Allelic Status of Chromosomes 17p, 18q, 22q, 3p and their Clinical Usefulness in Colorectal Cancer

SHIN FUJITA[1], HIDEO BABA[2,3], SEIICHIRO YAMAMOTO[1], TAKAYUKI AKASU[1], YOSHIHIRO MORIYA[1] and KOKICHI SUGANO[2,4]

[1]*Department of Surgery and* [2]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo;*
[3]*Department of Surgery, Saitama Municipal Hospital, Saitama;*
[4]*Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, Tochigi, Japan*

Abstract. *Background: To determine whether the allelic status of chromosomes is clinically useful in colorectal cancer, the allelic losses at chromosomes 17p, 18q, 22q and 3p and their relationships with the clinicopathological features in colorectal cancer (CRC) patients, who had undergone curative surgery without adjuvant chemotherapy, were examined. Materials and Methods: The allelic status at 17p, 18q, 22q and 3p was analyzed by PCR-SSCP (polymerase chain reaction single-strand conformation polymorphism) in 139 CRC from patients who had undergone curative surgery between October 1994 and June 1996. The relationships between these allelic losses and the clinicopathological features were investigated. Results: The lymph node status was significantly associated with the allelic status of 17p, 18q and 22q. The tumor site and tumor differentiation were significantly associated with the allelic status of 18q. When patients with more than two allelic losses were defined as the high allelic loss group and those with no, or only one allelic loss were defined as the low allelic loss group, it was found that the lymph node involvement was significantly higher in the high than in the low allelic loss group. Only three out of 25 patients in the low allelic loss group had lymph node metastasis, and 15 patients in this group without lymphatic invasion had no lymph node metastasis. There was no relationship between the allelic status and survival at any stage. Conclusion: The allelic status was significantly associated with lymph node metastasis. A combination of allelic status and lymphatic invasion assessment can predict the lymph node status before radical surgery.*

Correspondence to: Shin Fujita, Department of Surgery, National Cancer Center Hospital, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Tel: 81-3-3542-2511, ext. 7089, Fax: 81-3-3542-3815, e-mail: sfujita@ncc.go.jp

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There have been many reports on the relationships between the clinicopathological features of colorectal cancer (CRC) patients and the allelic status of chromosomes 1p (1, 2), 2p (3), 3p (4), 4p (5), 5q (6), 8p (7, 8), 17p (1, 8, 9) and 18q (8-15), or a combination of different allelic statuses (8, 16). Several reports have shown that the prognosis for patients with allelic losses is worse than for those without allelic losses. However, there have been conflicting results for chromosomes 5q (6), 17p (15, 17-19) and 18q (1, 17, 19, 20) and for combinations of chromosomal alterations (21). Therefore, these genetic alterations of allelic status are not clinically used for CRC.

To determine whether the allelic status is, in fact, clinically useful in CRC, four chromosomes were studied: 17p, 18q, 22q and 3p. Chromosomes 17p and 18q have tumor suppressor genes, p53 and DCC, respectively, and their allelic status has been suggested, in many reports, to be associated with clinicopathological features (1, 8, 10-15). The allelic loss of 22q is relatively frequent in CRC (22-24), but there have been no reports of a relationship between the clinical background and the allelic status of 22q. The allelic status of 3p was reported to be associated with survival prognosis (4), and preferential allelic loss of 3p was observed in metastatic tumors in comparison with primary CRC (25). The status of these four chromosomes in 139 cancers, obtained from CRC patients who had undergone curative surgery without adjuvant chemotherapy, was analyzed. Then, the relationship between the allelic status and clinicopathological features was examined.

Materials and Methods

Patients and tissues. A total of 139 CRC, from patients who had undergone curative surgery without adjuvant chemotherapy at the National Cancer Hospital, Tokyo, Japan, between October 1994 and June 1996, were examined. The primary tumors had been obtained immediately after surgery and stored frozen in liquid nitrogen until DNA extraction. All surviving patients had been followed for more than 5 years, initially at 3-month intervals for

2 years and at 6-month intervals thereafter. Adjuvant chemotherapy had not been given.

