

Original Article

Histone-modifying genes as biomarkers in hepatocellular carcinoma

Shih-Ya Hung^{1,2}, Hui-Hua Lin¹, Kun-Tu Yeh³, Jan-Gowth Chang^{1,2,4,5}

¹Epigenome Research Center, China Medical University Hospital, Taichung 40447, Taiwan; ²Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan; ³Department of Pathology, Changhua Christian Hospital, Changhua 50006, Taiwan; ⁴Department of Laboratory Medicine, China Medical University Hospital, Taichung 40447, Taiwan; ⁵School of Medicine, China Medical University, Taichung 40447, Taiwan

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Abstract: Hepatocellular carcinoma (HCC) is the world's fifth most common cancer and second leading cause of cancer-related death in Taiwan. Over 600,000 HCC patients die each year worldwide despite recent advances in surgical techniques and medical treatments. Epigenetic regulations including DNA methylation and histone modification control gene expressions and play important roles during tumorigenesis. This study evaluates association between histone-modifying genes and prognosis of HCC to ferret out new diagnostic markers. We collected 50 paired HCC and adjacent non-cancerous tissues from Taiwanese patients for survey by RT-qPCR and tissue microarray-based immunohistochemistry (TMA-based IHC) staining. RT-qPCR data showed four of twenty-four genes over eight-fold up-regulated in tumor tissues: e.g., histone phosphorylation gene-*ARK2*, methylation genes-*G9a*, *SUV39H2*, and *EZH2* ($n = 50$, all $p < 0.0001$). Results of TMA-based IHC staining showed proteins of *ARK2*, *EZH2*, *G9a*, and *SUV39H2* also overexpressed in tumor tissues. Staining intensity of *SUV39H2* correlated with HCV infection ($p = 0.025$). We further restricted the analysis only in tumor tissues, we found *EZH2* staining intensity associated with tumor stage ($p = 0.016$) and survival ($p = 0.007$); *SUV39H2* intensity associated with tumor stage ($p = 0.044$). Our findings indicate overexpression of histone-modifying genes *EZH2* and *SUV39H2* associated with prognosis of HCC cases. *EZH2* expression can serve as a novel prognostic biomarker during HCC progression among Taiwanese.

Keywords: Hepatocellular carcinoma, histone acetylation, histone methylation, histone phosphorylation, histone modification

Introduction

Hepatocellular carcinoma (HCC; also called liver cancer) is a highly lethal cancer (third most common cause of cancer mortality worldwide) with rising incidence; approximately 750,000 new cases reported each year around the globe and overall 5-year survival rate is only 0-14% [1, 2]. In addition, the Cancer Registry Annual Report in Taiwan of 2012 ranked HCC as second most common cancer-related death in Taiwanese. This illustrates the urgency to expose cellular mechanisms involved in this aggressive cancer to develop more effective treatments and better prognosis. Chief risk factors include chronic viral hepatitis of B- or C-virus infection (HBV or HCV), alcohol abuse, nonalcoholic steatohepatitis, aflatoxin B1 expo-

sure, and liver cirrhosis [3, 4]. Currently, HCCs are diagnosed by the invasive (biopsy) and non-invasive (images and tumor markers) methods [5]. The former include imaging diagnosis such as computed tomography (CT), magnetic resonance imaging (MRI), and tumor markers like α -fetoprotein and glypican-3 [5]. Yet detailed molecular mechanisms of hepatocarcinogenesis are not fully understood; molecular factors capable of predicting clinical outcome and acting as potential therapeutic targets remain limited.

Human genome is highly compacted by nucleoprotein complexes called chromatin, whose basic unit is a nucleosome; DNA is tightly packed in nuclei with the help of proteins called linker (H1) and packing (H2A, H2B, H3, and H4)

histones [6]. The term “epigenetics” means changes in gene expression and cellular phenotype without affecting DNA sequence [7]. Epigenetic mechanisms can be considered as an interface between the genome and risk factors of lifestyle and the environment [8]. Histone modification is one of the epigenetic mechanism belonging to post-translational modification of N-terminal tails of histone proteins by acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, biotinylation, and potentially other modifications; these appear to work together with other epigenetic mechanisms in establishing and maintaining gene activity states, thus regulating a gamut of cellular processes [9]. So that several families of enzymes catalyze post-translational modifications of histones, including acetyltransferases and deacetylases, methyltransferases and demethylases, etc. play key roles in histone modification [10]. Recent studies prove the importance of histone-modifying enzymes and chromatin remodeling complexes in regulating chromatin activities of transcription, replication, and DNA repair. Histone modification plays essential roles in generating the dynamic state of chromatin [6]. Alternations in the function of histone-modifying molecules and protein complexes are found to disrupt the pattern and levels of histone marks and consequently deregulate the control of chromatin-based processes leads to development of many cancers [11].

