Molecular alterations of the *NF2* gene in hepatocellular carcinoma and intrahepatic cholangiocarcinoma

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Received April 14, 2017; Accepted October 2, 2017

DOI: 10.3892/or.2017.6055

Abstract. Neurofibromatosis type 2 with mutations in the neurofibromin 2 (*NF2*) gene, encoding the Merlin protein, is an autosomal dominant disorder characterized by enhanced cancer predisposition, particularly tumors of the central nervous system. Recent animal studies indicate that disruption of *NF2*/Merlin function in oval cells, which are hepatic progenitor cells, may lead to the development of primary liver cancers including hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC); however, its role in human primary liver cancer remains unclear. In the present study, we explored the role of *NF2*/Merlin in human primary liver cancers. Tumor tissues (n=144) were used for the screening of *NF2* mutation, while whole blood samples from 219 HCC and 194 healthy control cases were used for analysis of single nucleotide polymorphisms (SNPs) in liver cancer. The expression and amplification of *NF2*/Merlin and its downstream gene in the Hippo pathway, Yes-associated protein (YAP), were also analyzed. Missense *NF2* mutations were identified in 2 of 106 (1.9%) HCCs and 2 of 38 (5.3%) ICCs. Allele frequency of *NF2* IVS4-39 A/A was significantly higher in the HCCs than that in the healthy controls. Noteworthy, *NF2*/Merlin showed a dual role as a tumorigenic gene and tumor-suppressor gene; Merlin was expressed at higher levels in tumors than in adjacent non-tumor tissues of HCC; while the rate of Merlin upregulation was significantly lower in poorly differentiated ICCs. In addition, a significant negative correlation between Merlin and YAP expression was observed in ICC. In conclusion, we provide initial evidence of human primary liver cancers characterized by molecular alterations of *NF2*/Merlin and the involvement of the Hippo pathway in the pathogenesis of human liver cancer.

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Key words: primary liver cancer, *NF2*/Merlin, YAP, genetic alteration, expression

Introduction

Primary liver cancer originates in the liver and comprises mainly hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), which account for approximately 90% and 5-15% of liver cancer cases, respectively (1). Hepatitis B virus (HBV), HCV, and aflatoxins have been identified as major causal factors that act individually and synergistically in the development of liver cancer (2,3). Other genetic factors such as genetic polymorphisms or gene mutations in tumor-associated genes may also play important roles in the development of liver cancer (4).

Neurofibromatosis type 2 is a devastating autosomal dominant disorder that includes schwannomas, meningiomas, and ependymomas (5). Neurofibromatosis type 2 with mutations in the neurofibromin 2 (*NF2)* gene is characterized by enhanced cancer predisposition. The *NF2* gene encodes Merlin, a cytoskeletal protein that also functions as a microtubule stabilizing protein (6). *NF2* mutations have been identified in the majority of sporadic and NF2-associated schwannomas (7). *NF2* is a regulator of the Hippo pathway, which controls organ size and regulates cell proliferation, motility, survival, and signaling pathways. Genetic mutations in *NF2* are frequently identified in tumors of the central nervous system; however, their presence in cancers of other organs has seldom been reported (8).

Recently, an animal study reported that liver-specific deletion of the *NF2* tumor-suppressor gene in the developing or adult mouse results in the progressive expansion of hepatic

progenitor/oval cells throughout the liver without affecting differentiated hepatocytes (8). All surviving mice eventually developed both cholangiocellular and hepatocellular carcinomas, suggesting that $Nf2^{-/-}$ progenitors can be a cell of origin for these tumors (8). Drvarov *et al* also proposed that the development of primary liver cancer may be directly regulated by the *NF2* gene (9). Although these studies indicated that loss of function of the *NF2* gene may be associated with the development of liver cancers, its role in human primary liver cancer remains unclear (8-11).

To explore the role of *NF2* in human liver carcinogenesis, we investigated the effects of molecular alterations of *NF2*/Merlin in human primary liver cancer. In addition, we analyzed the potential association of *NF2*/Merlin with Yes-associated protein (YAP), another Hippo pathway gene downstream of *NF2*, in the development of primary liver cancer.

Materials and methods

Tissue samples and cell line. A total of 144 patients with primary liver cancer were enrolled in this study, including 106 HCCs and 38 ICCs. The HCC patients included 92 men (86.8%) and 14 women (13.2%) aged 22-80 years with a mean age of 51.6 years, while the ICC patients included 31 men (81.6%) and 7 women (18.4%) aged 30-81 years with a mean age of 54 years. The patients underwent surgical treatment at the Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Department of Hepatology, Tianjin Infectious Disease Specialty Hospital and the Minimally Invasive Hepatobiliary Cancer Center, Beijing You-an Hospital between May 2011 and December 2015. All tumor samples were fixed in buffered formalin and embedded in paraffin; five matched pairs of frozen tumors were also included in the analysis (Table I).

