Experimental and Molecular Pathology 108 (2019) 156–163

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

The different expression of tumor suppressors, RASSF1A, RUNX3, and GSTP1, in patients with alcoholic steatohepatitis (ASH) vs non-alcoholic steatohepatitis (NASH)

Yue Jia, Ping Ji, Barbara French, Brittany Tillman, Samuel W. French*

Harbor-UCLA Medical Center, Department of Pathology, Torrance, CA 90502, United States

ARTICLE INFO

Keywords:
Tumor suppressor
RASSF1A
RUNX3
GSTP1
Hepatocellular carcinoma (HCC)
Alcoholic steatohepatitis (ASH)
Non-alcoholic steatohepatitis (NASH)

ABSTRACT

As the fifth most common cancer and the second leading cause of cancer related deaths worldwide, hepatocellular carcinoma (HCC) causes up to one million deaths annually. Alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) are becoming the two major risk factors because both may develop liver fibrosis and hepatocellular carcinoma (HCC) if left untreated. However, compared with 3–10% of patients with ASH may progress to HCC annually, about only 0.5% NASH patients may progress to HCC annually. The present study is to clarify the protein expression differences of tumor suppressor genes (TSGs) between ASH and NASH. In liver biopsied specimens from NASH and ASH patients, using an immunofluorescence method and morphometrically quantitating the fluorescence intensity, we studied the protein expression within hepatocytes cytoplasm of candidate TSGs including RUNX3, GSTP1, and RASSF1A. Compared with the control group of patients, the expression levels of all three proteins were upregulated in the ASH group of patients ($p < .001$ in all molecules). While RUNX3 was upregulated, GSTP1 and RASSF1 did not change in the NASH group of patients. The most important finding is that compared with the ASH group of patients, the expression levels of all three TSG proteins, RUNX3, GSTP1, and RASSF1, were significantly lower in the NASH group of patients ($p < .001$ in all three molecules). These results confirmed our previous finding that there are significant differences of many molecules including TSGs that changed in NASH compared to ASH. Thus, we conclude that there are significantly different TSGs and pathways involved during the pathogenesis of HCC development in NASH compared to ASH that may help to develop different strategies for prevention and treatment of NASH and ASH patients.

Abbreviations

ASH	alcoholic steatohepatitis
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
HCC	hepatocellular carcinoma
RASSF1A	Ras association domain family protein 1A
RUNX3	Runt-related transcription factor
GSTP1	glutathione S-transferase protein 1
MEK	Mitogen-activated protein kinase kinase
GPCR	G protein-coupled receptor
PI3K	phosphatidylinositol 3-kinase
Akt	also known as PKB, Protein Kinase B
TLR4	Toll-like receptor 4
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
STAT3	signal transducer and activator of transcription 3
FAT10	HLA-F-adjacent transcript 10
FOXO1	forkhead box protein O1

1. Introduction

Cell fate includes growth, division, differentiation and death. The whole procedure is controlled by negative and positive regulations mainly through two classes of genes: the tumor suppressor genes (TSGs) and the proto-oncogene genes, respectively. In physiological conditions, tumor suppressor genes repress the formation and development of tumor while damage in their expression or function leads to uncontrolled cell growth or cancer. The TSGs' function is impaired by mutations, loss of chromosome region or silencing by promoter methylation [Martin and Dufour, 2008].

As the fifth most common cancer and the second leading cause of cancer related deaths worldwide, hepatocellular carcinoma (HCC) is a major global healthcare burden and causes up to one million deaths

* Corresponding author.
E-mail address: sfrench@labiomed.org (S.W. French).

https://doi.org/10.1016/j.yexmp.2019.04.002
Received 13 September 2018; Received in revised form 26 February 2019; Accepted 1 April 2019
Available online 03 April 2019

annually [Ferlay et al., 2012]. Hepatitis B (HBV) and hepatitis C (HCV), obesity/metabolic syndrome, and alcoholism are the major risk factors which may lead to hepatocellular carcinoma (HCC) development. In the last two decades, the development of the non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) increased dramatically due to the rising rates of obesity/metabolic syndrome. The relationships between NASH and HCC development has been proved by both experimental and epidemic studies. NASH can also progress to HCC without cirrhosis [Cholankeril et al., 2017]. Alcoholic liver disease (ALD) and ASH are becoming the major causes of HCC in the US [Morgan et al., 2004] [Ramadori et al., 2017][Testino et al., 2014]. NASH and ASH have similar histological features, including significant lipid deposition and fat droplet storage in the hepatocytes. Although both NASH and ASH may progress to fibrosis, cirrhosis, and ultimately hepatocellular carcinoma (HCC), the ratios of NASH progressing to cirrhosis and to HCC are about 7–10% [Scaglioni et al., 2011] and 0.5% [Lindenmeyer and McCullough, 2018], respectively. But the ratios of ASH progressing to cirrhosis and to HCC are 10–20% [Schwartz et al., 2012][Dam-Larsen et al., 2004] and 3–10% [Schwartz and Reinus, 2012], annually.