Blunt-end SSCP analysis of allelic status. The allelic status was determined by blunt-end SSCP (single-strand conformation polymorphism) analysis (26, 27). Briefly, three intragenic polymorphic markers (intron 1, exon 4 and intron 7) of the *p53* gene, and two 17p13 markers (D17S695, D17S919), a 17p11 marker (D17S969), two 18q21 markers (D18S51, D18S499), a 22q12 marker (D22S685) and a 3p23 marker (D3S2396) were analyzed by blunt-end SSCP. For the amplification of these polymorphic markers, the primers shown in Table I were used. The forward and reverse primers were synthesized and labelled with indodicarbocyanine (Cy5) amidite reagent, a fluorescent dye (Pharmacia, Uppsala, Sweden), using an Oligo 1000 DNA synthesizer (Beckman, Fullerton, CA, USA). In PCR, the first denaturation step was done at 95˚C for 5 min. PCR amplification was performed for 30 to 40 cycles under the following conditions: denaturation at 95˚C for 30 to 60 sec, annealing at 50 to 67˚C for 30 to 60 sec and extension at 72˚C for 30 to 60 sec. For the blunting reaction, 0.5 units of Klenow fragment (TAKARA BIO, Shiga, Japan) was added to 5 μL of the PCR product and incubated at 37˚C for 30 min. One microliter of the reaction mixture was mixed with 10 μL of the loading buffer and denatured at 80˚C for 5 min. One microliter of the aliquot was electrophoresed on 15% polyacrylamide gel at 20˚C to 24˚C for 10 h at 20 W using an ALFred DNA sequencer (Pharmacia). The data were analyzed using the Fragment Manager (Pharmacia) software package. In the analysis of a normal heterozygote, the ratio of the peak heights of the signal from each allele was constant, with a variation of within 5% (27). Therefore, an allelic loss was defined as when one of the peak heights for a tumor sample was decreased by more than 10% of that of the corresponding normal tissue. Supposing the A1 allele is lost in a heterozygote carrying the A1 and A2 alleles, T is the peak height of the signal from the tumor samples, and N is the peak height of the signal from the normal control. Then, the percent peak height (%) is given as:

$$(N_{A1}/N_{A2} - T_{A1}/T_{A2}) \times 100 / (N_{A1}/N_{A2}) \quad (26).$$

If at least one of the markers of the same chromosome showed an allelic loss, the chromosome was defined as having an allelic loss.

Statistical analysis. Statistical analysis was carried out by the Chi-squared test. The survival rates were calculated by the Kaplan-Meier method and survival curves were compared by the log-rank test. Cox's proportional hazard model was used for multivariate analysis. The level of statistical significance was set at <0.05.

Table I. *Primers used for PCR-SSCP analysis.*

Forward	Reverse
17p11-13	
D17S695	
5'CTGGGCAACAAG AGCAAAATTC3'	5'TTTGTTGTTGTTCAT TGACTTCAGTCT3'
D17S919	
5'AGGCACAGAGT GAGACTTG3'	5'GCTTAATTTTCACGA GGTTCAG3'
p53 intron 1	
5'TCTTAGCTCGCG GTTGTTTC3'	5'ACTGGCGCTGTGT GTAAATG3'
p53 exon 4	
5'AGCTCCCAGAAT GCGAGAG3'	5'CTGGGAAGGGACA GAAGATG3'
p53 intron7	
5'AGGTCAGGAGCC ACTTGCC3'	5' GTGATGAGAGGTG GATGGGT3'
D17S969	
5'ATCTAATCTGTCA TTCATCTATCCA3'	5'AACTGCAGTGCTG CATCATA3'
18q21	
D18S51	
5'GAGCCATGTTCA TGCCACTG3'	5'CAAACCCGACTAC CAGCAAC3'
D18S499	
5'CTGCACAACATA GTGAGACCTG3'	5'AGATTACCCAGAA ATGAGATCAGC3'
22q12	
D22S685	
5'TTCTTAGTGGGGA AGGGATC3'	5'TGAGTTTGATGTTT TTGATAGACA3'
3p23	
D3S2396	
5'ACCTCTTACTTGT GTTCTTGGG3'	5'TGACCAAGCC AGTATTGGAT3'

