

Original paper

Title:

Oxidative Stress and Tumor Progression in Colorectal Cancer

Running title: Oxidative stress in colorectal cancer

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Acknowledgement of any financial support: none

A list of key words:

reactive oxygen species (ROS), thymidine phosphorylase (TP), colorectal cancer,
derivative-reactive oxygen metabolites (d-ROM)

A list of all abbreviations used:

Reactive Oxygen Species (ROS); Thymidine Phosphorylase (TP); Derivative-Reactive
Oxygen Metabolites (d-ROM)

ABSTRACT

Background / Aims: Elevated oxidative status has been found in many types of cancer cells. Recent studies have shown that the enzymatic product of thymidine phosphorylase (TP) generated reactive oxygen species (ROS) within cancer cells. The aim of this study was thus to evaluate the signal transduction pathway and the role of ROS in colorectal cancer. **Methodology:** Blood specimens were obtained from the drainage vein of the tumor during operation in 76 patients with colorectal cancer. Serum ROS levels were measured using the derivative-Reactive Oxygen Metabolites (d-ROM) test and serum TP levels were examined by a highly sensitive ELISA method. **Results:** There was no significant correlation between serum levels of ROS and TP. Serum ROS levels were elevated in proportion to tumor invasion and had a significant positive correlation with tumor size ($p < 0.05$). However, they did not increase in patients with liver metastasis. **Conclusions:** These findings suggest that ROS are independent of TP-triggered signaling transduction and are associated with increased tumor invasion, but not liver metastasis in patients with colorectal cancer. From this point of view, new strategies related to ROS may provide improved therapeutic results as well as a preventative effect on carcinogenesis of the colorectum.

INTRODUCTION

Reactive oxygen species (ROS) include superoxide radical anion (O_2^-), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) and play important roles in cell growth and intracellular signal transduction pathways (1-5). Excessive production of intracellular ROS has been reported to result in an oxidative environment which damages cellular molecules or modulates gene expression (6-8).

The colon is constantly exposed to ROS originating from endogenous and exogenous sources (9). It is well known that enhanced oxidative stress of the colon is associated with an increased risk of cancer (10-12). In addition, ROS have been proposed to be involved in tumor metastasis, which is a complicated processes including epithelial-mesenchymal transition (EMT), migration, invasion of the tumor cells and angiogenesis (13, 14). A more comprehensive understanding of ROS-triggered signaling transduction, transcriptional activation and regulation of gene expression will help strengthen our understanding of the critical role of ROS in tumor progression.

Recent studies have reported that the enzymatic product of thymidine phosphorylase (TP), an enzyme involved in pyrimidine metabolism, generates ROS within a TP-overexpressing cancer cell and free radical stress increases tumor cell production of

angiogenic factors and an intestinal collagenase (14-16). In our previous studies, we showed that the serum TP levels in venous blood drainage specimens reflect the prognosis of patients with colorectal cancer, particularly the risk of liver metastasis (17, 18). However, it remains unclear why the serum TP levels are elevated in patients with liver metastasis. Accordingly, the purpose of the present study was to investigate whether an oxidative stress mechanism is regulated by TP and to evaluate the role of ROS in colorectal tumor progression.

METHODOLOGY

Patients and Tumor Samples

Seventy-six patients (47 males and 29 females) with colorectal cancer, who underwent surgery between June 1997 and January 2002 in the Department of Surgery, Nagasaki University, were enrolled as subjects (**Table 1**). None had received chemotherapy or irradiation prior to surgery. Twelve patients had liver metastases (10 patients with synchronous metastasis and 2 patients with metachronous metastasis). The average age at surgery was 65.3 years (range, 34-87 years). The average follow-up period was 89.4 months. The extent of tumor invasion was based on TNM classification. Five patients

were classified as T1, 7 as T2, 55 as T3, and 9 as T4. Thirty-three patients had lymph node metastases. Serum samples were collected after obtaining informed consent from each patient in accordance with institutional guidelines. All patients had histologically verified adenocarcinoma of the colon or rectum.

Clinical Follow-Up

All patients were followed up after discharge by physical examination, routine serum chemistries and serum tumor marker tests every 1-3 months and by abdominal ultrasonography and computed tomography every 3-6 months.

