Table 1 continued

Study	Peginterferon type and dose	Ribavirin	Duration, weeks[a]	Patients, *N*	Male, *N*	Age, years (mean)	BW, kg (mean)	Ethnic group, *N*				HCV genotype, *N*			
								Caucasian	African	Asian	Other	G1	G2	G3	G4–6
Ferenci [43]	2a 180 μg	1000–1200 mg	48	95	65	44	NR	NR	NR	NR	NR	95	0	0	0
Ferenci [44]	2a 180 μg	400 mg, fixed	24	141	87	36.2	73.1	NR	NR	NR	NR	0	19	122	0
	2a 180 μg	800 mg, fixed	24	141	84	36.8	71.1	NR	NR	NR	NR	0	18	123	0
Ferenci [45]	2a 180 μg	1000–1200 mg	72[a]	150	98	44.3	76.9	NR	NR	NR	NR	134	0	0	16
	2a 180 μg	1000–1200 mg	48	139	90	45.1	78.5	NR	NR	NR	NR	127	0	0	12
Fried [4]	2a 180 μg	1000–1200 mg	48	453	324	42.8	79.8	372	27	28	26	298	54	86	13
Fried [46]	2a 180 μg	1200 mg, fixed	48	46	37	47.1	98.4	32	4	NR	10	46	0	0	0
	2a 180 μg	1600 mg, fixed	48	47	41	49.6	100.3	29	6	NR	12	47	0	0	0
	2a 270 μg	1200 mg, fixed	48	47	35	47.1	101	35	4	NR	8	47	0	0	0
	2a 270 μg	1600 mg, fixed	48	47	37	48.5	97	32	5	NR	10	47	0	0	0
Gish [47]	2a 180 μg	1000–1200 mg	24–48[a]	45	32	49	80	38	NR	NR	NR	NR	NR	NR	NR
Glue [48]	2b 0.35 μg/kg	600–800 mg	24	12	NR	39.8	65.6	NR	NR	NR	NR	9	NR	NR	NR
	2b 0.7 μg/kg	600–1200 mg	24	18	NR	39.8	65.6	NR	NR	NR	NR	5	NR	NR	NR
	2b 1.4 μg/kg	600–1200 mg	24	18	NR	39.8	65.6	NR	NR	NR	NR	4	NR	NR	NR
Hadziyannis [6]	2a 180 μg	1000–1200 mg	24	280	185	42	77.1	254	9	16	1	118	53	91	NR
	2a 180 μg	1000–1200 mg	48	436	287	43	77.3	394	11	26	5	271	66	87	NR
	2a 180 μg	800 mg, fixed	48	361	226	42.6	77	315	11	31	4	250	46	53	NR
	2a 180 μg	800 mg, fixed	24	207	140	41.2	78.3	183	7	14	3	101	39	57	NR
Hasan [49]	2b 1.5 μg/kg	1000–1200 mg	48	21	16	NR	NR	0	19	2	0	4	0	0	17
Helbling [50]	2a 180 μg	600–800 mg	48	60	36	47[b]	73	NR	NR	NR	NR	25	7	24	3
	2a 180 μg	1000–1200 mg	48	64	45	47[b]	74	NR	NR	NR	NR	30	11	18	4
Herrine [51]	2a 180 μg	800–1000 mg	48	32	24	48	NR	NR	NR	NR	NR	25	NR	NR	NR
Hezode [52]	2a 180 μg	1000–1200 mg	48	82	46	45[b]	NR	76	2	4	0	82	0	0	0
Ide [53]	2b 1.5 μg/kg	600–1000 mg	48	56	26	55.3	NR	0	0	56	0	56	0	0	0
	2b 1.5 μg/kg	600–1000 mg	48–68[a]	57	30	54.6	NR	0	0	57	0	57	0	0	0
Jacobson [54]	2b 1.0 μg/kg	1000–1200 mg	48	161	122	49.2	NR	118	23	NR	20	145	NR	NR	NR
	2b 1.5 μg/kg	800 mg, fixed	48	160	117	49.8	NR	106	27	NR	27	141	NR	NR	NR
Jacobson [55]	2b 1.5 μg/kg	800 mg, fixed	24–48[a]	2444	1560	45.8	83.8	1926	237	59	222	1506	526	386	23
	2b 1.5 μg/kg	800–1400 mg	24–48[a]	2469	1539	45.8	84	1993	208	59	209	1512	499	421	33
Jensen [56]	2a 180 μg	1000–1200 mg	48	313	212	48.5	80.9	282	25	NR	6	284	1	8	19
	2a 180 μg	1000–1200 mg	72	156	107	49.4	81.2	137	17	NR	2	142	1	5	8
	2a 360 μg	1000–1200 mg	48[a]	156	94	48.8	81.1	141	10	NR	5	142	3	4	5
	2a 360 μg	1000–1200 mg	72[a]	317	203	48.1	81.5	279	29	NR	9	288	2	7	19

Table 1 continued

Study	Peginterferon type and dose	Ribavirin	Duration, weeks[a]	Patients, *N*	Male, *N*	Age, years (mean)	BW, kg (mean)	Ethnic group, *N*				HCV genotype, *N*			
								Caucasian	African	Asian	Other	G1	G2	G3	G4–6
Kamal [57]	2b 1.5 μg/kg	1000–1200 mg	24	95	49	41.6	NR	NR	NR	NR	NR	0	0	0	95
	2b 1.5 μg/kg	1000–1200 mg	36	96	51	43.9	NR	NR	NR	NR	NR	0	0	0	96
	2b 1.5 μg/kg	1000–1200 mg	48	96	50	41.2	NR	NR	NR	NR	NR	0	0	0	96
Kamal [58]	2b 1.5 μg/kg	10.6 mg/kg	24[a]	69	37	41[b]	NR	NR	NR	NR	NR	69	0	0	0
	2b 1.5 μg/kg	10.6 mg/kg	36[a]	79	32	40.5[b]	NR	NR	NR	NR	NR	79	0	0	0
	2b 1.5 μg/kg	10.6 mg/kg	48[a]	160	100	42.2	NR	NR	NR	NR	NR	160	0	0	0
	2b 1.5 μg/kg	10.6 mg/kg	48	50	26	43.2	NR	NR	NR	NR	NR	50	0	0	0
Kawaoka [59]	2b 1.0 μg/kg	600–1000 mg	24	26	9	57[b]	53	0	0	26	0	0	26	0	0
	2b 1.5 μg/kg	600–1000 mg	24	27	15	55[b]	61	0	0	27	0	0	27	0	0
Khattab [60]	2b 1.5 μg/kg	800–1400 mg	48	49	34	37	NR	0	49	0	0	0	0	0	49
Kuboki [61]	2a 180 μg	600–1000 mg	48	100	74	52[b]	66.7	0	0	100	0	85	15	0	0
	2a 180 μg	600–1000 mg	48	99	62	52	62.8	0	0	99	0	99	0	0	0
Lagging [62]	2a 180 μg	800 mg, fixed	12	194	123	41.5	79.8	NR	NR	NR	NR	0	55	137	0
	2a 180 μg	800 mg, fixed	24	188	105	42	76.5	NR	NR	NR	NR	0	49	139	0
Langlet [63]	2a 180 μg	1000–1200 mg	24–48[a]	314	173	45.4	73.1	278	24	7	5	166	NR	NR	49
Lee [64]	2b 1.5 μg/kg	1000–1200 mg	24	76	53	44.6	67.8	0	0	76	0	38	38	0	0
Liu [65]	2a 180 μg	1000–1200 mg	24	154	88	54	67.6	0	0	154	0	154	0	0	0
	2a 180 μg	1000–1200 mg	48	154	87	53	65.8	0	0	154	0	154	0	0	0
Lodato [66]	2b 1.0–1.5 μg/kg	10.6 mg/kg	24–48[a]	43	23	49.6	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2b 1.5 μg/kg	10.6 mg/kg	24–48[a]	22	12	48.7	NR	NR	NR	NR	NR	NR	NR	NR	NR
Mangia [7]	2b 1.0 μg/kg	1000–1200 mg	12	133	NR	NR	NR	NR	NR	NR	NR	0	102	31	0
	2b 1.0 μg/kg	1000–1200 mg	24	80	NR	NR	NR	NR	NR	NR	NR	0	58	22	0
	2b 1.0 μg/kg	1000–1200 mg	24	70	39	49.7	69.5	NR	NR	NR	NR	0	53	17	0
Manns [3]	2b 1.5 μg/kg	1000–1200 mg	48[a]	514	346	44	83	NR	NR	NR	NR	349	NR	NR	12
	2b 1.5 μg/kg	800 mg, fixed	48	511	321	43	82	NR	NR	NR	NR	348	NR	NR	16
Marcellin [67]	2b 1.5 μg/kg	800–1200 mg	24[a]	10	8	51.4	75.8	10	0	0	0	10	0	0	0
Marcellin [68]	2a 180 μg	1000–1200 mg	24–48[a]	318	201	45.1	NR	256	17	11	34	212	47	47	NR
McHutchison [15]	2a 180 μg [15]	1000–1200 mg	48	1035	613	47.6	82.8	733	200	20	82	1035	0	0	0
	2b 1.0 μg/kg	800–1400 mg	48	1016	607	47.5	83.4	724	187	21	84	1016	0	0	0
	2b 1.5 μg/kg	800–1400 mg	48	1019	613	47.5	84	732	183	10	94	1019	0	0	0
McHutchison [69]	2a 180 μg	1000–1200 mg	48	114	76	50[b]	NR	100	10	2	2	114	0	0	0
Mecenate [70]	2a 180 μg	1000–1200 mg	12[a]	72	NR	42[b]	NR	NR	NR	NR	NR	0	NR	NR	0
	2a 180 μg	1000–1200 mg	24[a]	67	54	45[b]	NR	NR	NR	NR	NR	0	37	30	0
	2a 180 μg	1000–1200 mg	24[a]	71	NR	42[b]	NR	NR	NR	NR	NR	0	NR	NR	0

