**MICRO-SATELLITE INSTABILITY IN MULTIPLE AND SINGLE GASTRIC
CANCERS USING FLUORESCENT DNA AUTO-SEQUENCER**

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Abbreviations: MSI - Micro-satellite instability; RER - Replication error

Abstract

Micro-satellite instability (MSI) may underlie one of the etiologies in multistep gastric carcinogenesis, which is strongly related with the alteration of DNA mismatch repair genes. There are many reports for this abnormality in sporadic or familial gastric cancer, but only a few in multiple gastric cancer. In order to elucidate the difference between these two groups, we performed the assessment of MSI by 10 micro-satellite markers in 4 multiple and 25 single gastric cancers using fluorescent DNA auto-sequencer. We detected replication errors (RER) in one (25%) of 4 cases of multiple gastric cancer and 2 (8%) of 25 cases of single gastric cancer, which showed no statistical significance between these two groups. Both cases of single cancer were advanced cancers, of which histological types were poorly differentiated adenocarcinoma, and they showed less than 2 positive markers. On the other hand, in the RER positive case of multiple gastric cancer, histological types were well-differentiated adenocarcinoma and early cancers, in which of one lesion indicated that 7 micro-satellite markers were positive. And the other lesion in the same case showed RER negative, which showed that genetic heterogeneity was recognized. For clinicopathological factors according to RER status, no significant differences were observed in age, gender, tumor localization and size, histological type, depth of invasion, lymph node metastasis, venous invasion or pTNM classification. In conclusion, it is suggested that the carcinogenic etiology of these two groups may be different from our results.

Introduction

In recent years, molecular biological studies of hereditary non-polyposis colorectal cancer have revealed that there are some cancers in which abnormalities of the

mismatch repair gene can be recognized (Vogelstein, Fearon *et al.*, 1989; Ichili, Takeda *et al.*, 1993 and Aaltonen, Peltomaki *et al.*, 1993). It is believed that when a mismatch repair gene should be altered, the replication error (RER) of DNA might be passed onto the next cell along cell division, and this change sequentially causes alterations of oncogenes and/or cancer suppressor genes, resulting in carcinogenesis. In human DNA, there are 104-105 repeated sequences of single base, such as (A)_n or (CA)_n, and multiple base unit which they are designated as micro-satellites. Abnormalities of mismatch repair gene can readily lead to misreading of repeated sequences, and therefore changes to the length of the micro-satellite (micro-satellite instability; MSI) are induced. Analysis of MSI in various malignant tumors has been progressing and successive studies of genetic alterations in gastric cancer have been reported. In single gastric cancer, the frequency of MSI varies between 7% and 50%, with differences by countries or by reporters (Seruca, Santos *et al.*, 1995; Rhyu, Park *et al.*, 1994; Sepulveda, Santos *et al.*, 1999 and Kim, Woo *et al.*, 2000. Apart from single gastric cancer, reports on multiple primary cancer included gastric cancer and on familial gastric cancer have been increasing in recent years (Horli, Han *et al.*, 1994; Akiyama, Nagasaki *et al.*, 1996; Keller, Rudelius *et al.*, 1998 and Shinmura, Tani *et al.*, 1998), but there are only a few reports on multiple gastric cancer (Nakashima, Inoue *et al.*, 1995 and Nakashima, Honda *et al.*, 1995). In this study, MSI was examined in 4 cases of multiple gastric cancer and in 25 cases of single gastric cancer, using a fluorescent auto-sequencer, and the relevance of MSI in both types of cancer was investigated.

Materials and Methods

Patients

We analyzed 4 cases of multiple gastric cancer and 25 of single gastric cancer surgically resected in the Department of Surgery, the Makino Memorial Hospital (Yokohama City, Japan) between 1996 and 1998. Since the cases of multiple gastric cancer were all synchronous and accompanied with 2 lesions in each stomach, there were a total of 33 cancerous lesions. Clinicopathological features of these cases were summarized in Table I. Histological types were 17 cases of well-differentiated adenocarcinoma, 11 of poorly differentiated adenocarcinoma, 4 of signet-ring cell carcinoma and 1 of papillary adenocarcinoma. For the pTNM classification, Stage 0 were

found in 12 cases, stage I in 9, stage II in 4, and stage III in 8. None of the patients underwent chemotherapy or radiation therapy prior to surgery, and any familial history for cancer was recognized in none of the patients. Histopathological examinations were conducted in accordance with WHO classifications and Japanese classification of gastric carcinoma (2nd English edition).

