

• LIVER CANCER •

Thrombocytosis: A paraneoplastic syndrome in patients with hepatocellular carcinoma

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Abstract

AIM: Hepatocellular carcinoma (HCC) patients manifest a variety of paraneoplastic syndromes. Thrombocytosis was reported in children with hepatoblastoma. The aims of this study were to evaluate the prevalence and clinical significance of thrombocytosis in HCC patients and its relationships with serum thrombopoietin (TPO).

METHODS: We retrospectively reviewed clinical, biochemical and image data of 1 154 HCC patients. In addition, we measured platelet count and serum TPO in HCC patients with and without thrombocytosis, in patients with cirrhosis, chronic hepatitis and healthy subjects in a cross-sectional study.

RESULTS: Thirty-one (2.7%) of 1 154 HCC patients had thrombocytosis (platelet count $\geq 400 \text{ K/mm}^3$). HCC patients with thrombocytosis were significantly younger, had a higher serum α -fetoprotein, higher rate of main portal vein thrombosis, larger tumor volume, shorter survival, and were less likely to receive therapy than HCC patients without thrombocytosis. Multivariate logistic regression analyses showed that tumor volumes $\geq 30\%$ and serum α -fetoprotein $\geq 140 000 \text{ ng/mL}$ could significantly predict thrombocytosis. HCC patients with thrombocytosis had a significantly higher mean serum TPO than those without, as well as patients with cirrhosis, chronic hepatitis and healthy subjects. Platelet count and serum TPO dropped significantly after tumor resection in HCC patients with thrombocytosis and re-elevated after tumor recurred.

Furthermore, the expression of TPO mRNA was found to be more in tumor tissues than in non-tumor tissues of liver in an HCC patient with thrombocytosis.

CONCLUSION: Thrombocytosis is a paraneoplastic syndrome of HCC patients due to the overproduction of TPO by HCC. It is frequently associated with a large tumor volume and high serum α -fetoprotein.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignancy in Taiwan. During its clinical course, patients may manifest a variety of paraneoplastic syndromes, including hypercholesterolemia, hypoglycemia, hypercalcemia, and erythrocytosis^[1]. According to our previous reports, the prevalence of paraneoplastic syndromes was 11.4-12.1% for hypercholesterolemia, 2.8-5.3% for hypoglycemia, 1.8-4.1% for hypercalcemia, and 2.5-3.1% for erythrocytosis^[2-6]. Thrombocytosis has been found in children with hepatoblastoma and other malignancies^[7-9]. The prevalence and clinical significance of thrombocytosis in adult patients with HCC have not been previously reported.

Human thrombopoietin (TPO), also known as megakaryocyte growth factor, is known to play a key role in the development of megakaryocytes^[10,11]. TPO is secreted principally by hepatocytes and bone marrow stromal cells^[10-14]. In addition, the expression of TPO gene has been found in both rat and human hepatoma cell lines^[15,16]. The relationships between serum TPO levels and platelet counts in HCC patients, especially those associated with thrombocytosis, are of clinical interest. Our aim was to evaluate the prevalence of thrombocytosis in Chinese patients with HCC in a retrospective study. The clinical, biochemical and image characteristics of HCC patients with thrombocytosis were evaluated. Moreover, in order to evaluate the role of serum TPO in the manifestations of thrombocytosis in HCC patients, serum TPO levels were measured in HCC patients (with and without thrombocytosis), cirrhotic patients, chronic hepatitis patients, and normal subjects in a cross-sectional study.

MATERIALS AND METHODS

The clinical, laboratory and image data were retrospectively reviewed in 1 269 consecutive patients diagnosed with HCC at the Division of Gastroenterology, Taipei Veterans General Hospital, from January 1991 to December 1994^[6]. Of these patients, 1 253 were enrolled in this study who met the following criteria: (1) histological proof of HCC; or (2) at least two typical HCC image findings (ultrasonography, computerized tomography, celiac angiography or magnetic resonance imaging) along with a serum AFP of more than 20 ng/mL. Among

the 1 253 patients, data of 1 197 cases were analyzed after excluding the patients with incomplete examination and data for analysis. Finally, data of 1 154 patients were selected for analyses in this study after excluding 43 patients with an evidence of acute infections or gastrointestinal bleeding. Patients with polycythemia vera were also excluded.

