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Research Paper

Post-Resection Exhaustion of Intra-Platelet Serotonin: Also an Indicator of Early Hepatocellular Carcinoma Recurrence?

Bibek Aryal[1], Toshiaki Shimizu[2], Jun Kadono[1], Akira Furoi[3], Teruo Komokata[4], Iwao Kitazono[1], Chihaya Koriyama[5], Munekazu Yamakuchi[2], Teruto Hashiguchi[2*✉], Yutaka Imoto[1*]

1. Cardiovascular and Gastroenterological Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University 890-8520, Kagoshima, Japan
2. Department of Laboratory and Vascular Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8520, Japan
3. Department of Surgery, Kirishima Medical Center, Kirishima 899-5112, Japan.
4. Department of Surgery, Kagoshima Medical Center, National Hospital Organization, Kagoshima 892-0853, Japan
5. Department of Epidemiology and Preventive Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8520, Japan

* These authors share joint senior authorship

✉ Corresponding author: Teruto Hashiguchi; Department of Laboratory and Vascular Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan. Telephone: (+81)-99-275-5437; Fax: (+81)-99-275-5437. Email: terutoha@m3.kufm.kagoshima-u.ac.jp

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Abstract

Purpose: Serotonin (5-hydroxytryptamine, 5-HT) is well known for its growth stimulatory effect on several types of carcinoma and tumor cells. Since a large portion of 5-HT is stored and transported by platelets, the aim of this study was to assess the influence of platelet-sequestered 5-HT on post-resection hepatocellular carcinoma (HCC) recurrence.

Methods: This pilot study was conducted in a cohort of forty patients diagnosed with HCC undergoing partial hepatectomy. 5-HT levels in serum, plasma and intra-platelet (IP) were monitored preoperatively and four weeks after liver resection. The patients were followed every three months after the surgery.

Results: Follow-up was standardized to a fixed length of time. Fifteen patients (37.5%) developed HCC recurrence during 18 months follow-up. Patients with recurrence had significantly reduced serum and IP 5-HT levels at four weeks of liver resection (P = 0.003 and P = 0.014 respectively). Accordingly, in the Cox regression hazard model, serum and IP 5-HT were able to independently predict the recurrence (hazard ratio = 0.1, 95% confidence interval = 0.01 – 0.75 and hazard ratio = 0.1, 95% confidence interval = 0.01 – 0.89 respectively). The optimal cut-off value of 42.77 ng/ml for serum [area under the curve (AUC): 0.78, P = 0.003] and 0.3117 ng per 10^6 platelets (AUC: 0.733, P = 0.015), on receiver operating characteristic (ROC) curve corresponded to maximum sensitivity and specificity of prediction. The disease free interval was significantly worse in patients with low serum and IP 5-HT (P = 0.001 and P = 0.029 respectively).

Conclusion: IP 5-HT monitored during early follow-up, after liver resection may represent a useful marker of early HCC recurrence.

Key words: Hepatocellular Carcinoma; liver cirrhosis; liver resection; platelet; serotonin

Introduction

Beyond the confines of its role as a neurotransmitter, serotonin (5-hydroxytrypamine, 5-HT) has also been described for its mitogenic property in a wide range of normal and tumor cells. In this context, some studies have proposed associations between 5-HT or its receptors and different

cancers[1-5].

Primarily synthesized by the enterochromaffin cells in the intestinal mucosa, the circulating 5-HT is largely taken up and reserved in circulating platelets[6]. This platelet-sequestered 5-HT (intra-platelet 5-HT, IP 5-HT) serves critical functions in different pathophysiological events. Its role as an inducer of liver regeneration after partial hepatectomy (PH) has already been acknowledged in molecular investigations as well as clinical trials[7, 8]. However, 5-HT also seems to have a negative effect in liver pathophysiology. In particular, it contributes to liver fibrosis[9], mediates oxidative stress in non-alcoholic steatotic hepatitis[10], and perpetuates viral hepatitis[11].

