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Somatic Mutation during Metastasis of a Mouse Fibrosarcoma Line Detected by DNA Fingerprint Analysis

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Metastatic nodules were examined by DNA fingerprint analysis. The probes used, Pc-1 and Pc-2, detect mutations as shifts in bands of the minisatellite loci which are dispersed among chromosomes. Four clonal lines of a fibrosarcoma from an F_1 mouse (C57BL/Ka × C3H/He) were selected for various metastatic potentials upon inoculation into syngeneic mice. These four lines exhibited many extra bands resulting from recombination and/or DNA slippage, indicating accumulation of mutations during the successive passages in mice. One of the four, a 505 cell line which had been passaged extensively *in vitro* and consisted of a heterogenous population, was inoculated into thirteen syngeneic mice, and gave rise to six lung metastatic nodules in two mice. All the nodules showed band-patterns distinct from one another, although nodules within a given mouse tended to show similar patterns. When a genetically tagged 505-05-01 clone was analyzed, three of nine metastatic nodules obtained also revealed new bands. These results strongly suggest that somatic mutations occur at a high frequency during metastasis, providing direct evidence of genetic instability of the tumor cells.

Key words: Metastasis — Minisatellite — DNA fingerprinting — Tumor progression

Metastasis remains of great clinical significance. The process of metastasis involves a series of sequential steps.[1] It comprises growth of the primary neoplasm, local invasion, transport through lymphatic and/or blood vessels, attachment to the capillary bed, endothelial degradation, extravasation and proliferation. Initial non-invasive and non-metastatic tumors tend to go through malignant progression and become heterogeneous. Subsequently, a subpopulation(s) of cells appears with properties that enable them to give rise to metastasis.

The phenotypic diversity may be caused by the increased genetic instability of tumor cells. Treatments of tumor cells with mutagenic agents alter their phenotypes, resulting in appearance of clones with various tumorigenic potentials from non-tumorigenic to highly metastatic.[2–5] Since phenotypes of clones are heritable, tumor progression may occur as a result of genetic alterations. The high frequency of phenotypic changes can be accounted for by changes other than gene mutation, such as DNA methylation,[6] gene amplification, DNA rearrangement, and changes of chromosome composition.

We have previously isolated two probes for DNA fingerprinting which detect mutations due to homologous recombination and/or slippage during DNA replication.[7] DNA fingerprints represent multiple hypervariable restriction fragments of a large number of autosomal loci dispersed throughout mammalian genomes.[8,9] These provide a sensitive and effective assay for mutations and have been used to screen somatic DNA rearrangements in human tumors[10] and chemically induced murine tumors.[11] Using this method, we have examined tumor cells isolated from lung metastatic nodules. The results demonstrate that DNA rearrangements occur at a high frequency during tumor metastasis.

MATERIALS AND METHODS

Mice F_1 mice (BCF_1 mice) crossed between C3H/He females and C57BL/Ka males were used throughout the study. Mice were kept in plastic cages, in an air-conditioned room. They were given commercial pellets and tap water *ad libitum*.

Cells Three tumor lines and two *in vitro* tumor cell lines were mostly used in this experiment. The 505 tumor is a mouse fibrosarcoma line with low metastatic ability and was induced originally by methylcholanthrene (MCA) in BCF_1 mice.[12] 1153Pn and 1153Ln are two sublines of 505 tumor which exhibited high metastatic abilities via the bloodstream and lymphstream, respectively.[12] 505 cells are a culture line derived from 505 tumor and 505-05-01 cells are a clone of 505 cells. 505-05-01 cells were further transfected with 1 μg of pSV2neo plasmid by the method of Wigler *et al.*[13] and the resulting clones were described previously.[14] These cells were cultured 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. These cells retained the properties of the parental tumors during the period of study. The 505 and

505-05-01 cells are of fibrous morphology *in vitro* and produced a well-differentiated fibrosarcoma *in vivo* which metastasized spontaneously, mainly to the lung.

