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Frequent Expression of Midkine Gene in Esophageal Cancer Suggests a Potential Usage of Its Promoter for Suicide Gene Therapy

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We have examined the expression of midkine (MK), a neurotrophic factor with heparin-binding activity, in human esophageal cancer cells. Seven esophageal cell lines tested expressed the transcript and 8 out of 14 human esophageal tumor specimens were positively stained with anti-MK antibody, while surrounding normal esophageal tissues in these specimens were not stained. The 5′-flanking, 2.3 kb genomic region of the *MK* gene was shown to drive the transcription of a reporter gene in the esophageal cell lines in a *cis* acting manner. Forced expression in esophageal cancer cells of herpes simplex virus-thymidine kinase gene mediated by the flanking region of the *MK* gene conferred sensitivity to a prodrug, ganciclovir. The 5′-upstream region of the *MK* gene thus possesses putative promoter activity which can be used for suicide gene-based gene therapy for esophageal cancer.

Key words: Midkine — Suicide gene — HSV-TK — Esophageal cancer — Gene therapy

Midkine (MK) is a heparin-binding growth factor and its expression is conserved across species and is developmentally regulated.[1–3] It promotes neurite outgrowth and has a mitogenic activity towards fibroblasts and neuroectoderm cells.[4, 5] The biological function of MK during tumorigenesis is not clearly understood, though MK expression is elevated in various types of human cancer such as Wilms' tumor,[6] breast cancer,[7] and neuroblastoma.[8] Recent studies have shown that MK plays a possible role in enhancing angiogenesis[9] and that overexpression of the *MK* gene is a poor prognostic factor for patients with bladder carcinoma.[10] In normal adult tissues, MK is expressed solely in the kidney, lung and small intestine.[6] The limited expression in normal tissues implies a possible use of the promoter, which preferentially functions in tumor cells, for therapeutic strategies based on tumor-specific gene expression.

Esophageal cancer remains one of the most intractable tumors due to the higher incidence in aged patients and its rapid infiltration into neighboring tissues despite multimodal approaches.[11] Novel procedures, therefore, should be introduced to improve the prognosis, and gene therapy is a possible therapeutic approach.

Increased MK expression in human gastrointestinal tumors was reported,[12] but the expression in esophageal cancer has not been extensively studied. In this study, we examined the expression of the *MK* gene in human esophageal cancer and found that it was detectable solely in the tumors. We then tested whether the 5′-upstream region of the *MK* gene could be used as a promoter to generate transcriptional activation in esophageal tumors. For that purpose, the expressions of a reporter gene and the herpes simplex virus-thymidine kinase (*HSV-TK*) gene, a suicide gene frequently used for gene therapy,[13] were investigated in human esophageal cancer cell lines, using the upstream region of the *MK* gene.

MATERIALS AND METHODS

Cells Human esophageal cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum. T.Tn cells were from Japanese Cancer Research Resources Bank and ECGI10 cells from Riken Cell Bank (Tsukuba). TE1, TE2, TE10, TE11 and TE13 cells were kindly provided by Dr. T. Nishihara (Tohoku University, Sendai). A human melanoma line, A875 from Dr. A. Nakagawara (Chiba Cancer Center, Chiba) and a human foreskin fibroblast line, HFF from Dr. T. Yamakawa (Tokyo Metropolitan Institute of Gerontology, Tokyo) were

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Abbreviations used: MK, midkine; HSV-TK, herpes simplex virus-thymidine kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; CAT, chloramphenicol acetyltransferase; GCV, ganciclovir.

Table I. Expression of MK Protein in Human Esophageal Tumor Specimens and Clinicopathological Data of the Patients

Patient No.	Age/Gender	MK expression	TNM	Stage	Pathology (differentiation)
1	60/M	+	T3N0M0	II A	well
2	64/M	+	T1N0M0	I	moderately
3	65/F	+	T3N0M0	II A	moderately
4	67/M	+	T2N1M0	II B	well
5	68/M	+	T3N1M1b	IV B	poorly
6	70/M	+	T3N1M1a	IV A	well
7	70/M	+	T3N0M0	II A	well
8	76/M	+	T3N1M0	III	moderately
9	55/M	–	T2N1M1a	IV A	moderately
10	58/M	–	T1N1M1b	IV B	poorly
11	59/M	–	T2N1M0	II B	poorly
12	68/M	–	T1N0M0	I	moderately
13	75/M	–	T1N1M0	II B	poorly
14	77/F	–	T3N1M1a	IV A	moderately