We defined hypercholesterolemia in HCC patients as a serum cholesterol level greater than 250 mg/dL (two standard deviations above the mean value of age-and-sex-matched healthy controls); hypoglycemia as plasma glucose less than 60 mg/dL; hypercalcemia as corrected serum calcium level more than 11.0 mg/dL; and erythrocytosis as a hemoglobin level greater than 16.7 gm/dL or hematocrit greater than 50% as in our previous reports^[2-6]. Thrombocytosis was defined as having a platelet count greater than 400 K/mm³.

To compare the serum TPO level, 18 consecutive HCC patients with thrombocytosis and 72 age-sex-tumor volume matched HCC patients without thrombocytosis were consecutively collected in a cross-sectional study from January 1999 to December 2000. In addition, 42 age-sex-matched cirrhotic patients and 66 chronic hepatitis patients were randomly selected for comparison. The etiologies of chronic hepatitis and cirrhosis were either viral hepatitis B or hepatitis C, which were all confirmed by liver biopsies. None of the patients with chronic hepatitis or cirrhosis received interferon or other antiviral treatments before blood sampling. Alcoholic patients were not enrolled. Patients were also excluded if an acute infection or gastrointestinal bleeding was noted during enrollment. In addition, 62 healthy subjects who received their annual physical examinations at Taipei Veterans General Hospital, and whose age and sex were matched with the aforementioned 90 HCC patients, were randomly selected as healthy controls. Sera of the aforementioned subjects or patients were stored in aliquots at -70 °C until analyzed.

The underlying liver cirrhosis in HCC patients was diagnosed histologically or by characteristic image findings with the presence of ascites or esophageal varices. Patients with cirrhosis were given a score from 5-15 according to Child-Pugh's classification^[17]. Tumor volume was calculated from computerized tomographic films and was expressed as percentages of tumor volume in total liver volume. The grade of differentiation and arrangement of tumor cells were assessed by a liver pathologist according to the classification of Edmondson and Steiner^[18]. The pathologist was not given any clinical information pertaining to the biopsy specimens.

All clinical data of HCC patients including age, sex, Child-Pugh's scores, liver biochemistries (measured by a Hitachi Model 736 automatic analyzer, Tokyo, Japan), prothrombin time, and complete blood counts were recorded when an HCC was first diagnosed or at the time the thrombocytosis developed. Serum α -fetoprotein (AFP) was measured by a commercial kit (ELSA2-AFP, CIS bio-international, Cedex, France). Anti-HCV was measured by a second-generation enzyme immunoassay kit (Abbott Laboratories, Chicago, IL, USA). The serum markers of hepatitis B surface antigen (HBsAg) and antibody to hepatitis D virus (Abbott Laboratories) were recorded. Distant metastases to extrahepatic regional lymph nodes or other organs were evaluated by image studies. Methods of therapy for HCC, including surgical resection of tumors, transcatheter arterial chemoembolization (TACE), sono-guided percutaneous ethanol injection, or systemic chemotherapy and survival times were recorded in each patient. This study was approved by Taipei Veterans General Hospital, Taiwan.

Serum TPO levels were quantitatively measured by a solid phase enzyme immunoassay (Quantikine, R&D systems, Abingdon, UK). For the expression of TPO mRNA, an equal amount of fresh frozen tumors and non-tumor parts of the liver samples from a patient with thrombocytosis who received lobectomy

for HCC were homogenized with a pestle. Total RNA was purified by TRIZOL reagent (Invitrogen and Life Technologies, Rockville, MD, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to amplify 1 μ g RNA using the First-strain cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the instruction manual. PCRs were carried out with 35 cycles at 94 °C for 1 min, at 60 °C for 1 min and at 72 °C for 1 min. Primer sequences were sense: 5'-TGCGTTTCCTGATGCTTGAG-3' and anti-sense: 5'-AACCTTACCCTTCCTGAGACA-3'^[12]. β -actin was used as an internal standard and diethyl pyrocarbonate-treated water was used as a negative control. The analysis of PCR products was performed by 20 g/L agarose gel electrophoresis. For quantitation of TPO mRNA, the PCR product was purified by the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and $A_{260\text{ nm}}$ was measured to estimate the DNA contents. Serial dilutions of this standard were then included in the following real-time PCR along with samples to produce a standard curve. Quantitative PCR was performed in 20 μ L reaction capillaries using 1 \times DNA master SYBR Green I mix (Roche Diagnostics, Indianapolis, IN, USA), bovine serum albumin, 4 mmol/L MgCl₂, 200 μ mol/L dNTPs, 0.4 U InViTaq-polymerase (InViTec; Berlin, Germany), 1 μ L cDNA and 0.5 μ mol/L of the TPO-specific sense and antisense primers. DNA amplification, data collection and analyses were performed with LightCycler (Roche Diagnostics)^[19]. The program was optimized and performed finally as denaturation at 94 °C for 30 s followed by 40 cycles of amplification (at 94 °C for 0.1 s, 60 °C for 0.1 s, 72 °C for 20 s). The temperature ramp rate was 20 °C/s. At the end of each extension step, the fluorescence of each sample was measured to allow the quantification of the PCR product. After the PCR was completed, the melting curve of the product was measured by a temperature gradient from 60 °C to 96 °C at 0.2 °C/s with continuous fluorescence monitoring to produce a melting profile of the primers. For normalization, the amount of TPO amplicon was divided by the amount of β -actin amplicon of the respective sample.

