Successful Interferon Therapy Reverses Enhanced Hepatic Progenitor Cell Activation in Patients with Chronic Hepatitis C

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The enhanced accumulation of hepatic progenitor cells (HPCs) is related to the risk of progression to hepatocellular carcinoma (HCC). Interferon (IFN) treatment reduces HCC risk in patients with chronic hepatitis C virus (HCV) infection. However, the underlying mechanisms remain unclear. The aim of this study was to examine the effects of IFN treatment on HPC activation in HCV patients. Immunohistochemical detection and computer-assisted quantitative image analyses of cytokeratin 7 (CK7) were performed to evaluate HPC activation in paired pre- and post-treatment liver biopsies from 18 HCV patients with sustained virological response (SVR) to IFN-based therapy and from 23 patients without SVR, as well as normal liver tissues obtained from surgical resection specimens of 10 patients. Pretreatment HCV livers showed increased CK7 immunoreactivity, compared with normal livers (HCV: median, 1.38%; normal: median, 0.69%, $P=0.006$). IFN treatment reduced hepatic CK7 immunoreactivity (median, 1.57% pre-IFN vs. 0.69% post-IFN, $P=0.006$) in SVR patients, but not in non-SVR patients. The development of HCC following IFN treatment was encountered in 3 non-SVR patients who showed high post-IFN treatment CK7 immunoreactivity ($>4\%$). Successful IFN therapy can reverse enhanced HPC activation in HCV patients, which may contribute to the reduced risk of HCC development in these patients.

Introduction

HEPATIC PROGENITOR CELLS (HPCs) are bipotential cells that can differentiate into biliary and hepatocytic lineages (Roskams and others 2004). Differentiation into hepatocytes occurs through intermediate hepatobiliary cells (IHBCs), while differentiation into the biliary lineage leads to the formation of atypical reactive ductules. These 3 cell types, isolated HPCs, IHBCs, and reactive ductular cells (RDCs)], which can be referred to as the “HPC compartment,” express alpha-fetoprotein (AFP), gamma-glutamyl transpeptidase (GGT), and certain cytokeratin markers [eg, cytokeratin 7 (CK7)] (Dollé and others 2010). In various human liver diseases, including chronic hepatitis C, HPC activation [a term used to refer to an increase in the number of HPCs and differentiation of the HPCs toward hepatobiliary lineages (Libbrecht and Roskams 2002)] occurs only when hepatocyte damage and loss and/or cholangiocytes are combined with impaired regeneration of the mature cell types involved (Fausto 2004). HPC activation is frequently

observed predominantly as a ductular reaction (DR) at the portal interface. DR is a dynamic multicellular complex characterized by the presence of reactive ductular epithelial cells, arranged in irregular strings along the margins of the portal tract in close contact with mesenchymal, inflammatory, and endothelial cells. The epithelial elements of DR consist of isolated HPCs, IHBCs, and RDCs (Roskams and others 2004). In addition, the enhanced accumulation of activated HPCs seems to be related to the risk of progression to hepatocellular carcinoma (HCC) (Ziol and others 2010). HPCs have been suggested as targets of malignant transformation in chronic liver diseases, including chronic hepatitis C (Lo and Ng 2013).

Chronic hepatitis C virus (HCV) infection is a leading cause of HCC (Hajarizadeh and others 2013). Interferon (IFN) therapy reduces the risk of progression to HCC in HCV patients (Rebecca and others 2013). Patients with sustained virological response (SVR) to IFN therapy benefit the greatest in terms of reduced risk of HCC. This study represented the first step toward understanding the underlying

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mechanisms responsible for reducing the risk of HCC in patients with SVR following IFN treatment. To evaluate HPC activation in this study, the degree of DR was quantified by immunohistochemical detection of CK7 because this phenotypic marker is advantageous for the identification of all the epithelial elements of DR, although there are many different markers for HPCs (Dollé and others 2010).

