Lack of Allelic Preference in Amplification and Loss of the c-*myc* Oncogene in Methylcholanthrene-induced Mouse Sarcomas

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Sarcomas were induced in F1 mice between C57BL/6N and C3H/He strains by subcutaneous injection of methylcholanthrene. The c-*myc* oncogene was found to be amplified in 16 cases among 43 sarcomas of C57BL/6N × C3H/He mice and 1 case among 5 sarcomas of the reciprocal cross. The origin of the amplified allele was determined by the polymerase chain reaction single strand conformation polymorphism analysis. Among the 17 sarcomas, only one had both of the alleles amplified. The rest of the tumors carried the amplified c-*myc* allele coming either from C57BL/6N (9 cases) or from C3H/He (8 cases). These results indicate that the c-*myc* allele is amplified randomly in methylcholanthrene-induced mouse sarcomas irrespective of its origin, such as paternal or maternal allele and C57BL/6N or C3H/He allele. In addition to these changes, the unamplified c-*myc* oncogene was found to be lost in 12 cases out of the 17 sarcomas with the amplification.

Key words: MCA-induced sarcoma — F1 mouse — c-*myc* allele — PCR-SSCP analysis

The two alleles which constitute the diploid genome of somatic cells in mammals are not equivalent in their involvement in the process of tumorigenesis. Two mechanisms are known to underlie the non-equivalence of the alleles. The first derives from the difference in the DNA sequence between the alleles, which may in turn affect the frequency of mutation of the genes. Thus, a bias was noted for duplication of chromosome 15 in mouse thymomas. In certain crosses, two of the trisomic chromosomes of chemically induced and virally induced thymomas of F1 mice always originate from one particular side of the parental strains.[1-3] The second mechanism involves the differential imprinting of the alleles. Although the sequence may be the same, the paternally inherited allele of the Rb gene exhibits a higher frequency of somatic mutation in sporadic cases of human osteosarcomas.[4]

These data demonstrate that allelic preference does exist in activation of cancer-related genes and chromosomes. However, more data have to be accumulated before the generality of the phenomenon can be assessed. Technical difficulties in identification of each of the alleles has been the major obstacle to the study of allelic preference. Although restriction fragment length polymorphism has partially solved the problem, low frequencies of the polymorphism had limited the applicability of the method. Recently, the polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) analysis was developed and shown to be quite useful for detecting small base changes in DNA sequences.[5, 6] This provides an ideal method for detecting the allelic polymorphism.

In this report, we have analyzed the allele of the amplified c-*myc* gene in methylcholanthrene (MCA)-induced sarcomas of F1 mice between C57BL/6N and C3H/He by PCR-SSCP. It was found that there was no allelic preference in amplification of the c-*myc* oncogene. In addition, the unamplified counterpart was frequently lost in those sarcomas carrying the amplified c-*myc* oncogene.

MATERIALS AND METHODS

Mice and tumor induction C57BL/6N × C3H/He F1 (BCF1), female C3H/He and male C57BL/6N mice were purchased from Charles River Japan, Inc., Atsugi, Kanagawa Pref. C3H/He × C57BL/6N F1 (CBF1) mice were bred in our animal facility. Mice were injected subcutaneously with 0.5 to 1 mg of MCA dissolved in 0.1 ml of olive oil as described previously.[7, 8] CBF1 mice were injected at a single site on the back. In order to minimize the necessary number of mice, BCF1 mice were injected at 6 sites on the back. When tumors had grown to 1 cm in diameter, they were excised and processed for further studies.

