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Allelotype Analysis in Osteosarcomas: Frequent Allele Loss on 3q, 13q, 17p, and 18q[1]

Toshikazu Yamaguchi, Junya Toguchida, Takao Yamamuro, Yoshihiko Kotoura, Norihiko Takada, Noriyoshi Kawaguchi, Yasuhiko Kaneko, Yusuke Nakamura, Masao S. Sasaki, and Kanji Ishizaki[2]

Radiation Biology Center [To. Y., M. S. S., K. I.] and Department of Orthopedic Surgery, Faculty of Medicine [To. Y., J. T., Ta. Y., Yo. K.], Kyoto University, Yoshida-Konoecho, Sakyo-ku, Kyoto 606, Japan; Cancer Institute [N. K., Y. N.], Kami-ikebukuro 1-37-1, Toshima-ku, Tokyo 170, Japan; Chiba Cancer Center Hospital [N. T.], Nitona-cho 6-6-6-2, Chiba 280, Japan; and Saitama Cancer Center [Ya. K.], Omuro 818, Ina-cho, Kitaadachi-gun, Saitama 362, Japan

ABSTRACT

We have investigated the involvement of tumor suppressor genes in the genesis of osteosarcoma by analyzing allele losses at polymorphic loci in tumor tissues. Genotypes of DNA from primary osteosarcoma tissue and corresponding normal cells from 37 patients were analyzed at 58 polymorphic loci representing each autosomal chromosome arm except 5p and 20q. Allele losses were found at polymorphic loci on 36 of 37 chromosome arms analyzed. In particular, four of them showed frequencies of allele loss higher than 60%: 3q (75%); 13q (68%); 17p (72%); and 18q (64%). This result suggests that, in addition to the *RB* (retinoblastoma) gene on 13q and the *p53* gene on 17p, at least two more tumor suppressor genes located on 3q and 18q are frequently involved in the development of osteosarcoma. The extent of allele losses as defined by fractional allelic loss among 36 tumors was diverse, from 0 to 0.64. The median fractional allelic loss value of 0.32 was much higher than those previously reported in colorectal carcinoma and breast carcinoma. Although no definite association of fractional allelic loss value to clinical prognosis of each case was found in osteosarcoma, tumors with 17p loss were more prone to the early onset of lung metastasis than tumors without 17p loss, indicating that allele loss on chromosome 17p can be a useful measure of prognosis.

INTRODUCTION

Allele losses at specific polymorphic loci have been extensively analyzed in various types of cancer (1). The initial purpose of these studies was to identify the chromosome arms that harbor the tumor suppressor genes in each type of tumor. Mutations of tumor suppressor loci have been shown to be disclosed by chromosomal alterations such as a deletion or a homologous recombination that may involve some neighboring polymorphic loci (2). Therefore, allele loss in the tumor genome may indicate the presence of a tumor suppressor gene around the polymorphic locus. Several tumor suppressor genes have been successfully identified with this strategy (3–10), and a number of chromosome regions have been shown to be possible sites of the tumor suppressor genes (1).

A second application of allelic deletion analysis is the evaluation of biological properties of tumor cells based on the extent of allele loss. The multiple allele loss has been evaluated by Vogelstein *et al.* (11) as the FAL[3] value defined as the number of chromosome arms with allele loss divided by the number of chromosome arms with informative, *i.e.*, constitutionally heterozygous, polymorphic loci. In colorectal carcinoma, patients with more than the median FAL value or those with allele loss on 17p or 18q had a considerably worse prognosis than did the other patients (11, 12). This indicates the possibility of applying the allelic deletion analysis as a molecular hallmark to estimate patients' prognosis, although similar studies on some other types of tumors (13–16) have demonstrated no clear correlations between FAL value and clinical prognosis.

