Assessment of gankyrin mRNA expression in tissue and liquid biopsy as a sensitive biomarker for hepatocellular carcinoma

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Abstract. The early diagnosis of hepatocellular carcinoma (HCC) and the detection of disease recurrence are vital for the improvement of patient survival. The aim of the present study was to investigate the use of circulating plasma gankyrin mRNA expression as a potential biomarker for patient management. A total of 64 patients and healthy volunteers were recruited in the present study [54 individuals and 10 archived material of formalin-fixed paraffin-embedded samples (5 normal liver samples were obtained from transplant donors and 5 from patients undergoing cirrhotic liver biopsy)]. The blood plasma of patients with HCC (n=32), metastatic HCC (n=5), cirrhosis (n=7), hepatitis C virus$^+$ (HCV$^+$; n=5) and healthy individuals (n=5) was evaluated. The expression levels of gankyrin/PSMD10 mRNA in tissue samples from patients with HCC (tumor and adjacent; n=32), cirrhosis (n=5) and normal liver (n=5) were evaluated. RNA extraction from peripheral blood and formalin-fixed paraffin-embedded tissue samples and reverse-transcription-quantitative polymerase chain reaction were performed using commercially available kits according to standard procedures. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. In patients with cirrhosis, HCV$^+$ and healthy individuals, gankyrin/PSMD10 RNA was not determined. In all patients with either HCC or metastatic HCC, target RNA was detected. The mean gankyrin/PSMD10 expression in tissues exhibited significant differences between groups. Evidence was provided that gankyrin RNA expression was significantly increased in HCC compared with that in normal and cirrhotic tissues. Gankyrin RNA was not detectable in the blood plasma of healthy individuals and individuals without cancer, while its expression was significantly increased in the plasma of patients with HCC. Gankyrin RNA in liquid biopsy can thus be used for screening patients who belong to the risk groups for the development of HCC. Since gankyrin is not specific for HCC, it needs to be used with other markers for the diagnosis of HCC, although it can be used alone for the early detection of tumor recurrence.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies. It is the third leading cause of malignant tumor-related mortality; the fifth most common among males and the seventh most common among females (1). At the early stages of the disease (stages 0-A), the tumor is considered curable; however, in 60% of HCC cases, the disease is diagnosed at advanced stages when the average life expectancy, despite treatment, is only 8 months, while the relevant life expectancy at the middle stages of the disease is 26 months (2,3). Furthermore, surgical treatment as an ideal, curative treatment option is available only at the early stages of cancer. Therefore, the early diagnosis of HCC and the monitoring of disease recurrence are vital for the improvement of patient survival.

Over the past decade, a number of molecular techniques were introduced in basic and clinical oncology. Liquid biopsy (LB) is one such diagnostic modality (4). Initially, it was used for the detection of circulating tumor cells in blood; however, it now includes other tumor-related components, such as DNA, RNA, microRNA (miRNA/miR) and proteins (5,6). LB has not yet become a routine clinical test, although its high potential for tumor detection, molecular profiling, surgical radicality monitoring and treatment outcome prediction is evident (7). Compared with the tumor core biopsy technique, LB is a less invasive procedure using body fluids, including blood, for analyses (8). The development of the LB technique led to the

use of tumor-related nucleic acids as biomarkers for cancer. Plasma-derived tumor-related RNA was of particular interest.

Gankyrin, also known as PSMD10 or p28GANK, is a small molecular protein composed of seven ankyrin domains and is a component of the 26S proteasome (9-11). It is encoded by the PSMD10 gene and plays a crucial role in cell cycle progression, apoptosis and tumorigenesis. Initially, gankyrin was identified in human HCC (12); however, several studies have demonstrated that gankyrin expression is upregulated in other types of cancer (for example kidney, breast, testicular cancer and squamous cell carcinoma) as well (13-15).

Numerous studies have demonstrated that p28GANK is one of the main factors involved in hepatocellular carcinogenesis. Gankyrin protein expression is increased not only in HCC tissues, but also in cirrhotic liver, while it is absent in normal liver samples (16-20). In another study, the lowest level of gankyrin protein was found in the normal liver, while its expression was notably increased in HCC, and the level was highest in metastatic HCC (21). A previous study demonstrated that gankyrin was associated with cell cycle and tumor progression (22). As shown by several studies, gankyrin can promote HCC metastasis by enhancing epithelial-mesenchymal transition via the PI3K/Akt/HIF-1α signaling pathway (21,23-25). These findings indicate that gankyrin may be used as a diagnostic and prognostic marker for HCC and for monitoring tumor recurrence.