Table I. Clinicopathological parameters of investigated cases.

Subjects	PLC	HCC	ICC	P-value
No. of cases	144	106	38	-
Age (years)				
Mean	52.2	51.6	54.0	-
Range	22-81	22-80	30-81	
Sex				
Male	123	92	31	0.30
Female	21	14	7	
Tumor stage				
Stage I	72	55	17	0.29
>Stage I	72	51	21	
HBV DNA				
Positive	115	93	22	<0.001
Negative	29	13	16	
Differentiation				
Well	22	18	4	0.54
Moderate	73	54	19	
Poor	49	34	15	

PLC, primary liver cancer; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

Two hundreds and nineteen whole blood samples from patients with HCC and 194 whole blood samples, from healthy controls from the Beijing Friendship Hospital and Tianjin Infectious Disease Specialty Hospital, were enrolled to analyze the genetic susceptibility of the *NF2* single nucleotide polymorphism (SNP).

All patients provided informed consent and approval for the use of their clinical materials for research purposes. The study protocol was approved by the Clinical Research Ethics Committee of Beijing Friendship Hospital. The HCC cell line HepG2 was purchased from the National Platform of Experimental Cell Resource for Sci-Tech (Beijing, China).

Single-stranded conformational polymorphism (SSCP) analysis and direct DNA sequencing for NF2 mutations. SSCP analysis was performed to prescreen for mutations in the *NF2* gene. Primers for *NF2* were designed with the online software Primer3 (Table II). Extraction of genomic DNA from paraffin sections and PCR-SSCP analysis for *NF2* were performed as described previously (12,13). Genomic DNA of the whole blood was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Electrophoresis was performed using apparatus DYCZ-20E (Beijing Liu-Yi Biotechnology, Beijing, China) at 45 W for 4.5-5.5 h at room temperature with cooling by fan. Gels were silver stained as previously described (12,13). Samples exhibiting mobility shifts on SSCP analysis were subsequently re-amplified with the same primers as used for SSCP, and the PCR products were sequenced using a Big Dye Terminator cycle sequencing kit (ABI Prism; Applied Biosystems, Foster City, CA, USA) in an ABI 3730 DNA sequencer (Applied Biosystems). Mutations identified were verified by sequencing a second product of amplification on both strands. PolyChen-2 software (http://genetics.bwh.harvard.edu) was used to predict the functional consequence of the identified individual missense mutations.

Immunohistochemistry (IHC). Sections (4-μm) were cut for IHC. After deparaffinization of the slides, antigen retrieval was performed in antigen unmasking solution (Vector H-3300) with microwaving for 15 min, keeping the solution boiling, and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min followed by treatment with 5% skimmed milk in phosphate buffered saline (PBS)-0.1% bovine serum albumin for at least 1 h at room temperature to block nonspecific staining.

Immunohistochemical staining was performed using antibodies against Merlin (Santa Cruz Biotechnology, sc331) and YAP (Abcam, ab52771) at 4˚C overnight. Secondary antibody (Vector, MP-7401) was used at 37˚C for 1 h, and visualization of antigen-antibody reactions was achieved with 3,3'-diaminobenzidine (Vector, SK-4100). Tissue structures were visualized by counterstaining with hematoxylin.

IHC scoring for Merlin and YAP was based on the strength and distribution of staining by two independent observers blinded to the clinical data (14). The IHC reaction was scored by multiplying the percentage of positive tumor cells (PP: 1, <10% positive tumor cells; 2, 10-49%; 3, ≥50% positive

Table II. Primers for SSCP and direct DNA sequencing for the *NF2* gene.

Exon	Sequence		Product size (bp)
	Forward	Reverse	
1	GGTCCCGGGCCTGAGC	CTCGACTGTCACCGCAGCAG	179
2	TCCCCATTGGTTTGTTATTG	AGCCCCACCAGTTTCATC	182
3	CACAGGAGGAAGTGCCAATA	GGGGTAGCCTTGACTGATG	199
4	GTGAGGCCATCTGTTGTG	TTAACGCCCAGGAAAAATAC	205
SNP43608 A>C[a]			
5	CTCTCCCTTTCTTCTTTCC	GGTTAGCTTTCTTTTAGACCA	154
6	TAAAAGTGGCAAACAATACC	GCCCATAAAGGAATGTAAAC	191
7	GACAGTGTCTTCCGTTCTCC	GGCCCTCACTCAGTCTCTG	158
8	GGCGCTTACAGTAGCTGTTCTT	CACACATGTCCTACCTCCTTGTC	184
9	GGCTGTCGGACTGAAACTGT	GCGCCAAGTGAGATACCATT	150
10	AACCTTTTTGTCTGCTTCTG	CCAGGACTGACCACACAG	194
11	TCGAGCCCTGTGATTCAATGACT	AACCCCAGCCCCTCAGAAAT	190
12-1	ATCTGGGCGGGAGAACAG	ATTTCCTGCTCAGCCTCTGC	162
12-2	CCGAGGAGGAGGCAAAACTTC	CAGCCTCCTCGCCAGTCTG	207
13	CCCTCTTCTGTGAAGCTGACA	CCGGGAGGAAAGAGAACATC	208
14	CCAAGCTCCTAATCCGAAAT	GGCACAGGGGGCTACATA	180
15	TGATGCATGATACCCTCTTG	GAGACCCTGGGTACCTTTTTA	204
16	CCCTCTCAGCTTCTTCTCTGC	CCCTATGGATGGCTCTCTTGA	170
SNP 8240 G>C	AGAATATTCGCCGTGTGTCC	TGAGTGGCTACAGTGGCAGT	227
SNP 99632 C>T	TGCAATTGCCTTGAACTACG	GCTTCTCAGGGCTTCAGTGT	228

