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Isolation of a Novel Gene Showing Reduced Expression in Metastatic Colorectal Carcinoma Cell Lines and Carcinomas

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To investigate genes involved in metastatic stages of cancer, we analyzed expression of mRNAs in three cell lines derived from murine colon adenocarcinoma 26 by means of a differential display method. Each of these lines exhibits distinct metastatic characteristics. Among many bands representing different expression patterns in the display, we confirmed by northern analysis that a gene corresponding to one amplified fragment, termed *grm2* (gene related to metastasis 2), was expressed more abundantly in NL4, the derivative with the lowest metastatic potential, than in cell lines NL17, an experimentally metastatic derivative, and in NL22, a spontaneously metastatic derivative. Using the *grm2* fragment as a probe, we isolated murine cDNA clones and subsequently human cDNA clones corresponding to the *GRM2* gene. The human and mouse homologues both encode proteins of 600 amino-acid residues, which show weak homologies to proteins belonging to the myosin family. When we examined the expression levels of this novel gene in human colon cancers and in corresponding metastatic foci, we found that in more than half of these tissues, expression was significantly reduced in association with malignant potential. Our results imply that in humans the *GRM2* gene product may regulate the metastatic phenotype of some colorectal cancers.

Key words: Metastasis — Differential display — GRM2

Invasion of malignant cells from primary tumors and subsequent metastases critically influence mortality among cancer patients, but the genetic and biological mechanisms involved are still poorly understood. Metastasis is a complex series of events in which some genes function as stimulatory factors and others inhibit the process; presumably, an imbalance among several such genes induces metastasis.[1] Recently the *nm23* and *KAI1* genes were isolated as suppressors of metastasis[2,3]; an increase in the concentration of soluble CD44, on the other hand, seems to be associated with metastatic activity of gastric and colon cancers.[4] To investigate this multi-step process, it is useful to compare cancer cell lines that possess differing metastatic abilities. Three cell lines have been established from a murine colon adenocarcinoma (colon 26)[5,6]; one of them, NL4, shows a low potential for metastasis to the lung in recipient mice. On the other hand, cell lines NL17 and NL22 each produce a large number of tumors in the lung when inoculated intravenously, although the potency of NL22 to generate lung metastasis through intravenous inoculation is slightly lower than that of NL17. However, NL22 can form many metastatic foci in the lung after subcutaneous inoculation, whereas NL17 cannot. We chose these three cell lines for attempts to isolate genes related to metastasis by means of differential display. This method is designed to screen a defined subpopulation of transcripts through reverse transcriptase-polymerase chain reaction (RT-PCR) using arbitrarily selected primers, and to display the results as bands on a gel after electrophoretic separation of the amplified cDNAs.[7–11]

In the study reported here, using the differential display method we isolated a novel, possibly metastasis-related gene, *grm2*, and subsequently obtained its human homologue, *GRM2*. We also investigated the levels of *GRM2* mRNA expression in primary and metastatic colonic tumors from surgical patients. The results suggested that in tumor cells the *GRM2* gene may function as a negative regulator of progression.

MATERIALS AND METHODS

Cell lines Three cell lines with different metastatic abilities, NL4, NL17 and NL22, were established earlier

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from murine colon adenocarcinoma 26.[5,6] NL4 exhibits a low potential for metastasis to the lung in recipient mice. Cell lines NL17 and NL22 each produce a large number of tumors in the lung when inoculated intravenously, although the potency of NL22 to generate lung metastasis through intravenous inoculation is slightly lower than that of NL17. However, NL22 can form a large number of metastatic foci in the lung after subcutaneous inoculation and NL17 cannot.[5,6] We cultured all three cell lines in RPMI 1640 containing 10% fetal bovine serum and 2 m*M* glutamine.