Expression of MK protein was analyzed with anti-MK antibody. TNM was based on the classification by the International Union Against Cancer.

cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Immunohistochemical analysis Human specimens were surgically obtained from 14 Japanese esophageal cancer patients (summarized in Table I). The samples were fixed with 4% paraformaldehyde/phosphate-buffered saline at 4°C. The sections of 4–5-μm thickness were incubated with 10% skim milk for 20 min, and then with affinity-purified rabbit anti-MK antibody for 60 min. The sections were sequentially reacted with 0.3% H_2O_2/methanol, biotinylated anti-rabbit IgG, avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine; they ware washed with phosphate-buffered saline after each reaction. The sections were counterstained with hematoxylin.

Northern blot analysis Poly A^+ mRNA was extracted using an mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). One microgram of mRNA was subjected to electrophoresis in a denaturing formaldehyde-agarose gel and transferred to a nylon filter. The human MK or human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA[14] was labeled with [^{32}P]dCTP and used as a probe. The hybridization was performed in a solution of 50% formamide/5× SSC/50 m*M* NaH_2PO_4/2× Denharts'solution/0.1% sodium dodecyl sulfate (SDS)/0.1 mg/ml salmon sperm DNA at 42°C for 12 h. The filter was washed with a solution of 0.2× SSPE/0.1% SDS several times at 50°C.

Chloramphenicol acetyltransferase (CAT) assay Human genomic DNA containing the first exon of *MK* and the 5′-upstream flanking region (2.3 kb)[15] was inserted into pCAT-basic vector (Promega, Madison, WI). Two plasmids, CAT gene driven by the SV40 early promoter and CAT gene without promoters, were from Promega. Esophageal cancer cells were transfected with the respective plasmid DNAs containing the CAT gene (10 μg) and 1 μg of DNA which could constitutively express the β-galactosidase gene (pCH110, Amersham Pharmacia Biotech) using Lipofectin reagent (Life Technologies, Gaithersburg, MD). Two days later, cell extracts were prepared by sonication. To normalize the transfection efficiency, the amounts of cell extracts used for CAT assay were adjusted according to the β-galactosidase activity.[16] CAT activity was measured by the incubation of each extract with 50 μCi of [^{14}C]chloramphenicol and 1 m*M* acetyl CoA in 0.25 *M* Tris-Cl (pH 7.8) for 120 min at 37°C as described.[17]

Assay of *in vitro* sensitivity to ganciclovir (GCV) HSV-TK cDNA (from Dr. K. Ikenaka, National Institute for Physiological Science, Okazaki) was ligated into a retrovirus vector LXSN (from Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA)[18] next to a 2.3 kb *MK* genomic DNA fragment (see Fig. 4) and used in CAT assay. Esophageal and melanoma cells were transfected with the vector DNA bearing the *MK+HSV-TK* gene using Lipofectin reagent and were cultured in G418 (Life Technologies)-containing medium to obtain stably transfected cells. The G418-resistant or wild-type cells, placed in 96-well plates at the density of 5×10^2 cells/well, were cultured in the absence or presence of various concentrations of GCV (0.1–100 μg/ml) for 7 days. The viable cell number in each well was measured with a cell counting kit (Dojindo Laboratories, Kumamoto). The amounts of formazan produced were determined from the absorbance at 450 nm.

RESULTS

Immunohistochemical analysis We examined the expression of MK protein in human specimens of esophageal tumors. Eight out of 14 specimens were positively stained with anti-MK antibody, while none of the surrounding normal esophageal tissues in MK-positive specimens reacted with the antibody (Fig. 1). The tumor cells were cytoplasmically stained with the antibody. We noticed that all the specimens of well differentiated type were stained with the antibody. However, no other correlations of MK expression with clinicopathological features of the patients examined were evident (Table I).

