

P53 Inducible Gene-3: Potential Serum Marker for Hepatocellular Carcinoma Beyond Milan Criteria

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mL, respectively), as compared to healthy controls (3.71 ± 1.79 ng/mL, $p < 0.0001$). PIG3 values were significantly different across the following tumor diameter ranges: less than 2 cm, 18.47 ng/mL; 2 to 5 cm, 18.54 ng/mL; and greater than 5 cm, 38.72 ng/mL; $p = 0.0012$. The correlation coefficient between log (AFP) and PIG3 levels in HCC patients was $r = 0.4564$, $p = 0.005$.

CONCLUSION: Serum p53-inducible gene 3 levels were significantly elevated in patients with HCC beyond 5 cm, indicating potential as an adjunct to imaging in determining eligibility for liver transplantation. The potential for PIG3 as a biomarker of HCC warrants further prospective study.

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Key words: p53 inducible gene; Hepatocellular carcinoma; Milan criteria; Serum marker

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ABSTRACT

AIMS: The diagnosis of Hepatocellular carcinoma (HCC) is usually made late, precluding curative resection or liver transplantation in over 70% of HCC patients. P53 inducible gene 3 (PIG3) is a p53 downstream gene and a key component of the DNA damage response pathway, which is relevant in conditions with rapid cell turnover such as HCC. Our aim was to assess whether PIG3 could serve as a novel biomarker for HCC.

METHODS: A pilot study of 37 consecutive patients with the diagnosis of HCC, 42 patients with hepatic cirrhosis versus healthy controls was conducted. Serum AFP and PIG3 levels measured with enzyme-linked immunosorbent assay were compared across groups using student t-test and correlation coefficient (R) between AFP and PIG3 was calculated.

RESULTS: Clinical characteristics were similar across the HCC and cirrhosis groups. Serum PIG3 levels were significantly higher among patients with cirrhosis or HCC (23.27 ± 13.26 and 24.1 ± 18.61 ng/

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with around 750,000 cases diagnosed worldwide in 2008^[1-2]. The etiologies of HCC are varied, although over 80% occur in patients with hepatitis B and C or alcoholic cirrhosis. In fact, the incidence in North America has nearly doubled over the last two decades, paralleling the rising incidence of hepatitis C virus (HCV) related cirrhosis^[3-5]. Despite improvements in the treatment of cirrhosis and decreases in cirrhosis-related mortality, mortality related to HCC has continued to increase globally with an estimated 500,000

deaths every year^[6]. It carries a very poor prognosis, with a median survival time of approximately 8 months and a 5-year survival rate of 11% in the U.S.^[7]. This poor prognosis is mainly attributed to the lack of widespread screening of patients with chronic liver disease and the lack of serum biomarkers to detect early disease.

Curative treatment in the form of surgical resection or liver transplantation is available if HCC is detected at an early stage, and results in a 5-year disease-free survival exceeding 50%. It is anticipated that 50-60% of those patients diagnosed with HCC between 2010 to 2015 will be eligible for curative treatment, as a result of more widespread surveillance programs^[8]. A standard biannual surveillance protocol incorporating ultrasound and serum alphafetoprotein (AFP) levels has traditionally been performed in patients with cirrhosis or selected patients with chronic hepatitis B, if followed at a liver clinic. However, the sensitivity of serum AFP in detecting HCC is variable depending on the threshold value used: 22% with a cut-off value of 200 ng/mL, and 60% with a cut-off of 20 ng/mL^[9]. The latter value seems to provide the best balance between sensitivity and specificity. The positive predictive value varies based on prevalence of HCC: with an AFP of 20 ng/mL and a prevalence of HCC of 5% in a liver clinic population, this value is only 41.5%^[9-10]. Given these weaknesses, AFP is now no longer recommended by experts as part of the screening protocol for HCC^[10]. Hence, there is a dire need for improved diagnostic tools in HCC^[10].

