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Difference in Allelic Expression of Genes Probably Associated with Tumor Progression in Murine Fibrosarcomas and Cell Lines

Hiroshi Ohtsuka,[1,2] Yoshiro Mafune,[1] Katsumasa Tsunashima,[1] Hideaki Takahashi[2] and Ryo Kominami[1]

[1]*First Department of Biochemistry and* [2]*Department of Orthopaedic Surgery, Niigata University School of Medicine, Asahimachi-doori 1-757, Niigata 951*

Allelic expression was examined by single-strand conformation polymorphism analysis in murine fibrosarcomas from inter-subspecific F_1 mice between C57BL/6 and MSM. Ten genes encoding p53, mdm2, E-cadherin, 72 kD metalloproteinase and its inhibitor (Timp2), thymidine kinase and four glucose transporters (Gluts) were examined. These genes were chosen because of their probable association with tumor development and progression. In some of the tumors and cell lines, p53, E-cadherin and Glut3 genes showed remarkable differences in allelic expression, one allele being poorly expressed. The allele-specificity persisted in nine cell lines obtained by repeated transplantations from one tumor. These results suggest that expression of some genes is allele-specific in tumor cells and the pattern of specificity is stable. Such a decrease or a loss of expression in one of the alleles may be functionally equivalent to the loss of heterozygosity of the gene, and therefore this may confer malignant properties on tumor cells. It is also suggested that differential expression of two alleles is a common event in tumor cells.

Key words: Allelic expression — Inter-subspecific F_1 mouse — Polymorphism — Tumor suppressor gene

Neoplastic cells often show alterations in gene expression. Deregulated expressions of the p53 tumor suppressor gene and the c-*myc* oncogene have been recognized as factors in the development of human and animal cancers.[1,2] Mechanisms of the alterations include point mutations in regulatory elements, deletions and loss of chromosomes, and possibly epigenetic mechanisms. Loss or decrease of gene expression may play roles in carcinogenesis and malignant progression in the cases of tumor suppressor genes and genes which function in the suppression of tumor progression.[3] In fact, the p53 gene is not expressed in many human cancers.[4,5]

Recently, allele-specific expression of imprinted genes, such as H19 and IGF2, was investigated in tumors[6,7]; some tumors showed bi- or mono-allelic expression due to relaxation of imprinting. This raises the possibility that gene expression may undergo an allele-specific change in tumor cells. If monoallelic expression of tumor suppressor genes frequently occurs, it may be functionally equivalent to allelic loss. Also, low expression of genes suppressing tumor metastasis may favor metastasis. However, allele-specific expression has rarely been examined.

For an analysis of allelic expression, we have developed four primary murine fibrosarcomas in F_1 mice and have obtained a series of tumor cell lines with different malignancies by repeated transplantations. The tumor cells are derived from inter-subspecific F_1 mice between C57BL/6 and MSM, and therefore show polymorphisms in many loci. In this study, we examined ten genes which may be involved in the development and malignant progression of tumors: p53, mdm2, E-cadherin, 72 kD metalloproteinase and the inhibitor (Timp2), thymidine kinase and four glucose transporters. The results show allelic differences in expression of some of the genes. This suggests that allele-specific expression may be a common feature in tumor cells.

MATERIALS AND METHODS

Tumor induction and cell culture F_1 mice were obtained by mating C57BL/6 females with MSM males. MSM mice are an inbred strain derived from Japanese wild mice, *Mus musculus molossinus.*[8] Mice were kept in plastic cages in an air-conditioned room and given commercial pellets and tap water *ad libitum.* Eight- to twelve-week-old mice were given a single subcutaneous injection of 0.1 mg of methylcholanthrene dissolved in olive oil. Mice were killed on the appearance of sarcomas, generally 3–4 months after the injection, and then tumors were collected. Four fibrosarcomas (MST) were isolated, cut into pieces, and cultured *in vitro* (Fig. 1). In total, eleven primary culture lines were obtained. The MST cultures were maintained at 37°C in 5% CO_2 and 95% air in alpha-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. Primary cultures were similarly obtained from fetuses of the F_1 mice.