Results

Allelic status and clinicopathological backgrounds. The allelic status of 17p was informative in all the patients, the allelic status of 18q was informative in 136 patients (98%), that of 22q was informative in 122 patients (88%) and that of 3p was informative in 106 patients (76%). Representative electropherogram profiles from the SSCP analyses are shown in Figure 1. The clinicopathological backgrounds of the informative cases are shown in Table II. The lymph node status was significantly associated with the allelic status of 17p, 18q and 22q ($p<0.01$, <0.01 and 0.01, respectively). The tumor site and tumor differentiation were significantly associated with the allelic status of 18q ($p<0.01$ and 0.03, respectively). To examine the relationships between the number of allelic losses and the clinicopathological backgrounds, the examined patients were classified into high and low allelic loss groups. The high allelic loss group contained patients with more than two allelic losses. The low allelic loss group contained patients with no, or only one allelic loss. Patients with more than two non-informative alleles or with one allelic loss and one non-informative allele were excluded, because these patients' allelic status could not be classified into either group. In this way

Table II. *Clinicopathological backgrounds for informative cases.*

Chromosomes	17p		18q		22q		3p	
	Loss	Retained	Loss	Retained	Loss	Retained	Loss	Retained
Gender								
Male	68	16	68	15	41	31	28	37
Female	38	17	41	12	27	23	15	26
p	0.11		0.51		0.75		0.51	
Age								
<60	40	11	43	8	23	20	16	23
60≤	66	22	66	19	45	34	27	40
p	0.65		0.35		0.71		0.94	
Tumor site								
Colon	63	25	63	23	42	38	30	39
Rectum	43	8	46	4	26	16	13	24
p	0.09		< 0.01		0.32		0.40	
Tumor differentiation								
Well	46	20	47	18	38	24	19	26
Moderate	60	13	62	9	30	30	24	37
p	0.08		0.03		0.21		0.77	
Lymphatic invasion								
Negative	47	19	50	14	30	31	17	31
Positive	59	14	59	13	38	23	26	32
p	0.18		0.58		0.14		0.33	
Venous invasion								
Negative	56	22	57	19	39	32	24	33
Positive	50	11	52	8	29	22	19	30
p	0.16		0.09		0.83		0.73	
Depth of invasion (pT)								
pT1, pT2	18	10	23	5	11	13	9	14
pT3, pT4	88	23	86	22	57	41	34	49
p	0.10		0.77		0.28		0.87	
Lymph node status (pN)								
Negative	49	25	49	22	31	37	22	34
Positive	57	8	60	5	37	17	21	29
p	<0.01		<0.01		0.01		0.77	

seven patients were excluded. The clinicopathological backgrounds of patients in the high and low allelic loss groups are shown in Table III. The lymph node status was significantly associated with high and low allelic status ($p<0.01$). In the low allelic loss group, only three CRC patients out of 25 (12%) patients had lymph node metastases, while 15 patients without lymphatic invasion had no lymph node metastasis.

Allelic status and disease-free survival. The disease-free survival rates are shown in Table IV. In stages I and II, the high allelic loss group showed slightly worse survival than the low allelic loss group. In stage III, patients with allelic loss at 18q showed worse survival than those without allelic loss at 18q, and the high allelic loss group also showed worse survival than the low allelic loss group (Figure 2). Patients with allelic loss at 3p and those with allelic loss at 22q showed better survival than those without these allelic losses. However, these differences were not significant. In multivariate analysis, only the lymph node status was selected as a significant prognostic factor.

Discussion

Many reports have shown relationships between the clinicopathological background or prognosis of CRC patients and their allelic status (1-16). However, these allelic status relationships are not used clinically because the results have not been fully validated. Of the four chromosomes examined here, allelic loss at chromosome 18q has been suggested to have a strong association with poor prognosis for CRC patients in many reports (8-15). However, some reports, including our study, did not show a significant association between the allelic status of 18q and prognosis (1, 17, 19, 20). Barratt *et al.* suggested that there was an interaction between the allelic status and response to adjuvant therapy (19). Their results showed that only patients without allelic loss gained survival benefits from adjuvant therapy, while those with allelic loss did not. This explains the conflicting results of the association between allelic status and prognosis, because many studies into allelic status included patients who either did or did not receive