P53 is a tumor suppressor gene encoded by the TP53 gene in humans, and is known to play a role in the regulation of cell division^[11]. Increased intracellular concentration of the P53 protein has been seen in HCC tumors with p53 mutations, which has been correlated with poor patient survival rates^[12]. Recent studies have indicated that an overexpression of P53 may be related to more aggressive tumor growth, a high risk of tumor recurrence and overall poor survival rates in patients with HCC^[13]. P53 inducible gene 3 (PIG3) was originally isolated as a P53 target gene, with evidence that it was a component of the DNA damage response pathway^[14]. It shares significant homology with oxidoreductases involved in apoptosis. The induction of PIG3 is dependent on P53, and it has been shown that PIG3 is a long-lived reporter^[15]. This longer half-life may prove to be useful in detecting transient activation of P53^[16]. Thus, the aim of our study was to determine whether serum PIG3 could potentially be a screening blood test for HCC, and whether it correlated with the size of HCC tumors.

METHODS

Patients with a diagnosis of HCC were identified and recruited prospectively between October 2008 and July 2010 in the HCC and Hepatology clinics at the McGill University Health Centre. There were three groups for comparison: (1) patients with clinical and radiologic diagnosis of hepatocellular carcinoma; (2) patients with a diagnosis of cirrhosis but without HCC; and (3) healthy controls working in our laboratory. The latter individuals had no history of medical illness, including malignancy, liver or renal conditions. Serum samples of approximately 5 mL were obtained from routine blood tests performed at regularly scheduled follow-up appointments. These were stored at -80 degrees Celsius and tested for AFP and PIG3.

A commercially available ELISA kit for PIG3 (Exalpha Biologicals, MA, USA) was used to carry out specimen testing according to the manufacturer's instructions. AFP levels were measured using the Centaur Automated Chemiluminescence System (Bayer Diagnostics, USA). PIG3 levels in the study patients were correlated with AFP levels, radiological imaging and when

applicable, biopsy or explant pathology (size, number and histologic characteristics of the tumors). All laboratory values, imaging, and pathology reports were retrieved from the MUHC Transplant Database or computerized patient records.

Statistical Analysis

Stata (StataCorp, Version 10.1, Texas, USA) was used to perform the student's *t*-test and one way analysis for variance (ANOVA) for continuous variables as appropriate, and the chi-square test for categorical variables. PIG3 levels were compared between healthy controls, cirrhotic patients without HCC, and patients with HCC. Pearson's correlation coefficient was used to assess the relationship between AFP and PIG3 levels. Results are presented as mean±SD.

RESULTS

Patient Population

A total of 38 HCC patients, 32 hepatic cirrhosis patients without HCC and 18 healthy controls consented to the study between 2008 and 2010. Of the HCC patients, 29 also had cirrhosis. According to our power calculation, this number of patients should be adequate to demonstrate a significant difference in PIG3 expression between cases and controls if one exists. This was based on a sample size calculation assuming significance level 0.05, power 0.8 and data from previous studies to make an assumption that only 55% of cases and up to 10% of controls would over-express PIG3.

Demographic characteristics are listed in table 1, and were not significantly different between the groups.

Average PIG3 scores

Serum PIG3 levels were significantly higher among patients with cirrhosis, HCC or HCC with cirrhosis (23.65±13.26, 24.1±18.61 and 25.18±19.87 ng/mL, respectively), as compared to those among healthy controls (3.71±1.79 ng/mL, $p<0.0001$), shown in figure 1.

We also compared serum PIG3 levels between various ranges of tumor sizes. We performed a similar analysis to compare PIG3 values across the following tumor diameter ranges less than 2 cm, 18.47 ng/mL; 2 to 5 cm, 18.54 ng/mL; and greater than 5 cm, 38.72 ng/mL; $p=0.0012$ (Figure 2).