All data are expressed as mean \pm SD. Results were compared between groups using the Chi-square test, Fisher's exact test, Student's *t*-test, Mann-Whitney test or cross tabulation depending on the type of data analyzed. Survival adjusted for therapy was analyzed using the Kaplan-Meier method and was compared using the log rank method. Univariate and multivariate logistic regression using SPSS software (SPSS Inc., Chicago, IL, USA) were performed to evaluate the predictive values of the patients' clinical, laboratory, and tumor features associated with thrombocytosis. For all tests, only the results with *P* values less than 0.05 (two-tail test) were considered to be statistically significant.

RESULTS

Among the 1 154 HCC patients (1 005 males, 149 females, mean age 62.0 \pm 12.0 years, with a range of 13 to 84 years), 735 (63.7%) patients were HBsAg positive, 180 (15.6%) were anti-HCV positive, 49 (4.3%) were positive for both HBsAg and anti-HCV and 19 (1.6%) were positive for HBsAg and antibody to hepatitis D virus. The mean serum AFP level was 43 983 \pm 163 097 ng/mL (median 268 ng/mL, range 3-1 892 500 ng/mL).

Totally, 243 (21.1%) of 1 154 patients had paraneoplastic syndromes during the clinical course of HCC, in which 175 had a single paraneoplastic manifestation and 68 had multiple paraneoplastic manifestations. Thirty-one (2.7%) of 1 154 HCC patients had thrombocytosis (mean platelet count 484 \pm 87 K/mm³, range 403-688 K/mm³). The prevalence of hypercholesterolemia was 12.6%, hypoglycemia was 5.4%, hypercalcemia was 4.4%, and erythrocytosis was 3.2%. Eighteen of 31 HCC patients with thrombocytosis had other paraneoplastic manifestations

(11 with hypercholesterolemia, 1 with hypoglycemia, 4 with hypercholesterolemia and hypoglycemia, 1 with hypercholesterolemia and hypercalcemia, and 1 with hypercholesterolemia, hypoglycemia and hypercalcemia). Nine hundred and eleven HCC patients were free of paraneoplastic manifestations.

In comparison of the clinical, laboratory data and tumor features between HCC patients with thrombocytosis and those without, HCC patients with thrombocytosis were significantly younger in age, had a higher mean serum AFP level, higher rate of main portal vein (MPV) thrombosis, bilobar tumor involvement, larger tumor volume, were less likely to receive or be suitable for HCC therapy, and had a shorter survival time than those without thrombocytosis (Table 1). HCC patients with thrombocytosis tended to have more extra-hepatic metastases than those without thrombocytosis (32% vs 19%, $P = 0.059$). There were no significant differences in sex distribution, viral etiologies, mean Child-Pugh's scores, and rates of cirrhosis between the two groups. Two hundred and fifty-eight (22%) of 1 154 HCC patients and 10 (32%) of 31 patients with thrombocytosis had their HCC tissues for histological analyses. Among the 10 HCC patients with thrombocytosis, tumor cell arrangement showed a trabecular pattern in 7 patients, and a mixed trabecular and acinar pattern in 3. Tumor cell differentiation revealed grade I in 3 patients, grade II in 4 patients, and grade III in 3 patients. The tumor cell arrangement and differentiation showed no significant differences between patients with and without thrombocytosis.

