Serum monomeric laminin-γ2 as a novel biomarker for hepatocellular carcinoma

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The diagnosis of hepatocellular carcinoma (HCC) in the early stages is important for successful clinical management. Laminin (Ln)-γ2 expression has been reported in various types of malignant carcinomas. We recently developed a highly sensitive method to measure serum monomeric Ln-γ2 levels using a fully automated chemiluminescent immunoassay (CLIA). Using our CLIA, we evaluated its diagnostic value in sera from patients with chronic liver disease (CLD) and patients with hepatocellular carcinoma (HCC). Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were also examined in these subjects. Median levels of Ln-γ2 were significantly higher in patients with HCC (173.2 pg/mL; range: 39.5–986 pg/mL) compared with patients with CLD (76.7 pg/mL; range: 38.7–215.9 pg/mL) and with healthy volunteers (41.1 pg/mL; range: 10.9–79.0 pg/mL). The optimal cutoff value for Ln-γ2 that allowed us to distinguish between HCC and nonmalignant CLD was 116.6 pg/mL. Elevated Ln-γ2 levels were observed in 0% of healthy volunteers, 17% of patients with CLD, and 63% of patients with HCC. The positivity rate in patients with HCC for the combination of Ln-γ2 and DCP was 89.5%, which was better than that for either of the two markers alone (63% and 68%, respectively). Among patients with early-stage HCC (T1 or T2), the positivity rates for monomeric Ln-γ2, AFP and DCP were 61%, 39% and 57%, respectively. Serum Ln-γ2 may be a potential biomarker for HCC surveillance. The combination of Ln-γ2 and DCP may be more sensitive for laboratory diagnosis of HCC than the combination of AFP and DCP.
γ2 chains, which are encoded by three distinct genes (LAMA3, LAMB3 and LAMC2, respectively). Ln-332 mediates epithelial cell adhesion to the BM. In particular, Ln-γ2, a component of Ln-332, is frequently expressed as a monomer in several types of malignant cancer cells and tissues without simultaneous expression of Ln-α3 and Ln-β3 chains. Ln-γ2 expression has been immunohistochemically detected in various malignant carcinomas (stomach, esophageal, colon, biliary and pancreatic tumors) in areas where Ln-α3 and Ln-β3 are not expressed, indicating that Ln-γ2 as a monomer might be a specific marker of invasion. Although Ln-332 is widely expressed in human tissues, it is not detected in the normal liver. Interestingly, several reports indicated that the γ2 chain of Ln-332 is expressed in HCC tissues. Therefore, the presence of monomeric Ln-γ2 in the serum may be useful as a biomarker for HCC. In an earlier study, we developed a monoclonal antibody that preferentially recognizes the monomeric form of Ln-γ2 in the serum may be useful as a biomarker for HCC. In an earlier study, we developed a monoclonal antibody that preferentially recognizes the monomeric form of Ln-γ2.

**Patients and Methods**

**Patients.** Study subjects were enrolled from the liver clinic at the St. Marianna University School of Medicine Hospital (Kanagawa, Japan) between January 2007 and April 2015. The study protocol was approved by the institutional review boards of St. Marianna University School of Medicine (Res-2302), the Institute of Medical Science, University of Tokyo (24-44-1017), the Kanagawa Cancer Center (Res-24) and Abbott Japan (OSE2014-006). Written informed consent was obtained from each patient. Demographic and clinical information was collected from each subject. Healthy volunteers with no history of liver disease, alcohol consumption of less than 40 g/week, and no risk factors for viral hepatitis were recruited among the hospital staff and were used as controls (Table 1). The etiology of the underlying liver disease was attributed to hepatitis C virus (HCV) based on the detection of HCV antibody/HCV RNA in the serum, to hepatitis B virus (HBV) based on the detection of hepatitis B surface antigen in the serum, or to alcohol based on a daily alcohol intake of more than 40 g/ethanol per day for more than 15 years. The CLD group consisted of patients with no evidence of hepatic mass lesions suspicious for HCC on ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). To establish the diagnosis of HCC, at least two liver imaging results indicating a mass lesion with characteristics resembling an HCC (arterial enhancement ± washout on CT with contrast medium, MRI and/or hepatic angiography) were required. Typical HCC shows hypervascularity in the arterial phase and washout of contrast medium in the portal venous phase. For few cases, in which the diagnosis of HCC was still equivocal despite liver imaging studies, percutaneous liver biopsy was performed. Tumors were staged based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan). In this classification, the T factor includes three criteria: solitary tumor, maximum tumor diameter <2 cm and no vascular invasion. Tumor stage T1 meets all the three criteria, T2 meets two of the three criteria, T3 meets one of the three criteria, and T4 does not meet any of the criteria. Serum samples were stored at −80°C until analysis.