Expression of the *MK* gene in human esophageal cell lines In order to establish an experimental system using cell lines, we examined the expression of the *MK* gene in 7 human esophageal cancer cell lines by northern blot analysis. All the lines tested expressed the transcripts but the amounts of the transcript varied among the lines (Fig.

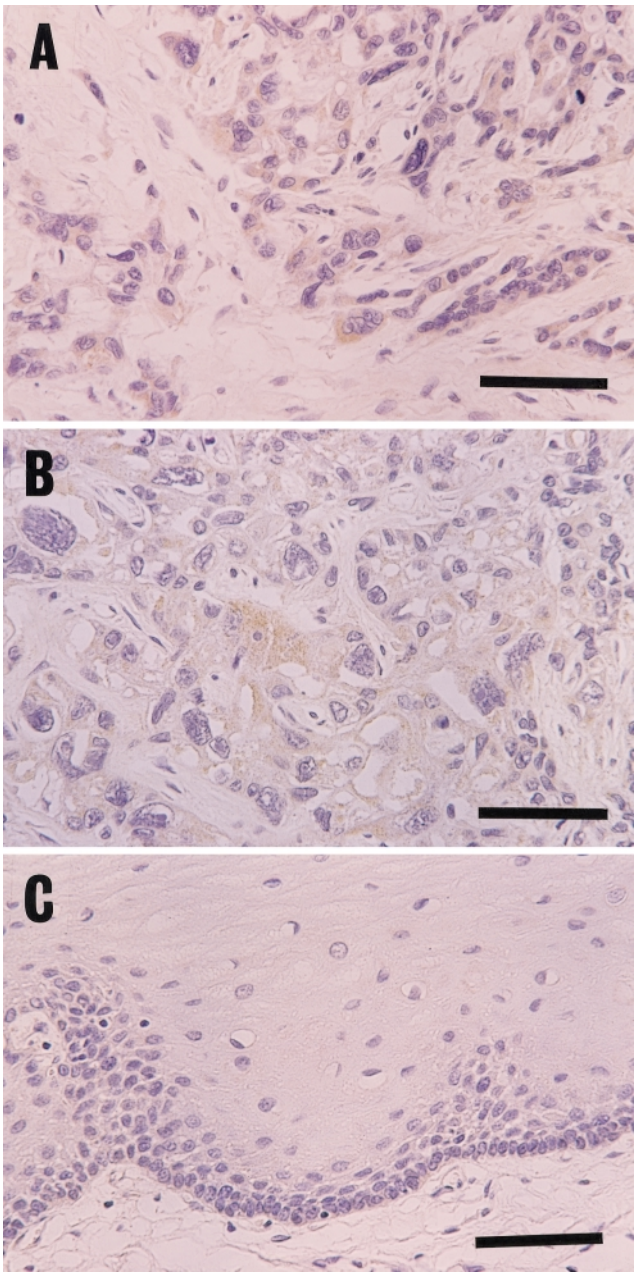

Fig. 1. Representative human esophageal specimens immunohistochemically analyzed with anti-MK antibody. Esophageal tumor (A), metastatic foci in liver (B) and surrounding normal esophagus (C) from the same patient (Patient 5 in Table I). The bars indicate 100 μm.

2). In contrast, a human melanoma line, A875, was negative for midkine expression, and a human fibroblast line, HFF, expressed the *MK* gene but the level of the expression was markedly lower than that of esophageal lines tested (Fig. 2).

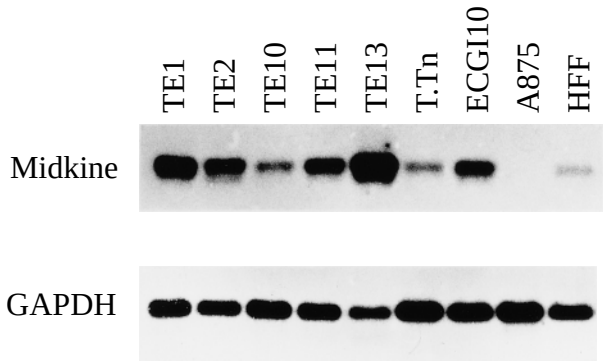

Fig. 2. MK expression in human esophageal and other cell lines. One microgram of poly A^+ RNA was hybridized with ^{32}P-labeled MK cDNA and the same filter was rehybridized with ^{32}P-labeled GAPDH cDNA. The approximate sizes of the MK and GAPDH transcripts are 0.9 kb and 1.8 kb, respectively.