Hepatocarcinogenesis is viewed as a complex and multi-step process entailing epigenetic and genetic events [12]. Recent reports show histone-modifying genes associate with HCC prognosis. Besides histone acetylation-related proteins, epigenetic aberrations which caused by histone phosphorylation (like ARK2 and MSK1) and lysine or arginine methylation (like G9a, EZH2, SUV39H1, and SUV39H2) demonstrably associate with progression of many tumors [13-15]. For example, histone phosphorylation enzymes-Aurora kinases (subfamily of serine/threonine mitotic kinases) are primary molecules for maintaining accurate cell cycling and genomic stability [16]. In HCC, ARK1 (Aurora-A kinase; centrosome-associated serine/threonine kinase) is overexpressed frequently in HCC patients, and correlated with high grade and high stage [17]. ARK2 (Aurora-B kinase, which phosphorylates histone H3 at serine 10) is a chromosomal passenger protein and that is

essential for chromosome segregation and cytokinesis; mRNA level of ARK2 is an independent molecular marker predicting tumor invasion of HCCs [18]. Likewise, Aurora kinase inhibitors are potential therapeutic agents for HCC treatment [18, 19]. G9a, a histone methyltransferase for lysine 9 of histone 3 (H3K9), is also a major player in gene silencing and essential for early embryogenesis to regulate developmental gene expression [20, 21]. EZH2 (a histone methyltransferase for lysine 27 of histone 3) belongs to the polycomb genes crucial for transcriptional regulation via nucleosome modification, chromatin remodeling, and interaction with other transcription factors [22]. Positive EZH2 expression as a diagnostic biomarker detects aggressive phenotype or a phenotype with poor prognosis in patients of China population [23]. SUV39H1 was the first SET domain-containing histone lysine methyltransferase (HKMT) discovered in 2000; SUV39H1 and SUV39H2 knockout mice have shown genomic instability [24]. The mRNA level of *SUV39H1* has been shown no different in 23 paired HCC and non-cancerous liver tissues from Japanese population [25]. On the other hand, *SUV39H1* mRNA level shows up-regulated in 38 HCC patients from Hong Kong [26].

The present report examines expressions of histone-modifying genes in 50 HCC patients to derive a relationship between gene expressions and clinicopathological parameters in these Taiwanese patients. Here, we assessed mRNA expression of 24 histone-modifying genes further studied protein expressions in HCC and corresponding non-cancerous liver tissues by RT-qPCR and immunohistochemistry staining. Once we better understand the clinical relevance of those histone-modifying genes in HCCs among the Taiwanese population, clinicians can offer effective cancer treatments, biomarkers for early detection, better prognosis, and novel therapeutic targets. Identifying molecular markers related to hepatocarcinogenesis would benefit patients and serve as potential therapeutic target for novel HCC drug treatments.

Materials and methods

Patients and clinical methods

This study was approved by the Institutional Review Board of the China Medical University

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Table 1. Primer and probe sequences of RT-qPCR assays

Gene	Forward primer	Reverse primer	Probe
ADA	ccttcgacaagcccaagta	gcccctctgctgtgttagc	ctcctgcc
ARK2	attgctgacttcggctggt	gtccagggtgccacacat	cttcctcc
ATF2	cgaccgcagtcattacaaca	gtggagttgtgtgagctgga	tgtggctg
CARM1	cacaccgacttcaaggacaa	aaaaacgacaggatcccaga	tggctgtg
CBP/CREBBP	gagatccaggggcagaatg	aactgatcctttgaaattgtcgt	ctggggct
DOT1	ccccactcacctcagacg	ttgcaatTTTTctcaattgc	cccagcag
ESA1	aaggagctcatcgtcacagt	caagaggacctgcttcaact	gctggatc
EZH2	tgcacatcctgacttctgtga	tcatctccataaaggaatgttatg	ggaggtgg
G9a	gggaccttcatctgcgagta	tctcacatcagcctcagcat	gggagctg
GAPDH	agccacatcgtctcagacac	gcccaatacagccaaatcc	cttcagcc
Gcn5	ccgaccagctctacacaacc	aggcactggggtgagactt	gggagctg
HAT1	tctaaagttgatgagaactttgactgt	ttgtctaattttgccctcaaca	ctgcctct
MLL/TRX	gcgccaagctctttgcta	catcatcatcataacatttgcacag	cttcctcc
MOF	gaagttcctcatcgtcttcag	tcagacagcggcttctcc	ctccagct
MSK1	tggctgctgacagatcttgggt	caaaaggaaatgatcctttcagtttc	cctggcgc
P300	gcagcctgcaactccact	gaggatttgatacctgcttca	cctggagc
PCAF	cagagaccctgaccagcttt	gcgctttgatggctcttc	acctgctg
PRMT1	ggagaattttgtagccacttg	cctggccacaggacactt	ctccagcc
SET1	cactgggtccatcacacaat	ctgagcctgtctggtgctc	ctggggcc
SETDB1	tgaaaaagagcccttgaagc	ccaggaagggaagacatgc	tcctctcc
SET7	atggatagcgcgacgagaga	taatccgtcatcgtccaggt	tggaggag
SET8	ggatttctaccctgtccgaag	ttcaatcaattcatctattctttcc	cctggagc
SRC1	tctcaaaacagaagcagatgga	gacgtcagcaaacactgaa	acctgctg
SUV39H1	tcattggagtacgtgggagaga	cctgacggctgtagatctgg	ctctgctt
SUV39H2	gcattgtttccacaagaacc	tgggctgtggtcaatagaatc	ctccagca