Despite increased evidence of the role of gankyrin in HCC and other tumors, to the best of our knowledge, there are currently no studies available on circulating cell-free RNA for gankyrin in the blood plasma of patients with cancer. As a biomarker, circulating cell-free RNA has some advantages due to its stability, the non-invasive method used for its collection and the accuracy of the obtained quantitative data.

The aim of the present study was to investigate the feasibility of plasma circulating gankyrin mRNA expression as a potential biomarker for HCC. The expression p28GANK RNA was analyzed in the blood plasma, as well as in the tumor and non-tumor liver tissue of patients with HCC and cirrhosis, and in healthy individuals.

Materials and methods

Blood and formalin-fixed paraffin-embedded (FFPE) tissue samples-study design. A total of 64 patients and healthy volunteers were recruited in the present study, 54 individuals from whom blood was collected and 10 archived material of FFPE (5 normal liver samples were obtained from transplant donors and 5 from patients undergoing cirrhotic liver biopsy) (Tables I and II). All participants provided written informed consent prior to enrolment. The present study was approved by the Institutional Ethics Committee of the Alexandre Natishvili Institute of Morphology (Ivane Javakhishvili Tbilisi State University). All patients were admitted to the 'Israeli-Georgian Medical Research Clinic Healthycore'.

To investigate the expression of the gankyrin/PSMD10 gene in the plasma of patients with HCC, blood samples from patients with HCC (n=32), metastatic HCC (n=5), cirrhosis (n=7), hepatitis C virus$^+$ (HCV$^+$; n=5) and healthy individuals (n=5) were evaluated. All patients with HCC had cirrhosis and sustained virologic response (SVR) following previous direct-acting antiviral treatment. In the group 'HCV$^+$ individuals', patients with newly-diagnosed active HCV hepatitis without cirrhosis and HCC were included. In the group 'patients with cirrhosis', patients without HCC and with SVR were included. The 'HCC group' consisted of 32 patients, 5 females (15.6%) and 27 males (84.4%). The mean age of the patients was 55.7 years (±5.7; range, 46-66 years). The group of patients with metastatic HCC consisted of 5 male individuals with mean age 57.4 years (±3.6; range, 52-62 years). The mean age of the 7 patients with cirrhosis was 57.1 years (±2.9; range, 53-61 years), and among these, there was 1 female patient (14.3%) and 6 male patients (85.7%). The group of patients with HCV infection consisted of 5 individuals, including 1 female (20%) and 4 males (80%) with a mean age of 49.4 years (±1.7; range 48-52 years). Among the 5 healthy volunteers, there was 1 female (20%) and 4 males (80%) without a history of neoplasm, liver and other organ chronic disease. The mean age of the healthy volunteers was 50.0 years (±4.1; range, 45-55 years). The clinical characteristics of the patients in all the study groups are presented in Table I.

The expression of gankyrin/PSMD10 RNA was analyzed in tumor tissue samples from patients with HCC (tumor and adjacent; n=32), cirrhosis (n=5) and normal tissues obtained from living donors prior to transplantation for liver graft quality assessment (n=5). The HCC group consisted of the same patients as in the case of blood plasma. Tumor adjacent tissue (ADJ T) was collected from only 27 cases, as 5 patients only had biopsy samples, as surgery was not indicated. The group of patients with cirrhosis consisted of 5 male individuals (100%). The mean age was 50.4 years (±2.1; range, 48-53 years). The mean age of the healthy liver donors was 35.6 years (±3.04; range, 32-40 years), including 1 female (20%) and 4 males (80%). The clinical characteristics of all the involved individuals are presented in Table II.

