Supplementary Table 5. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
1291722996[a]	EMID2	7	13	20	insG	4
1292538685	LOC100289561	7	1	14	insA	3
1295252926	MLL5	7	12	21	insG	3
1298402792	NRCAM	7	1	17	insT	3
1319055841[a]	KCP	7	10	62	insC	1
1319073009	KCP	7	1	30	delC	2
1333766379	LOC441294	7	1	46	insA	4
1334380185	CTAGE4	7	1	39	insA	3
1334381975	ARHGEF5L	7	1	19	insA	1
1339923632[a]	KRBA1	7	12	76	insC	2
1339973995[a]	SSPO	7	9	44	insC	1
1340003537[a]	SSPO	7	60	15	insC	4
1340012514	SSPO	7	76	23	delA	2
1340015859	SSPO	7	83	14	delC	2
1340525483	C7orf29	7	1	24	delC	1
1341211228[a]	ATG9B	7	10	49	insC	1
1341434558	SMARCD3	7	10	21	delC	3
1342197228	GALNTL5	7	5	71	delT	4
1342442397[a]	MLL3	7	14	208	insT	4
1356372261[a]	XKR5	8	6	55	delAG	1
1374409954[a]	NEFL	8	3	38	delG	4
1380219728[a]	UBXN8	8	7	83	insT	1
1380304215	TEX15	8	1	23	insA	3
1388426070[a]	PLEKHA2	8	11	28	delC	2
1395399601[a]	PRKDC	8	31	17	insG	1
1398930064	PXDNL	8	14	27	insA	3
1410692513[a]	YTHDF3	8	4	24	insG	1
1415952398	C8orf34	8	2	32	insG	3
1445261384	LAPTM4B	8	2	16	insC	3
1490189877[a]	JRK	8	1	12	delCA	3
1490189878[a]	JRK	8	1	19	delA	2
1491176363	ZNF623	8	1	29	insT	3
1492082552[a]	RECQL4	8	14	20	delG	3
1498992866	LOC645969	9	1	155	insT	4
1527437913[a]	C9orf144B	9	4	20	delC	4
1543290663[a]	FOXD4L5	9	1	39	delG	1
1546032104	TRPM3	9	22	19	insT	3
1552818643	VPS13A	9	48	29	insG	3
1574648314	COL15A1	9	13	29	insC	3
1586295095	MUSK	9	1	62	insT	3
1608846803[a]	ABO	9	6	117	insC	4
1620006324	GDI2	10	7	14	insG	3
1620254092	IL2RA	10	4	14	insC	3
1621795546[a]	ITIH5	10	14	23	delC	1
1633127998	NSUN6	10	2	26	insA	3
1647389817	ITGB1	10	13	22	insA	3
1652560241[a]	LOC340947	10	2	25	delT	1
1653671683[a]	LOC642424	10	3	117	delT	1
1657313101	AGAP4	10	7	23	delT	2
1658942495	FAM25G	10	3	48	insC	3
1662197526	LOC100287932	10	6	22	insA	4
1662338998[a]	AGAP6	10	1	50	insC	2
1666373362	PCDH15	10	19	56	insC	3
1673760921	TMEM26	10	6	25	insT	3
1685560504	FAM149B1	10	7	26	insT	3
1701949711	PANK1	10	3	30	insA	3
1708407108	CCNJ	10	3	18	insC	3
1708510568	ZNF518A	10	1	30	insC	1
1708668598	DNTT	10	2	18	insA	3
1709332414	C10orf12	10	1	18	insG	3
1728973932[a]	PNLIPRP2	10	3	52	insG	1

Supplementary Table 5. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
1733216553	BRWD2	10	8	28	insT	3
1737221823	ZRANB1	10	1	17	insA	3
1738045786	MMP21	10	7	33	insG	3
1748226082	C11orf21	11	4	63	insG	1
1750053615	RRM1	11	14	17	insT	3
1753342600	SYT9	11	4	18	insG	3
1760006709[a]	SPON1	11	5	72	insC	4
1764016157	SAAL1	11	7	40	delT	4
1771005317	LUZP2	11	12	34	insA	3
1782417168	TRAF6	11	6	24	delG	3
1782519665	RAG2	11	1	21	insA	3
1792247476[a]	CREB3L1	11	12	40	insG	1
1802120506	TCN1	11	7	13	insA	3
1802663567[a]	MS4A14	11	2	61	delTT	4
1802663568[a]	MS4A14	11	2	22	delT	3
1803663946[a]	TMEM216	11	3	54	insA	4
1804797590	AHNAK	11	3	12	insG	3
1805556025	SLC22A10	11	1	17	insC	3
1810263379[a]	UNC93B1	11	7	53	insG	3
1810284280[a]	ALDH3B1	11	2	63	insC	2
1810287509[a]	ALDH3B1	11	6	18	insC	1
1810293595[a]	ALDH3B1	11	9	28	insC	4
1814065554	LOC729523	11	1	22	delT	3
1826743977	DLG2	11	5	23	insT	3
1832107207	LOC642446	11	1	33	delT	4
1837197723[a]	CWC15	11	5	152	insT	1
1837299118[a]	SFRS2B	11	1	36	insC	4
1850549218	ATM	11	49	24	insT	3
1852355678	ZC3H12C	11	2	25	insC	3
1854201323[a]	DIXDC1	11	7	16	insC	1
1860877259[a]	TREH	11	15	28	insG	2
1861246651[a]	SLC37A4	11	3	37	delC	1
1861288156	VPS11	11	2	13	insC	4
1867800518	EI24	11	9	14	insC	4
1867851321	CHEK1	11	5	44	insC	3
1888645169[a]	PRB3	12	4	34	delG	4
1888731023[a]	PRB1	12	3	136	delC	1
1891856090	ATF7IP	12	11	19	insG	3
1893735417[a]	MGST1	12	2	12	delAA	3
1893735418[a]	MGST1	12	2	18	delA	3
1898574937	SLCO1B1	12	7	17	insC	3
1902256413	BCAT1	12	5	22	insG	3
1913975525	KIF21A	12	10	20	insT	3
1914378775	SLC2A13	12	10	17	insA	3
1927092176	KRT6C	12	1	15	insG	2
1930622534	SUOX	12	3	14	insG	3
1931678522	TMEM194A	12	9	23	insG	3
1932337710	OS9	12	12	17	insA	3
1959863488	LRRIQ1	12	26	12	delA	3
1962616654	C12orf50	12	3	28	insA	3
1978598568[a]	TDG	12	3	14	insA	3
1986789153	LOC100287839	12	9	35	insC	3
1997115077	RSRC2	12	10	28	insG	3
1999523126	UBC	12	1	29	delT	3
2009256491	ZMYM5	13	5	14	insC	3
2012756904	SACS	13	9	20	insT	3
2012761230	SACS	13	9	23	insT	3
2017859185	FLT1	13	4	36	insA	3
2022550582	STARD13	13	5	85	delT	1
2026525487	CSNK1A1L	13	1	13	insC	2
2038965626	RCBTB1	13	8	17	insG	3