Measurement of the Serum TP Levels

The serum samples were obtained from venous blood drainage specimens of the primary tumor immediately after laparotomy, and then were stored at -80°C until use. The serum TP levels were determined by enzyme-linked immunosorbent assay (ELISA) using the previously reported method with the following modifications (17, 18). The same pair of monoclonal antibodies - 104B and 232-2 - was used, but 232-2 was labeled with biotin and its binding was detected by peroxidase-conjugated streptavidin. Due to these modifications, the accuracy of the ELISA was improved 25-fold in comparison to

the original procedure. In serum from the peripheral blood of 16 healthy volunteers, the mean TP level was 14.1 ± 5.2 ng/ml.

Measurement of the Serum d-ROM Levels

The oxidative status was studied by measuring hydroperoxides in the serum using the d-ROM (derivatives of reactive oxygen metabolites) test. The test was carried out using the Free Radical Analytical System 4 (FRAS4; Wismerll Co. Ltd. Tokyo, Japan). The test is based on the concept that the amount of organic hydroperoxides present in serum is related to the free radicals from which they are formed. When the serum sample is dissolved in an acidic buffer, the hydroperoxides react with the transition metal ions liberated from the proteins in the acidic medium and are converted to alkoxy and peroxy radicals. These newly formed radicals are able to oxidize an additive (N, N-diethyl-para-phenyldiamine) to the corresponding radical cation. It was reported that the value of serum ROS had a high positive correlation with the serum d-ROM value of measured by the d-ROM test (19-21). The concentration of this persistent species can be easily determined through spectrophotometric procedures (absorption at 505 nm), and are generally expressed in conventional units (Carratelli units; U.CARR), in which 1U.CARR corresponds to $0.8\text{mg}/\text{H}_2\text{O}_2$. The normal values of the test are less

than 300U.CARR. Values >300U.CARR indicate a condition of oxidative stress (301-320U.CARR: borderline, 321-340U.CARR: mild oxidative stress, 341-400U.CARR: moderate oxidative stress, 401-500U.CARR: strong oxidative stress, 501U.CARR and higher: very strong oxidative stress).

Statistical Analyses

All data were expressed as the means \pm standard deviation. The Mann-Whitney test was used to determine statistically significant differences between two groups. Spearman's correlation was used to examine correlations. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Relationship between the serum levels of d-ROM and TP

The serum d-ROM and TP levels in venous blood drainage specimens were successfully measured in all patients in this study. The mean of the serum d-ROM levels was 317.4 ± 68.6 U.CARR, and the mean of the serum TP levels was 52.1 ± 51.9 ng/ml. As shown in **Figure 1**, the serum d-ROM levels were not significantly correlated with

the serum TP levels ($p=0.77$).

Relationship between the serum d-ROM and TP levels and tumor growth

First, the correlation between the serum d-ROM or TP levels and the clinicopathologic features was evaluated. A statistically significant correlation was observed between the serum d-ROM levels and tumor size ($p=0.036$). The serum d-ROM levels were 329.9 ± 77.1 U.CARR in patients with a tumor of more than 40mm and 301.0 ± 52.5 U.CARR in patients with a tumor of less than 40mm (**Figure 2A**). No significant correlation was noted between the serum TP levels and tumor size (**Figure 2B**).

The serum d-ROM levels of each depth of invasion group are shown in **Figure 3A**. The serum d-ROM levels were 291.4 ± 78.8 U.CARR in the T1 group, 305.1 ± 38.1 U.CARR in the T2 group, 320.1 ± 72.8 U.CARR in the T3 group, and 324.9 ± 59.1 U.CARR in the T4 group. As the tumor invasion progressed, there was a trend toward a greater increase in the serum d-ROM levels. On the other hand, there was no correlation between the serum TP levels and the depth of invasion (**Figure 3B**). None of the other variables, including age, gender, tumor site, and lymph node metastasis, showed a statistically significant correlation with the serum d-ROM or TP levels.

Serum d-ROM and TP levels and liver metastasis

The serum d-ROM levels in the 12 patients with liver metastasis were not significantly correlated with those in the 64 patients without liver metastasis (326.1 ± 73.5 U.CARR vs. 315.8 ± 68.2 U.CARR) (**Figure 4A**).