Analysis of MSI

Paraffin-embedded tissue sections cut to a thickness of several micrometers were prepared from both cancerous and non-cancerous tissue in the resected specimens, and DNA was extracted using Takara DEXPATM (Takara Shuzo Co., Kyoto, Japan). Of the DNA material obtained, 40 ng was taken as a template, and PCR reaction was performed using primer marked with fluorescent dye of three colors as blue, green, and yellow. PCR conditions were as indicated below. With a reaction liquid volume at a total of 20.0 μ l, including 10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM MgCl₂, 0.001% w/v gelatin, 50 μ M dNTP, 250nM each primer, 0.5U AmpliTaq Gold; after preheating for 10 minutes at 95° C, the following three-step cycle was repeated 35 times: 95° C, 15 seconds; 60° C, 15 seconds; 72° C, 15 seconds.

Then a prolonged reaction of 30 minutes at 72° C was added. Ten kinds of micro-satellite marker were divided into the following 2 groups of 5 types each, and PCR product was mixed; Group 1: D2S123, D2S136, D3S1067, BAT-25, BAT-26 and Group 2: D17S796, TP53, D17S786, D17S787, TGF β -R II. The loading sample consisted of 1 μ l of mixture fluid from each group, 0.8 μ l of loading buffer (50mg/ml blue dextran, 25mM EDTA), 2.4 μ l of deionized formamide and 0.8 μ l of size standard.

For gel preparation, urea (10.8g), 50% Long Ranger solvent (3.0ml) and 10 $\times$ TBE buffer (3.0ml) were dissolved in 15.6ml of distilled water, and after degassing, 10% APS (150 μ l) and TEMED (15 μ l) were added and mixed. This mixture was poured onto a 36cm glass plate and left to stand for 2 to 6 hours after setting up a 34-space, square-toothed comb.

Next, the loading sample was denatured at 95° C for 5 minutes, then 1.5 μ l of each sample was applied and electrophoresis was conducted for 2 hours by means of an ABI-377 DNA auto-sequencer (PE Biosystems, Inc., Foster City, CA, USA). A com-

parison was made of the peaks of the same marker arising from normal tissue and cancerous tissue, using the GeneScanTM as waveform analyzed software, and shift and LOH were assessed. For shift, when a new peak not recognized with normal tissue was confirmed with cancerous tissue, it was assessed as shift (+), that is, MSI positive. LOH assay was done in the case of heterozygote on the marker of No.17 chromosome. Of the two sizes of amplified product originating from the cancerous DNA, the value of the peak area of the large size was taken as "A" and that of the small size, as "a," and when the values of the two sizes of amplified product originating from the normal tissue DNA were taken as "B" and "b," respectively, LOH (+) was assessed when (A/a)/(B/b) was less than 50% or (A/a)/(B/b) was more than 200%.

Statistical analysis

Statistical analyses were performed using the Chi-squared test with Yates' correction, Fisher's exact test, or Student's t-test using Statview-J 4.5 software. A p value of <0.05 was considered statistically significant.

Results

In 3 (10.3%) of 29 cases RER positive was recognized with at least one or more markers (Table II). They were 2 of 25 cases of single gastric cancer (8%) and 1 of 4 cases (25%) of multiple gastric cancer, which showed no statistical significance between these two groups. The details of the 3 cases of MSI positive were presented as follows. Case 1: single gastric cancer, depth of invasion was proper muscle, poorly differentiated adenocarcinoma, pTNM was III, positive marker was BAT-25. Case 13: single gastric cancer, depth of invasion was subserosa, poorly differentiated adenocarcinoma, pTNM was II, positive markers were D3S1067 and TGF β -R II. Case 27: multiple gastric cancer, depth of invasion were mucosa and submucosa, well-differentiated adenocarcinoma, pTNM was I, positive markers were D3S1067, BAT-25, BAT-26, D17S796, TP53, D17S786 and TGF β -R II in only one of two lesions. Both cases of single gastric cancer were advanced cancers, of which histological types were poorly differentiated adenocarcinoma, and there were no more than two positive markers. On the other hand, in the case of multiple gastric cancer, histological types were well-differentiated adenocarcinoma and early cancers, in which of one indicated that seven micro-satellite