Age, sex, viral hepatitis markers, serum AFP, MPV thrombosis, metastases, bilobar tumor involvement, and tumor volumes were

all selected as independent variables in logistic regression analyses, with the presence of thrombocytosis as the dependent variable. The continuous variables were transformed to categorical variables with the cut-off points determined by the Receiver Operating Characteristic Curve. In an univariate analysis, an age <60 years, serum AFP >140 000 ng/mL, tumor volume >30% of the total liver volume, bilobar tumor involvement, and MPV tumor thrombosis were significantly correlated with the presence of thrombocytosis (Table 2). In a stepwise multivariate analysis, a tumor volume >30% (odds ratio: 9.901, 95% confidence interval: 2.187-44.402, $P = 0.003$) and serum AFP >140 000 ng/mL (odds ratio: 2.660, 95% confidence interval: 1.090-6.487, $P = 0.031$) were significant predictive variables associated with the presence of thrombocytosis in HCC patients.

In the cross-sectional study, the mean platelet count was 440 ± 43 K/mm³ (range 401 to 540 K/mm³) in 18 HCC patients with thrombocytosis, 174 ± 100 K/mm³ (range 102 to 396 K/mm³) in 72 HCC patients without thrombocytosis, 72 ± 29 K/mm³ (range 18 to 154 K/mm³) in 41 patients with cirrhosis, 178 ± 48 K/mm³ (range 105 to 300 K/mm³) in 66 patients with chronic hepatitis, and 212 ± 35 K/mm³ (range 125 to 347 K/mm³) in 62 healthy controls. The mean serum TPO level was significantly higher in HCC patients with thrombocytosis (404 ± 196 pg/mL), when compared with HCC patients without thrombocytosis (181 ± 85 pg/mL), cirrhotic patients (103 ± 34 pg/mL), chronic hepatitis patients (113 ± 46 pg/mL), and healthy subjects (123 ± 53 pg/mL) (Figure 1). Serum TPO levels were positively correlated with platelet counts among 90 HCC patients ($r = 0.474$, $P < 0.001$). Among the 10 HCC patients with thrombocytosis, the

Table 1 Comparison of clinical and laboratory data, and tumor features between hepatocellular carcinoma (HCC) patients with and without thrombocytosis

| | HCC patients with thrombocytosis (n = 31) | HCC patients without thrombocytosis (n = 1 123) | P value |
|---|---|---|---------|
| Age (yr) | 52±19 | 62±12 | <0.001 |
| Sex (male: female) | 25:6 | 980:143 | 0.201 |
| HBV: HCV: HBV+HCV-related | 24:1:0 | 711:179:49 | 0.114 |
| Mean Child-Pugh's scores | 7.2±2.1 | 6.8±2.2 | 0.336 |
| Mean platelet counts (k/mm ³) | 484±87 | 154±80 | <0.001 |
| Median (range) | 441 (403-688) | 133 (11-396) | |
| Mean α-fetoprotein (ng/mL) | 251 042±458 734 | 57 879±212 788 | <0.001 |
| Median (range) | 4 859 (4-1 892 500) | 252 (3-1 621 700) | |
| Cirrhosis (+/-) | 23:8 | 913:210 | 0.445 |
| MPV tumor thrombosis (+/-) | 11:20 | 171:952 | 0.005 |
| Tumor metastases (+/-) | 10:21 | 196:927 | 0.059 |
| Tumor volumes % | 63.5±21.2 | 30.7±26.9 | <0.001 |
| Both lobes with tumor involvements (+/-) | 22:9 | 439:684 | <0.001 |
| Therapy for HCC (+/-) | 10:21 | 583:540 | 0.048 |
| Mean survival (d) | 144±208 | 373±521 | <0.001 |
| Median (range) | 77 (4-852) | 147(2-3 666) | |

Data were expressed as mean±SD. HBV: hepatitis B virus, HCV: hepatitis C virus, MPV: main portal vein.

Table 2 Significant predictive variables in association with thrombocytosis in hepatocellular carcinoma patients using univariate logistic analyses

| Variables | Odds ratio | 95% confidence interval | P value |
|---|------------|-------------------------|---------|
| Age <60 yr | 2.905 | 1.415-5.967 | 0.004 |
| α-fetoprotein >140 000 ng/mL ¹ | 6.802 | 3.515-14.663 | <0.001 |
| Tumor volume >30% ¹ | 16.1 | 3.757-68.333 | <0.001 |
| Both lobes with tumor involvements | 3.788 | 1.732-8.306 | <0.001 |
| Main portal vein tumor thrombosis | 3.408 | 1.435-6.467 | 0.004 |

¹Significantly predictive variables in multivariate analyses.

mean platelet count dropped significantly from 420 ± 48 K/mm³ to 278 ± 38 K/mm³ ($P < 0.001$) after a surgical tumor resection or TACE, while the mean platelet count for HCC patients with thrombocytosis, who did not receive any therapy, showed a progressive increase during follow up.