One cell line, MST1-4, was subcloned to obtain cell lines with different malignancy in the following way (Fig. 1). MST1-4 was cloned *in vitro* by plating into 96-well flat-bottomed microtiter plates at an average concentration of 0.75 cell/well. The clones were designated as C clones. One clone (C-23) was cultured for three months, and the P-4 cell line was obtained after *in vitro* cloning. Cells of the P-4 clone (5×10^5) were given intraveneously to F_1 mice, and metastatic nodules were obtained from the lung (2L8) and liver (2HC). Each of the cell lines was given intravenously and/or subcutaneously, and four metastatic nodules (SCL1, L-b, 3K4 and 1-L-1) were obtained as shown in Fig. 1. K4-2-1 cells were similarly obtained from 3K4 cells (Fig. 1). C-23 and P-4 did not form metastatic nodules after subcutaneous injection. In contrast, 2L8, 3K4 and K4-2-1 metastasized (Fig. 1).

Isolation of DNA and RNA Cellular DNA was extracted as described previously.[9] Cultured cells were collected by centrifugation for 5 min at 1,200 rpm at room temperature and washed twice with PBS (0.14 *M* NaCl, 5 m*M* KCl and 20 m*M* phosphate, pH 7.5). The cell pellet was resuspended in a buffer containing 0.1 *M* NaCl, 10 m*M* EDTA, 10 m*M* Tris-HCl, pH 7.6, and 100 mg/ml proteinase K, and lysed by addition of 1/20 vol. of 10% sodium dodecyl sulfate (SDS). After incubation at 50°C overnight, the lysate was extracted with phenol-chloroform and DNA was recovered by ethanol precipitation.

Total cellular RNA was obtained from tumors and cell lines with guanidine thiocyanate followed by CsCl gradient ultracentrifugation as described.[10] In brief, washed cells were homogenized at 4°C in 4 *M* guanidine thiocyanate, 0.1 *M* Tris-HCl, pH 7.5, 0.14 *M* β-mercaptoethanol, 0.5% Sarkosyl (sodium lauryl sarcosinate). The homogenate was then layered onto 3.5 ml of 5.7 *M* CsCl-0.01 *M* EDTA, pH 7.5 cushion in a polyallomer tube. The tube was centrifuged, and RNA pellets were dissolved in TE buffer.

Preparation of cDNA Total cellular RNA of each sample (10 μg) was denatured at 73°C for 3 min and annealed with 40 pmol of oligo-dT primers or 10 pmol of sequence-specific anti-sense-strand primers.[11] Nine units of avian myeloblastosis virus (AMV) reverse transcriptase (Life Science, Inc.) was used to transcribe mRNA into cDNA. The reaction was carried out in 50 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 8 m*M* $MgCl_2$, 4 m*M* DTT, and 1 m*M* dNTP at 42°C for 2 h.

PCR-SSCP analysis Primers for polymerase chain reaction (PCR) were synthesized on an Applied Biosystems 380B synthesizer, based on reported sequences. Sequences for forward and reverse primers are as follows: p53, F-TTCAGGGCTGAGACACAATCCT, R-ATTGTAGGTGCCGAGGTCCCAA[12]; mdm2, F-TCTACCTCATCTAGAAGGAGAT, R-TAAGCTTCTTTGCACACGTGAAA[13]; E-cadherin, F-TCTCCCTACTGGACTGTCTG, R-GGGCAGATCATAGCTCAAAAG[14]; 72 kD metalloproteinase, F-ACTGGATGGAGGAGAACCAA, R-GTCACTCAATGGGTGTCTTTAT[15]; tissue inhibitor of proteinase (Timp2), F-CCCCATGAATCCACAGACTT, R-AAGCACCCCTCACCACAGAT (GenBank accession #M82858); thymidine kinase, F-CCAAGATGCCTCAATGCAGA, R-GCTCACTAAGAGCTGAGGTT[16]; glucose transporters 1 (Glut1), F-CCTGCCCTGCTGTGTATAGA, R-GCTTAGGTAAAGTTACAGGAGT,[17] Glut2, F-CTGCAGCTACAACTGCTATC, R-GTACGCAAAACCCGAAGTCT,[18] Glut3, F-GGAGACCTCATCAGGATGA-

