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Cloning of Differentially Expressed Genes in Highly and Low Metastatic Rat Osteosarcomas by a Modified cDNA-AFLP Method

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To identify differentially expressed genes between highly and low metastatic rat transplantable osteosarcomas, we applied a modified AFLP (amplified fragment length polymorphisms) method for cDNA subtraction. The specific point of our modification is selective amplification using suppression PCR technique after restriction enzyme cutting. Our cDNA-AFLP gave high reproducibility (about 95%) in mRNA patterns and enabled us to clone four dominantly expressed genes in a highly metastatic tumor line. Three showed homology with known genes, encoding Ki-67, a proliferation-associated effective marker of malignancy, type IV collagen alpha-3, a major component of basement membrane, and KIAA77 for which the function is unknown. Although one fragment showed no database homology, we revealed a derivation from the rat homologue of the *Drosophila melanogaster* diaphanous gene (Dia) by cloning of longer cDNA. Dia genes, known to affect actin filament formation, are downstream effectors of Rho small GTPase. The results suggest that alterations in the expression of cytoskeletal protein, basement membrane elements, and proliferative markers may be important for metastasis of osteosarcomas.

Key Words: **metastasis; subtraction; cDNA-AFLP; osteosarcomas; metastasis.**

Metastasis consists of multiple steps including invasion, transportation and proliferation in target organs (1). Metastasis associated molecules, like extracellular matrix proteinases (2, 3) and their inhibitors (4) have been identified for some time, but we are still far from understanding the complex mechanisms operating in metastasis. The lack of sufficient predictive markers is one reason for the continued poor prognosis of many neoplasms. We previously established a spontaneously derived transplantable osteosarcoma (SOS) and a highly lung metastatic variant (S-SLM) (5–8) as an animal model in osteosarcoma. Despite a more than 100-fold increase in the metastatic potential of S-SLM to lung, the common origin and lack of any major differences in histopathological morphology and doubling time in host animals between SOS and S-SLM are major advantages of this model (7). Using these tumor lines, we previously reported NDP kinase/nm23 was upregulated in S-SLM (7), p53 tumor suppressor gene was intact in both tumor lines (9) and telomerase activity did not correlate with metastatic potential (10).

In the present study, we performed subtractive cDNA cloning for the two transplantable osteosarcomas using the cDNA-AFLP (amplified fragment length polymorphisms) approach. For this purpose, we employed a genomic AFLP method that was originally developed to isolate genomic markers in plant genetics (11). To our knowledge, this is the first report of gene cloning of possible metastasis associated genes in osteosarcomas.

MATERIALS AND METHODS

Tumor materials. Total RNAs were extracted from transplantable subcutaneous tumors by the acid guanidine phenol chloroform method (12) using ISOGEN (Nippon Gene, Tokyo, Japan), five weeks

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Abbreviations used: PCR, polymerase chain reaction; cDNA-AFLP, cDNA-amplification fragment length polymorphisms; SOS, osteosarcoma-spontaneous origin; S-SLM, selected lung metastasis lesion.

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after transplantation. Poly(A) tailed RNA was isolated with Oligotex dT super 30 (Takara, Kyoto, Japan), and used as the material for cDNA synthesis. Detailed information on the general nature of SOS and S-SLM are available in our previous reports (5–8).

The strategy of the modified cDNA-AFLP method using suppression PCR. The AFLP method was originally developed to obtain polymorphic genomic markers for the study of plant genetics (11). The basic strategy of our cDNA-AFLP method is shown in Fig. 1. Our method mainly consists of two PCR steps. The first step is suppression PCR with overhung primers and the second is nested PCR with selective primers having selective nucleotides at their 3′ end. After two amplification steps, electrophoresis on sequencing gels, exposure of the dried gels to films, excision of differentially expressed bands and their cloning were performed.