DNA extraction and Southern blotting High-molecular-weight cellular DNA was extracted as described previously[15] with some modifications. Tissue-cultured cells were lysed in a buffer containing 0.5% sodium dodecyl sulfate (SDS), 0.1 *M* NaCl, 1 m*M* EDTA, 10 m*M* Tris-HCl pH 7.5, and 100 μg/ml proteinase K. After incubation at 50°C overnight and phenol-chloroform extraction of the lysate, DNA was recovered by ethanol precipitation. Pulmonary metastatic nodules were asceptically cut off and minced with scissors. Tissue explants were cultured with Dulbecco's modified Eagles's medium supplemented with 15% fetal bovine serum and L-glutamate. When cells migrating and proliferating from tissue explants were near confluency, they were transferred to culture bottles and DNA was isolated later.

DNA was digested with restriction enzymes, subjected to electrophoresis through a 1.25% agarose gel and transferred to a nylon filter according to the method of Southern.[16] Prehybridization was done in 4×SSC, 1%SDS, 10 m*M* Tris-HCl pH 7.5, and 10 μg/ml yeast RNA at 60°C for 2 h. Filters were then hybridized in the prehybridization buffer containing ^{32}P-labeled Pc-1 or Pc-2 at 65°C for 16–24 h. Filters were washed in 1×SSC/1%SDS at 65°C for 30 min, and in 0.2×SSC/1%SDS at 65°C for 30 min to get DNA fingerprints. The locus-specific bands were detected by further washing of the filter in 0.05×SSC/1%SDS at 65°C for 30 min.

Minisatellite probes Two minisatellite probes used in this study, Pc-1 and Pc-2, are locus-specific polymorphic clones isolated from the mouse genome by hybridization to a mo-1 minisatellite clone.[7] They each contain a repeated sequence, GGGCA and AGGCAGG, respectively. Sau3A inserts of the two clones were subcloned into the *Bam*HI site of pUC19 and used as probes for DNA fingerprint analysis.

Spontaneous metastasis BCF_1 mice were inoculated subcutaneously at the back with 2×10^6 cells.[14] The genetically tagged 505-05-01 cells were mixed (1×10^5 cells each) and used for inoculation. The mice were killed 4 to 6 weeks after the inoculation and the lungs were examined for spontaneous metastases of the cells.

Cloning of cells 505 cells were distributed into 96-well flat-bottomed microtiter plates at an average concentration of 0.75 cell/well. Each clone was expanded *in vitro* and subjected to DNA extraction.

RESULTS

Changes in minisatellite sequences among 505 tumor lines A 505 tumor induced by methylcholanthrene in F_1 mice between C57BL/Ka and C3H/He exhibited a low metastatic ability. 1153Pn and 1153Ln were established from 505 tumor cells by successive transplantations of pulmonary and lymphatic nodules, respectively. They showed high and tissue-specific metastasis.[12] In order to determine whether mutations accumulated during serial transplantations, DNAs were isolated from these tumors, digested with *Hae*III, and subjected to DNA fingerprinting with Pc-1 and Pc-2 minisatellite probes.