Hospital (CMUH103-REC1-033). Fifty Taiwanese patients diagnosed with HCC at Changhua Christian Hospital (Changhua, Taiwan) during 2002-2006 enrolled. All underwent surgery with or without radiotherapy and/or chemotherapy. Their 50 paired HCC tissues and corresponding adjacent non-cancerous liver tissues were stored at -80°C until RNA extraction and IHC staining, their medical records reviewed. All tumors were classified according to the 7th edition of *TNM Staging Manual of American Joint Committee on Cancer* [27].

RNA extraction, reverse-transcription, and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues according to instructions of the High Pure RNA Isolation Kit (Roche Applied Science; Mannheim, Germany). Single-strand cDNA was synthesized by high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City,

CA). The RT-qPCR was performed to analyze mRNA expression in 4 groups: {1} histone acetylation genes- such as ADA (adenosine deaminase), ATF2 (activating transcription factor 2), CREBBP (CREB binding protein), ESA1 (histone acetyltransferase ESA1), GCN5 (general control of amino-acid synthesis 5-like 2, yeast), HAT1 (histone acetyltransferase 1), MOF (lysine acetyltransferase 8), p300/CBP (E1A binding protein P300), PCAF (TAF6-like RNA polymerase II, P300/CBP-associated factor), and SRC1; {2} histone lysine methylation genes such as MLL/TRX (myeloid/lymphoid or mixed-lineage leukemia/tri-

thorax homolog, drosophila), SET1 (SET domain containing 1A), SET7 (SET domain containing 7), G9a (euchromatic histone-lysine N-methyltransferase 2), SETDB1 (SET domain, bifurcated 1), SUV39H1 (suppressor of variegation 3-9 homolog 1, drosophila), SUV39H2 (suppressor of variegation 3-9 homolog 2, drosophila), EZH2 (enhancer of zeste homolog 2, drosophila), DOT1 (disruptor of telomeric silencing-1), and SET8 (SET domain containing lysine methyltransferase 8); {3} histone arginine methylation genes such as CARM1 (coactivator-associated arginine methyltransferase 1) and PRMT1 (protein arginine methyltransferase 1); {4} histone serine/threonine phosphorylation genes such as ARK2 (aurora kinase B) and MSK1 (ribosomal protein S6 kinase, 90 kDa, polypeptide 5). We used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the RT-qPCR internal control. **Table 1** shows primer and probe sequences for each RT-qPCR reaction. The thermal cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s,

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Table 2. Overview of different classes of histone-modifying genes in this study

Histone modifications	Residues modified	Genes-included in this study	Functions regulated
Acetylation of lysine	Lysine	ADA, ATF2, CREBBP, ESA1, GCN5, HAT1, MOF, p300/CBP, PCAF, SRC1	Transcription; repair; replication; condensation
Methylation of lysine	Histone H3 Lysine 4	MLL/TRX, SET1, SET7	Transcription; repair
	Histone H3 Lysine 9	G9a, SETDB1, SUV39H1, SUV39H2	
	Histone H3 Lysine 27	EZH2	
	Histone H3 Lysine 79	DOT1	
	Histone H4 Lysine 20	SET8	
Methylation of arginine	Histone H3 on Arginine 17	CARM1	Transcription
	Histone H4 on Arginine 3	PRMT1	
Phosphorylation of serine/threonine	Histone H3 on Serine 10	ARK2, MSK1	Transcription; repair; condensation