Supplementary Table 5. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
2046563396	PRR20	13	2	28	delC	2
2063234131	KLF12	13	4	47	insT	3
2066482633	MYCBP2	13	75	17	insT	3
2066508358	MYCBP2	13	62	14	insC	3
2066632819	MYCBP2	13	22	23	delC	3
2066717508	MYCBP2	13	2	33	delAA	3
2066717509	MYCBP2	13	2	33	delA	3
2088603872	GPR18	13	1	20	insT	3
2105948032	NDRG2	14	1	34	delG	1
2106009532	FLJ10357	14	18	14	delG	3
2108927297	DHRS4L2	14	6	41	insA	4
2109139875[a]	MDP-1	14	6	13	delA	1
2117359342	AKAP6	14	1	20	insA	3
2117747539	AKAP6	14	12	21	insA	3
2137979011	DDHD1	14	10	22	insC	3
2148241015[a]	GPHB5	14	1	18	insG	4
2158414589[a]	C14orf169	14	1	19	insC	3
2159993929[a]	FAM164C	14	1	14	insA	1
2160606560	TTLL5	14	4	17	insA	3
2179419547	SERPINA12	14	2	54	insC	3
2179491154	SERPINA4	14	3	14	insG	3
2181450460	PAPOLA	14	5	33	insC	3
2202211427[a]	CHRFAM7A	15	4	191	delCA	1
2202211428[a]	CHRFAM7A	15	4	252	delA	4
2203996021[a]	CHRNA7	15	6	166	delTG	1
2203996022[a]	CHRNA7	15	6	50	delG	2
2204534873	SCG5	15	5	24	insC	3
2212460825	CASC5	15	10	14	insA	3
2220067652	SLC12A1	15	5	21	insA	3
2237036677	LOC100287371	15	3	32	insG	3
2243652079[a]	NR2E3	15	6	34	delC	1
2251295853	KIAA1024	15	1	14	insT	3
2252413491	ARNT2	15	14	24	insC	3
2256610001	ZSCAN2	15	2	14	insC	3
2257065252	PDE8A	15	4	20	delT	3
2261248094	FANCI	15	2	19	insC	3
2261584966	C15orf42	15	7	21	insT	3
2270957952[a]	LOC145814	15	4	23	insC	4
2271092254[a]	SYNM	15	1	19	insG	3
2274046312[a]	C16orf35	16	12	89	insG	4
2274304546	AXIN1	16	1	20	delC	3
2277509768[a]	NLRC3	16	7	81	delG	1
2285935013[a]	LOC729978	16	4	20	delAT	4
2285935014[a]	LOC729978	16	4	44	delT	1
2292434768	NOMO2	16	24	22	insG	3
2294397443	ACSM2A	16	9	23	delA	3
2294883814	DNAH3	16	53	18	insC	3
2304906998	HSD3B7	16	6	48	delC	2
2332868773	CLEC18C	16	3	24	insA	3
2333553555[a]	HYDIN	16	68	29	delA	4
2338969142[a]	CNTNAP4	16	1	82	insT	1
2351412465[a]	LOC100289580	16	2	67	delC	2
2354387432	PRPF8	17	4	11	insG	3
2356396572[a]	P2RX5	17	3	13	delG	1
2359357840	C17orf100	17	1	14	insG	2
2360272579[a]	SENP3	17	6	20	delA	4
2361527508[a]	PIK3R6	17	16	42	insG	1
2363416732	C17orf48	17	3	19	insA	3
2371198121	LGALS9C	17	9	16	insA	4
2376394518[a]	SEBOX	17	1	29	insG	4
2376430014[a]	SLC46A1	17	4	15	delA	1