SSCP, single-stranded conformational polymorphism; SNP, single nucleotide polymorphisms. [a]Same primers as exon 4.

tumor cells) by their prevalent degree of staining (SI: 0, negative; 1, weak; 2, strong staining). Immunoreactive scores (IRS = PP x SI) ranging from 3 to 6 were defined as IHC++, whereas IRS value <3 was defined as IHC 0 to +. Cases with Merlin IHC level ++ were defined as having increased Merlin expression. Cases with YAP IHC level ++ were defined as having increased YAP expression (14). When there was a discordance between the observers, a pathologic peer review was undertaken to determine a consensus score.

Western blot analysis. Western blot analysis was performed as previously described (14,15). Briefly, frozen tissues or cell cultures were lysed and clarified by centrifugation. The protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). Each protein extract (80 μg) was loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed with the primary antibody against Merlin (Santa Cruz Biotechnology, sc331), with β-actin (Sigma, St. Louis, MO, USA) as the loading control. The membrane was then incubated with species-specific secondary horseradish peroxidase-conjugated antibodies (Sigma). Protein bands were revealed by chemiluminescence (Pierce) and detected on X-ray film (Kodak, Rochester, NY, USA).

Real-time PCR for YAP amplification. For the detection of *YAP* gene amplification, real-time PCR was performed with the *Homo sapiens* actin, H *(HACTIN)* sequence as the reference. The sequences of primers were as follows: 5'-GTTTGGA TGATGGATGCCATT-3' (forward) and 5'-ATGCTGTGAC ATGAAGCATCTGA-3' (reverse) for *YAP* and 5'-GCAAAG ACCTGTACGCCAACA-3' (forward) and 5'-TGCATCCTG TCGGCAATG-3' (reverse) for *HACTIN*. qPCR was performed using the SYBR® Premix Ex Taq™ in an ABI PRISM 7500 detection system according to the manufacturer's instructions. For the reactions, annealing was carried out at 65˚C, and 40 cycles of amplification were performed. Data were normalized using *HACTIN* as a reference gene. The relative levels of expression of the target gene among the different samples were calculated accordingly.

The gene copy numbers of the samples were calculated according to the following formula: ΔCt = [Ct (target) - Ct (reference)] and ΔΔCt = [ΔCt(tumor) - ΔCt (normal)]. The relative gene copy numbers were calculated using the expression 2 x $2^{-\Delta\Delta Ct}$, with a ΔΔCt ratio 2 x $2^{-\Delta\Delta Ct}$ >2.88 considered to indicate amplification (16).

Statistical analysis. The Chi-square test (for expected values >5) and the Fisher's exact test (for expected values ≤5) were used to determine the molecular associations using SAS v9.2 software (SAS Institute, Inc., Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant result for all tests.

Table III. Identified *NF2* missense mutations in liver cancer cases and the allele frequency of SNP IVS4-39 A/A.

NF2 missense mutations			
Case no.	Age/Sex	Tumor type	*NF2* mutation
310	50/M	HCC	Codon 319, CAG→CAC, Gln→His
353	43/M	HCC	Codon 339, CTC→TTC, Leu→Phe
276	47/M	ICC	Codon 355, GAG→AAG, Glu→Lys
312	61/M	ICC	Codon 562, CAC→CGC, His→Arg

SNP IVS4-39				
Alleles	HCC	Health controls	χ^2	P-value
C/C+A/C	146 (66.67%)	154 (79.38%)	7.74	0.005401
A/A	73 (33.33%)	40 (20.62%)		

HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; SNP, single nucleotide polymorphism.