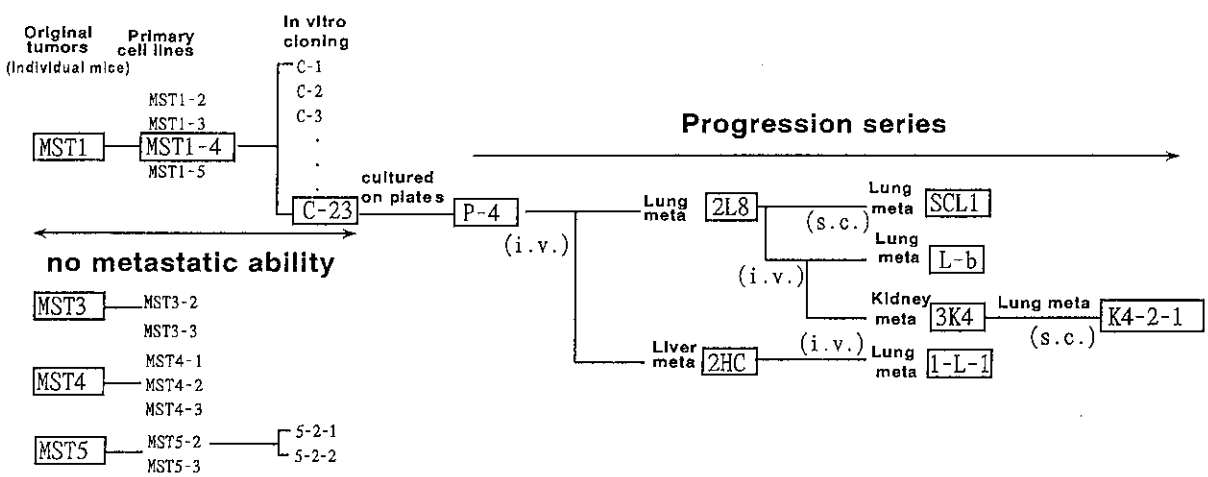

Fig. 1. Pedegree and relationship of MST tumors and cell lines. MST tumors and a series of cell lines with a variety of degrees of malignancy were obtained as described in "Materials and Methods."

A, R-CAAGGAAGTATCCCCAAATCAA (GenBank accession #M75135) and Glut4, F-GAAGGGTGCTAAACCCGAAA, R-TCTGCTCCCTATCCGTTCTT.[19]

PCR was carried out for cDNA and cellular DNA in a 10–20 μl volume under the reported conditions.[20] One primer was end-labeled with ^{32}P and used for amplification. In brief, the reaction was processed through 30–35 cycles of amplification consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with the last elongation step lengthened to 10 min. In some cases, Mg^{2+} ion in the buffer was increased to 3 m*M*. For single strand conformation polymorphism (SSCP) analysis, the products were heat-denatured and separated by 8% polyacrylamide gel electrophoresis.[21]

As for cDNA analysis, we confirmed the absence of contamination of cellular DNA in RNA samples by control PCR without the reverse transcriptase treatment; no PCR products were detectable.

The fidelity of PCR products showing polymorphism was examined by segregation analysis using 61 backcross mice (data not shown). The strain distribution patterns each conformed to the reported chromosomal location of the gene,[22] indicating that the polymorphism detected here does represent the respective gene.

Sequence analysis The primers used to amplify the noncoding region of the p53 gene (5′-TTCAGGGCTGAGACACAATCCT-3′ and 5′-ATTGTAGGTGCCGAGGTCCCAA-3′) are specific to the p53 gene because the p53 pseudogene has deletions and base-substitutions in the region. The PCR products were heat-denatured and electrophoresed in 8% polyacrylamide gel containing 5% glycerol. DNA fragments in shifted bands were recovered by suspending the gel in 10 m*M* Tris-HCl, pH 7.6, 100 m*M* NaCl and 0.1 m*M* EDTA, and were subjected to sequence analysis using the Sequenase sequencing kit (Toyobo Co., Tokyo) according to the manufacturer's specifications.

RESULTS

We conducted a search for polymorphism in the p53 cDNA region in two mouse strains, C57BL/6(B6) and MSM. A polymorphism was detected at a trailer region by SSCP analysis. Two primers were synthesized and used for reverse transcription and polymerase chain reaction (RT-PCR). Template RNAs were isolated from liver of B6, MSM and their F_1 mice and also from normal fibroblasts of four F_1 mouse fetuses. This amplification procedure gave a 185 bp fragment from +1230 to +1414 nucleotide position. Here, the first letter of the initiation codon is numbered nucleotide position 1.[12] The RT-PCR products were heat-denatured and separated by polyacrylamide gel electrophoresis (Fig. 2). B6 strain gave a slower-migrating band than MSM did, and all six samples of the F_1 mice showed both of the bands. The polymorphism relevant to the mobility difference was determined by directly sequencing the PCR products; B6