Although there are many theories such as chromosomal loss of tumor suppressor genes, oxidative stress, decreased liver retinoic acid level, altered DNA methylation, and genetic susceptibility [Morgan et al., 2004][Stickel et al., 2002][Stickel, 2015], the molecular mechanisms underlying hepatocarcinogenesis are not understood in NASH and ASH patients. Our previous work found that there are different molecular gene/protein changes in ASH compared with NASH [Jia et al., 2018][Nguyen et al., 2018][Lu et al., 2018], including the TLR/NFKB/CXCR4/7 [Liu et al., 2014][Liu et al., 2015a][French et al., 2012a, 2012b][Nan et al., 2005][French et al., 2010], PI3K/AKT/mTORC1 [Afifiyan et al., 2017a, 2017b], and Tec kinase signaling pathways in both ASH and NASH [Afifiyan et al., 2017a, 2017b]. The present study tried to reveal the association of tumor suppressor genes (TSGs)/tumor suppressor proteins, such as Ras association domain family protein 1A (RASSF1A), Glutathione S-transferase P1 (GSTP1), and Runt-related transcription factor 3 (RUNX3), in ASH and NASH patients. Using liver biopsy specimens from NASH and ASH patients and a control group, we found that the RASSF1A, RUNX3, and GSTP1, may play important but different roles in tumorigenesis of HCC in ASH or NASH patients.

2. Methods

Formalin-fixed paraffin-embedded biopsies of ASH liver (n = 60 in RASSF1A, 50 in RUNX3 and in GSTP1), NASH liver (n = 30), and normal liver (n = 30) were collected from Harbor-UCLA Medical Center and from the Long Beach Veterans Affairs' clinical trial in treatment of alcoholic hepatitis. The study was conducted following the principals of the Declaration of Helsinki and was designated as exempt by our institutional ethics review board and the data was analyzed anonymously. The reference number of the ethics review is #040958. The “controls” liver biopsy specimens are from a clinical practice and archive without ASH, NASH, or HCC diagnosis. The “controls” specimens do not show significant fat accumulation in hepatocytes; fibrosis stages; duct metaplasia; PMN or lymphocytes infiltration; or MDBs. The primary antibodies were rabbit anti-RASSF1A (Cat# ab126764; Abcam, CAMBRIDGE, MA) and anti-RUNX3 (Cat# ab49117; Abcam, CAMBRIDGE, MA), and anti-GSTP1 (Cat# HPA019869; SIGMA, ST. LOUIS, MO). For each protein studied, the liver biopsy sections were probed first with antigen-specific primary antibody followed by a secondary fluorescence antibody. Either donkey anti-mouse or anti-rabbit Alex Fluor (Jackson Labs, West Grove, PA) was used as the second antibody.

Table 1
Histopathological features of ASH and NASH cases (percentage in each group).

		ASH	NASH
Macrovesicular lipid droplets	0	5.0%	6.7%
	1+	35.0%	20.0%
	2+	15.0%	6.7%
	3+	20.0%	33.3%
	4+	25.0%	33.3%
Microvesicular lipid droplets	0	75.0%	60.0%
	1+	25.0%	0.0%
	2+	0.0%	20.0%
	3+	0.0%	20.0%
	4+	0.0%	0.0%
PMN	0	20.0%	66.7%
	1+	20.0%	26.6%
	2+	10.0%	0.0%
	3+	30.0%	6.7%
	4+	20.0%	0.0%
Lymphcytes	0	25.0%	13.3%
	1+	30.0%	13.3%
	2+	15.0%	13.3%
	3+	10.0%	40.0%
	4+	20.0%	20.1%
Fibrosis	0	0.0%	13.3%
	1+	0.0%	0.0%
	2+	0.0%	20.0%
	3+	10.0%	26.7%
	4+	90.0%	40.0%
MDBs	0	0.0%	0.0%
	1+	30.0%	40.0%
	2+	5.0%	6.7%
	3+	20.0%	6.7%
	4+	45.0%	46.6%

These slides were double-stained with anti-ubiquitin antibody to detect Mallory-Denk Bodies and DAPI was used to stain nuclei. The staining of all the specimens was performed together at the same time to provide accurate comparison between groups. Positive and negative controls were used for the antibody validation. For each candidate molecule, the intensity of the fluorescent staining of the liver cells was measured with 40× magnifications and standard exposure time in at least three different areas on each section by using a Nikon 400 fluorescent microscope. By using the Nikon morphometric system, 10 representative fluorescence intensities from each of three separate areas of the liver biopsy sections were measured under microscope. The average number of these 30 measurements was used to represent the data (the florescent intensity measurement) of each case and was analyzed. The mean value, standard error, and statistical differences of data achieved from the Nikon were analyzed by Graph pad statistical software (version 6.01). Controls vs ASH, controls vs NASH, and ASH vs NASH were compared by ANOVA with a p-value of < 0.05 considered statistically significant.