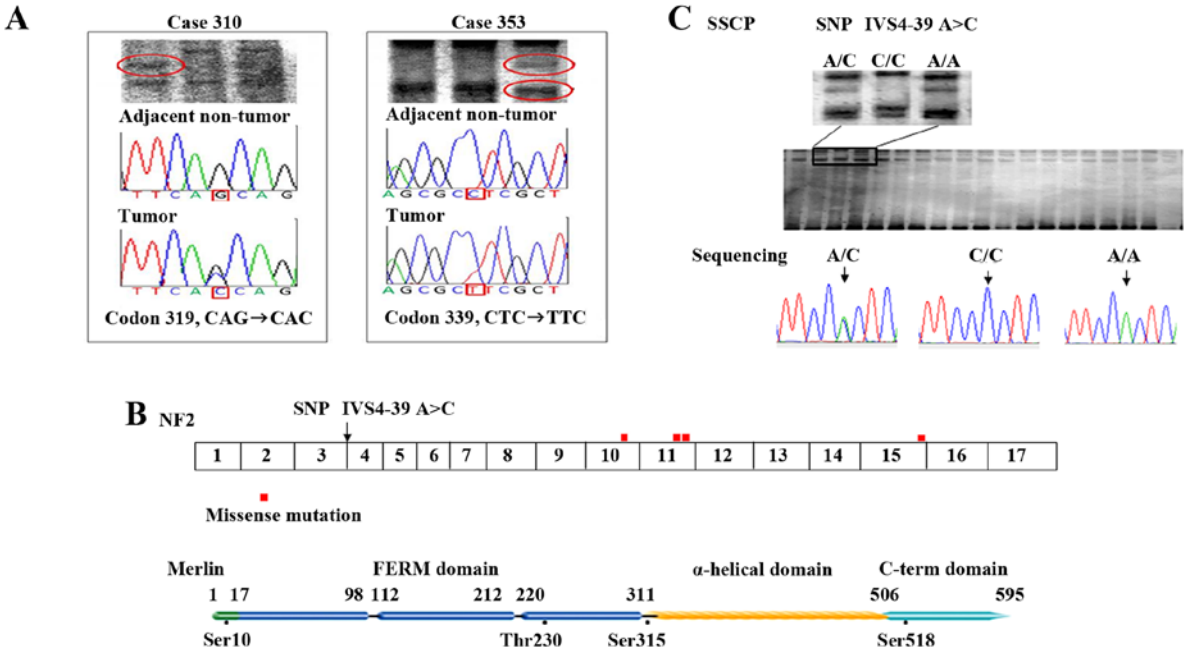

Figure 1. *NF2* missense mutations identified in primary liver cancer cases and allele frequency of *NF2* IVS4-39 A/A. (A) Representative SSCP and direct sequencing of the *NF2* gene in HCC. Right panel, mutation at codon 319 (CAG→CAC, Gln→His) in an HCC case (case 310). Left panel, mutation at codon 339 (CTC→TTC, Leu→Phe) in an HCC case (case 353). The reverse complement sequence is given. The mutations were not identified in the corresponding adjacent non-tumor tissue, indicating that the mutations are somatic. (B) Distribution of *NF2* mutations in the primary liver cancer cases. Mutation are located preferentially in exon 10 to 11 (the joint part of FERM domain and α-helical domain), but also in other functional domains (close to phosphorylation site, Ser518/Ser315). (C) SSCP analysis and direct sequencing of SNP IVS4-39 A>C, with three patterns of bands identified by SSCP electrophoresis, corresponding to genotype A/C, C/C, and A/A. HCC, hepatocellular carcinoma; SSCP, single-stranded conformational polymorphism; SNP, single nucleotide polymorphism.

Results

NF2 mutations and single nucleotide polymorphisms in HCC and ICC. A total of 144 cases of primary liver cancer, including 106 cases of HCC and 38 cases of ICC, were screened for *NF2* mutations. Since *NF2* mutations have been identified in exons 1-15, but less identified in exons 16-17, and due to the very large size of exon 17 (17), we screened only mutation in exon 1-16 of the *NF2* gene.

SSCP followed by direct sequencing of the 16 exons of *NF2* revealed a total of four missense *NF2* point mutations in 2 of 106 (1.9%) HCCs and 2 of 38 (5.3%) ICCs. (Table III, Fig. 1A), distributed among exons 10, 11, and 15 (Fig. 1B), including one G→C, one C→T, one A→G, and one G→A transition (Table III). In addition, two splicing variants and one synonymous mutation: i.e. IVS7+36 G→C, IVS16+24 C→T and E89E (GAA→GAG) were identified in three ICC cases, respectively. As predicted by PolyChen-2 software, the

