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Increased Frequency of *p53* Mutation in Sporadic Colorectal Cancer from Cigarette Smokers

Michiko Miyaki[1,2], Takeru Iijima[1,2], Reiko Ishii[1], Yumi Kita[1], Morio Koike[3], Toshio Kuroki[2] and Takeo Mori[4]

[1]Hereditary Tumor Research Project, [4]Department of Surgery and [3]Department of Pathology, Tokyo Metropolitan Komagome Hospital, Tokyo and [2]Institute of Molecular Oncology, Showa University, Tokyo, Japan

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Background: Cigarette smoking has been shown to increase the risk of colorectal cancer. However, the relation between smoking and genetic alterations has not been clarified in this type of cancer.
Methods: Mutations of *p53*, *APC*, *β-catenin* and *K-ras-2* genes were analyzed in colorectal carcinomas from 28 smokers and 33 non-smokers. Frequencies and types of mutations were compared between smokers and non-smokers.
Results: The frequency of carcinomas with *p53* mutation was higher in smokers (20/28, 71%) than in non-smokers (15/33, 45%) ($P = 0.037$). The common type of mutation was single-base substitution including G:C to A:T transition in both groups (68% in smokers and 67% in non-smokers). With respect to G:C to A:T transitions, mutation at CpG sites was less frequent in smokers (9/15, 60%) than in non-smokers (10/10, 100%), whereas mutation at non-CpG sites was more frequent in smokers (6/16, 40%) than in non-smokers (0/10, 0%) ($P = 0.028$). The frequency of *APC* mutation was not significantly different between smokers (14/28, 50%) and non-smokers (15/33, 45%). No *β-catenin* mutation was detected in carcinomas from smokers. *K-ras-2* mutation occurred in smokers at a similar frequency (9/28, 32%) to that in non-smokers (13/33, 39%). Concerning pathological aspects, Dukes' A carcinomas were less frequent in smokers (11%) than in non-smokers (33%), whereas Dukes' D carcinomas were more frequent in smokers (25%) than in non-smokers (15%).
Conclusion: The present results suggest that an increased frequency of *p53* gene mutation, including G:C to A:T transitions at non-CpG sites, is associated with an increased risk of colorectal carcinogenesis in cigarette smokers.

Key words: cigarette smoking – p53 mutation – APC mutation – K-ras-2 mutation

INTRODUCTION

Recent large cohort and case control studies have demonstrated that cigarette smoking is a risk factor for colorectal cancer incidence (1,2) and many previous studies have also suggested an association between cigarette smoking and colon cancer or adenoma (3–10). Smoking has been found to form many carcinogenic compounds which reach various tissues directly or indirectly. Carcinogens are known to cause genetic changes in multiple tumor suppressor genes and oncogenes resulting in cancer formation. Of these genes, *p53* gene is commonly mutated in human cancer including colon, stomach, lung, head and neck, bladder cancers and various other types of cancer. Accordingly, *p53* gene is considered to be suitable as a marker in the examination of the effect of tobacco carcinogens on molecular pathogenesis of various cancers. In the case of lung cancer, a strong association with smoking has been shown (11,12) and one of the genetic targets for the risk of lung cancer has been revealed to be the *p53* gene (13–16). Increased frequency and altered spectrum of *p53* mutation have been observed in lung cancer from not only smokers, but also non-smokers exposed to environmental tobacco smoke (13–15). An effect of smoking on the *p53* gene has also been observed in bladder cancer (17). In head and neck cancer, heavy smokers have been shown to have an elevated *p53* expression (18). However, in the case of colorectal cancer, genetic targets of smoking are still unknown, although DNA adducts with compounds formed by tobacco smoking have been detected in the colonic mucosa (19). With respect to colorectal carcinogenesis, we have previously demonstrated that it proceeds through multiple steps, including genetic change of *APC*, *p53*, *Smad4*,

For reprints and all correspondence: Michiko Miyaki, Hereditary Tumor Research Project, Tokyo Metropolitan Komagome Hospital, 3–18–22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan.
E-mail: mmiyaki@opal.famille.ne.jp

β-catenin and *K-ras-2* genes (20–24). To clarify which genes are targets of cigarette smoking, we analyzed *p53*, *APC*, *β-catenin* and *K-ras-2* genes in colorectal cancer from cigarette smokers and non-smokers. The results indicated an increased frequency of *p53* gene mutation in smokers.