On the other hand, the serum TP levels in the patients with liver metastasis were significantly higher than those in the patients without liver metastasis (111.6 ± 81.1 ng/ml versus 40.9 ± 35.5 ng/ml: $p < 0.001$) (**Figure 4B**).

DISCUSSION

We previously indicated that serum TP levels were significantly elevated in colorectal cancer patients with liver metastasis and reflected the prognosis (17, 18). TP, which is identical to platelet-derived endothelial cell growth factor, is an angiogenic factor and catalyzes the reversible phosphorolysis of thymidine, deoxyuridine and their analogs (22-24). However, it remains uncertain how TP associates with the cascade of liver metastasis in colorectal cancer.

Recent studies have reported that 2-deoxy-D-ribose-1-phosphate (2dDR1P) released from thymidine by TP generates oxygen radicals, and that free radical oxidative stress causes angiogenesis and metastasis (14, 16). ROS can stimulate cell proliferation,

promote genetic instability, and induce adaptive responses that enable cancer cells to maintain their malignant phenotypes. ROS-mediated DNA damage has long been thought to play an important role in carcinogenesis initiation and malignant transformation by leading to mutations in tumor suppressor genes (25). The gastrointestinal tract, especially the colon, is constantly exposed to ROS originating from endogenous and exogenous sources, and ROS have been associated with an increased risk of colon cancer (14, 26).

Therefore, we hypothesized that a TP-triggered signal transduction pathway plays a role in the liver metastasis of patients with colorectal cancer. The mechanism of this association would proceed as follows. (1)The downstream mediators of TP function give rise to oxidative stress. (2)ROS increase tumor cell production of the angiogenic factors interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF), in addition to inducing production of matrix metalloproteinase-1 (MMP-1). This gives rise to (3)liver metastasis of the colorectal cancer.

To examine this hypothesis, we measured serum ROS levels using the d-ROM test and compared them with serum TP levels. The results showed that there was no significant correlation between the serum levels of ROS and TP. In addition, serum ROS levels were not significantly elevated in colorectal cancer patients with liver metastasis. In

contrast, serum ROS levels were elevated in proportion to tumor invasion and had a significant positive correlation with tumor size. These results suggest that TP may be involved in liver metastasis by regulating another intracellular signal transduction, and that the mechanism of ROS-triggered signal transduction has relation to tumor invasion, but not tumor metastasis in colorectal cancer (**Figure 5**). In fact, with respect to factors other than TP, several previous reports have demonstrated that ROS generation may be induced intracellularly, in either an NADPH oxidase-dependent or a mitochondria-dependent manner, by growth factors and cytokines (TGFbeta1 and HGF) and tumor promoters (such as TPA) capable of triggering cell adhesion, EMT and migration (25, 27, 28).

In conclusion, it may be possible to prevent tumor progression by regulating ROS, which enhance cell proliferation and apoptosis suppression. For example, one possible therapeutic strategy targeting ROS-triggered signal transduction would be to increase ROS scavenging, thereby dampening H₂O₂ signaling and depressing tumor growth. In general, clarification of the true role of ROS in various cancers will lead to new strategies for cancer prevention and therapy.

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Figure legends

Figure 1 Correlation between the serum thymidine phosphorylase (TP) levels and the serum derivatives of reactive oxygen metabolites (d-ROM) levels in venous blood drainage specimens from colorectal cancer patients.

Figure 2 Relationship between the serum derivatives of reactive oxygen metabolites (d-ROM) levels (A) and the serum thymidine phosphorylase (TP) levels (B) and tumor size. Data points represent the means \pm standard deviation. NS, not significant.

Figure 3 Relationship between the serum derivatives of reactive oxygen metabolites (d-ROM) levels (A) and the serum thymidine phosphorylase (TP) levels (B) and depth of invasion of colorectal cancer. *Bars* represent the standard deviation.

Figure 4 Plot depicting the serum derivatives of reactive oxygen metabolites (d-ROM) levels (A) and the serum thymidine phosphorylase (TP) levels (B) according to presence or absence of liver metastasis. *Points* (○) are the observed serum d-ROM or TP levels in individual patients. *Bars* are the mean values for each group.

Figure 5 Schematic representation of the roles of thymidine phosphorylase (TP) and reactive oxygen species (ROS) in tumorigenesis.