RNA extraction from peripheral blood samples. Plasma samples were isolated from 4 ml peripheral blood using BD Vacutainer® Blood Collection Tubes (EDTA-K2 was used as an anticoagulant; BD Biosciences). The tubes were centrifuged at 1,610 x g for 15 min, and 1 ml supernatant plasma was transferred to 1.5 ml tubes and stored at -80˚C until use. Fresh total DNA/RNA/miRNA was extracted from blood plasma using the RecoverAll™ Total Nucleic Acid Isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. Total nucleic acid concentration was measured using a NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Tumor RNA extraction from FFPE tissue samples. The starting material for RNA purification was freshly cut sections of FFPE tissue, each with a thickness of up to 20 μm. Up to 4-20 μm sections from each FFPE tissue block were combined and placed in a 1.5-ml microcentrifuge tube. Total DNA/RNA was extracted from the FFPE tissues of tumor, adjacent liver and control samples using the RecoverAll™ Total Nucleic Acid Isolation kit for FFPE (Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. The RNA concentrations and purity were determined using a Nanodrop1000 spectrophotometer (NanoDrop Technologies;

Table I. Clinicopathological characteristics of the patients from whom plasma was collected.

Characteristic	Patients with HCC (n=32)	Patients with metastatic HCC disease (n=5)	Patients with cirrhosis (n=7)	HCV-positive patients (n=5)	Healthy individuals (n=5)
Sex, n (%)					
Male	27 (84.4)	5 (100)	6 (85.7)	4 (80)	4 (80)
Female	5 (15.6)	0	1 (14.3)	1 (20)	1 (20)
Age, years					
Mean (SD)	55.7 (5.7)	57.4 (3.6)	57.1 (2.9)	49.4 (1.7)	50.0 (4.1)
Median	57.5	58	56	49	50
Range	46-66	52-62	53-61	48-52	45-55
Child-Pugh class, n (%)					
A	18 (56.3)	NA	NA	NA	NA
B	10 (31.3)	NA	NA	NA	NA
C	4 (12.5)	NA	NA	NA	NA

HCC, hepatocellular carcinoma; SD, standard deviation; NA, not applicable.

Table II. Clinicopathological characteristics of the patients undergoing liver tissue biopsy.

Characteristic	Patients with cirrhosis (n=5)	Healthy individuals (n=5)
Sex, n (%)		n (%)
Male	5 (100)	4 (80)
Female	0	1 (20)
Age, years		
Mean (SD)	50.4 (2.1)	35.6 (3.04)
Median	50	35
Range	48-53	32-40

SD, standard deviation.

Thermo Fisher Scientific, Inc.). The A260/280 and A260/A230 ratios were recorded along with the RNA concentration (ng/ml). The RNA samples with a A260/A280 ratio ranging from 1.8 to 2.1 were considered of good purity.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR)-p28/gankyrin. RT-qPCR was performed using TaqPath™ 1-Step Multiplex Master Mix (No ROX; cat. no. A28522; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Human 18S ribosomal RNA was used as an internal reference gene. Each sample was run in duplicate. The RT-qPCR protocol included an initial denaturation step at 95˚C for 20 sec and 40 cycles of denaturation at 95˚C for 3 sec and annealing at 60˚C for 30 sec. The relative expression levels were calculated using $2^{-\Delta\Delta Cq}$ method as previously described (26). cDNA was amplified using the following Taqman® assays: p28/Gankyrin-(Assay ID Hs01100439_g1) and human 18S ribosomal RNA-(Assay ID Hs99999901_s1; Thermo Fisher Scientific, Inc.).

Statistical analysis. The Mann-Whitney U test was used to determine the statistical significance for comparisons data of $2^{-\Delta\Delta Cq}$ and fold change. For multiple comparisons, the non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test was. Statistical analyses were carried out using SPSS (version 23.0; IBM Corp.) and GraphPad Prism (version 8.0; Dotmatics).

Results

The plasma gankyrin/PSMD10 RNA level was examined using RT-qPCR in the patients with HCC (n=32), metastatic HCC (n=5), cirrhosis (n=7), HCV^+ (n=5) and healthy individuals (n=5). In patients with cirrhosis, HCV^+ and healthy individuals, gankyrin/PSMD10 RNA was not detected by the method described in the present study. ΔCq (target gene Cq - housekeeping gene Cq) was calculated for every sample. The mean ΔCq in HCC group was 11.69±1.6, and in patients with metastatic HCC it was 10.16±0.4. A statistically significant difference in the ΔCq was found between the groups (mean difference, 1.532; 95% CI of difference, 0.7444-2.3200; P<0.0002; Fig. 1).