MATERIALS AND METHODS

Tumor Samples

Sixty-one sporadic primary colorectal carcinoma samples were obtained from 61 Japanese patients at Tokyo Metropolitan Komagome Hospital after informed consent. All samples were invasive adenocarcinomas. Patients were divided into two groups: non-smokers (33 cases) and smokers (28 cases). In the latter group, amounts of cigarette smoking (number of cigarettes per day × years) ranged from 400 to 4080 (mean 961). Surgically removed tumors were histopathologically diagnosed and appropriate areas of tumor tissues were frozen at –80°C until they were used for mutation analysis.

Mutation Analysis

DNA was isolated from fresh frozen tumor tissue samples and corresponding normal tissues by the proteinase K–phenol–chloroform method. Exons 4 (codon 33) through 10 (codon 367) of the *p53* gene, exons 14 (codon 582) through 15I (codon 1640) of the *APC* gene, exon 3 of the *β-catenin* gene and exon 1 (including codons 12 and 13) of the *K-ras-2* gene were analyzed by the PCR-SSCP method. DNA fragments in abnormal bands were amplified by asymmetric PCR and then sequenced by the dideoxy termination method as described previously (21). PCR primers for exons 4–8 of *p53* gene were the same as those previously reported (22) and primers for exon 9 were sense TGATGAGAATTCGCCTCTTTCCTAGCACTG and antisense TGATGAGAATTCCCAAGACTTAGTACCTGA and for exon 10 they were sense TGATGAGAATTCCTCTGTTGCTGCAGATCC and antisense TGATGAGAATTCGCTGAGGTCACTCACCT. Primers for the *APC* gene were the same as those reported previously (25). Primers for exon 3 of the *β-catenin* gene were the same as those used previously (24). Primers for the *K-ras-2* gene were sense ATGACTGAATATAAACTTGT and antisense TCCACAAAATGATTCTGAAT.

Statistical Method

Frequencies of mutations were compared between smokers and non-smokers using Fisher's exact test. A P-value of <0.05 was considered significant.

RESULTS

p53 Gene Mutation

DNA samples of 28 colorectal carcinomas from cigarette smokers and those of 33 colorectal carcinomas from non-smokers were analyzed for exons 4–10 of the *p53* gene by PCR-SSCP and direct sequencing methods. All *p53* mutations detected are listed in Table 1. *p53* mutations were detected in 20 of 28 carcinomas (71%) from smokers and in 15 of 33 carcinomas (45%) from non-smokers (Table 2). This difference was statistically significant ($P = 0.037$). Two carcinomas from smokers (MY223 and MY250) exhibited two mutations, the latter case having two mutations in one allele. Accordingly, total mutations detected in smokers were 22 in 28 carcinomas. Eight of 22 mutations (36%) from smokers and nine of 15 mutations (60%) from non-smokers existed at the previously observed hot spot codons in colorectal carcinomas (codons 175, 245, 248, 273 and 282). The spectrum of *p53* mutations was compared between smokers and non-smokers (Table 3). The common type of *p53* mutation was single-base substitution in both smokers (19/22, 86%) and non-smokers (13/15, 87%). The main mutation direction was G:C to A:T transitions in both groups: this was found in 15 of 22 mutations (68%) in smokers and in 10 of 15 (67%) in non-smokers. With respect to G:C to A:T transitions, the frequency of occurrence at CpG sites was less in smokers (9/15, 60%) than in non-smokers (10/10, 100%). In contrast, the frequency of G:C to A:T transitions at non-CpG sites was higher in smokers (6/15, 40%) than in non-smokers (0/10, 0%) ($P = 0.028$).