Supplementary Table 5. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
2382300534	CCL7	17	2	24	insT	3
2383802642[a]	MMP28	17	4	28	insC	4
2384283858	TBC1D3C	17	13	31	insG	1
2388631071	KRT10	17	1	14	delC	3
2392844842[a]	PLCD3	17	10	24	insC	1
2393016586[a]	MAP3K14	17	4	16	insG	2
2407377091	CLTC	17	3	28	insT	3
2409792732	MED13	17	2	24	insA	3
2411313186[a]	WDR68	17	5	42	delG	1
2412151377	DDX5	17	8	23	insT	3
2434290839	MYOM1	18	8	16	insA	3
2448603454	RBBP8	18	14	22	insC	3
2451555232	LOC100287386	18	2	31	insA	1
2471234235	SLC14A2	18	4	39	delC	3
2492044315	CDH19	18	11	24	insA	3
2501962862	ZNF516	18	2	27	delG	2
2508173565[a]	SPPL2B	19	7	26	insC	2
2510788089	UHRF1	19	14	13	insC	3
2514792389	MUC16	19	3	18	insA	3
2514803399	MUC16	19	3	29	insT	3
2518236406	ZNF799	19	4	25	insA	3
2521463907[a]	CYP4F8	19	4	79	insC	1
2522001621[a]	HSH2D	19	5	71	delA	2
2538892348	C19orf55	19	9	17	delG	2
2543188059	ZNF780B	19	2	24	insC	3
2543756504[a]	LTBP4	19	24	14	insG	1
2544255517[a]	CYP2F1	19	1	53	insC	4
2544853028	CEACAM5	19	4	26	insT	3
2547650400[a]	CEACAM20	19	8	54	delT	1
2547930257[a]	CBLC	19	8	18	insC	3
2552076265[a]	DHDH	19	4	55	insG	2
2552600822	ALDH16A1	19	10	73	insC	2
2554469302[a]	LOC147645	19	10	37	insG	4
2555437083[a]	ZNF480	19	1	51	delG	1
2555750854	ZNF83	19	1	26	insG	3
2559350849	ZSCAN5C	19	1	50	insA	2
2560590155	ZNF749	19	3	34	insA	1
2560866399	ZNF671	19	4	14	insA	3
2561351770[a]	ZNF274	19	4	78	insG	2
2562070563	DEFB126	20	2	20	delCC	3
2562070564	DEFB126	20	2	20	delC	3
2567847490[a]	CHGB	20	4	28	delGA	2
2567847491[a]	CHGB	20	4	70	delA	2
2580083985	CSRP2BP	20	4	17	insG	3
2583130413[a]	NCRNA00153	20	7	49	insG	1
2606534089	DDX27	20	4	21	insA	3
2608270909	MOCS3	20	1	23	insG	3
2640900547[a]	KRTAP7-1	21	1	27	delA	4
2643647262[a]	SON	21	12	40	insA	1
2643647273[a]	SON	21	12	33	delA	4
2654166830	TRAPPC10	21	21	24	insT	3
2656193953[a]	LOC100288508	21	5	14	insC	1
2670991039	HORMAD2	22	2	23	insG	3
2681753989	DNAJB7	22	1	66	insA	1
2683020374	CYP2D6	22	5	18	insG	4
2701397266	WWC3	X	7	15	insG	3
2708217926	RBBP7	X	2	16	insC	3
2709924320	CDKL5	X	4	26	insC	3
2711314268	CXorf23	X	3	22	delG	3
2713575280	PHEX	X	19	29	insG	3
2736210225	KDM6A	X	17	17	insC	3

Supplementary Table 5. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
2736225869	KDM6A	X	24	16	insA	3
2737802282	SLC9A7	X	7	21	insC	3
2739406485	SSX1	X	6	45	insT	3
2739444455[a]	SSX9	X	2	11	delC	2
2741301867[a]	DGKK	X	22	55	insG	1
2743920170	SSX2B	X	6	35	insC	3
2745406637	WNK3	X	16	40	insA	3
2755460714	OPHN1	X	8	18	insC	3
2757668459	KIF4A	X	28	24	insG	3
2758547967	NONO	X	6	22	insT	3
2761842615	RLIM	X	3	18	insG	3
2771580011	HDX	X	5	18	insA	3
2779112744	PCDH11X	X	2	18	insT	3
2788398590	CENPI	X	20	19	insC	3
2789376503	TCEAL6	X	1	24	insG	2
2789554171	NXF2	X	7	14	insT	3
2789554906	NXF2	X	10	29	insA	3
2802179110	IL13RA2	X	4	18	insT	3
2823639589	ARHGEF6	X	18	16	insA	3
2840930428[a]	LCAP	X	1	57	insC	2
2841794077	MPP1	X	7	14	insG	3

[a]These indels commonly occurred in more than one HCC.

Supplementary Table 6. List of 81 Nucleotide Positions in 77 Genes With Indels at a Frequency of >20% of Reads in 4 Nontumorous Tissues From 4 Patients