Promoter activity in human esophageal tumor cells To investigate whether the 5′-upstream region of *MK* genomic DNA fragment can be used as a transcriptional promoter in human esophageal tumors, we tested CAT activity in 7 esophageal cancer cell lines transfected with the CAT gene-containing plasmid. All the lines tested could transcribe the reporter gene under the control of 2.3 kb *MK* genomic region (Fig. 3). However, the transcriptional activites varied among the lines and were not related with the amounts of the MK transcript (Fig. 2). Relative CAT activity powered by the putative MK promoter was less than that of the SV40 promoter in 5 lines. The activity of the MK promoter judged in terms of acetylation rate was 6.0–30% of that of the SV40 promoter. In contrast, 2 lines (TE13 and T.Tn) showed CAT activity comparable to that of the SV40 promoter (85–160%).

***In vitro* sensitivity** We then examined the antitumor effect in terms of the expression of the *HSV-TK* gene controlled by the putative MK promoter. To avoid the effect of the promoter present in the 5′-long terminal repeat of the retrovirus vector, the putative MK promoter-driven *HSK-TK* gene was inserted in an antisense orientation opposite to that of the retroviral promoter (Fig. 4A).[19, 20] The plasmid was then transfected into TE13, TE1 and A875 cells, and the pooled G418-resistant cells were tested for their sensitivity to GCV. The transfected TE13 and TE1 cells showed increased sensitivity compared with the respective parent cells (Fig. 4, B and C), whereas the sensitivity of MK-negative A875 cells remained the same as that of untransfectecd cells (Fig. 4D). In each experiment, the cells transfected with control DNA bearing the *HSV-TK* gene without the putative MK promoter showed

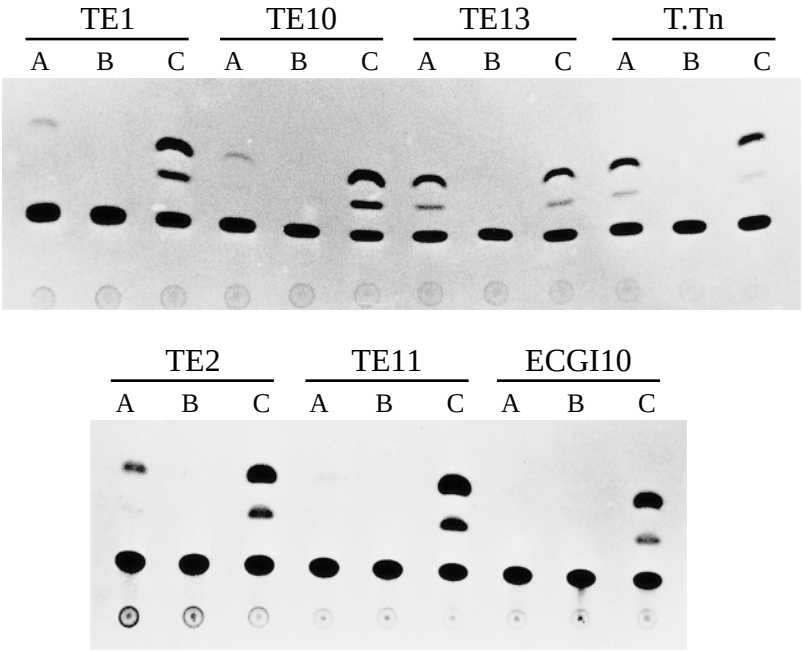

Fig. 3. Autoradiogram of CAT assay in human esophageal cell lines. Each cell line was transfected with putative MK promoter-driven (A), promoter-less (B) or SV40 promoter-driven (C) CAT gene.

the same sensitivity as found in wild-type cells (data not shown).

