Inserting Chromosome 18 into Pancreatic Cancer Cells Switches Them to a Dormant Metastatic Phenotype

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ABSTRACT

We demonstrated previously that restoration of chromosome 18 suppressed growth of pancreatic cancer cells in vitro, as well as that of tumors inoculated into nude mice. We also demonstrated that loss of 18q was associated with poor prognosis. Hence there is the possibility that the 18q arm harbors a gene(s) implicated in tumor progression and/or metastasis. In this study, we evaluated the effect of restoring chromosome 18 on metastasis in a few human pancreatic cancer cell lines with and without inactivation of SMAD4. After microcell-mediated chromosome 18 transfer, hybrid showed more than a 10-fold weaker metastatic ability than corresponding parental cells; mice injected with 1.25 × 10^6 hybrid clones via tail vein had less than one-tenth of the number of macroscopic metastases in the lung when compared with the control cells. Microscopic examination confirmed the decrease in the number of metastatic lesions. After inoculation of hybrid cells, more than 80% of the high-power fields showed no micrometastases, contrasting with their abundance after using the parental cells. Hybrid cells restored maspin expression irrespective of SMAD4 status in corresponding parental cells. On the other hand, significantly lower vascular endothelial growth factor and matrix metalloproteinase 2 secretion was observed by measuring levels in the conditioned media (CM); the averages were 22% and 20%, respectively. Angiogenesis assays using in vivo Matrigel plugs demonstrated that less neovascularization was observed in nude mice with hybrid cells than with corresponding parental cells. When cells were treated with CM from hybrids, the migration of human umbilical vascular endothelial cells was decreased, but it was partially restored with anti-vascular endothelial growth factor neutralizing antibody, as compared with CM from parental cells. These data represent the first functional evidence suggesting that chromosome 18q encodes a gene that strongly suppresses metastatic activity, possibly through dormancy.

INTRODUCTION

Pancreatic cancer is one of the worst diseases worldwide in terms of prognosis; its mean 5-year survival rate is <5% (1). The mortality rate virtually equals its incidence rate; the reasons for this biological aggressiveness include the latency of early symptoms and a lack of efficient detection methods at the curable stages. Thus, acquisition of efficient approaches and markers able to accurately detect the earliest stages of pancreatic cancer should be prioritized.

The molecular pathology of pancreatic carcinogenesis is characterized by a broad spectrum of mutations in various genes and chromosomal alterations; some of these seem to be specific. The oncogene KRAS2 and tumor suppressor genes TP53, MTS1, and SMAD4 are mutated in many pancreatic cancers (2–4). Various chromosomal gains (5p, 7p, 8q, 17q, and 20q) and losses (1p, 6q, 9p, 12q, 17p, and 18q) have also been observed in cytogenetic and allelotype studies (5–9). Among these foci for genetic imbalances, loss of 18q is an early event in pancreatic carcinogenesis (10), and the restoration of chromosome 18 can suppress the growth of pancreatic cancer cells in vitro (11). Furthermore, LOH of 18q is associated with a poor prognosis (12). LOH of 18q is a common event in over 90% of pancreatic carcinomas; only 50% of them are characterized by a biallelic inactivation of the SMAD4 gene (4). Although 18q LOH is frequent in intraductal papillary mucinous tumors, one of the premalignant lesions of the pancreas, the SMAD4 protein is expressed in these tumors (13). Moreover, loss of SMAD4 expression occurs biologically later in the neoplastic progression that leads to the development of infiltrating pancreatic cancer, at the stage of histologically and clinically recognizable carcinoma (14).

Metastasis is one of the most important factors in poor prognosis. The regulatory genes involved can be broadly cate-
orized as either metastasis promoting or metastasis suppressing. Because chromosome 18 has been proven to harbor a cluster of candidate tumor suppressor and metastasis suppressor genes, including SMAD2, SMAD4, DCC, SERPINB5 (maspin), and PAI-2, and because poor prognosis is associated with 18q LOH (12), it is of great interest to examine whether genes on chromosome 18 play roles in metastatic processes. Therefore, we herein compared the metastatic ability of pancreatic cancer cells after restoration of chromosome 18 with that of their corresponding parental cells.