Preparation of DNA Tumor tissue was excised, minced with scissors and transferred into a 3 cm culture dish. Tumor cell explants were trypsinized after 10 days and the cells were seeded onto 10 cm dishes. This procedure effectively eliminated stromal cells such as fibroblasts, macrophages and other lymphocytes in the tumor tissues. After confluency was reached, tumor cells were stored for future study. DNA was purified from the cultured cells according to the procedure described previously.[9]

Southern blotting and PCR-SSCP analysis Amplification of the c-myc oncogene was assessed by Southern blotting of DNA[10] using a probe of the 10 kb *Kpn*I fragment of the genomic c-myc oncogene.[9] The mouse α-globin gene was used as an internal marker for the amount of DNA applied, for which the 2 kb genomic fragment of the mouse α-globin gene was used as a probe. PCR-SSCP analysis was performed according to the procedure reported by others.[5, 6] A set of oligonucleotides, MMMYCE12, spanning a mouse microsatellite locus was used as the primers for PCR-SSCP analysis of the c-myc oncogene.[11]

RESULTS

Amplification of the c-myc oncogene in MCA-induced sarcomas Sarcomas developed at the site of injection with a latency of 70 to 120 days.[8] Histological examination of tumors revealed that they were malignant histiocytomas. In the case of BCF1 mice, 53 sarcomas were formed at 120 injection sites. Thus, the frequency of sarcoma formation was 44% (53/120). Out of these 53, 43 cases were successfully cultured for 10 days. DNA was purified from these short-term cultures; contamination with DNA from normal host cells was minimized in the present system.

Southern blotting analysis of DNA from 48 sarcomas (5 cases of CBF1 and 43 cases of BCF1 mice) demon-

strated frequent amplification of the c-myc oncogene in these tumors. Some of the results are shown in Fig. 1. The degree of amplification was estimated to be around 2 to 10 times. Table I summarizes these results. Frequency of amplification was 35%. The level of amplification was in the same range as those reported previously using other strains of mice.[8] In our previous study, the c-myc oncogene was amplified 5 to 10 times in 4 cases out of 24 MCA-induced sarcomas (17%) of NFS, BALB/c and CBF1 strains of mice. Five sarcomas of CBF1 mice of the previous study were included in the present analysis.
PCR-SSCP analysis We utilized PCR-SSCP analysis to determine the allele of the amplified c-myc oncogene in these sarcomas. The primers used here detected a 107 bp sequence of the mouse c-myc gene which contained a $(CA)_n$ repeat and was polymorphic among laboratory strains of mice.[11]

Fig. 2 demonstrates some of the results of such studies. Here, two of the c-myc fragments of the C57BL/6N allele detected by these primers form bands migrating more slowly than those of the C3H/He allele (Fig. 2, lanes 1 and 2). Analysis of DNA from normal tissues of BCF1 and CBF1 mice revealed four bands (Fig. 2, lanes 3 and 4). These four bands were always present in sarcomas in which the c-myc oncogene was not amplified (data not shown). In one of the sarcomas with the amplified c-myc oncogene, BCF1 7274-2, strong C57BL/6N bands and faint C3H/He bands were detected (Fig. 2, lane 11).
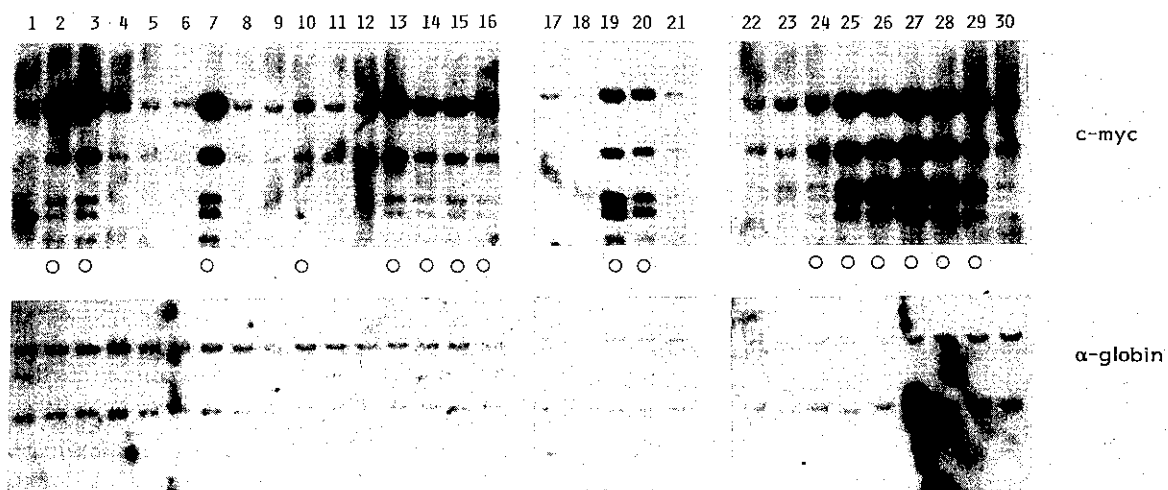