Resection is the recommended first line treatment for patients with single hepatocellular carcinoma (HCC) with well-preserved liver function[12]. Unfortunately, resection of very small tumors is also associated with higher recurrence rates[13]. Despite the relevance of disease recurrence in terms of overall survival, diagnostic tools have merely been described to predict HCC recurrence.

5-HT has already been identified as a marker for early detection of breast cancer recurrence [3]. Specific to HCC, 5-HT promoted tumor growth both in vivo and in vitro models[4, 5]. 5-HT has also been proposed as a diagnostic marker of HCC[14]. Concurrently, a diametrically opposite effect of 5-HT has been reported on its tumor inhibitory properties; a substantial exhaustion in the platelet uptake of 5-HT is evidently observed in clinical and translational studies[15, 16].

An intricate defect in platelet functions has been described in patients with cirrhosis or underlying liver disease, including storage pool defect, defective signal transduction, glycoproteins dysfunction and impaired thromboxane synthesis[17-20]. Concomitantly, the presence of circulating exhausted platelets with depleted dense granules substances were found in patients with a heterogeneous group of malignant tumors[21, 22]. Recent advances in understanding platelet biology for its implications in molecular diagnostics in patients with different types of cancer have gained substantial attention in the oncology research.

Through this pilot project, we sought to investigate if IP 5-HT concentration represents a marker of HCC recurrence. Accordingly, we evaluated the feasibility of IP 5-HT for assessing early recurrent HCC from two perspectives: before and after resection of the primary tumor. We cross-examined the validity of IP 5-HT's association with HCC recurrence by evaluating some other platelet granule-released growth factors.

Patients and Methods

Prospective Study Cohorts

A total of forty patients with pathologically proven primary HCC who went on to have liver resection were enrolled in the study from May 2013 to June 2015. All the patients belonged to Child Pugh class A subgroup. Patient inclusion criteria were based on Clinical Practice Guidelines for Hepatocellular Carcinoma (Japanese Society of Hepato-Biliary-Pancreatic Surgery), 2013. This trial is registered in UMIN Clinical Trial Registry (UMIN000026380).

The institutional ethics committee (Kagoshima University # 24-155/ 26-77, Kirishima Medical Center # 2505 and Kagoshima Medical Center # 25-30) approved analyses of blood samples and patient data; all patients gave signed, informed consent. Study was conducted in accord with the ethical standards of the Committee on Human Experimentation of the institution in which the experiments were done or in accord with the ethical standards of the Helsinki Declaration of 1975.

Before liver resection, all patients underwent a thorough laboratory evaluation, including serum α-fetoprotein (AFP) and protein induced by vitamin K antagonist-II (PIVKA-II). Hepatic functional reserve was assessed by indocyanine green (ICG) clearance test, 99mTc-galactosyl human serum albumin (GSA) scintigraphy and Child-Pugh score. The diagnosis and staging of HCC were established with triple-phase computed tomography (CT) of the abdomen.

Follow up

Patients were followed with ultrasonography (USG) every 3 months after resection, and if USG showed any evidence of tumor, subsequent contrast-enhanced CT or magnetic resonance imaging (MRI) of the abdomen and non-contrast CT of the chest were performed. The diagnosis of intrahepatic recurrence was made on the basis of imaging alone if the tumor exhibited the typical enhancement characteristics.

Sample Preparation

Venous blood was collected preoperatively (PRE OP) and four weeks after surgery (POST OP). During this period, none of the patients received selective serotonin reuptake inhibitors (SSRIs). Complete blood count (CBC) was performed with an automated hematology analyzer, Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan).

Serum and Plasma

Whole blood was collected in the serum

separating tube and a citrate tube, containing 0.5 ml of sodium citrate (for plasma and platelet preparations), and an EDTA-2k tube (for cell count; Venoject II, Terumo Corp., Tokyo, Japan). Serum tube was incubated at room temperature for 30 minutes to allow clotting. Serum and plasma tubes were centrifuged at 1710 × g for 10 minutes. Only the top 75% of the resultant supernatant was carefully pipetted to avoid contamination.