A

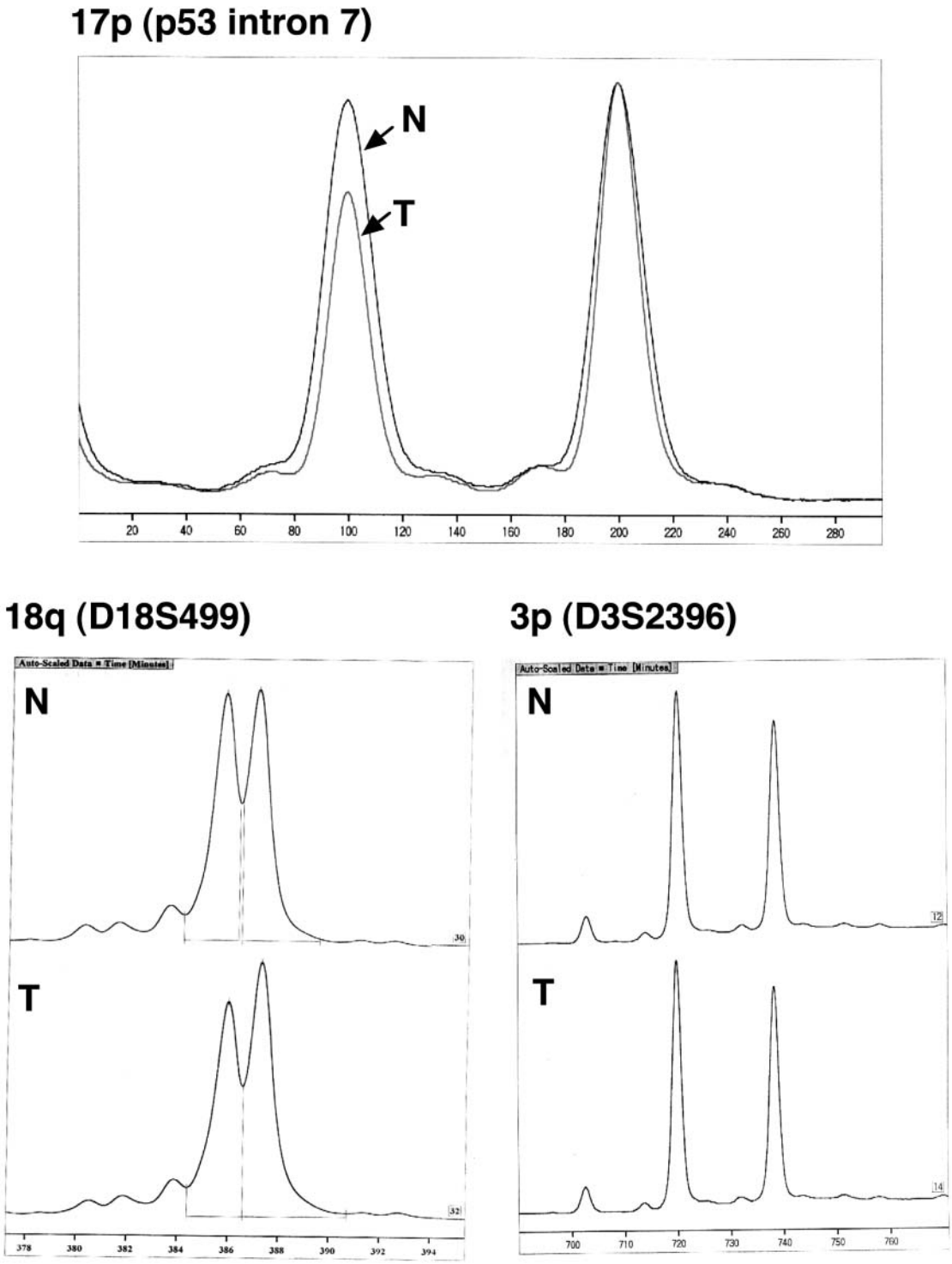