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
36247083	THRAP3	1	4	18	delG	1
75174421	SLC44A5	1	16	19	delT	1
114430296	TRIM33	1	20	23	delC	1
133132499	YY1AP1	1	7	37	insT	1
133844355	RHBG	1	9	48	delC	4
201121914	CAPN2	1	3	14	delC	1
201173637	TP53BP2	1	13	22	delG	1
247664301	C2orf43	2	4	18	delA	2
319394229	SNRNP200	2	37	20	delA	1
322653290	AFF3	2	14	35	delA	1
331834049	RANBP2	2	20	16	delG	1
332901065	RGPD5	2	20	22	delT	1
374789589	NEB	2	4	37	insT	4
382950536	LY75	2	5	19	delA	4
401635744	TTN	2	274	25	delA	1
409835043	FAM171B	2	8	17	delT	1
412064501	COL3A1	2	14	21	insA	4
454784716	PTMA	2	4	14	delT	1
463724350[a]	AQP12B	2	1	27	delC	3
463734336	AQP12A	2	2	14	delG	2
503335742	DLEC1	3	4	20	delT	1
503335743	DLEC1	3	4	20	delA	1
735406533	CNOT6L	4	10	21	delG	1
785877214	LARP2	4	14	23	delA	1
798021293	SCOC	4	1	18	insC	1
810971969	TRIM2	4	5	18	delC	1
883256725	PRLR	5	3	29	delG	4
939146286	ANKRD32	5	16	15	insC	1
985314450[a]	LOC100288105	5	1	27	delC	3
1033746568	BMP6	6	5	24	delC	1
1068664364	KIAA0240	6	4	17	insT	4
1193244025	FAM120B	6	1	43	insA	1
1222659823	KIAA0644	7	1	463	delC	4
1282877394	CDK6	7	3	21	delA	1
1289880816	CYP3A4	7	12	45	delG	1
1333766765	LOC441294	7	1	13	delA	4
1340012514	SSPO	7	76	53	delA	3
1356372261	XKR5	8	6	130	delA	4
1490189877[a]	JRK	8	1	29	delC	3
1490189878[a]	JRK	8	1	15	delA	2
1492082552[a]	RECQL4	8	14	43	delG	3
1505961686	MPDZ	9	2	28	insG	4
1526015264	NFX1	9	3	36	delT	1
1573925487	GABBR2	9	17	59	insT	4
1580509237	ABCA1	9	4	24	insT	1
1637516682	ARMC3	10	18	19	delT	1
1637516683	ARMC3	10	18	19	delT	1
1657313100[a]	AGAP4	10	7	19	delT	2
1657313101[a]	AGAP4	10	7	14	delT	3
1807397040	SYVN1	11	7	15	insA	1
1832107207	LOC642446	11	1	18	delT	4
1855967268	ZW10	11	8	40	delC	1
1861246651	SLC37A4	11	3	143	delC	4
1884320486	ATN1	12	4	15	delA	1
1929584670	KIAA0748	12	6	31	delC	4
1955959709	PPFIA2	12	18	52	delA	4
1994379801	CIT	12	17	28	delG	1
1995110709	DYNLL1	12	2	14	delG	1
1997144069	KNTC1	12	2	31	delC	4
2105624179	RNASE4	14	1	15	delC	1

Supplementary Table 6. Continued

Reference position	Gene	Chromosome	Coding sequence	Coverage	Allele change	Patient no.
2162358340	C14orf133	14	13	15	delT	1
2243652079	NR2E3	15	6	129	delC	4
2256057594	ADAMTSL3	15	12	26	delT	1
2277509768[a]	NLRC3	16	7	12	delG	2
2302633200	EIF3C	16	4	18	delG	4
2303380808	SULT1A4	16	3	24	delA	1
2351412465	LOC100289580	16	2	103	delC	4
2356396572	P2RX5	17	3	40	delG	4
2376621991	SPAG5	17	3	13	delC	1
2386619109	CCDC49	17	5	14	delT	1
2413869089	APOH	17	5	24	delC	1
2501962862	ZNF516	18	2	29	delG	3
2507200605	MUM1	19	8	36	delG	1
2538892348	C19orf55	19	9	20	delG	2
2565046537	UBOX5	20	2	15	delG	1
2587599923	ZNF337	20	4	19	delT	1
2598525448	ZHX3	20	1	19	delT	1
2625038622	NRIP1	21	1	24	delG	1
2661518554	FAM108A5	22	2	13	delG	3
2748277559	SPIN2B	X	1	13	delG	2
2792445004	TEX13A	X	2	18	delC	3

[a]These indels commonly occurred in more than one HCC.

Supplementary Table 7. List of 40 Somatic Mutations With Amino Acid Changes Commonly Detected in Both the Tumor (at a Frequency of More Than 20% of Reads) and Matched Nontumorous Cirrhotic Liver (at a Frequency of More Than 5% of Reads) of the Same Patient

					Tumor		Nontumor	
Gene	Reference position	Chromosome	Reference nucleotide	Mutation nucleotide	Mutation frequency (%)	Patient no.	Mutation frequency (%)	Patient no.
LEPR	65548341	1	C	A	25.8	3	15.0	3
							21.9	1
ZNF408	1792629936	11	T	A	20.4	2	16.0	2
							15.8	4
HRNR	129676984	1	G	C	28.9	3	5.4	3
PXDN	228577682	2	G	C	45.1	4	47.2	4
POTEF	353150970	2	T	A	41.8	4	31.0	4
ALPP	455451136	2	C	T	32.5	4	37.5	4
GPR125	682521774	4	C	A	38.1	2	40.0	2
HERC6	746068457	4	T	A	36.5	4	44.9	4
EGFLAM	886579974	5	T	G	23.3	3	5.3	3
C4A	1057829599	6	T	G	25.0	2	11.5	2
WISP3	1134999625	6	T	G	43.3	4	64.3	4
C7orf10	1234451360	7	T	A	25.0	3	8.3	3
PVRIG	1290339880	7	C	T	23.5	1	21.3	1
MUC17	1291200140	7	G	A	21.2	4	12.5	4
PLOD3	1291376235	7	G	C	48.2	4	51.7	4
COL27A1	1589933932	9	A	G	56.8	4	54.6	4
AGAP9	1658906463	10	T	G	36.7	4	16.2	4
POLL	1713935693	10	G	T	44.8	4	38.6	4
MUC5AC	1747183167	11	G	A	43.9	4	43.8	4
MRGPRX3	1764064669	11	T	C	40.0	4	42.5	4
TMEM133	1843211533	11	A	C	59.5	4	83.3	4
TMEM123	1844621025	11	G	A	27.3	2	7.3	2
TMPRSS4	1860336319	11	C	T	54.4	4	41.3	4
DHRS4L2	2108914889	14	G	T	20.5	3	11.5	3
GOLGA6C	2247104814	15	A	T	21.7	4	9.6	4
PRSS22	2276813235	16	C	T	50.0	4	36.7	4
FAM38A	2351390771	16	C	T	21.4	4	54.3	4
GGT6	2357265990	17	G	A	92.3	4	41.7	4
COX10	2366897810	17	C	T	55.2	4	36.3	4
KIAA0100	2376657621	17	A	C	47.8	4	60.0	4
TBC1D3B	2384202011	17	C	T	63.0	4	27.4	4
TBC1D3D	2385938140	17	A	G	45.9	4	21.0	4
ERBB2	2387531879	17	A	G	66.7	4	54.6	4
CSH2	2411602334	17	C	T	90.9	4	79.5	4
QRICH2	2423941144	17	T	G	50.0	4	60.4	4
MOCOS	2461870479	18	T	C	72.0	4	62.2	4
CPAMD8	2522819358	19	G	A	21.8	3	15.2	3
MAP4K1	2541732174	19	G	A	36.0	4	54.3	4
PSG8	2545901763	19	C	A	28.3	3	9.5	3
KRTAP12-2	2654734983	21	C	T	59.3	4	43.5	4