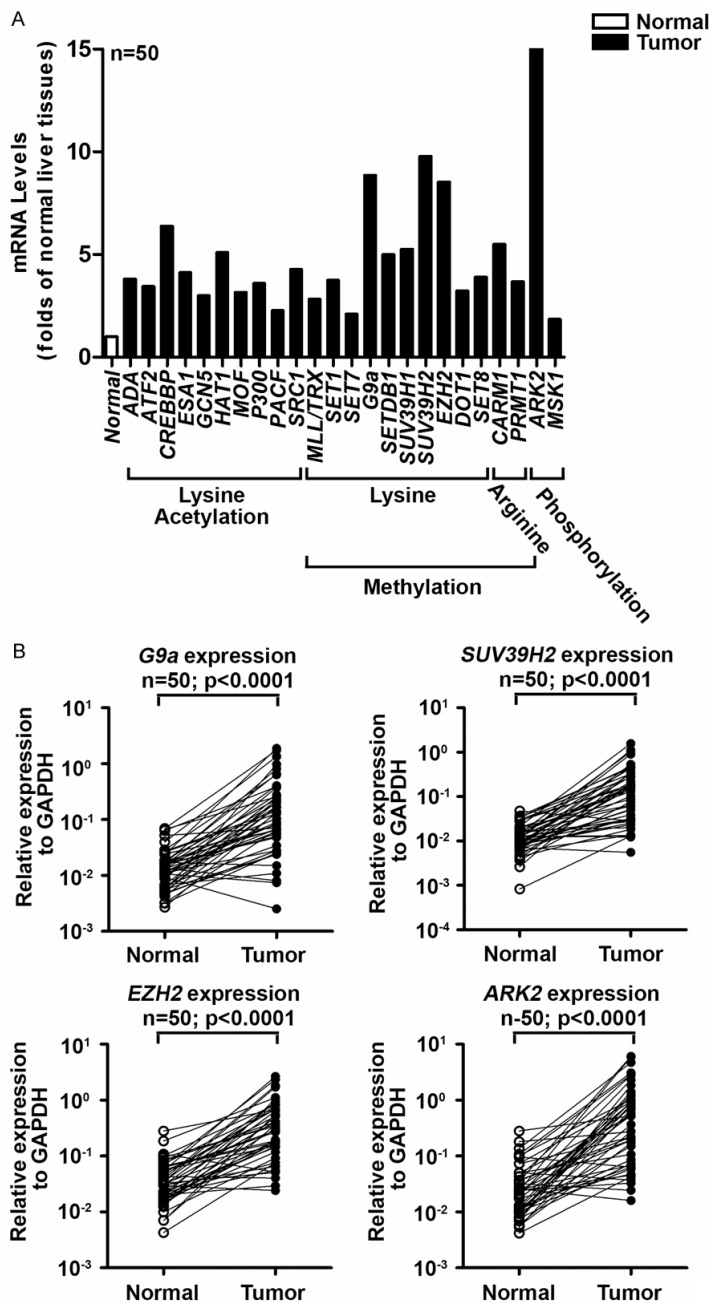


Figure 1. Enhanced mRNA expression levels of 24 histone-modifying genes in tumorous liver tissues of 50 HCC patients. Twenty-four histone-modifying genes including acetylation of lysine, methylation of lysine, methylation of arginine, and phosphorylation of serine/threonine were selected for RT-qPCR study. A: RT-qPCR shows all 24 genes significantly up-regulated in tumorous versus non-tumorous tissues of liver in 50 HCC patients ($P < 0.05$ in all 24 genes). B: G9a, SUV39H2, EZH2, and ARK2 were more than eightfold up-regulated in tumorous tissues of fifty HCC patients. Gene expression is presented as relative expression to GAPDH for each paired liver tissues.

60°C for 30 s, and 72°C for 10 s. Expression levels of mRNA were first normalized to mRNA of GAPDH and then expressed as folds of normal by $2^{-\Delta\Delta Ct}$ method according to Liu et al [28].

Tissue microarray-based immunohistochemistry (TMA-based IHC) staining analysis and antibodies

TMA-based IHC staining for ARK2, EZH2, G9a, and SUV39H2 proteins was performed as follows. Primary antibodies for the study were ARK2 (Santa Cruz; sc-14327), EZH2 (Invitrogen; 49-1043), G9a (Santa Cruz; sc-22877), and SUV39H2 (Abgent; AP1281a). Briefly, the 5- μ m paraffin-embedded sections (5 \times 8 = 40 cores/slide tissue array; each slide has 20 paired of normal and tumor tissues from HCC patients) were deparaffinized, retrieved with heat in 10 mM