TABLE I Summary of the clinicopathological data in 29 patients

No.	Age	Gender	Location ^a	Histological type ^b	pTNM stage	RER status
1	68	M	A	por	III	positive
2	67	F	A	tub	0	negative
3	74	F	C	sig	I	negative
4	39	M	C	por	III	negative
5	46	F	B	sig	0	negative
6	71	M	B	tub	0	negative
7	55	M	A	tub	0	negative
8	52	M	A	tub	0	negative
9	54	M	A	tub	0	negative
10	59	F	B	por	0	negative
11	66	M	B	tub	I	negative
12	67	M	A	tub	0	negative
13	83	M	AB	por	II	positive
14	58	M	A	tub	I	negative
15	32	F	B	por	III	negative
16	73	M	B	tub	0	negative
17	56	M	B	tub	III	negative
18	53	M	B	por	I	negative
19	63	M	A	por	III	negative
20	43	M	B	por	I	negative
21	71	M	AB	por	III	negative
22	67	M	AB	por	III	negative
23	87	M	A	pap	II	negative
24	62	M	AB	por	III	negative
25	64	F	B	tub	I	negative
26-1	57	F	B	sig	II	negative
2			A	sig	II	negative
27-1	78	F	A	tub	I	positive
2			B	tub	I	negative
28-1	79	F	A	tub	0	negative
2			B	tub	0	negative
29-1	71	M	A	tub	I	negative
2			B	tub	I	negative

^a Location: A, antrum; B, body; C, cardia

^b Histological type: por, poorly differentiated adenocarcinoma; tub, well-differentiated adenocarcinoma; sig, signet-ring cell carcinoma; pap, papillary adenocarcinoma

TABLE II Microsatellite instability of RER positive cases

NO.	D2S123	D2S136	D3S1067	BAT-25	BAT-26	D17S796	TP53	D17S786	D17S787	TGFbeta-R II
1	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
13	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
27-1	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)

(+), RER positive; (-), RER negative

TABLE III Clinicopathological characteristics and MSI of gastric cancers according to RER status

	RER—	RER +	p value
Number of cases	26	3	
Age	61.0±12.5	76.3±7.6	0.05
Gender			
Male	18	2	0.69
Female	8	1	
Number of Cancers	30	3	
Location			
Cardia	2	0	0.21
Body	14	0	
Antrum	14	3	
Size of tumor			
<2cm	13	0	0.21
≥2cm	17	3	
Histologic type			
Well differentiated tubular	16	1	0.61
Poorly differentiated tubular	9	2	
Signet-ring cell	4	0	
Papillary	1	0	
Depth of invasion *			
m or sm	16	1	0.48
mp or deeper	14	2	
Lymph-node metastasis (n)			
n+	8	1	0.83
n-	22	2	
Vascular invasion (v)			
v+	8	2	0.21
v-	22	1	
Lymphatic invasion (ly)			
ly+	11	2	0.34
ly-	19	1	
pTNM stage			
0	11	0	0.57
I	9	1	
II	3	1	
III	7	1	

* m, mucosa; sm, subserosa; mp, proper muscle

markers were positive. The other lesion in the same case showed RER negative, and genetic heterogeneity was recognized. For the positive markers, two identical markers of D3S1067 and TGF β -R II were recognized in 2 of 3 RER positive cases.

Clinicopathological factors according to RER status were examined, and no significant differences were observed in age, gender, tumor localization and size, histological type, depth of invasion, lymph node metastasis, venous invasion or pTNM classification (Table III).

With respect to LOH of No.17 chromosome, no abnormalities were recognized in any of the specimens retrieved.