NOTE. The first 2 genes listed were recurrently mutated in the nontumorous inflamed livers of 2 patients.

Supplementary Table 8. Overview of Selected Exome Sequencing Data From 22 Patients With HCV Infection

		Aligned reads	Aligned sequence (*base pairs*)	Median read depth
TP53	Tumor	29,334	2,035,570	1476.2
	Nontumor	31,848	2,200,641	1575.3
	Lymphocytes	36,690	2,539,944	1917.2
CTNNB1	Tumor	90,022	6,215,000	2344.3
	Nontumor	75,785	5,282,450	1991.2
	Lymphocytes	100,430	7,013,325	2710.8
LEPR	Tumor	34,328	2,390,335	538.3
	Nontumor	60,128	4,219,089	1025.6
	Lymphocytes	86,830	6,085,511	1423.0

NOTE. Selected exome sequencing of *TP53*, *CTNNB1*, and *LEPR* was performed for 22 nontumorous cirrhotic liver tissues, 10 HCC tissues, and matched peripheral lymphocytes from each patient. Aligned reads, aligned sequences (base pairs), and median read depth are shown for each sample.

Supplementary Table 9. Clinical Features and Overview of Deep Sequencing Data of Patients Who Underwent Deep Sequencing of the *LEPR* Gene

	Chronic hepatitis (n = 15)	Normal liver (n = 9)
Age (*y*)	59.3	55.9
Sex (male/female)	6/9	7/2
Aligned reads	4290	3956
Aligned sequence (*base pairs*)	1,044,737	1,275,068
Median read depth	2838	3440
No. of mutations in the *LEPR* gene	0	0

NOTE. We determined the sequences of the *LEPR* gene in the liver of 15 noncirrhotic patients with HCV-associated chronic hepatitis. In addition, normal liver tissues were obtained from 9 liver donors at the time of the operation. Age, sex, aligned reads, aligned sequences (base pairs), median read depth, and numbers of mutations are shown.

Supplementary Table 10. Mean Body Weights and Serum Levels of Insulin, Triglyceride, Total Cholesterol, and Alanine Aminotransferase of C57BL/KsJ-*db/db* (*db/db*) Mice and Misty (Control) Mice After 4 Weeks of Treatment With TAA

	db/db	Control
Body weight (*g*)	46.5 ± 0.6	23.5 ± 0.4
Insulin (*ng/mL*)	30.6 ± 28.3	1.6 ± 0.2
Triglyceride (*mg/dL*)	95.0 ± 5.0	50.0 ± 20.0
Total cholesterol (*mg/dL*)	215.0 ± 15.0	95.0 ± 15.0
Alanine aminotransferase (*IU/L*)	1325.0 ± 1085.0	75.0 ± 35.0

NOTE. All data are presented as mean ± SD.

Supplementary Table 11. Categorization of the Mutated Genes Detected by Whole Exome Sequencing of the AID-Expressing Hepatocyte Cell Line Using the Kyoto Encyclopedia of Genes and Genomes Database

Pathway				
Metabolic pathways	ATP6V0A4	DMGDH	HSD17B3	PGD
	ATP6V1C2	GALNT1	HYAL2	PHGDH
	BCMO1	GATM	NDST1	POLR3B
	CPS1	HKDC1	PAH	
PI3K-Akt signaling pathway	BCL2L11	IBSP NOS3	PRKCZ	TEK
	COL27A1			
MAPK signaling pathway	FLNB	SP1	CACNA1F	PTPN7
Cytokine-cytokine receptor interaction	LEPR	TNFRSF8	TNFRSF10A	
Transcriptional misregulation in cancer	EYA1	GZMB	JMJD7-PLA2G4B	
Proteoglycans in cancer	FLN	ITGB3	TIMP3	VTN
PPAR signaling pathway	CPT1B	CYP4A22	PPARD	
Cell cycle	E2F2	ESPL1	MCM7	
Pathways in cancer	FLT3	TRAF4	PDGFA	
Hedgehog signaling pathway	GLI3	LRP2	CSNK1A1L	
Others	95 genes			

NOTE. The genes categorized in multiple pathways are shown in only one representative pathway. Constitutive AID expression resulted in the accumulation of nucleotide alterations in various genes, including LEPR, of the cultured hepatocyte–derived cells. Whole exome sequencing was performed on DNA derived from established non-neoplastic human primary hepatocyte cells[6] with constitutive AID expression. AID expression in the cultured hepatocytes was performed using a lentiviral system.[5] After 8 weeks of AID expression, the DNA was extracted and subjected to whole exome sequencing as described in Materials and Methods. Overall, a total of 460 nucleotide positions in 380 different genes were defined as mutated in the AID-expressing cultured hepatocytes through the variant filtering process. Among them, pathway analyses by the Kyoto Encyclopedia of Genes and Genomes revealed that many genes, including LEPR, were categorized into well-known signaling pathways: the metabolic pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, cytokine-cytokine receptor interaction pathway, and transcriptional misregulation in cancer pathway. Only categorized genes are shown.