MATERIALS AND METHODS

Pancreatic Cancer Cell Lines. The pancreatic cancer cell lines used in the present study were PCI-35, BxPC3, Panc-1, and MIAPaCa2. PCI-35 was a generous gift from Dr. Hiroshi Ishikura (Hokkaido University School of Medicine), and the other three were purchased from ATCC (Manassas, VA). MRC-5 normal human fibroblast cells, HUVECs (ATCC), and pancreatic cancer cells were cultured according to the protocols of the suppliers and were already well characterized mutagenically (15). For each cell line, five stable hybrids containing a normal copy of chromosome 18 were established [PCI-35H(18)-1 through PCI-35H(18)-5, BxPC3H(18)-1 through BxPC3H(18)-5, Panc-1H(18)-1 through Panc-1H(18)-5, and MIAPaCa2H(18)-1 through MIAPaCa2H(18)-5, respectively]. These cell lines were generated by the MMCT technique described in our previous study (11) and grown in medium containing 400 μg/ml G418. The A9H(18) mouse fibroblast cell line carrying a single copy of human chromosome 18 tagged with an integrated neomycin-resistant gene was maintained in the same selective medium.

Analysis of Metastatic Ability. To estimate the metastatic ability of the hybrids, we used a lung colonization model (16). Briefly, parental and hybrid cells were prepared as single-cell suspensions in sterile PBS at a concentration of 5 × 10⁶ cells/ml, and a volume of 250 μl (1.25 × 10⁵ cells; viability of 95% as determined by trypan blue exclusion) was injected i.v. via the tail vein into 8-week-old male athymic nude mice (BALB/c-nu/nu; purchased from Japan Clea Inc., Tokyo, Japan). Animals were sacrificed on a day between days 30 and 35, when the majority of the control mice became moribund. The left lungs were snap frozen and used for molecular biological analysis, and the right lungs were fixed in formalin overnight at 4°C. The white surface metastatic tumors were counted, and microscopic studies were also performed. This study was approved by the Ethical Committee of the Tohoku University School of Medicine. All of the animal experiments were performed according to Tohoku University Institutional and NIH guidelines.

Immunohistochemical Analysis. Right lungs were excised, fixed in formalin overnight, sectioned discontinuously into five portions, and immunostained with mouse anti-human cytokeratin monoclonal antibody (Cosmo Bio, Tokyo, Japan) developed with the AEC system (Zymed Immunomouse Kit). At least 100 random HPFs were microscopically examined. Nude mice tumors generated in our previous study (11) as well as 10 primary human pancreatic tumors were immunostained using goat anti-human maspin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (17). Immunohistochemical staining of nude mice for frozen tumors with anti-PECAM-1 antibody (clone MEC13.3; PharMingen, San Diego, CA) was performed as described previously (18).

Microsatellite Analysis. To test whether or not the introduced chromosome 18 was retained in each cell line, we performed microsatellite analyses by methods described previously (19). Genomic DNAs from the A9H(18) and each parental cell line and its injected hybrid and the corresponding nude mice lung metastatic tumors were analyzed with a panel containing seven highly polymorphic microsatellite markers: D18S1104; D18S463; D18S1144; D18S483; and D18S58. For each marker, PCR amplification was carried out at least twice. Nucleotide sequences and conditions for PCR have been described previously (11).

RT-PCR. RT-PCR was performed according to methods described previously (20). Primers and conditions used for RT-PCR have been described elsewhere (11, 20, 21). Ten primary human pancreatic cancers as well as their corresponding normal pancreatic tissues were also used. These tissues were obtained at Tohoku University Hospital with informed consent. Tissue sections from all patients were reviewed by a board-certificated pathologist (T. F.), and the diagnosis was reconfirmed histologically in all specimens.

Southern Blot Analysis. The SERPINB5 RT-PCR products were run on a 3% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled SERPINB5-specific probe (22). Hybridization procedures were carried out as described previously (23). Each blot was visualized and quantified by the BAS 1500 and Image Gauge 3.3 software (FUJI Photo Film Co., Ltd., Minamiashigara, Japan).