Differential display Each preparation of total mRNAs (2.0 μg) from the three cell lines was mixed with 25 pmol of 3′-anchored oligo-dT primer ($GT_{15}MG$, $GT_{15}MA$, $GT_{15}MT$, $GT_{15}MC$, where M represents a mixture of G, A, and C) in 8 ml of diethylpyrocarbonate(DEPC)-treated water, and heated at 65°C for 5 min. To this solution were added 4 μl of 5×first-strand buffer (0.25 *M* Tris-HCl, pH 7.5, 0.375 *M* KCl, 0.05 *M* DTT, 0.015 *M* $MgCl_2$), 2 μl of 0.1 *M* DTT, 1 μl of 250 μ*M* dNTPs, 1 μl of ribonuclease inhibitor (40 units; Toyobo, Tokyo) and 1 μl of Superscript II reverse transcriptase (200 units; BRL, Gaithersburg, MD), to a final volume of 20 μl. This RT reaction mixture was incubated at 37°C for 1 h, diluted 2.5-fold by addition of 30 μl of DEPC-treated water, and stored at −20°C until use. The cDNAs were amplified by PCR in the presence of [α-^{35}S]dATP (10 mCi/μl; Amersham, Buckinghamshire, England), as follows: each 20-μl PCR mixture contained 2 μl of the RT reaction mixture, 2 μl of 10×PCR buffer (100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 500 m*M* KCl, 1 mg/ml gelatin, pH 8.5), 1.2 μl of 25 μ*M* dNTPs, 1 μl of [α-^{35}S]dATP, 0.25 μl of Taq DNA polymerase (5 units/μl; Boehringer Mannheim, Mannheim, Germany), 25 pmol of 3′-anchored oligo-dT primer, and 25 pmol of 5′-primer (10-mer deoxy-oligonucleotide primer with arbitrary sequences). Reactions were performed under the following conditions: one cycle of 3 min at 95°C, 5 min at 40°C, and 5 min at 72°C, then 40 cycles of 30 s at 95°C, 2 min at 40°C, and 1 min at 72°C, followed by 5 min at 72°C. Samples were precipitated with ethanol, resuspended in formamide sequencing dye, and electrophoresed on a 6% acrylamide/7.5 *M* urea sequencing gel. The gel was dried without fixation and autoradiographed overnight.

Cloning and DNA sequencing of amplified fragments The autoradiogram and the dried gel were oriented with radioactive ink, and cDNA bands showing different intensities among the three cell lines were located by marking with a pencil. Each selected gel slice, along with the 3 mm paper, was shaken in 300 μl of dH_2O for 1 h. After the polyacrylamide gel and paper were removed, cDNA was recovered by ethanol precipitation in the presence of 0.3 *M* NaOAC with 1 μl of 10 mg/ml glycogen as a carrier, and redissolved in 10 μl of dH_2O. For reamplification, 5 μl of the solution was used for PCR under the same conditions and with the same primers as used for the first PCR. The second PCR products were electrophoresed, dried without fixation, and subjected to overnight autoradiography. Amplified products of the appropriate sizes were recovered in the same manner as the first PCR products. The reamplified DNAs were cloned into the EcoRV site of pBluescript SK(−). Both strands were sequenced by the dideoxy chain-termination method, using T7 DNA polymerase.

Screening of cDNA A cDNA library of NL4 was constructed with oligo(dT) primer, and cloned in Uni-ZAPTMXR (Stratagene, La Jolla, CA). A total of 5×10^5 clones was screened with an [α-^{32}P]dCTP-labeled cDNA fragment isolated by differential display. Positive clones were selected and their insert DNAs were excised *in vivo* in pBluescript II SK(−) according to the supplier's recommendation. A human testis cDNA library was constructed using oligo(dt)-primed human testis cDNA and Uni-ZAPTMXR (Stratagene). Finally, 1×10^6 clones were screened with an [α-^{32}P]dCTP-labeled cDNA fragment that had been isolated by screening the murine NL4 cDNA library.

Tissue samples Specimens of 10 colorectal cancers that had metastasized (two to the lung and eight to the liver) and the adjacent normal mucosae were obtained from patients undergoing surgery. The materials were immediately snap-frozen in liquid nitrogen and stored at −80°C.