APC Gene Mutation

APC mutation was analyzed for the region (exons 14–15I) where >90% of total *APC* mutations were detected in the case of sporadic colorectal tumors (21,26). The frequency of carcinomas with *APC* gene mutations was 50% (14/28) in smokers and 45% (15/33) in non-smokers (Table 2). More than 85% of mutations detected (12 of 14 in smokers and 13 of 15 in non-smokers) existed between codons 1300 (exon 15G) and 1580 (exon 15I) (Table 1). The frequencies of deletions were 57% (8/14) and 53% (8/15), insertions 14% (2/14) and 20% (3/15) and single-base substitutions 29% (4/14) and 27% (4/15) in smokers and non-smokers, respectively. All mutations formed stop codons resulting in truncated APC proteins, except for one mutation resulting in deletion of exon 14. Occurrence of *APC* mutation was independent of the occurrence of *p53* mutation.

β-Catenin Gene Mutation

β-Catenin mutation from ACC (Thr) to GCC (Ala) at codon 41 was detected in one (MY90) of 33 carcinomas from non-smokers; it was not detected in any of 28 carcinomas from smokers.

K-ras-2 Gene Mutation

K-ras-2 mutation was detected in nine of 28 carcinomas (32%) from smokers and in 13 of 33 carcinomas (39%) from non-smokers (Tables 1 and 2). Both codons 12 and 13 were mutated in these two groups. The direction of mutation was predominantly G to A (glycine to aspartic acid) in both smokers (8/9, 89%) and non-smokers (12/13, 92%). Occurrence of *K-ras-2* mutation was independent of the occurrences of *p53* and *APC* mutations.

Table 1. Mutations of *p53*, *APC* and *K-ras-2* genes in primary colorectal carcinomas from smokers and non-smokers