Chronic Rejection Associated with Antiviral Therapy for Recurrent Hepatitis C after Living-Donor Liver Transplantation

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Background. Chronic rejection (CR) has been reported to be associated with antiviral therapy for recurrent hepatitis C in liver transplant (LT) recipients. The aims of this study were to clarify the details of antiviral therapy-associated CR after living-donor liver transplantation (LDLT) and to identify the factors associated with CR.

Methods. A retrospective chart review was performed on 125 recipients who had received antiviral therapy for recurrent hepatitis C after LDLT between January 2001 and September 2012. The characteristics of patients who developed CR during or within 6 months after antiviral therapy were compared with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year.

Results. Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. In five patients, rejection progressed rapidly and resulted in death within 3 months after diagnosis. Analysis revealed two significant factors associated with CR: reduction of the immunosuppressant dose during antiviral therapy and a low fibrosis score as the indication for antiviral therapy.

Conclusions. CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. This complication may be prevented by ensuring that the immunosuppressant dose is not reduced during antiviral therapy.

Keywords: Chronic rejection, Hepatitis C, Liver transplantation, Living donor, Antiviral therapy.

(*Transplantation* 2014;97: 344–350)

Hepatitis C virus (HCV) infection, which leads to liver cirrhosis and hepatocellular carcinoma, is the most common indication for liver transplantation (LT) in Japan, the United States, and western Europe. Most patients who undergo LT for HCV-related liver disease develop recurrent viral infection, and 70% to 90% suffer from histologically proven recurrent hepatitis (*1–6*). The progression of recurrent hepatitis C is often rapid. Without appropriate antiviral therapy, 10% to 25% of patients develop cirrhosis within 5 years after transplantation, and this explains the relatively poor prognosis for HCV-positive recipients compared with HCV-negative recipients (*7*). Interferon (IFN)–based combination therapy is commonly administered to prevent the progression of hepatitis C after LT (*8, 9*), but its efficacy in LT recipients is limited. The mean (range) sustained virologic response (SVR) rate in patients with recurrent hepatitis C after LT is only 30% (8%–50%) (*10*). One reason for the low SVR rate is the high rate of treatment withdrawal, particularly because of the unique adverse effects of IFN therapy for transplant recipients, including chronic rejection (CR) (*11, 12*).

CR is characterized by progressive ductopenia, with atrophy and loss of the bile ducts in the portal tracts and by arteriopathy with foamy cell infiltration (*13–15*). A cholestatic liver enzyme pattern suggests the diagnosis of CR. If bile duct enlargement and/or hepatic artery changes are excluded by imaging studies as potential causes of abnormal liver function tests, then CR is confirmed or excluded by liver biopsy examination. The incidence of CR after LT is

This work was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research 21229009 and 23590972, Health and Labor Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.
The authors declare no conflicts of interest.

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Received 5 August 2013. Revision requested 13 August 2013.
Accepted 28 August 2013.

ISSN: 0041-1337/14/9703-344
DOI: 10.1097/01.TP.0000435702.61642.0a

approximately 3% to 5%. This event does not simply represent end-stage acute cellular rejection (ACR), although the two may be temporally related. The pathogenesis of CR is not completely understood, although its association with donor-specific human leukocyte antigen antibodies was recently reported (*16*). Additional immunosuppressive therapy is unlikely to be beneficial for CR patients, particularly those with late disease in which bile duct loss affects more than 50% of the portal tracts, and retransplantation is required (*15*).

Several studies have suggested an association of CR with IFN-based antiviral therapy (*17–20*). Two recent reports found that CR was associated with antiviral therapy for recurrent hepatitis C after LT (*11, 12*). Stanca et al. (*12*) reported that 12 of 70 LT recipients with HCV infection treated with pegylated IFN (peg-IFN) and ribavirin developed CR. Their study indicated that ACR and CR are not strongly associated and that CR progresses rapidly, terminating in graft failure. Fernandez et al. (*11*) reported that 7 of 79 (9%) patients developed CR during antiviral therapy. They found that the use of cyclosporine in immunosuppression therapy, achievement of an SVR, and ribavirin discontinuation were factors associated with CR development.

Although the details of patients with antiviral therapy-associated CR after deceased-donor liver transplantation (DDLT) have been reported (*11, 12*), no study of antiviral therapy-associated CR in patients receiving living-donor liver transplantation (LDLT) has been published thus far. The features specific to LDLT, including blood-relative donors, posttransplantation liver regeneration, and ABO-incompatible LT, might result in characteristic differences between LDLT and DDLT patients.

We aimed to clarify the details of antiviral therapy-associated CR after LDLT and to identify the factors associated with CR.