3. Results

All ASH and NASH specimens showed significant fat accumulation in hepatocytes (microvesicular fat score 0–3^+ and macrovesicular fat score 1^+-4^+); Fibrosis stages (0–4^+); Duct Metaplasia (all positive); PMN (polymorphonuclear neutrophils score 0–4^+); lymphocytes (score 0–4^+); and MDBs (Mallory-Denk body score 1^+-4^+). There was no statistically significant difference between the ASH and the NASH specimens (Table 1).

The protein expression level of several candidate molecules including RASSF1A, RUNX3, and GSTP1 in the specimens from patients with ASH, NASH, and normal controls were compared. Representative

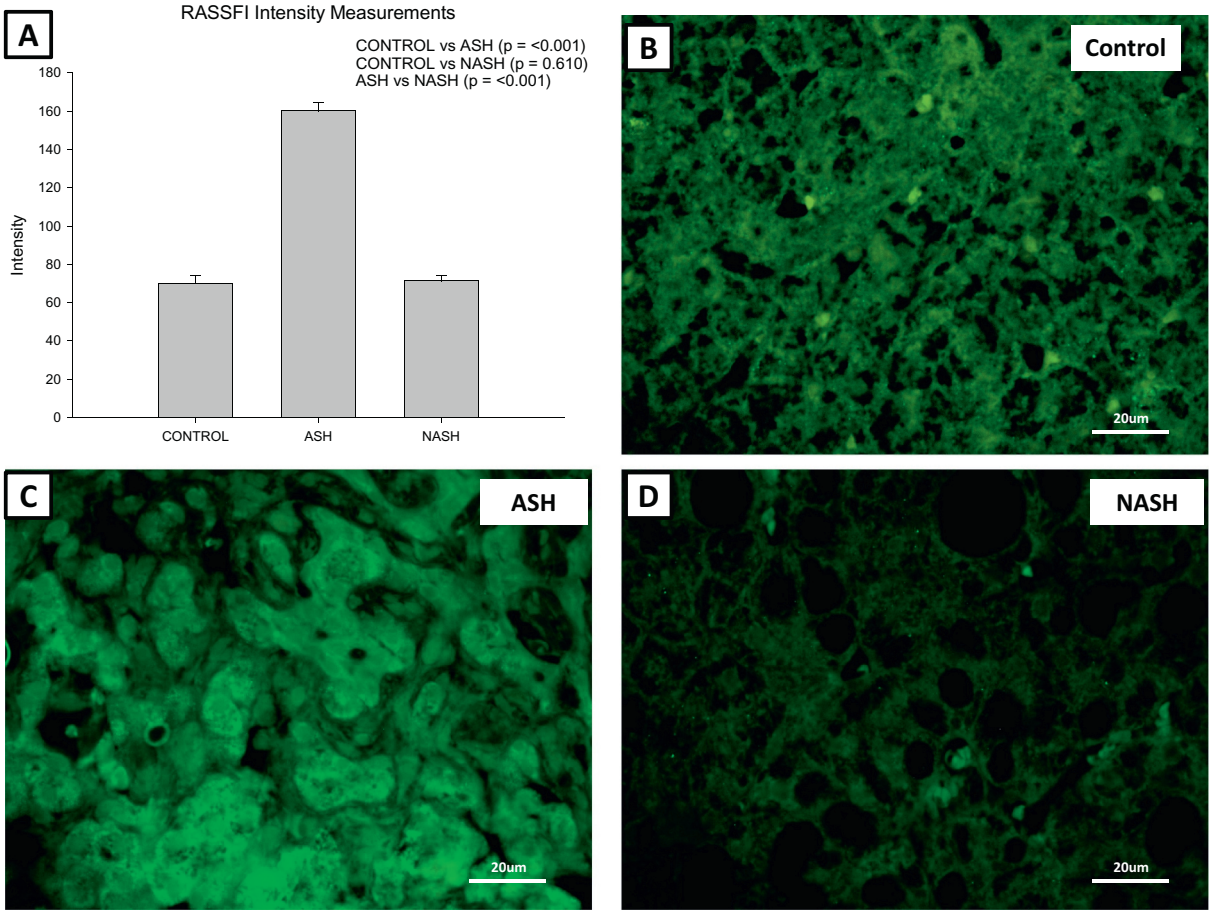

Fig. 1. Different changes of RASSF1A in ASH and NASH specimens. (A) Level of expression of RASSF1A protein upregulated in ASH, NASH or normal controls. Expression is measured as fluorescence intensity and displayed as mean +/− standard deviation. Representative images of fluorescence intensity to measure RASSF1A expression in normal control (B), ASH (C) and NASH (D) liver specimens. A line is drawn through the image to yield a fluorescence intensity graph; the intensity of the ten highest peaks are measured, excluding nuclear regions (not shown). Three separate areas per slide were measured in this manner including Figs. 2 and 3. Scale bars: 20 μm.

fluorescent intensity data are shown in Fig. 1–3.

In ASH patients, levels of all tested candidate proteins including RASSF1A, RUNX3, and GSTP1 (Fig. 1–3) were markedly increased compared with the control group ($p < .001$ in all groups). In NASH patients, RASSF1A and GSTP1 were not changed when compared to the control group ($p > .05$, Fig. 1 and Fig. 3), while the RUNX3 level was increased ($p < .05$, Fig. 2).