Table 1. Patient characteristics

Characteristics	No. of patients	%
Age (yr)		
Median	65.3	
Range	34–87	
Gender		
Male	47	62
Female	29	38
Tumor site		
Colon	41	54
Rectum	35	46
Depth of invasion		
T1	5	7
T2	7	9
T3	55	72
T4	9	12
Tumor size		
≤ 40 mm	33	43
> 40 mm	43	57
Lymph nodes metastasis		
(–)	43	57
(+)	33	43
Liver metastasis		
(–)	64	84
(+)	12	16

Figure 1

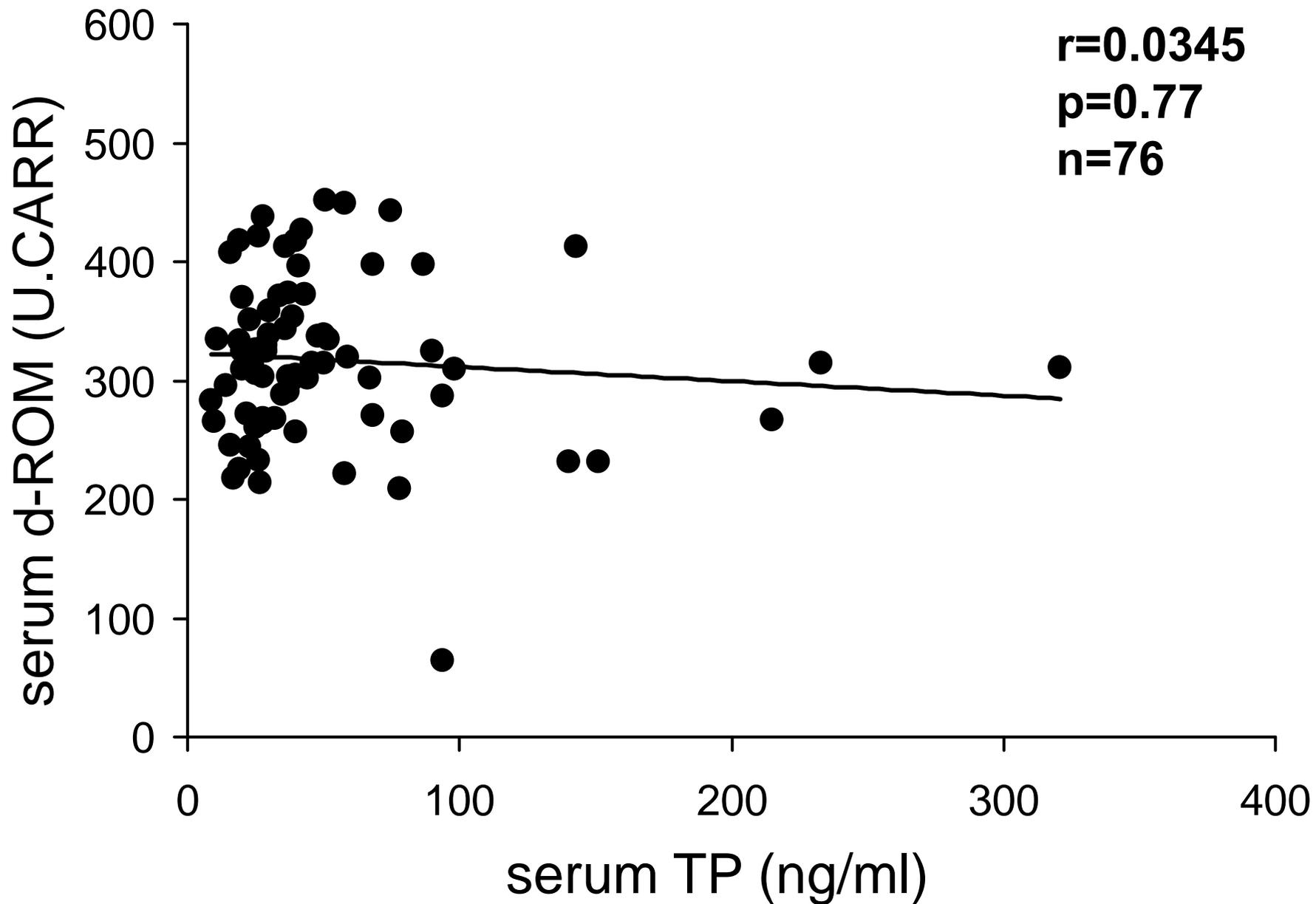
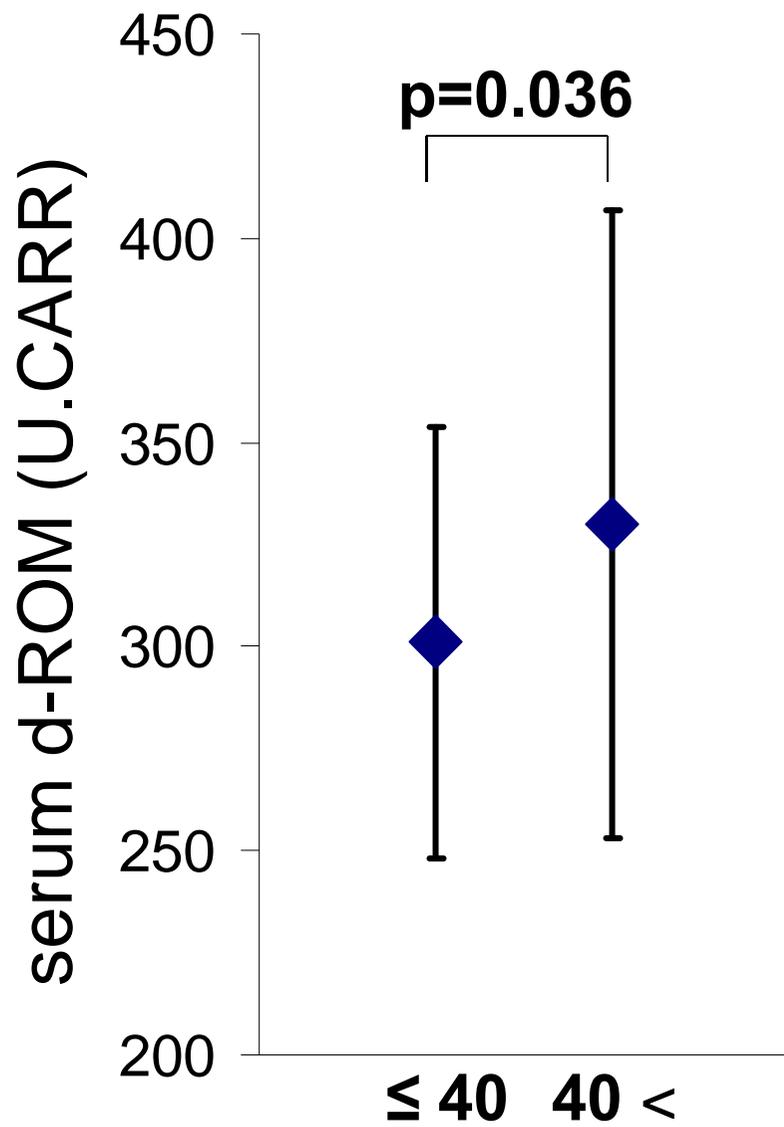
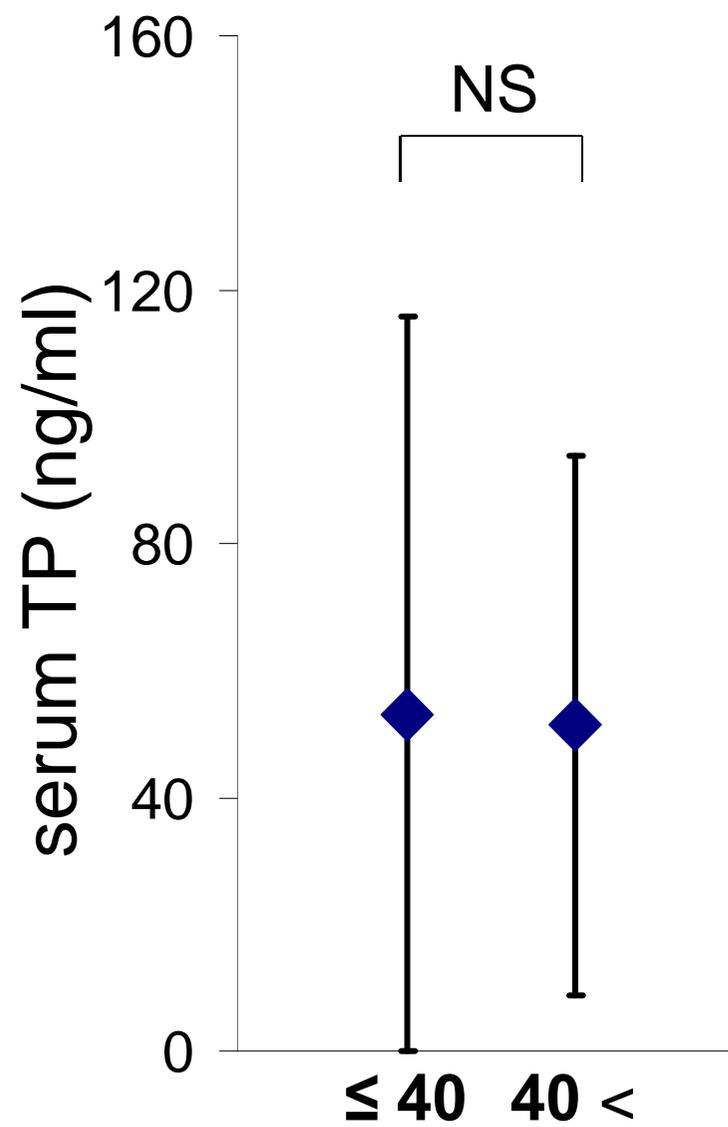