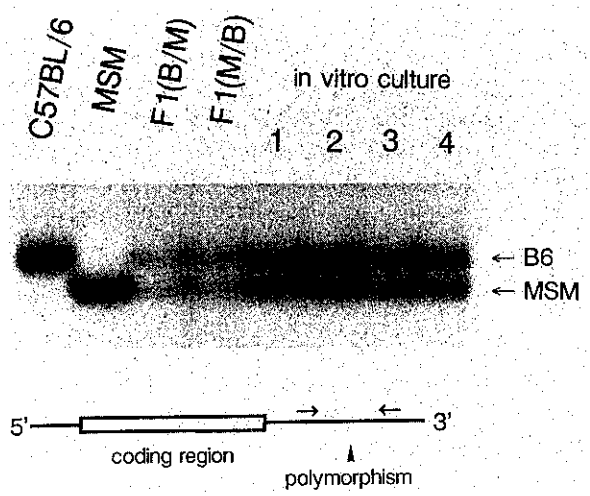

Fig. 2. RT-PCR-SSCP analyses of the p53 gene. PCR was carried out for RNA obtained from liver and four independent *in vitro* culture cells of F_1 mice as described in "Materials and Methods." One primer was end-labeled with ^{32}P and used for the amplification. PCR products were heat-denatured and separated by 8% polyacrylamide gel electrophoresis. After electrophoresis, the gel was dried and autoradiographed. No band signals were obtained from RNA templates without the reverse transcriptase treatment. DNAs and RNAs used are indicated above the gel lanes. The diagram illustrates the structure of p53 mRNA and the positions of two primers used (marked by arrows).

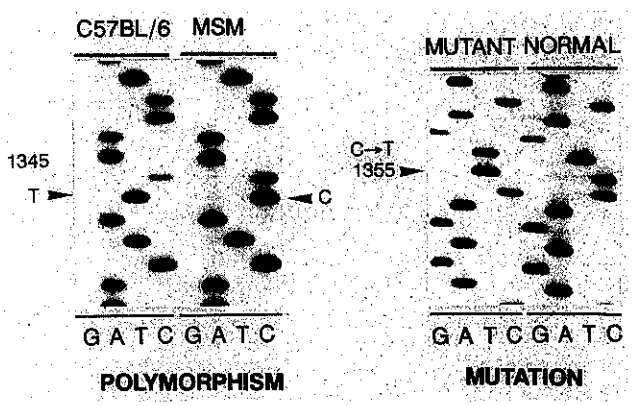

Fig. 3. Sequence analysis of a polymorphism in the p53 gene between C57BL/6 and MSM (left) and a mutation responsible for the band shift (right). PCR was carried out for the noncoding exon in the p53 gene. The products from C57BL/6 and MSM were both directly sequenced as described in "Materials and Methods" (left). DNA was recovered from the mobility-shifted band in MST1-4 and subjected to direct sequencing (right).

showed the thymidine residue at nucleotide position 1345, whereas MSM exhibited cytosine (Fig. 3). This polymorphism was used for determining respective allelic expressions of the p53 gene in four newly isolated fibrosarcomas and their eleven cultured cell lines. The fibrosarcomas were developed in F_1 mice between B6 and MSM (Fig. 1, and see "Materials and Methods").

DNA and RNA were isolated and subjected to PCR-SSCP and RT-PCR-SSCP analyses, respectively. Most of the PCR products for DNA showed a pattern similar to that of F_1 except for MST1-2, MST1-3, MST1-4 and MST1-5, in which a mobility-shifted band was detected (Fig. 4A). Since MST1-4 and MST1-5 did not have the band derived from the normal B6 allele, a mutation had occurred in the B6 allele and existed in some cells of the MST1 tumor. The mutation was identified; the base C at position 1355 in the non-coding region was changed to T (Fig. 3). The bands derived from B6 and MSM alleles showed the same or similar band-intensity in all samples, indicating the absence of allelic loss.

