

Efficacy of an Angiogenesis Inhibitor, TNP-470, in Xenotransplanted Human Colorectal Cancer with High Metastatic Potential

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BACKGROUND. The summation of gene mutations increases the metastatic potential of colorectal cancer. The genetic characterization and hepatic metastatic potential of five xenotransplanted human colon carcinoma strains were investigated. Furthermore, the therapeutic effect of the angiogenesis inhibitor, TNP-470, was evaluated.

METHODS. The correlation between gene mutation and rate of hepatic metastases of five colon cancer strains transplanted orthotopically or subcutaneously was evaluated. The strain with the highest hepatic metastatic rate from orthotopical tumors, TK-4, was used in the experiment with TNP-470 treatment. Mice were given tumor transplants orthotopically or subcutaneously followed by 30 mg/kg of TNP-470 on alternate days from Day 10 or Day 21 after transplantation, respectively.

RESULTS. The rate of hepatic metastases from orthotopically transplanted tumors of 5 strains was 38 to 79%. Interestingly, TK-4 with *K-ras* and p53 mutations and overexpression of p53 protein induced hepatic metastases from both orthotopical (79%) and subcutaneous tumors (44%). Although TNP-470 only significantly inhibited subcutaneous tumor growth, its antimetastatic effect was significantly demonstrated on the hepatic metastases of both orthotopical and subcutaneous tumors.

CONCLUSION. p53 mutation is thought to enhance angiogenesis, favoring the growth of hepatic metastases. TNP-470 proved the excellent antimetastatic effect of TK-4 on hepatic metastases. TK-4 has the highest metastatic potential and p53 mutation. An antiproliferative effect was observed on the rapidly growing primary tumors in which angiogenesis may be dominant. *Cancer* 1996; 77:1736-40.

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KEYWORDS: colorectal cancer, *K-ras*, p53, DCC, angiogenesis, TNP-470, doubling time, orthotopical transplantation, hepatic metastases.

Hepatic metastases are one of the most common forms of recurrence of colorectal cancer. The prognosis for patients with hepatic metastases is dismal. Thus, prevention is needed to improve prognosis.

The genetic basis of colorectal cancer and the correlation between gene mutations and metastases or prognosis have been investigated.¹ The mutation of p53 is considered to influence angiogenesis.²⁻⁴ In this respect, an angiogenesis inhibitor, TNP-470, has attracted considerable interest. TNP-470 is an analog of fumagillin derived from *Aspergillus fumigatus*.^{5,6} It has been reported to show both antiproliferative and antimetastatic effects on various human or rodent tumors.⁷⁻⁹ We also reported the therapeutic effects of TNP-470 on orthotopically transplanted human colon cancer.^{10,11} In the present study, TK-4 was selected as a target tumor because it had mutant p53 and could induce hepatic metastases regard-

less of whether or not the tumor was transplanted orthotopically or subcutaneously. The antiproliferative effect of TNP-470 on both primary lesions, which had different doubling times, and its antimetastatic effect on hepatic metastases from both primary tumors (with different metastatic routes) was investigated.

MATERIALS AND METHODS

Animals

Male BALB/c nu/nu mice were obtained at age 4 weeks from Clea Japan, Inc. (Tokyo, Japan). The animals were used in this study at age 5 weeks.

Human Colorectal Cancer Strains

Five human colon cancer strains, TK series consisting of TK-3, -4, -9, -10, and -11, were established from hepatic metastatic lesions in our department and maintained by subcutaneous passage.

Preparation of TNP-470

The angiogenesis inhibitor TNP-470 (Takeda Chemical Industries, Ltd., Osaka, Japan) was suspended in a vehicle of 1% ethanol and 5% gum arabic in saline.

Gene Analysis

The tumor tissue from five strains, resected from the tumors transplanted subcutaneously, were immediately frozen in liquid nitrogen and later utilized for direct sequencing and RNA extraction. The exact mutations of *K-ras* and *p53* were determined by the direct nucleotide sequencing of abnormally migrating amplified products. mRNA expression of DCC in the strains was determined by reverse transcription polymerase chain reaction (RT-PCR) of DCC using the total RNA of the tumors. RT-PCR of DCC has been reported elsewhere.¹²

Immunohistochemistry

The modified antigen retrieval method¹³ was used to enhance the immunoreactivity of p53 protein in the paraffin sections of the tumor tissue from 5 strains transplanted subcutaneously. Briefly, the sections were boiled in distilled water for 10 minutes by microwaving. Sections were then incubated with DO7 (Novocastra Lab, Newcastle, U.K.) for 1 hour and incubated with biotinylated anti-mouse immunoglobulin followed by peroxide-conjugated streptavidin for 10 minutes at room temperature.