Figure 2

A



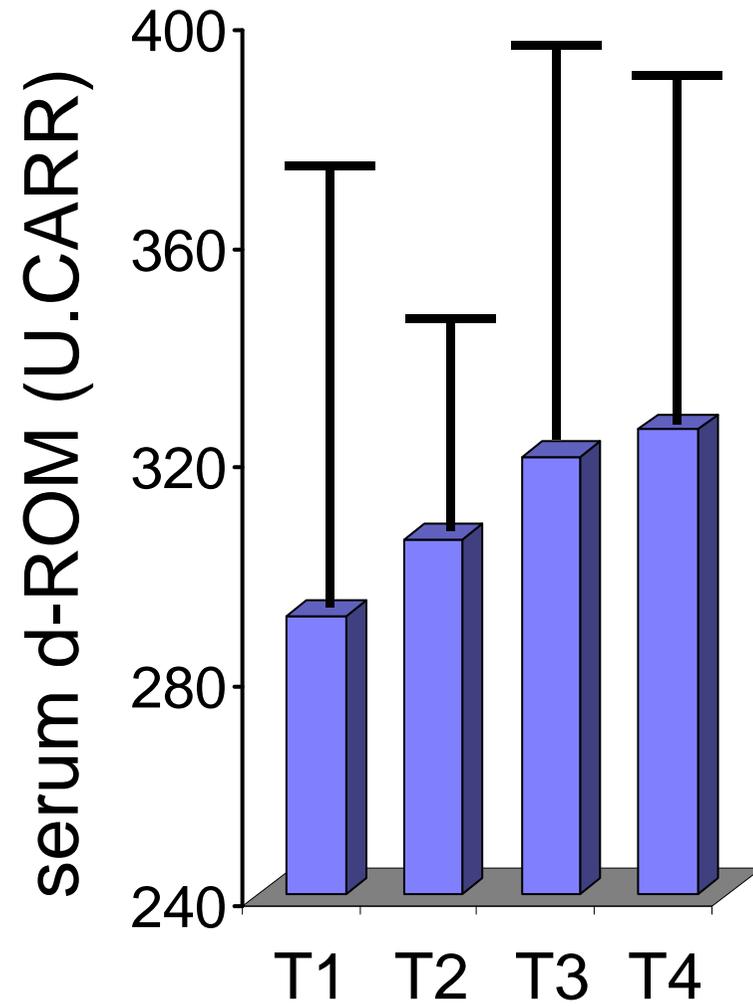
B



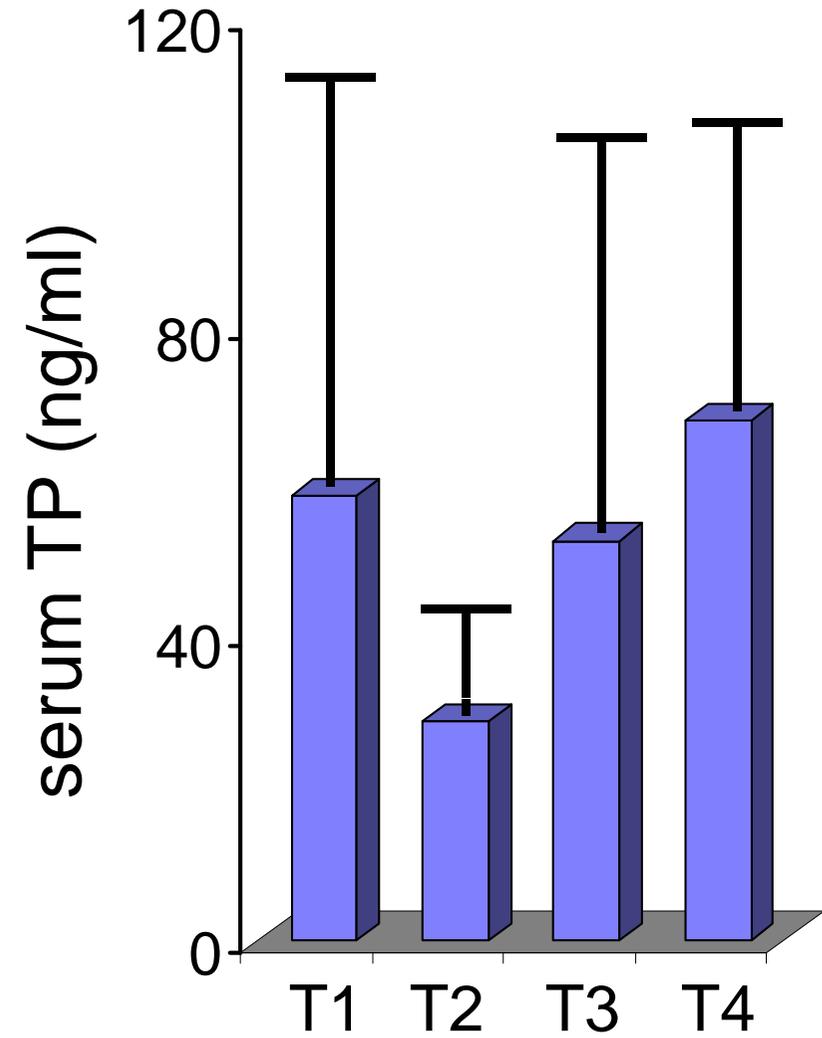
Tumor size (mm)