Northern blot analysis

Expression of transcripts in colon 26 cell lines: Samples of poly(A)-containing RNA (5.0 μg) from colon 26 derivative cell lines were analyzed by electrophoresis on 1% agarose gels containing 6% formalin. Separated RNAs were transferred onto charged-nylon filters. Prehybridization and hybridization were performed in a solution containing 50% formamide, 5×Denhardt solution, 6×SSC and 1% salmon sperm DNA. cDNA fragments that had been isolated in the differential display were labeled with [α-^{32}P]dCTP using the random oligonucleotide priming method. Filters were washed twice for 20 min in 0.1×saline sodium citrate/0.1% sodium dodecyl sulfate at 50°C. Radioactivity was measured with a BAS 1000 Bioimage Analyzer (Fujix, Tokyo).

Expression of transcripts in human tissues: Northern blot analyses were performed using Multi-tissue blots obtained from Clontech Labs. (Palo Alto, CA). Prehybridization, hybridization and probe labeling were performed in the same manner as in the northern analyses of the colon 26 cell lines. Almost full-length GRM2 cDNA was used as the probe.

Expression of transcripts in metastatic tumors: From each resected specimen frozen in liquid nitrogen, total RNA was prepared by using TRIZOL (BRL). Ten micrograms of isolated RNA was transferred onto nylon filters.

Almost full-length GRM2 cDNA labeled with ^{32}P was used as the probe. The filter was reprobed with glyceraldehyde 3-phosphate dehydrogenase to control for integrity and quantity of the RNA loaded.

Fluorescence *in situ* hybridization (FISH) We isolated a cosmid clone by colony hybridization of a human genomic cosmid library, using the [α-^{32}P]dCTP-labeled GRM2 cDNA as a probe. The cosmid clone was confirmed to contain the actual gene by Southern blot analysis. We applied direct R-banding FISH, a technique based on FISH combined with replicated prometaphase R-bands as described previously.[12-14] Provia 100 film (Fuji, ISO 100) was used for the microphotography (filter combination, Nikon B-2A).

RESULTS

Differential display and cloning of an amplified fragment To detect genes that might be involved in metastatic processes of cancer, we used the differential display method, using as templates mRNAs isolated from the three derivative murine cell lines, NL4, NL17 and NL22. By using 120 different primer sets, we identified a large number of bands that were likely to represent different levels of expression among the three cell lines. One band that was detected more intensely in NL4 than in the other two cell lines, as shown in Fig. 1A, was isolated, cloned, and sequenced. The cloned PCR product, designated *grm2*-1, consisted of 285 nucleotides. Differences in *grm2* expression among the three cell lines were confirmed by northern-blot analysis using the cloned PCR fragment as the probe (Fig. 1B). Although a transcript of about 2.2 kb long was seen in all three cell lines, it was most abundant in NL4; expression of this gene in NL17 cells was hardly more than half that of NL4, and that in NL22 was scarcely more than one-fourth.

Isolation of cDNA clones in mouse and human We screened a cDNA library (5×10^5 plaques) constructed from mRNA isolated from the NL4 cell line, using the amplified fragment obtained from the differential display as a probe, and isolated five positive clones. The cDNA clone with the largest insert that represents the murine *grm2* consisted of 2102 bp, encoding a protein of 600 amino acids with a calculated molecular weight of 69,112 Da. Subsequently, we screened a human testis cDNA library (1×10^6 plaques) to isolate a human homologue using the murine cDNA insert as a probe, and identified two positive clones. Sequencing of these two overlapping cDNA clones revealed that the *GRM2* gene consisted of 1,983 bp, with an open reading frame of 1,800 bp encod-