**Antibodies and antigens.** Recombinant monomeric Ln-γ2 protein was purified from the culture medium of Madin-Darby canine kidney cell transfectants, as described previously (Nakagawa M, manuscript in preparation). In the present study, we used CLIA to evaluate the diagnostic value of monomeric Ln-γ2 in sera from patients with CLD and patients with HCC.

### Table 1. Demographic information and etiology of liver diseases

|                      | Healthy control (n = 52) | Chronic liver disease (n = 24) | Hepatocellular carcinoma (n = 57) |
|----------------------|--------------------------|------------------------------|----------------------------------|
| Gender               |                          |                              |                                  |
| M:F                  | 32/20                    | 11/13                        | 38/19                            |
| Age (y)              | 33 (25–58)               | 59 (35–74)                   | 71 (49–80)                       |
| Etiology (%)         |                          |                              |                                  |
| HCV                  | 20 (83)                  | 30 (52)                      |                                  |
| HBV                  | 3 (13)                   | 8 (14)                       |                                  |
| HBV+HCV              | 1 (2)                    |                               |                                  |
| Alcohol              | 12 (21)                  |                               |                                  |
| NBNCNAL              | 1 (4)                    | 5 (9)                        | 1 (2)                            |
| Autoimmune           |                          |                              |                                  |

All data are expressed as median (range).

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two-step sandwich assay using paramagnetic microparticles coated with 2H2 antibody (Nakagawa M et al., manuscript in preparation) and rabbit polyclonal antibody labeled with acridinium. The procedure was adjusted to be applied to a fully automated detection machine (ARCHITECT of Abbott Laboratories, Chicago, IL, USA).

Analysis of tumor markers for hepatocellular carcinoma. Serum AFP levels were measured by using the ARCHITECT system, and DCP levels were measured by using LUMIPULSE (Fujirebio, Tokyo, Japan) in SRL Tokyo Laboratories (Tokyo, Japan).

Statistical analysis. Serum monomeric Ln-γ2 levels were reported as mean ± SD and median (and range). Serum AFP and DCP levels were reported as median: range. Log transformation was used on the AFP and DCP values to account for the large range of values for both markers among patients with HCC. Continuous variables were compared between the two groups using the Mann–Whitney U-test and Student’s t-test; P-values less than 0.05 were considered significant. GraphPad Prism 6 Software (San Diego, CA, USA) was used to generate all scatter plots, receiver operating characteristic (ROC) curves, and the calculation of the area under the ROC (AUC) curves. The optimal cutoff point included the point on the ROC curve closest to the (0, 1).