Western Blot Analysis. Western blot analysis was performed as described previously (24). A total lysate of human normal fibroblast MRC-5 was used as the control. CM were obtained, stored, and analyzed as described previously (25). Antibodies used were goat anti-human MMP2 and MMP9 (Santa Cruz Biotechnology) and rabbit anti-human VEGF (Sigma, St. Louis, MO). The relative intensities of signals were analyzed using the Luminescent Image Analyzer LAS-1000 Plus and Image Gauge 3.3 software (FUJI Photo Film Co., Ltd.).

Matrigel Plug Assays. Each Matrigel plug assay was carried out by abdominal midline s.c. injection of a 500-μg Matrigel plug alone (negative control) or containing 25% CM. Plugs were removed after 12 days, fixed in formalin, embedded in paraffin, and stained with H&E as described previously (26). The Matrigel plugs and nude mice tumor vascularization were quantitated by NIH 1.62 software. VEGF concentrations in CM were quantitated using a VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Invasion and Migration Assay. The effects of maspin on the invasion through Matrigel and motility of pancreatic cancer cell lines were analyzed using either a simple modified Boyden chamber or one precoated with Matrigel growth factor (Becton Dickinson Labware, Franklin Lakes, NJ) reduced as described previously (25). Five thousand cells, serum-starved for 3 h, were seeded in the serum-free medium into the upper compartment. In the lower compartment of the chamber, media supplemented with 10% fetal bovine serum with and without fibronectin (5 μg/ml) were added. Antibodies were diluted in cell culture medium and added to the lower compartment in concentrations ranging from 10 to 30 μg/ml. Endothelial cell
RESULTS

First, a panel consisting of seven selected highly polymorphic microsatellite markers was used to monitor whether or not the introduced chromosome 18 remained in each hybrid cell line. Genomic DNAs from the parental cell line and its derived hybrid, A9H(18), were used for PCR. Typical examples are shown in Fig. 1. A complete copy or at least a great majority of human chromosome 18 was transferred and maintained in each hybrid cell; the band originating from A9H(18) was seen in the DNA of the hybrid cell line in each case.

Once the presence of the introduced chromosome 18 in the hybrids was verified, we estimated the metastatic ability of the hybrids using a lung colonization model. As shown in Table 1, we recorded a significantly decreased number of surface metastases/total number of mice in their hybrids, Panc-1(18); lane H, their hybrids, Panc-1(18); lane M, nude mice lung metastatic tumors after injection of Panc-1(18); A9H(18), the donor cell for chromosome 18 in MMCT experiment. Arrows indicate bands corresponding to the chromosome 18 originating from A9H(18). The cytogenetic position of each marker is indicated in parentheses below each marker.

In our previous work, we demonstrated growth suppression induced by introduction of chromosome 18 into these pancreatic cancer cell lines (11). There were at least two possibilities for the reduction of metastasis in hybrid cells: one was simply suppression of cell growth in mice lungs, and the other was involvement of factors regulating the metastatic ability; angiogenesis is one such factor. One of the possible targets located on 18q and involved in angiogenesis is SERPINB5 (maspin), so we examined its levels first to consider their functional importance in metastasis. Expression of SERPINB5 mRNA was observed initially only in parental cells having inactivated SMAD4, PCI-35 and BxPC3, respectively (Fig. 3, Lanes 5 and 7). In sharp contrast, maspin mRNA was undetectable in SMAD4wt cells but was expressed in the hybrids containing an extra copy of chromosome 18, namely, Panc-1H(18) and MIAPaCa2H(18) (Fig. 3, A and B). Furthermore, low expression of maspin was observed in 8 of 10 normal human pancreatic tissues by RT-PCR and confirmed by Southern hybridization (see Fig. 4, A and B). In contrast, overexpression of maspin was observed in all 10 of the human pancreatic primary tumor specimens studied (see Fig. 4C). Additionally, a subcutaneous skin metastasis detected in one of the patients showed abundant maspin expression (Fig. 4, D and E).