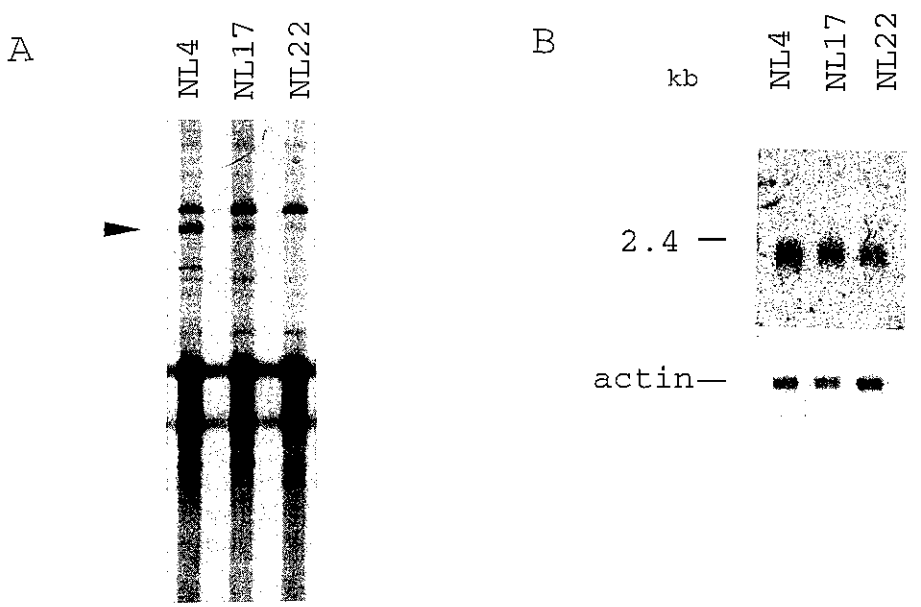

Fig. 1. A, Differential display pattern using mRNAs isolated from three cell lines derived from murine colon 26 adenocarcinoma. The arrowhead indicates a band that is more intense in NL4 than in either of the other two cell lines. The length of this fragment is approximately 285 bp. The primers used for this experiment were 5′-TCGGTCATAG-3′ (5′ primer) and 5′-GT_{15}MC-3′, where M represents a mixture of G, A, and C (3′ primer). B, Northern analysis of *grm2* expression in the three derivatives of colon 26 adenocarcinoma. A size marker is shown on the left. As a quantity control, expression of the beta-actin gene was examined using the beta-actin cDNA to probe the same blot (lower). Radioactivities were measured with a BAS 1000 Bioimage Analyzer (Fujix, Tokyo).