Osteosarcoma is a malignant bone tumor occurring in the extremities of young adolescents in most cases. With the aid of the advancement of the diagnostic imaging system, it has become feasible to define the extent of tumors before the operative treatment and, hence, to minimize the incidence of local recurrence that would result in poor prognosis. Therefore, the occurrence of distant metastasis, particularly lung metastasis, is now regarded as a major determining factor for patients' prognosis. Several characteristics of osteosarcomas, such as the level of serum alkaline phosphatase (17) or the activity of bone morphogenetic protein in tumor tissues (18), have been suggested to provide useful measures for the metastatic potential of tumors. However, no particular genetic alterations have been shown to be predictive of the patients' prognosis. In this study, we examined the extent and variety of allelic deletions in osteosarcoma by using polymorphic DNA markers which covered all of the autosomal chromosome arms, except 5p, 20q, and acrocentric short arms, and addressed the question of whether the extent of allelic deletions has any significant relationship with tumor phenotypes such as histopathological types, grade of malignancy, or patients' prognosis. This study also revealed possible tumor suppressor loci which may be involved in the development of osteosarcoma.

MATERIALS AND METHODS

DNA Samples. Osteosarcoma samples were obtained from the primary focus of tumors in 37 patients, consisting of 27 males and 10 females, and the ages at diagnosis ranged from 5 to 52 yr old, with an average age of 18 yr. All cases were clinically and histopathologically confirmed as conventional osteosarcoma except two cases (KS-141 and KS-147) of parosteal osteosarcoma. According to a surgical staging based on the criteria described by Enneking *et al.* (19), there were 34 cases in Stage IIb and 3 cases in Stage III. Tumor tissues were frozen immediately after surgical removal and stored at −80°C before the isolation of DNA. Peripheral leukocytes were isolated from each patient by the use of Ficoll-Hypaque from heparinized blood.

Polymorphic DNA Markers. All probes used in this study are listed in Table 1. The polymorphic loci detected by these probes are described in Human Gene Mapping 11 (34). The probes 6929, HF12–32, SW50, 9F11, AW101, MS1-14, HF12–1, HF12–62, 4.1H2, and R12.21 were obtained from Japanese Resources for Cancer Study (Gene Bank); c-*myb* was kindly provided by Dr. H. Yuasa; p68RS2.0 was from Dr. T. Dryja; and 7F12, 9D11, 1E8, and 9A7 were from Dr. W. Cavenee. The probe pHRVNTR was previously described (20).

Southern Blot Analysis. High-molecular-weight DNA was isolated according to standard procedure with phenol extraction. Restriction endonuclease digestion of DNA samples, agarose gel electrophoresis, labeling the probes by nick translation, Southern hybridization, and autoradiography was performed as previously described (21).

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[2] To whom requests for reprints should be addressed.

[3] The abbreviation used is: FAL, fractional allelic loss.