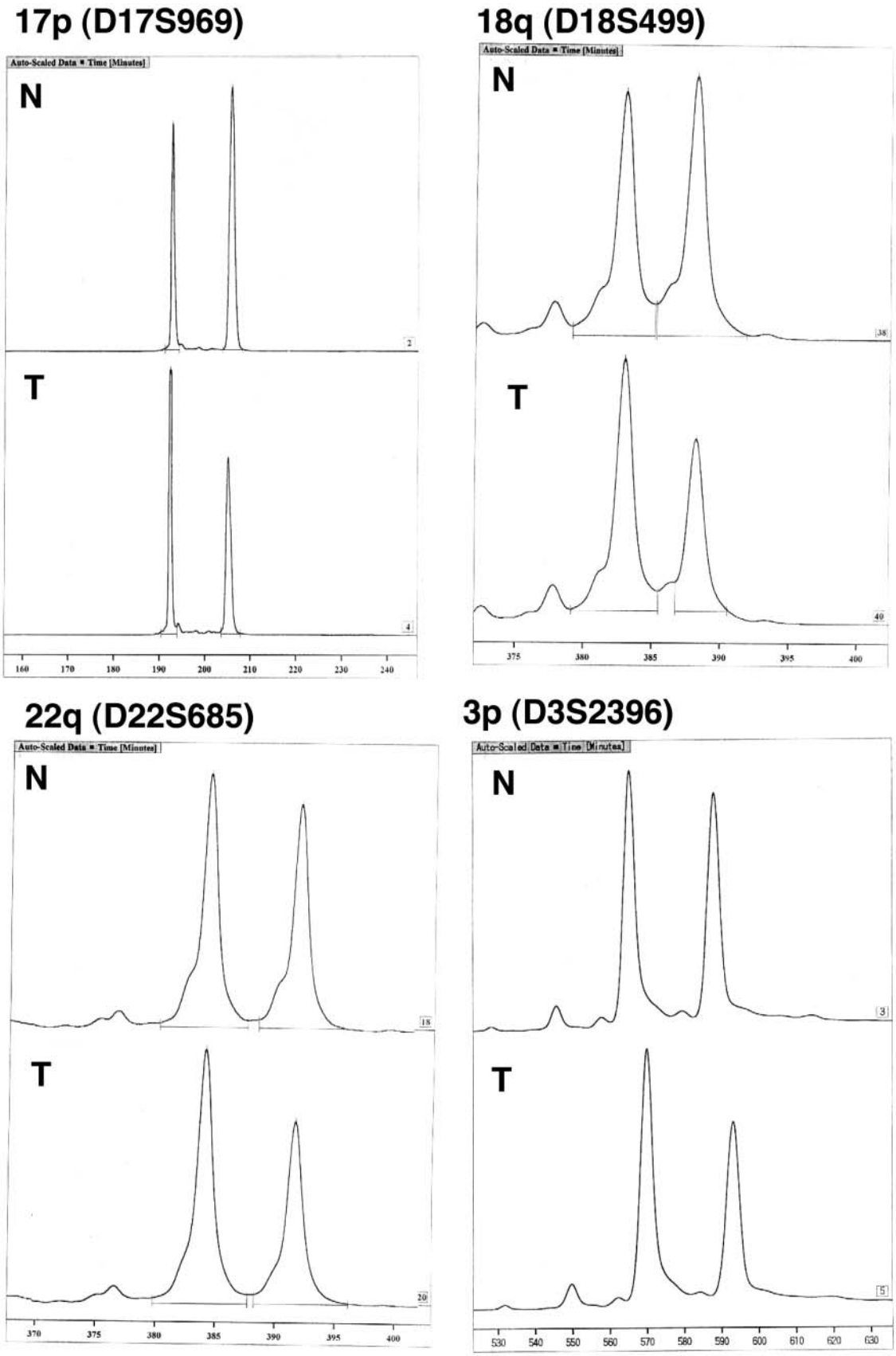