Platelet Isolation

Venous blood in citrate tubes was centrifuged at 90 × g for 15 minutes. Again, only the top 75% of the resultant platelet rich plasma (PRP) was gently pipetted to avoid contamination. The PRP was centrifuged at 2810 × g to isolate platelets. The supernatant, platelet poor plasma (PPP), was collected precisely and decanted for the complete removal of the plasma from the pellets. Platelet pellets isolated from each 200 µl of PRP were suspended in 220 µl of lysis buffer (150 mM sodium chloride, 25 mM Tris-HCl pH 7.6, 1% Tergitol-type NP-40 and 0.1 % sodium dodecyl sulfate, 1 % sodium deoxycholate in distilled water to make 100 ml solution); after incubating for 20 minutes, the lysate solution was pipetted and vortexed until the pellets were completely dissolved in the solution. CBC was carried out in 3 preparations: whole blood, PRP and PPP.

Quantification of Cytokines

Serum, plasma and platelet extracts were analyzed together by commercially available enzyme-linked immunosorbent assay (ELISA) tests for human serotonin (Enzo LifeSciences Inc., Farmingdale, NY, US) and, Platelet derived growth factor- BB (PDGF-BB), Epidermal growth factor (EGF) and Angiopoetin-1 (Ang -1) (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's guidelines.

Calculation of IP 5-HT

We have established different methods to calculate the intra-platelet growth factors[23, 24]. Platelet content of 5-HT per 10^6 platelets was calculated using our previous equation[23]:

$$V_1 \times C_1 / V_2 \times C_2$$

V_1 = Volume of lysate solution

C_1 = 5-HT concentration in platelet lysate solution

V_2 = Volume of PRP

C_2 = Platelet count in PRP

Briefly, 220 µl of lysis buffer was added to the platelets isolated from each 200 µl of PRP. The concentration was adjusted to the platelet count obtained from the PRP.

Statistical Analysis

Statistical analyses were conducted with SPSS software (version 21; SPSS, Inc., Chicago, IL) and Graph Pad Prism (version 6.0d for Mac OS X, USA, GraphPad Software, San Diego California, USA), and were based on nonparametric tests (Mann-Whitney's U test, Wilcoxon's signed rank test, and Spearman's correlation). The Fisher's exact test was used to evaluate frequencies between categorical variables. Patients were divided in lower and higher postoperative serum and IP 5-HT group, and platelet counts based on the median value obtained from the non-recurrent group; on this basis, Cox's proportional hazards regression model was used for the univariable (UVA) and multivariable analyses (MVA) to determine the variables independently associated to recurrence. Because of the collinearity between serum and IP 5-HT, MVA was separately run for these two variables. Receiver operating characteristic (ROC) analysis was performed to assess the specificity and sensitivity of IP 5-HT levels to predict recurrence. Two-tailed P values of less than 0.05 were considered statistically significant. IP 5-HT was expressed per 10^6 platelets.

Results

During the study period, forty patients underwent liver resection for HCC. Since this study aimed to identify the early recurrence, we adopted 18 months follow-up providing a uniform exposure to the cohort. During this period, fifteen patients (37.5%) developed recurrence, and the characteristics of patients stratified according to the recurrence status are shown in Table 1. Of all the variables, only gender and preoperative INR were significantly different between the groups.

Diminution in Serum and IP 5-HT Concentration after Liver Resection

To investigate the impact of tumor resection on IP 5-HT concentrations, we examined the 5-HT concentrations just prior to the surgery and after four weeks of surgery. A significant decrease in serum (P = 0.013) and IP 5-HT (P < 0.001) was observed four weeks after liver resection (Figure 1, A and C). No significant difference was observed between pre and postoperative plasma 5-HT baseline values (P = 0. 253; Figure 1B).

Postoperative Serum or IP 5-HT can Independently Predict Early HCC Recurrence

In Table 2, we stratified the 5-HT concentrations and platelet counts based on recurrence. As shown, the postoperative 5-HT concentration was significantly lower in serum and IP of patients with

recurrence (P = 0.003 and P = 0.014). Similarly, patients with recurrence had a significantly lower postoperative platelet count (P = 0.018). There was no difference in postoperative AFP concentration between the groups; however, we observed a weak positive correlation between the serum AFP and IP 5-HT concentrations (Figure S1).