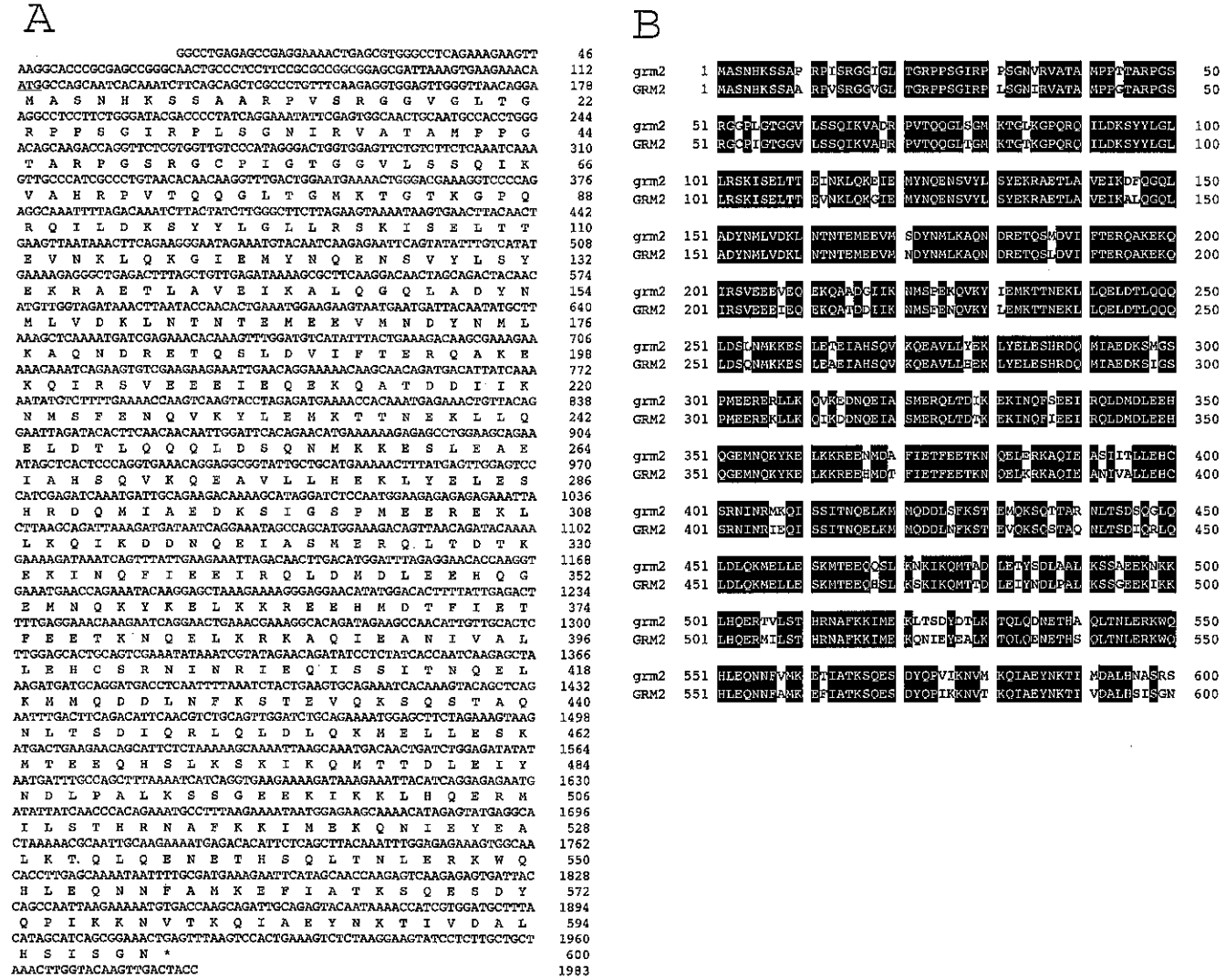

Fig. 2. A, Alignment of nucleotide and deduced amino acid sequences of human GRM2. The initiation methionine (ATG) of this gene is underlined. The termination codon (TGA) is indicated by an asterisk. B, Comparison of the predicted amino acid sequences of the *GRM2* cDNA with the deduced product of murine (*grm2*) cDNA. Identical residues are marked by shading.

ing a protein of 600 amino acids with a calculated molecular weight of 69,144 Da (Fig. 2A). The deduced amino acid sequence of human GRM2 product showed 88% homology to that of the murine homologue (Fig. 2B). Screening for homology of the GRM2 amino acid sequence with known sequences in the public database using the FASTA program[15] detected a low degree of homology (24% identity) between human GRM2 and nonmuscle myosin heavy chain.

Expression of *GRM2* in human cancers Expression of *GRM2* was examined using mRNAs isolated from various human tissues (Fig. 3A). A 2.5-kb transcript was observed in all human tissues examined but it was relatively abundant in testis and also, although to a lesser extent, in skeletal muscle. To investigate whether expression of the *GRM2* gene is altered in human carcinomas or in their metastatic foci, we performed northern blot analyses of tissues from ten colorectal cancers and corresponding normal colorectal mucosae; two of these tumors had metastasized to the lung and eight had metastasized to the liver. Among the ten cases examined, four showed no differences in expression of *GRM2* but six, including one with lung metastasis, revealed a significant reduction of *GRM2* expression in primary tumors and