Fig. 1. Amplification of the c-myc oncogene in methylcholanthrene-induced sarcomas of F1 mice. Two micrograms of DNA was digested with *Hind*III, electrophoresed and analyzed by Southern blotting hybridization. The upper panel shows the filter probed for the c-myc oncogene and the lower panel shows the duplicate probed for the α-globin gene. Open circles indicate the amplification. Lanes 1, 17 and 22 are control DNA. Lanes 2–16 are, BCF1 7412-3, BCF1 7413-1, CBF1 6328, CBF1 6329, CBF1 6330, CBF1 6334, BCF1 7199-3, BCF1 7210-2, BCF1 7214-2, BCF1 7354-1, BCF1 7354-2, BCF1 7200-2, BCF1 7273, BCF1 7274-2 and BCF1 7353-2, respectively. Lanes 18–21 are BCF1 7413-3, BCF1 7415-4, BCF1 7419-4 and BCF1 7424-5, respectively. Lanes 23–30 are BCF1 7412-1, BCF1 7412-2, BCF1 7412-3, BCF1 7413-1, BCF1 7413-2, BCF1 7413-4, BCF1 7413-5 and BCF1 7415-2, respectively.

Table I. Amplification·and Loss of Heterozygosity of the c-*myc* Locus

Case of sarcoma	Number of cases	Level of amplification	Amplified and lost allele	
			C57BL/6N	C3H/He
CBF1	5			
CBF1 6334		×10	×10	— a)
BCF1	43			
BCF1 7200-2		×5	×5	—
BCF1 7214-2		×2	×2	—
BCF1 7273		×5	—	×5
BCF1 7274-2		×5	×5	×1
BCF1 7353-2		×5	×5	—
BCF1 7354-2		×2	×1	×2
BCF1 7412-2		×2	×1	×2
BCF1 7412-3		×5	—	×5
BCF1 7413-1		×10	×10	—
BCF1 7413-2		×10	×10	—
BCF1 7413-4		×10	×5	×5
BCF1 7413-5		×10	—	×10
BCF1 7415-4		×5	×5	—
BCF1 7419-4		×5	—	×5
BCF1 7422-3		×5	—	×5
BCF1 7423-3		×10	×10	—
Total number	48			
Cases with amplification	17			
Frequency of amplification	17/48			
Frequency of co-amplification	1/17			
Allele involved in amplification			10	8
Frequency of LOH	13/17			
Allele lost			5	8

a) — indicates loss of the allele.

This indicates that the amplified allele was of C57BL/6N origin and the PCR product of the normal C3H/He allele formed faint bands. Some sarcomas with a low level of amplification of the c-*myc* oncogene, such as BCF1 7214-2, BCF1 7354-2 and BCF1 7412-2, also had bands of both alleles (data not shown). In contrast, only two of the four bands were detected in DNA of all other sarcomas with high-level amplification of the c-*myc* oncogene, except BCF1 7413-4. Southern analysis of BCF1 7413-4 indicated that its c-*myc* oncogene was amplified 10 times (Fig. 1, lane 28). In PCR-SSCP analysis, bands of both alleles were present with equal intensities (Fig. 2, lane 14). Thus, both alleles of the c-*myc* gene were amplified about 5 times in this particular sarcoma.