Metastatic Potential

Orthotopical transplantation

Small pieces of tumor tissue (5 mm in greatest dimension) were resected from the lesions transplanted subcutaneously in the exponential growth phase. These tumor pieces were sutured to the cecal wall of nude mice with 6-0 Dexon (Davis-Geck Inc., Manati, P.R.) after the serosa

TABLE 1
Metastatic Rate of Human Colon Cancer Strains

Strain	Hepatic metastases			
	Orthotopical transplantation	n	Subcutaneous transplantation	n
TK-3	68%	28	0%	16
TK-4*	79%	28	44%	16
TK-9	64%	25	0%	12
TK-10	50%	12	0%	12
TK-11	38%	13	0%	12

*Strain used in the present study.

was removed. Six weeks after transplantation, hepatic metastases were identified both macroscopically and microscopically. The rate of hepatic metastases was expressed by dividing the number of mice with hepatic metastases by the total number of mice. The number of mice in each strain ranged from 12 to 28.

Subcutaneous transplantation

Small pieces of the tumor tissue were transplanted subcutaneously in the right axillary region. Hepatic metastases were identified 5 weeks after transplantation and the metastatic rate was calculated in the same manner as for the orthotopical transplantation. The number of mice in each strain ranged from 12 to 16.

Therapeutic Effect of TNP-470

In the experiment with TNP-470, TK-4 was used among 5 strains because it demonstrated the highest hepatic metastatic rate from orthotopical tumors and because it showed the only hepatic metastases from subcutaneous tumors. After transplantation, the mice were divided into a control group and a TNP-470 group. The mice with orthotopic transplants in the TNP-470 group (n = 10) received 0.2 mL of TNP-470 solution (30 mg/kg) subcutaneously and the control group (n = 9) received saline on alternate days from Day 10 after tumor transplantation. Treatment was continued until the mice were sacrificed 6 weeks after tumor transplantation and hepatic metastases were evaluated both macroscopically and histologically. The transplanted tumors were also removed and weighed. The mice with subcutaneous transplants in the TNP-470 group (n = 10) received 0.2 mL of TNP solution (30 mg/kg) subcutaneously and the control group (n = 9) received saline on alternate days from Day 21 after tumor transplantation. The mice were sacrificed 5 weeks after tumor transplantation and were evaluated in the same manner as those with orthotopic transplants.

TABLE 2
Gene Analysis of Human Colon Cancer Strains

Strain	K-ras sequence	p53 sequence	p53 staining	DCC mRNA
TK-3	codon 13 GGC → GAC	—	—	not detected
TK-4	codon 12 GGT → GAT	exon 7 codon 248 CGG → TGG	diffuse	+
TK-9	—	—	—	not detected
TK-10	codon 13 GGT → GTT	—	sparse	+
TK-11	—	—	—	+

Statistical Analysis

The Student's *t* test was used to compare actual tumor weight with estimated tumor weight with body weight. The chi-square test was used to compare the number of mice with hepatic metastases in each group.

RESULTS

Rate of Hepatic Metastases

Table 1 shows the rates of hepatic metastases in the TK series. For the tumors transplanted orthotopically, the rates of hepatic metastases ranged from 38 to 79%. Hepatic metastases from subcutaneous tumors were observed only in TK-4 and the metastatic rate was 44%.

Doubling Time of TK-4

Doubling time for the primary tumors was calculated by dividing the actual tumor weight at sacrifice by the initial tumor weight at implantation. The doubling time of TK-4 was 10.56 days for tumors transplanted orthotopically (*n* = 28) and 5.68 days for those transplanted subcutaneously (*n* = 16).

Gene Analysis of Strains

Table 2 shows the occurrence of K-ras and p53 mutation and the expression of DCC mRNA in the TK series. K-ras mutation was demonstrated in 3 out of 5 strains and p53 mutation was demonstrated in TK-4. mRNA expression of DCC was detected in 3 strains.