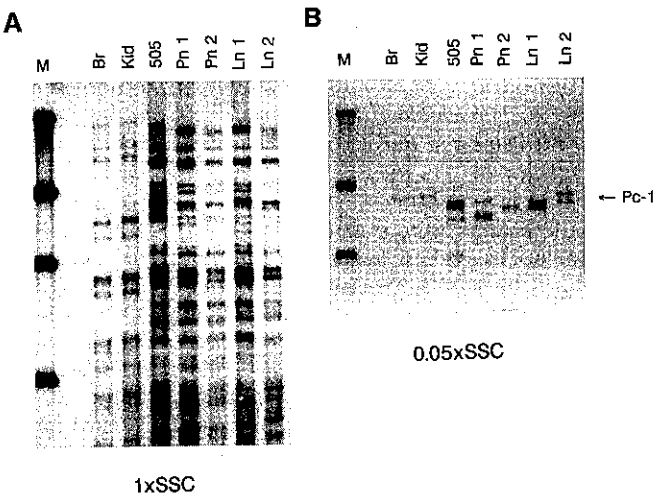

Fig. 1. DNA fingerprints (A) and the Pc-1 locus (B) of normal tissues and metastatic tumors. DNA was extracted from tissues and tumors, digested with *Hae*III and subjected to DNA fingerprint analysis using the Pc-2 minisatellite as a probe. Washing was performed at 65°C in 1×SSC (A). The same filter was rehybridized to Pc-1 and washed at 65°C in 0.05×SSC (B). Br and Kid: brain and kidney of an F_1 mouse (C3H/He×C57BL/Ka), 505: 505 tumor, Pn1 and Pn2: 1153Pn tumors at the 23rd and 43rd transplantation, respectively. Ln1 and Ln2: 1153Ln tumors at the 30th and 58th transplantation, respectively. M indicates the position of the size markers; 23.1 kb, 9.4 kb, 6.6 kb and 4.4 kb from top to bottom.

Fig. 1A shows the fingerprint patterns of normal tissues, 505 cells, 1153Pn (23rd and 43rd transplantations) and 1153Ln (30th and 58th transplantations) using Pc-2 probe. All tumors showed more bands than the normal tissues did, which partly reflected polypoidy of the tumor cells. Our preliminary karyotype analysis revealed that 505 tumor cells mainly consisted of triploid cells (data not shown). Many extra bands observed in the tumor cells were not present in normal tissues. This indicates that some of the fragments which shared homology to the Pc-2 repeat unit sequence, AGGCAGG, underwent recombinational mutation. The same filter was rehybridized to Pc-1 and washed under a more stringent condition (Fig. 1B). The Pc-1 locus was also subject to mutation in 505, 1153Pn and 1153Ln tumors.

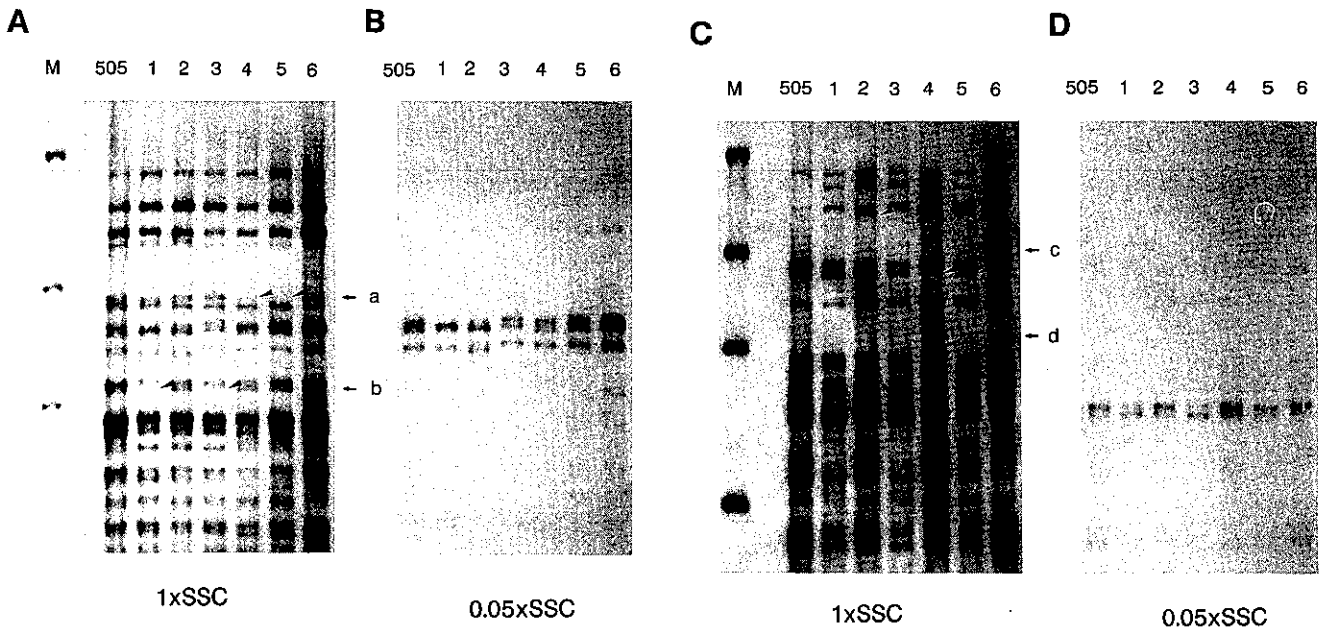

Fig. 2. DNA fingerprints and the Pc-1 and Pc-2 loci of 505 cells and lung metastatic nodules. Metastatic nodules were surgically removed, minced with scissors and cultured for a short period of time. DNA was purified from the cells, digested with *Hin*fI and subjected to DNA fingerprinting. Lanes 1–2 and 3–6 were lung metastatic nodules derived from different mice. Panels A and B show the results of Pc-1 probe and panels C and D of Pc-2 probe. Arrowheads and arrows indicate the bands shifted.

