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Tryptase serum levels in patients suffering from hepatocellular carcinoma undergoing intra-arterial chemoembolization: Possible predictive role of response to treatment

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Abstract. Tryptase is a serin protease stored in mast cell granules that has recently been found to be involved in tumor angiogenesis. Data from experimental tumor models have suggested that prior to the onset of angiogenesis mast cells were accumulated near tumor cells and were required for the macroscopic expansion and metastatic spread of primary tumor cells. Hepatocellular cancer (HCC) is a well-established, highly angiogenesis-dependent hypervascular tumor. The aim of this preliminary study was to assess tryptase serum levels in 30 HCC patients prior and subsequent to hepatic transarterial chemoembolization (TACE). In this study, patients with intermediate stage (B) HCC, according to the Barcelona Clinic Liver Cancer (BCLC) staging classification, were enrolled. Additional patient features were adequate liver functional reserve and A or B status, according to the Child-Pugh classification. Tryptase levels were measured using the UniCAP-Tryptase fluoroimmunoassay. TACE was performed by loading doxorubicin on microspheres. The mean ± standard deviation (SD) tryptase level pre-TACE was 7.74±3.62 μg/l, and post-TACE 4.67±2.79 μg/l. A statistically significant difference ($P<0.001$) was detected, using the Student's t-test, between pre- and post-TACE tryptase level concentrations. No correlations were found between tryptase levels and other important clinicopathological features of patients. This is the first preliminary study analyzing the potential significance of serum tryptase levels in HCC patients. The results demonstrated higher serum tryptase levels in HCC patients, suggesting tryptase release from HCC tissue. As expected, after TACE, serum tryptase levels were decreased. Therefore, we suggested that tryptase was a potential biomarker of response to TACE treatment in HCC patients.

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Introduction

Tryptase is a neutral serine protease with a molecular weight of 134 kDa and a tetrameric structure consisting of non-covalently linked subunits. Tryptase is stored mainly in the cytoplasmic granules of mast cells, and in small amounts in stem cells and basophils. Four different forms of tryptase have been identified in human mast cells thus far: α-, β-, γ- and δ-tryptase (1). Of these, α- and β-tryptase are the two best circulating isoforms described. α-tryptase is being constantly released from mast cells in the bloodstream and, therefore, no increase in release was observed during the activation and degranulation of mast cells. β-tryptase is selectively concentrated in the secretory granules of mast cells and is released only after degranulation, thus the presence of a high concentration in the bloodstream is a clear expression of mast cell activation (2).

Mast cells have multiple functions, including allergic and anaphylactic reactions, immune modulation (3,4) and extracellular matrix remodeling, tissue repair (5) and regulation of vascular permeability (6), and in particular angiogenesis, which is highly important in cancer development (7). Angiogenesis is of crucial importance in the development of tumors both *in situ* and at distance; moreover, it is a powerful anticancer target (8). Data from experimental tumor models suggested that mast cells were accumulated near tumor cells prior to the onset of angiogenesis and were required for the macroscopic expansion and metastatic spread of primary tumor cells (9). Studies in the literature have reported that mast cells exert neoangiogenic activity in a number of malignancies, such as colorectal (10), breast (11), endometrial (12) and uterine cervical cancers (13), as well as haemangiomas (14) and multiple myeloma (15).

Notably, experimental studies showed the release of tryptase from mast cells to be a potent angiogenic factor, able to stimulate the neovascolarization both *in vitro* (16) and *in vivo* (17), and therefore directly involved in cancer cell proliferation. Consequently, human tryptase induces vascular tube

formation either by direct mitogen action on endothelial cells (18-21) or by indirect proteolytic action on the surrounding matrix (22-24). In the latter case, tryptase activates matrix metalloproteinases (MMPs) and plasminogen activator (PA) (25-28) which, by degrading the extracellular matrix, facilitates an increase of the space available for neovascular growth, while contributing to the release of pro-angiogenic factors, such as VEGF and FGF-2 from the matrix. VEGF is also indicated as a circulating biomarker of tumor angiogenesis (8,29,30). Thus, tryptase has been suggested to be an agonist of a receptor activated by an endogenous protease, PAR-2 expressed on mast cells and involved in cell proliferation, as well as angiogenesis (31).