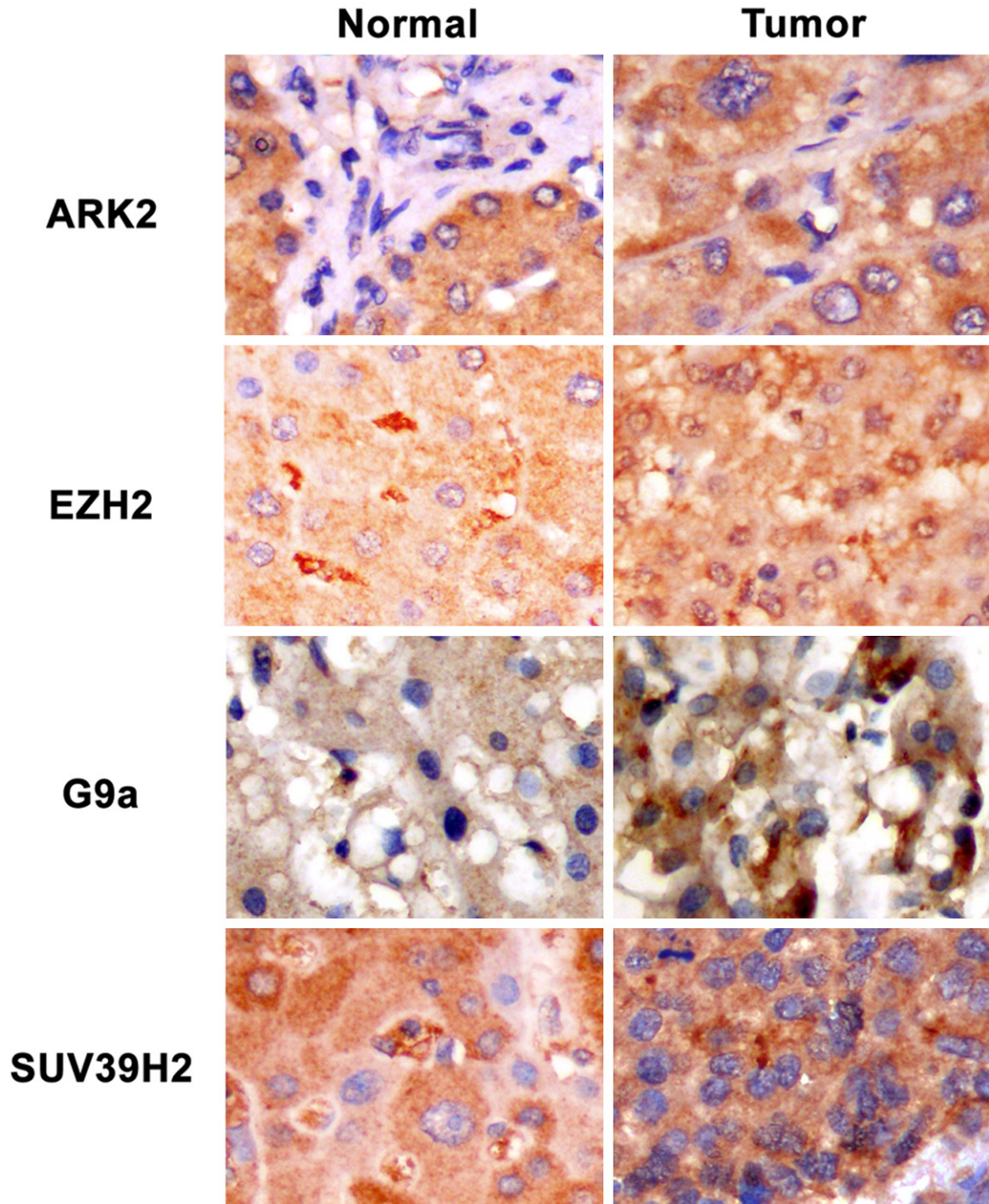


Figure 2. Tissue microarray-based immunohistochemical staining patterns of four histone-modifying proteins. Immunohistochemical staining results of ARK2, EZH2, G9a, and SUV39H2 in tissues from hepatocellular carcinoma (HCC) patients. Original magnification, $\times 400$.

citrate buffer (pH 6.0) at 121°C for 10 min, then treated with 3% hydrogen peroxide to remove endogenous peroxidase activity. Primary antibodies were used as suggested by manufacturers and were used at dilution of

1:50 after optimization. Tissue sections treated with secondary antibodies and with biotin-streptavidin complex for 30 min each at 37°C. Diaminobenzidine served as chromogene for immunoperoxidase reaction, slides counter-

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Table 3. Correction of ARK2 and EZH2 protein intensity with clinicopathological parameters in 50 Taiwanese patients with HCC

Characteristics		ARK2				EZH2			
		Tumor ≥ Normal	Tumor < Normal	Total	P-value	Tumor ≥ Normal	Tumor < Normal	Total	p-value
Tumor Size	≤ 2 cm	1	5	6	1.000	0	6	6	1.000
	> 2 cm	4	28	32		1	30	31	
Grade	Well	0	4	4	0.309	0	3	3	0.827
	Moderate	5	22	27		1	26	27	
	Poor	0	7	7		0	7	7	
Stage	I, II	4	17	21	0.349	1	20	21	0.474
	III, IV	1	17	18		1	17	18	
Survival	≤ 2 years	1	16	17	0.363	1	16	17	0.447
	> 2 years	4	18	22		0	21	21	
HBV infection	No	2	10	12	0.594	0	12	12	0.647
	Yes	2	21	23		1	21	22	
HCV infection	No	1	19	20	0.303	0	19	19	0.458
	Yes	3	13	16		1	15	16	
Virus infection	No	0	2	2	1.000	0	2	2	1.000
	Yes	4	31	35		1	33	34	
Cirrhosis	No	1	15	16	0.618	1	15	16	0.444
	Yes	3	18	21		0	20	20	

stained with hematoxylin and examined for intensity by the pathologist in a blind manner. More than 50% of cells displayed positive staining and intensity scores of moderate and strong, was considered to be protein overexpression in this study. Tissue specimens were broadly distributed by immunohistochemical staining category (0, 1+, 2+, or 3+) according to the guidelines based on Hofmann et al. [29].