Figure 3

A



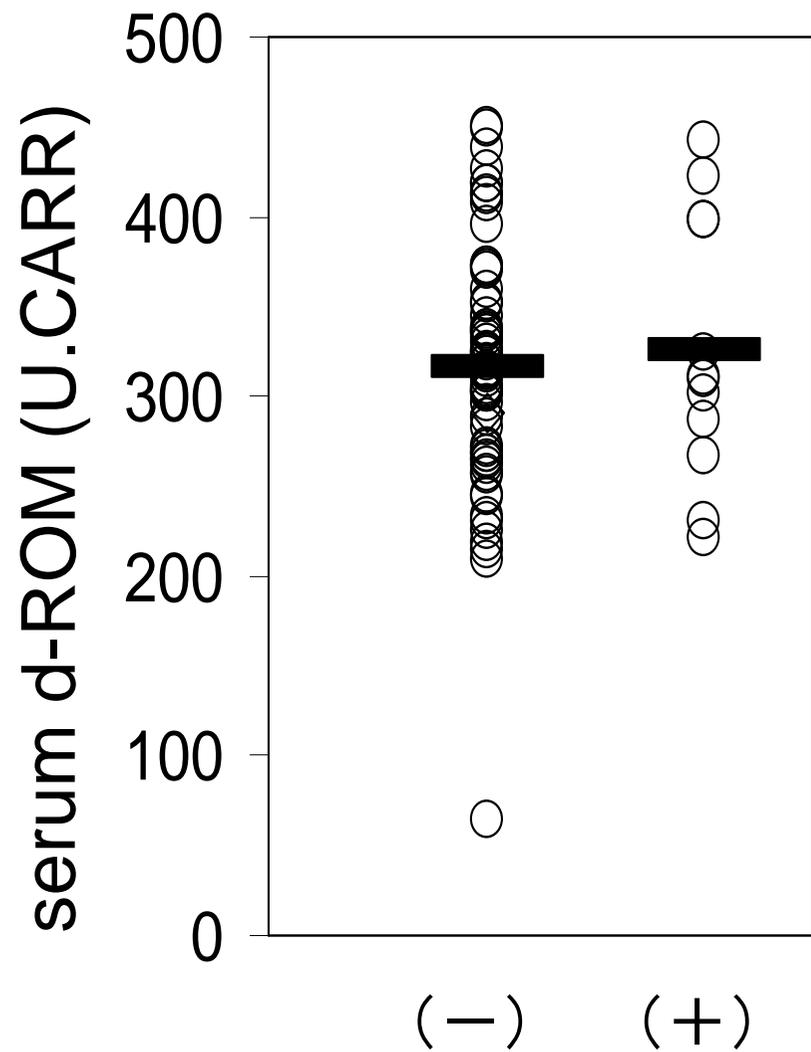
B



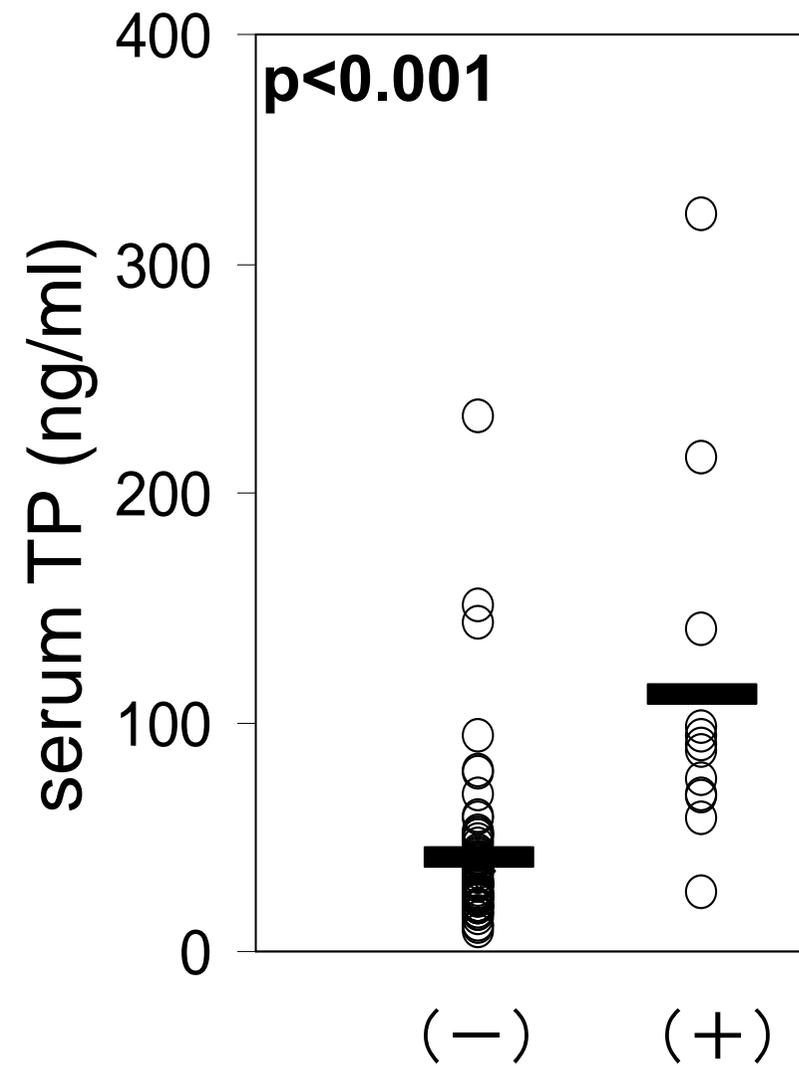
Depth of invasion

Figure 4

A



B



liver metastasis

Figure 5