Table 1 continued

Study	Peginterferon type and dose	Ribavirin	Duration, weeks[a]	Patients, *N*	Male, *N*	Age, years (mean)	BW, kg (mean)	Ethnic group, *N*				HCV genotype, *N*			
								Caucasian	African	Asian	Other	G1	G2	G3	G4–6
Mendez-Navarro [71]	2a 180 μg	1000–1200 mg	48	63	26	46.2	70.4	0	0	0	63	63	0	0	0
Meyer-Wyss [72]	2b 1.0 μg/kg	800 mg, fixed	24–48[a]	113	64	39[b]	72	NR	NR	NR	NR	49	14	41	9
	2b 1.5 μg/kg	800 mg, fixed	24–48[a]	106	76	42[b]	73	NR	NR	NR	NR	64	10	26	6
Napoli [73]	2b 1.5 μg/kg	800–1200 mg	48[a]	14	10	46.9	NR	NR	NR	NR	NR	14	0	0	0
	2b 1.5 μg/kg	800–1200 mg	48	17	11	47.3	NR	NR	NR	NR	NR	17	0	0	0
Pearlman [74]	2b 1.5 μg/kg	800–1400 mg	48	49	23	56[b]	NR	NR	23	NR	26	49	0	0	0
	2b 1.5 μg/kg	800–1400 mg	72	52	34	54[b]	NR	NR	25	NR	27	52	0	0	0
Roberts [75]	2a 180 μg	1000–1200 mg	48	438	285	43.3	78.7	365	55	0	1	436	0	0	0
	2a 360 μg	1000–1200 mg	48[a]	433	298	43.6	77.3	355	61	0	2	432	0	0	0
Roffi [76]	2b 1.0 μg/kg	1000–1200 mg	48	57	36	56[b]	75	NR	NR	NR	NR	33	15	9	0
Rossignol [77]	2a 180 μg	1000–1200 mg	48	40	36	39	NR	NR	NR	NR	NR	0	0	0	40
Rumi [16]	2a 180 μg	1000–1200 mg	24–48[a]	212	128	51.6	72.2	NR	NR	NR	NR	91	69	34	18
	2b 1.5 μg/kg	1000–1200 mg	24–48[a]	219	120	52.8	68.9	NR	NR	NR	NR	87	74	32	26
Rustgi [78]	2a 180 μg	1000–1200 mg	24–48[a]	117	81	50	89.7	79	26	0	12	117	0	0	0
Sanchez-Tapias [79]	2a 180 μg	800 mg, fixed	48	165	113	42.8	73.3	NR	NR	NR	NR	149	1	7	8
	2a 180 μg	800 mg, fixed	72	161	102	43.2	74.4	NR	NR	NR	NR	142	1	8	8
	2a 180 μg	800 mg, fixed	24	148	88	39.3	67.9	NR	NR	NR	NR	45	18	75	10
	2a 180 μg	800 mg, fixed	48	36	20	42.4	68.7	NR	NR	NR	NR	35	0	0	1
Scotto [17]	2a 180 μg	15 mg/kg	48	71	42	45.8	80.7	NR	NR	NR	NR	45	6	8	12
	2b 1.5 μg/kg	15 mg/kg	48	72	40	47.8	78.9	NR	NR	NR	NR	47	5	9	11
Shiffman [80]	2b 1.5 μg/kg	800–1400 mg	48	48	27	49[b]	82	NR	17	NR	31	48	0	0	0
Shiffman [81]	2a 180 μg	800 mg, fixed	16	732	448	46	81.5	635	22	21	54	NR	372	358	NR
	2a 180 μg	800 mg, fixed	24	731	461	45.6	81.6	638	21	18	54	NR	356	369	NR
Shiffman [82]	2a 180 μg	1000–1200 mg	48	936	673	50	NR	693	157	NR	NR	936	0	0	0
Sjogren [83]	2b 1.5 μg/kg	1000–1200 mg	48	29	19	46[b]	82	17	9	2	1	29	0	0	0
Sood [84]	2b 1.0 μg/kg	1000–1200 mg	24	76	67	43.1	NR	0	0	76	0	0	0	76	0
	2b 1.5 μg/kg	1000–1200 mg	24	27	21	37.3	NR	0	0	27	0	0	0	27	0
Tang [85]	2a 180 μg	1000–1200 mg	20[a]	11	4	42[b]	70	8	NR	NR	3	11	0	0	0
	2a 180 μg	1000–1200 mg	32[a]	10	5	38[b]	69	10	0	0	0	10	0	0	0
	2a 180 μg	1000–1200 mg	44[a]	11	8	41[b]	79	10	NR	NR	1	11	0	0	0
	2a 180 μg	1000–1200 mg	48[a]	13	9	41[b]	71	11	NR	NR	2	13	0	0	0
Toyoda [86]	2b 1.5 μg/kg	600–1000 mg	8[a]	15	5	53.6	NR	0	0	15	0	0	15	0	0
	2b 1.5 μg/kg	600–1000 mg	24[a]	28	16	57.8	62.2	0	0	28	0	0	28	0	0
	2b 1.5 μg/kg	600–1000 mg	24[a]	17	7	NR	NR	0	0	17	0	0	17	0	0

Table 1 continued

Study	Peginterferon type and dose	Ribavirin	Duration, weeks[a]	Patients, *N*	Male, *N*	Age, years (mean)	BW, kg (mean)	Ethnic group, *N*				HCV genotype, *N*			
								Caucasian	African	Asian	Other	G1	G2	G3	G4–6
Wagner [87]	2a 180 μg	800–1200 mg	16	71	52	38	75.3	NR	NR	NR	NR	0	19	51	0
	2a 180 μg	800–1200 mg	24	71	41	39	74.6	NR	NR	NR	NR	0	19	52	0
	2a 180 μg	800–1200 mg	24	11	4	42	80.1	NR	NR	NR	NR	0	1	10	0
Wagner [88]	2a 180 μg	1000–1200 mg	48	352	183	45.4	74	344	0	0	8	352	0	0	0
Yenice [18]	2a 180 μg	800–1200 mg	48	37	13	49.9	NR	NR	NR	NR	NR	37	0	0	0
	2b 1.5 μg/kg	800–1200 mg	48	37	10	50.8	NR	NR	NR	NR	NR	37	0	0	0
Yu [89]	2b 80–100 μg	1000–1200 mg	24[a]	45	28	45.4	68.3	0	0	45	0	45	0	0	0
	2b 80–100 μg	1000–1200 mg	48[a]	15	11	45.1	68.6	0	0	15	0	15	0	0	0
Yu [90]	2a 180 μg	1000–1200 mg	16	50	32	50.8	67.7	0	0	50	0	0	50	0	0
	2a 180 μg	1000–1200 mg	24	100	58	49.9	65.8	0	0	100	0	0	100	0	0
Yu [91]	2a 180 μg	1000–1200 mg	24	100	57	49.7	65.5	0	0	100	0	100	0	0	0
	2a 180 μg	1000–1200 mg	48	100	58	49.1	67.5	0	0	100	0	100	0	0	0
Zeuzem [92]	2a 180 μg	800 mg, fixed	24	212	90	43.8	73.9	183	17	5	7	144	38	20	10
	2a 180 μg	800 mg, fixed	48	210	82	43.9	73.7	180	20	4	6	141	41	18	10
Zeuzem [93]	2a 180 μg	1000–1200 mg	24[a]	43	33	39.1	74.7	NR	NR	NR	NR	23	NR	NR	1
	2a 180 μg	1000–1200 mg	48	134	83	43.2	73.8	NR	NR	NR	NR	90	NR	NR	6
	2a 360 μg	1000–1200 mg	48	11	6	42.6	79	NR	NR	NR	NR	9	NR	NR	2
Zeuzem [94]	2b 1.5 μg/kg	800–1200 mg	24	237	127	42.2	71.3	225	NR	NR	NR	237	0	0	0
Zeuzem [95]	2a 180 μg	1000–1200 mg	48	114	66	41.9	73.4	105	NR	NR	9	114	0	0	0

HCV hepatitis C virus, *BW* body weight, *NR* not reported

[a] Details of pegylated (Peg) interferon dose and duration of treatment are described in Supplementary Table 1