The most interesting finding is that all three molecules, RASSF1A, RUNX3, and GSTP1 were significantly lower in the NASH group of specimens compared with the ASH group (Fig. 1–3).

The representative merged co-immunofluorescence (Co-IF) staining pictures of target molecule (green): RASSF1, RUNX3, GSTP1, with MDB (red) and DAPI (blue) are provide in Fig. 4. Compared with control group, both ASH and NASH groups show MDBs presenting and co-expressing with target molecules. These merged Co-IF pictures are consistent with the quantitative data analysis of target molecules presented in Figs. 1–3.

4. Discussion and conclusions

The growing rates of obesity and the metabolic syndrome have become a major health burden worldwide which is leading to an increased number of patients suffering from NASH to cirrhosis and even hepatocellular carcinoma (HCC). In patients receiving liver transplantation due to HCC, NASH is the most rapid growing risk factor [Wong et al., 2014, 2015]. The risk to develop HCC in patients drinking alcohol chronically (longer than 10 years and > 80 g/day) increases approximately 5 fold [French, 2013]. The most common cause of HCC is ALD which leads to around ⅓ of all HCC cases in US and Italy [Morgan et al., 2004] [Testino et al., 2014]. Both NASH and ASH could progress to cirrhosis and even HCC, but the detailed mechanisms remain unknown. Clinical data and genetic mutations studies confirmed the connection between NAFLD and NASH, and increased HCC risk. The cellular and murine models have also shown the importance of gene modifications, cellular stress and inflammation in driving the HCC progression from NASH [Charrez et al., 2016]. It has been reported recently that obesity-associated hepatic oxidative stress can independently contribute to the pathogenesis of NASH, fibrosis, and HCC via STAT-1 and STAT-3 signaling pathways [Grohmann et al., 2018]. It is well known that the rates of those progressing to cirrhosis or HCC annually are lower in the NASH if compared with those in ASH. Over the last decade, studies of the genetic changes and molecular signaling pathways in hepatocellular carcinoma has progressed substantially [Frenette and Gish, 2011]. Our published data support that different molecules and pathways may be