Table 1 *Loss of heterozygosity in primary 37 osteosarcomas*

Chromosome	Probe	Locus	Enzyme	Tumors with loss/informative cases
1p	6929	*MYCL*	*Eco*RI	0/9
	YNZ2	*D1S57*	*Rsa*I	2/20
1q	HBI40	*D1S66*	*Msp*I	0/11
2p	TBAB5.7	*D2S47*	*Pvu*II	2/10
2q	YNH24	*D2S44*	*Msp*I	7/26
3p	HF12-32	*D3S2*	*Msp*I	4/12
3q	EFD64.2	*D3S46*	*Msp*I	9/12
4p	MCOC14	*D4S124*	*Pst*I	1/13
4q	EFD139	*D4S163*	*Pst*I	3/17
5q	Pi227	*D5S37*	*Pst*I	5/12
6p	YNZ132	*D6S40*	*Taq*I	7/13
6q	c-*myb*	*MYB*	*Eco*RI	6/20
	JCZ30	*D6S37*	*Hin*dIII	3/14
7p	RM7.4	*D7S370*	*Msp*I	2/17
7q	JCZ67	*D7S396*	*Rsa*I	0/18
	met H	*MET*	*Taq*I	1/14
8p	sw50	*D8S7*	*Hin*dIII	1/10
8q	MCT128.2	*D8S39*	*Pst*I	1/14
9p	MCT112	*D9S15*	*Msp*I	3/10
9q	EFD126.3	*D9S7*	*Taq*I	2/11
10p	MHZ15	*D10S17*	*Msp*I	5/14
10q	EFD75	*D10S25*	*Taq*I	9/17
11p	HRVNTR	*HRAS1*	*Msp*I	5/11
11q	MCT128.1	*D11S144*	*Msp*I	4/21
12p	EFD33.2	*D12S14*	*Taq*I	4/16
12q	YNH15	*D12S17*	*Msp*I	5/26
	9F11	*D12S4*	*Taq*I	1/5
13q	7F12	*D13S1*	*Msp*I	9/12
	68RS2.0	*RB1*	*Rsa*I	5/7
	9D11	*D13S2*	*Msp*I	6/8
	1E8	*D13S4*	*Msp*I	11/15
	9A7	*D13S3*	*Hin*dIII	4/9
14q	CMM101	*D14S13*	*Msp*I	1/29
	AW101	*D14S1*	*Hin*dIII	0/7
15q	MS1-14	*D15S1*	*Msp*I	4/9
	THH55	*D15S27*	*Msp*I	5/12
16p	EKMDA2.1	*D16S83*	*Rsa*I	6/15
16q	79.2.23	*D16S7*	*Taq*I	9/32
17p	YNZ22	*D17S5*	*Taq*I	16/22
	MCT35.1	*D17S31*	*Msp*I	12/17
	MCT35.2	*D17S31*	*Taq*I	9/12
	HF12-1	*D17S1*	*Msp*I	7/8
	10.5	*MYH2*	*Hin*dIII	6/12
	A10-41	*D17S71*	*Msp*I	5/9
17q	THH59	*D17S4*	*Taq*I	2/9
	CMM86	*D17S74*	*Taq*I	3/22
	HHH152	*D17S32*	*Bam*HI	2/14
	HHH202	*D17S33*	*Rsa*I	2/7
18p	HF12-62	*D18S1*	*Taq*I	7/15
	HHH163	*D18S21*	*Pvu*II	2/10
18q	EFZ10	*D18S22*	*Pvu*II	7/11
19p	4.1H2	*D19S7*	*Msp*I	1/3
	JCZ3.1	*D19S20*	*Taq*I	5/21
19q	EFD4.2	*D19S22*	*Pvu*II	3/9
20p	R12.21	*D20S5*	*Msp*I	4/13
	CMM6	*D20S19*	*Taq*I	2/8
21q	MCT15	*D21S113*	*Msp*I	1/15
22q	MS3-18	*D22S1*	*Bgl*II	8/21

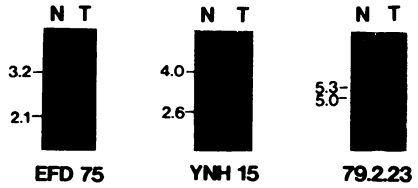

Fig. 1. Southern blot analysis demonstrating LOH in KS-159. DNA samples were obtained from tumor tissue (*T*) and normal tissue (*N*). The probes used are indicated below each panel.

RESULTS

Frequency of Allele Loss on Each Chromosome Arm. Most of the loci analyzed (54 of 58 loci) showed allele losses in at least one tumor. Fig. 1 shows examples of the analysis in one case (KS-159). These allele losses were observed with various frequencies on each chromosome arm (Fig. 2). The frequency of allele losses at polymorphic loci on each chromosome arm ranged from 0% (1q, 0 of 11) to 75% (3q, 9 of 12) (Table 1). Since we have not isolated tumor cells from tumor tissue, it is possible that we could not detect allele loss in some samples because of contaminated nonneoplastic cells. However, since all tumor samples were histologically confirmed as osteosarcoma with a high proportion of tumor cells, the underestimation of frequency of allele loss might be small, if any. As we reported previously (22, 23), allele losses were frequently observed at polymorphic loci on 13q (67.6%, 21 of 31) and 17p (71.4%, 25 of 35). However, the highest frequency of allele loss was observed at the polymorphic locus on 3q (75%, 9 of 12), and that on 18q also showed frequent allele loss (63.6%, 7 of 11). In addition to these four chromosome arms, three chromosome arms (6p, 10q, and 15q) showed allele losses with a frequency higher than 50% (53.8%, 52.9%, and 50%, respectively).