Patients and Methods

Patients

This study retrospectively investigated 41 chronic HCV patients (22 male, 19 female; mean age 56 ± 15 years old) without HCC who were evaluated at the Liver Unit of the Hamamatsu University School of Medicine Hospital between January 2001 and December 2012. Diagnosis of chronic HCV infection was based on elevated serum transaminase levels for at least 6 months in the presence of serum HCV RNA. Of the 41 patients, 18 showed SVR to IFN-based therapy and 23 did not. The selection of patients was based on the availability of both pre- and post-treatment liver biopsies. The pretreatment clinical and demographic characteristics of the 41 patients are summarized in Table 1. Normal liver tissues were obtained from the surgical resection specimens of 10 patients (9 male, 1 female; mean age 63 ± 15 years old). All control subjects were HCV antibody negative and had metastatic liver tumors from colon cancer (8 patients) or gastric cancer (2 patients). All patients provided written informed consent before participating in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), as reflected by the a priori approval of the institution's human research committee.

Liver samples

Liver tissue was obtained by percutaneous needle biopsy or by surgical resection. All liver specimens were fixed in formalin and were paraffin embedded for hematoxylin and eosin and Azan–Mallory staining and for subsequent immunohistochemical analyses. The histological characteristics of chronic hepatitis were evaluated using the standard criteria proposed by Desmet and others (1994).

TABLE 1. CLINICOPATHOLOGICAL CHARACTERISTICS OF HCV PATIENTS BEFORE INTERFERON THERAPY

Variables	
Clinical characteristics	
Age (year) mean $\pm$ S.D.	56 ± 15
Sex (male/female)	22/19
Biological characteristics	
Platelets ($\times 10^4$ /mL)	15.4 (7.8–26.1)
ALT (IU/L)	64 (21–302)
GGT (IU/L)	31 (16–216)
AFP (ng/mL)	5 (2–333)
Pathological characteristics	
Hepatic necroinflammation (A1/A2-3)	11/30
Hepatic fibrosis (F0-1/F2-3)	20/21

Unless otherwise indicated, values represent medians (ranges) or frequencies.

ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; AFP, alpha-fetoprotein; HCV, hepatitis C virus.

Immunohistochemistry for the detection of CK7 was performed using the EnVision System (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Briefly, deparaffinized sections (4 μ m in thickness) were subjected to an autoclave heating treatment in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. The sections were incubated with a mouse monoclonal anti-CK7 antibody (1:200 dilution, clone OV-TL 12/30; Dako, Carpinteria, CA), followed by a goat anti-mouse EnVision-HRP enzyme conjugate (Dako). Diaminobenzidine served as the chromagen. Nuclear staining was performed using Mayer's hematoxylin.

Quantification of HPC activation

HPC activation was evaluated according to the accumulation of the epithelial elements of DR by quantifying the immunoreactivity of CK7 in portal and periportal areas using computer-assisted image analysis (ImageJ 1.48v; National Institutes of Health, Bethesda, MD). The measured sections contained at least 2 complete portal tracts. The sections were screened at low power, and the corresponding circumferences were determined for areas with complete portal tracts. The percentage of the positive area was defined as the ratio of the immunoreactive area to the total area of each field multiplied by 100.

Statistical analyses

Statistical analyses were performed using the SPSS Statistics software package, release 21 for Windows (IBM, Armonk, NY). The distributions of all variables were assessed using the Shapiro–Wilk normality test. If the data were not normally distributed, nonparametric statistics was used for the analysis. Quantitative values were compared between the pretreatment and post-treatment phases using the paired *t*-test or Wilcoxon signed-rank test. Correlation coefficients were calculated using Pearson's product-moment correlation analysis or Spearman's rank correlation analysis. All tests of significance were 2-sided, and a *P*-value less than 0.05 was considered to be statistically significant.

Results

Pre- and post-IFN treatment HPC activation in HCV patients

The CK7 immunoreactivities of the pre- and post-treatment HCV livers and normal livers are shown in Figure 1. The HCV livers showed increased CK7 immunoreactivity, compared with the normal livers (HCV: median, 1.38%; normal: median, 0.69%, *P*=0.006); the portal and periportal CK7-immunoreactive areas consisted of anatomical bile ducts and the epithelial elements of DR in the HCV livers (Figs. 1A, C), whereas the immunoreactive areas consisted of anatomical bile ducts only, without DR in the normal livers (Fig. 1E). The associations of hepatic CK7 immunoreactivity with various clinicopathological characteristics in the HCV patients are shown in Table 2. Hepatic CK7 immunoreactivity was positively correlated with serum AFP (*r*=0.584, *P*<0.001), alanine aminotransferase (ALT) (*r*=0.485, *P*=0.001), and GGT levels (*r*=0.464, *P*=0.002), degree of hepatic necroinflammation (*r*=0.454, *P*=0.003), and fibrosis (*r*=0.509,