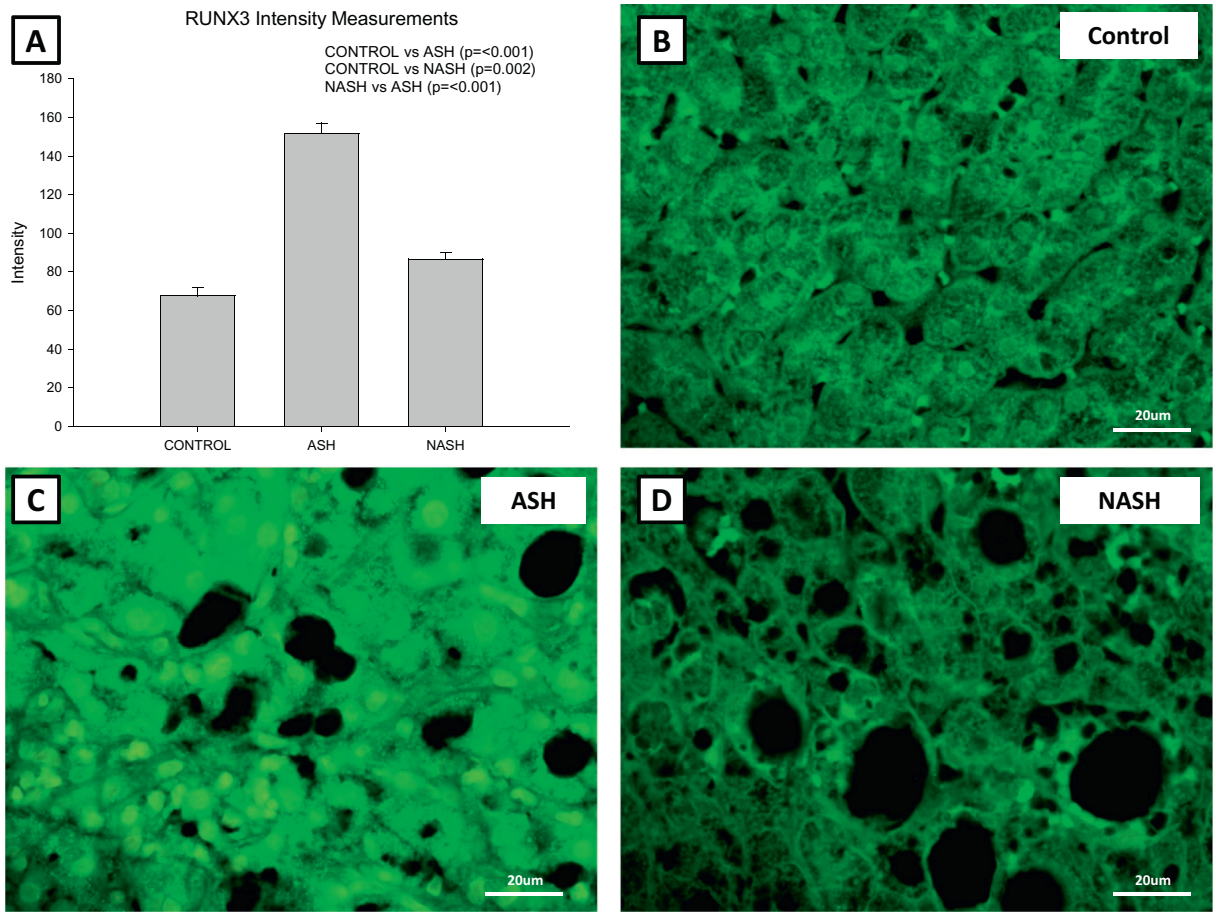

Fig. 2. Different changes of RUNX3 in ASH and NASH specimens. (A) Level of expression of RUNX3 protein upregulated in ASH, NASH or normal controls. Expression is measured as fluorescence intensity and displayed as mean +/− standard deviation. Representative images of fluorescence intensity to measure RUNX3 expression in normal control (B), ASH (C) and NASH (D) liver specimens. Scale bars: 20 μm.

involved in NASH compared with ASH during the tumorigenesis [Nguyen et al., 2018][Lu et al., 2018][Jia et al., 2018]. We showed that the TLR/NFKB/CXCR4/7 [Liu et al., 2014][Liu et al., 2015a][French et al., 2012a, 2012b] [Nan et al., 2005], PI3K/AKT/mTORC1 signaling pathways, and Tec kinase signaling pathway connected to each other in both ASH and NASH [Afifiyan et al., 2017a, 2017b].

There are 10 core Ras association domain family proteins (RASSF1–10) may serve as Ras effectors [Donninger et al., 2016] [Fernandes et al., 2013]. In addition to pro-apoptotic signaling pathway, RASSF proteins connect Ras to a broad range of pathways including modulating cell cycle, inflammation, autophagy, DNA repair, ubiquitination, protein acetylation, and Raf pathway [Vos et al., 2000]. RASSF1A is one of the best characterized and the most commonly expressed isoform from RASSF members. K-Ras can use RASSF1 proteins as pro-apoptotic effectors [Vos et al., 2000]. Inactivated RASSF1A by aberrant promoter methylation is often seen in a broad range of human tumors [Donninger et al., 2007][van der Weyden and Adams, 2007] [Agathanggelou et al., 2005][Gao et al., 2012][Zhan et al., 2017][Pan et al., 2013] including HCC [Araújo et al., 2016]. Up to 15% of tumors may carry RASSF1A mutations [Pan et al., 2005][Kashuba et al., 2009]. In Ras driven tumors, RASSF1A promoter methylation correlates with the worst prognosis [Kim et al., 2003]. Suppression of RASSF1A by shRNA promotes metastasis in mutant Ras cell lines and overexpression of RASSF1A by the transgenic method inhibits K-Ras induced proliferation [Chamberlain et al., 2014]. Our present data showed that in ASH patients, levels of RASSF1A protein were significantly increased compared with the control group. In NASH patients, comparing with the control group, RASSF1A was not changed (Fig. 1). The most interesting finding is that RASSF1A was significantly lower in the NASH group of specimens compared with the ASH group (Fig. 1).

Runt-related transcription factor (RUNX) family members play pivotal roles in both normal development and neoplasia [Shiraha et al., 2013]. As a downstream effector of the transforming growth factor-beta (TGF-β), RUNX3 is an important tumor suppressor gene in human neoplasia [Subramaniam et al., 2009][Lotem et al., 2017][Lotem et al., 2015] including HCC [Nakanishi et al., 2011][Tanaka et al., 2012][Gou et al., 2017][Sun et al., 2017]. RUNX3 acts as a tumor suppressor via multiple mechanisms. The meta-analysis suggests that RUNX3 hypermethylation may be implicated in the pathogenesis of HCC [Yang et al., 2014]. In response to DNA damage, RUNX3 facilitates p53 acetylation by p300 and p53 phosphorylation. When oncogenes are activated, RUNX3 induces ARF which stabilizes p53 (RUNX3-ARF-MDM2-p53) (Fig. 5) [Lee et al., 2017]. The ARF-MDM2-p53 pathway constitutes an effective mechanism for protecting cells from upstream