[b] Data values are expressed as medians

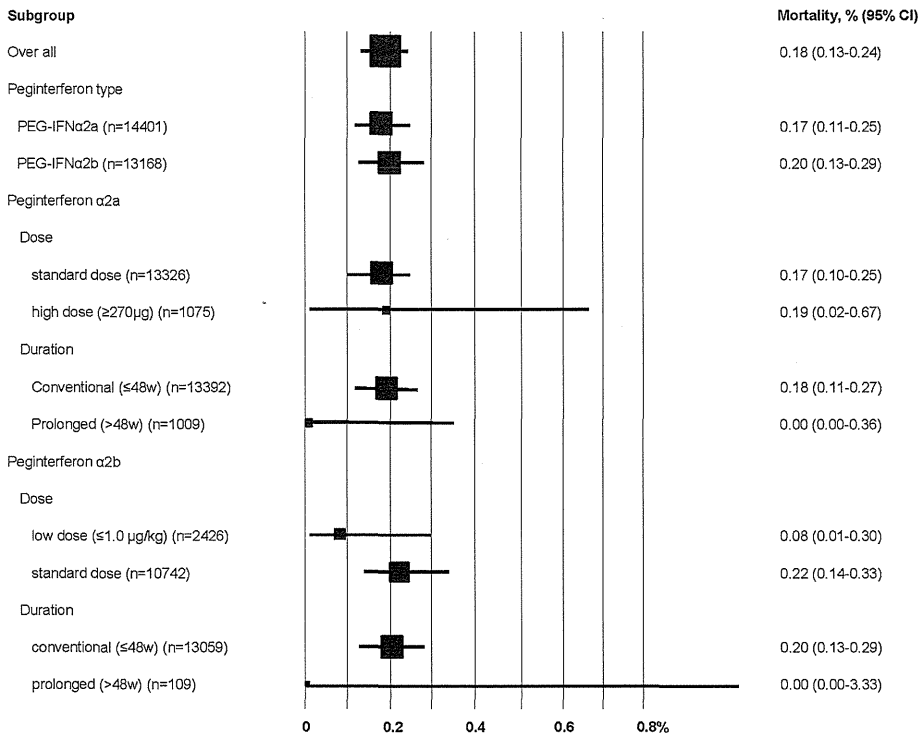

Fig. 2 Forest plot of mortality, comparing treatment regimens. Sizes of the *boxes* reflect sample sizes, with the *bars* showing the 95 % confidence interval (*CI*)

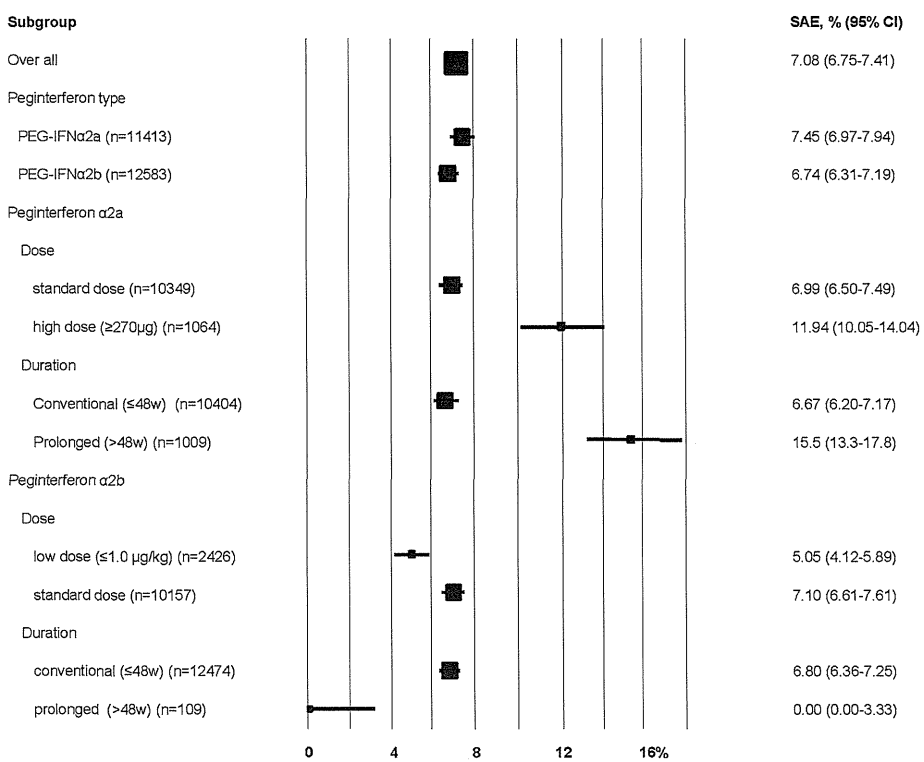

Fig. 3 Forest plot of SAEs, comparing treatment regimens. Sizes of the *boxes* reflect sample sizes, with the *bars* showing the 95 % confidence interval

Meta-regression analysis showed that greater body weight, an increased proportion of male patients, an increased proportion of HCV genotype 1, and an increased proportion of Caucasian patients and decreased proportion of Asian patients were significantly associated with increased SAE rates (Table 2). There was no significant

association between increased SAE rates and the mean patient age or the proportion of African patients.

Discussion

According to a report by the World Health Organization (WHO), age-specific annual death rates from all causes in individuals aged 45–49 years in the United States, Italy, and Japan (locations of the majority of our enrolled studies) were 409, 216, and 248 per 100000, respectively [11]. The crude mortality of 0.18 % (180 per 100000) found in the present study is low by comparison, even allowing for the biased population tolerable to PEG-IFN/RBV. Furthermore, the annual mortality rate could have been lower than the crude mortality rate, considering that the study period was longer than 1 year (including the follow-up period) in most enrolled studies. The annual treatment-related mortality rate could have been lower than our finding of a treatment-related mortality of 0.06 %. However, the treatment-related mortality rate may be an underestimate, as assessment of the causal relationship between treatment and mortality can be subjective and/or biased. Nonetheless, these PEG-IFN/RBV-related mortality rates would be acceptable considering the high SVR rates and considering that SVR drastically reduces adverse events related to chronic hepatitis C infection. In the present study, the most common cause of mortality was suicide, and all of the suicides were considered as treatment-related. This finding should alert treating physicians when they are treating patients with a history of psychiatric illness.

Two types of PEG-IFNs (i.e., PEG-IFN alpha-2a and 2b) are approved for the treatment of chronic hepatitis C. PEG-IFN alpha-2a has a molecular mass of 40 kDa and PEG-IFN alpha-2b a mass of 12 kDa. In comparison with PEG-IFN alpha-2b, PEG-IFN alpha-2a is less effectively

Table 2 Meta-regression analysis for continuous variables

Variables	Slope[a]	Standard error	*P* value
Mean age, per year increase			
All studies ($N = 100$)	−0.00244	0.00380	0.52
Alpha-2a ($N = 60$)	−0.00049	0.00513	0.93
Alpha-2b ($N = 40$)	−0.00203	0.00488	0.68
Mean body weight, per 1 kg increase			
All studies ($N = 95$)	0.00343	0.00147	0.02
Alpha-2a ($N = 64$)	0.00584	0.00242	0.02
Alpha-2b ($N = 31$)	0.00067	0.00178	0.71
Proportion of male patients, per 1 % increase			
All studies ($N = 125$)	0.00305	0.00130	0.02
Alpha-2a ($N = 73$)	0.00218	0.00182	0.23
Alpha-2b ($N = 52$)	0.00257	0.00166	0.13
Proportion of Caucasian patients, per 1 % increase			
All studies ($N = 75$)	0.00167	0.00043	<0.001
Alpha-2a ($N = 46$)	0.00102	0.00062	0.11
Alpha-2b ($N = 29$)	0.00201	0.00061	0.003
Proportion of African patients, per 1 % increase			
All studies ($N = 72$)	−0.00092	0.00113	0.42
Alpha-2a ($N = 41$)	0.00781	0.00394	0.55
Alpha-2b ($N = 31$)	−0.00030	0.00109	0.79
Proportion of Asian patients, per 1 % increase			
All studies ($N = 58$)	−0.00092	0.00042	0.03
Alpha-2a ($N = 32$)	−0.00042	0.00061	0.50
Alpha-2b ($N = 26$)	−0.00106	0.00053	0.06
Proportion of genotype 1 patients, per 1 % increase			
All studies ($N = 129$)	0.00143	0.00036	<0.001
Alpha-2a ($N = 73$)	0.00179	0.00048	<0.001
Alpha-2b ($N = 56$)	0.00075	0.00046	0.104

[a] Slope values indicate increases (decreases) in the rates of serious adverse events (SAEs) per unit. For example, a 1-year increase in mean age in a study results in 0.00464 (0.464 %) decrease in the SAE rate

cleared by the kidneys and therefore has a longer half-life. In fact, pharmacokinetic analysis in 22 patients showed that PEG-IFN alpha-2a was still detectable in 10 patients 168 h after the administration of 180 μg/week, whereas the administration of 1.0 μg/kg/week of PEG-IFN alpha-2b was undetectable in 11 of 12 patients at the same time point [12]. PEG-IFN alpha-2a is thought to be more effective than PEG-IFN alpha-2b because of its longer half-life. A recent meta-analysis showed a higher SVR rate after treatment with PEG-IFN alpha-2a than after treatment with PEG-IFN alpha-2b [13]. On the other hand, the half-life of each PEG-IFN may be related to its safety profile. However, among studies that have directly compared the safety of the two PEG-IFNs, only one reported a significant difference between SAE rates for PEG-IFN alpha-2a and PEG-IFN alpha-2b (11.7 vs. 8.6 %, $P = 0.02$) [14–18]. The inability of the other studies to detect such a difference may have been due to small sample sizes. In fact, a difference in SAE rates between the two PEG-IFNs was observed in pooled samples in our study.