Figure 2 illustrates the relationship of platelet counts with the serum levels of cholesterol, AFP and TPO in a 36-year-old HCC male patient. The serum AFP level was 164 000 ng/mL and serum HBsAg was positive. Image studies revealed a large tumor mass over the right lobe of liver with an invasion to the left medial segment. Hypercholesterolemia (serum cholesterol level 602 mg/dL) and thrombocytosis (platelet count 403 K/mm³) were noted at the diagnosis of HCC. He received a surgical removal of tumors followed by chemotherapy with 5-fluorouracil for 12 wk. Platelet counts, serum levels of cholesterol and AFP dropped significantly after a surgical removal of the tumors. However, all re-elevated when the tumor recurred 18 wk after surgical treatment. Serum TPO level was 360 pg/mL before surgery (platelet count 403 K/mm³), and fell to 130 pg/mL one week after operation (platelet count 120 K/mm³). Figure 3 shows the qualitative expression of TPO mRNAs using RT-PCR in tumor and matched non-tumor tissues of the resected liver samples. The expression of TPO mRNAs was more intensive in tumor than in the non-tumor liver tissues. The results from the quantitative real-time PCR also revealed that the concentration of TPO mRNAs in tumors was 1.92 times greater than in matched non-tumor liver tissues.

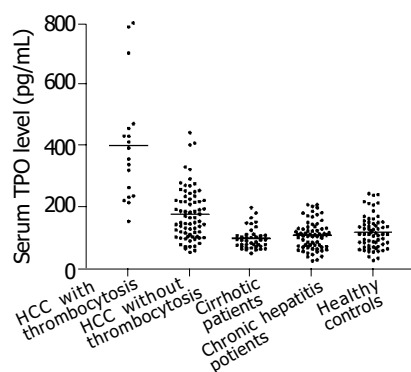


Figure 1 Distribution of serum thrombopoietin levels in hepatocellular carcinoma (HCC) patients with and without thrombocytosis, patients with cirrhosis, chronic hepatitis and healthy subjects. The mean serum thrombopoietin level in HCC patients with thrombocytosis was significantly higher than in HCC patients without thrombocytosis, patients with cirrhosis, chronic hepatitis and healthy subjects.

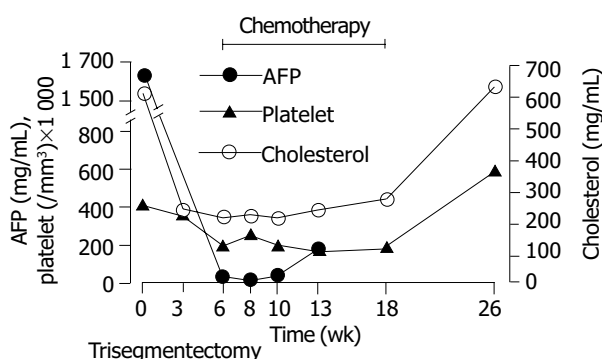


Figure 2 Clinical course of a hepatocellular carcinoma patient with thrombocytosis. The serum alpha-fetoprotein (AFP) level was 164 000 ng/mL before tumor resection. Hypercholesterolemia (serum cholesterol level: 602 mg/dL) and thrombocytosis (platelet count: 403 K/mm³) were noted before operations. The platelet counts, serum levels of cholesterol

and AFP fell significantly after a surgical removal of the tumors. However, all re-elevated when the tumor recurred 18 wk after surgical treatments.

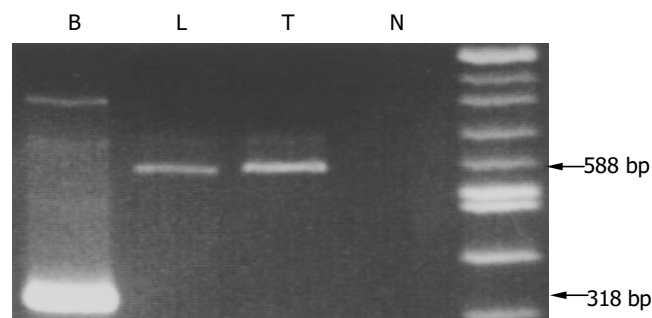


Figure 3 Analyses of thrombopoietin from tumor tissues (T) and non-tumor liver tissues (L) in a hepatocellular carcinoma patient with thrombocytosis using reverse-transcription polymerase chain reaction. β -actin was used as an internal standard (B). N: negative control. Results from an agarose gel electrophoresis showed a more intense density of thrombopoietin band from tumor tissues when compared with non-tumor liver tissues.