In the light of these results, several trials have evaluated the association between tumor angiogenesis and tryptase-positive mast cells in hematological and solid cancers. Ribatti *et al* (32) presented trials showing that tryptase-positive mast cells are involved in the angiogenesis of β-cell non-Hodgkin's lymphomas, myelodysplastic syndrome (33) and β-cell chronic lymphocytic leukemia (34,35). In fact, angiogenesis correlated with the tryptase-positive and total mast cell counts, and both increased proportionally with the increasing degree of malignancy. In their study, Ranieri *et al* (36) reported that tryptase-positive mast cells positively correlated with angiogenesis in breast cancer. Further experimental evidence confirmed a correlation between angiogenesis and tryptase-positive mast cell counts in human endometrial (37) and invasive cervical cancers (38). Both parameters increased in accordance with tumor progression.

A previous study investigating the presence of tryptase-positive mast cells in human hepatocellular carcinoma (HCC) cells (39) is of particular interest. Findings of that study demonstrated that HCC tissues with different histological grades showed a different number of mast cells, with the highest number shown in well-differentiated HCC. Mast cells decreased in poorly-differentiated HCC, suggesting a possible involvement in the early stages of development of the disease.

As yet, no studies are available on the direct activity of tryptase released from mast cells in HCC albeit HCC is known to be a well-established hypervascular tumor with only a few antiangiogenic therapeutic indications (40), while tumor growth is known to be highly dependent on angiogenesis.

The present prospective study aimed to assess the tryptase levels in HCC serum prior and subsequent to hepatic transarterial chemoembolization (TACE) treatment, with a view to predict the response to locoregional treatment and evaluate the possibility of tryptase being a circulating biomarker surrogate of the presence of neoplastic disease.

Materials and methods

Study population and treatment procedure. Between May 2009 and July 2010, 30 patients (8 females and 22 males; median age 74 years; range, 48-86) with intermediate grade [stage B, according to the Barcelona Clinic Liver Cancer (BCLC) staging classification] unresectable HCC, underwent intra-arterial chemoembolization of the liver at the U.O.C. Interventional Radiology of National Cancer Centre 'Giovanni Paolo II' (Bari, Italy). The patients were enrolled in this prospective study and underwent measurement of serum tryptase prior and subsequent to TACE.

Table I. Eligibility criteria for treatment with TACE.

Criteria	Value
HCC	Cytohistologically confirmed Unresectable (technical reasons, comorbidities, refusal of treatment)
Liver function level	Adequate
Child-Pugh class	A or B
Bilirubin	≤2.4 mg/dl
Ascites	Absence
BCLC stage	B, Intermediate
No. 1 tumor nodule	>3.0 cm
Max no. 3 tumor nodules	≤3.0 cm
ECOG performance status	0-2

TACE, transarterial chemoembolization, HCC, hepatocellular carcinoma; BCLC, Barcelona Clinic Liver Cancer; ECOG, European Cooperative Oncology Group.

The study was approved by the Institutional Scientific and Ethics Committee and the participants signed written informed consent.

The pre-treatment evaluation included: biochemical liver function, complete blood count, coagulation profile, dose serum α-fetoprotein (AFP), chest X-ray, liver ultrasound with contrast medium (CEUS) and computed tomography (CT) scan of the abdomen.

The diagnosis of HCC was cytohistologically confirmed by echo-guided fine-needle aspiration biopsy (FNAB) or, alternatively, based on classic imaging findings for HCC associated with pathological increase of AFP levels higher than the cut-off 200 ng/ml.

The selection criteria for TACE at our Institute included: i) absence of extrahepatic metastases, ii) patency of the portal vein and iii) adequate functional reserve of the liver (stage A or B, according to the Child-Pugh classification, serum bilirubin ≤2.4 mg/dl, absence of ascites and hepatic encephalopathy) (Table I).