PIG3 levels in HCC patients with one lesion were lower than in those with multiple lesions, but this difference did not reach statistical significance (18.80 ng/mL versus 29.42 ng/mL; $p=0.34$). HCC patients with cirrhosis had higher PIG-3 levels than those without cirrhosis; however, this difference was not statistically significant (20.18±13.39 ng/mL versus 25.18±19.87 ng/mL, respectively, $p=0.51$).

In order to assess the practical value of PIG3, we attempted to correlate the expression of this novel biomarker to that of AFP, the standard in HCC. When AFP was less than 9 ug/L, the average PIG3 level was 16.16 ng/mL. When AFP was greater than 9 ug/L, the average PIG3 level was 28.40 ug/L ($p=0.020$). The correlation coefficient between log (AFP) and PIG3 levels in HCC patients was $r=0.4564$, $p=0.0051$ (Figure 3).

The average survival of patients with HCC was 835±129 days. However, the correlation coefficient of p53-inducible gene 3 values and survival was -0.402, indicating that there was no good correlation between this serum marker and survival.

DISCUSSION

The incidence of HCC is rising in North America, predominantly due to Hepatitis C and NASH cirrhosis. A standard surveillance regimen is

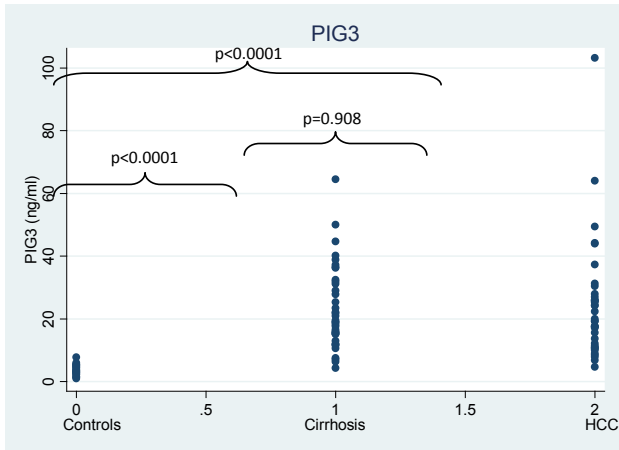


Figure 1 Comparison of PIG3 among healthy controls, patients with cirrhosis, and patients with HCC/HCC: Hepatocellular carcinoma; PIG3 levels in HCC without cirrhosis: 20.2±13.4; PIG3 levels in HCC with cirrhosis: 25.2±19.9 (*p*-value=0.5084).

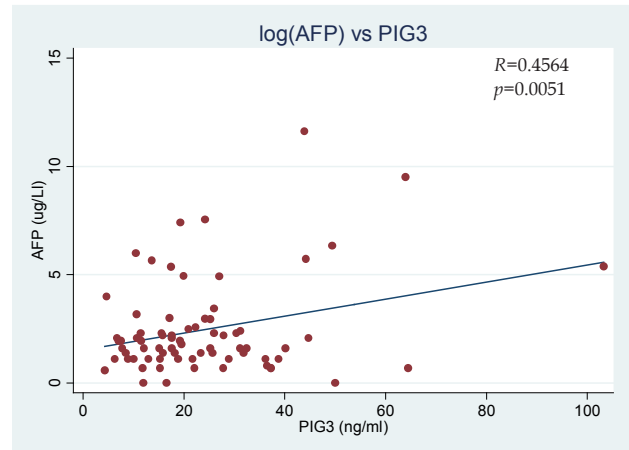


Figure 3 Positive Linear Correlation of serum P53 inducible gene-3 (PIG3) with Alphafetoprotein (AFP) level.