Increasing the dose intensity of PEG-IFN and prolonging treatment duration have been attempted to achieve higher IFN levels in blood for longer periods, eventually resulting in a higher SVR rate. Treatment dose and duration are also expected to be related to the safety profile. The higher SAE rates in regimens with more intensive dosing observed for PEG-IFN alpha-2a and 2b and longer treatment duration observed for PEG-IFN alpha-2a support this hypothesis. The higher SAE rates in regimens with longer treatment duration were not observed for PEG-IFN alpha-2b, probably due to small sample sizes in regimens with longer treatment duration of PEG-IFN alpha-2b.

As mortality and SAE during PEG-IFN/RBV treatment are rare, most studies reported no such events. Therefore, the proportion calculated using the DerSimonian and Laird weight for the random-effect model showed considerable discrepancies between crude and pooled rates. In fact, pooled and treatment-related mortalities calculated using the random-effects (DerSimonian and Laird) model were 0.30 % (0.24–0.37 %) and 0.17 % (0.12–0.22 %), respectively, which were considerably different from the crude rates of each outcome (data not shown). Thus, we adopted crude instead of pooled rates for mortality and SAE.

Our meta-regression analysis showed a significant association between increased SAEs and HCV genotype 1. It is plausible that patients with genotype 1, which is difficult to treat, received a higher dose and longer duration of treatment. This is consistent with the results of the subgroup analysis.

A significant positive association between the SAE rate and the proportion of Caucasian patients, and an inverse relationship between SAEs and the proportion of Asian patients were also observed. This result may suggest a role of genetic diversity in the mechanisms underlying the adverse effects of PEG-IFN/RBV. Indeed, inosine triphosphate pyrophosphatase (*ITPA*) gene variants are associated with RBV-induced hemolytic anemia, and genetic polymorphisms near the interleukin-28B (*IL-28B*) gene were reported to be associated with response to HCV treatment with PEG-IFN and RBV, and the frequency of the variants differed between ethnic groups [19, 20].

We found that greater body weight was associated with a higher SAE rate. Of note, in the PEG-IFN alpha-2a-based regimen, the starting dose was fixed regardless of body weight; thus, with the PEG-IFN alpha-2a regimen, there might have been an overdose for patients of lower weight, leading to SAEs. However, whether such overdosing occurred was not clear in this study because there was a positive correlation between body weight and the SAE rate in patients receiving the PEG-IFN alpha-2a regimen. The reason for this positive relationship remains unclear; however, it may be because obesity is itself associated with various medical comorbidities.

We also found that an increased proportion of male patients in a study was associated with a higher SAE rate. It has been reported that female gender was an independent factor contributing to severe anemia [21], so the reason for the present finding of the increased proportion of male patients remains unclear; it may be correlated with increased body weight which caused a higher SAE rate. However, whether the proportions of individuals with obesity differed between male and female patients is not clear, because data on body mass index was often lacking.

In the present study increased mean age was not associated with a higher SAE rate, whereas discontinuation, dose reduction, and grade 3 adverse events were more frequent in older patients in previous studies [22, 23]. The lack of an association between mean age and the SAE rate in the present study could be due in part to the patients' mean age of 45.9 years, and the proportion of patients over 60 being small. Low-risk patients tend to be included in RCTs. This is one of the limitations of this study.

Recently, the use of HCV nonstructural 3/4A serine protease inhibitors combined with PEG-IFN and RBV were reported to achieve higher SVR rates in genotype 1 patients compared with conventional PEG-IFN/RBV. These triple therapies are considered to be the next standard of care for chronic hepatitis C [24, 25]. Adverse events during triple therapies could include those related to PEG-IFN/RBV, as these regimens include PEG-IFN/RBV.

We extracted only RCTs for our analysis in order to obtain highly reliable data and minimize the influence of recall bias because RCTs are prospectively designed, and SAEs should be defined a priori. However, several limitations are still worth noting. The latent limitation of this study is inter-study variability in the definition of SAE. The

precise meaning of 'serious' has not been determined, and some discrepancies between studies exist. These discrepancies may diminish the accuracy of the pooled SAE rate in this study. Second, even by choosing only RCTs, we could not completely exclude the influence of publication bias.

Overall, PEG-IFN/RBV treatment is relatively safe, with low mortality, considering the fact that chronic hepatitis C patients carry a high risk of cirrhosis and HCC. Nevertheless, the SAE rate with this treatment is not negligible and the development of safer regimens should be, and is, encouraged.

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Table 1. Characteristics of the Randomized Cohorts and SVR Rates of Heterozygous Genotype rs12979860CT With Additional Genotyping of rs8099917

Random Sample Size	Sample Number	Mean Age ±SD	Male	HCV RNA ≥400.000 IU/mL	Severe Fibrosis	SVR		P-value
						rs12979680CT/ rs8099917TT	rs12979680CT/ rs8099917TG	
10%	96	47±11	58%	69%	55%	48%	36%	0.408
20%	192	48±11	59%	80%	43%	43%	32%	0.379
30%	295	48±11	60%	72%	48%	50%	38%	0.154
40%	396	47±11	63%	66%	55%	57%	39%	**0.012**
50%	474	47±11	60%	68%	53%	56%	37%	**0.003**
60%	588	48±11	58%	71%	52%	57%	35%	**0.0001**
70%	654	47±11	58%	72%	52%	56%	39%	**0.002**
80%	754	48±11	58%	70%	51%	55%	39%	**0.002**
90%	835	48±11	59%	71%	52%	56%	40%	**0.001**
100%	942	48±11	59%	70%	52%	55%	40%	**0.001**

SD, standard deviation; IU, international units; SVR, sustained virological response; $P < 0.05$ considered to be statistically significant.

fibrosis stage on the SVR rates of genotype rs12979860CT/rs8099917TT and rs12979860CT/rs8099917TG (Supporting Table 1). Again, it becomes obvious that the impact of additional genotyping of rs8099917 on the prediction of SVR is improved in patients with heterozygous genotype of rs12979860 who have high baseline HCV RNA levels ($P = 3.7 \times 10^{-5}$), HCV subtype 1a ($P = 3.3 \times 10^{-5}$), or severe fibrosis stages ($P = 0.001$), being female ($P = 0.023$), or of younger age ($P = 0.029$). Thus, the different patient characteristics most likely explain the differences in the SVR rates.

From that, one possibly may conclude that two SNPs are good in large cohorts but not relevant for clinical practice. However, the idea of large studies is to inform individual clinical practice. Our results derived from a large cohort suggest that algorithms and models that include both rs12979860 and rs809917 as well as baseline parameters and viral factors are informative to guide therapeutic decision making.[3]

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Plasma Lysophosphatidic Acid Levels and Hepatocellular Carcinoma

To the Editor:

We read with interest the article by Mazzocca et al.,[1] showing that serum lysophosphatidic acid (LPA) levels are increased in hepatocellular carcinoma (HCC) patients correlated with tumor burden, while not enhanced in cirrhosis patients. However, we think that their LPA values in serum samples need to be carefully evaluated, because of some technical issues in the measurement of LPA levels in blood samples. First, because LPA is released from platelets, LPA has been measured in plasma but not in serum when evaluating its clinical significance.[2,3] Second, as we previously demonstrated,[4] LPA levels in plasma samples are markedly increased after sample preparation unless the temperature is kept under strict control, potentially because the synthetic enzyme autotaxin (ATX) and the substrate lysophosphatidyl choline coexist in plasma samples to abundantly produce LPA. LPA was once reported as a biomarker of ovarian cancer,[2] but contrary data were later demonstrated, in which a distinct sampling of plasma may explain this discrepancy.[3] Indeed, LPA levels in serum reported by Mazzocca et al. were approximately 10 times higher than the previously reported LPA levels in plasma.[2,3] If their LPA values in serum were increased after sampling similarly in each sample, plasma LPA levels might be correlated with HCC burden as reported. To clarify this, we have newly measured plasma LPA levels in HCC patients,

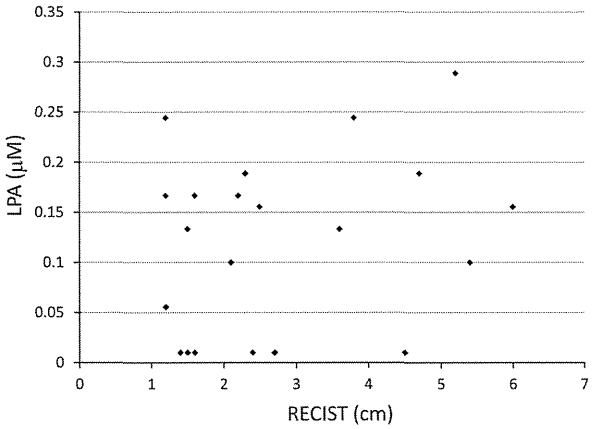

Fig. 1. Plasma LPA levels and HCC burden. Plasma LPA levels, measured in 21 HCC patients (13 males and 8 females; 2 patients with chronic hepatitis B, 15 with chronic hepatitis C, and 4 with non-B non-C chronic liver disease), were not significantly correlated with HCC burden as evaluated by RECIST (Response Evaluation Criteria in Solid Tumors; Spearman rank, $r = 0.158$, $P = 0.4937$). This study was approved by the Institutional Research Ethics Committee and informed consent was obtained for the use of the samples.

and found that they were not correlated with tumor burden, as shown in Fig. 1. Moreover, plasma LPA levels in HCC patients (0.12 ± 0.09 mM, mean $\pm$ SD, n = 21), were not different from the previously reported levels in non-HCC patients with chronic hepatitis C (0.10 ± 0.05 mM).[5] Although Mazzocca et al. reported no enhancement of serum LPA levels in cirrhosis patients, we[5] and others[6] previously showed that plasma LPA levels and serum ATX activity were increased in chronic liver diseases in association with fibrosis and cholestatic pruritus, from which HCC frequently arises. Collectively, a role of LPA in HCC should be cautiously analyzed.