Lack of bands corresponding to the unamplified allele in most of sarcomas might be due to some artifact of the system. For example, it is possible that one allele is amplified to such an extent that the PCR product of the normal allele becomes relatively too small to be detected in these sarcomas. However, as has been shown in Fig. 1 and Table I, even for sarcomas with a similar level of
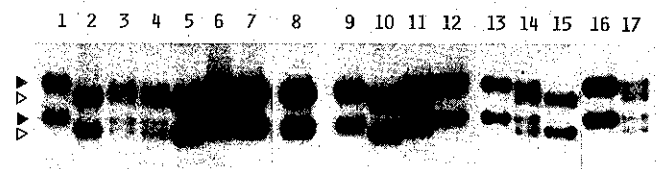

1 2 3 4 5 6 7 8   9 10 11 12 13 14 15 16 17

Fig. 2.   PCR-SSCP analysis of the c-*myc* alleles. Cellular DNA was amplified through 30 cycles of PCR with terminally labeled primers. SSCP analysis was done using 8% polyacrylamide gel containing 10% glycerol. Closed triangles indicate the c-*myc* bands of the C57BL allele and open triangles are those of the C3H/He allele. Lanes 1–17 are C57BL/6N control, C3H/He control, BCF1 control, CBF1 control, BCF1 7412-3, BCF1 7413-1, BCF1 7413-2, BCF1, BCF1 7200-2, BCF1 7273, BCF1 7274-2, BCF1 7353-2, BCF1 7413-2, BCF1 7413-4, BCF1 7413-5, CBF1 6334 and BCF1 control, respectively.

amplification of the c-*myc* oncogene such as BCF1 7274-2 and BCF1 7412-3, bands of the normal allele were detectable for the former but not for the latter. There-

fore, inability to detect two bands of normal alleles in most of the samples is not readily explicable in terms of a difference in the amounts of the amplified products. The lack of detectable bands in PCR-SSCP analysis is likely to be due to the loss of the normal allele in these sarcomas.

The results of PCR-SSCP analysis are summarized in Table I. Of all sarcomas of CBF1 and BCF1 mice, 10 cases of amplification involved the C57BL/6N allele and 8 cases the C3H/He allele. One of these sarcomas had both alleles of the c-*myc* oncogene amplified. Three of sarcomas with the amplified c-*myc* oncogene had the normal allele intact and all the rest, 13 out of 16 sarcomas, had lost the unamplified counterpart. Out of these 13 tumors, the loss involved the C3H/He allele in 8 cases and the C57BL/6N allele in 5 cases. The difference in the frequency of the loss between the C3H/He allele and the C57BL/6N allele was not statistically significant by the *t* test.

DISCUSSION

In this study, it was demonstrated that a significant fraction of primary sarcomas induced by MCA carries the amplified c-*myc* oncogene. We have previously observed amplification of the c-*myc* oncogene in MCA- and α-tocopherol-induced sarcomas in several strains of mice.[8, 12] The significance of the amplification in the development of sarcomas is yet to be elucidated. Nevertheless, it was noticed that sarcoma cells with the amplified c-*myc* oncogene tend to have smaller and more refractile morphology in culture. In addition, for successful transplantation into syngeneic hosts, fewer cells were required for sarcomas with the amplified c-*myc* gene than those without (O. Niwa, unpublished). Thus, the amplification observed here may contribute to the growth advantage and aggressiveness of the tumor cells. The frequency of the amplification in this study is higher than that of the previous study.[8] This might be due to the method used here for isolating the tumor cells and DNA. We applied short-term culture of the tumor cells, while we had isolated DNA directly from tumors in the previous study. Sarcomas with the amplified c-*myc* oncogene may have a better chance of being established *in vitro* and the fraction of amplification-positive tumors may increase under the present conditions.