These results indicate that DNA rearrangements occurred at multiple minisatellite loci during the metastasis of the tumor cells.

Changes of minisatellite sequences in cells from metastatic nodules We next tested whether or not somatic changes occur in a single metastatic process. *In vitro*-adapted 505 cells were inoculated subcutaneously into the back of mice. Mice were killed 28 days after the inoculation. Two out of thirteen mice injected gave two and four lung metastatic nodules. The nodules were isolated and cultured for a short period of time to remove contaminating lymphocytes and fibroblasts. DNA was extracted and examined by DNA fingerprinting (Fig. 2). Every nodule examined showed a different band-pattern from that of the parental 505 cell line; they had gained and/or lost a band(s) (indicated by arrowheads in Fig. 2A and 2C). The Pc-1 locus of cells from nodules comprised two or three bands (Fig. 2B). The band-patterns of nodules were similar among nodules in one mouse but distinct from those of the other mouse. The Pc-2 locus, however, exhibited different band-patterns even within the same mouse (Fig. 2D). The DNA fingerprint patterns of Pc-1 and Pc-2 were also distinct among nodules; bands a and b (indicated by arrows a and b in Fig. 2A) were absent in lanes 4 and 5, and lanes 1 and 3, respectively. Two additional bands c and d (marked by arrows in Fig. 2C) were found in a nodule shown in lane 6.

These findings can be interpreted as follows; a subpopulation of the 505 cell line which carried different band-patterns of the Pc-1 locus developed or pre-existed in the population. Within this population, subclones appeared which had lost band a or gained band b. One clone in a nodule further underwent mutations giving rise to bands c and d. These events seem to have occurred during metastasis, since it is believed that a single clone usually dominates metastatic nodules within a mouse.[14, 17] These is, however, another possibility, i.e., that the 505 cell line had undergone mutations before inoculation and was already genetically heterogeneous in the population. The present result may represent only a selection of the cells with high metastatic potential.

Clonal difference in minisatellite patterns In order to determine whether the 505 cell line is genetically heterogeneous, the cells were cloned and analyzed by DNA fingerprinting. Of the nine clones thus examined, five differed in band-pattern from each other (lanes 1, 2, 3, 6 and 8 in Fig. 3A). The result implies that somatic rearrangements had occurred in 505 tumor cells probably during transplantations and/or during maintenance in culture, and that genetic heterogeneity had existed in the