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Potential conflict of interest: Nothing to report.

Reply:

Ikeda et al. remark that platelets are a main source of lysophosphatidic acid (LPA) and therefore the interpretation of LPA serum concentrations deserves careful attention. However, the same authors previously reported[1] an inverse correlation between plasma LPA concentrations and the number of platelets in patients with chronic C hepatitis. Therefore, it is possible that in physiologic conditions platelets remain the main source of LPA, while in chronic inflammation such as hepatitis C, liver cirrhosis, or hepatocellular carcinoma (HCC), the platelet contribution to LPA production may likely become less relevant. In our study we analyzed sera for LPA detection in healthy donors, liver cirrhosis, and HCC patients, performing well-standardized procedures of collection for each sample. Thus, the contribution of platelets to the LPA concentration was, in reality, normalized. On the contrary, the authors should consider that even in plasma or whole blood, platelet activation is an extremely difficult problem to deal with and control. For example, prolonged tourniquet application, or twisting of the needle in the vein, are major factors interfering with the function of platelets during blood withdrawal, as reviewed by Ruggeri.[2] Unfortunately, these limitations are common for a number of molecules involved both in cancer and in blood cell biology.[3]

Moreover, Ikeda et al. investigated patients with chronic hepatitis C, in whom the inflammatory response is a key component of the tissue microenvironment. In their study, the fibrotic status was also questionable, due to their choice of statistical method (comparison among groups should be done with Kruskal-Wallis tests), and because of the very limited number of patients (14), further stratified into four different groups, which means the conclusions were affected by low power.[1] In our study,[4] we compared liver cirrhosis versus HCC. In the former case, the inflammation is reduced while the fibrotic response is increased, consequently inducing a different microenvironment response.[5] This could explain why patients with liver cirrhosis display relatively low levels of LPA. In addition, it is conceivable that when HCC develops in cirrhotic liver, LPA levels rise once more, as in cases of active inflammatory states (i.e., viral hepatitis). Another key point is patient selection. Ikeda et al. do not provide any information with regard to the clinical features of the patients, i.e., etiology, BCLC stage, previous therapy, etc., as well as how they calculated the size of the tumor in patients with multifocal disease, for instance. Finally, some differences between Caucasian and Asian patients with HCC are to be expected, since the natural history is completely different in Western and Southeast Asian countries.[6] In our study,[4] we demonstrated that LPA has a role in promoting tumor progression and we did not attempt to speculate about the use of LPA as a clinical biomarker. To validate LPA as a potential biomarker for HCC a different study design is required, as well as first considering the power of the study. The enhancement of serum LPA levels reported by Watanabe et al.[1] referred to a relatively small number of patients with chronic hepatitis C. In addition, the

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MicroRNA-140 Acts as a Liver Tumor Suppressor by Controlling NF-κB Activity by Directly Targeting DNa Methyltransferase 1 (Dnmt1) Expression

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MicroRNAs (miRNAs) are small RNAs that regulate the expression of specific target genes. While deregulated miRNA expression levels have been detected in many tumors, whether miRNA functional impairment is also involved in carcinogenesis remains unknown. We investigated whether deregulation of miRNA machinery components and subsequent functional impairment of miRNAs are involved in hepatocarcinogenesis. Among miRNA-containing ribonucleoprotein complex components, reduced expression of DDX20 was frequently observed in human hepatocellular carcinomas, in which enhanced nuclear factor-κB (NF-κB) activity is believed to be closely linked to carcinogenesis. Because DDX20 normally suppresses NF-κB activity by preferentially regulating the function of the NF-κB-suppressing miRNA-140, we hypothesized that impairment of miRNA-140 function may be involved in hepatocarcinogenesis. DNA methyltransferase 1 (Dnmt1) was identified as a direct target of miRNA-140, and increased Dnmt1 expression in DDX20-deficient cells hypermethylated the promoters of metallothionein genes, resulting in decreased metallothionein expression leading to enhanced NF-κB activity. MiRNA-140-knockout mice were prone to hepatocarcinogenesis and had a phenotype similar to that of DDX20 deficiency, suggesting that miRNA-140 plays a central role in DDX20 deficiency-related pathogenesis. ***Conclusion*****: These results indicate that miRNA-140 acts as a liver tumor suppressor, and that impairment of miRNA-140 function due to a deficiency of DDX20, a miRNA machinery component, could lead to hepatocarcinogenesis. (HEPATOLOGY 2013;57:162-170)**

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide.[1] Although multiple major risk factors have been identified, such as infection with hepatitis viruses B or C, the molecular mechanisms underlying HCC development remain poorly understood, hindering the development of novel therapeutic approaches. Therefore, a better understanding of the molecular pathways involved in hepatocarcinogenesis is critical for the development of new therapeutic options.

Nuclear factor-κB (NF-κB) is one of the best-characterized intracellular signaling pathways. Its activation is a common feature of human HCC.[2-4] It acts as an inhibitor of apoptosis and as a tumor promoter[4,5] and is associated with the acquisition of a transformed phenotype during hepatocarcinogenesis.[6] In fact, studies using patient samples suggest that NF-κB activation in the liver leads to the development of HCC.[7] Although there are conflicting reports,[8] activation of the NF-κB pathway in the liver is crucial for the initiation and promotion of HCC.[4]

Abbreviations: DEN, diethylnitrosamine; Dnmt1, DNA methyltransferase 1; HCC, hepatocellular carcinoma; miRNA; microRNA; miRNP, miRNA-containing ribonucleoprotein; MT, metallothionein; NF-κB, nuclear factor-κB; RT-PCR, reverse-transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; UTR, untranslated region.

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MicroRNAs (miRNAs) are small RNA molecules that regulate the expression of target genes and are involved in various biological functions.[9-12] Although specific miRNAs can function as either suppressors or oncogenes in tumor development, a general reduction in miRNA expression is commonly observed in human cancers.[13-22] In this context, it can be hypothesized that deregulation of the machinery components involved in miRNA function may be related to the functional impairment of miRNAs and the pathogenesis of carcinogenesis.

In this study, we show that the expression of DDX20, an miRNA-containing ribonucleoprotein (miRNP) component, is frequently decreased in human HCC. Because DDX20 is required for both the preferential loading of miRNA-140 into the RNA-induced silencing complex and its function,[23] we hypothesized that DDX20 deficiency would lead to hepatocarcinogenesis via impaired miRNA-140 function. MiRNA-140 knockout mice were indeed more prone to hepatocarcinogenesis, and we identified a possible molecular pathway from DDX20 deficiency to liver cancer.

Materials and Methods

Mouse and Liver Tumor Induction. MiRNA-140$^{-/-}$ mice have been described.[24] Recombinant murine tumor necrosis factor-α (TNF-α) (25 μg/kg; Wako, Osaka, Japan) was injected into the tail vein, and the mice were sacrificed 1 hour later. To induce liver tumors, 15-day-old mice received an intraperitoneal injection of diethylnitrosamine (DEN) (25 mg/kg body weight), and were sacrificed 32 weeks later. All animal experiments were performed in compliance with the regulations of the Animal Use Committee of the University of Tokyo and the Institute for Adult Disease, Asahi Life Foundation.

Plasmids. FLAG-tagged human DDX20-expressing plasmids were as described.[23] The pGL3-based reporter plasmid containing Dnmt1 3′ untranslated region (UTR) sequences was provided by G. Marucucci.[25]

Detailed Materials and Methods. The detailed experimental procedures of clinical samples, cells, plasmids, reporter assays, reverse-transcription polymerase chain reaction (RT-PCR) analysis, antibodies, western blotting, cell assays, immunohistochemistry, microarray analysis, methylation analysis, and electrophoretic mobility-shift assay are described in the Supporting Information.

Statistical Analysis. Statistically significant differences between groups were determined using a Wilcoxon rank-sum test. A Wilcoxon signed-rank test was used for statistical comparisons of protein expression levels between HCC and surrounding noncancerous tissues.

Table 1. Cases with Differential Expression Levels of miRNP Components in HCC (n = 10)

Gene ID	Gene Symbol	Decreased	Increased	No Change
23405	Dicer1	2	1	7
27161	EIF2C2 (AGO2)	1	1	8
6895	TARBP2(TRBP2)	2	0	8
11218	DDX20 (GEMIN3)	8	0	2
50628	GEMIN4	1	0	9

The expression levels of each miRNP component were determined via immunohistochemistry.

The numbers indicate the number of cases that had the differential expression levels (decreased, increased, or no change) in HCC tissues compared with those in surrounding liver tissues.