In brief, double stranded cDNAs were synthesized from 1 μg of each mRNA. cDNAs were digested by *Eco*RI and *Taq*I (Takara, Kyoto, Japan) under suitable conditions following the manufacturer's recommendation. After ethanol precipitation, digested fragments were ligated with 6 μg of specific adaptors at each end in a volume of 30 μl at 16°C by T4 ligase (Takara, Kyoto, Japan). E-A1, 5′-CTCGTAGACTGCGTACC-3′ and E-A2, 5′-AATTGGTACGCAGTCTAC-3′ were used as the EcoRI adaptors, T-A1, 5′-GACGATGAGTCCTGAG-3′ and T-A2, 5′-CGCTCAGGACTCAT-3′ as the *Taq*I adaptor. Three kinds of cDNA fragments would be expected after ligation of the two kinds of adaptors, *Eco*RI–*Eco*RI (EE), *Eco*RI–*Taq*I (ET) and *Taq*I–*Taq*I (TT). Since EcoRI recognizes 6 base pairs and *Taq*I recognizes 4 base pairs for cutting, the relative incidence of the three kinds of fragments is rare for EE, moderate for ET, and abundant for TT. We selectively amplified ET fragments with overhung primers (primers that anneal with template in the form of overhanging 5′ end: see Fig. 1). While the ET fragments can be exponentially amplified, this is not the case for EE or TT fragments because they dominantly form pan-like structures (self-annealing) at the annealing step of the PCR cycle (suppression PCR) with inverted sequences at the ends (13) (see Fig. 1). Sequences of overhung PCR primers were as follows: TAQ-1, 5′-GATCGACGATGAGTCC-3′ and Eco-1, 5′-GCTCGTAGACTGCGTA-3′. The conditions for suppressive PCR were according to Siebert *et al.* (13).

For the second selective PCR step, first PCR products were diluted five fold with distilled water and amplified with nested PCR primers with selective nucleotides at their 3′ end. Although the number of selective nucleotides was changed in the primary experiment, the basic sequences of primers with selective nucleotides were as follows: TDS, 5′-GATGAGTCCTGAGCGAMN-3′ and EMS, 5′-GACTGCGTACCAATTCX-3′ (MN and X are A, T, C, or G). PCR conditions were as follows: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 200 μM dNTPs, 1 μM each primers, 1.5 mM $MgCl_2$, 1.25 units *Taq* polymerase in a volume of 25 μl. Cycling conditions for the second PCR were: 94°C 2 min, 65°C 1 min, 72°C 1 min for five times in the precycle, then the annealing temperature was decreased to 60°C for 30 cycles in the main amplification with a thermal cycler (Perkin–Elmer). The hot start technique was performed at the start of PCR so that products were labeled by [^{32}P]dCTP.

For the electrophoresis step, labeled PCR products were denatured in formamide dye at 100°C and run on a 6 M urea 6% polyacrylamide gel at 65 W until xylene cyanol reached about 5 cm above the bottom of the 45-cm gel. After drying without fixation, cDNA-AFLP patterns were detected by exposure to Kodak X-Ray Films at −50°C overnight.

Bands dominantly expressed in either the highly or the low metastatic tumor were excised, eluted in 100 μl of distilled water, then reamplified with the same nested primers and cloned into the *Eco*RV site of the pBlueScript SK+ plasmid by TA cloning (14). To prevent biased subcloning, we picked up five independent clones and sequenced them. Northern blotting was performed to confirm differential expression between SOS and S-SLM. The cloned cDNAs were ^{32}P labeled by a random hexamer method, and used as probes. Two hundred nanograms of Poly(A) tailed RNA or 5 μg of total RNA derived from SOS and S-SLM was run on formalin denaturing gels and transferred to Biodyne B nylon membranes (Pole). A human glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe (Clontech) was employed as the loading control for Northern blot analysis. Radioactivity and intensity of signals were measured with the BAS2000 imaging system (Fuji).