RESULTS

Patient Characteristics and Treatment Outcomes

The study included 125 HCV-infected LT patients treated with standard IFN and/or peg-IFN in combination with ribavirin for recurrent hepatitis C after LDLT. Of these, 69 (55%) were men (median [range] age at the beginning of therapy, 57 [32–70] years). Most patients were infected with HCV genotype 1b (n=101 [81%]). The HCV genotype for the remaining patients was 2a (n=14), 2b (n=6), 3a+3b (n=1), and indeterminate (n=2). Genotype was not examined in one patient. The median (range) serum HCV RNA load at the beginning of antiviral therapy after LDLT was 3980 (31 to <69,000) kIU/mL. The median (range) donor age was 42 (19–65) years. Seventy-three (58%) donors were men, and 84 (67%) were blood relatives of the recipients. The graft type was the right lobe for 108 (86%) patients and the left lobe for 17 (14%) patients. The blood type combination was incompatible for 27 (22%) patients. Thirty-six (29%) patients had histologically diagnosed ACR before antiviral therapy, 16 of whom had moderate or severe ACR. No patient had shown ACR findings in the liver biopsy examination immediately before antiviral therapy. The median (range) time to treatment initiation after LDLT was 8.9 (1.1–72.4) months. Before treatment, necroinflammatory activity of levels A1, A2, and A3 based on the METAVIR score was found in 82 (66%), 40 (32%), and 3 (2%) patients, respectively. Fibrosis scores of F0, F1, F2, and F3 were found in 19 (15%), 82 (66%), 19 (15%), and 5 (4%) patients, respectively. Tacrolimus-based immunosuppression was administered to 117 (94%) patients and cyclosporine was administered to 7 (6%) patients. Mycophenolate mofetil (MMF) without calcineurin inhibitor (CNI) was administered to one patient because of renal failure at the beginning of antiviral therapy. In the patients who received tacrolimus, the mean (range) serum trough level at therapy initiation was 6.2 (2.0–12.7) ng/mL. In addition to CNIs, MMF and prednisolone were administered at the start of the antiviral treatment to 39 (31%) and 21 (17%) patients, respectively.

Of the 123 patients in whom the final treatment outcomes could be evaluated, 54 (44%) patients achieved SVR, 12 (10%) relapsed, 30 (24%) were nonresponders, and 27 (22%) withdrew from treatment. The remaining two patients were still undergoing treatment during the analysis.

Characteristics of Patients with Antiviral Therapy-Associated CR

Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. The characteristics and clinical courses of these seven patients are shown in Table 1. Although four patients had a history of ACR before antiviral therapy was initiated (three of whom had moderate or severe ACR), three had no previous ACR episodes. The METAVIR score-based fibrosis level before antiviral therapy was F0 in three of the seven patients, F1 in three patients, and F2 in one patient, indicating that the antiviral therapy had been initiated at an early stage of fibrosis. The median (range) time from transplantation to initiation of antiviral therapy in these seven recipients was 9 (2–72) months. Tacrolimus was administered to five patients and cyclosporine was administered to one patient when the antiviral therapy was initiated. One patient did not receive a CNI because of renal failure (patient 7). Four patients received MMF, and one patient received prednisolone in combination with tacrolimus and MMF. The trough levels of tacrolimus and cyclosporine were within the therapeutic range. Standard amounts of immunosuppressant were therefore used for all patients, except for patient 7 who received MMF only. Immunosuppressant doses were reduced during therapy in five of seven patients. The tacrolimus dose was reduced for two patients (patients 2 and 3), as a result of which the blood trough level of tacrolimus decreased by approximately 2 ng/mL. In patient 3, MMF (500 mg/day) was also stopped during treatment. In patient 4, the MMF dose was reduced from 1000 to 250 mg per day, and prednisolone treatment (2.5 mg/day) was also terminated during treatment. In patient 5, MMF (1000 mg/day) was stopped immediately after initiation of antiviral therapy. Patient 6 received no CNI, and MMF dose was reduced from 500 to 250 mg per day during treatment. Three patients received standard IFN, and four received peg-IFN. Ribavirin was not administered to three patients immediately before the diagnosis of CR because of anemia.

CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. Two patients were diagnosed

TABLE 1. Characteristics of patients with CR associated with antiviral therapy

Patient	1	2	3	4	5	6	7
Age (years)	62	41	45	67	50	59	49
Gender	Female	Male	Female	Female	Female	Male	Male
ABO mismatch with donor	Match	Match	Match	Mismatch	Match	Mismatch	Match
Relation to donor	Related	Related	Nonrelated	Related	Nonrelated	Nonrelated	Nonrelated
Graft type (lobe)	Right	Right	Right	Right	Left	Right	Right
Splenectomy	No	No	No	No	Yes	Yes	No
Previous ACR	Yes	Yes	Yes	No	Yes	No	No
Previous moderate/severe ACR	Yes	No	Yes	No	Yes	No	No
Previous steroid pulse	Yes	No	No	No	Yes	No	No
HCV genotype	1b	1b	1b	2a	1b	1b	1b
HCV RNA (kIU/mL) before IFN	>850	3620	1790	>5000	<5000	<5000	16,000
METAVIR score before IFN	A2 F2	A2 F0	A1 F0	A1 F1	A2 F1	A1 F0	A1 F1
Months from LT to IFN	13	2	5	13	7	9	72
Months from initiation of IFN to diagnosis of CR	9	1	16	10	15	8	7
Immunosuppressant at initiation of IFN	Tacrolimus	Tacrolimus,	Tacrolimus, MMF	Tacrolimus, MMF, PSL	Cyclosporine, MMF	Tacrolimus, MMF	MMF
Trough level of CNI	7.8	7.9	7.9	6.8	152	5.9	—
Reduction of immunosuppressant during IFN (reduced drugs)	No	Yes (tacrolimus)	Yes (tacrolimus, MMF)	Yes (MMF, PSL)	Yes (MMF)	No	Yes (MMF)
Type of IFN	Standard	Standard	Standard	Pegylated	Pegylated	Pegylated	Pegylated
Ribavirin discontinuation	No	No	Yes	Yes	No	No	Yes
IFN at diagnosis of CR	On treatment	On treatment	1 month after end of IFN	5 months after end of IFN	On treatment	On treatment	On treatment
At diagnosis of CR							
Liver biopsy	Foam cell arteriopathy, bile duct atrophy	Bile duct atrophy	Bile duct atrophy	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Foam cell arteriopathy, bile duct atrophy
AST (IU/L)	121	90	53	73	331	124	36
ALT (IU/L)	67	37	43	63	288	52	32
ALP (IU/L)	2034	906	494	1751	2143	528	1164
γ-GTP (IU/L)	561	768	155	209	515	27	1489
Bilirubin (mg/dL)	18.6	18.8	31.5	38.1	11.8	16.4	22.6
HCV RNA (kIU/mL)	Undetectable	460	Undetectable	Undetectable	16,000	Undetectable	0.40
Treatment for CR	Tacrolimus, MMF	Tacrolimus	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, PSL	Tacrolimus, MMF, rapamycin, steroid pulse	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, rapamycin, steroid pulse
Outcome	Died	Alive	Died	Died	Died	Died	Died
Months from diagnosis of CR to death	64	—	1	1	1	3	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; PSL, prednisolone.