Results

DDX20 Expression Is Frequently Decreased in HCC. The expression levels of proteins reported to be miRNP components (Dicer, Ago2, TRBP2, DDX20 [also known as Gemin3], and Gemin4)[26] were initially determined via immunohistochemistry in HCC and noncancerous background liver tissues from 10 patients. DDX20 expression was lower in HCC tissue compared with the surrounding noncancerous tissue in 8 of 10 cases, whereas expression of the other genes was unchanged (Table 1 and Supporting Fig. 1). Therefore, and because DDX20 was recently identified as a possible liver tumor suppressor in mice,[27] we determined its role as a human HCC suppressor.

DDX20 protein expression was lower in several HCC cell lines, such as Huh7 and Hep3B (Fig. 1A), compared with normal hepatocytes. DDX20 protein levels were also lower in human HCC needle biopsy specimens than in surrounding noncancerous liver tissue (Fig. 1B). Immunohistochemical analysis

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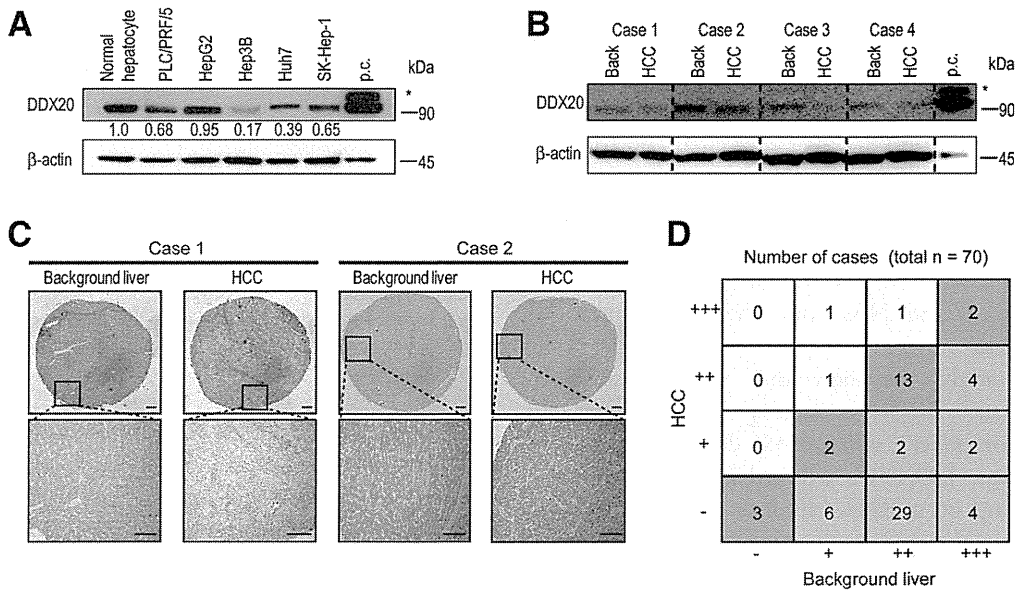

Fig. 1. Reduced DDX20 expression levels in hepatocellular carcinoma. (A) DDX20 protein expression in HCC cell lines. Numbers between the panels indicate DDX20 protein levels normalized to β-actin levels. Lysates of 293T cells transiently transfected with a FLAG-tagged DDX20-expressing plasmid yielded two DDX20 bands corresponding to the endogenous DDX20 protein and the transfected FLAG-tagged DDX20 protein (*) as a positive control (p.c.; far right lane). Data represent the results of three independent determinations. (B) DDX20 protein expression in four HCC needle biopsy specimens and in the surrounding noncancerous background liver tissue (Back). *Positive control. (C) Immunohistochemical analysis of DDX20 protein expression in HCC and surrounding tissues (background liver). Two representative cases are shown. Scale bars, 500 μm. The lower panels display magnified images of the boxed areas in the upper panels. (D) Grid summarizing DDX20 immunohistochemical staining data from 70 cases. In 47 cases (pink shading), DDX20 protein levels were lower in the HCC tissues than in the surrounding tissues ($P < 0.05$; Wilcoxon signed-rank test).

confirmed that DDX20 expression was frequently lower in HCC than in surrounding noncancerous liver tissue (Fig. 1C,D). Specifically, 47 of 70 cases examined showed reduced DDX20 protein expression in HCC versus background noncancerous liver tissue (Fig. 1D and Supporting Table 1). These results indicate that the expression of DDX20, an miRNP component, is frequently reduced in human HCC, and suggest that this reduced DDX20 expression might be involved in the pathogenesis of a subset of HCC cases.

NF-κB Activity Is Enhanced by DDX20 Deficiency. Because DDX20 knockout mice are embryonic-lethal,[28] DDX20 has been suggested to have important biological roles. DDX20, a DEAD-box protein,[29] was originally found to interact with survival motor neuron protein.[30] Later, it was identified as a major component of miRNPs,[31] which may mediate miRNA function. As we have reported, DDX20 is preferentially involved in miRNA-140-3p function,[23] acting as a suppressor of NF-κB activity in the liver.[32] DDX20-knockdown PLC/PRF/5 cells exhibit enhanced NF-κB activity[23] (Fig. 2A). Whereas the proliferation rates of DDX20-knockdown cells and control cells were comparable (Fig. 2B), apoptotic cell death after stimulation with TNF-related apoptosis-inducing ligand (TRAIL), which induces both cell apoptosis and NF-κB activation,[33] was significantly reduced in DDX20-knockdown cells (Fig. 2C). Similar results were obtained using DDX20-knockdown HepG2 cells (Supporting Fig. 2A-D). Conversely, NF-κB activity was reduced, but cell proliferation remained unchanged, in Hep3B cells stably overexpressing DDX20 (Fig. 2D,E). Sensitivity to TRAIL-induced apoptosis was restored in these cells (Fig. 2F). Similar results were also obtained using Huh7 cells (Supporting Fig. 2E-H). These data confirm a previous report that DDX20 deficiency enhances NF-κB activity and the downstream events of this pathway.

Metallothionein Expression Is Decreased by DDX20 Deficiency. Next, to investigate the biological consequences of DDX20 deficiency, we examined the changes in transcript levels in DDX20-knockdown cells using microarrays (GEO accession number: GSE28088). The expression of genes driven by NF-κB that are related to carcinogenesis, such as FASLG, IRAK1, CARD9, and Galectin-1, were enhanced significantly in DDX20-knockdown cells, as expected (Table 2). To determine the mechanism underlying the enhanced NF-κB activation in DDX20-deficient cells, we searched for candidate genes and noticed that the

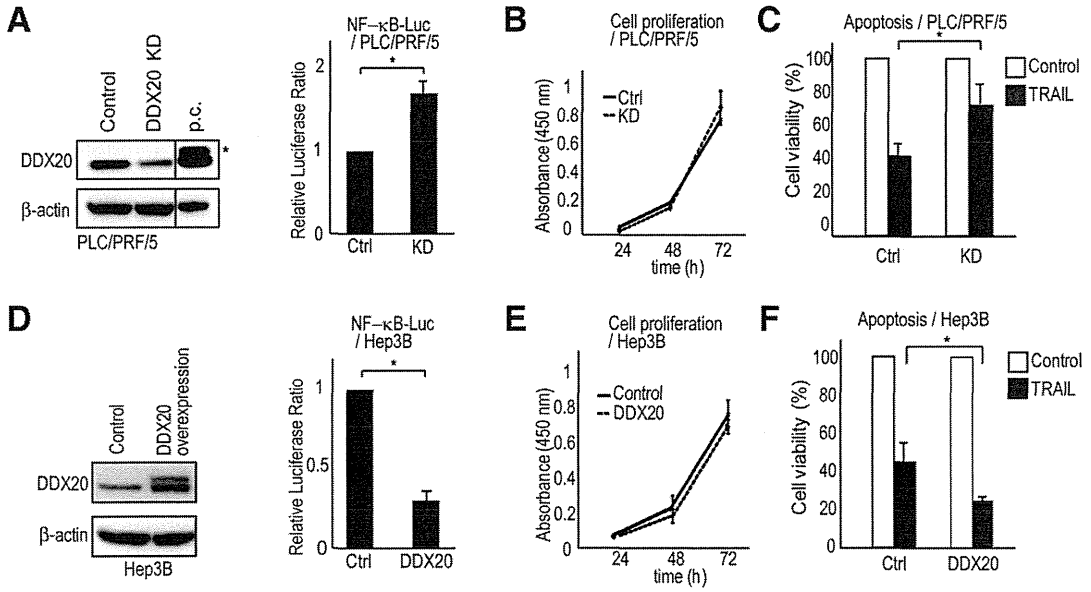

Fig. 2. Modulation of downstream events of the nuclear factor-κB pathway by DDX20. (A) Left: Establishment of stable DDX20-knockdown (DDX20 KD) PLC/PRF/5 cells. *Positive control (p.c.). Right: DDX20 deficiency enhances TNF-α–induced NF-κB activity. NF-κB reporter plasmids were transiently transfected into control (Ctrl) or DDX20-knockdown (KD) PLC/PRF/5 cells. The cells were then treated with TNF-α (5 ng/mL) or vehicle for 6 hours. *$P < 0.05$. Data are presented as the mean ± SD of three independent determinations. (B) Cell proliferation rates were comparable for control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells. Data are presented as the mean ± SD of three determinations. (C) DDX20 deficiency reduces TRAIL-induced apoptotic cell death. Control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells were incubated with 25 ng/mL TRAIL. Data represent cell viability after TRAIL stimulation (gray bars) relative to the number of vehicle-treated cells (white bars). *$P < 0.05$. Data are presented as the mean ± SD of triplicate determinations. (D) Left: Establishment of stable DDX20-overexpressing cells. Hep3B cells were infected with control or FLAG-tagged DDX20-overexpressing lentiviruses and selected on puromycin. Western blot analysis confirmed increased expression of DDX20 protein. Right: DDX20 overexpression suppresses TNF-α–induced NF-κB activity. NF-κB reporter plasmids were transiently transfected into Hep3B control (Ctrl) and DDX20-overexpressing (DDX20) cells treated with TNF-α for 6 hours. Data are presented as the mean ± SD of three independent determinations. *$P < 0.05$. (E) Proliferation of control (Ctrl) and DDX20-overexpressing (DDX20) Hep3B cells was measured as described in (B). (F) DDX20 overexpression reduces TRAIL-induced apoptotic cell death. Data for control (Ctrl) and DDX20-overexpressing (DDX20) Hep3B cells are shown. *$P < 0.05$.