Functional assessment of maspin is depicted in Fig. 5. Basal migration with serum-free medium in the lower chamber was the same for both parental and hybrid cells. On the other hand, stimulated migration of hybrid clones toward bFGF was significantly enhanced upon maspin antibody treatment but was seen to a lesser
extent in parental cells. This property was only seen in the hybrids generated by parental cells originally lacking maspin expression (Fig. 5A), and no effect was seen, regardless of the titer of antibody used, in hybrids whose parental cells were expressing maspin (Fig. 5B). In contrast, the invasiveness of hybrids was significantly reduced and then restored upon the same treatment, regardless of the initial status of maspin expression (Fig. 5D). However, neither motility nor invasion of hybrids was restored to the levels of untreated parental cells used as controls. This result suggests that maspin expression can explain only part of the reduction of hybrid motility and invasiveness.

As some of the most important steps in the establishment of metastasis cascade, angiogenesis and invasion were studied by focusing on activities of some soluble factors such as VEGF or MMPs. When cells were treated with CM from hybrids, the migration of HUVECs was decreased, but it was partially restored with anti-VEGF neutralizing antibody, as compared with CM from parental cells (Fig. 5C). Significant decreases in secretion of VEGF (average, 20.5%; see Fig. 6, A and B) and MMP2 (average, 24.7%; Fig. 6C) in the culture media from hybrids were observed when the levels were compared with those of the corresponding parental cells. A relatively similar rate of reduction in migrated endothelial cells was observed in response to either antihuman VEGF treatment or Matrigel plugs. These results were confirmed by microvessel assessment in tumors that developed in nude mice (Fig. 7); a significant reduction in the hybrid cells was seen when compared with parental cells [by 22±2.75% for PCI-35H(18)].

**DISCUSSION**

Our recent studies have shown that restoration of chromosome 18 status suppressed the *in vitro* growth of human pancreatic cancer cells and that *in vivo* growth was also suppressed in nude mice (11). In the present study, we observed a significant suppression of metastatic ability in pancreatic cancer cells.

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**Table 2** Comparison of metastatic abilities between parental and hybrid cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>No metastases</th>
<th>Microscopic metastases</th>
<th>Macroscopic metastases</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCI-35</td>
<td>4.9 ± 1.9</td>
<td>24.6 ± 3.1</td>
<td>77.9 ± 9.1</td>
<td>4</td>
</tr>
<tr>
<td>PCI-35H(18)</td>
<td>82.4 ± 7.4</td>
<td>3.9 ± 1.1</td>
<td>6.9 ± 2.1</td>
<td>15</td>
</tr>
<tr>
<td>Panc-1</td>
<td>6.1 ± 1.9</td>
<td>32.8 ± 6.7</td>
<td>72.6 ± 5.6</td>
<td>4</td>
</tr>
<tr>
<td>Panc-1H(18)</td>
<td>78.4 ± 9.7</td>
<td>2.8 ± 0.91</td>
<td>5.5 ± 1.9</td>
<td>15</td>
</tr>
<tr>
<td>BxPC3</td>
<td>5.3 ± 1.1</td>
<td>33.5 ± 3.1</td>
<td>69.8 ± 6.2</td>
<td>4</td>
</tr>
<tr>
<td>BxPC3H(18)</td>
<td>73.5 ± 7.6</td>
<td>4.1 ± 1.2</td>
<td>5.9 ± 1.6</td>
<td>15</td>
</tr>
</tbody>
</table>

*a* Results represent the mean of 100 HPF ± SD. Experiments were performed in triplicate, and five discontinuous lung sections were analyzed (×100 magnification) in each experiment.

*b* The threshold for macroscopic metastases was considered to be >300 cells/positive stained focus; *P* < 0.001.

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![Fig. 2](https://clincancerres.aacrjournals.org)
in which we restored a normal copy of chromosome 18. We also recorded a significant suppression in the number of surface metastases developing in hybrid cell-injected mice as compared with parental cell-injected mice; at least one of every hybrid clone had no countable macroscopic metastases. Furthermore, microscopic examination showed that animals injected with hybrid cells developed a significantly lower number of micrometastases and only very rare macrometastases when compared with those injected with parental cells. Moreover, in lungs showing no metastases on surface examination, we recorded

Fig. 3 Expression of SERPINB5 (maspin) in pancreatic cancer cell lines was analyzed by RT-PCR (A) and Southern blotting (B). Expression of SMAD4 transcripts by RT-PCR is shown in C. Lane 1, Panc-1; Lane 2, Panc-1H(18); Lane 3, MIAPaCa2; Lane 4, MIAPaCa2H(18); Lane 5, PCI-35; Lane 6, PCI-35H(18); Lane 7, BxPC3; Lane 8, BxPC3H(18); Lane 9, normal colon (A), PCR-amplified maspin cDNA (B), or fibroblast cell line MRC-5 (C).