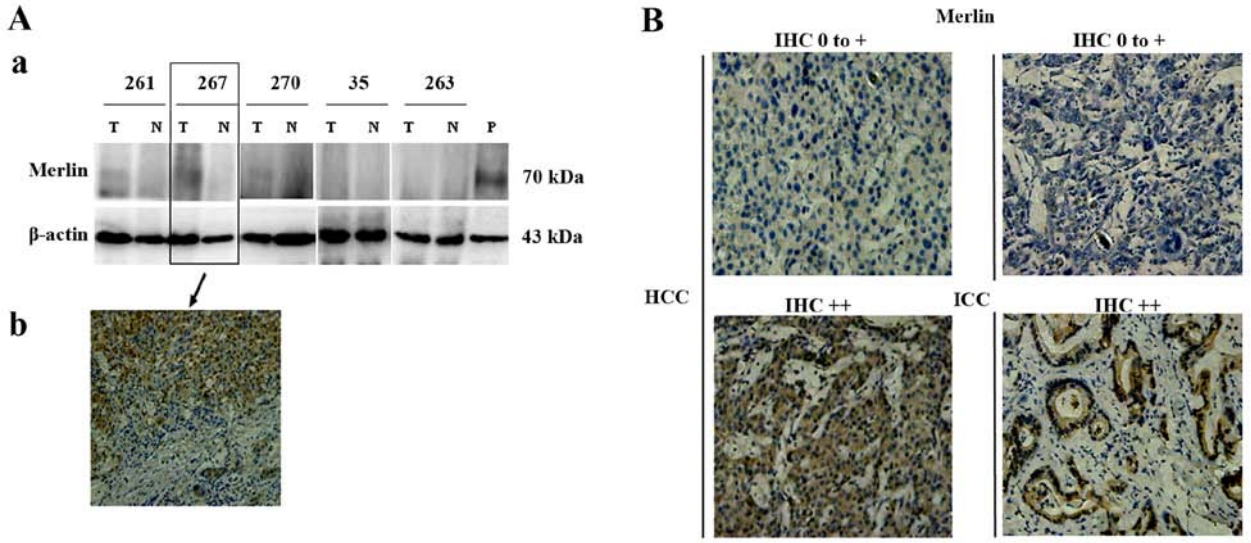

Figure 2. Merlin protein expression in primary liver cancer cases. (A) Merlin protein expression in tumor (T) and adjacent non-tumor tissue (N) of representative HCC cases. (a) Five pairs of tumor cases were subjected to western blot analysis to determine Merlin expression; (b) representative IHC of Merlin expression in tumor and adjacent non-tumor tissue of one HCC case (case 267). Original magnification, x10. (B) Representative IHC of Merlin protein expression. Left panels, IHC staining of Merlin in HCC; right panels, IHC staining of Merlin in ICC. Merlin expression level of IHC ++ was defined as increased Merlin expression. Original magnification, x20. HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

mutation Q319H and H562R were predicted to be probably damaging, while the L339F and E355K were predicted to be benign mutations but with minor impairments of function with scores of 0.441 and 0,031, respectively.

Three specific SNPs of the *NF2* gene were chosen for analysis based on their intragenic location, the validation status in ethnically diverse population, and a preliminary analysis showing their polymorphic nature in the Chinese population (18,19). There were no significant differences between HCC and healthy controls in the rare allele frequency of the SNPs 8240 G>C (promoter region, G/G: 74.18% vs. 76.65%, P=0.66) and 99632 C>T (3' non-coding region, C/C: 10.00% vs. 13.57%, P=0.33), except IVS4-39 A>C identified by the present study, of which the allele (A/A) frequency was significantly higher in tumors than in controls (Table III and Fig. 1C).

Merlin expression is increased in tumor tissues compared with that in adjacent non-tumor tissues of HCC. Of the 144 cases of primary liver cancer with paraffin sections, 131 (94 HCCs and 37 ICCs) were available for analysis of Merlin IHC expression. In the 94 cases of HCC and 37 cases of ICC, adjacent non-tumor tissue was available for 80 HCCs and 31 ICCs. The IHC analysis showed that 26 of 80 HCCs or 10 of 31 ICCs (32.5 or 32.3%) had increased Merlin expression in tumors compared with that in adjacent tissues. In 49 of 80 HCCs or 21 of 31 ICCs (61.3 or 67.7%), the expression levels of Merlin were low in both tumors and adjacent tissues. None of the ICCs showed increased Merlin expression in adjacent non-tumor tissues compared with that in tumor tissues, and increased Merlin expression was not observed in both adjacent non-tumor tissues and tumor tissues of the ICCs. However, the results showed that the rate of increased Merlin expression was significant higher in tumor tissues than in adjacent non-tumor tissues of HCC (P=0.013, Table IV), suggesting that Merlin may play a role as an oncogene in HCC development.

Table IV. Merlin expression in tumor and adjacent non-tumor tissue in HCC.

	Non-tumor (IHC ++)	Non-tumor (IHC 0-+)	Total	χ^2	P-value
Tumor (IHC ++)	9	17	26	6.16	0.013
Tumor (IHC 0-+)	5	49	54		
Total	14	66	80		

HCC, hepatocellular carcinoma.