DISCUSSION

In this study we have shown that human esophageal tumors express MK. At the transcriptional level all the human esophageal cancer cell lines tested expressed a large amount of MK mRNA compared with a melanoma and a fibroblast line (Fig. 2). In immunohistochemical staining, more than half of the human specimens from esophageal tumors reacted with anti-MK antibody, in contrast to the surrounding normal esophageal tissues (Fig. 1). Aridome *et al.* analyzed the MK expression in 2 human esophageal tumors and detected the transcript in tumors, but not in non-cancerous regions.[12] We did not find any correlation between the MK expression in esophageal tumors and the clinical outcome of the patients (data not shown). However, high MK expression in tumors was shown to be a poor prognostic marker in neuroblastoma[8] and bladder cancer patients.[10] Further investigations are necessary to assess the clinical value of the MK expression in esophageal cancer.

Recently we have shown that forced expression of the *MK* gene could transform NIH3T3 cells[21] and that MK expression was elevated in the early stage of carcinogenesis in human colorectal cancer.[22] However, the biological significance of the MK expression in tumor cells remains unclear. Nevertheless, the 5′-flanking region of the *MK* gene could be useful for tumor-specific expression. Usefulness of the expression of the *HSV-TK* gene in tumors, followed by GCV administration, has been investigated in various animal models[23, 24] and its clinical application is being tested.[25] We found that the 2.3 kb-upstream region of the *MK* gene contained a promoter activity which could initiate transcription of the fused CAT gene in esophageal cancer cell lines (Fig. 3). Among the lines tested, two (TE13, T.Tn) showed strong transcriptional activity which was comparable to that of the SV40 promoter. A cytotoxic effect caused by GCV was seen in 2 cell lines, TE13 and TE1, when they were transfected with the *HSV-TK* gene driven by the MK promoter (Fig. 4, B and C). However, their CAT activity generated by the putative MK promoter was not correlated with their sensitivity (Fig. 3). A low level of promoter activity may be enough to transcribe the *HSV-TK* gene and to convert GCV to phosphorylated GCV. The specificity of MK-based transcription was confirmed by the finding that MK-negative A875 cells did not show increased sensitivity to GCV after transfection with MK-driven *HSV-TK* gene (Fig. 4D). We observed discordant results between the amount of transcript and the promoter activity in esophageal cancer cell