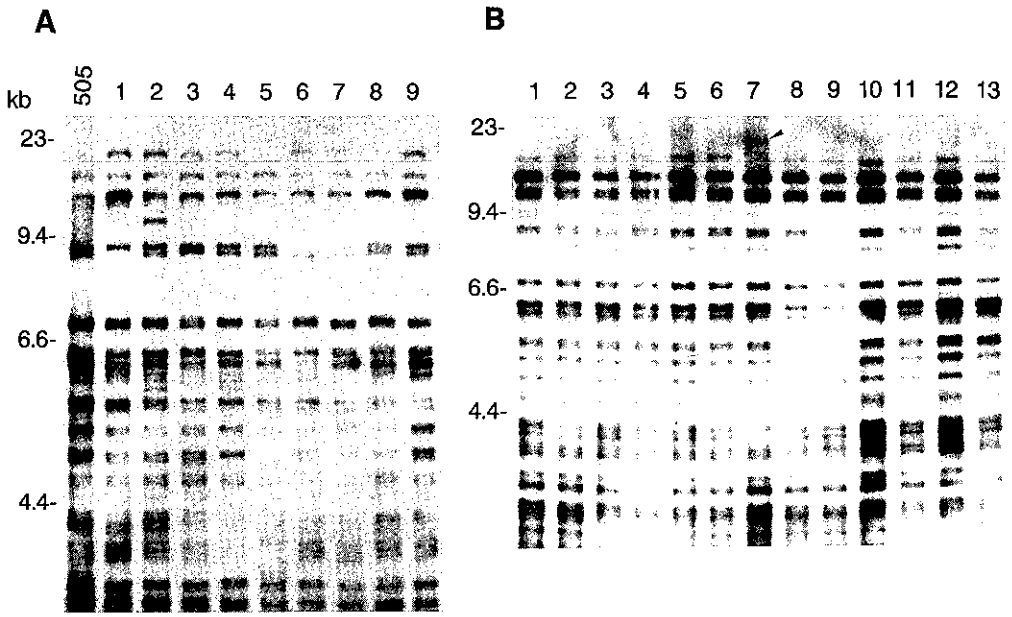

Fig. 3. Genetic heterogeneity of 505 cells. 505 cells were cloned to give rise to nine clones (A). One clone (505-1) was further cloned to yield thirteen subclones (B). DNA was extracted from these clones, digested with *Hae*III (A) or *Hin*fI (B) and probed with Pc-1. Washing was performed in 1×SSC at 65°C. An arrowhead indicates a band not shared by any other clone *in vitro*.

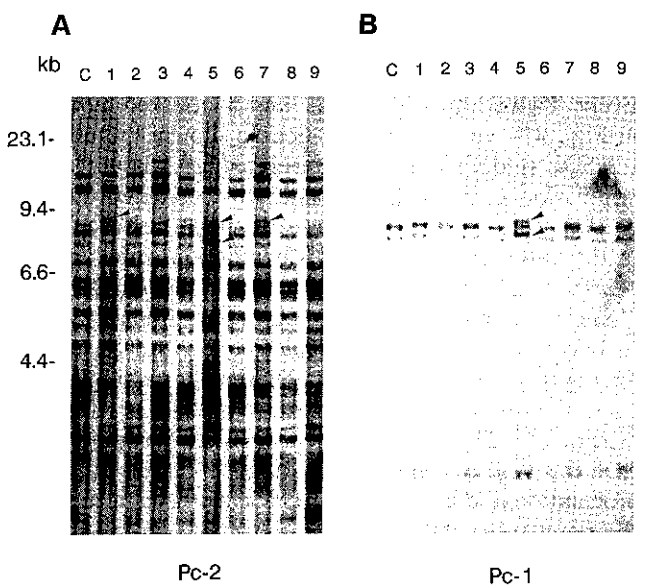

Fig. 4. Mutations in metastatic nodules of genetically tagged clones of 505-05-01 cells. Genetically tagged clones with pSV2neo were mixed and inoculated subcutaneously into the back of BCF_1 mice, yielding nine nodules. DNA was extracted, digested with *Hae*III and subjected to Southern analysis. A: the filter hybridized to Pc-2 was washed in 1×SSC/1%SDS at 65°C. B: the same filter rehybridized to Pc-1 was washed in 0.2×SSC/1%SDS at 65°C. An arrowhead indicates new mutations in the nodules.

population before inoculation. One clone (clone 505-1) was further recloned to give rise to thirteen subclones, the patterns of which were identical except for one (indicated by an arrowhead in Fig. 3B). One may note that the band-pattern in Fig. 3B differs from that of Fig. 2A, although the enzyme and probe used are the same. The difference is probably caused by the different length of the probe used. Nevertheless, the result indicates that the frequency of mutation at the minisatellite loci is unusually high in 505 cells.

In order to address the question of whether new mutation was induced during metastasis, we analyzed 505-05-01 clones genetically tagged with pSV2neo. These clones can be distinguished by the specificity of the integration pattern of the plasmid. A mixture of ten such clones was inoculated subcutaneously into the back of mice. Nine lung metastatic nodules were isolated from three mice and cultured in a medium containing G418. A previous experiment had demonstrated that one clone, no. 11, dominated in this type of mixed inoculation.[14] The band-pattern of the nodules was similar to that of the original clone 11 (Fig. 4A and 4B). However, three nodules (two of which gave the same result) showed extra bands (Fig. 4A) and one of them exhibited mutations at the Pc-1 locus as well (marked by arrowheads in Fig. 4B). The result implies that DNA rearrangements frequently occur in the process of cancer metastasis, including *in vitro* growth of the tumor cells.