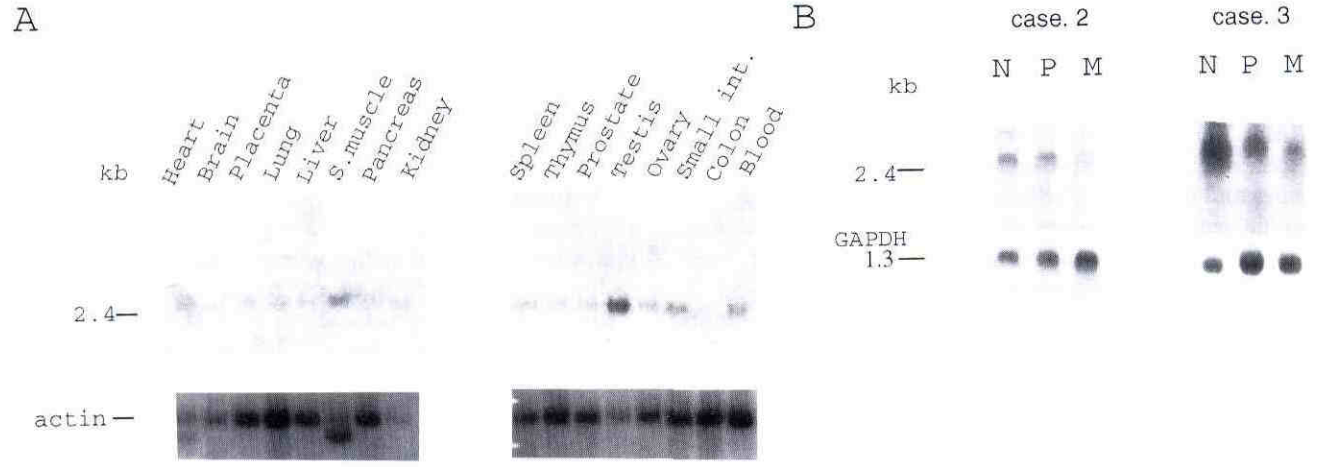

Fig. 3. Northern blot analysis of *GRM2*. A, Expression of *GRM2* in various normal human tissues. Size markers are shown on the left. For control purposes a beta-actin probe was used to measure expression of beta-actin on the same blot (lower). B, Expression of the *GRM2* gene in normal colonic tissue (N), corresponding primary colorectal tumor (P), and metastatic liver tumor (M) in cases 2 and 3. GAPDH cDNA was used as a control of the amount of RNA (lower).

Table I. *GRM2* mRNA Expression in Colorectal Cancer with Metastasis

No.[a]	Metastasis	Expression pattern[b]		
		Normal tissue	Primary tumor	Metastatic tumor
1	Liver	100	21	14
2	Liver	100	51	24
3	Liver	100	44	10
4	Liver	100	46	37
5	Liver	100	43	29
6	Lung	100	68	58
7	Liver	100	121	89
8	Liver	100	93	104
9	Liver	100	70	65
10	Lung	100	94	76

a) Cases in which >40% reduction of *GRM2* expression in primary or metastatic tumor was judged as reduction of the expression.
b) Comparison of expression by northern analysis. Shown is the ratio of *GRM2* to GAPDH. Expression in normal tissue among the three samples of the same patient was standardized as 100. Radioactivity was determined with a BAS 1000 Bioimage Analyzer (Fujix, Tokyo).

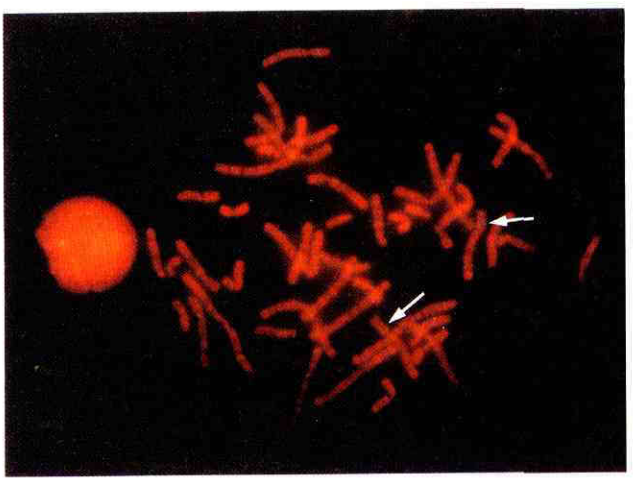

Fig. 4. Localization of *GRM2* on metaphase chromosomes by fluorescence *in situ* hybridization. The arrows indicate the signals on 9p21.1.