Tumor sample	Smoking status[a]	Gender	Age (years)	*p53* mutation		*APC* mutation		*K-ras-2* mutation	
				Codon	Nucleotide change	Codon	Nucleotide change	Codon	Nucleotide change
Smokers									
MY15	1040	F	72	175	CGC→CAC	–[b]		13	GGC→GAC
MY39	800	M	55	248	CGG→TGG	–		–	
MY44	400	F	66	282	CGG→TGG	1397–8	GA deletion	–	
MY49	500	M	55	196	CGA→TGA	–		–	
MY69	1120	M	52	211	ACC→ATT	1415	T deletion	12	GGT→GAT
MY72	750	M	53	157	GTC→TTC	1554–6	A deletion	12	GGT→GAT
MY88	4080	M	88	245	GGC→GAC	1450	CGA→TGA	12	GGT→GAT
MY92	420	F	80	248	CGG→CAG	1310–11	GATT deletion	–	
MY115	800	M	69	156–9	4 bp deletion, 1 bp insertion	–		–	
MY122	880	M	64	173	GTG→ATG	–		–	
MY148	920	M	66	175	CGC→CAC	–		–	
MY162	795	M	73	245	GGC→AGC	1487–8	T deletion	–	
MY172	800	M	64	159	GCC→GTC	–		12	GGT→GAT
MY176	450	M	54	248	CGG→TGG	935	TAC→TAA	–	
MY204	1050	M	55	204	GAG→TAG	–		–	
MY206	630	M	62	237	ATG→ATA	1429	CAA→TAA	13	GGC→GAC
MY223	2250	M	65	196	CGA→TGA	–		–	
				342	CGA→TGA				
MY231	1800	M	55	253–5	6 bp deletion	642–5	3 bp change, 7bp deletion	–	
MY250	680	M	54	278	CCT→GCT	1300–04	11 bp deletion	–	
				264–5	51 bp insertion				
MY287	1050	M	56	220	TAT→TGT	–		–	
MY37	400	M	60	–		1577–80	TATT deletion	12	GGT→CGT
MY83	500	M	45	–		–		–	
MY157	715	M	75	–		–			
MY229	1000	M	70	–		–		12	GGT→GAT
MY235	400	F	59	–		1361	C insertion	–	
MY237	750	M	51	–		1554–6	A insertion	13	GGC→GAC
MY259	915	M	81	–		–		–	
MY284	1000	M	70	–		1310	AAG→TAG	–	
Non-smokers									
MY6	0	M	66	176	TGC→TTC	–		12	GGT→GAT
MY22	0	F	55	213	CGA→TGA	–		12	GGT→GAT
MY53	0	M	63	249	AGG→GGG	1397	CAG→TAG	–	
MY62	0	F	70	248	CGG→TGG	–		13	GGC→GAC
MY65	0	F	83	175	CGC→CAC	–		–	
MY78	0	F	73	213	CGA→TGA	1376	TAT→TAA	12	GGT→GAT
MY112	0	F	74	174–180	18 bp deletion	1411	T deletion	–	
MY153	0	F	68	273	CGT→CAT	1458	C deletion	12	GGT→GAT
MY155	0	M	65	245	GGC→GTC	1374–5	AC deletion	–	
MY160	0	F	64	273	CGT→CAT	–		–	
MY209	0	F	65	273	CGT→CAT	653	G/g→G/a	12	GGT→GTT
MY239	0	M	45	282	CGG→TGG	1319	C deletion	–	
MY243	0	F	65	282	CGG→TGG	–		–	
MY279	0	F	59	248	CGG→TGG	1407	T deletion	–	
MY292	0	M	52	271–6	16 bp deletion	–		12	GGT→GAT
MY27	0	M	66	–		–		–	
MY41	0	F	78	–		–		13	GGC→GAC
MY57	0	F	81	–		1487–8	T insertion	–	
MY75	0	M	67	–		–		–	
MY85	0	F	48	–		941	GAA→TAA	–	
MY86	0	F	48	–		–		–	
MY90	0	M	66	–		–		12	GGT→GAT
MY94	0	M	65	–		–		–	
MY96	0	F	70	–		–		–	
MY120	0	F	61	–		–		–	
MY142	0	M	60	–		1465–6	GT insertion	–	
MY174	0	F	86	–		1370–1	AA deletion	12	GGT→GAT
MY221	0	M	58	–		1488	TATTA insertion	–	
MY225	0	M	57	–		–		12	GGT→GAT
MY227	0	F	61	–		1420–3	8 bp deletion	12	GGT→GAT
MY233	0	M	58	–		1356–60	14 bp deletion	–	
MY294	0	F	79	–		–		12	GGT→GAT
MY300	0	F	66	–		–		–	

[a]Number of cigarettes per day × years.
[b]–, Mutation was not detected.

Table 2. Frequencies of *p53*, *APC* and *K-ras-2* gene mutations in primary colorectal carcinomas from smokers and non-smokers

Smoking status	No. of tumors with mutation/No. of tumors analyzed (%)		
	p53	*APC*	*K-ras-2*
Smokers	20/28 (71)	14/28 (50)	9/28 (32)
Non-smokers	15/33 (45)	15/33 (45)	13/33 (39)
P-Value[a]	0.037	0.35	0.38

[a]Smokers vs non-smokers

Pathological Aspects

The frequencies of differentiation types and Dukes' stages of colorectal carcinomas were compared between smokers and non-smokers, as shown in Table 4. Well differentiated carcinomas were the most frequent in both groups and the distribution of carcinomas by differentiation type was not obviously different between the two groups. With respect to Dukes' stages, Dukes' A tumors were less frequent in smokers (3/28, 11%) than in non-smokers (11/33, 33%), whereas Dukes' D tumors occurred more frequently in smokers (7/28, 25%) than in non-smokers (5/33, 15%) (Table 4). Moreover, the frequency of G:C to A:T transitions at non-CpG sites in Dukes' D tumors with p53 mutations was higher in smokers (3/4, 75%) than in non-smokers (0/3, 0%).