Immunohistochemical Staining

Strong immunoreactivity and diffuse staining were observed in TK-4 (Fig. 1). Sparse staining was observed in TK-10, whereas no staining was demonstrated in the other 3 strains.

Antiproliferative effect of TNP-470

Table 3 shows actual tumor weight at sacrifice. Although TNP-470 had no antiproliferative effect on orthotopically transplanted tumors, it did significantly inhibit the growth of subcutaneously transplanted tumors.

Antimetastatic Effect of TNP-470

Table 4 shows the effect of TNP-470 on hepatic metastases. Administration of TNP-470 significantly inhibited he-

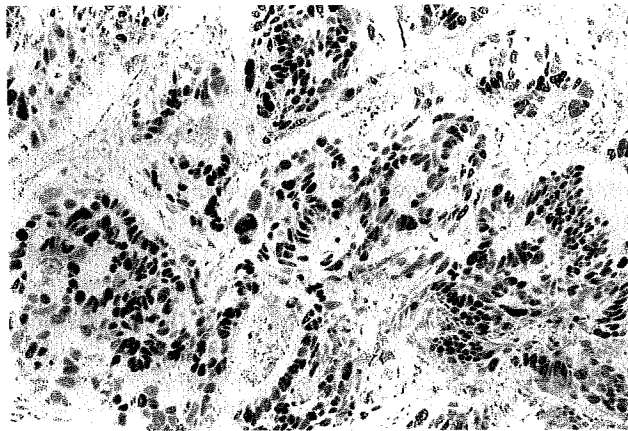


FIGURE 1. Immunohistochemical staining of tumor tissue with TK-4 transplanted subcutaneously. Diffuse staining with strong intensity of p53 protein was demonstrated (original magnification $\times 200$).

patic metastases in tumors transplanted by both methods. All of the metastatic lesions were confirmed histologically.

Body Weight Loss

In the orthotopical experiment, there was a significant loss of body weight at sacrifice (19.2 ± 1.65 g for control vs. 17.0 ± 2.01 g for TNP-470 group). In the subcutaneous experiment, no significant loss was observed (20.0 ± 1.32 g for control vs. 19.6 ± 1.41 g for TNP-470 group).

DISCUSSION

In the present study, orthotopic transplantation was used to evaluate the metastatic potential of colorectal cancer strains since it was conducive for establishing hepatic metastases.¹⁴⁻¹⁶ The metastatic foci of the strains were easily detected macroscopically and were confirmed histologically. The metastatic potential of the 5 strains was evaluated simply by counting the number of mice with hepatic metastases at sacrifice, because the number of metastatic foci were similar at this time (data not shown). Differences in the duration of occurrence of hepatic metastases among strains after transplantation could not be evaluated in the present experiment.

TABLE 3
Antiproliferative Effect of TNP-470 on Tumor Growth

Group	Control (mg)	TNP-470 (mg)
Orthotopical tumors	619 ± 236	625 ± 247
Subcutaneous tumors	1139 ± 436	687 ± 189 ^a

^a $P < 0.05$.

TABLE 4
Inhibitory Effect of TNP-470 on Hepatic Metastases

Primary tumor	Control	TNP-470
Orthotopical transplantation	7/9	1/10 ^a
Subcutaneous transplantation	4/9	0/10 ^b

^a $P < 0.01$.

^b $P < 0.05$.

The numbers represent the amount of mice with hepatic metastases.

Among common human cancers, genetic implication in carcinogenesis is well explained for colorectal cancer. As for the summation of gene abnormalities, development of colorectal tumor was postulated by Vogelstein, et al.¹⁷ Tumor progression is considered to be a successive clonal expansion. The summation of gene abnormalities increases malignant potential. In the present study, TK-4 had high metastatic potential (79% from orthotopical tumors and 44% from subcutaneous tumors) and both *K-ras* and p53 mutation, however, it expressed mRNA of DCC. With immunohistochemical staining, diffuse overexpression of mutant p53 protein was demonstrated in TK-4, sparse distribution of immunoreactive was observed in TK-10, and no p53 mutation was detected. It is possible that the immunoreactivity to p53 antibody of the cells was not due to p53 mutation, it was instead due to heterogeneous control of p53 expression in tumor cells.¹⁸ Because the pattern of immunoreactivity to p53 was the same in 3 strains (including TK-4) as the original primary tumors, hepatic metastatic lesions, and xenotransplanted tumors (unpublished data), it was maintained after they were transplanted into nude mice. Tumor metastases occurs by a complex process that includes angiogenesis.¹⁹ The production of angiogenic factors by tumor cells or host cells leads to degradation of the basement membrane, followed by endothelial cell migration and proliferation,²⁰ and the formation of tubular structures. It was reported that p53 mutation induced angiogenesis in brain tumors² and induced vascular endothelial growth factor (VEGF) production.³ It was also reported that loss of wild-type allele of p53 reduced the expression of thrombos-