Table 1. Clinical and Pathological Data at the Time of Resection of Primary Tumor

Variable Demographical and Clinical Data (N=40)	Recurrent Cases (n = 15)	Non-Recurrent Cases (n = 25)	P -Value
Age, Median (Min., Max.)	71 (55, 83)	74 (51, 80)	0.726
Sex, n (%)			0.002
Male	9 (60%)	21 (84%)	
Female	6 (40%)	4 (16%)	
Etiology, n (%)			0.254
HBV	4 (26.66%)	8 (32%)	
HCV	7 (46.77%)	5 (20%)	
None	4 (26.66%)	12 (48%)	
Fibrosis, n (%)			0.083
0	0 (0%)	4 (16%)	
1-2	6 (40%)	12 (48%)	
3-4	9 (60%)	9 (36%)	
Total bilirubin, mg/dl			0.725
Median (Min., Max)	0.8 (0.6, 1.5)	0.7 (0.5, 2.5)	
≥ 1.0	1.3 (1, 1.5)	1.2 (1.1, 2.5)	
INR			0.026
Median (Min., Max)	1.09 (0.92, 2.43)	1.02 (0.89, 1.29)	
≥ 1.0	1.09 (1, 2.43)	1.04 (1, 1.29)	
AFP, ng/ml			0.791
Median (SD)	24.30 (2072)	15.7 (2344)	
≥ 20	102 (2703)	914.8 (3671)	
Extent of resection, n (%)			0.502
Minor hepatectomy	11 (73.33%)	15 (60%)	
Major hepatectomy	4 (26.67%)	10 (40%)	
Tumor stage, n (%)			0.543
I-II	11 (73.33%)	17 (68%)	
III-IV	4 (26.67%)	8 (32%)	
Tumor size, n (%)			0.730
< 5 cm	11 (73.33%)	18 (72%)	
≥ 5 cm	4 (26.67%)	7 (28%)	
Histological grade, n (%)			0.686
Well to moderate	13 (86.67%)	19 (76%)	
Poor	2 (13.33%)	6 (24%)	

Min.: Minimum, Max.: Maximum, n/N: numbers, HBV: Hepatitis B Virus, HCV: Hepatitis C Virus, INR: International Normalised Ratio

Table 2. 5-HT Concentrations and Platelet Count on the Basis of Recurrence. Association Between Cytokine of Interest (Serum, Plasma and IP 5-HT), POST OP Platelet Count and AFP, and HCC Recurrence by Mann-Whitney Test

Variables	Recurrent Cases	Non-Recurrent Cases	P-Value
PRE OP Serum 5-HT, ng/ml			0.100
Median (Min., Max.)	56.78 (24.3, 591.9)	109.4 (22.8, 389.2)	
PRE OP Plasma 5-HT, ng/ml			0.470
Median (Min., Max.)	25.68 (4, 72)	29.40 (5.1, 116.1)	
PRE OP IP 5-HT, ng			0.630
Median (Min., Max.)	0.62 (0, 3)	0.79 (0, 5)	
PRE OP Platelet count, $\times 10^3/\mu l$			0.121
Median (Min., Max.)	134 (61, 255)	156 (69, 387)	
POST OP Serum 5-HT, ng/ml			0.003
Median (Min., Max.)	6.06 (0, 204)	91.14 (0, 525)	
POST OP Plasma 5HT, ng/ml			0.065
Median (Min., Max.)	20.40 (0, 67.7)	40.9 (0, 164)	
POST OP IP 5-HT, ng			0.014
Median (Min., Max.)	0.2 (0, 1.4)	0.50 (0, 2)	
POST OP Platelet count, $\times 10^3/\mu l$			0.018
Median (Min., Max.)	13.60 (12, 29)	17.30 (10, 29)	
POST OP AFP, ng/ml			0.548
Median (Min., Max)	4.5 (1.4, 844)	3.7 (1.3, 382)	