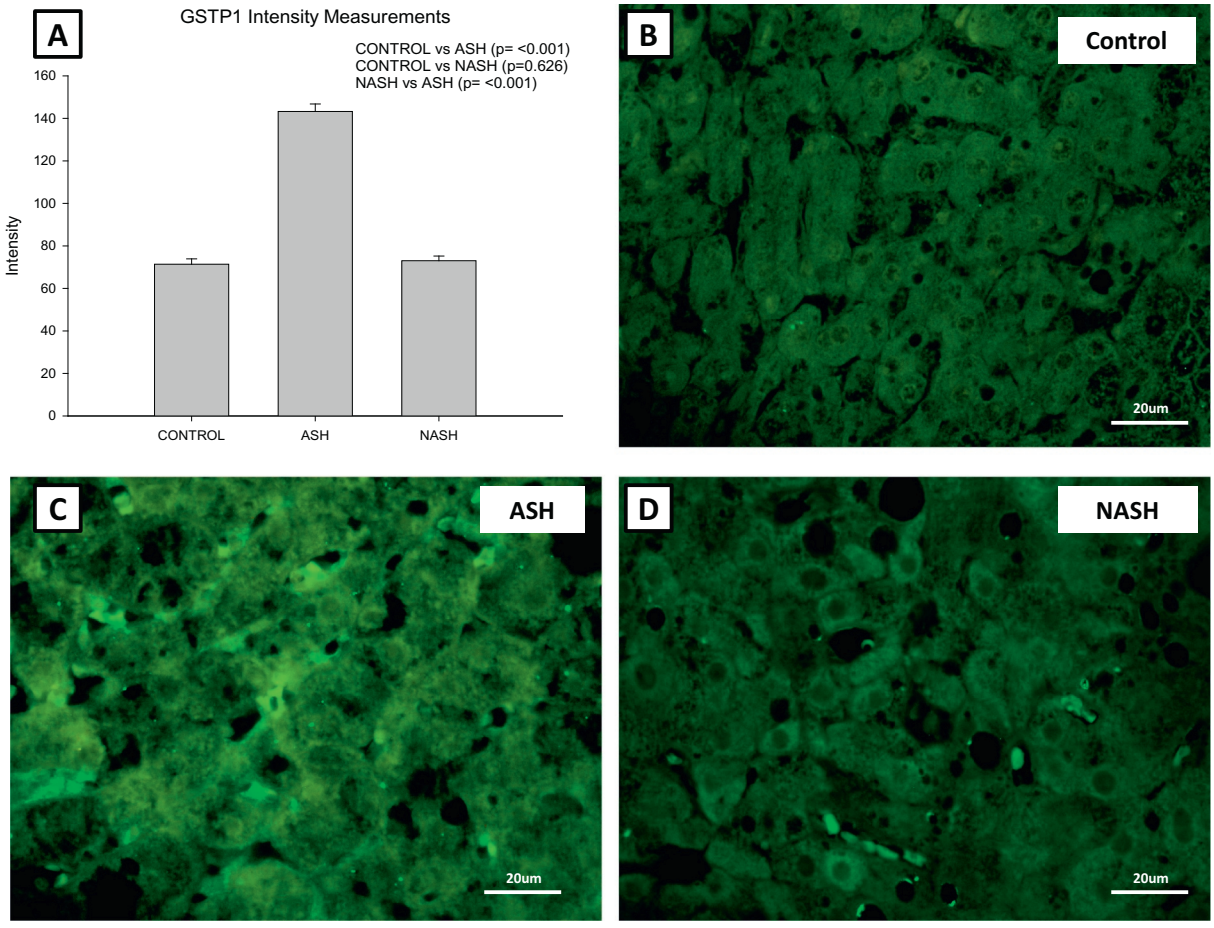

Fig. 3. Different changes of GSTP1 in ASH and NASH specimens. (A) Level of expression of GSTP1 protein upregulated in ASH, NASH or normal controls. Expression is measured as fluorescence intensity and displayed as mean +/− standard deviation. Representative images of fluorescence intensity to measure GSTP1 expression in normal control (B), ASH (C) and NASH (D) liver specimens. Scale bars: 20 μm.

oncogenic stimuli such as activated Ras [Chi et al., 2009]. Importantly, Ras activation induces ARF and often occurs earlier than p53 inactivation during cancer development. MDM2 directly binds the RUNX3 and blocks RUNX3 transcriptional activity. RUNX3 and the ubiquitous p53 protein are both principal responders of the ARF-MDM2 cell surveillance pathway that prevents pathologic consequences of abnormal oncogene activation [Chi et al., 2009]. In HCC, RUNX3 expression is frequently lost or decreased due to hemizygous deletion or hypermethylation of its promoter lesion. The significance of the decreased expression of RUNX3 in HCC is likely related to dysfunction of cell cycle regulation, apoptosis, angiogenesis, and development of epithelial-mesenchymal transition [Shiraha et al., 2011]. Our previous work and the literature has confirmed the relationship of the transcription factor RUNX3 may be altered in ASH and NASH patients [Nguyen et al., 2018][Liu et al., 2015b]. Although ERK activation and PTEN down regulation in NASH are highly dependent on the lipid accumulation and are related to growth factors such as IGFs, it has been reported that the MEK/ERK pathway is related to RUNX3 gene expression directly [Liu et al., 2012] or indirectly [Chen et al., 2016]. The present data showed that in ASH and NASH patients, levels of RUNX3 protein were significantly increased compared with the control group. Although the increase of RUNX3 levels in NASH group are statistically significant, the change between NASH and control group may not be clinically significant. RUNX3 was significantly lower in the NASH group specimens compared with the ASH group (Fig. 2).