On the other hand, the pattern of allelic expression was changed in some samples (Fig. 4A). Five samples, MST1-2, MST1-3, MST1-4, MST1-5 and MST5-3 showed an MSM-derived band less intense than the B6

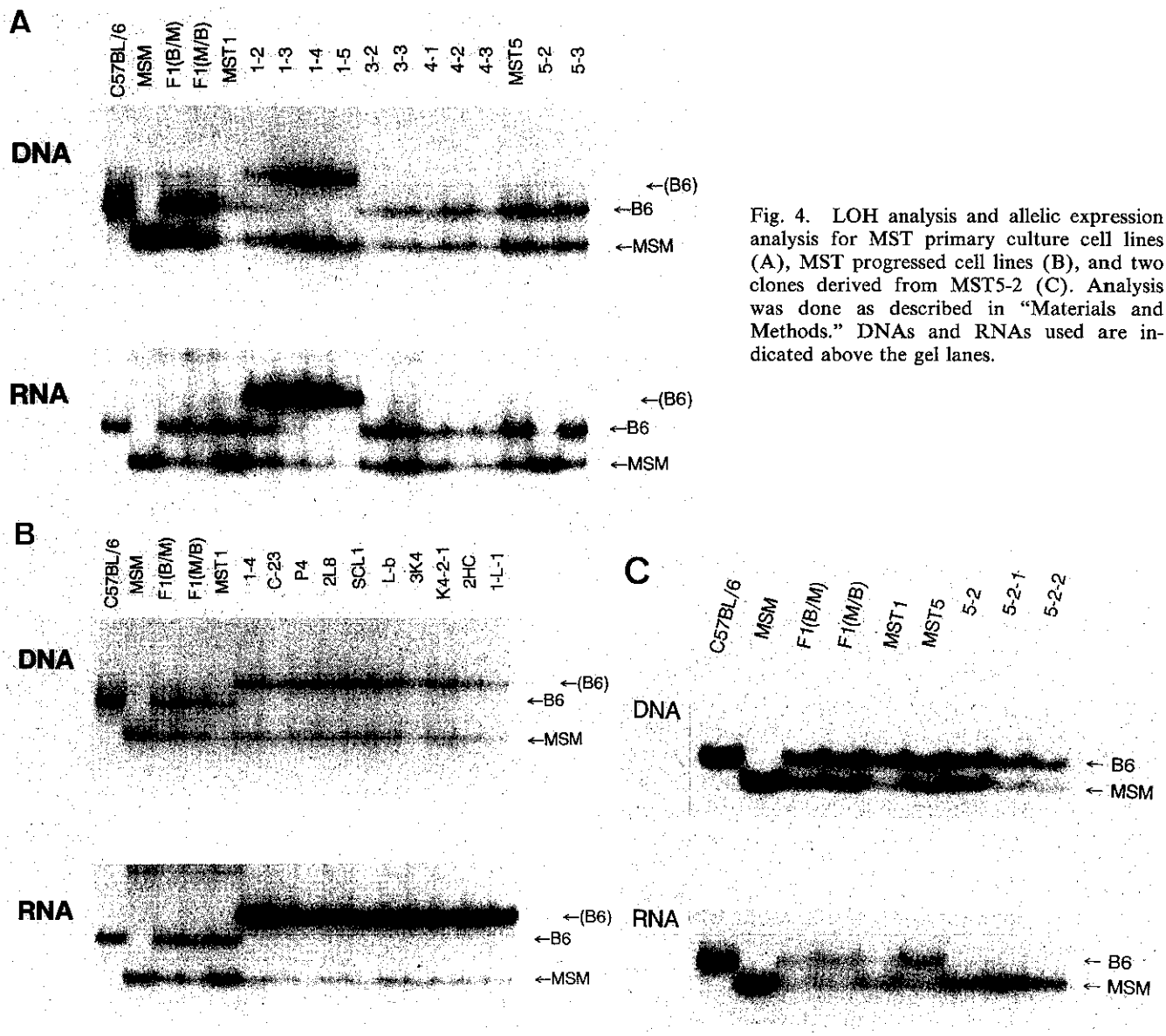

Fig. 4. LOH analysis and allelic expression analysis for MST primary culture cell lines (A), MST progressed cell lines (B), and two clones derived from MST5-2 (C). Analysis was done as described in "Materials and Methods." DNAs and RNAs used are indicated above the gel lanes.

band, and conversely samples MST1 and MST5-2 showed decreases in the B6-derived band. This suggested that there were cells in MST1 that expressed the p53 gene of the MSM allele much less than that of the B6 allele. Similarly, MST5 tumor contained some cells with decreased expression of the B6 allele. These results suggest that allele-specific expression of the p53 gene frequently occurs in the tumor cells.