Min.: Minimum, Max.: Maximum, PRE OP: Preoperative, POST OP: Postoperative, IP: Intra-Platelet, 5-HT: 5-hydroxytryptamine, IP 5-HT expressed per 10^6 platelets

Table 3. Univariable and Multivariable Analyses by Cox Proportional Hazard Model

Variables	Univariable			Multivariable		
	HR	95 % CI	P-Value	HR	95 % CI	P-Value
Sex (M/F)	1.03	0.33-3.33	0.95			
Fibrosis Grade (0-2/ 3-4)	0.50	0.18-1.41	0.19			
PRE OP INR (<1/ ≥ 1)	0.17	0.02-1.29	0.09			
POST OP Serum 5-HT	0.09	0.01-0.69	0.02	0.1	0.01-0.75	0.03
POST OP IP 5-HT	0.10	0.01-0.78	0.02	0.11	0.01-0.89	0.03
POST OP Platelet count	0.36	0.11-1.13	0.08	0.41	0.13-1.32	0.13

M: Male, F: Female, PRE OP: Preoperative, POST OP: Postoperative, IP: Intra-Platelet, 5-HT: 5- hydroxytryptamine, HR: Hazard ratio

Since we observed a significant association of postoperative serum and IP 5-HT concentrations with HCC recurrence, it was of interest to investigate, if postoperative serum or IP 5-HT level could independently predict early HCC recurrence. Therefore, we performed univariable and multivariable analyses using the Cox proportional hazard model (Table 3). To specifically test for independence of serum and IP 5-HT from platelets,

platelet count was always included as a covariate in MVA. Strikingly, postoperative serum and IP 5-HT were able to predict early recurrence independently (hazard ratio = 0.1, confidence interval = 0.01 – 0.75 and hazard ratio = 0.1, confidence interval = 0.01 – 0.89 respectively).

5-HT is stored in the dense-granules of platelet; to examine if a similar phenomenon occurs with the alpha-granules secreted growth factors, we analyzed platelet derived growth factor- BB (PDGF-BB), epidermal growth factor (EGF) and Angiopoetin-1, in serum, which were previously studied for their role in liver pathophysiology. We observed a similar association between HCC recurrence and serum PDGF-BB, Angiopoetin-1 and EGF (Table S1); moreover, PDGF-BB also yielded a statistically significant result. Furthermore, we found, a moderately weak, yet positive correlation between serum 5-HT and PDGF-BB concentrations in both pre and postoperative samples (r = 0.35, P = 0.02 and r = 0.32, P = 0.04 respectively; Figure S2).

Determination of Cut-off Serum and IP 5-HT Concentration to Predict Early HCC Recurrence

Because we had observed a significantly lower postoperative serum and IP 5-HT concentrations in patients with recurrence, we aimed to further characterize the potential of postoperative 5-HT values to predict early recurrence. Receiver operating characteristic (ROC) curves for serum and IP 5-HT was plotted (Figure 2, A and B), revealing significant predictive values of serum [area under curve (AUC) = 0.78; P = 0.003] and IP 5-HT (AUC = 0.733; P = 0.015). With this ROC plot, a cut-off level of 42.77 ng/ml of serum and 0.311 ng (per 10^6 platelets) of IP 5-HT was chosen to identify patients likely to develop recurrence with a specificity of 72% for serum and 68% IP, and sensitivity of 80% or 66.7% for serum and IP respectively. This holds the positive predictive value (PPV) of 63.16% and negative predictive value (NPV) of 85.71 % for serum 5-HT, and PPV of 56 % and NPV of 77.8% for IP 5-HT.

Based on the cut-off values obtained from the ROC curve, patients were divided in high and low 5-HT in serum and IP. A log rank test was run to determine the differences in disease free interval (DFI) distribution between groups with high and low 5-HT (Figure 3, A and B).

The DFI patterns differed significantly between the patients with high and low 5-HT; patients with lower serum {$\chi 2$ (2) = 10.118, P = 0.001} or IP {$\chi 2$ (2) = 4.729, P = 0.029} 5-HT displayed a significantly shorter DFI.