The glutathione S-transferases (GSTs) family of enzymes is well known for their cytoprotective role and their involvement in the development of anticancer drug resistance [Shiraha et al., 2013] [Singh et al., 2015]. The GSTs, especially GSTP1 isoform, play important roles, not only in tumor drug resistance, but also in promoting neoplastic transformation [Schnekenburger et al., 2014] in different organs [Zhang et al., 2016][Kuang et al., 2016][Song et al., 2014][Yu et al., 2013][Zhao et al., 2017] including liver [Usami et al., 2005][Jain et al., 2012][Liu et al., 2018]. It has been reported that the GSTP1 gene is a downstream transcriptional target of the tumor suppressor p53 [Lo et al., 2008]. The wild-type p53 protein binds to the GSTP1-p53 motif and the motif to be transcriptionally functional in human tumor cells. High levels of GSTP1 transcripts and protein were associated with wild-type p53 and, conversely, low GSTP1 levels with mutant p53. The ability of wild-type p53 to transcriptionally activate the human GSTP1 gene defines a novel mechanism of protecting the genome and, potentially, of tumor drug resistance [Lo et al., 2008]. In the present study, the change of GSTP1 was similar to RASSF1A. Compared with the control group,

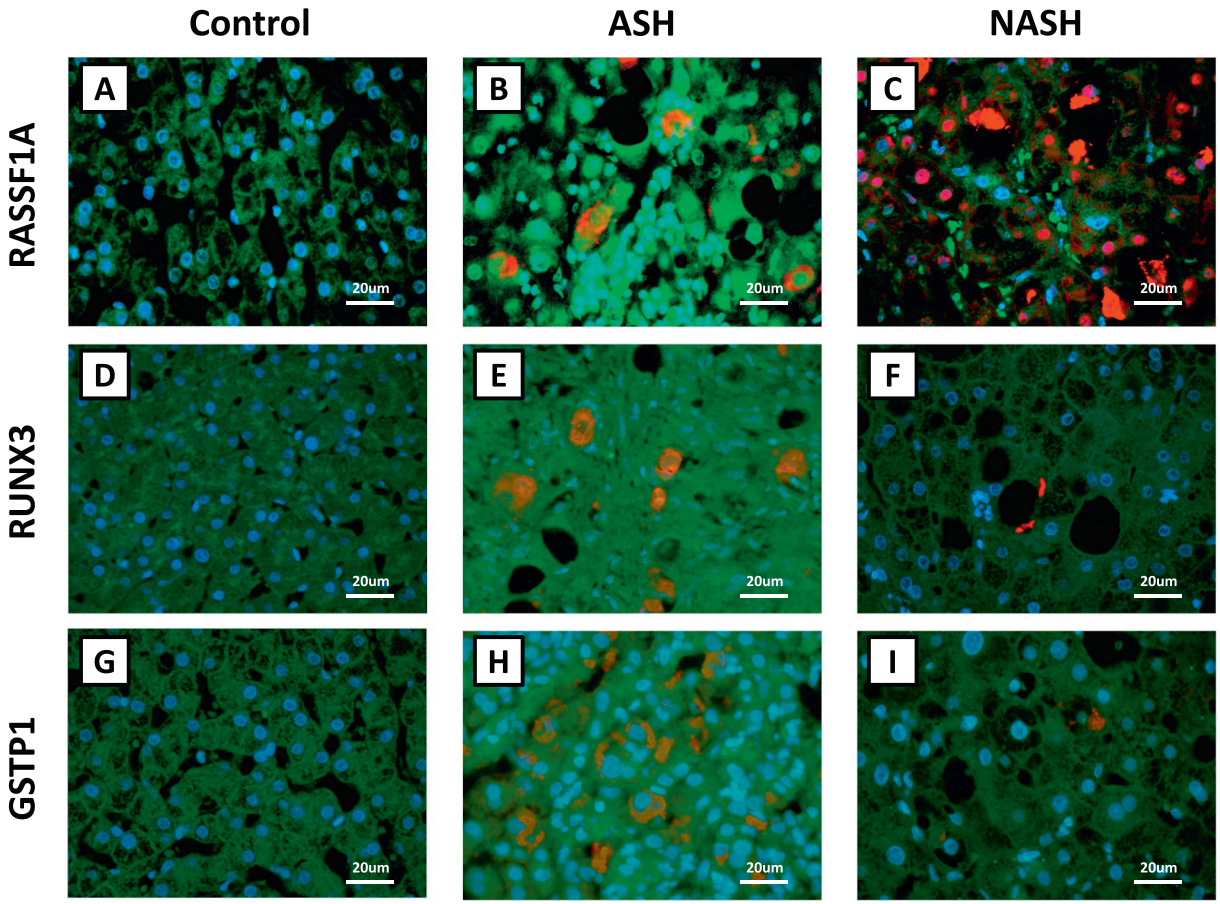

Fig. 4. Merged co-immunostaining of target molecule: RASSF1, RUNX3, GSTP1, with DAPI and MDB. (A,B,C) Co-immunostaining for RASSF1A (green) and MDB (red) with DAPI nuclear counterstain (blue) in control, ASH, and NASH liver biopsy specimens. (D,E,F) Co-immunostaining for RUNX3 (green) and MDB (red) with DAPI nuclear counterstain (blue) in control, ASH, and NASH liver biopsy specimens. (G,H,I) Co-immunostaining for GSTP1 (green) and MDB (red) with DAPI nuclear counterstain (blue) in control, ASH, and NASH liver biopsy specimens. Quantitative data analysis of target molecules are presented in Figs. 1 to 3. Scale bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein levels of GSTP1 in ASH patients were significantly higher than that in the control group, but there was no change in the NASH patients. GSTP1 was also significantly lower in the NASH group specimens compared with the ASH group (Fig. 3).

