M1, M7 and M12), using the two CA repeat microsatellite markers near the *APC* (Table III).

Analysis of K-*ras* mutations K-*ras* mutations were observed in only one of 12 (8.3%) MHAPs, while they were observed in 10 of 27 (37.0%) adenomas and in 1 of 8 (12.5%) hyperplastic polyps (Tables II and III).

Analysis of *p53* mutations and LOH We performed an immunohistochemical study of M1 (carcinoma in MHAP) using anti p53 antibody (data not shown). The carcinoma lesion was strongly stained, but the surrounding MHAP lesion was hardly stained. Further, as shown in Fig. 2 and Tables II and III, *p53* mutation was identified only in the focal carcinoma, not in the surrounding MHAP portion. LOH of *p53* was identified in the cancer, but not in the surrounding MHAP of M1. In M7, neither *p53* mutation nor LOH was identified in the benign portion of the MHAP or in the synchronized cancer (Tables II and III), and in M12, no LOH of *p53* was identified in the MHAP.

DISCUSSION

In order to determine the role of the *APC* gene in MHAPs, we examined MHAPs, adenomas and hyperplastic polyps. It has been reported that 32 to 75% of somatic mutations of adenomas occur in the MCR.[4, 17] Therefore, we examined the MCR in this study. However, no *APC* mutation was identified in 12 MHAPs. It is likely that adenomatous appearance is not always related to *APC* gene mutation, and MHAPs may involve mutations other than *APC* gene mutation.

In our study, the frequency of K-*ras* mutation in MHAPs was less than that of adenomas. Jen *et al.*[5] found K-*ras* mutations in 22% of hyperplastic polyps and in 25% of adenomas. Thus, K-*ras* mutation may be implicated in MHAP, but K-*ras* is not a key gene for MHAP formation.

We also examined *p53* mutations in two cases where MHAP was associated with cancer (M1 and M7). *p53* mutation was identified only in the cancerous portion in M1, in which the cancer appeared to have developed within the MHAP, not in the benign MHAP portions. Thus, *p53* may play an essential role in the transformation of MHAP to carcinoma.[18]

Recently, Allen[3] proposed that colorectal carcinogenesis occurs through the LOH pathway and the RER pathway. The LOH pathway is followed by *APC* inactivation, and a typical disease is familial adenomatous polyposis. The RER pathway is initiated by an inherited or somatic mutation within one of the DNA mismatch repair genes such as *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6*.

Jeevaratnam *et al.*[7] examined RER of giant hyperplastic polyps, adenomas and cancers of a giant hyperplastic polyposis family. Some patients of the family developed MHAPs, but RER analysis was not carried out for their MHAPs. RERs were detected at one or two of three microsatellite loci in 3 of 19 tumors (15%). The frequency of RER was almost the same as that of sporadic colorectal cancers. In contrast, RER of major HNPCC cancers was detected at all three loci. Therefore, giant hyperplastic polyposis may not involve the RER pathway. In our series, RER was examined in 3 MHAP cases (M1, M7 and M12) simultaneously with LOH analysis of *APC* and *p53*. Two of the three cases had a carcinoma: one in MHAP and one associated colon carcinoma. No RER was identified in either the MHAP portions or carcinomas (Table III). However, we examined only 3 MHAPs using 3 microsatellite markers, so further analysis will be necessary to examine whether the RER pathway has any involvement in MHAP development.

It has been suggested that hyperplastic polyps may, very infrequently, transform into adenomas,[19] which means that hyperplastic polyps change into hyperplastic polyps with adenomatous aberrant crypt foci. But a true MHAP is different from a hyperplastic polyp with adenomatous aberrant crypt foci or a polyp with admixed hyperplastic glands and adenomatous glands.[6] Polyps with admixed hyperplastic glands and adenomatous glands were excluded from this study. As mentioned above, the molecular characteristics of MHAPs are similar to those of hyperplastic polyps in terms of *APC* and K-*ras* mutational analysis. The mean ages of the MHAP group and the hyperplastic polyp group were slightly different at 56.9±8.4 and 51.8±12.8 (Student's *t* test; $P>0.05$), respectively. The mean size of MHAPs was 1.3±0.68 cm and that of hyperplastic polyps was 0.9±0.9 cm (Student's *t* test; $P>0.05$). These data are consistent with the idea that hyperplastic polyps may develop into MHAP. A clonal change may originate in a hyperplastic polyp and progress into MHAP, or an admixed hyperplastic polyp may be a transient lesion in the formation of an MHAP. By examining the gene alterations of MHAPs, hyperplastic polyps and admixed hyperplastic polyps, the question may be solved.

Although MHAP is rare, analysis of gene abnormalities associated with this tumor may reveal a novel pathway from normal mucosa to preneoplastic lesions. In view of the possibility that large MHAPs, hyperplastic polyps and hyperplastic polyposis may become malignant, it should be considered whether treatment is appropriate.

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