Discussion

Through this study, we introduce post-resection IP 5-HT as one of the indicators of early HCC recurrence. In this study, we monitored IP 5-HT in two distinct events: with the primary tumor and after resection of the tumor. The concentrations of postoperative 5-HT in serum or platelets, four weeks after liver resection, predicted the early HCC recurrence; the peculiar finding that stems from this study is the explicit low concentrations of postoperative 5-HT in all three-blood preparations from patients with recurrence, which is further supported by the results obtained from other platelet-sequestered growth factors.

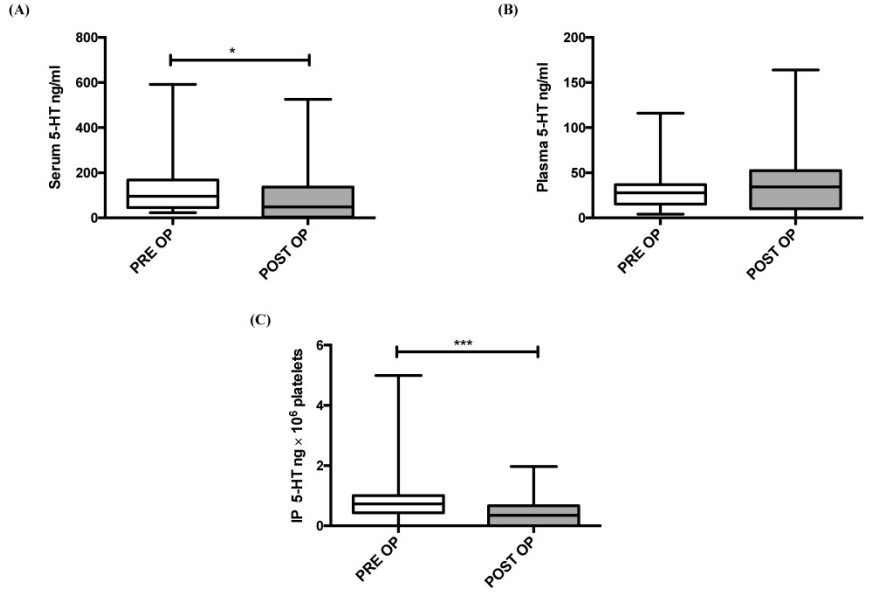

Figure 1. 5-HT concentration before (PRE OP) and 4 weeks after liver resection (POST OP) in: serum (A), plasma (B) and IP (C). IP 5-HT concentration was expressed per 10^6 platelets. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

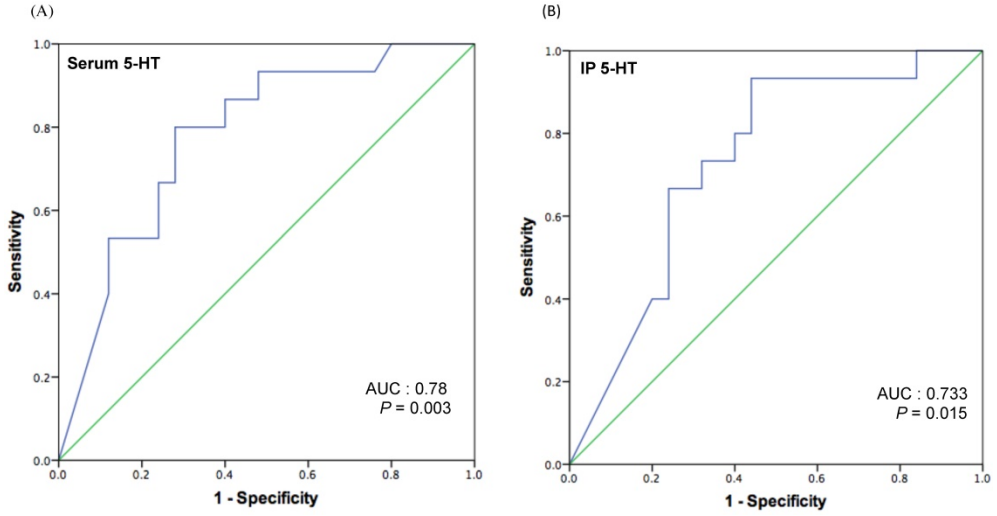

Figure 2. ROC curve analysis for serum (A) and IP 5-HT (B) levels to determine a cut-off value to predict early HCC recurrence after liver resection.