Figure 1. *Electropherogram profiles in SSCP analysis. A: Percent peak height of tumor tissue profile was 23%, 14% and 1% at p53 intron 7, D18S499 and D3S2396, respectively. As defined in Materials and Methods, this patient had allelic loss of 17p and 18q, while the allele of 3p was retained. The allele of 22q was not informative (data not shown). B: Percent peak height of the tumor tissue profile was 43%, 36%, 18% and 22% at D17S969, D18S499 D22S685 and D3S2396, respectively. All the alleles examined were lost in this patient.*

B


17p (D17S969)
Auto-Scaled Data ■ Time [Minutes]
N
T
160 170 180 190 200 210 220 230 240
18q (D18S499)
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375 380 385 390 395 400
22q (D22S685)
Auto-Scaled Data ■ Time [Minutes]
N
T
370 375 380 385 390 395 400
3p (D3S2396)
Auto-Scaled Data ■ Time [Minutes]
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530 540 550 560 570 580 590 600 610 620 630

Table III. *Clinicopathological backgrounds of low and high allelic loss groups.*

Allelic loss	Low (n=25)	High (n=107)	*p*
Gender			
Male	12	68	
Female	13	39	0.29
Age			
<60	8	40	
60≤	17	67	0.61
Tumor site			
Colon	19	63	
Rectum	6	44	0.11
Tumor differentiation			
Well	15	47	
Moderate	10	60	0.15
Lymphatic invasion			
Negative	15	49	
Positive	10	58	0.20
Venous invasion			
Negative	17	57	
Positive	8	50	0.18
Depth of invasion (pT)			
pT1, pT2	7	19	
pT3, pT4	18	88	0.25
Lymph node status (pN)			
Negative	22	47	
Positive	3	60	<0.01

Table IV. *Disease-free survival according to allelic status.*

		5-year disease-free survival rate			
		Stage I, II	*p*	Stage III	*p*
17p	Loss	80% (n=49)		56% (n=57)	
	Retained	80% (n=25)	0.96	63% (n=8)	0.80
18q	Loss	81% (n=49)		54% (n=60)	
	Retained	85% (n=22)	0.62	71% (n=5)	0.34
22q	Loss	83% (n=31)		68% (n=37)	
	Retained	83% (n=37)	0.79	47% (n=17)	0.19
3p	Loss	82% (n=22)		67% (n=21)	
	Retained	81% (n=34)	0.79	45% (n=29)	0.36
High and low allelic loss status					
	High	77% (n=47)		59% (n=60)	
	Low	86% (n=22)	0.30	67% (n=3)	0.83

adjuvant therapy. Another explanation for the conflicting data is non-specific allelic loss. Because chromosomal losses and gains are driven by chromosomal instability that persists throughout the lifetime of the tumor cells (28), some of the allelic losses may not affect the malignant potential of cancer cells, and these non-specific alterations may decrease the prognostic importance of the allelic losses, *i.e.*, these non-specific alterations may obscure the effects of allelic loss.

We showed that the allelic status was significantly related to the lymph node status. If the lymph node status could be predicted before radical surgery, it would be useful for clinical decision making. Taking the high-risk factor for lymph node metastasis, lymphatic invasion (29, 30), into account, patients without allelic loss and without lymphatic invasion had a very low incidence of lymph node metastasis. Among 14 patients without allelic loss at 18q or lymphatic invasion, only one patient (7%) had lymph node metastasis. Among 19 patients without allelic loss at 17p or lymphatic invasion, only one patient (5%) had lymph node metastasis. Fifteen patients in the low allelic loss group without lymphatic invasion had no lymph node metastasis. However, the presence of lymphatic invasion cannot be determined before resection, only after. These results suggested that the combination of allelic loss status and lymphatic invasion status can predict lymph node metastasis before radical surgery. This is particularly useful information, especially for T2 or more so for rectal cancer because, *e.g.*, in the absence of these risk factors, such tumors can be treated by local excision, by endoscopic resection or transanal resection. Therefore, further examination of the relationship between allelic status and lymph node status is warranted in future studies.

The DNA of tumor tissues is inevitably not homogeneous because of stromal cell contamination or the genetic heterogeneity of tumor cell populations, which have also been proposed to cause a wide range of allelic losses. In conventional RFLP (restriction fragment length polymorphism) or PCR-based RFLP analysis, to detect allelic loss the proportion of tumor cells in the sample must exceed at least 50% of the total cells, and a large amount of DNA is required. Clinical samples are often contaminated with normal cells, and the tumor cellularity is sometimes less than 50%. In such cases, conventional techniques cannot detect allelic loss and the allelic status is considered to be retained. This suggests that conventional techniques cannot be used to detect clear associations between allelic loss and prognosis. Here, blunt-end SSCP analysis, which can detect allelic losses when the tumor cellularity of the sample is around 10% and requires only a small amount of DNA, was used. These advantages enabled the detection of allelic losses using small amounts of DNA obtained from biopsy specimens, surgical materials and formalin-fixed, paraffin-embedded sections. The method is clinically very useful, because surgical materials and biopsy samples of cancer are usually contaminated with many normal cells.