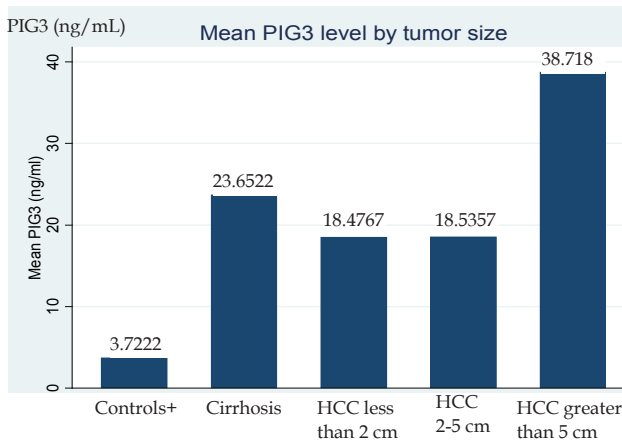


Figure 2 Comparison of serum P53 inducible gene-3 (PIG3) among patient groups with cirrhosis and hepatocellular carcinoma (HCC) tumor diameter. The classification of tumor size is based on a single nodule. Where multiple nodules are present, the size of the largest nodule is considered.

recommended for patients with liver cirrhosis. However, only a small proportion of those at-risk patients currently undergo surveillance in the United States^[17-18]. The HCC tumors diagnosed in the context of a surveillance regimen are at a much earlier stage than those diagnosed following onset of symptoms, offering a better chance for cure^[19]. The standard surveillance regimen was biannual ultrasound and AFP until recently, when AFP was dropped from the AASLD guidelines on HCC management given its poor sensitivity. There is therefore a greater need to discover novel serum biomarkers that enable earlier HCC detection.

The primary aim of our study was to determine the efficacy of PIG3 as a biomarker for HCC. The expression of this biomarker was no different between patients with HCC and cirrhotic patients without HCC. However, serum PIG3 levels were significantly increased among those patients with tumors greater than 5 cm in diameter, and correlated very well with AFP levels. Serum PIG-3 could thus potentially be used as an adjunct to imaging modalities (particularly ultrasound) in determining which patients have HCC tumors beyond Milan criteria, and thus eligibility for liver transplantation. The

Table 1 Demographic and clinical characteristics of patients with cirrhosis and HCC versus those without HCC.				
Demographics	Control (n=18)	HCC (n=37)	Cirrhosis without HCC (n=42)	p-value ²
Age (in years)	32.5±10.6	63.6±9.9	55.0±10.6	0.0004*
Sex (M/F)	4/7	27/10	23/19	0.094
Ethnicity	Caucasian: 15 Asian: 3 African: 0	Caucasian: 29 Asian: 7 African: 1	Caucasian: 35 Asian: 5 African: 1 Native: 1	
Etiology of Liver Disease		Hepatitis B: 7 Hepatitis C: 12 Alcoholic: 9 NASH: 1 Unclear: 8	Hepatitis B: 6 Hepatitis C: 15 Alcoholic: 2 NASH: 8 Primary sclerosing cholangitis: 2 Primary biliary cirrhosis: 2 Unclear: 7	
Tumor size (of largest tumor)	Not applicable	4.3+/-2.9	Not applicable	
Grade of tumor ¹				
1	Not applicable	3	Not applicable	
2		2		
3		1		

NASH: Non-alcoholic steatohepatitis; ¹This data was only available on 6 patients; ²p-value comparing characteristics between HCC patients and cirrhotics. Significant at *p*<0.05.

theoretical basis for PIG3 as a biomarker for dysplasia is predicated on it being induced by the p53 tumor suppressor gene, which is a cell mediator of apoptosis. p53 is the most commonly mutated gene in malignancies^[20]. In particular, p53 has been shown to be overexpressed in a significant proportion of HCC tumors by immunohistochemistry^[11,12,14,16]. These tumors are distinguished by being larger sized (>5 cm) and poorly differentiated. Nuclear accumulation of p53 on immunohistochemistry has been associated with more aggressive growth and poor survival outcomes^[11,13,16]. Overexpression of p53 mRNA and protein correlated strongly with very high alphafetoprotein levels^[16], and mutations in p53 have been detected in the serum of HCC patients^[21]. Serum anti-p53^[22,23] levels have been associated with survival of HCC patients, tumor size and tumor grade. Similarly, overexpression of p53 has correlated with overall survival rates of HCC patients^[24-26], tumor size and grade^[22]. Thus, both serum p53 and anti-p53 have been suggested as biomarkers complementary to AFP^[27-28]. However, another study discovered that the expression of anti-p53 antibodies was not specific to HCC, but was equally prevalent among patients with cirrhosis and chronic liver disease^[29]. This again likely reflects the rapid cell turnover seen in this context.