DISCUSSION

Recent studies have indicated an association between cigarette smoking and an increased risk of colorectal cancer (1–8,10) and several reports have suggested that smoking is also related to the risk of colorectal adenomas (5,6,9). These observations suggest that smoking causes DNA lesions in the colon that lead to cancer. In the case of lung, an association of smoking with a high risk of cancer has been shown and one target of smoking has been revealed to be the *p53* gene (13–16). A similar relationship between smoking and *p53* mutation has also been observed in the cases of bladder cancer (17) and head and neck cancers (18). In colorectal cancer, however, genetic alterations related to smoking have not yet been clarified.

The present comparative examination of mutations of *p53*, *APC*, *β-catenin* and *K-ras-2* genes in primary colorectal carcinomas from smokers and non-smokers demonstrated that the frequency of *p53* mutation was higher in smokers (71%) than in non-smokers (45%). Although the spectrum of mutations was not significantly different between smokers and non-smokers and the main mutation direction was G:C to A:T transition in both groups, the frequency of mutations at non-CpG sites was significantly higher in smokers than in non-smokers. Previous estimation of mutation at the CpG site within G:C to A:T transition was 64% (22) or 76% (27) in colorectal carcinomas, but these studies did not appear to separate carcinomas clearly into smoker and non-smoker categories. The present results indicated that 100% of G:C to A:T transition occurred at the CpG site (and none at the non-CpG site) in non-smokers,

Table 3. Spectrum of *p53* gene mutations in primary colorectal carcinomas from smokers and non-smokers

Mutation	Smokers: No. of mutations detected (%)[a]	Non-smokers: No. of mutations detected (%)[b]
G:C→A:T	15 (68)	10 (67)
G:C→T:A	2 (9)	2 (13)
G:C→C:G	1 (5)	0 (0)
A:T→G:C	1 (5)	1 (7)
Deletion, insertion	3 (14)	2 (13)
G:C→A:T	15	10
At CpG site	9	10
At non-CpG site[c]	6	0

[a]Percentage of total number of mutations (n = 22) in smokers.
[b]Percentage of total number of mutations (n = 15) in non-smokers.
[c]*P*-Value (smokers vs non-smokers) = 0.028.

Table 4. Pathological aspects of primary colorectal carcinomas from smokers and non-smokers

Pathological aspects	Smokers: No. of tumors (%)[a]	Non-smokers: No. of tumors (%)[b]
Histology:		
Well differentiated	17 (61)	21 (64)
Moderately differentiated	10 (36)	8 (24)
Poorly differentiated	0 (0)	1 (3)
Mucinous	1 (3)	3 (9)
Dukes' stage:		
A	3 (11)	11 (33)
B	7 (25)	8 (24)
C	11 (39)	9 (27)
D	7 (25)	5 (15)

[a]Percentage of total number of tumors (n = 28) in smokers.
[b]Percentage of total number of tumors (n = 33) in non-smokers.

which is consistent with the assumption that either 5-methylcytosine in the CpG site is spontaneously deaminated, to result in a thymine (28), or that mutagens unrelated to smoking tend to cause mutation at the CpG site (29,30). In contrast, a significantly high frequency of G:C to A:T transition at the non-CpG site occurring only in smokers suggests the participation of some tobacco-specific mutagens.