FIG. 1. Immunohistochemical staining for cytokeratin 7 (CK7) in paired pre- and post-interferon treatment liver biopsies from hepatitis C virus (HCV) patients with and without sustained virological response (SVR) and in normal liver tissue from a patient with a metastatic tumor. **(A)** In a representative patient with SVR, the pretreatment HCV liver shows increased CK7 immunoreactivity; the portal and periportal immunoreactive areas (*brown color*) consist of anatomical bile ducts and the epithelial elements of ductular reaction (DR). **(B)** In the same patient, the post-treatment liver shows less abundant CK7 immunoreactivity. **(C, D)** In a representative patient without SVR, no significant difference in CK7 immunoreactivity was observed between the pretreatment **(C)** and post-treatment **(D)** HCV liver tissues. **(E)** In the normal liver, the portal and periportal immunoreactive areas consist of anatomical bile ducts only, without DR. Original magnification 200 $\times$.

$P=0.001$) and was negatively correlated with platelet count ($r = -0.387$, $P=0.012$). The multiple regression analysis indicated that serum GGT levels (standard partial regression coefficient=0.401, $P=0.005$) and hepatic necroinflammation (standard partial regression coefficient=0.373, $P=0.008$) were predictive factors of hepatic CK7 immunoreactivity.

The clinical characteristics of the SVR and non-SVR HCV patients before and after IFN therapy are shown in Table 3. IFN therapy significantly improved serum levels of AFP (median of 4.0–2.5 ng/mL, $P=0.002$), ALT (median of 59–15 IU/L,

$P<0.001$), and GGT (median of 31–18 IU/L, $P=0.001$) and platelet counts (median of 15.3–17.2 $10^4/\mu\text{L}$, $P=0.004$) in the 18 SVR patients. The hepatic necroinflammatory activity decreased in 14 of the SVR patients (77.8%). The degree of hepatic fibrosis decreased in 13 of the SVR patients (72.2%). As shown in Figures 1B and 2A, hepatic CK7 immunoreactivity was also significantly reduced (median of 1.57%–0.69%, $P=0.001$). There was no significant difference in CK7 immunoreactivity between the post-treatment livers of the SVR patients and the normal livers ($P=0.533$).

In contrast, serum levels of AFP (median of 8.0–4.0 ng/mL, $P=0.001$) and ALT (median of 75–65 IU/L, $P=0.023$) were reduced by IFN therapy in the 23 non-SVR patients. The hepatic necroinflammatory activity decreased in 8 of the non-SVR patients (34.8%). The degree of hepatic fibrosis decreased in 6 of the non-SVR patients (26.1%). CK7 immunoreactivity did not change (median of 1.32–1.63%, $P=0.732$), as shown in Figures 1D and 2B. Both the serum AFP and ALT levels of the SVR patients were significantly lower than those of the non-SVR patients following IFN therapy ($P<0.001$, respectively).

Predictive factors for post-IFN treatment normalization of HPC activation in HCV patients

As shown in Table 4, univariate analyses identified that post-IFN treatment AFP (<5 ng/mL, $P=0.024$), ALT (<30 IU/mL, $P=0.013$), hepatic necroinflammation (A0–1

TABLE 2. ASSOCIATIONS OF HEPATIC CYTOKERATIN 7 IMMUNOREACTIVITY WITH VARIOUS CLINICOPATHOLOGICAL CHARACTERISTICS IN HCV PATIENTS BEFORE INTERFERON TREATMENT

Variables	Univariate analysis		Multivariate analysis	
	r	P-value	r	P-value
Platelets ($\times 10^4/\text{mL}$)	-0.387	0.012		
ALT (IU/L)	0.485	0.001		
GGT (IU/L)	0.464	0.002	0.401	0.005
AFP	0.584	<0.001		
Hepatic necroinflammation	0.454	0.003	0.373	0.008
Hepatic fibrosis	0.509	0.001		