Discussion

Since mismatch repair genes such as hMSH2, hMLH1, hPMS1 and hPMS2 are linked to each other and caused to function as a single unit, it is supposed that if mutations should be noted in any of these genes, normal function would be lost (Prolla, Christie *et al.*, 1994). MSI reflects abnormalities in these mismatch repair gene systems, and it is thought to be a cause of DNA replication error, resulting in the increase of frequency for genetic mutations and sequentially potential for carcinogenesis.

In this study, the ten most widely used types of marker were selected from micro-satellites investigated in past reports. In the analysis for RER assessment, when there were alterations in one or more markers, it was assessed as shift (+), that is RER positive, although there were differences in judgement of RER used by various reporters. Therefore, the overall incidence of RER was 3 out of 29 patients (10.3%), which was relatively low in comparison to those in other reports (Seruca, Santos *et al.*, 1995; Rhyu, Park *et al.*, 1994; Sepulveda, Santos *et al.*, 1999 and Kim, Woo *et al.*, 2000). It has been recognized that there is a higher incidence of RER in gastric cancer in Japan, Korea or Portugal, as compared to Europe and United States (Sepulveda, Santos *et al.*, 1999 and Wirtz, Muller *et al.*, 1998), and this tendency is probably based on inherent factors. Variations in positive rates can be observed even among reports from the same region, and this is ascribed to deviations in background factors of the cases, as well as to differences in the kinds and numbers of micro-satellite investigated (Wirtz, Muller *et al.*, 1998).

Several characteristic points have been identified for MSI in the carcinogenetic process of gastric cancer. Rhyu, Park *et al.*, (1994) reported that in 2 of 16 cases of RER positive gastric cancer, MSI of the same pattern as that in cancer was recognized in dysplasia adjacent to cancer. In the investigation by Semba, Yoshizaki *et al.*, (1996), the rate of positive MSI was 42% in 12 patients with gastric adenoma, and 33% in 9 with intestinal metaplasia. RER was observed even in intestinal metaplasia, which should be a precancerous lesion, so they pointed out that some intestinal metaplasia might be genetically unstable and might be the starting point of multistep carcinogenesis. Hamamoto, Yokozaki *et al.*, (1997) also reported that RER was positive in 4 of 15 patients with incomplete-type intestinal metaplasia adjacent to gastric cancer. Therefore, MSI is thought to be an event that occurs in the early stage of the carcinogenetic process.

Differences in the number of MSI positive locus can be present as the second characteristics. In our study, of the cases of MSI positive, 2 patients with advanced cancer had up to 2 loci, but 1 patients with early stage cancer had RER phenotype with 7 loci. In the report by Keller, Ruselius *et al.*, (1998), 4 of 14 patients with of RER positive single gastric cancer had 6 or more loci, and the remaining 10 patients had 3 loci or less. Moreover, of the patients with low level MSI, many were of significantly advanced tumor stage (pT3+pT4). In contrast, in patients with high level MSI of 6 loci or more, only earlier tumor progression was noted (pT1+pT2). These results were consistent with our own research findings. Dos Santos, Seruca *et al.*, (1996) reported that carcinomas with multiple RER positive loci predominantly showed intestinal or atypical histological differentiation, expanding growth pattern, absent or minimal desmoplastic response, abundant lymphoid infiltration, low DNA content, relatively low frequency of lymph node metastasis, and better prognosis, as compared to those with a few RER positive loci. Furthermore, Gleeson, Sloan *et al.*, (1996) pointed out that low-level instability could be a reflection of inherent instability, and differences in the locus count of RER positive might suggest that there could be differences in carcinogenetic process and in genetic pathway.

There are some reports concerning overall RER frequency, clinicopathological factors and prognosis. In our study, no correlation was found between RER and various factors such as age, gender, histological type, venous invasion, lymph node metastasis or

pTNM classification. The results obtained by Wirts, Muller *et al.*, (1998) were also consistent with our own findings, indicating that no differences were observed between early stage cancer (pT1) and advanced cancer (pT2-pT4). In addition, they pointed out that MSI occurred in the early stage of tumor progression. In the series by Keller, Ruselius *et al.*, (1998), among clinicopathological factors a significant difference was noted only in tumor localization, but similar differences were not found with other factors. Respecting prognosis, it was reported that the prognosis was significantly better in patients with RER positive than in RER negative (Oliveira, Seruca *et al.*, 1998) and that no significant differences could be noted (Wirts, Muller *et al.*, 1998). A definite agreement could not be obtained for prognosis.