Table 2. Increased Expression of NF-κB–Related Genes in DDX20-Knockdown HepG2 Cells Compared with Wild-Type Cells

RefSeq ID	Symbol	Description	Ratio	Representative Gene Function
NM_000639	FASLG	Fas ligand	3.5	NF-κB target, apoptosis
NM_052813	C9orf151	CARD9	2.5	NF-κB cascade, NF-κB target
NM_014959	CARD8	Tumor up-regulated CARD-containing antagonist of CASP9 (TUCAN)	2.2	NF-κB target
NM_131917	FAF1	FAS-associated factor 1 (hFAF1)	1.9	Cytoplasmic sequestering of NF-κB, NF-κB target
NM_020644	TMEM9B	Transmembrane protein 9B precursor	1.9	Positive regulation of NF-κB transcription factor activity
NM_017544	NKRF	ITBA4 protein	1.9	Negative regulation of transcription
NM_006247	PPP5C	Protein phosphatase T	1.8	Positive regulation of NF-κB cascade
NM_020345	NKIRAS1	KappaB-Ras1	1.8	NF-κB cascade
NM_001569	IRAK1	IRAK-1	1.7	Positive regulation of NF-κB transcription factor activity
NM_177951	PPM1A	Protein phosphatase 1A	1.7	Positive regulation of NF-κB cascade
NM_018098	ECT2	Epithelial cell-transforming sequence 2 oncogene	1.6	Positive regulation of NF-κB cascade
NM_002305	LGALS1	Galectin-1 (putative MAPK-activating protein MP12)	1.6	Positive regulation of NF-κB cascade
NM_015093	TAB2	TAK1-binding protein 2	1.6	Positive regulation of NF-κB cascade
NM_004180	TANK	TRAF-interacting protein	1.5	NF-κB cascade
NM_014976	PDCD11	Programmed cell death protein 11	1.5	rRNA processing
NM_015336	ZDHHC17	Putative NF-κB–activating protein 205	1.5	Positive regulation of NF-κB cascade
NM_002503	NFKBIB	IKB-β	1.5	Cytoplasmic sequestering of NF-κB
NM_138330	ZNF675	Zinc finger protein 675	1.5	Negative regulation of NF-κB transcription factor activity

The genes were identified as NF-κB–related based on the Gene Ontology and the GeneCodis Databases.

Table 3. Decreased Expression Levels of MT Genes in DDX20 Knockdown HepG2 Cells Compared with Wild-Type Cells

Symbol	Description	Ratio
MT1E	Metallothionein-1E	**0.12**
MT1F	Metallothionein-1F	**0.36**
MT1H	Metallothionein-1H	**0.16**
MT1G	Metallothionein-1G	**0.06**
MT1M	Metallothionein-1M	**0.24**
MT1X	Metallothionein-1X	**0.27**
MT2A	Metallothionein-2	**0.28**
MT3	Metallothionein-3	0.84
MTL5	Metallothionein-like 5 (Tesmin)	1.12

Numbers in boldface type indicate values <0.5.

expression levels of a group of metallothioneins (MTs), such as MT1E, MT1F, MT1G, MT1M, MT1X, and MT2A, were all significantly decreased when DDX20 was deficient (Table 3). The decreased expression of MTs in DDX20-knockdown HepG2 and PLC/PRF/5 cells was confirmed via quantitative RT-PCR (Fig. 3a and Supporting Fig. 3). Expression of MT-3, which was not altered in the microarray analysis, was similarly unaltered in quantitative RT-PCR analysis. Notably, it was already known that MTs are frequently silenced in human primary liver cancers.[34-36] In addition, MT knockout mice have enhanced NF-κB activity, likely due to reactive oxygen species, and these mice are more prone to hepatocarcinogenesis.[37] These results suggest that DDX20 deficiency enhances NF-κB activity by decreasing the expression of MTs, which could facilitate the development of liver cancer.

MiRNA-140 Directly Targets Dnmt1. Because MT expression is regulated principally by CpG island methylation in their promoter regions,[38,39] we examined the quantitative methylation status of MT promoters in DDX20-knockdown cells. The CpG islands of the MT1E, MT1G, MT1M, MT1X, and MT2A promoters, and the CpG shores of the MT1F promoters, were significantly more highly methylated under DDX20-deficient conditions, as determined by the comprehensive Illumina Quantitative Methylation BeadChip method (Table 4, Supporting Table 2, and GSE 37633). A crucial step in DNA methylation involves DNA methyltransferase (Dnmt), which catalyzes the methylation of CpG dinucleotides in genomic DNA.[40] The methylation status of MT promoters is mediated specifically by Dnmt1.[41] Because Dnmt1 contains a predicted miRNA-140-3p target site in its 3′ UTR, with a perfect match to its seed sequences (Fig. 3B), and because the effects of miRNA-140-3p activity were impaired in DDX20-knockdown cells,[23] it was hypothesized that whereas miRNA-140 normally targets and suppresses Dnmt1 protein expression, miRNA-140-3p dysfunction due to DDX20 deficiency results in enhanced Dnmt1 expression, leading to hypermethylation of MT promoters. Consistent with this hypothesis, Dnmt1 expression was increased significantly in DDX20-knockdown cells (Fig. 3C). miRNA-140 precursor overexpression suppressed activity of the Dnmt1 3′ UTR reporter construct, the effect of which was lost when two mutations were introduced into its seed sequences (Fig. 3D). MiRNA-140 precursor overexpression suppressed Dnmt1 protein expression (Fig. 3E). These results indicate that miRNA-140 directly targets Dnmt1 and suppresses its expression in the normal state. Consistently, decreased DDX20, increased Dnmt1, and decreased MT expression were detected together in human clinical HCC samples, as determined via immunohistochemistry (Fig. 3F). By contrast, miRNA-140 precursor-overexpressing Huh7 cells showed increased expression of MTs and reduced NF-κB activity *in vitro* (Supporting Fig. 4A,B). Moreover, the increase in the number of spheres formed from PLC/PRF/5 cells due to DDX20 knockdown was antagonized by treatment with an NF-κB inhibitor or a demethylating agent (Supporting Fig. 5). Taken together, these results suggest that the up-regulated Dnmt1 protein expression caused by functional impairment of miRNA-140-3p due to DDX20 deficiency results in decreased expression of MTs *via* enhanced methylation at the CpG sites in their promoters. This may lead to enhanced NF-κB activity and cellular transformation at least *in vitro*.

MiRNA-140 Is a Liver Tumor Suppressor. To further examine the biological consequences of functional impairment of miRNA-140 due to DDX20 deficiency, we determined the phenotypes of miRNA-140 knockout (miRNA-140$^{-/-}$) mice (Fig. 4A). Similar to the *in vitro* DDX20 knockdown results, Dnmt1 expression was increased and MT levels decreased in the liver tissue of these mice (Fig. 4B). NF-κB–DNA binding activity was enhanced in the livers of miRNA-140$^{-/-}$ mice after tail-vein injection of TNF-α, a crucial cytokine that induces NF-κB activity and hepatocarcinogenesis (Fig. 4C). As was found in MT knockout mice, phosphorylation of p65 at serine 276, which is critical for p65 activation, was significantly increased in the livers of miRNA-140$^{-/-}$ mice after DEN exposure, which induces NF-κB activation and liver tumors[37] (Fig. 4D). Notably, the size and number of liver tumors that developed 8 months after DEN exposure were markedly elevated in miRNA-140$^{-/-}$ mice compared with control mice (Fig. 4E,F). These results indicate that miRNA-140$^{-/-}$ mice are indeed