Statistical analysis

Statistical analyses were performed using SPSS v17 (SPSS Inc., Working, UK). Paired *t*-test was used for analysis RT-qPCR assays. Correction between protein expression and clinicopathological features of 50 HCC patients were drawn by Pearson's Chi-squared test, *P*-values < 0.05 considered statistically significant.

Results

Four mRNAs of histone-modifying genes more than eightfold overexpressed in HCC tissues

To rate mRNA expression of histone-modifying genes in HCC, we performed RT-qPCR analysis

on total RNA extracted, classifying 24 histone-modifying genes as {1} acetylation of lysine, {2} methylation of lysine, {3} methylation of arginine, or {4} phosphorylation of serine/threonine. **Table 2** shows an overview of these histone-modifying genes. RT-qPCR results show all 24 genes significantly up-regulated in HCC tissues (tumors) from 50 patients as compared to adjacent non-cancerous tissues (normal) (*P* < 0.05; **Figure 1A**). Since all these genes showed statistically significant increase in tumors, we set eightfold up-regulation as threshold of mRNA overexpression. We found G9a (↑8.85 folds; methylation of lysine), SUV39H2 (↑9.78 folds; methylation of lysine), EZH2 (↑8.52 folds; methylation of lysine), and ARK2 (↑15.53 folds; phosphorylation of serine/threonine) were more than eightfold overexpressed in tumor (*P* < 0.0001; *n* = 50; **Figure 1B**). **Figure 1B** shows gene expression patterns in 50 paired tissues.

Increase protein expressions of ARK2, EZH2, G9a, and SUV39H2 in tumors

Since mRNAs of ARK2, EZH2, G9a, and SUV39H2 were more than eightfold overexpressed in tumor tissues of 50 HCC patients.

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Table 4. Correction of ARK2 and EZH2 protein intensity in tumor tissues with clinicopathological parameters in 50 Taiwanese patients with HCC

Characteristics		ARK2 in tumor				EZH2 in tumor			
		≤ 1+	≥ 2+	Total	P-value	≤ 1+	≥ 2+	Total	P-value
Tumor Size	≤ 2 cm	2	4	6	1.000	2	4	6	0.668
	> 2 cm	11	24	35		17	18	35	
Stage	I	3	4	7	0.780	3	4	7	0.344
	II	3	11	14		4	10	14	
	III	4	9	13		8	5	13	
	IV	3	6	9		5	4	9	
Grade	Well	2	2	4	0.578	1	3	4	0.635
	Moderate	8	22	30		15	15	30	
	Poor	3	5	8		4	4	8	
Survival	≤ 2 years	7	14	21	0.747	14	7	21	0.015*
	> 2 years	6	16	22		6	16	22	

*, $p < 0.05$.

Table 5. Correction of G9a and SUV39H2 protein intensity with clinicopathological parameters in 50 Taiwanese patients with HCC

Characteristics		G9a				SUV39H2			
		Tumor ≥ Normal	Tumor < Normal	Total	P-value	Tumor ≥ Normal	Tumor < Normal	Total	P-value
Tumor Size	≤ 2 cm	2	4	6	1.000	2	4	6	1.000
	> 2 cm	10	20	30		8	20	28	
Grade	Well	1	2	3	0.108	1	3	4	0.710
	Moderate	11	15	26		8	16	24	
	Poor	0	7	7		1	5	6	
Stage	I, II	7	12	19	0.728	7	12	19	0.285
	III, IV	5	13	18		3	13	16	
Survival	≤ 2 years	5	12	17	1.000	4	11	15	1.000
	> 2 years	7	13	20		6	14	20	
HBV infection	No	5	6	11	0.434	5	7	12	0.24
	Yes	6	17	23		4	16	20	
HCV infection	No	5	16	21	0.153	2	17	19	0.025*
	Yes	7	7	14		7	8	15	
Virus infection	No	0	2	2	0.536	0	2	2	1.000
	Yes	12	21	33		9	22	31	
Cirrhosis	No	4	11	15	0.721	3	12	15	0.451
	Yes	7	13	20		7	12	19	

*, $p < 0.05$.