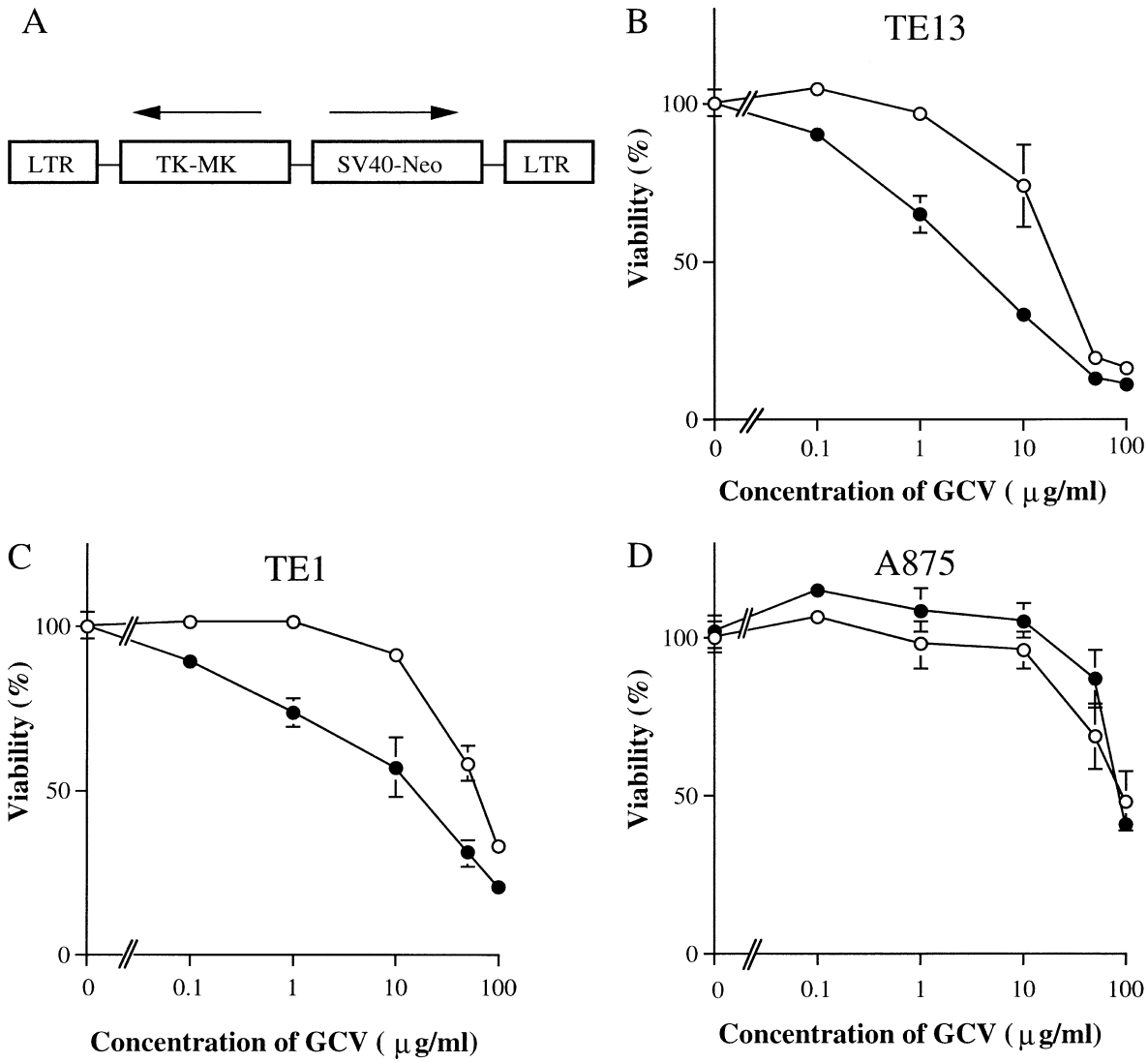

Fig. 4. *In vitro* sensitivity to various concentrations of GCV. (A) The plasmid construct used for transfection. The arrows indicate the orientation of the transcripts. Three samples of wild-type cells (○) or cells transfected with the *MK* gene-driven *HSV-TK* gene (●) in each group [TE13 (B), TE1 (C) or A875 (D)] were tested. Standard error bars are also shown.

lines (Figs. 2 and 3). Since the CAT assay solely detects *cis*-acting elements, the steady-state level of the transcript may be influenced by other elements.

Analysis of the promoter region of the *MK* gene revealed that it contained a retinoic acid-responsive element.[26] Upregulation of the transcriptional activity by retinoic acid can be observed in tumor cells. The expression of the *WT1* gene, a tumor suppressor gene identified in Wilms' tumor, was shown to suppress the MK expression and two possible WT1 binding sites were identified in the promoter region.[27] This is an intriguing clue to the mechanism of elevated expression of the *MK* gene in Wilms' tumor.[6] However, the expression of the *WT1* gene is restricted,[28] and expression in the esophagus has not been reported. Recently, loss of WT1 expression in colon carcinoma and in breast cancer, whose MK expression was elevated,[7, 12] were demonstrated.[29, 30] Therefore, the biological relevance of the expression of WT1 and that of MK requires further study.

Esophageal tumors occur frequently in aged patients, in whom intensive therapeutic maneuvers may be inappropriate. Several strategies such as combinations of conven-

tional therapies have had limited success in improving the prognosis and the quality of life of patients. The present study suggests a possible gene therapy using a suicide gene/prodrug system. Tumor-specific expression of suicide gene(s) followed by the administration of prodrug(s) can destroy tumor cells without causing serious damage to normal tissues. Obstruction of the esophagus, a complication often found in patients, might be treatable in part by endoscopic injection of vectors bearing the MK promoter-driven suicide gene(s) and non-toxic prodrug administration. The treatment is less invasive than surgery, and should alleviate the patient's discomfort. Gene therapy with tumor specificity achieved by the usage of the MK promoter, whose activity may be enhanced by retinoic acid, seems to be worth testing in patients with esophageal cancer.

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