TABLE 3. PRE- AND POST-TREATMENT CLINICOPATHOLOGICAL CHARACTERISTICS IN SUSTAINED VIROLOGICAL RESPONDERS AND NONRESPONDERS TO INTERFERON TREATMENT FOR CHRONIC HCV INFECTION

Variables	SVR (n = 18)			Non-SVR (n = 23)		
	Pretreatment	Post-treatment	P-value	Pretreatment	Post-treatment	P-value
Platelets ($\times 10^4/\text{mL}$)	15.3	17.2	0.004	15.4	16.0	NS
ALT (IU/L)	59	15	<0.001	75	65	0.023
GGT (IU/L)	31	18	0.001	44	38	NS
AFP	4.0	2.5	0.002	8.0	4.0	0.001
Hepatic necroinflammation (A0-1/A2-3)	5/13	17/1	<0.001	6/17	10/13	NS
Hepatic fibrosis (F0-1/F2-3)	9/9	15/3	0.034	11/12	11/12	NS
CK7 immunoreactivity	1.57	0.69	0.001	1.32	1.63	NS

Unless otherwise indicated, values represent medians (ranges) or frequencies.
SVR, sustained virological response; CK7, cytokeratin 7.

vs. A2-3, $P=0.019$), hepatic fibrosis (F0-1 vs. F2-4, $P<0.001$), and IFN response (SVR vs. non-SVR, $P=0.001$) were predictive factors of the post-IFN treatment normalization of CK7 immunoreactivity ($<0.8\%$) following IFN therapy. Multivariate analysis revealed that SVR status (odds ratio=0.024; 95% CI=0.028–0.777, $P=0.024$) and a lower degree of hepatic fibrosis following IFN treatment (odds ratio=0.050; 95% CI=0.005–0.495, $P=0.010$) were significant independent factors for the post-IFN treatment normalization of CK7 immunoreactivity.

Post-IFN treatment HCC development and HPC activation in HCV patients

By June 2014, the median follow-up durations were 11.2 years after the post-IFN treatment liver biopsy for all 41 patients, 10.4 years for the SVR patients, and 11.7 years for the non-SVR patients. During the follow-up period, 3 patients developed HCC. None of them exhibited SVR. In these patients, reductions in serum AFP levels were observed between pre- and post-IFN treatment biopsies (pretreatment 35.0 ng/mL vs. post-treatment 6.0 ng/mL), whereas no consistent change in hepatic CK7 immunoreactivity was found after treatment (Fig. 2B). The post-IFN treatment clinical

characteristics of these patients are shown in Table 5. The degree of post-IFN treatment hepatic CK7 immunoreactivity was higher in these patients than in the non-SVR patients without HCC development (median, 5.87 vs. 1.48, $P=0.008$).

Discussion

HCV infection is a primary cause of chronic hepatitis, which often progresses to liver cirrhosis and HCC. The eradication of HCV by IFN treatment is the most promising therapeutic intervention for preventing the progression to liver cirrhosis and HCC in HCV patients. However, the underlying mechanisms have not yet been fully clarified. As the first step toward understanding these mechanisms, this study focused on the comparison of HPC activation before and after IFN treatment in SVR and non-SVR HCV patients, considering that HPC activation is involved in hepatic fibrogenesis and hepatocarcinogenesis (Lo and Ng 2013; Williams and others 2014). In this study, HPC activation was evaluated by quantifying the epithelial elements of DR, which are isolated HPCs, RDCs, and IHBCs, with the immunohistochemical detection of CK7 as described previously (Libbrecht and Roskams 2002). CK7 and cytokeratin 19 (CK19) are

FIG. 2. Changes in hepatic CK7 immunoreactivity in sustained virological responders and nonresponders after interferon treatment for chronic HCV infection. (A) Following SVR, hepatic CK7 immunoreactivity was significantly reduced. (B) Non-SVR patients showed no significant changes in hepatic CK7 immunoreactivity after interferon treatment. The solid lines denote patients with post-treatment development of hepatocellular carcinoma (HCC); the dotted lines denote patients without HCC.