Results

Detection of Ln-γ2 expression in hepatocellular carcinoma tissues. To investigate the expression of Ln-γ2 in HCC, immunohistochemical analyses were performed. In accordance with previous reports,(17,19) cytoplasmic staining of Ln-γ2 was observed in 5/10 of surgically-resected HCC nodules (Fig. 1a). Ln-γ2 immunoreactivities were observed in marginal, poorly differentiated parts of the tumor nodules in most of cases (Fig. 1a), but not in well-differentiated parts of HCC and adjacent normal tissues. Moreover, expression of Ln-α3 was not observed in HCC nodules (Fig. 1b) and their adjacent normal tissues. To further identify the molecular composition of Ln-γ2 in HCC, Ln-γ2 protein was detected by western blotting using HCC tissue lysates from patients. When purified monomeric Ln-γ2 was electrophoresed on SDS-PAGE under nonreducing conditions, two proteolytic fragments (140 and 100 kDa) were detected by an anti-Ln-γ2 polyclonal antibody that detects both Ln-γ2 isoforms (Fig. 2a). Ln-γ2 is known to be processed by metalloproteinases, such as MMP2 and MT1-MMP, and two different fragments (named γ2α of 140 kDa, and γ2γ of 100 kDa, under nonreducing conditions) have been previously described.(24) In contrast, recombinant Ln-332 was detected as a large 450-kDa band, which corresponds to a complex of three polypeptide chains cross-linked by disulfide bonds. Western blotting under reducing conditions indicated that Ln-332 was the source of monomeric Ln-γ2 and of the proteolytic fragment(s) (Fig. 2b). Two HCC cell lines, Alexander and HepG2, also expressed both forms of monomeric Ln-γ2 fragments (Fig. 2c). Then, we examined Ln-γ2 in tissues from surgically-removed HCC nodules. Similar to HCC cell lines, HCC tissue lysates also contained both forms of monomeric Ln-γ2 (Fig. 2d). Heterotrimeric Ln-332-γ2 was undetectable in HCC cell lines or surgically-removed HCC nodules (data not shown). Taken together, our immunohistochemical and western blotting studies indicate that monomeric, but not heterotrimeric, Ln-γ2 was expressed in HCC nodules.

Monomeric Ln-γ2, alpha-fetoprotein and des-gamma-carboxy prothrombin levels among healthy volunteers, patients with chronic liver disease, and patients with hepatocellular carcinoma. Because monomeric Ln-γ2 rather than heterotrimeric Ln-γ2 was expressed preferentially in HCC nodules, the

Fig. 1. Immunohistochemical staining for Ln-γ2 and Ln-α3 in hepatocellular carcinoma (HCC). (a) A representative example of positive staining for Ln-γ2. Cytoplasmic staining of Ln-γ2 was observed in 5/10 of surgically resected HCC nodules. (b) Expression of Ln-α3 was not observed in HCC nodules. (c) HE staining. Scale bars, 50 μm.
diagnostic value of monomeric Ln-γ2 was evaluated in sera from patients with HCC and CLD using the automated CLIA. To establish the cutoff value for Ln-γ2, control sera from 52 healthy Japanese volunteers were analyzed. Demographic and clinical characteristics of healthy volunteers and patients with CLD or HCC are shown in Table 1. Child–Pugh scores of patients with HCC are shown in Table 2. Clinical characteristics of patients with HCC are presented in Table 3.

The mean and median serum monomeric Ln-γ2 levels of healthy volunteers were 44.3 ± 17.6 pg/mL (mean ± SD) and 41.1 pg/mL (range: 10.9–79.0 pg/mL), respectively. Serum monomeric Ln-γ2 levels were measured in 24 patients with CLD and 57 patients with HCC (Fig. 3a). Infection with HCV was the most common etiologic factor between the two groups of subjects with liver disease. The median monomeric Ln-γ2 concentrations were 76.7 pg/mL, with a range between 38.7 and 215.9 pg/mL (patients with CLD) and 173.2 pg/mL, with a range between 32.4 and 986 pg/mL (patients with HCC). A significant increase in monomeric Ln-γ2 levels was observed in patients with HCC when compared with patients with CLD and healthy volunteers (P < 0.01). ROC curves were plotted to identify cutoff values for serum monomeric Ln-γ2 to distinguish between patients with HCC and healthy controls (Fig. 3b). When the cutoff value was set at 75.9 pg/mL, the sensitivity and specificity were 86% (95% confidence interval [CI], 74–94%) and 98% (95% CI, 90–99%), respectively. The discriminative ability of monomeric Ln-γ2 (ROC curve AUC = 0.952; 95% CI, 91–99%) significantly surpassed that of DCP (ROC curve AUC = 0.825; 95% CI, 73–92%, P < 0.05) and it was as effective as AFP (ROC curve AUC = 0.929; 95% CI, 88–98%) when comparing healthy volunteers and patients with HCC. When healthy volunteers were compared with patients with nonmalignant CLD, this cutoff value yielded a discriminative ability for monomeric Ln-γ2 (ROC curve AUC = 0.819; 95% CI, 72–92%), with a sensitivity of 50% (95% CI, 29–71%) and specificity 96% (95% CI, 87–99%). With this cutoff value, monomeric Ln-γ2 positivity was found in 1 (2%), 12 (50%) and 50 (86%) subjects among healthy volunteers, patients with CLD, and patients with HCC, respectively. These results indicate that this cutoff value (>75.9 pg/mL) is useful to discriminate patients with CLD, with or without HCC, from healthy subjects.