It is conceivable that the amplification may have occurred during the short-term cultivation of the cells. However, doubling time of the sarcoma cells is around 24 h and the cells can replicate around 10–15 times during the 15 day culture period. This seems to be too short a time for a clone of sarcoma cells with the amplified c-*myc* oncogene to dominate other clones. We believe that the amplification observed in the present study precedes the cultivation of the cells.

With the use of PCR-SSCP analysis, alleles involved in amplification of the c-*myc* oncogene were determined. The analysis demonstrated that the amplification was random with respect to the parental strain and the paternal/maternal origin. Thus, both of the c-*myc* alleles seem to have an equal chance of amplification during development of primary sarcomas. The mouse c-*myc* oncogene has been mapped on chromosome 15[13] and analysis of mice with Robertsonian translocation indicated that this chromosome is not imprinted. Even under the condition of paternal or maternal disomy, embryos develop to term and the mice are apparently normal in every respect.[14] Thus, lack of allelic preference with respect to the paternal/maternal origin in amplification of the c-*myc* gene suggests that this locus may be devoid of imprinting at least in somatic tissues of mesodermal origin. Indeed, *Hpa*II sensitivity of DNA from mouse liver of various stages revealed no evidence of differential DNA methylation of the locus.[15]

Our results also indicated that there is no allelic preference in relation to the parental strain of mice for the amplification. The c-*myc* oncogene has been implicated in many types of malignancies in mice, including B cell lymphomas and thymomas. These tumors are known to be highly dependent on the genetic background of mice. In most cases, molecular mechanisms of genetic susceptibility to tumor development are still not clear, except for some chemically induced tumors where the highly polymorphic drug-metabolizing enzyme, P450, plays a major role.[16] A recent study has indicated that the c-*myc* locus in BALB/c mice is less susceptible to DNA repair after UV irradiation than that in DBA/2N mice, suggesting its possible involvement in the high incidence of B cell lymphomas in BALB/c mice.[17] Mutability of tumor-related genes may differ among strains because of differences in the structures of genes. Indeed, the human c-Ha-*ras* transgene was shown to be more mutable than the endogenous mouse counterpart in transgenic strains of mice.[18] The effect of genetic background on the susceptibility to cancer was shown to be expressed at the cellular level in the case of hepatocarcinomas in mice.[19] Lack of allelic preference in our present study indicates that strain difference in DNA sequence of the c-*myc* gene does not affect the frequency of amplification.

Several mechanisms of gene amplification have been proposed.[20] Our present data indicate that, except for sarcoma BCF1 7413-4, amplification affected only one of the alleles of the c-*myc* oncogene. Therefore, the event which triggers gene amplification does not operate on both alleles at the same time.

Loss of the unamplified c-*myc* allele was unexpected. This loss of heterozygosity at the c-*myc* locus occurred too frequently to be a simple coincidence. In addition, the loss occurred predominantly in sarcomas with high-level

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amplification of the gene. Although there may still be a possibility of the artifactual origin of the loss due to technical problems intrinsic to PCR-SSCP analysis, it seems most likely that the loss is advantageous for development of sarcomas. One possibility is that the presence of the normal c-myc gene somehow suppresses tumor progression. This seems to be unlikely because high-level expression of the normal c-myc sequence has been shown to participate in transformation of cells in collaboration with the activated *ras* oncogene.[21] Another possibility is that there may be an as-yet unidentified tumor suppressor gene in close proximity to the c-myc oncogene. Indeed, the loss of the normal c-myc allele was reported for thymomas and this was taken as evidence for a tumor suppressor gene on mouse chromosome 15.[22] The same group has also shown the suppressive effect of chromosome 15 derived from normal mouse fibroblasts in a cell fusion study.[23] The region of deletion involved in the loss should be assessed to allow elucidation of the biological meaning of this phenomenon.

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