Glypican-3 as a Serum Marker for Hepatocellular Carcinoma

To the Editor: Hippo et al. (1) have recently reported in an article published by Cancer Research that glypican-3 (GPC3) is a serologic marker of hepatocellular carcinoma (HCC). The results published by Hippo et al. basically confirm the findings reported previously by our laboratory (2) and by Nakatsura et al. (3). The fact that three independent laboratories using different anti-GPC3 antibodies have reached similar results strongly suggests that GPC3 is a useful marker for HCC. Furthermore, the three laboratories have also found that, due to the lack of correlation between serologic concentrations of GPC3 and α-fetoprotein in HCC patients, the simultaneous use of both markers significantly increases the sensitivity of the test. Despite our general agreement with the results of Hippo et al., we disagree with the conclusions reached by these authors with regard to the biochemical analysis of the results.

Basically, their claim that only the NH₂-terminal portion of GPC3 is present in the sera of HCC patients is not correct. It is well established that GPC3 is composed of two subunits that are linked by one or more disulfide bonds (4, 5). The two subunits are produced by cleavage of GPC3 at residue R358 by a convertase (5), which generates a NH₂-terminal fragment of ~40 kDa and a COOH-terminal fragment of ~30 kDa. It is important to note that in most cases the COOH-terminal fragment of GPC3 will carry the heparan sulfate chains and will produce a high molecular weight smear instead of a band in a Western blot. If GPC3 is run through a reducing gel, the two GPC3 subunits will be separated, and an antibody directed against the NH₂-terminus will only detect the 40-kDa band, which does not have the heparan sulfate chains, as was shown by Hippo et al.

Currently, we do not have an explanation as to why Hippo et al. did not detect GPC3 in their Western blot analysis of serum from HCC patients and in the supernatant of HCC cell lines when they used their own anti-GPC3 monoclonal antibody against the COOH-terminal antibody. We have already shown that our COOH-terminal antibody detects the smear corresponding to glycanated GPC3 both in the conditioned medium from HCC cells (2) and in the serum of HCC patients (Fig. 1).

In summary, we believe that the GPC3 ELISA tests done by Hippo et al. and by our laboratory in the serum of HCC patients are detecting the same biochemical entity, although we have used an antibody that recognizes the COOH-terminal fragment and Hippo et al. used an antibody against the NH₂-terminal portion of GPC3. This conclusion is clearly supported by the fact that both laboratories found a similar proportion of GPC3-positive sera (51% in the case of Hippo et al. and 53% in our laboratory).

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References

In Response: We have reported previously overexpression of glypican-3 (GPC3), glycosylphosphatidylinositol-anchored proteoglycan, at protein level in hepatocellular carcinoma (1). Recently, we further showed elevated levels of serum GPC3 in patients with hepatocellular carcinoma (2), which is grossly similar with a previous report by Capurro et al. (3). However, a target molecule in the serum measurement by ELISA seems different: (a) soluble GPC3 (sGPC3), NH₂-terminal fragment of GPC3 detected with NH₂-terminal antibodies in our study, and (b) glycanated GPC3 detected with COOH-terminal antibodies in their study. They claim that sGPC3 is still linked via disulfide bond to glycanated COOH-terminal fragment of GPC3 even after cleavage, and they could detect this complex with their COOH-terminal antibody, suggesting they and we are measuring the identical molecule.

In a previous study, we independently identified a cleavage site at R358 and showed that sGPC3 of 40 kDa was mainly detected with NH₂-terminal antibodies in the culture supernatant of hepatoma cells and sera of patients with hepatocellular carcinoma, whereas core proteins without cleavage at R358 were not detectable unlike in the total cell lysates (2). We detected glycanated form in serum as well with both NH₂- and COOH-terminal
antibodies, but only when the blotting membranes were exposed for longer time compared with when detecting sGPC3 (data not shown). These observations led us to conclude that sGPC3, but not the core protein or glycanated form without cleavage, is the major diagnostic target (2).

We did not directly examine existence of sGPC3 linked to glycanated COOH-terminal fragment because Western blotting was done in reducing condition in our study; here, we then examined Huh6 hepatoma cell culture supernatant in nonreducing condition. A dense band corresponding to sGPC3 detected in reducing condition becomes faint in nonreducing condition (Fig. 2A), suggesting a major portion of sGPC3 was linked to glycanated COOH-terminal fragment. Thus, signal discrepancy in ELISA between COOH- and NH$_2$-terminal antibodies may be partly attributable to the difference in affinity between our antibodies.

Based on the opinion by Capurro et al, only smearing should be detected; however, a band slightly smaller than 50 kDa was detected solely with NH$_2$-terminal antibody in addition to smearing corresponding to glycanated GPC3 detected with both NH$_2$- and COOH-terminal antibodies in the long exposure (Fig. 2A and B). This 50-kDa band was also detected with another antibody that reacts with regions near R358 within the COOH-terminal fragment, suggesting presence of another cleavage site within COOH-terminal fragment. Considering this band was detected in reduced condition as well in Huh6 (Fig. 2C) and HepG2 (2), a fragment containing sGPC3 without cleavage at R358 but with cleavage at a yet unidentified site is present in the culture supernatant. It remains to be determined whether this fragment is derived from membrane-bound GPC3 or secreted GPC3; however, in either case, this fragment is not detectable with COOH-terminal antibodies.

In conclusion, it is likely that what we measured with our sandwich ELISA using combination of NH$_2$-terminal antibodies is a mixture of sGPC3 linked to glycanated COOH-terminal fragment and a 50-kDa fragment containing sGPC3 without cleavage at R358 and glycanation. NH$_2$-terminal antibodies that we were the first to generate were useful in dissecting different molecules derived from GPC3. We believe our serum assay system directed toward sGPC3 may be advantageous in detecting all these potential molecular species of sGPC3.

Thank you for the opportunity to respond to Dr. Cappuro’s letter.

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