Western blot analysis of five pairs of representative HCC cases with available IHC data (cases 261, 267, and 270, IHC ++; case 35 and 263, IHC 0-+) showed consistent results with those of IHC regarding Merlin expression, confirming that Merlin levels were higher in tumors than in adjacent non-tumor tissues of HCC (Fig. 2A).

Increased Merlin expression is associated with the degree of differentiation of tumor cells. Merlin expression was mainly localized in the cytoplasm of tumor cells (Fig. 2B), and upregulated in 20.2% (19/94) of the HCCs and 45.9% (17/37) of the ICCs. There was no correlation between Merlin expression and the clinicopathological characteristics of HCC (Table V). However, of the 37 ICC cases, 3 (13.6%), 7 (31.8%), and 12 (54.5%) with IHC scores of 3-6, 1-2, and 0, respectively, had well and medium differentiated tumors, whereas 9 (60.0%), 1 (6.7%), and 5 (33.3%) cases with IHC scores of 3-6, 1-2, and 0, respectively, had poorly differentiated ICCs, indicating that low Merlin expression was associated with a worse degree of differentiation of tumor cells (P=0.009; Table V). These results indicated that alterations in Merlin expression are associated

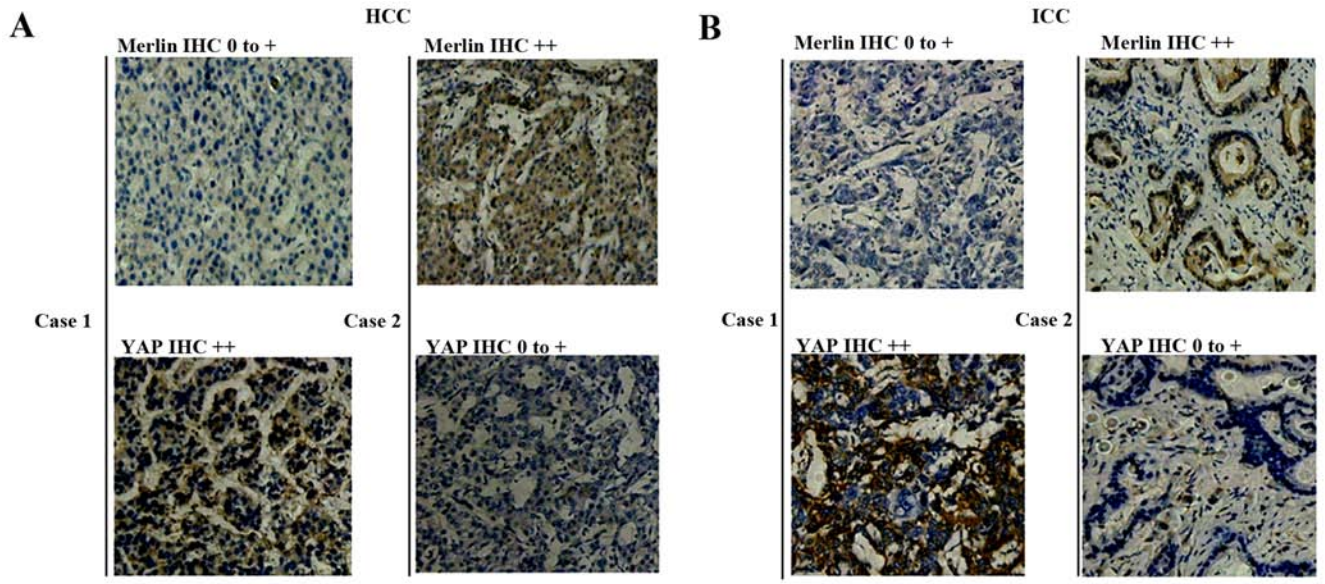

Figure 3. Association between Merlin and YAP protein expression in primary liver cancer cases. (A) Representative IHC of Merlin and YAP protein expression in HCC. Left panel, IHC staining of Merlin and YAP in a representative case (case 1) with Merlin expression level of IHC 0-+ but YAP expression level of IHC ++; right panel, IHC staining of Merlin and YAP in a representative case (case 2) with Merlin expression level of IHC ++ but YAP expression level of IHC 0-+. (B) Representative IHC of Merlin and YAP protein expression in ICC. Left panel, IHC staining of Merlin and YAP in a representative case (case 1) with Merlin expression level of IHC 0-+ but YAP expression level of IHC ++; right panel, IHC staining of Merlin and YAP in a representative case (case 2) with Merlin expression level of IHC ++ but YAP expression level of IHC 0-+. Original magnification, x20. HCC, hepatocellular carcinoma.

with the degree of malignancy of ICC cells and that Merlin may play a tumor-suppressor role.