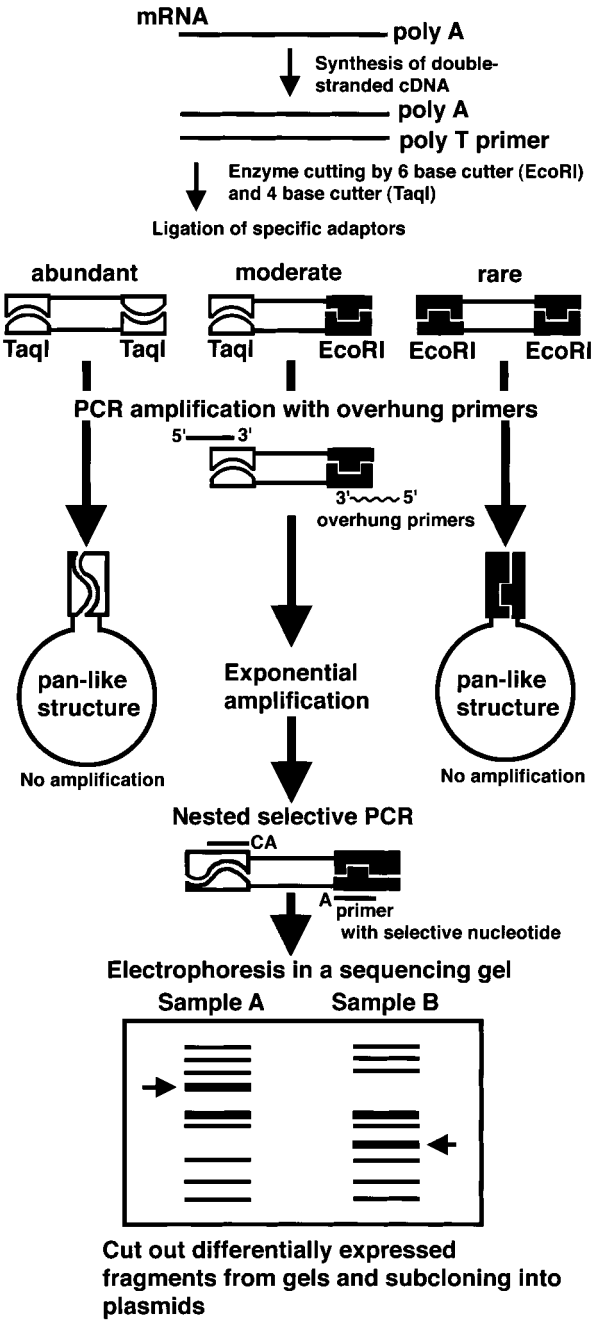

FIG. 1. A flow chart of the cDNA-AFLP method using suppression PCR. White and black adaptors can ligate with *Taq*I and *Eco*RI sites in cDNA fragments, respectively. Note that the adaptors at the ends are elongated after the amplification with overhung primers. With the elongated adaptors, fragments that have same adaptors at both ends prefer to form a pan-like structure (self-annealing) than annealing with primers. The T_m value for the self annealing is higher than that for annealing between adaptor and primer.

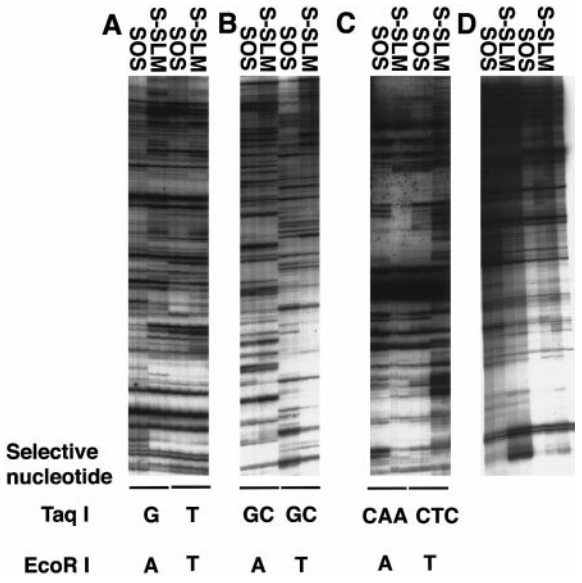

FIG. 2. cDNA-AFLP patterns obtained with different selective nucleotide *Taq*I primers. Results obtained with one selective nucleotide (A), two selective nucleotides (B), three selective nucleotides (C) are shown. (D) The mRNA patterns for the same samples by the traditional differential display method using arbitrary 10 mer and poly(A) primers are represented. In all mRNA patterns, two amplified products obtained from independent PCRs were loaded to confirm the reproducibility. The "best" pattern was obtained with primers with two selective nucleotides.

For sequencing of obtained clones, a cycle sequencing reaction system (Perkin–Elmer) and Genetic Analyzer 310 (Perkin–Elmer) were used. BLAST and FASTA homology searches were performed with the nucleotide information. For the unknown clone, longer cDNAs were obtained using the Marathon cDNA amplification system (Clontech) based on rapid amplification of cDNA ends (RACE) with long distance PCR or the ZAP–cDNA synthesis system (Stratagene).