We then studied protein expressions of ARK2, EZH2, G9a and SUV39H2 in 50 HCC patients by IHC staining on tissue microarray. **Figure 2** shows the typical staining patterns of ARK2, EZH2, G9a, and SUV39H2 in respective normal and tumor tissues from HCC patients. We found that protein levels of ARK2, EZH2, G9a, and SUV39H2 were increase only in tumor but not

normal tissues. These results concur with our mRNA expression data.

Association of ARK2, EZH2, G9a, and SUV39H2 protein levels with clinicopathological parameters

Table 3 shows relationship between protein intensity of ARK2 or EZH2 and clinicopathologi-

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Table 6. Correction of G9a and SUV39H2 proteins expression levels in tumor tissues with clinicopathological parameters in 50 Taiwanese patients with HCC

Characteristics	G9a in tumor				SUV39H2 in tumor				
	≤ 1+	≥ 2+	Total	P-value	No	Yes	Total	P-value	
Tumor Size	≤ 2 cm	1	5	6	1.000	2	4	6	0.206
	> 2 cm	6	29	35		4	31	35	
Stage	I	2	5	7	0.143	3	4	7	0.044*
	II	0	14	14		0	14	14	
	III	2	11	13		1	12	13	
	IV	3	6	9		2	7	9	
Grade	Well	0	4	4	0.170	0	4	4	0.098
	Moderate	4	26	30		3	27	30	
	Poor	3	5	8		3	5	8	
Survival	≤ 2 years	5	16	21	0.240	3	18	21	1.000
	> 2 years	2	20	22		3	19	22	

*, $p < 0.05$.

cal parameters of 50 HCC patients. ARK2 and EZH2 intensity in normal and tumor liver tissues were not significant associated with any clinicopathological parameters: i.e., tumor size, grade, stage, survival, HBV infection, HCV infection, virus infection, and cirrhosis. We then restricted the analysis to ARK2 and EZH2 intensity in tumor tissues and found EZH2 but not ARK2 intensity in tumor tissues associated with survival ($P = 0.015$; **Table 4**). **Table 5** shows the relationships between G9a and SUV39H2 intensity and clinicopathological parameters in 50 HCC patients; SUV39H2 intensity was associated with HCV infection ($P = 0.025$); G9 intensity was not linked with any clinicopathological parameters: e.g., tumor size, grade, stage, survival, virus infection, and cirrhosis. If we further restricted the analysis to G9a and SUV39H2 intensity in tumors, we found SUV39H2 but not G9a intensity associated with tumor stage ($P = 0.044$; **Table 6**).

Discussion

In 2012, HCC ranked as second leading cause of cancer-related deaths in Taiwan (Department of Health, Executive Yuan). The hepatitis B vaccination program launched in 1984 has successfully reduced HCC incidence in children, yet incidence and mortality in the general Taiwanese population remain high (overall 5-year survival rate was 15% during 1987-1992) and lack diagnostic markers for early detection [30-32]. Histones originally believed

to function as a scaffold for DNA packing, have now emerged in post-translational modifications that regulated chromatin condensation and DNA accessibility. Histone modifications affect chromatin structure further gene expression, and play a vital role in establishment of gene silencing during tumorigenesis [33]. N-terminal tails of histone proteins are subject to a wide range of covalent modification at many sites. Over 60 residues on histones are modified; the most studied are acety-

lation and methylation of specific lysine residues on histone H3 and H4 [34]. Kondo et al. showed that multiple epigenetic silencing mechanisms inappropriately active in HCC cells and mRNAs of histone methyltransferases-*EZH2* and *G9a* (but not *SUV39H1*) higher in malignant liver tissues of 23 HCC patients of Japan [25]. Suggesting histone methylations contribute to gene silencing in HCC. In this report, we have studied four groups of histone modifying genes including lysine acetylation, lysine methylation, arginine methylation, serine/threonine phosphorylation. This is the first report discussing expressions of 24 histone-modifying genes in hepatocarcinogenesis.

To assess histone-modifying genes from mRNA to protein levels, both mRNAs and proteins were measured in tissue pairs by RT-qPCR and tissue microarray-based immunohistochemistry (TMA-based IHC) staining. Since RT-qPCR accurately monitors mRNA expressions and immunohistochemistry staining allowed us to generate staining profiles for various antibodies specific to the same fixation and staining procedures for 40 cores normal and tumor tissues from HCC patients on the same slide. Although immunohistochemistry staining is essential for diagnostic pathology, it exhibits inconsistency of quality assurance in data interpretation. Despite widespread use of immunohistochemistry, significant problems remain with regard to variability in tissue fixation, processing, staining methodologies and

reagents, and interpretation of staining results persists between different slides [35]. Combining RT-qPCR and immunohistochemistry staining in this study, we monitor histone-modifying gene's mRNA expression and its' coding protein level to glean more information to interpret our data.