Reports for MSI in multiple primary cancers including gastric cancer and in multiple gastric cancer are quite few. Horii, Han *et al.*, (1994) reported that RER positive was observed in 34 of 38 patients with multiple primary cancer (89%), which showed a significantly higher incidence in comparison to those with single cancer (11%). And in 22 of 38 patients with gastric cancer, 5 patients had MSI positive with low loci, but the remaining 17 patients had high frequency of at least 2 loci. They pointed out that the RER positive cases might be believed as a high risk of having additional primary carcinomas. To our knowledge, there were only reports for multiple gastric cancer by Nakashima, Inoue *et al.*, (1995) and Nakashima, Honda *et al.*, (1995). In their investigation of 14 patients with multiple gastric cancer, RER was positive in 11 patients (78.5%), but no significant differences could be observed between these and RER negative cases in clinicopathological factors such as age, gender, histological type or clinical stage. Since RER was significantly higher in multiple gastric cancer as compared to single gastric cancer (Nakashima, Honda *et al.*, 1995 and Nakashima, Inoue *et al.*, 1995), they concluded that there was a high potential of additional carcinogenesis in patients with multiple gastric cancer as in multiple primary cancers, and that a genetic predisposition that encouraged carcinogenesis was obtained. In our study, RER was found at a high frequency in multiple gastric cancers in 1 of 4 cases of multiple gastric cancer (25%) as compared to 2 of 25 cases of single gastric cancer (8%). These differences were not statistically significant, but our positive rate was lower than that of Nakashima, Honda *et al.*, (1995). The reason was thought to be based on the different

kinds of micro-satellite investigated. In our multiple cases, only one cancer showed RER positive, but the other had RER negative, which showed heterogeneity of MSI. In 11 patients with RER positive multiple gastric cancer, Nakashima *et al.*, also recognized heterogeneity of MSI in 8 cases (72.7%), indicating that this was not a rare phenomenon in multiple cancers (Nakashima, Honda *et al.*, 1995). As they pointed out, even in the same microenvironment or the same genetic background, the generation of separate tumors might occur along different genetic pathways. It is also suspected that heterogeneity of MSI might be due to random occurrence of MSI in each tumor.

Of the 10 kinds of micro-satellite used in this study, TGF β -type II receptor (TGF β -R II) was positive in 2 of 3 patients with RER positive (66.7%). In previous reports, the incidence of TGF β -R II positivity in RER positive was recognized at high rates of approximately 50-90% (Oliveira, Seruca *et al.*, 1998; Souza, Antar *et al.*, 1996; Guo, Wang *et al.*, 1998; Wu, Lee *et al.*, 1998 and Chung, Kim *et al.*, 1999). It was also reported that mutations of TGF β -R II were abundant at a higher rate with multiple loci RER positive than with RER positive of a few loci (Wu, Kee *et al.*, 1998 and Ottini, Falchetti *et al.*, 1998). Of the abnormalities to mismatch repair gene, it is suspected that there is a close relationship to abnormalities of hMSH6 (Chung, Park *et al.*, 1997). In normal cells, TGF β acts as a strong inhibitor of cellular progression through TGF β receptor. It is supposed that TGF β -R II, unlike micro-satellite within intron, is deactivated selectively by RER because this micro-satellite exists within the coding region, and it thus contributes more directly to carcinogenesis. Therefore, it is regarded as one of the target genes of carcinogenesis caused by MSI. The results of our study supported these hypotheses.

Unfortunately, details of the relationship between RER and carcinogenesis are still unclear because most of the reports, including our own, cover only a small number of cases. Nevertheless, although it is impossible to explain the carcinogenetic process in terms of alterations to each single genes, the mechanism of RER carcinogenesis must be elucidated by establishing uniform standards of evaluation and accumulating cases based on the same.

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