TABLE 4. PREDICTIVE FACTORS FOR POST-INTERFERON TREATMENT NORMALIZATION OF HEPATIC CYTOKERATIN 7 IMMUNOREACTIVITY IN HCV PATIENTS

Category			Univariate		Multivariate	
			P-value	Odds ratio	95% C.I.	P-value
Post-treatment	Age	0: ≤ 60 year 1: > 60 year	0.050			
	ALT	0: < 30 U/L 1: ≥ 30 U/L	0.013			
	GGT	0: < 30 U/L 1: ≥ 30 U/L	0.051			
	AFP	0: < 5 ng/mL 1: ≥ 5 ng/mL	0.024			
	Hepatic necroinflammation	0: 0–1 1: 2–3	0.019			
	Hepatic fibrosis	0: 0–1	< 0.001	0.050	0.005–0.495	0.010
		1: 2–3				
IFN treatment		0: SVR 1: Non SVR	0.001	0.024	0.028–0.777	0.024

IFN, interferon.

generally recognized as reliable phenotypic markers for HPCs (Chougule and Sumitran-Holgersson 2012), but the immunohistochemical detection of CK7 is more advantageous for the identification of IHBCs.

Our main findings can be summarized as follows: (1) varying degrees of enhanced HPC activation existed before IFN treatment in the HCV patients; (2) HPC activation was ameliorated in the SVR patients following IFN treatment; (3) the normalization of HPC activation was associated with the eradication of HCV by IFN treatment and by reduced hepatic fibrosis following IFN treatment; and (4) HCC was encountered in the non-SVR patients along with a greatly enhanced HPC activation.

This study confirmed previous reports demonstrating that enhanced HPC activation existed in HCV livers. HPC activation has been described in a variety of chronic liver diseases (Lowes and others 1999; Fotiadu and others 2004). The replacement of hepatocytes lost from normal hepatic parenchyma is known to occur by the replication of mature hepatocytes. The inhibition of this replication promotes the

activation of a secondary replicative pathway through HPCs. HCV infection causes cell cycle arrest, impairing mature hepatocyte replication (Marshall and others 2005; Rathi and others 2011). Potential mediators of this response include reactive oxygen species, inflammatory cytokines, and HCV proteins. In the current study, the degree of hepatic necroinflammation and serum GGT levels were found to be independent predictive factors of the degree of HPC activation. In chronic HCV infection, HPC activation can be induced by the inhibitory effects of IFN-gamma and tumor necrosis factor-alpha, which are inflammatory cytokines overexpressed in HCV livers (Napoli and others 1996; Gochee and others 2003) during mature hepatocyte replication (Fausto 2004). GGT plays an important role in antioxidant defense, participating in the metabolism of glutathione (Zhang and Forman 2009), and it is upregulated following exposure to oxidative stress (Ravuri and others 2011). Oxidative stress inhibits the replication of mature hepatocytes, resulting in the activation of HPCs (Roskams and others 2003). In addition, the HCV protein-mediated impairment of the mature hepatocyte cell cycle

TABLE 5. POST-IFN TREATMENT CLINICOPATHOLOGICAL CHARACTERISTICS OF HCV PATIENTS HAVING SUSTAINED VIROLOGICAL NONRESPONSE TO INTERFERON TREATMENT WITH AND WITHOUT POST-TREATMENT DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

Variables	Non-SVR patients	
	With HCC (n=3)	Without HCC (n=20)
Sex (male/female)	2/1	9/11
Age (year)	53 (58–50)	59 (68–44)
Platelets (×10 ⁴ /mL)	12.8 (13.5–10.7)	17.4 (29.7–7.8)
ALT (IU/L)	70 (222–52)	61 (131–15)
GGT (IU/L)	98 (316–28)	36 (152–11)
AFP (ng/mL)	6.0 (14.0–3.0)	4.0 (15.0–2.0)
Hepatic necroinflammation (A0-1/A2-3)	0/3	10/10
Hepatic fibrosis (F0-1/F2-4)	0/3	11/9
CK7 immunoreactivity (%)	5.87 (7.62–4.36)	1.48 (7.57–0.46)
Period from end of IFN treatment to HCC development (year)	5.6 (10.3–3.8)	
Period from post-treatment liver biopsy to HCC development (year)	2.4 (4.7–1.0)	

Values represent medians (ranges) or frequencies.

non-SVR, sustained virological nonresponse; HCC, hepatocellular carcinoma.

likely contributes to HPC activation. The degree of cell cycle arrest was shown to be associated with viral protein abundance in HCV-infected cultured cells (Rathi and others 2011).