When p53 is activated by oncogenic stimuli, various genes involved in apoptosis, cell cycle control and DNA repair are in turn activated^[30]. PIG3 is one such gene that was found to be induced just before the onset of apoptosis^[31]. It is known to mediate generation of reactive oxygen species, which are essential in the apoptotic response. PIG3 was recently shown to participate in the early cellular response to DNA damage. A few studies have been conducted to assess whether there is a link between genetic polymorphisms of PIG3 and susceptibility to cancer. This is particularly interesting, because the promoter region of PIG3 consists of a microsatellite induced by p53, unique among p53-induced genes^[32-33]. This microsatellite has arisen recently in human evolutionary history, and is thought to be an adaptation of the tumor suppressor mechanism^[32]. The number of variable number tandem repeats (VNTR) has been shown to correlate with activation by p53. In a Japanese population of patients with bladder cancer, there was a correlation between a lesser number of VNTRs in the PIG3 promoter and higher grade and stage of bladder cancer^[34]. A similar study of patients with breast and lung cancer revealed no association between genetic polymorphisms in the PIG3 promoter with cancer^[35]. Induction of PIG3 via upregulation of its promoter has been used to induce cell apoptosis in tumor cells *in vitro*^[36]. Similarly, PIG3 negatively regulated growth of neuroblastoma cells *in vitro*^[37]. Although serum PIG3 per se has never been evaluated, the above studies suggest that serum PIG3 would be elevated in malignancies where there is a rapid cell turnover, with release of this protein into the circulation upon cellular apoptosis. This in fact was seen, with levels of PIG3 being significantly higher among those with tumors larger than 5 cm in diameter as compared to those with cirrhosis or less sizeable tumors.

The principal limitation of our pilot study was the inability to correlate serum PIG3 levels with survival data and other general outcomes, given the recent obtention of serum samples and therefore shorter follow-up period. As well, this was a relatively small study, although we did meet the numbers of samples required as per our initial power calculations. We also did not have pathology specimens on many of the HCC patients, as the diagnosis of HCC does not require histological confirmation.

p53-inducible gene is a novel biomarker whose expression is clearly different between those patients with diseased livers (either cirrhosis or HCC) and people with healthy livers. Our study is the first to evaluate the potential of PIG3 as a serum biomarker for malignancy. We hypothesize that temporal changes in this marker

may reflect the dynamic state of increased cell turnover in diseased livers. Additionally, PIG3 was also significantly elevated in patients with HCC tumors beyond 5 cm, potentially indicating greater aggressivity. Recently, the histologic grade of HCC was used to determine candidacy for liver transplant rather than the standard Milan criteria^[8]. We surmise that PIG3 may serve as a serum marker that can reflect the grade of the HCC without biopsy, although this would need to be verified prospectively in HCC patients undergoing transplantation. Serum PIG3 could therefore serve as an adjunct to imaging modalities in determining eligibility for liver transplantation. PIG3 has potential as a biomarker because of its long half-life, as opposed to more transiently expressed genes. Further research should be conducted to better characterize PIG3 in the context of dysplasia, and to better correlate this marker with HCC tumor behavior. Such characterization could potentially enable incorporation of PIG3 as a serum biomarker into hepatology practice, particularly by helping to determine eligibility for liver transplantation in the context of HCC.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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