Fig. 4 Expression of maspin in 10 normal and 10 cancerous tissues by RT-PCR (A and C). Results in B represent Southern hybridization in 10 pancreatic normal tissues. Lanes 1–10 are normal (A) or primary tumor (C) specimens. Lane 11 in C is a positive control, MRC-5. Expression of maspin is shown by immunostaining with maspin monoclonal antibody in a skin metastatic lesion in one of the pancreatic cancer patients (D, ×100; E, ×200).
only a few, hardly detectable micrometastases that appeared to be dormant. However, the dramatic suppression of macrometastases and the presence of apparently dormant micrometastases in hybrid clone-injected animals may simply suggest important suppression in cell proliferation.

In an attempt to gain more insight into this effect, we previously analyzed the expression of known genes residing on chromosome 18 and related to the metastasis process. MMCT could be approximated, at least theoretically, with transfection of a panel encompassing a single allele of each gene residing on the chromosome 18. Among these genes, only SERPINB5 (maspin) has been shown to functionally suppress metastasis of breast cancer cells in an in vivo model (27). Interestingly, in the parental cells, maspin expression showed a tendency toward inverse association with a simultaneous inactivation of SMAD4; specifically, only SMAD4-null cells expressed maspin at levels similar to those found in human tumors; no expression was detected in SMAD4** cells. Maspin and SMAD4 are closely located on chromosome 18 (18q21.1 and 18q21.3, respectively) and share a critical region for pancreatic cancer. Despite the abundance of data concerning SMAD4, there are no detailed studies describing the frequency of maspin inactivation or its implications for pancreatic cancer. To date, LOH of 18q is a common event in over 90% of pancreatic carcinomas, but only 50% of them are characterized by a biallelic inactivation of the SMAD4 gene (4). Inoue et al. (13) reported that SMAD4 protein is expressed in virtually all intraductal papillary mucinous tumors, suggesting inactivation of SMAD4 as a rather late event in pancreatic tumorigenesis. Based on our findings, we could hypothesize that SMAD4 and maspin are inactivated or rather mutated at different stages of pancreatic tumorigenesis. However, to elucidate this attractive aspect, additional studies will be necessary. A low to moderate level of maspin expression was observed in the majority of normal human pancreatic tissues by RT-PCR. Maass et al. (17) found by Northern blotting that the mRNA level of SERPINB5 in normal pancreas tissue is low. This could be due to a low level of SERPINB5 or to a higher sensitivity of the RT-PCR method. Additionally, rather high levels of SERPINB5 expression have been observed in primary pancreatic tumors. In fact, the latter finding makes SERPINB5 an unlikely target of chromosome 18 loss. To date, it has been suggested that maspin may be useful in separating ductal adenocarcinoma from acinar cell carcinoma, pancreatic endocrine tumor, solid pseudopapillary tumor, and chronic pancreatitis (28). Overall, there is a completely different pattern of maspin expression in pancreatic tumorigenesis than in breast and prostate cancers. This paradoxical expression of maspin has been reported recently by Sood et al. (29) in ovarian carcinoma.