FAL in Each Tumor. Except for a case of KS-163 in which only 4 loci were informative, the FAL values were calculated in the remaining 36 cases with more than 10 informative loci as described by Vogelstein *et al.* (11). Distribution of the FAL value in 36 cases is summarized in Fig. 3. The FAL values varied among 36 cases, ranging from 0 (three cases; 0 of 26, 0 of 24, and 0 of 20) to 0.64 (7 of 11) with a median value of 0.32. The average FAL value of tumors in each histopathological subtype was 0.32 in the osteoblastic type (23 cases), 0.35 in the chondroblastic type (7 cases), 0.20 in the fibroblastic type (3 cases), and 0.15 in the telangiectatic type (one case). There

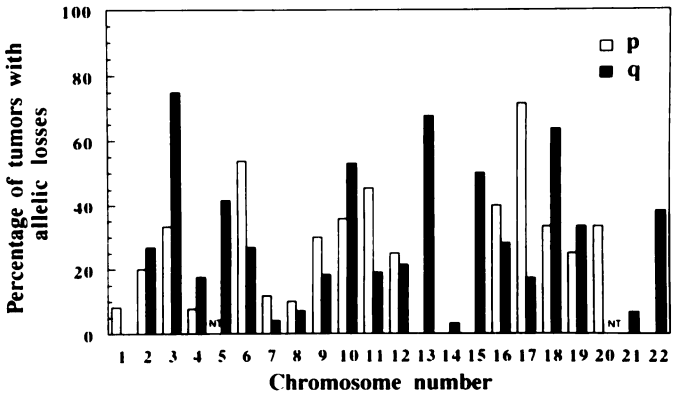

Fig. 2. Frequency of allele loss on each chromosomal arm in 37 osteosarcomas. The probes used are listed in Table 1.

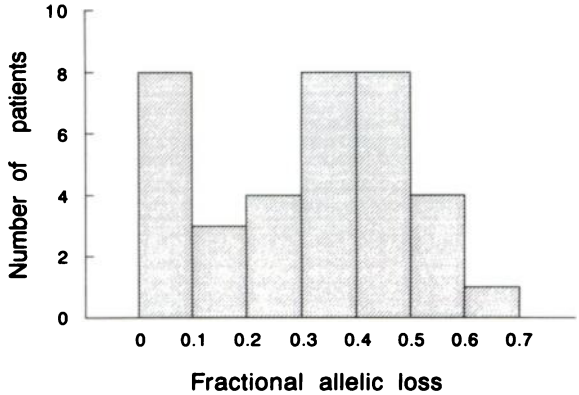

Fig. 3. FAL values of 36 osteosarcomas. The FAL value of each case which showed more than 10 informative arms by restriction fragment length polymorphism analysis was defined according to the definition of Vogelstein *et al.* (11).

Table 2 *Correlation of gene alterations with lung metastasis*

Thirty-six osteosarcoma patients were divided into two groups with lung metastasis (+) and without lung metastasis (−). The FAL value and loss of 13q have no statistically significant relationship with lung metastasis ($P = 0.176$, $P = 0.811$, respectively), but loss of 17p correlates with lung metastasis ($P = 0.032$, χ^2).