We next examined a series of MST cell lines; one cell line, MST1-4, was subjected to repeated subcutaneous transplantation to obtain more malignant cell lines, designated as MST progression cell lines (Fig. 1, and see "Materials and Methods"). Some of the obtained lines acquired an ability to form metastatic nodules. DNA analysis of the cells revealed no allelic loss of the p53 gene, and RT-PCR showed maintenance of an allelic expression pattern similar to that in MST1-4 (Fig. 4B). This indicated that the expression ratio and possibly the expression level of the two alleles persisted in these cell lines through repeated transplantations. Since MST5-2 also showed a different expression of the two alleles, two *in vitro* clones were obtained from MST5-2 and examined (Fig. 4C). DNA analysis revealed an increase in the B6-derived band for both clones, suggesting imbalance in the copy number of the two alleles. However, RNA analysis showed a remarkable decrease in the B6 band. This expression pattern was similar to that of MST5-2.

In order to examine the generality of differential allelic expression, we investigated nine other genes as well: mdm2, E-cadherin, 72 kD metalloproteinase and its inhibitor (Timp2), thymidine kinase and four glucose transporter (Glut) genes. The nine genes were chosen from among sixteen genes examined which were considered likely to affect the malignant properties of tumor cells, because these nine exhibited polymorphisms in transcribed non-coding regions.

As regards mdm2 gene expression, no difference between alleles was detected for MST primary cell lines or MST progression cell lines (Fig. 5). Similar results were obtained for 72 kD metalloproteinase, Timp2, and Glut1 genes (data not shown). On the other hand, differences in allelic expression were observed for E-cadherin and Glut3 in MST primary cell lines (Figs. 6 and 7). MST1-2, MST1-3, MST4-2 and MST4-3 showed a preference of the expression of MSM-derived E-cadherin mRNA, and MST3-3 expressed more of MSM mRNA than

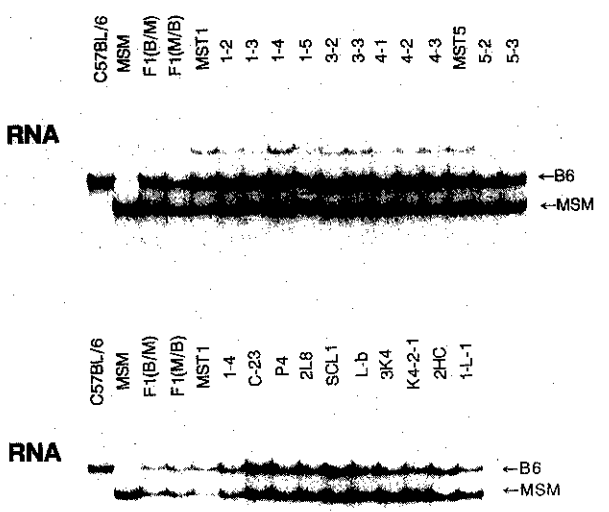

Fig. 5. RT-PCR-SSCP analysis of the mdm2 gene. RNAs were subjected to RT-PCR as described in "Materials and Methods." The products were analyzed by the SSCP method.