metastatic foci in comparison to corresponding noncancerous tissues. Fig. 3B shows two examples, and Table I summarizes the results of these experiments in all 10 cases examined. In half of the metastatic tissues, we detected reductions of 63–90% in expression of *GRM2*.

FISH To determine the chromosomal location of GRM2, we performed a direct R-banding fluorescence *in situ* hybridization with the genomic cosmid clone as the probe. More than 100 typical R-banded (pro)metaphase plates were examined. The visible signals were localized to the p21.1 band of chromosome 9 (Fig. 4).

DISCUSSION

We attempted to identify metastasis-related genes by analyzing mRNA expression profiles of three derivatives

of a murine colon cancer, each with distinct metastatic characteristics. By means of the differential display method we identified a candidate gene related to metastasis, and subsequently isolated its human counterpart, *GRM2*. This approach is very powerful for identification of genes that are expressed differently among various tissues and cell lines. For example, human breast cancer-specific genes, a human prostate cancer-specific gene, and a mouse developmental stage-specific gene have been isolated through differential display.[16–19] Expression of murine *grm2* in NL17 and NL22, cell lines with high metastatic potential, was significantly reduced in comparison to that in NL4, a colon cancer 26 derivative with a much lower metastatic potential. Expression of *grm2* in NL22, a derivative with spontaneous metastatic characteristics, was lower than that in NL17, an experimentally metastatic derivative. These results suggested that expression of *grm2* could be correlated with the metastatic potentials and phenotypes of murine colon-cancer cell lines. A similar tendency was evident in human colon cancers as well, since primary tumors and metastatic tissues excised from six of ten colorectal-cancer patients showed reduced expression of the *GRM2* gene while three of ten cases without metastasis showed reduced expression of the *GRM2* gene (data not shown). Expression levels were lower in metastatic tissues than in primary tumors, a result which supported the notion that the *GRM2* gene product may play a role in suppressing metastasis of colorectal cancers. However, as expression of the *GRM2* gene was reduced to some degree in the primary foci as well, information concerning *GRM2* expression levels in primary tumors might be a useful predictor for their metastatic potential. Although no significant differences with respect to *GRM2* expression were detected in four of the ten cases, those tumors may have sustained mutations causing functional inactivation of the *GRM2* gene or dysfunction of other metastasis-related genes such as *nm*23,[20–25] *KAI1*,[3] and CD44.[26,27] In fact, evidence that point-mutational inactivation of the *nm*23 gene may be related to metastasis of colorectal carcinomas has been reported.[28]

A noteworthy finding is our assignment of *GRM2* to chromosomal band 9p21.1, a region where chromosome losses frequently occur in cancers in various tissues, such as lung,[29] bladder,[30] head and neck,[31] and esophagus.[32] Recently p16, that was localized to 9p21 and found to be within the commonly deleted region, was isolated as a candidate tumor suppressor gene.[33] However, a low frequency of somatic point mutations in primary tumors that had loss of heterozygosity in 9p21[34] raised the possibility of involvement of other 9p genes. Although it has not been fully examined, *GRM2*, which is localized in a critical area of chromosomal loss at region 9p, might be a candidate gene for tumor progression and metastasis in these types of tumor.

Metastasis is a multi-step process involving detachment of tumor cells from the primary tumor, invasion of adjacent structures, adhesion to the capillaries, and proliferation in distant organs. Although the biological functions of the novel gene reported here are unknown, *GRM2*, may regulate one or more of these steps.

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