	Lung metastasis	
	+	−
FAL value		
Above median	13	5
Below median	8	10
Allele loss on 13q		
+	13	8
−	5	5
Allele loss on 17p		
+	18	7
−	3	8

is no statistically significant difference among those subtypes. Each of the tumors in Stage III (KS-103, KS-118, and KS-131) showed a relatively high FAL value of 0.48, 0.48, and 0.33, whereas two parosteal osteosarcomas, which are supposed to be less malignant than conventional osteosarcoma, showed lower values (0 in KS-141 and 0.2 in KS-147). In four cases (KS-114, KS-152, KS-111, and KS-165), the allele loss was found on only one chromosome arm (1 of 19, 1 of 12, 1 of 22, and 1 of 14, respectively), but the location of unique allele loss was not consistent, *i.e.*, on 17p in KS-114 and KS-152, on 15q in KS-111, and on 3p in KS-165.

Correlation of FAL with Clinical Prognosis. During the period of 2-yr follow-up after the initial treatment, 21 of 36 patients developed lung metastasis. We compared the frequency of allele losses in primary tumors of 21 cases who developed lung metastasis during this period (designated as Group +) and 15 cases without lung metastasis (designated as Group −). The number of cases with FAL values above the median value in Group + is higher than that in Group −, but there is no statistically significant difference between the two groups ($P = 0.176$) (Table 2) Allele loss on chromosome 13q also showed a similar tendency, although it was not statistically significant ($P = 0.811$) (Table 2). The number of cases with allele loss on 17p in Group + was significantly higher than that in Group − ($P = 0.032$), indicating that primary tumors with allele loss on 17p have a higher chance of developing lung metastasis within 2 yr (Table 2). Although such a tendency was observed at long-term follow-up analysis with Kaplan-Meier survival curves in terms of the occurrence of lung metastasis (Fig. 4), the difference was not statistically significant ($P = 0.08$ by the generalized Wilcoxon test).

DISCUSSION

Multiple allele loss has been reported in several types of tumors including malignant melanoma (24), colorectal cancer (25), small cell lung cancer (26), and breast cancer (27). We previously reported the frequent occurrence of concerted allele loss on chromosomes 13q and 17p in osteosarcoma (22, 23). These genetic alterations have been shown to relate to the mutation of the *RB* gene and the *p53* gene, respectively (3, 22, 28, 29). Although the frequency of allele loss was lower than that of 13q or 17p, allele losses at polymorphic loci on other chromosome arms were also observed, suggesting the possible involvement of other tumor suppressor genes (22). In this study we analyzed allele loss on almost all chromosome arms and found frequent allele loss (>50%) on seven chromosome arms (3q, 6p, 10q, 13q, 15q, 17p, and 18q).

Frequent allele loss on chromosome 10q has been reported in glioblastoma (30) and prostate cancer (31). The entire copy of chromosome 10 appeared to be lost in glioblastoma, whereas the common region of deletion was shown to be in 10q24-qter in prostate cancer. The common region of deletion in osteosarcoma seems to be in 10q, because some tumors with allele loss on 10q retained constitutional heterozygosity at the locus on 10p. The relationship between the alterations on chromosome 10 in glioblastoma or prostate cancer and allele loss on 10q in osteosarcoma remains to be determined.

Allelic deletions on chromosome 18q were initially found in colorectal carcinoma (32), and this finding resulted in the isolation of the *DCC* (deleted in colorectal carcinoma) gene (7). As far as we know, mutations of this gene have been reported only in colorectal carcinomas. However, it was recently shown that 13 of 49 breast carcinomas lost one allele at the polymorphic locus located within the *DCC* region, suggesting the involvement of the *DCC* gene in breast carcinoma (33). Although the location of the probe that we used in this study relative to the *DCC* gene was not known, the mutation analysis of the *DCC* gene in osteosarcoma with 18q allele loss seems to be an intriguing matter.