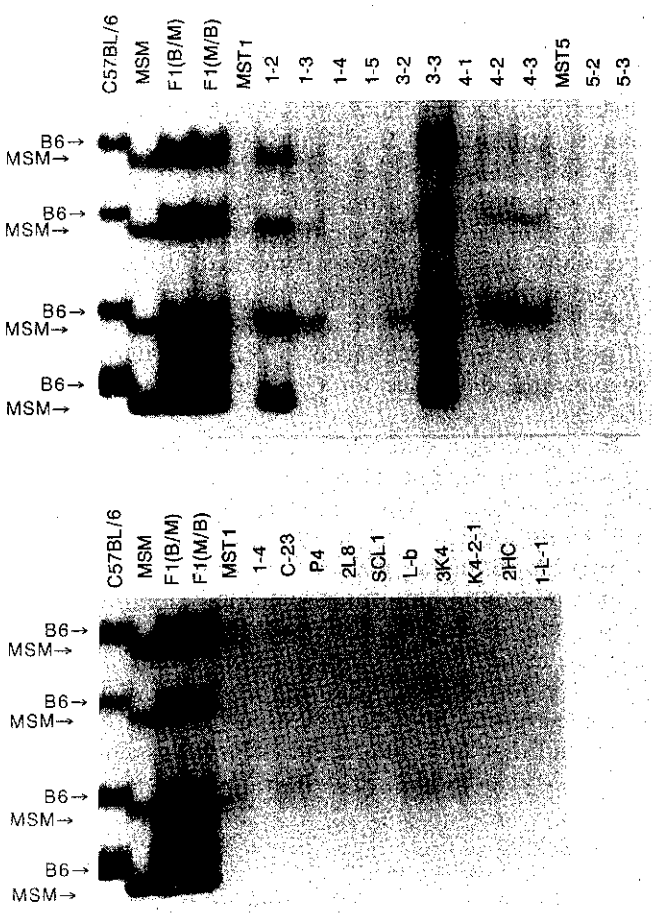

Fig. 6. RT-PCR-SSCP analysis of the E-cadherin gene. The analysis was carried out as described in "Materials and Methods." SSCP analysis exhibited four bands for B6 and MSM PCR products, probably due to their ability to form different conformers.

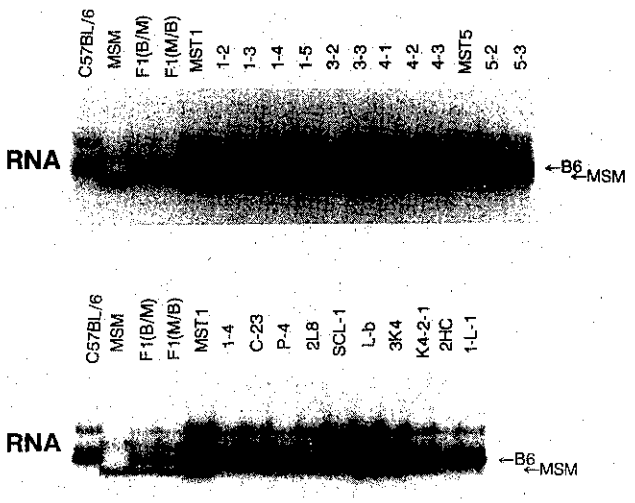

Fig. 7. RT-PCR-SSCP analysis of the Glut3 gene. The analysis was carried out as described in "Materials and Methods."

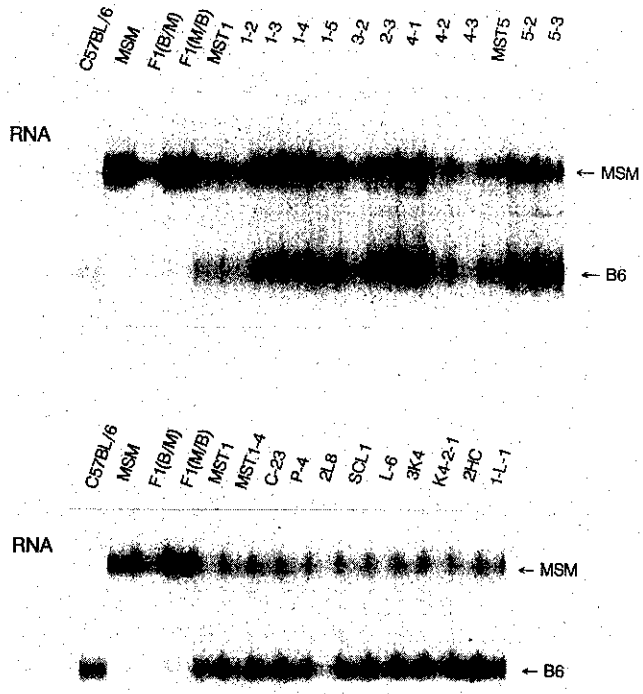

Fig. 8. RT-PCR-SSCP analysis of the thymidine kinase gene. The analysis was carried out as described in "Materials and Methods." The PCR products consistently showed broad bands in SSCP analysis.

B6 mRNA. As for Glut3, MST1-2, 1-4, 1-5 and MST5 showed expression of the B6 allele much more than that of the MSM, and MST3-2, 3-3, 4-1, 4-2 and 4-3 exhibited an inverse pattern of expression. The TK gene exhibited a unique change in expression. In two reciprocal F_1 mice, the MSM allele was expressed more than the B6 allele. However, the expression of the two alleles was similar in MST tumors and the cell lines (Fig. 8). Probably because of poor expression, we failed to detect enough RNA to evaluate the difference for Glut2 and Glut4 (data not shown).