TACE was performed under general anesthesia by binding DC-Beads® (Biocompatibles, Farnham, UK) to a total dose of doxorubicin of 100 mg/50 ml and injecting by percutaneously inserting a microcatheter into the femoral artery of the patient under fluoroscopic guidance (X-ray) that corresponds to the artery of the liver. When applicable, the artery feeding the tumor was cannulated in a superselective approach. In the case of bilobar tumor involvement, the chemoembolic agent was injected two subsequent time points, after 30-60 days, starting with the lobe more extensively involved.

Sample preparation. The peripheral blood samples were obtained between 7:00 and 9:00 am one day prior and subsequent to TACE treatment. The samples were immediately

Table II. Baseline clinical characteristics of 30 patients with hepatocellular carcinoma.

Characteristics	No.
Age, years (range)	74 (48-86)
Gender (males/females)	22/8
Etiology (HCV/HBV)	28/2
Child-Pugh grade (A/B)	22/7
Serum AFP, ng/ml (range)	650 (2-5,800)
Serum bilirubin, mg/dl (range)	1.6 (0.3-2.4)
Serum AST, IU/l (range)	47 (19-191)
Serum ALT, IU/l (range)	33.5 (8-151)

Data shown are the median values. HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; AST, aspartate aminotransferase; IU, International Unit; ALT, alanine aminotransferase.

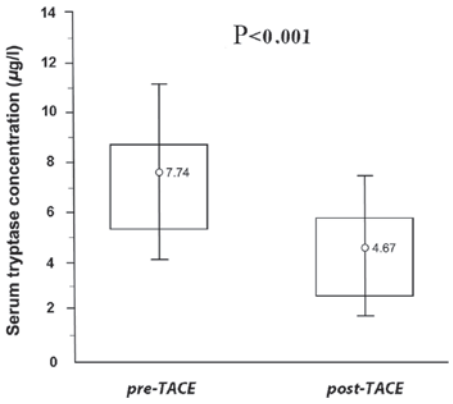

Figure 1. Comparison of the mean concentrations of serum tryptase at baseline and after treatment of intra-arterial chemoembolization (TACE) is shown. In the box, plot values are expressed as the means and standard deviations in μg/l.

Table III. Serum tryptase levels in 30 patients with HCC measured 1 day prior and subsequent to treatment with TACE.

Sample collection time	n	Mean concentrations of serum tryptase (μg/l)	Standard deviation (μg/l)	P-value
24 h before TACE	30	7.74	3.62	<0.001
24 h after TACE	30	4.67	2.79	

TACE, transarterial chemoembolization, HCC, hepatocellular carcinoma.

dispensed in test tubes with serum separator tubes without additives (Becton Dickinson Vacutainer Systems Hemogard, Plymouth, UK) and left for at least 30 min at room temperature to allow for a complete clotting process. The samples were subsequently centrifuged at 1,500 x g for 15 min at room temperature and the supernatant was recovered. Patient sera obtained were collected, aliquoted and frozen at -80˚C until the analysis phase.

Prior to starting the analytical phase, the samples were thawed to room temperature and mixed thoroughly by vortexing at low speed, in order to eliminate any residues of fibrin or other particulate matter potentially affecting reproducible results. Lipemic or hemolyzed samples potentially interfering with the assay were excluded from the analytical evaluation.

Serum tryptase levels were measured by fluoroenzyme immunoassay (FEIA) for each sample, using Uni-CAP100 (Pharmacia Diagnostics AB, Uppsala, Sweden) at the Institute of Allergy and Clinical Immunology of the University Hospital of Bari (Bari, Italy).

Statistical analysis. Statistical analysis was carried out using SPSS software (version 17.0). Descriptive statistics of serum tryptase levels were used to calculate the means and ranges of distribution [range and standard deviation (SD)]. The Kolmogorov-Smirnov test was used for the evaluation of the normal distribution of data. The correlation between the serum tryptase values pre- and post-locoregional treatment and other continuous variables were evaluated using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

The baseline clinical data of the 30 patients studied are shown in Table II. Twenty-eight patients (94%) were positive for the hepatitis C antibody, while 2 patients (6%) were positive for the hepatitis B surface antigen (HBsAg). The serum AFP median level of patients was 12.9 ng/ml. Twenty patients (67%) had normal AFP levels (<20 ng/ml), while the remaining 10 patients (33%) had higher levels.