As far as we know, frequent (>50%) allele loss on 3q, 6p, and 15q was not reported in any types of cancer. The frequency of allele loss on 3q was the highest among polymorphic loci analyzed in this study and strongly suggests the existence of a tumor suppressor gene on this chromosome arm. Further studies including deletion mapping to localize the precise regions on these chromosomes are required.

After the original report on colorectal carcinoma (11), the allelotype studies have been performed in several different types of cancers including breast carcinoma (13, 14), renal cell cancer (15), and hepatocellular carcinoma (16). Comparing the results of this study with those in literature, we found several interesting features of osteosarcoma. The median value of FAL in 36 osteosarcomas (0.32) was much higher than that of colorectal carcinoma (0.20) (11) or breast carcinoma (0.15) (14). This high FAL value in osteosarcoma may be relevant to the fact that the majority of cases in this study (33 of 36 cases) were found at relatively advanced stage (IIb). Considering the fact that all three cases with Stage III showed a much higher value, it seems that the high FAL value may be associated with the progression of osteosarcoma, although our results are not conclusive because of the lack of tumors at early stage.

In colorectal carcinoma, both the high FAL value and the allele loss on 17p and 18q showed the strong association with poor prognosis (11, 12), although it is not yet clear whether the allele loss on a specific chromosome arm (17p or 18q) has a

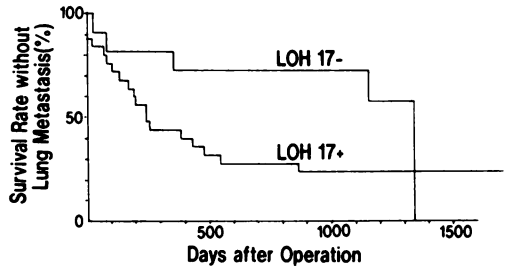

Fig. 4. Kaplan-Meier survival *curves* relating the occurrence of lung metastasis. *LOH 17−* shows the group of patients without 17p loss, and *LOH 17+* shows that of patients with 17p loss.

causative role in the development of metastasis or represents the multiple alterations of genome which may endow cells with metastatic potential. In this study, we found that patients with higher FAL values are much likely to develop lung metastasis than patients with lower FAL values. Although this trend is not statistically significant, it might be due to the small number of samples. Further studies with a larger number of patients are required for a warranted conclusion. Among allele losses on each chromosome arm, the loss on 17p was significantly associated with the occurrence of early lung metastasis. This may support the causative role of 17p allele loss for lung metastasis. However, as shown in Fig. 4, the correlation with 17p loss and lung metastasis was not statistically significant with the Kaplan-Meier method, because several cases without 17p loss had lung metastasis later than 2 yr from initial treatment. These discordant results between two statistical analyses may be also caused by a small number of patients or an insufficient period of follow-up study. Multivalent analysis including clinical factors such as the treatment is obviously required to draw any conclusions.

The common region of deletion on 17p was shown to be in 17p13 where the *p53* locates (23). Approximately 40% of tumors in this study have either gross rearrangements in the *p53* genomic locus or point mutations in the coding sequence of the *p53* gene.[4] Therefore, the association of 17p allele loss with early lung metastasis may indicate the causative role of the *p53* gene mutations in the development of lung metastasis. However, it does not exclude a possible involvement of another gene in 17p13, as suggested in other types of tumors (14). Nevertheless the association of loss with early lung metastasis may encourage the use of molecular genetic analysis in the prediction of prognosis and associated treatment schemes.

The data we presented in this paper have some future implications to be tested. Heterogeneous FAL values among tumors at the same stage (IIb) indicate that the multistep process of the development and progression of osteosarcoma may be extremely heterogeneous. Precise understanding of this process has to await the identification of responsible genes on the chromosome with frequent allele loss, for example, on 3q and 18q. Furthermore, we could not analyze the correlation of allele loss in these chromosome regions with prognosis, because not all cases are informative for the polymorphic probes. Using multiple markers on these chromosome arms will enable us to define the significance of allele loss on these chromosome arms.

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