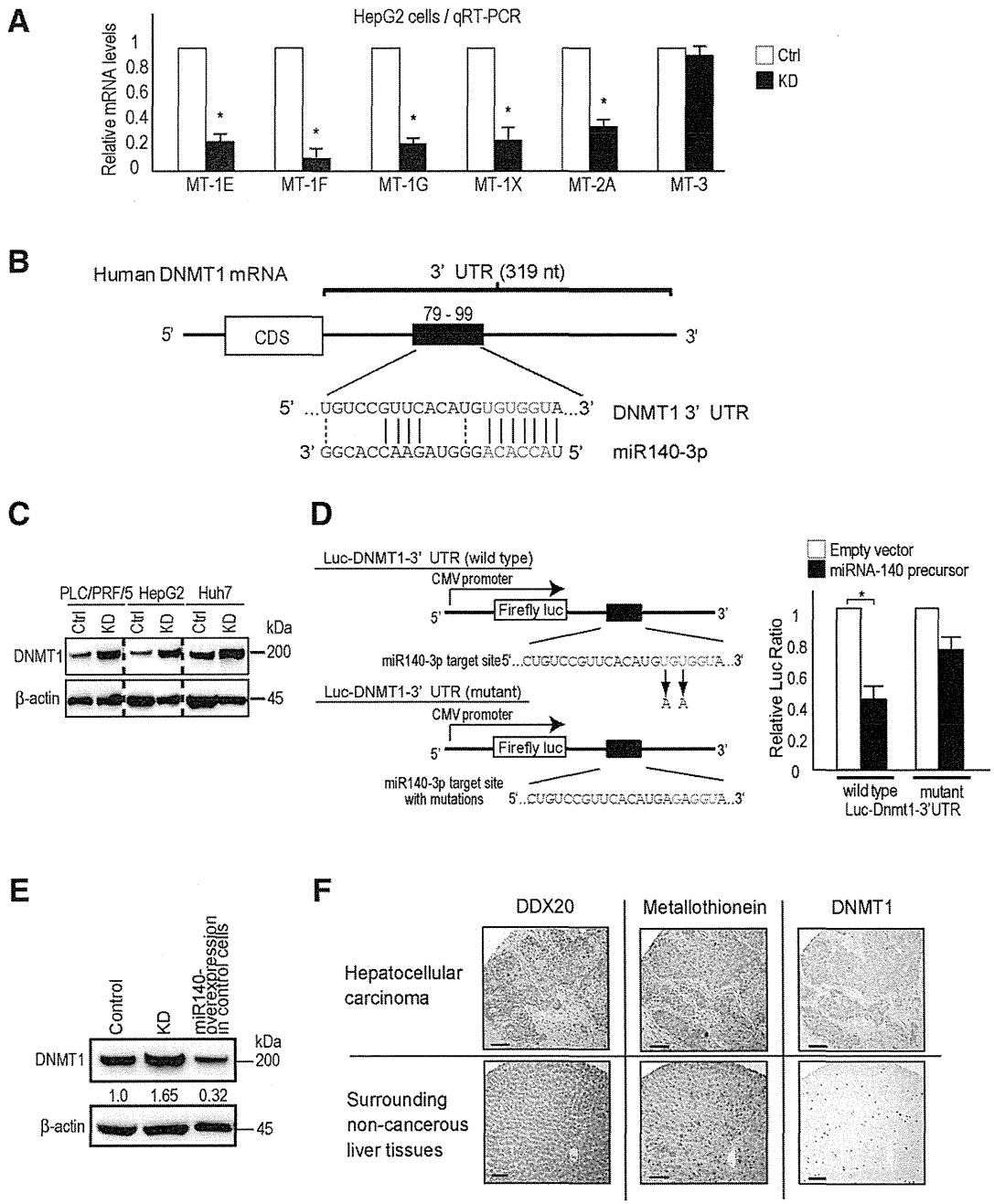

Fig. 3. Targeting of Dnmt1 by miRNA-140-3p and reduced MT expression. (A) The expression levels of MTs were determined using quantitative reverse-transcriptase polymerase chain reaction. The relative expression ratios of the MTs in control (white bars) and DDX20-knockdown (black bars) HepG2 cells were calculated by normalizing control cell values to 1.0. The data represent the mean ± SD of three independent determinations. *$P < 0.05$. (B) Putative miRNA-140-3p target sites in the 3′ UTR of human Dnmt1. Seed sequences are indicated in red. (C) Dnmt1 expression was increased in DDX20-knockdown cells. Ctrl, control cells; KD, DDX20-knockdown cells. (D) Left: Schematic diagrams of wild-type (upper) and mutant (lower) luciferase reporter constructs (Luc-Dnmt1-3′ UTRs) carrying the Dnmt1 3′ UTR region harboring the putative miRNA-140-3p target site. The mutant seed sequence contained two nucleotide substitutions. Right: The Dnmt1 3′ UTR is targeted directly by miRNA-140-3p. Cells were cotransfected with Luc-Dnmt1-3′ UTR (wild-type or mutant) plus either an empty vector (white bars) or a plasmid expressing the miRNA-140 precursor (black bars). Data are the mean ± SD of three independent determinations. (E) Overexpression of miRNA-140 reduces Dnmt1 expression in control cells. Values between the panels indicate Dnmt1 protein levels normalized to those of β-actin. KD, DDX20 knockdown cells. (F) Representative histochemical images showing expression of DDX20, Dnmt1, and MT proteins in HCC (upper three panels) and surrounding tissue (lower panels). Compared with adjacent noncancerous liver tissue, HCCs exhibited decreased DDX20 and MT expression and increased Dnmt1 expression. Note that adjacent sections were stained for each protein. Scale bar, 50 μm.

Table 4. Methylation Levels in CpG Islands of the MT Genes in DDX20-Knockdown HepG2 Cells Compared with Control Cells

Symbol	CpG Island Methylation Ratio	Target ID
MT1E	**1.14**	cg00178359
	1.29	cg06463589
	3.65	cg02512505
	1.02	cg15134649
MT1G	**2.14**	cg16452857
	1.03	cg27367960
	1.00	cg03566142
	0.99	cg07791866
MT1M	**1.16**	cg02132560
	0.98	cg02160530
	1.03	cg04994964
MT1X	**1.24**	cg05596720
	1.05	cg26802333
	1.06	cg09147880
	1.01	cg08872713
MT2A	**2.06**	cg07395075
	0.94	cg20430434

Values were determined using the quantitative Illumina Human Methylation BeadsChip. Boldface values indicate increased methylation levels in DDX20 knockdown cells.

more prone to liver cancer development and suggest that miRNA-140 acts as a liver tumor suppressor, probably by suppressing NF-κB activity, although we cannot completely exclude other molecular mechanisms. Nonetheless, these results also suggest that the impairment of miRNA-140 function due to DDX20 deficiency may lead to hepatocarcinogenesis in humans, as we have observed in miRNA-140$^{-/-}$ mice (Supporting Figs. 6 and 7).

Discussion

Here, we report that miRNA-140$^{-/-}$ mice have increased NF-κB activity and are more prone to HCC development. In addition, we show that DDX20, an miRNP component, is frequently decreased in human HCC tissues. Because DDX20 deficiency preferentially causes impaired miRNA-140 function,[23] the functional impairment of miRNA-140 may result in phenotypes similar to those of miRNA-140$^{-/-}$ mice and may lead to hepatocarcinogenesis. In support of the hypothesis that DDX20 dysfunction is involved in hepatocarcinogenesis, DDX20 is located at 1p21.1-p13.2, a frequently deleted chromosomal region in human HCC,[27] and DDX20 was recently identified as a possible liver tumor suppressor in a functional screen in mice.[27] Although the possibility that intracellular signaling pathways other than miRNA-140 may also be involved in the biological consequences of DDX20 deficiency cannot be denied, we believe that functional impairment of miRNA-140 plays a major role in the phenotypes induced by DDX20 deficiency, based on the phenotypic similarities.

Changes in miRNA expression levels have been reported in various tumors.[7,12,42] However, in this study, we found that reduced expression of an miRNA machinery component might lead to carcinogenesis, at least in part, through functional impairment of miRNAs. Recent studies have shown that components of the RNA interference machinery are associated with the outcome of ovarian cancer patients,[43] and that single-nucleotide polymorphisms in miRNA machinery genes can be used as diagnostic risk markers.[44,45] Therefore, the impairment of miRNA function caused by deregulated miRNA machinery components may also be involved in carcinogenesis.

Our study identified Dnmt1 as a critical target of miRNA-140. The decreased MT expression due to the CpG promoter methylation induced by Dnmt1 resulted in enhanced NF-κB activity. This finding was consistent with the results obtained using MT gene knockout mice, in which enhanced NF-κB activation promoted hepatocarcinogenesis.[37] The decrease in MT expression that results from increased Dnmt1 expression caused by functional impairment of miRNA-140, together with increased NF-κB activation and hepatocarcinogenesis in MT knockout mice,[37] supports the concept that the DDX20/miRNA-140/Dnmt1/MT/NF-κB pathway may play a crucial role in hepatocarcinogenesis. However, we cannot fully exclude the possibility that other intracellular signaling pathways are also involved in the induction of hepatocarcinogenesis by miRNA-140 or DDX20 deficiency, because the precise role of NF-κB in hepatocarcinogenesis has not been clearly defined,[8] although constitutive activation of NF-κB signaling has been frequently detected in human HCCs.[46] The mechanisms by which DDX20 expression is initially decreased and the reason its locus is frequently deleted in HCC remain to be elucidated. However, because DDX20 expression is also regulated by methylation of its CpG promoter,[47] once this pathway is deregulated, decreased DDX20 expression could be maintained by a positive feedback mechanism, even without deletion of its locus.[27]

In conclusion, this study shows that miRNA-140 acts as a liver tumor suppressor. We show that DDX20, an miRNP component, is frequently decreased in human HCC, which may induce hepatocarcinogenesis via impairment of miRNA-140 function. These results suggest the importance of investigations of not only aberrant miRNA expression levels,[12,14,17,48] but also deregulation of miRNP