The serum tryptase levels in patients studied at the time of pre-treatment (24 h prior to TACE) had a mean value and SD of 7.74±3.62 μg/l. The concentrations of serum tryptase were correlated with clinical and laboratory variables. No significant correlation was observed between the tryptase serum levels and age (P=0.354), serum bilirubin (P=0.198), albumin (P=0.923), aspartate aminotransferase (P=0.112), alanine amino transferase (P=0.076) and AFP (P=0.634) levels. In addition, there was no significant difference in the serum tryptase concentrations between males and females (P=0.692).

The mean tryptase levels in serum determined at the time of the post-treatment (+24 h after TACE) were found to be 4.67±2.79 μg/l. The comparison between the mean values of tryptase concentration pre-treatment and post-locoregional treatment showed a statistically significant difference (P<0.001) (Fig. 1 and Table III).

Discussion

HCC is the fifth leading cause of cancer mortality in the world. The identification of biomarkers of easy execution and indication of the presence of HCC are likely to significantly improve early diagnosis. Early diagnosis of HCC is of crucial importance, as survival rates vary considerably in relation to the clinical stage of the disease (41). The prognosis of the 3-year survival rate was 50% for stage B patients (BCLC classification). Thus, identifying a new predictive marker with complete therapeutic response, or one indicating a persistent disease that facilitates subsequent follow-up aiming to identify a relapse of a neoplastic disease, is an extremely interesting approach to optimization of treatment and prognosis.

In the present study, endogenous tryptase was considered to be a possible biomarker surrogate for the presence of a neoplastic disease, since both *in vitro* and *in vivo* data (29-35) indicated that it was significant in neovascularization and ultimately tumor progression. This observation was based on HCC being a hypervascular tumor with a high rate of neoangiogenesis.

Findings of the present study have shown high serum tryptase levels in patients with stage B HCC. Therefore, the serum tryptase levels are likely to be a surrogate indicator of the magnitude of the angiogenic process and of HCC tumor tissue presence.

Confirming this hypothesis, the results of this study showed that following TACE and subsequent tumor tissue necrosis, serum tryptase levels decreased (Fig. 1), as if due to the destruction of mast cell content in the tumor nodule the source production of tryptase itself ceased. Thus, these serin-proteases acted as a predictive factor of response to locoregional treatment in HCC patients.

In the present study, the tryptase level was measured 24 h prior to treatment as a circulating biomarker surrogate for the presence of a neoplastic disease, and again 24 h after treatment to evaluate the reduction of the concentration of this level. Tryptase levels were assessed after 24 h considering their approximately 4-h long life-cycle. Therefore, with the primary source of tryptase production no longer existing, after 24 h a significant reduction in serum tryptase concentration should be expected.

The resulting data were consistent with this assumption, as the difference between average pre- and post-treatment concentrations (μg/l), was statistically significant.

Constant tryptase levels during post-treatment in patients are likely to indicate residual disease and, therefore, patients should undergo further diagnostic studies.

Therefore, the use of such a marker in the follow-up of treated patients is likely to allow for an early detection of HCC relapse highlighted by a rebound of serum tryptase levels, since in the presence of neoplastic tissue, there is a new share of mast cells capable of releasing tryptase.

Consequently, the range of patients with HCC should be broadened to confirm the preliminary data in this study. In particular, tryptase concentrations were aimed to be assessed in a series of patients with stage A, C and D HCC, according to BCLC staging, in order to evaluate possible statistically significant differences between those stages and stage B, which has already been studied.

Whether the cut-off tryptase average in pre-treatment patients differs significantly from the average serum tryptase concentrations in healthy patients was also determined.

In case the differences between tryptase levels in HCC patients and healthy subjects, and in subgroups of patients with different stages of the disease prove to be statistically significant, serum tryptase might be considered a simple execution biomarker indicating the presence of HCC, as well as the possible stage of disease.

To the best of our knowledge, no published data are currently available on serum tryptase as a circulating biomarker of disease activity in patients with HCC. The preliminary results of this study suggest that serine protease tryptase is likely to be a new circulating biomarker, indicating the presence of HCC notably improving early diagnosis of the disease.However, additional investigations are required to confirm this hypothesis.

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