It was found that the number of allelic losses was not associated with the prognosis of CRC. However, Choi *et al.* showed that the number of allelic losses was associated with prognosis, this factor still being significant in multivariate analysis (8). Because they had examined eight chromosomes (3p, 4p, 5q, 8p, 9p, 13q, 17p and 18q), this conflicting result might be explained by the difference in the chromosomes examined. If the level of chromosomal loss is an important

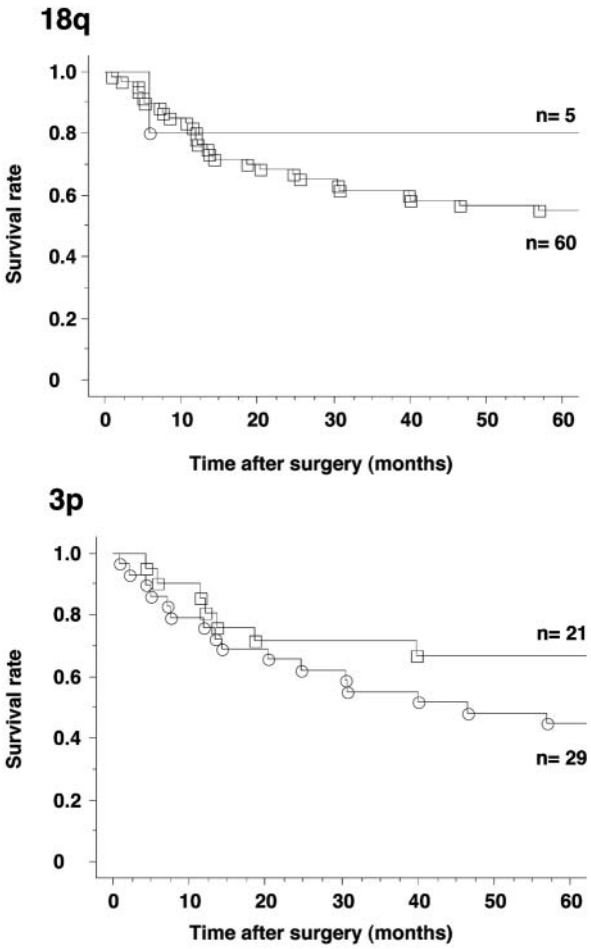

Figure 2. *Disease-free survival curves in stage III CRC. Patients with allelic loss at 18q showed worse survival than those without allelic loss at 18q, and patients with allelic loss at 3p showed better survival than those without this allelic loss. These differences were not significant. □: Lost, ○: Retained.*

prognostic factor, then it is of importance to determine which chromosomes are important for prognosis and how many chromosomes are to be examined. On the other hand, Rooney *et al.* obtained contrary results using comparative genomic hybridization (21). In their study, Dukes' C patients with more than two genomic aberrations had a better survival rate than did patients with fewer regions. Rooney *et al.* also showed that single genomic instabilities were not correlated with survival.

The allelic loss of chromosome 17p is a very common event in CRC. Although the allelic status of chromosome 17p is correlated with some clinicopathological backgrounds, only a small number of reports have suggested the prognostic importance of this allelic loss (1, 8), while other reports, including this study, showed no correlation between prognosis and allelic loss (15, 17-19). For p53, intragenic polymorphic markers were used. Even where the intragenic markers were informative, there was no correlation between prognosis and allelic loss of p53 (data not shown).

The allelic loss of chromosome 22q is relatively frequent in CRC (22, 23, 25, 31). However, there is no report of a tumor suppressor gene on 22q. Although Iino *et al.* have shown that allelic loss of chromosome 22q was correlated with lymph node metastasis (31), there have been no reports of a relationship between the allelic loss of chromosome 22q and prognosis. No relationship was found between the allelic loss of 22q and the clinicopathological background or prognosis, meaning that it probably is not a prognostic factor in CRC patients.

The allelic loss of chromosome 3p is also relatively frequent in CRC, and detailed deletion mapping studies have suggested the existence of tumor suppressor genes on this chromosome, although non have been reported. Iniesta *et al.* showed that allelic loss of 3p was significantly associated with worse prognosis in CRC patients (4). Although theirs was the first report to demonstrate the prognostic significance of the allelic loss of 3p, our study revealed no relationship between the clinicopathological background and allelic status. Choi *et al.* suggested that allelic loss of 3p was correlated with cancer-related death (8). Blaker *et al.* (25) showed preferential loss of chromosome 3p in CRC. However, no additional studies have supported this result, and we were unable to show a relationship between the clinicopathological background or prognosis and allelic loss of 3p.

In summary, although allelic status was not associated with prognosis in CRC patients without adjuvant chemotherapy, it was significantly associated with lymph node metastasis, and a combination of the allelic status and lymphatic invasion status can be used to predict the lymph node status before radical surgery. When allelic loss and lymphatic invasion are not detected after local excision, additional lymph node resection is not required.

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