A significant difference in the gankyrin expression level was found in the tissue samples. The $2^{-\Delta\Delta Cq}$ mean for gankyrin/PSMD10 in HCC, HCC ADJ T, cirrhosis and control group was 418.5±220.6, 78.68±49.40, 20.62±11.32 and 1.02±0.23, respectively. The differences between groups were statistically significant (P<0.001; Fig. 2).

Discussion

Several studies have used LB in the clinical management of HCC, including the research for early detection, monitoring of disease and prediction of response (27-34). In these studies, different molecular panels were used; however, satisfactory levels of specificity and sensitivity were not achieved. Thus, the identification of novel potential targets is critical in order to obtain desirable results for clinical implementation.

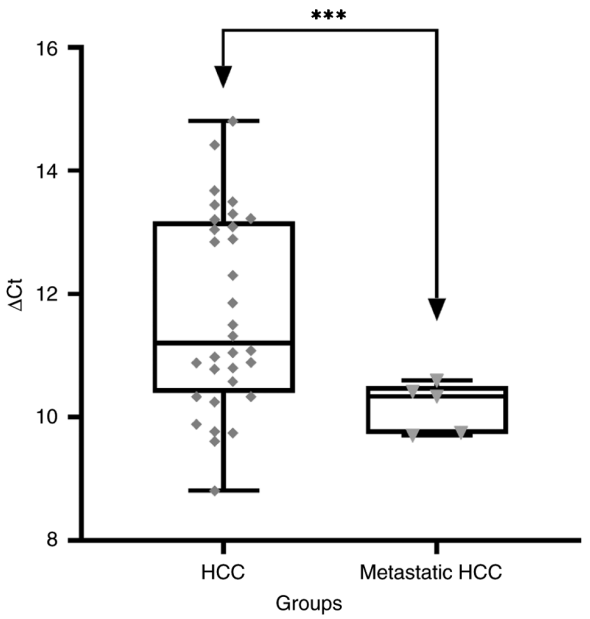

Figure 1. ΔCt of gankyrin mRNA in the blood plasma of patients with HCC and metastatic HCC. ***P<0.001; HCC, hepatocellular carcinoma.

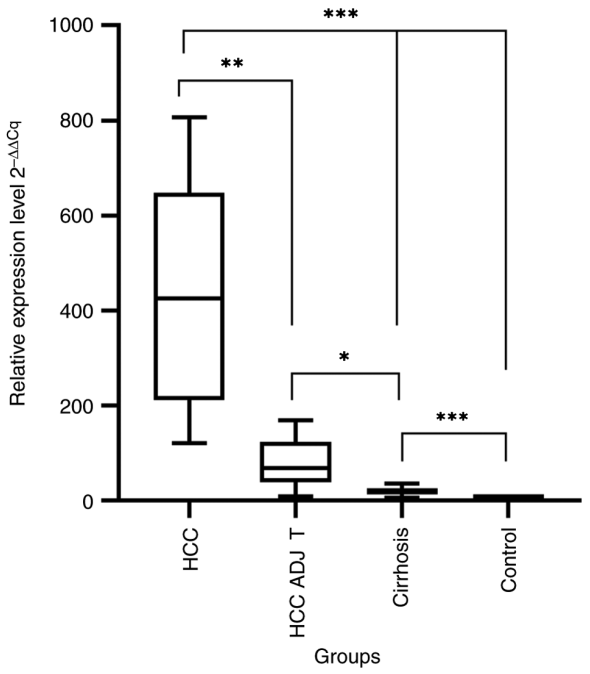

Figure 2. Relative expression level ($2^{-\Delta\Delta Cq}$) of gankyrin mRNA in tissue samples. *P<0.05, **P<0.01 and ***P<0.001. HCC, hepatocellular carcinoma; HCC ADJ T, hepatocellular carcinoma tumor adjacent tissue.

In the present study, to the best of our knowledge, the differential expression of gankyrin RNA in the serum and liver tissues samples from patients with HCC and from healthy individuals was reported for the first time, identifying novel potential biomarkers with a high diagnostic capacity.