Cigarette smoke contains many mutagenic compounds, such as nitrosamines, aromatic amines and polynuclear aromatic hydrocarbons (31,32). The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been described to be metabolized to methanediazohydroxide, which methylates DNA forming at least 13 different adducts including 7-methylguanine and O^6-methylguanine. These DNA adducts lead to G to A transitions (31,33). This compound may cause G:C to A:T transitions at non-CpG sites of *p53* gene in colorectal carcinomas in smokers. In spite of the high frequency of G:C to A:T transition, G:C to T:A transversion was low in colorectal carcinomas from non-smokers and it was not increased by smoking. In contrast, in lung cancers G:C to T:A

transversion of *p53* gene was increased by smoking and this type of mutation was predominant in smokers (16,27). Benzo[*a*]pyrene has been reported to form adducts at lung cancer mutational hot spots in *p53* gene (34), which is assumed to be a cause of G:C to T:A transversion in lung cancers in smokers. Such different effects of smoking on the mutation direction of *p53* gene in different organs suggests that different carcinogens in smoke are related to carcinogenesis in different organs.

Increased amounts of DNA-adducts have been detected not only in lung but also other tissues, including gastric mucosa and colonic mucosa, from smokers (19,34–37). It has been suggested that the stomach is exposed to tobacco carcinogens through saliva or bronchial secretions (37), and therefore it is possible that the colon is also exposed to such carcinogens in a similar manner.

DNA adducts formed by smoking are assumed to cause mutations in other genes besides *p53*. Previous observations of an increased risk of colorectal adenoma in smokers (5,6,9) suggested that smoking causes genetic lesions involved in adenoma formation, such as mutation at the *APC*, *β-catenin* or *K-ras-2* gene. In the present study, the frequency of carcinomas with *APC* mutation was not significantly different between smokers and non-smokers. *β-Catenin* gene is not assumed to be the target of smoking, since mutation of this gene was very rare in sporadic colorectal carcinomas and no mutation was detected in carcinomas from smokers. The frequency and direction of *K-ras-2* mutation in colorectal carcinomas were not different between smokers and non-smokers, similar to the case of *APC* mutation. These results seem to be inconsistent with the previous observations of increased risk of adenomas. However, smoking has also been associated with a risk of larger adenomas (5,6) and large adenomas often include carcinomatous components that are assumed to be produced by *p53* mutation. If this is the case, the present results of an increased frequency of *p53* mutation in smokers may be related to the increased risk of larger adenomas. Alternatively, some *de novo*-type minute carcinomas, which are formed by *p53* mutation in the early stage of carcinogenesis, may be included in adenomatous samples from smokers. Although our present data suggest the association of *p53* mutation of colorectal cancer with smoking, one previous report on immunohistochemical analysis for *p53* overexpression suggested that colorectal tumors developing through a *p53*-positive dependent pathway were not associated with smoking exposure (38). This discrepancy may be due to the difference between analytical methods, sequence analysis and immunohistochemical staining. For the determination of *p53* gene mutation, the sequence analysis appears to produce more definitive results than immunohistochemical analysis, since immunohistochemical staining of p53 protein is not always equivalent to a mutational change.

There has been evidence that colonic cancers of smokers were at more advanced stages than those of non-smokers (39). It was observed that Dukes' stage A tumors occurred more frequently among non-smokers and stage D tumors occurred more frequently among smokers. The present results show similar tendencies in their data with respect to the Dukes' stage of carcinomas. In addition to this, G:C to A:T transition mutations at non-CpG sites were detected in Dukes' D tumors from smokers, but not in Dukes' D tumors from non-smokers. Our previous examinations indicated that *p53* mutations contribute to the conversion of colorectal adenoma into early carcinoma (21). Moreover, the frequency of *p53* mutations was higher in invasive carcinomas than in intramucosal carcinomas (21), which suggests that *p53* gene mutations also contribute to the progression of colorectal carcinomas. Accordingly, the higher frequency of carcinomas at a more advanced stage in smokers may reflect a higher frequency of *p53* gene mutations in smokers, although the contribution of other unknown factors to the tumor stage in smokers cannot be excluded.

Recently, passive smoking was also assumed to influence carcinogenesis. However, in the present study, the degree of passive smoking in non-smokers could not be examined.

The present results suggest that an increased frequency of *p53* mutation, including G:C to A:T transitions at non-CpG sites, is associated with an increased risk of colorectal carcinogenesis in cigarette smokers.

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