pondin-1 in fibroblasts.⁴ Thus, p53 mutation may enhance the angiogenesis of tumors. Further studies should be carried out to clarify the role of angiogenic factors such as VEGF and basic fibroblast growth factor, and to identify the angiogenesis inhibitor in the enhancement of metastatic potential of colorectal cancer induced by p53 mutation.

TNP-470 specifically inhibits the formation of the capillary-like structures in angiogenesis.⁶ TNP-470 may inhibit both tumor growth and metastases by means of its antiangiogenic activity. The present study showed that TNP-470 has an antiproliferative effect on subcutaneous tumors, whereas no antiproliferative effect was observed on tumors transplanted orthotopically. It has been reported that the administration of TNP-470 has an antiproliferative effect on subcutaneous rodent tumors and human tumor cell lines,^{8,9} so TNP-470 may affect orthotopical tumors at a higher dose. However, the total dose of TNP-470 administered in the orthotopical tumor experiment was about three-fold of that used in the present experiment with subcutaneous tumors. Accordingly, our results suggest that the tumor is more sensitive to TNP-470 when transplanted subcutaneously than when transplanted orthotopically. Yamaoka et al.⁸ concluded that the inhibitory effect of TNP-470 on tumor growth was not mediated by direct cytotoxic activity, but by the inhibition of angiogenesis. In general, the growth of solid tumors depends on angiogenesis²¹ and a larger blood supply is required for rapidly growing tumors. In the present study, the doubling time of orthotopical tumors was about twice that of subcutaneous tumors, which may be why the antiproliferative effect of TNP-470 was greater in subcutaneous tumors in which angiogenesis may play a greater role. Kim et al performed a study using an antivascular endothelial growth factor antibody and found that the response of rapid proliferating tumors to the antibody was greater.²² Their results are consistent with our findings. Thus, in tumors that grow rapidly, TNP-470 can be expected to demonstrate an antiproliferative effect by means of its antiangiogenic activity.

An antimetastatic effect of TNP-470 on hepatic metastases was clearly demonstrated in the present study. Besides hepatic metastases, 1 out of 9 mice in the control group had metastases of the paraaortic lymph nodes in the experiment of orthotopical tumors and lung metastases in the experiment of subcutaneous tumors, respectively. In the TNP-470 group of both experiments, neither lymph nodes metastases nor lung metastases were demonstrated. The inhibition of angiogenesis has the following beneficial effects to inhibit metastases: (1) an antiproliferative effect on the primary tumor which decreases the number of tumor cells migrating into the tumor vessels; (2) a decrease in the production of proteases and other factors produced from endothelial cells that en-

hance metastases; and (3) the inhibition of angiogenesis in the target organ. It has been reported that one endothelial cell can support more than 100 tumor cells.²³ Since the present results revealed that TNP-470 inhibited hepatic metastases independently of its inhibitory effect on the growth of primary tumors, and since angiogenesis in micrometastatic foci may be more active than that in primary tumors, it is suggested that the antiangiogenic effect of TNP-470 on micrometastatic lesions mainly induces the antimetastatic effect. Further studies, especially histochemical investigations, are needed to clarify the mechanism of TNP-470 on metastases.

The major adverse effect of TNP-470 appears to be weight loss, which was also demonstrated in the previous study.¹¹ In the present study, 30 mg/kg of TNP-470 suppressed body weight gain in the mice transplanted orthotopically, and this toxicity appeared to be dose-dependent, since it was much higher in the orthotopical tumor experiment than in the subcutaneous experiment.

In conclusion, TNP-470 showed an excellent antimetastatic effect on hepatic metastases of colorectal cancer, and an antiproliferative effect on primary tumors was only observed in rapidly growing tumors.

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