DISCUSSION

DNA fingerprint analysis provides a useful approach for studying somatic changes and detects mutations in minisatellite loci.[18] We have used two minisatellite clones that show polymorphisms among mouse strains and even in BALB/c sublines.[7] The 505 tumor cells used in this study originated in an F_1 mouse between C57BL/Ka and C3H/He, and carry two distinct alleles at many minisatellite loci. Therefore, disappearance of bands in DNA fingerprints can be detected more efficiently in these tumor cells than in those of homozygous mice. Changes in DNA fingerprints could result from a number of processes: for example, loss of chromosomes or chromosomal regions through deletion, mitotic nondisjunction, and mitotic recombination. In the present study this method was used for studying mutations that occur during cancer metastasis.

We have observed mutations due to recombination in metastatic nodules at a high frequency. Metastasis consists of sequential steps involving multiple host-tumor interactions (see introduction). Genetic instability in tumor cells may be responsible for the progression of tumor cells through each step. Tumor cells become genetically heterogeneous and a subpopulation endowed with certain properties is selected to metastasize. However, mutations indicating the genetic instability have not been detected at the DNA level. Using DNA fingerprint analysis, we have now shown the existence of novel mutations in cells from metastatic nodules, arising from unequal homologous recombination and/or from DNA slippage during replication.

An enhanced level of recombination as revealed in the present study could result in activation of oncogenes and/or impairment of tumor suppressor genes, leading to tumor progression. Since telomeres and centromeres also consist of tandem repeat sequences as in the case of minisatellites, it is probable that mutations of this type also occur at these regions. Their dysfunction could cause abnormality in behavior of chromosomes including telomeric fusion and nondisjunction of chromosome partition, which are frequently observed in a number of clinical and experimental tumors. Synthetic probes which hybridize to either telomeres or centromeres have been prepared and used for the analysis. Our preliminary experiments have demonstrated frequent occurrence of such mutations during metastasis (Takada *et al.*, unpublished).

It is not clear, however, whether or not these changes in DNA fingerprints are directly related to the tumor progression. Even though such somatic changes are not relevant to phenotypic changes of the tumor, they could at least provide markers for studying tumor clonality. Analyses of tumors tagged with retrovirus vectors[19] and with plasmid DNA,[13, 17, 20] demonstrated a remarkable conversion of heterogeneous tumors toward clonal homogeneity. It may then be concluded that metastatic nodules in an animal are dominated by a single or a few clones. Such experiments, however, have a disadvantage intrinsic to gene transfer studies, i.e., the elements of the transfection and subsequent selection procedures themselves may cause phenotypic alterations. It was indeed demonstrated that calcium phosphate-mediated transfection itself was frequently associated with the appearance of variants with altered aggressiveness.[21]

A DNA fingerprint analysis exploits endogenous sequences of minisatellites as a marker so that phenotypes of tumor cells are not interfered with.[22] Our present results showed that most metastatic nodules within a mouse were derived not from a single clone but from several distinct clones (Fig. 2). This suggests the polyclonal origin of metastatic tumors. On the other hand, the inoculation of a mixture of genetically tagged 505-05-01 clones yielded nodules originating from a single clone, no. 11 (Fig. 4).[14] These results indicate that the size of the metastatic subpopulation determined with minisatellite probes is larger than that provided by experiments using DNA-tagged tumor cells. It is of clinical importance to estimate the effective size of the metastatic subpopulation, because a prognostic assay based on sampling a small part of the primary tumor would be inaccurate, if the aggressive subpopulation was a very small portion of the total number of tumor cells being sampled. The present study provides evidence that the metastatic subpopulation represents a relatively large proportion of the total number of tumor cells. This is consistent with clinical studies indicating that the general progressive propensity measured in a small sample of the primary tumor can be correlated with clinical parameters of metastasis and recurrence.

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