Besides the well-known alteration of histone acetylation patterns, cancer cells also display widespread changes in histone phosphorylation and methylation patterns [24, 36-39]. We selected genes that showed more than eight-fold overexpression in tumor tissues for further immunohistochemistry staining: histone phosphorylation protein (ARK2) and methylation proteins (G9a, EZH2 and SUV39H2). We previously showed that overexpression of histone methylation- and phosphorylation-related proteins-ARK2, G9a, EZH2 (but not SUV39H2) associated with prognosis of oral squamous cell carcinoma in 215 male of Taiwan [40]. ARK2 may serve as both an effective prognostic factor and a biomarker for predicting various clinical outcomes of oral cancer [40]. In the present report, our data shows all mRNAs of 24 histone-modifying genes increasing in tumor tissues, suggesting that histone modification (includes acetylation of lysine, methylation of lysine, methylation of arginine, and phosphorylation of serine/threonine) plays a lead role in HCC carcinogenesis. Within these 24 genes, mRNAs of *G9a*, *SUV39H2*, *EZH2*, and *ARK2* were more than eightfold overexpressed in tumor tissues. In spite of mRNAs expression, we found proteins of ARK2, EZH2, G9a, and SUV39H2 consistently up-regulated in tumors of 50 HCC patients.

ARK2 (a kinase for H3S10) overexpression proves associated with stages of malignant progression in thyroid carcinomas and with poor prognosis in endometrial carcinomas [13, 41]. Lin et al. showed that *ARK2* mRNA frequent overexpressed in 160 Taiwanese HCC cases, which can be used as an important molecular marker of early recurrence and poor prognosis [18]. In addition, they suggest that ARK2 selected inhibitors-AZD1152-HQPA and VE-465 had anticancer effects in HCC cells [18, 19]. Sorafenib (Nexavar®) is an orally active multikinase inhibitor approved in Europe for treatment of HCC, showing greater survival duration in combination therapy with doxorubi-

cin in advanced HCC [42]. We found mRNA and protein of *ARK2* were both overexpressed in 50 HCCs, yet staining intensity of ARK2 not correlated with any clinicopathologic characteristics. Possible reasons of this variation may be differences in selected antibody, sample size, genetic background, and/or risk factors between selected populations.

The polycomb group protein-EZH2 functions as a histone methyltransferase for H3K27 (histone H3 lysine 27) as catalytic subunit of polycomb repressive complex 2 (PRC2) and mediates transcription silencing [43]. Increased expression of EZH2 is frequently detected in HCC tissues and it is associated with the aggressiveness and/or poor prognosis [44-46]. Knockdown of EZH2 expression in HCC cells could reverse tumorigenicity in mice, which suggests that EZH2 inhibition has therapeutic value in HCC [47]. In addition, EZH2 expression, as examined by IHC, has been correlated with aggressiveness and poor prognosis of HCC in the China population, indicating EZH2 as a diagnostic biomarker for HCC [23]. Our results prove EZH2 intensity of tumor tissues also associated with survival in the 50 HCC patients, such that EZH2 might serve as an efficient prognostic biomarker.

Histone H3K9 di-methylation (H3K9me₂) and tri-methylation (H3K9me₃) can be mediated by histone methyltransferase (HMT)-G9a and SUV39H1, respectively. G9a promotes lung cancer cell invasion and may play an early role in metastasis cascade [48]. Nuclear intensity of G9a protein correlates with reduced overall survival and disease-free interval in lung cancer [48]. Kondo et al. showed mRNA expressions of *EZH2* and *G9a* but not *SUV39H1* were higher in cancerous tissues of 23 HCC patients in Japan [25]. We found both mRNA and protein expressions of ARK2, EZH2, G9a, and SUV39H2 in tumor tissues significantly enhanced, suggesting these histone methylation- and phosphorylation-related genes involves in hepatocarcinogenesis. But G9a protein intensity from immunohistochemistry staining shows no link with clinicopathological parameters in 50 HCC cases. On the other hand, SUV39H2 intensity correlated with HCV infection and tumor stage (the intensity in tumor tissues). Ours is the first report to link SUV39H2 expression with HCV infection and tumor stage in HCC cases.

This article focuses on markers of histone methylation and phosphorylation proteins: ARK2, G9a, EZH2, and SUV39H2. Findings suggest EZH2 and SUV39H2 as highly correlated with survival and tumor stage, respectively, and may yield important biomarkers that predict patient survival and prognosis in HCC patients of Taiwan.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jan-Gowth Chang, Epigenome Research Center, China Medical University Hospital, 2 Yude Road, Taichung 40447, Taiwan. Tel: +886-4-22052121 ext. 2008; Fax: +886-4-22037690; E-mail: d6781@mail.cmuh.org.tw

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