DISCUSSION

Common paraneoplastic syndromes seen in HCC patients include hypercholesterolemia, hypoglycemia, hypercalcemia, and erythrocytosis^[1]. Thrombocytosis has been reported in children with hepatoblastoma^[7-9]. The prevalence of thrombocytosis in HCC patients has not been previously reported. Our results showed that 2.7% of HCC patients had thrombocytosis which was defined as a platelet count >400 K/mm³. The prevalence of thrombocytosis might be underestimated because most HCC patients were associated with liver cirrhosis, and thrombocytopenia was frequently seen in these patients.

The clinical significance of thrombocytosis in HCC patients were similar to HCC patients with other paraneoplastic syndromes, including hypercholesterolemia, hypoglycemia, hypercalcemia, and erythrocytosis^[2-6]. Large tumor volumes, high serum AFP, high rates of MPV tumor thrombosis, low rates of receiving therapy and poor prognosis have been identified in HCC patients with thrombocytosis. According to the multivariate logistic regression analyses, HCC patients with thrombocytosis were characterized by a large tumor volume and high serum AFP. HCC patients with thrombocytosis seemed to have a similar life expectancy as HCC patients with erythrocytosis or hypercholesterolemia, and had a better prognosis than patients with hypoglycemia and hypercalcemia which were usually pre-terminal events^[6].

Before the cloning of TPO, the cause of thrombocytosis in children with hepatoblastoma and in other malignancies remains unclear. TPO, a glycoprotein hormone, is a potent stimulator of the growth and maturation of megakaryocytes and platelet production^[10,11]. The main sites of TPO production are the liver and, to a lesser degree, the kidneys, bone marrow and spleen. Messenger RNA transcripts of TPO have been found mainly in the liver and released into circulation^[14-16]. Most TPO is bound with and degraded by circulating platelets and megakaryocytes in the bone marrow, and the serum level is low. Circulating TPO levels are inversely correlated with the number of TPO receptors (c-Mpl-molecules) in regulating megakaryocytopoiesis and platelet production. When thrombocytopenia develops, binding receptors decrease and serum TPO levels increase. Elevated TPO levels stimulate megakaryocytopoiesis and result in increased platelet production^[20-25]. This reverse relationship of platelet counts and serum TPO levels has been found in patients

with hematological diseases^[26,27]. Patients with cirrhosis were frequently associated with low platelet counts. However, serum TPO levels in cirrhotic patients were found to be lower than chronic hepatitis patients or normal subjects due to inadequate TPO production by the diseased livers^[28-32]. Serum TPO of cirrhotic patients increased after orthotopic liver transplantation, which was followed by an increase in platelet counts^[14,33-35]. Our results revealed serum TPO levels were significantly lower in cirrhotic patients than in patients with chronic hepatitis or normal controls, and were consistent with previous reports.

Despite the fact that hepatoma cells have been found to express TPO *in vitro* and in animal models, the mechanisms by which thrombocytosis develops in HCC patients have not been studied. According to our results, HCC patients with thrombocytosis had a significantly higher mean serum TPO level than HCC patients without thrombocytosis. In addition, the platelet counts and serum TPO levels in HCC patients with thrombocytosis dropped after a surgical removal of the tumor or TACE, and re-elevated when a tumor recurred. Changes of platelet counts and serum TPO levels were parallel to the changes of serum AFP. Furthermore, by using the reverse transcription and real-time PCR methods, we demonstrated that the expression of TPO mRNA was more in tumor tissues than in matched non-tumor tissues in an HCC patient with thrombocytosis. Our results were consistent with the report that thrombocytosis in patients with hepatoblastoma was related to increased TPO production in tumors^[9]. Thus, we speculate that thrombocytosis in HCC patients is due to the overproduction of TPO by tumors, and mostly in patients with a large tumor burden. The mechanisms of thrombocytosis in HCC patients are similar to those for other paraneoplastic manifestations. Hypoglycemia has been related to the overproduction of insulin-growth-factor II with insulin-like activities^[2]. The cause of hypercalcemia has been related to overproduction of a parathyroid-related protein which interacts with parathyroid hormone receptors^[4,36]. Elevation of serum erythropoietin has been seen in HCC patients with erythrocytosis^[5,37].

In conclusion, thrombocytosis is one of the paraneoplastic syndromes in patients with HCC, due to the overproduction of TPO by HCC. HCC patients with thrombocytosis are associated with a large tumor volume and high serum AFP level.

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