Several findings can be highlighted in the results of the present study. First, the gankyrin RNA level in HCC tumor tissue was significantly higher, not only compared with that in healthy liver tissue, but also compared with that in adjacent and cirrhotic livers. Thus, it can be added as an ancillary study for the differential diagnosis of liver malignant and benign or reactive nodules on biopsy. Furthermore, in the liver tissue adjacent to the tumor, which is naturally cirrhotic liver, the gankyrin RNA level was also higher than that in the cirrhotic liver tissues of patients without HCC. It is not clear if this is a consequence of adjacent 'malignant' process, or whether the upregulation is a first step of 'malignant' transformation of hepatocytes. If the latter was confirmed, a good prediction marker for patients with cirrhosis would have been identified to determine the risk of HCC development.

Secondly, the data from the expression of gankyrin RNA in blood plasma revealed promising results. In healthy individuals, individuals with an HCV infection and in patients with cirrhosis, gankyrin RNA was not detectable at all in blood plasma, while it was detectable in all patients with either local HCC or metastatic disease. These findings indicate the potential of gankyrin RNA alone or in combination with other genetic markers to be used as a clinical biomarker for early detection, the monitoring of disease and the prediction of the response in patients with HCC.

Although additional research on gankyrin RNA is required, the findings of the present study, together with other clinical and instrumental data, may be informative for the management of HCC, particularly in cases where other studies did not provide a clear result. In addition, this marker would enable the detection of HCC at a stage when it is impossible to make a full diagnosis with computed tomography, ultrasound and MRT data.

For example, in the case of the Liver Imaging Reporting and Data System-3 (LiRads-3) lesion during cirrhosis, the patient is monitored for a long period of time, since the aforementioned type of lesion does not indicate a malignant tumor, but due to the presence of pathogenic risks, it is considered a potential danger. With the result of the LB, it may be possible to differentiate a malignant tumor in LiRads-3 lesions from a hyperplastic nodule, which occurs as an ordinary case in liver cirrhosis.

Although the present study included a relatively small number of patients, it provided promising and solid results, and could lay the foundation for further research conducted towards this direction, as well as for the detection of an improved molecular panel together with gankyrin to diagnose early-stage HCC.

The method described in the present study is less invasive and enables the determination of the molecular profile using blood plasma. LB is a simple diagnostic tool for early detection of malignancy in risk groups, such as those infected with HCV and hepatitis B virus for a long time, cirrhosis of the liver and non-alcoholic fatty liver disease. In addition, gankyrin RNA as a biomarker may be suitable for monitoring HCC recurrence.

The present study has certain limitations, which should be mentioned. First, the present study was conducted on a relatively small group of patients and needs to be confirmed in a larger case series involving different centers. Second, the present study did not include follow-up data of patients, which would be proof of the ability of gankyrin RNA in LB to be used for the early detection of HCC recurrence. As an LB marker, gankyrin RNA must be validated in patients with different liver tumors and also secondary ones.

In conclusion, the data of the present study provide strong evidence that the expression of gankyrin RNA is significantly increased in HCC compared with that in normal and cirrhotic

tissues. In the tumor adjacent liver tissue, the gankyrin RNA level was higher than that in the cirrhotic liver tissues of patients without HCC. Gankyrin RNA was not detectable in the blood plasma of healthy individuals and in that of patients without cancer; however, its expression was significantly increased in the plasma of patients with HCC. The detection of gankyrin RNA in LB can be practically used for screening patients who belong to the risk groups for the development of HCC.

Since gankyrin is not specific for HCC, it must be used with other molecular markers for the diagnosis of HCC, but it can be used alone for the monitoring and early detection of tumor recurrence. To prove this hypothesis, future studies are warranted, which must include data regarding gankyrin RNA expression levels in the blood samples before and after the resection of HCC and validation at the protein level.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors (LG, GL, HJS, ZB, LS, NG and MJ) contributed to the conception and design of the study. Material preparation was performed by LG, LS, NG and MJ. Data collection and analysis were performed by MJ, ZB and LG. Analysis was performed by ZB, HJS and GL. ZB and NG confirm the authenticity of all the raw data. The first draft of the manuscript was written by LG and all authors commented on previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the Alexandre Natishvili Institute of Morphology (Ivane Javakhishvili Tbilisi State University; approval no. N06/18) and written informed consent was provided by the patients. The present study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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