RESULTS

Optimization of number of selective nucleotides for a stable cDNA-AFLP pattern. For successful subtraction, we first changed and optimized the number of selective nucleotides in the *Taq*I primer (Fig. 2). The most clear and reproducible pattern was obtained when nested primers with two base selective nucleotides were used (Fig. 2B). Although more bands were obtained with primers for one base selective nucleotide, bands were fainter, and more difficult to identify and cut out (Fig. 2A). Primers with three base selective nucleotides showed prominent bands, but numbers were much fewer and a reduction in reproducibility was noted (Fig. 2C). Although the number of selective nucleotides in the EcoRI primer was changed, the intensity and number of bands detected per sample was not dramatically altered compared to the *Taq*I primer case (data not shown). From these data, we conclude that one base selective nucleotide for the *Eco*RI primer and two base selective nucleotides for the *Taq*I primer produce the best results with our method. Furthermore, comparison with traditional differential display (DD) method using the same materials demonstrated easier distinction of bands with our cDNA-AFLP (Fig. 2D).

Cloning of differentially expressed genes in two transplantable osteosarcomas. cDNA-AFLP analysis of SOS and S-SLM was performed using 16 kinds of *Taq*I and 4 kinds of *Eco*RI selective nested primers, making a total of 64 combinations. Clear patterns were obtained in all cases, with a high reproducibility of 95% (number of reproducible bands, 2747 and number of counted bands, 2885) between independent PCRs. About 40 to 50 clear bands were detected in one examination. Therefore, in total, we screened approximately 3200 bands in this experiment.

Comparison of cDNA-AFLP patterns revealed 43 different cDNA fragments between SOS and S-SLM. These bands were excised from gels and cloned into the plasmid. Northern blot analysis demonstrated 13 of the 43 fragments to show differences in expression compatible with the cDNA-AFLP patterns (two fragments were preferred to expressed in SOS, and the others showed dominant expression in S-SLM). We picked out, and further analyzed four fragments that exhibited especially prominent differences, with greater expression in the high metastatic tumor line, S-SLM (Fig. 3).

Sequencing and homology with the BLAST and FASTA programs in the Gene bank database (http://www.genome.ad.jp/) revealed homology of clone 27

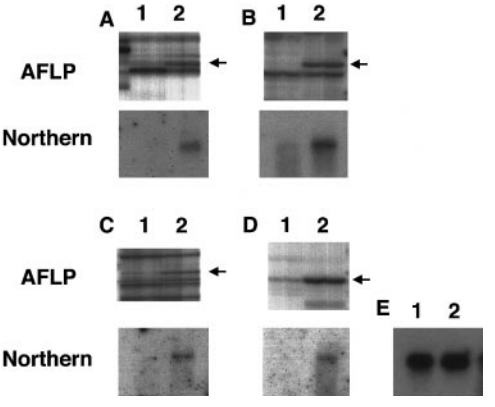

FIG. 3. Representative cDNA-AFLP patterns and Northern blots using differentially expressed fragments between highly and low metastatic rat osteosarcomas (SOS and S-SLM). Results for clone 16 (no homology) (A), clone 27 (KIAA77) (B), clone 29 (Ki-67) (C) and clone 31 (Type IV collagen α3) (D) are shown. For the cDNA-AFLP patterns, two independent PCR products were loaded to confirm the reproducibility. In all panels, lanes 1 and 2 represent the expression in the low metastatic (SOS) and in the highly metastatic line (S-SLM), respectively. Note that all genes were more strongly expressed in S-SLM than in SOS. Northern blot analysis with a GAPDH probe is also shown for comparison in E. Two hundred nanograms of each mRNA was run and transferred.

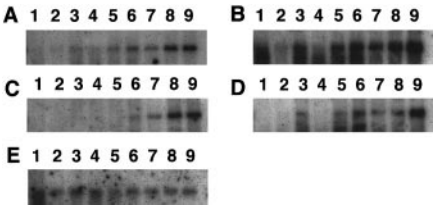

FIG. 4. Northern blot analysis in multiple tumor tissues with cloned fragments by the cDNA-AFLP method. Expression levels for clone 16 (unknown), clone 27 (KIAA77) (A), clone 29 (Ki-67) (B), clone 31 (Type IV collagen α3) (C) and GAPDH (D) are shown. Five micrograms of total RNAs from SOS (lanes 1–4), S-SLM (lanes 5–7), and lung metastatic nodules of S-SLM (lanes 8 and 9) was examined.

with the human KIAA77 gene cloned by Nomura *et al.* (90% identity in 134 bp). Clone 29 showed high homology with mouse Ki-67 (79% identity in 125 bp), and the clone 31 sequence matched type IV collagen alpha-3 in man (87% identity in 103 bp). Clone 16 had no match with known genes. The observed sizes of the known genes on Northern blotting were in good agreement with previously reports (KIAA77: more than 7 kb, Ki-67: 9.5 kb, type IV collagen alpha-3: 10 kb).