Fig. 5  A and B, motility assays. The indicated pancreatic cancer cells and their hybrids were plated in the upper chambers and allowed to migrate for 8 h toward either fetal bovine serum-free medium or medium containing 10 μg/ml bFGF (basal and stimulated migration, respectively). Ant.maspin antibodies were diluted in cell culture medium and added to the lower wells to final concentrations ranging from 10 to 20 μg/ml, as indicated. C, endothelial cell migration assay. HUVECs were allowed to migrate toward CM from either parental cells, hybrids, or CM neutralized with anti-VEGF polyclonal antibodies placed in the lower chambers of the wells. Normal human serum medium (IG) was used as a negative control. D, invasion assays. The indicated cells were allowed to invade through a Matrigel reconstituted membrane for 18 h. Antibodies were diluted in cell culture medium and added to the lower wells at 20 μg/ml. Normal human serum medium was used as a negative control. Migrated or invaded cells were counted, averaged, and expressed either as migrated cells/HPF (×100 magnification) or as relative invasion compared with control cells (D). Data represent the mean ± SD of at least three independent experiments. *, P < 0.05; §, P > 0.05.
Maspin overexpression was significantly associated with a high tumor grade and a shorter duration of overall survival. In our study, however, functional assessments of maspin expression cannot fully explain the observed effect on suppression of metastatic abilities. It is possible that the maspin expressed in these tumors may be inactive (29). Degradation and penetration of the extracellular matrix is a hallmark of tumor invasion and metastasis (30). In our study, stimulated migration was found to be significantly enhanced by maspin antibody treatment, but to a lesser extent than that seen with parental cells. This latter effect can be explained only by the hypothesis that the hybrids generated by the parental cells originally lacked maspin expression, and the phenomenon suggests a dose-independent mechanism. Notably, the invasiveness of hybrids was significantly reduced and then restored upon the same treatment, regardless of the initial status of maspin expression in the parental cells. However, neither the motility nor the invasion of hybrids could be restored to the levels of untreated parental cells used as controls. These results suggest that maspin expression can explain only a part of the reduction of hybrid motility and invasiveness.

Angiogenesis and invasion, both required steps in the metastatic process, are under the control of growth factors such as VEGF and proteinases, especially those from MMP members (30, 31). The aggressive phenotype of pancreatic carcinoma may arise from overexpression of MMP2 (32). VEGF may promote the distribution of metastases, leading to early cancer recurrence and the poor outcome of pancreatic cancer (33). Therefore, we examined the expression of these factors and showed a significant reduction in VEGF and MMP2 secretion in the culture media from hybrids as compared with the levels produced by parental cells. The differences among these cells in their expression of VEGF were more pronounced when the expression was measured by ELISA than when measured by Western blotting. The rate of protein secretion is probably more relevant to the rate of tumor neovascularization than the intracellular concentration of protein because angiogenesis is induced by the binding of protein to endothelial cell receptors (34). However, no significant differences were found among the cells for secretion of MMP1, MMP9, bFGF, or u-PA (data not shown). Furthermore, relatively similar reduction rates were observed in the endothelial cells that migrated through the antihuman VEGF-treated CM and into Matrigel plugs; these data suggest that VEGF status is responsible for the majority of changes in the angiogenic phenotype throughout the hybrid cells. These results were confirmed by PECAM-1 immunoreactivity in the nude mice tumors, which showed a significant reduction in the hybrid cells. This fact is concordant with a previous study showing that SMAD4 restoration in pancreatic cancer cells reduces angiogenesis rates through the down-regulation of VEGF expression (35). Taken together, these results suggested that the dramatic reduction in the metastatic abilities of hybrid cells containing a normal copy of chromosome 18 is only partially attributable to the changes demonstrated in VEGF or MMP2 secretion rates.

To date, MMCT has been proven to be useful for providing functional evidence of the chromosome location of tumor or metastasis suppressor genes in a variety of cancers, including pancreatic cancer (11), Nijmegen breaking syndrome (36), and prostate cancer (37). As stated earlier, a whole chromosome...
transfer raises at least two presumable effects: (a) direct effects of the known or unknown genes located on chromosome 18; and (b) indirect effects through possible interactions among the transferred chromosome and the other genes. Although, in this setting, these effects could not be clearly delineated, it is conceivable that the introduction of an extra copy of chromosome 18 confers a less aggressive metastatic phenotype to the pancreatic cancer cells. In other words, the metastatic inhibition encoded by 18q can explain the dormant status of the hybrids and, in turn, the presence of micrometastases unable to form macrometastases.

In summary, metastasis was suppressed when an intact chromosome 18 was transferred into human pancreatic cancer cells. This study provides the first functional evidence that one or more metastasis suppressor gene(s) is located on chromosome 18. Although this study clearly implicates the important role of gene(s) on chromosome 18, the precise subchromosomal localization of the metastasis suppressor gene(s) is still an open question. In the absence of spontaneous revertant hybrids, the precise localization and identification of a putative metastasis suppressor gene will require further and additional efforts.

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