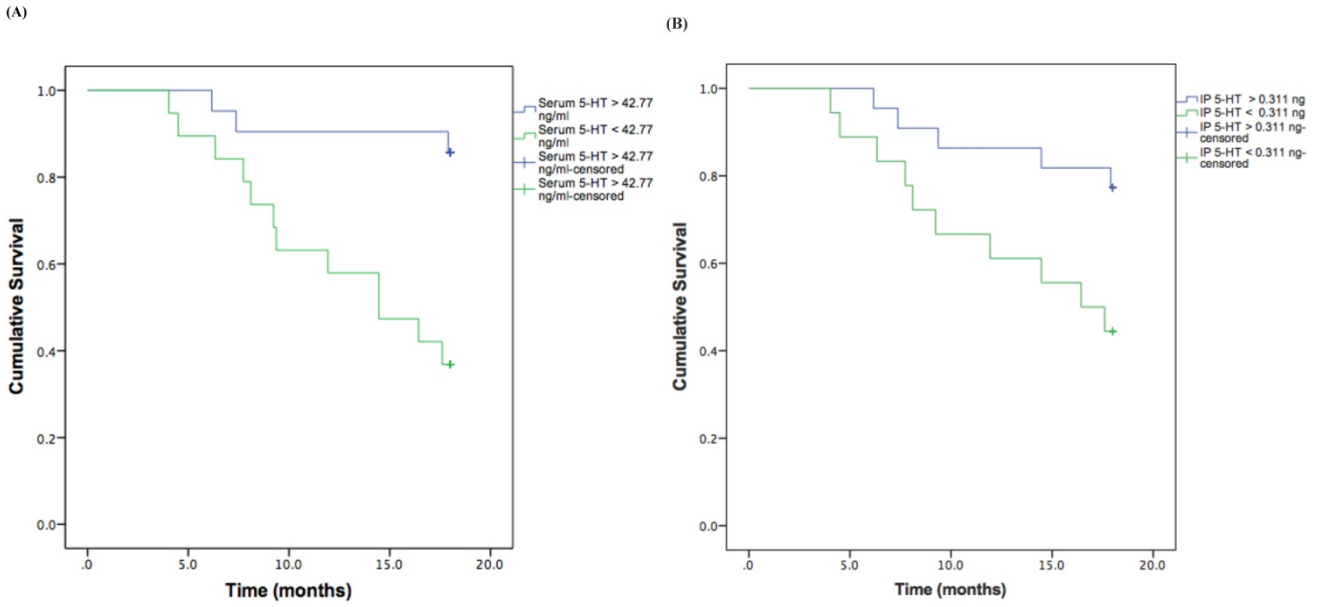

Figure 3. Kaplan-Meier disease-free interval curves according to serum 5-HT (A) (42.77 ng/ml; *P* = 0.001, log-rank test) or IP 5-HT (B) (0.311 ng × 10^6 platelets; *P* = 0.029 log-rank test).

An emerging body of evidence suggests both deleterious and beneficial actions of platelet on liver function. In our previous study, we have demonstrated how IP VEGF-A shifts its kinetics from cancer to liver regeneration[23]. Accordingly, there are fairly contradictory reports on platelet 5-HT's involvement in liver diseases[25]. For instance, IP 5-HT is known to exert both pro-regenerative and pro-fibrotic effect in the liver[26]. These signals are, however, dependent on the cellular and cytokine microenvironment specific to the stage and type of liver injury[27].