We performed Northern blot analysis of tumor tissues from a number of animals using the above four fragments as probes to confirm differential expression. Four subcutaneous SOS tumors, three S-SLM tumors and two S-SLM lung metastatic lesions were examined. All cloned genes were confirmed to be more strongly expressed in S-SLM and its lung metastatic lesions compared with SOS (Figs. 4A–4C). The control, human GAPDH probe showed no difference (Fig. 4E). Quantitative analysis with the BAS 2000 system revealed values for these four genes in S-SLM about four to sixfold higher than that in SOS.

Cloning of longer cDNA fragments and identification of clone 16. Since clone 16 showed no homology in the database search using its sequence information, we obtained longer cDNA fragments from traditional library screening and 5′ or 3′-RACE reactions based on long distance PCR. From the RACE reaction, four independent 5′ upstream (520 bp) and three 3′ downstream fragments (about 4.0 kb) were obtained. From traditional library screening, two independent cDNA clone (3.0 and 1.5 kb) were isolated. Sequence information and a subsequent homology search of the long cDNA showed high homology with mouse Dia2, the p140mDia gene, human Dia-12c, and the Dia-156 gene. In particular, the sequence of clone 16 showed 94% identity to the mouse Dia2 gene (Fig. 5A). Although we could not obtain a full length of open reading frame, 94% identity was observed in the putative amino acid sequence (Fig. 5B). Thus, we concluded clone 16 is the rat homologue of the mouse Dia2 gene. Dia genes (*Drosophila melanogaster* diaphanous gene) have conservative FH1/FH2 domains and form a gene family. They were first identified in Drosophila, as a result of screening for male-sterility mutations (15). Dia genes are conserved through many organisms including the yeast, human, and mouse (16).

DISCUSSION

We have applied the cDNA-AFLP technique to rat osteosarcomas that show large difference in metastatic potential. For cDNA subtraction, the traditional differential display (DD) method has often been used. Since this DD method basically depends on PCRs between poly(T) and arbitrary primers, the 3′ non coding region is often amplified. Therefore, it is very difficult to identify the genes with the sequence information from DD fragments. In contrast, although cDNA-AFLP is somewhat more laborious, it allows us to obtain sequence information in the coding region with higher probability.

For the cDNA application of the AFLP method, the use of selectively synthesized cDNA (17), or cutting by single restriction enzyme have been reported (18) in plant genetics. Although these studies gave stable mRNA patterns, there were also several problems (e.g., the necessity for repeated cDNA synthesis or difficulty of band detection in the low molecular weight area). With our method, there is the limitation that the detection of bands depends on the presence of 6-bp recognition sites, but this method would be the new approach for cDNA-AFLP.

We previously reported elevated level of the mRNA for NDP kinase/nm23 in S-SLM (7). We could not isolate nm23 gene as up regulated in the present study, but this was to be expected because this gene has *Taq*I but no *Eco*RI sites within its cDNA sequence in rat. In this study, although we could only obtain dominantly expressed genes in S-SLM, the use of other pairs of restriction enzymes would allow isolation of dominantly expressed genes in low metastatic tumor.

Of the four differentially expressed genes between high and low metastatic rat osteosarcomas here, we identified type IV collagen alpha 3 which forms trimers *in vivo*, and is a major component of basement membrane. Related proteins such as laminin or type IV collagen have been reported to be decreased in invasive tumor cells because of the high expression of matrix proteinases (19). However, with regard to their mRNA expression, Nakagawa *et al.* reported high expression of type IV collagen and metalloproteinase-2 mRNAs detected by Northern blot analysis in lung squamous cell carcinomas (20). In addition, laminin and type IV collagen in serum are known to be helpful markers for metastasis in murine sarcomas (21, 22).