Receiver operating characteristic curves were plotted to evaluate the cutoff value for monomeric Ln-γ2 that would distinguish between patients with HCC and patients with CLD. The optimal cutoff value was 116.6 pg/mL (Fig. 3c), with a sensitivity of 63% (95% CI, 49–76%) and a specificity of 83% (95% CI, 63–95%). When discriminating patients with CLD from those with HCC, DCP (ROC curve AUC = 0.845; 95% CI, 76–93%) outperformed monomeric Ln-γ2 (ROC curve AUC = 0.793; 95% CI, 69–89%; differences were not statistically significant) and AFP (ROC curve AUC = 0.788; 95% CI,
Table 3. Clinical characteristics of patients with hepatocellular carcinoma

|                | n  | Numbers of tumors | Diameter of largest tumor | AFP     | DCP     |
|----------------|----|-------------------|---------------------------|---------|---------|
| TNM I          | 10 | 1 (1)             | 15.0 ± 3.1 (15; 10–19)    | 12.3 ± 15.6 (5.3; 2.2–44.6) | 97 ± 110 (55; 14–302) |
| TNM II         | 18 | 4.9 ± 12 (2; 1–52) | 25.9 ± 10.0 (26.5; 13–44) | 98.7 ± 211.8 (16.2; 1.5–899) | 483 ± 1417 (47.5; 13–5380) |
| TNM III        | 21 | 23 ± 32 (5; 1–113) | 36.9 ± 18.0 (32.5; 21–85) | 345.9 ± 598.7 (72.5; 3.2–2364) | 1842 ± 5487 (162; 11–20 779) |
| TNM IV         | 8  | 58 ± 69 (14; 1–225)| 51 ± 20.0 (48.0; 26–82)   | 54 209 ± 146 719 (1116; 5.3–417 198) | 18 400 ± 29 309 (683; 32–75 000) |
| TNM IVA        | 5  | 42 ± 76 (6; 2–177) | 50.0 ± 17.3 (46; 33–71)   | 86 583 ± 146 719 (1522; 5.3–417 198) | 29 152 ± 33 435 (17 300; 32–75 000) |
| TNM IV B       | 3  | 86 ± 122 (31; 1–225)| 52.7 ± 28.1 (50; 26–82)   | 919 ± 1278 (371.5; 5.3–2379) | 481 ± 362 (589; 78–777) |

Numbers of TNM stages (I/II/III/IV-A/IV-B), numbers of tumors (mean ± SD, median; range), largest tumor diameter (mean ± SD, median; range), AFP (mean ± SD, median; range) and DCP (mean ± SD, median; range) are presented. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

Monomeric Ln-γ2 positivity (>116.6 pg/mL) was observed in 0/52 (0%), 4/24 (17%) and 36/57 (63%) subjects among healthy volunteers, patients with CLD, and patients with HCC, respectively. AFP positivity (>20 ng/mL; the upper normal limit reported in Japanese subjects) was observed in 0/52 (0%), 4/24 (17%) and 30/57 (53%) subjects among healthy volunteers, patients with CLD, and patients with HCC, respectively. The median concentrations (range) of AFP were 23.9 ng/mL (healthy controls, range: 0.9–8.2 ng/mL), 3.8 ng/mL (patients with CLD, range: 1.2–19.7 ng/mL), and 24.4 ng/mL (patients with HCC, range: 1.5–417 199 ng/mL) (Fig. 4a). DCP positivity (>40 mAU/mL, the upper normal limit reported in Japanese subjects) was observed in 1/52 (1.9%), 1/24 (4.2%) and 39/57 (68%) subjects among healthy volunteers, patients with CLD, and patients with HCC, respectively. The median concentrations (range) of DCP were 45.0 mAU/mL (healthy controls, range: 13.0–44.6 ng/mL), 16.2 ng/mL (patients with CLD, range: 1.5–899 ng/mL), 72.5 ng/mL (patients with HCC, range: 11.0–75 000 mAU/mL) (Fig. 4b). Monomeric Ln-γ2, AFP and DCP levels were found to be significantly elevated (P < 0.001) in sera from patients with HCC compared with sera from both healthy volunteers and patients with CLD. Moreover, Ln-γ2 and AFP levels were found to be significantly elevated (P < 0.01) in sera from patients with CLD compared with sera from healthy volunteers. When Ln-γ2 levels of patients with HCC were compared with log-transformed AFP or DCP levels of those patients, significant positive correlation was observed between Ln-γ2 and AFP (r = 0.41; P = 0.0015).