Merlin expression is significantly negatively correlated with YAP expression in tumor cells. YAP, which acts downstream of *NF2*/Merlin, was analyzed by IHC and gene amplification to examine the association between the two genes. Similar to Merlin, the YAP protein was expressed predominantly in the cytoplasm of tumor cells (Fig. 3A and B). YAP was upregulated in 48 of the 94 HCC cases (51.1%) and in 27 of the 37 ICC cases (73.0%) analyzed. No correlation was observed between Merlin and YAP expression in HCCs, although a tendency towards a negative correlation was observed (P=0.165, Table VI, Fig. 3A). However, a significant negative correlation was observed between Merlin and YAP expression in ICC (P=0.016, Table VI, Fig. 3B), suggesting that they may play antagonistic roles in tumorigenesis.

Of the 94 cases of HCC and 37 cases of ICC, *YAP* amplification was found in 33.0% (31/94) of the HCCs and 32.4% (12/37) of the ICCs. No significant correlation was observed between *NF2* mutations and *YAP* amplification in HCCs and ICCs.

Discussion

The Hippo signaling pathway, also known as the Salvador/Warts/Hippo (SWH) pathway, controls organ size in animals through regulation of cell proliferation and apoptosis (6,8). The involvement of the Hippo pathway in organ size determination and tumor suppression has been confirmed in genetically engineered mouse models. The Hippo pathway regulates growth by phosphorylating and inactivating YAP, an evolutionarily conserved transcriptional coactivator (8). *NF2* is the only Hippo pathway gene that is mutationally inactivated in cancer, and YAP is a major downstream effector of the *NF2* tumor suppressor with a role in mammalian growth control. The Hippo pathway functions in the proliferation, differentiation, and self-renewal of tissue-specific stem cells (8).

NF2 is involved in cytoskeleton development and is a microtubule stabilizer (6). As a Hippo pathway gene involved in the regulation of cell proliferation, motility, survival, and signaling pathways, genetic mutations in *NF2* have been detected in tumors of the central nervous system. However, *NF2* mutations have seldom been reported in tumors of other organs (7). Recent animal studies have shown that the disruption of *NF2* function in hepatic progenitor/oval cells may lead to the development of primary liver cancers including HCC and ICC (8). The diverse roles of *NF2*/Merlin in the pathogenesis of cancer have been described.

NF2 gene mutations have been identified in the majority of sporadic and *NF2*-associated schwannomas (20-23). Neurofibromatosis type 2 is a tumor-prone disorder characterized by the development of multiple schwannomas and meningiomas. Affected individuals inevitably develop schwannomas, typically affecting both vestibular nerves leading to hearing loss and deafness. The frequency of the *NF2* gene mutation is approximately 71% (12/17 cases), 24% (21/28 cases), and 41.7% (5/12 cases) in sporadic schwannomas, meningiomas, and ependymomas, respectively (21-23).

Recent studies have revealed that mutation in the *NF2* gene is also present in other human cancers, such as renal cell carcinoma, breast carcinoma, malignant mesotheliomas, lung carcinoma, colorectal carcinomas, and prostate carcinomas (23-26). These studies suggested that *NF2* mutations may contribute to tumorigenesis in multiple human cancers. In a study by Yoo *et al*, one *NF2* mutation was detected in 45 HCC samples (11), whereas Kanai *et al* did not detect any mutations in six exons of the *NF2* gene (10). These previous studies were confined to HCC (10,11). The present study is the

Table V. Association between Merlin IHC expression and clinical parameters in HCC and ICC.

Tumor type	Parameters	No.	Merlin IHC scoring 0 (n=12) n (%)	1-2 (n=8) n (%)	3-6 (n=17) n (%)	χ^2	P-value
HCC	Tumor stage						
	TNM I	54	2 (3.7)	42 (77.8)	10 (18.5)	0.356	0.837
	>TNM I	40	2 (5.0)	29 (72.5)	9 (22.5)		
	HBV infection[a]						
	Positive	80	4 (5.0)	61 (76.3)	15 (18.8)	0.504	0.777
	Negative	9	0 (0)	7 (77.8)	2 (22.2)		
	Differentiation						
	Well+moderate	61	3 (4.9)	46 (75.4)	12 (19.7)	0.205	0.903
	Poor	33	1 (3.0)	25 (75.8)	7 (21.2)		
ICC	Tumor stage						
	TNM I	16	5 (31.3)	4 (25.0)	7 (43.7)	0.191	0.909
	>TNM I	21	7 (33.3)	4 (19.1)	10 (47.6)		
	HBV infection[b]						
	Positive	20	7 (35.0)	5 (25.0)	8 (40.0)	0.944	0.624
	Negative	16	4 (25.0)	3 (18.8)	9 (56.3)		
	Differentiation						
	Well+moderate	22	3 (13.6)	7 (31.8)	12 (54.5)	9.394	0.009
	Poor	15	9 (60.0)	1 (6.7)	5 (33.3)		

[a]Five cases with HCV infection were not included, [b]one case with HCV infection was not included. HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

Table VI. Association of Merlin and YAP protein expression in HCC and ICC.