Human KIAA77 was cloned by Nomura *et al.* (23). Its mRNA, longer than 7 kb, is ubiquitously expressed in human tissue and comparatively highly in skeletal

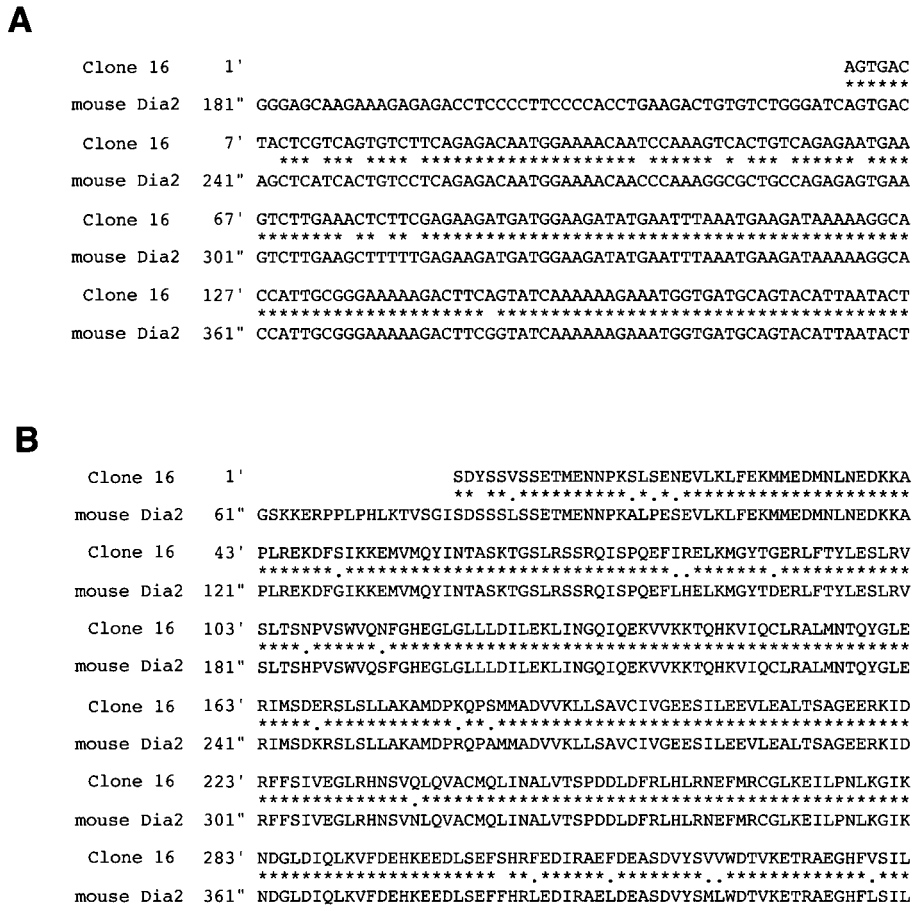

FIG. 5. Nucleotide (A) and predicted amino acid (B) sequence comparisons of the longer cDNA of clone 16 (upper sequence) and mouse Dia2 (lower sequence). Identical nucleotides or amino acids are indicated with asterisks.

muscle. A predicted hydrophobic region exists, but there is still no information on its biological functions.

Ki-67 is a proliferation-associated antigen which is used for immunohistochemical studies of tumors (24–26). Choong *et al.* reported the Ki-67 labeling index in malignant fibrous histiocytoma (MFH) to strongly correlate with metastasis in man (27). The present result suggests that it may have predictive potential for osteosarcomas.

The other gene corresponding to the Drosophila melanogaster diaphanous 2 (Dia2) have found to be dominantly expressed in S-SLM. Dia in *Drosophila,* formin in mouse, and Bni1p and Fus1 in yeast encode proteins that have a conserved polyproline region (FH1/FH2) (15). The protein has important roles in egg formation of *Drosophila,* in limb pattern formulation of the mouse, and in bud formulation and cell wall fusion during conjugation of yeast. In man, Dia is reported to be one of the genes responsible for premature ovarian failure (28). Recently, the Dia2 gene product was identified as a binding protein, an effector molecule for Rho small GTPase, in yeast two hybrid system (29). Furthermore, the mouse Dia protein, p140 Dia, is reported to be a target protein for Rho and to affect actin filament formulation and cell morphology (16). The signal pathway of Dia through small GTPase appears to play an important role in tumor invasion and actin mediated cell movement (16). Altered expression of Dia genes might have an influence on the cytoskeletal structure, and contribute to the metastasis of osteosarcomas.

In conclusion, the present study demonstrated that the cDNA-AFLP technique is a useful tool for molecular metastatic studies. Molecular analysis of other animal models should provide new insights into cancer metastasis (30).

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