We also evaluated whether a combination of monomeric Ln-γ2 (>116.6 pg/mL) and AFP (>20 ng/mL) or DCP (>40 mAU/mL) could improve the diagnostic efficacy for HCC (Fig. 5). Monomeric Ln-γ2 and DCP were detected in 51/57 patients (89.5%), monomeric Ln-γ2 and AFP in 46/57 (80.7%), and DCP and AFP in 47/57 (82.5%). The combination of all three markers was detected in 54/57 patients (94.7%). Interestingly, 67% (12/18) of patients who were negative for DCP were positive for Ln-γ2.

Monomeric Ln-γ2, alpha-fetoprotein and des-gamma-carboxy prothrombin values according to tumor staging. According to the TNM classification, 10 patients were diagnosed with stage I, 18 with stage II, 21 with stage III and 8 with stage IV tumors. Monomeric Ln-γ2 levels increased from stage I to stage IV with median levels (range) of 114.3 pg/mL (range: 65.3–323.6 pg/mL), 184.3 pg/mL (range: 39.5–549.1 pg/mL), 174.6 pg/mL (range: 32.4–985.8 pg/mL) and 248.1 pg/mL (range: 59.8–529.4 pg/mL), respectively (Fig. 6a). The AFP values were 5.3 ng/mL (range: 2.2–44.6 ng/mL), 16.2 ng/mL (range: 1.5–899 ng/mL), 72.5 ng/mL (range: 3.2–2364 ng/mL) and 1116 ng/mL (range: 5.3–417 198 ng/mL) for stage I, II, III and IV HCC, respectively (Fig. 6b). The DCP values were 55.0 mAU/mL (range: 14.0–302 mAU/mL), 47.5 mAU/mL (range: 13.0–5380), 162 mAU/mL (range: 11.0–20 779), and 683 mAU/mL (range: 32.0–75 000 mAU/mL) for stage I, II, III and IV HCC, respectively (Fig. 6c). Among patients with early-stage HCC (T1 or T2), the positivity rates for monomeric Ln-γ2, AFP and DCP were 17/28 (61%), 11/28 (39%) and 16/28 (57%), respectively.

Discussion

In our study, the clinicopathological significance of monomeric Ln-γ2 levels in the serum of patients with HCC was...
investigated. By using a fully automated CLIA, a significant increase in serum monomeric Ln-γ2 levels was observed in patients with HCC when compared with patients with CLD and healthy volunteers. Our results indicate the potential efficacy of serum monomeric Ln-γ2 levels as a marker for HCC surveillance.

Although trimeric Ln-332 is absent in the normal liver tissue, (16) increased expression of Ln-γ2 has been reported in metastatic and primary HCC nodules (17). Recent reports showed that the γ2 chain of Ln-332 was highly expressed in HCC expressing the biliary marker keratin 19. (19) In our study, western blot analysis indicated that monomeric Ln-γ2 was predominantly expressed in surgically removed HCC nodules and HCC cell lines. Notably, using a CLIA, we observed a significant increase in serum monomeric Ln-γ2 levels in patients with HCC when compared with patients with CLD and healthy volunteers. Aberrant activation of Wnt/β-catenin signaling is a common genetic abnormality in human HCC. (25) Accordingly, β-catenin targets LAMC2 through T-cell factor-binding elements, (18) thus supporting our data showing that serum and cytoplasmic expression of monomeric Ln-γ2 levels in HCC patients were increased. Etiologies of HCC in patients with elevated serum monomeric Ln-γ2 levels include HBV and HCV, but also alcoholic liver disease, NASH and autoimmune hepatitis. We also observed that monomeric Ln-γ2 levels increased according to the stepwise progression of liver disease (i.e. from chronic hepatitis to HCC) and according to tumor stages. We observed a modest increase in serum monomeric Ln-γ2 levels in patients with CLD without HCC. The ROC curve indicated a cutoff value for monomeric Ln-γ2 of 75.9 pg/mL for discriminating patients with HCC from healthy controls. A value of 116.6 pg/mL for monomeric Ln-γ2 yielded the best sensitivity and specificity for differentiating patients with HCC from those with CLD. Of the patients with CLD without HCC, four (16.7%) had elevated monomeric Ln-γ2 levels (>116.6 pg/mL). They also showed elevated AFP (>20 ng/mL, three of them) or DCP (>40 mAU/mL, one of them) levels. Increased levels of monomeric Ln-γ2 in such patients may reflect the hepatocellular regeneration that occurs during chronic liver injury, a mechanism suggested by other biomarkers.