This study showed that HPC activation was ameliorated following IFN treatment in HCV patients with SVR. Similar responses after successful IFN treatment were recently reported in cirrhotic HCV patients (D'Ambrosio and others 2012). The finding in the present study that the eradication of HCV by IFN treatment was a predictive factor for the post-treatment normalization of HPC supported the notion that this eradication could improve the HCV-related impairment of mature hepatocyte replication, leading to the amelioration of HPC activation. In addition, IFN- α was previously shown to promote the inhibition of proliferation, the induction of apoptosis, and the differentiation of HPCs in experimental murine models, both *in vitro* and *in vivo* (Lim and others 2006). These findings suggested that the direct effects of IFN on the activities of HPCs might contribute to the reversal of enhanced HPC activation following successful IFN treatment in HCV patients. Furthermore, the maturation-modulatory effects of IFN on HPCs could explain the downregulation of serum AFP without any changes in hepatic CK7-immunoreactivity observed between pre- and post-treatment biopsies in non-SVR patients, considering that the expression of phenotypic markers (AFP, hepatocyte lineage marker; CK7, biliary lineage marker) by HPCs depends on cell maturation and differentiation.

A lower degree of hepatic fibrosis after IFN treatment was also found to be a significant independent predictive factor for the post-IFN treatment normalization of HPC activation in this study. The degree of HPC activation was associated with the severity of hepatic fibrosis, independent of the underlying pathology in human chronic liver diseases (Lowes and others 1999; Clouston and others 2005). Recent experimental studies have suggested a close link between HPC expansion and the fibrogenic response (Chobert and others 2012; Kuramitsu and others 2013).

This study suggested that post-IFN treatment HPC activation potentially influenced the subsequent occurrence of HCC in HCV patients. It was recently demonstrated that post-IFN treatment AFP levels could predict the future risk of HCC in HCV patients (Asahina and others 2013). Considering that AFP is a HPC marker, it is plausible that post-IFN treatment HPC activation is associated with an increased risk of developing HCC in HCV patients. In addition, several studies have provided evidence in support of a role for HPC in hepatocarcinogenesis. These experimental studies have suggested that genetic alterations associated with the excessive and persistent self-renewal of HPCs might induce liver cancer stem cells, eventually leading to HCC (Dumble and others 2002; Chiba and others 2007; Tang and others 2008). Immunohistochemical studies of humans have shown that a substantial number of HCC cases and the precursor lesions of HCC, such as dysplastic foci and hepatocellular adenoma, express HPC markers, suggesting that HCC might originate in HPCs (Libbrecht and others 2000). Hepatocarcinogenesis may affect not only the levels of HPC marker expression but also post-translational modification (eg, phosphorylation and glycosylation) of HPC markers. Identification of these quantitative and qualitative alterations of HPC markers may contribute to the establishment of predicting markers for HCC development.

A recent prospective study found a link between the immunohistochemical detection of IHBCs, which are the immediate hepatocyte progeny of HPCs, and the risk of developing HCC in patients with HCV-related cirrhosis (Ziol and others 2010). In the present study, a lack of development of HCC was also found in HCV patients with SVR. The eradication of HCV by IFN treatment was demonstrated to decrease the risk of HCC development in HCV patients (Cardoso and others 2010). The underlying mechanisms likely involve the post-IFN treatment amelioration of HPC activation. However, a large-scale, long-term cohort study is necessary to clarify whether post-IFN treatment HPC activation can predict the future risk of HCC development in HCV patients.

In conclusion, enhanced HPC activation occurs during chronic HCV infection. Successful IFN therapy can reverse enhanced HPC activation in patients with chronic hepatitis C. The successful reduction of HPC activation in these patients by IFN treatment may contribute to reduced risk of HCC development.

Author Disclosure Statement

No competing financial interests exist.

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