In addition to high alcohol intake and the metabolic syndrome, HCC is also related to various etiological stressors including hepatitis B, hepatitis C, and aflatoxin B1. Aflatoxin B1 and hepatitis B have a clear direct oncogenic role through point mutations in the TP53 tumor suppressor gene and insertional mutagenesis respectively [Nault, 2014]. The “guardian of the genome”, p53 which is associated with modulations in metabolic pathways and induction of apoptosis, plays a unique role in the physiology and pathophysiology of the liver. The stressors frequently leads to the activation of p53 in the liver. Dysfunction of p53 may eventually cause progressing of liver simple steatosis to steatohepatitis, fibrosis, cirrhosis, and even HCC. The aflatoxin may lead to oncogenic mutation of p53 gene [Charni et al., 2014]. The ras/raf/MEK and Wnt signal-transduction pathways, plus the p53 tumor suppressor pathways significantly contribute to liver carcinogenesis (Fig. 5) [Staib et al., 2003]. Regeneration and tumorigenesis share common molecular pathways, nevertheless the outcome of regeneration is life, whereas tumorigenesis leads to death. Although the process of regeneration is strictly controlled, malignant transformation is unrestrained [Charni et al., 2017]. The three molecules with the same expression pattern in control, ASH, and NASH patient specimens are all able to connect with p53 pathway (Fig. 5). The upregulations of the three tumor suppressors in the ASH group may suggest that the hepatocytes under stress are trying to prevent the cells from tumorigenesis as compensation or rescue pathways involving p53. Although we found that mRNA level of p53 was downregulated in ASH liver biopsies, the downstream effector p27 was significantly upregulated in same specimens [Liu et al., 2015a, 2015b]. The different levels of the tumor suppressors between ASH and NASH show that different molecules and pathways are involved during the tumorigenesis in ASH and NASH.

In summary, in addition to the GPCR/PI3K/Akt and/or TLR4/NF-κB/STAT3 pathways connected by FAT10/FOXO1 [Jia et al., 2018], our present data and previous published studies demonstrates the different expression of tumor suppressor proteins RASSF1A, RUNX3, and GSTP1, cross-talking with p53, may explain the different rate of progression to fibrosis, cirrhosis, and eventually HCC in ASH and NASH patients. These findings may be very helpful to clarify the tumorigenesis of HCC and to guide possible therapeutic target direction, although more dedicated studies are needed.

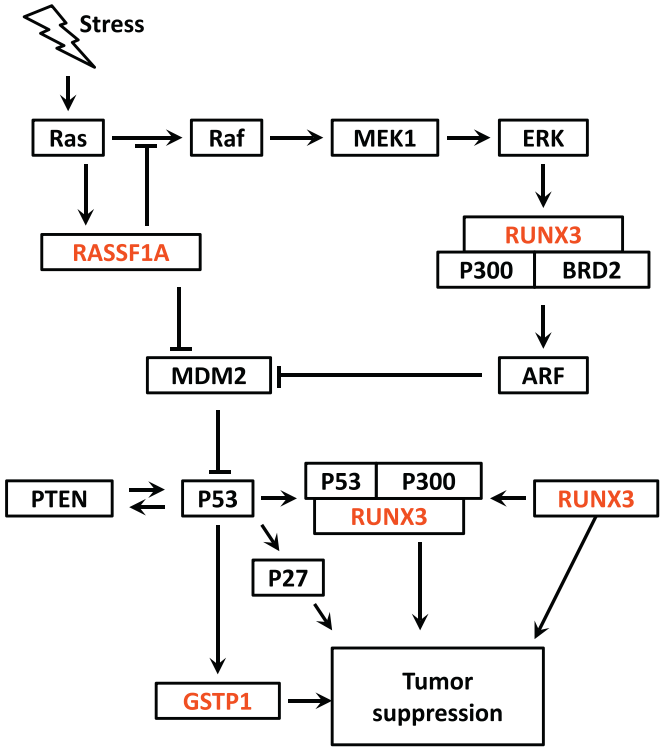

Fig. 5. Putative pathway for RASSF1A, RUNX3, and GSTP1 in the HCC tumorigenesis. The different expression of RASSF1A, RUNX3, and GSTP1 proteins in ASH and NASH suggest different pathways, including p53 and Ras/Raf/MEK pathways in HCC tumorigenesis due to different stressors.

Acknowledgements

This study was funded by NIH/AAA grant # UO-21898-05

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