AFP and DCP are the two most commonly used biomarkers for HCC. (7) Several studies indicate that each of these biomarkers alone is not sufficient for HCC surveillance. (9) We found that serum monomeric Ln-γ2 appeared to be more effective than AFP in differentiating patients with HCC from those...
with nonmalignant CLD. Among patients with early-stage HCC (T1 of T2), positivity rates for monomeric Ln-γ2 (>116.6 pg/mL), DCP and AFP were 61%, 57% and 39%, respectively. These results indicate that serum monomeric Ln-γ2 is potentially a useful biomarker to detect early-stage HCC. The combination of DCP and AFP has been reported to be useful for the early detection of HCC in patients with CLD. (26) Accordingly, the Asian Pacific Association for the Study of the Liver stated that the simultaneous measurement of AFP and DCP for the diagnosis of HCC provides higher sensitivity without decreasing specificity. (27) Interestingly, the positivity rate for the combination of monomeric Ln-γ2 and DCP was higher (89.5%) than that of either of the markers alone (63% and 68%, respectively, \( P < 0.02 \) or of the combination of DCP and AFP (80.7%).

In addition to HCC, monomeric Ln-γ2 is also involved in a number of other malignancies. Several immunohistochemical studies indicated that Ln-γ2 is expressed in various malignant carcinomas. (13-19) Our preliminary results indicated that elevated serum monomeric Ln-γ2 levels could be observed in patients with various malignancies, such as pancreatic adenocarcinoma (data not shown) and bladder carcinoma. (21)

Although further analysis is required, serum monomeric Ln-γ2 is a potentially useful biomarker for the screening of cancer in various situations, similar to the carcinomembryonic antigen.

The higher diagnostic positive rate shown by the combination of serum monomeric Ln-γ2 with DCP may improve the efficiency of surveillance programs for HCC. Approximately 75–80% of HCC cases are attributable to persistent HBV or HCV infections. While the rate of patients with HCC with viral infection has been decreasing, the rate and number of cases of patients with non-B non-C (NBNC)-HCC have been increasing, during the last two decades. (22) Most NBNC-HCC cases tend to be discovered in advanced stages when compared with patients with HBV-HCC and HCV-HCC, because patients with NBNC-HCC (i.e. patients who do not have viral hepatitis) do not receive regular HCC surveillance, whereas patients with HBV or HCV receive regular HCC surveillance. Accordingly, monomeric Ln-γ2 is potentially a useful biomarker for HCC surveillance in patients who are less likely to benefit from regular ultrasound screening, such as patients with metabolic syndrome and/or alcoholic liver disease.

Our results are very encouraging despite several limitations. First, because this was a single-center observational study, multi-center prospective studies are needed to evaluate the usefulness of serum monomeric Ln-γ2 for HCC surveillance in patients with CLD. Second, blood samples were collected only at one time point before treatment for HCC. However, despite the limitations, the use of serum monomeric Ln-γ2 as a biomarker to monitor therapeutic effects is of particular interest.

In conclusion, serum monomeric Ln-γ2 levels significantly increased in patients with HCC when compared with patients with CLD and healthy volunteers. The diagnostic value of the combination of monomeric Ln-γ2 with DCP levels was better than that of either of these markers alone, suggesting the potential usefulness of serum monomeric Ln-γ2 in HCC surveillance.

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