Previous studies have demonstrated elevated 5-HT concentrations in patients with cancer[1, 14, 28]. Our finding is apparently in contrast with most of the previous studies, showing low postoperative (IP) 5-HT in patients with early HCC recurrence. This discrepancy could be attributed to different inclusion criteria, different neoplastic conditions and existence of liver disease. The degree of liver fibrosis doesn't only affect the platelet count but also account for intrinsic platelet hypofunction[19, 20, 29]. Furthermore, storage pool defect has been observed in patients with cirrhosis, shrinking the effect of granules release on activation. The 'cirrhotic platelets' have decreased platelet factor 4 (PF4) and P-selectin in the alpha granules, and reduced ATP and 5-HT in the dense bodies[17, 18, 20]. Patients with chronic liver injury (cirrhosis) tend to develop early HCC recurrence after liver resection[30]. Likewise, we observed a positive, provisionally significant, association between fibrosis grade and early

recurrence in our cohort. Moreover, a similar tendency obtained with alpha-granule secreted growth factors and a positive correlation obtained between PDGF-BB and 5-HT prompt us to speculate the existence of platelet storage granule defects in patients with early recurrence. Thus, these results also must take into account the dynamic changes in platelet function augmented by stressful events, like post liver resection, in patients with advanced fibrosis.

Dysfunction of serotonergic systems with gastrointestinal microbiota dysbiosis resulting in an impaired uptake of serotonin by platelets from gut has been implicated in different conditions[31, 32]. It is now known that serotonergic mechanisms are altered in patients with underlying liver diseases. A decrease in the number of platelet membrane acceptors for 5-HT occurs in some neoplastic diseases[33]. Likewise, it is worth taking into consideration the mechanism, which could also interpret the functional significance of depleted IP 5-HT in patients with early HCC recurrence.

In a recent study, Shehta et al. has reported that the recurrence rate increases at lower platelet counts, and decreases at higher platelet counts after liver resection in cirrhotic patients[34]. In consonance with these findings, we also observed an increase recurrence rate in patients with post-resection exhaustion of platelet count and IP 5-HT. In addition to IP 5-HT exhaustion, some other platelet-sequestered growth factors also demonstrated a similar tendency in patients with early HCC recurrence. Taken together, another plausible speculation that cannot be fully excluded is that the phenomenon associated with recurrence might influence both the qualitative and quantitative properties of the platelets, or the reverse phenomenon may provoke the recurrence.

An increment in the concentration of 5-HT is known to exhibit a pro-mitogenic effect in cancers[4, 5, 35], while some studies describe a significant reduction in the kinetics of serotonin uptake by platelets in some cancers[15, 16]. Intriguingly, there are several reports, which found, even if not unequivocally, that using 5-HT or serotonergic agonists can be implemented in tumor growth inhibition[16, 36, 37]. From these diverging opinions, it makes sense to argue that based on the tumor type, associated events, the dose and the receptors involved, 5-HT might hinder the process of tumor growth; a great deal of revised evidence will be needed before we can know how this mechanism actually ensues.

This pilot study has some limitations that need to be pointed out. Main among these is the relatively small sample size. Furthermore, our study doesn't focus on the mechanistic evidence. Despite identical results in all three-blood preparations, we couldn't achieve statistically significant results with plasma preparations, and it could be attributed to our suboptimal plasma preparation technique; a slight deviation from the absolute technique described earlier by Starlinger et. al.[38]. However, we have shown a similar phenomenon with other platelet-alpha granules sequestered growth factors that strengthen the findings obtained from this small cohort. From another perspective, evaluation of 5-HT concentrations after resection of the tumor might represent a different spectrum of pathomechanisms to cancer recurrence.

Taken together, depleted IP 5-HT during early follow-up, after liver resection, was found to be a useful marker for prediction of early HCC recurrence. Since 5-HT is readily accessible in serum and reflects the platelet 5-HT, it may represent a potential biomarker to identify patients that require monitoring and consideration for recurrence. Nevertheless, the predictive potential of post-resection exhausted IP 5-HT in HCC recurrence needs to be further validated in large-scale studies. Moreover, the presence of 5-HT depleted platelets in patients with early HCC recurrence also emphasizes the need to delineate the precise molecular basis of platelet's pleiotropic actions and its functional consequences in the process of carcinogenesis.

Supplementary Material

Supplementary figures and tables.
http://www.jcancer.org/v08p3984s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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