Tumor type	Merlin/Yap expression	YAP IHC ++	YAP IHC 0-+	Total	χ^2	P-value
HCC	Merlin IHC ++	7	12	19	1.93	0.165
	Merlin IHC 0-+	41	34	75		
	Total	48	46	94		
ICC	Merlin IHC ++	9	8	17		0.011[a]
	Merlin IHC 0-+	18	2	20		
	Total	27	10	37		

[a]Fisher exact test. HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

first to examine the association between *NF2* mutations and the development of ICC. Since the *NF2* mutation rate in the population is estimated to be 6.5×10^{-6} (27), our findings indicated that *NF2* mutations may be associated with the pathogenesis of primary liver cancer ($P<0.05$), especially ICC. In addition, our results suggested that the genotype of *NF2* IVS4-39 A/A may be a potential risk factor for development of HCC in the Chinese population. The *NF2* splicing variant IVS4-39 A>C was close to the 5' end of exon 4 in FERM domain, and may have functional consequence on the splicing of *NF2* mRNA. However, further functional study of the variant would be helpful to elucidate the exact role of the variant.

Similar to ezrin-radixin-moesin (ERM) proteins, Merlin is a critical component that provides a regulated linkage between membrane proteins and the cortical cytoskeleton, in addition to its involvement in signal-transduction pathways (28,29). The main functional domains of Merlin are the FERM domain, α-helical domain, and C-terminal domain,

which contains an F-actin binding site, and the protein contains several critical phosphorylation sites, such as Ser10, Ser315, Ser518, and Thr230. Merlin can switch between open and closed conformations, which determine its activity and are regulated by changes in phosphorylation status (28,29). Similar to other ERM proteins, the function of Merlin is modulated by the association between the FERM domain and C-terminal domain. Its head-to-tail folding results in a closed active conformation upon dephosphorylation of Ser518 in the C-terminal domain, while in the open conformation upon phosphorylation of Ser518, Merlin is inactive and incapable of acting as a tumor-suppressor protein (30). The *NF2* mutations identified in liver cancer are located mainly in the FERM and C-terminal domains. In particular, mutation in Ser567, which is proximal to the Ser518 phosphorylation site, may affect the phosphorylation status of Ser518, as well as the association between the FERM domain and C-terminal domain. The *NF2* mutations identified in the present study may play a significant roles in the pathogenesis of primary liver cancer.

Both the upregulation and downregulation of Merlin in tumor tissues have been reported in different types of cancer (20,31,32). The results of the present study suggest that the *NF2* gene may play a dual role as an oncogene and tumor-suppressor gene, as reported previously in other cancers (33,34). Chen *et al* reported that Merlin could be overexpressed in some ovarian cancer (OVCA) cell lines and OVCA ascites cells, suggesting that Merlin could be an oncoprotein rather than a tumor-suppressor protein in certain OVCA cells, and that the functional duality of Merlin might represent a paradigm in proteome complexity and is especially important in investigating multifactorial diseases such as cancer (33). Similarly, it has been reported that the activator protein-1 (AP-1) protein acts as either an oncogene or an anti-oncogene by regulating genes involved in cell proliferation, differentiation, apoptosis, angiogenesis and tumor invasion depending on the cell type, cellular condition (e.g., differentiation stages), tumor stage and genetic background of the tumor (34). It has a double-edged activity; it can be anti-oncogenic by inducing apoptosis and it can be oncogenic by signaling cell survival (34). Thus, we deduced that Merlin may act as an oncogene in the development of HCC with a similar mechanism. Further functional study of Merlin in HCC development would be helpful to elucidate the underlying mechanism.

Recent reseach has shown that YAP is a key driver of tumorigenesis and tissue overgrowth caused by loss of *NF2* in the murine liver (35). Using conditional knockout mice, Zhang *et al* demonstrated that the *NF2*/Merlin tumor suppressor and the YAP oncoprotein function antagonistically to regulate liver development. While inactivation of YAP led to loss of hepatocytes and biliary epithelial cells, inactivation of Nf2 led to hepatocellular carcinoma and bile duct carcinoma. The suppression of *NF2* mutant liver phenotypes by loss of YAP suggest that YAP is a major effector of the *NF2* tumor suppressor in mammalian growth control (35). Consistently, the present study identified a significant negative correlation between Merlin and YAP expression in ICC, suggesting that they may function in an antagonistic manner in tumorigenesis.

In summary, the present study provides initial evidence of the involvement of *NF2*/Merlin in HCC and ICC, which could be of value to improve our understanding of the molecular pathogenesis of primary liver cancer.

Acknowledgements

The authors would like to thank Dr Xiao-Yan Shi (Department of Pathology, Beijing Friendship Hospital, Capital Medical University) for her critical pathological review. This study was supported by a grant from the Beijing Natural Science Foundation (no. 7132058), and a grant from the Nature Science Foundation of China (no. 81650014).

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