In MST progression cell lines, allelic expression ratios of Glut3 and thymidine kinase persisted during transplantations (Figs. 7 and 8). The mRNA of E-cadherin was hardly detected in these cell lines (Fig. 6). DNA analysis of the nine genes gave B6 and MSM bands of equal intensity in both the primary and progression cell lines (data not shown). This indicated no allelic loss of these gene loci.

The results obtained here suggested that MST tumors had some genes, one allele of which was not expressed fully, and that such differential allelic expression tended to be fixed at an early stage of the MST tumor progression.

DISCUSSION

Loss or decrease of gene expression is frequently observed in human and animal tumors and may affect the properties of the tumor cells. The expression loss of a gene can occur by inactivation of both alleles through a variety of combinations of the following mechanisms: point mutations, deletions, a chromosome loss, and probably epigenetic modifications, such as DNA methylation and alteration in chromatin structure, that impair gene expression. Although allelic deletion has been extensively investigated by means of loss of heterozygosity (LOH) analysis in various types of tumors,[4, 23, 24] the possibility of allele-specific loss of expression has rarely been studied except for recent reports on imprinted genes.[6, 7] This can be ascribed in part to difficulty in detecting polymorphisms at the mRNA level. For the purpose of easy detection of such polymorphisms, we have newly developed primary fibrosarcomas and cell lines that are derived from inter-subspecific heterozygous mice between C57BL/6 and MSM. MSM is an inbred strain derived from Japanese wild mouse, *Mus musculus molossinus*.[8]

We have chosen ten genes for examination of allele-specific expression, p53, mdm2, E-cadherin, 72 kD metalloprotease (MMP) and the inhibitor (Timp2), thymidine kinase, and four glucose transporters, because they include a tumor suppressor gene and genes that are supposed to affect tumor growth, malignant progression or metastasis. p53 is a nuclear phosphoprotein encoded by a tumor suppressor gene that can inhibit the transformation of cultured cells by various oncogenes.[25] Loss of wild-type p53 is a major event in the formation of

tumors in humans[4] and rodents.[26] Hyperexpression of the mdm2 gene inhibits this p53 function through the binding of mdm2 proteins to normal p53 proteins.[27] E-cadherin is a cell adhesion molecule whose expression is lost during processes such as tumor progression and metastasis.[28,29] MMP and Timp2 are a protease and the inhibitor, respectively, affecting tumor invasiveness and metastasis.[30,31] Thymidine kinase and glucose transporter genes are involved in thymidine uptake and efficient glucose transport, respectively, and therefore high expression of the genes is expected to give a growth advantage to tumor cells.[32,33]

Of the ten genes examined, those for p53, E-cadherin and Glut3 showed differences in allelic expression. The differences varied in MST tumors and MST primary cell lines. E-cadherin showed an allelic preference, i.e., MSM allele was expressed more than B6 allele (Fig. 6), whereas no preference was observed for either p53 or Glut3 (Figs. 4 and 7). The thymidine kinase gene showed an allelic expression pattern in MST tumors and the cell lines different from that in a normal tissue (Fig. 8). These results suggest that tumor cells have genes with an allele specific change of expression. We also examined ten cell lines, designated as MST progression cell lines, that were obtained by repeated transplantations (Fig. 1). The allele-specific expression pattern of these three genes did not change in these lines (Figs. 4, 6 and 7), suggesting that the change is stable through transplantations. This implies that the change of expression tends to be fixed at an early stage of the tumor progression. It is also suggested that allele-specific expression may be a common event in tumor cells.

Loss or decrease in expression of one allele may be functionally equivalent to allelic loss as detected by means of LOH analysis; i.e., the allelic change may lead to total function-loss of a gene together with inactivation of the remaining allele. Therefore, alteration in allelic expression must be important for genes that are associated with tumor development and malignant progression. Change in the function of these genes would affect the properties of tumor cells. However, we have not demonstrated such cases yet. Further studies are required to verify involvement of loss or decrease of allelic expression in carcinogenesis and malignant progression.

The mechanism of alteration in allelic expression is not clear. Since the frequency was high, it is likely that loss or decrease of genes was caused by epigenetic alterations. Errors in modification processes such as DNA methylation and chromatin structure might occur in tumor cells, resulting in inactivation of genes. It is also possible that the change was due to DNA mutations that occurred in regions controlling gene expression or those affecting the mRNA stability. It is important to note that epigenetic or genetic changes responsible for the impaired expression should occur in *cis*-acting elements.

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