with CR after antiviral therapy was terminated. Antiviral therapy was discontinued in the remaining five patients. Of note, six patients were treated with IFN for more than 7 months, suggesting that long-term administration of IFN is associated with CR. Liver biopsy was performed for diagnosis of CR because of abnormal liver function tests in all cases. All patients with documented CR had high levels of alkaline phosphatase (ALP). Total bilirubin levels were extremely high (11.8–38.1 mg/dL) at diagnosis, suggesting a delayed diagnosis of CR. All liver biopsies showed atrophy affecting most bile ducts as well as hepatocanalicular cholestasis. Two patients (patients 1 and 7) showed foam cell obliterative arteriopathy. Bile duct loss was shown in 100%, 67%, and 20% of the portal tracts in patients 4, 5, and 6, respectively. In none of the seven patients was evidence of ACR found in the biopsy specimens. Hepatic artery or biliary tract obstruction or structuring was excluded by imaging in all patients.

Serum HCV RNA was undetectable in four patients at CR diagnosis and remained undetectable in all four patients during the follow-up period. Two of the four patients were considered to have SVR. Final outcomes could not be determined in the remaining two patients who died within 24 weeks after termination of treatment.

Various intensive treatment protocols were used for these seven patients after CR diagnosis, including increase of tacrolimus dose, addition or increase in MMF and/or prednisolone dose, administration of steroid pulse therapy, and inclusion of rapamycin in the therapy. CR progressed rapidly to liver failure in five patients (patients 3–7). These five patients died within 3 months after diagnosis of CR due to liver failure and infection. The liver damage in patient 1 gradually progressed to liver failure, and the patient died at 64 months after CR was diagnosed. Only one patient (patient 2) recovered from CR and survived, although a follow-up liver biopsy showed chronic hepatitis C.

Risk Factors of CR Associated with Antiviral Therapy

Factors associated with the development of CR during and after antiviral therapy were analyzed by comparing the features of 7 CR patients with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year (Table 2). A reduction of the immunosuppressant dose during antiviral therapy (P=0.034) and a low fibrosis stage before antiviral therapy (P=0.045) were significantly associated with antiviral therapy-related CR. No significant associations were found with other variables, including donor factors, ribavirin discontinuation, and undetectable HCV RNA. The rate of previous ACR (P=0.065), rate of previous moderate or severe ACR (P=0.059), ALP level (P=0.121), and γ-glutamyl transpeptidase (γ-GTP) level (P=0.051) before antiviral therapy was higher in the patients who developed CR, but the differences from patients without CR were not significant.

DISCUSSION

Of the 125 patients, 7 (6%) who received antiviral therapy for hepatitis C after LDLT developed CR. CR progressed rapidly, resulting in death within 3 months after diagnosis, in 5 of these 7 patients.

The risk of rejection have been suggested to increase with IFN administration because of the drug's theoretical immunomodulatory actions, such as up-regulation of human leukocyte antigen class II antigens and induction of proinflammatory cytokines (*21*). Previous studies have reported that the frequency of CR in patients who received IFN was substantially higher compared with patients who did not receive antiviral therapy (*11, 12, 17*). In the present study, the rate of antiviral therapy-associated CR was 6%. This rate is high, because no CR occurred in the entire study period other than during or within 6 months after termination of antiviral therapy in the 230 HCV-positive recipients analyzed. Some cases showed sudden onset of CR after a long transplantation period in the absence of preexisting ACR, supporting the association of antiviral therapy with CR.

In our analysis, the two significant risk factors for CR were reduction of the immunosuppressant dose during antiviral therapy and low fibrosis score at antiviral therapy initiation. Additional characteristics associated with CR were elevated cholestatic enzyme levels at the time of diagnosis, onset of CR more than 7 months after treatment initiation (excluding one patient) and poor prognosis after the diagnosis. The MMF dose was reduced or stopped during antiviral therapy in four of five patients who had received MMF at the start of the treatment. We had initially tried to reduce the MMF dose during antiviral therapy, because MMF is known to suppress the bone marrow and could therefore augment the cytopenic effects of IFN and ribavirin. We had reduced immunosuppressant according to our reduction protocol even during antiviral therapy. Based on the data, we subsequently changed our strategy to maintaining the MMF dose and increasing the trough level of CNIs during antiviral therapy. The reason for the association between the low fibrosis score and CR is currently unclear. Although some institutions recommend early introduction of antiviral therapy (*8, 9*), our data suggest that antiviral therapy should not be administered to patients with no or mild fibrosis. On the contrary, it is reported that tolerance to therapy decreases significantly in patients with a fibrosis stage ≥3 on baseline liver biopsy (*22*). Therefore, the antiviral therapy should be initiated in patients with a fibrosis stage 2, as the recent review articles recommended (*23, 24*).

All our patients underwent LDLT, but no characteristics specific to LDLT, including blood-relative donors, graft size, and ABO incompatibility, were identified as risk factors for CR in our study. This appears to indicate that LDLT and DDLT patients do not differ with respect to antiviral therapy-associated CR.

Early diagnosis of CR, as well as prevention, is important for ensuring improved outcomes in LT recipients. CR was diagnosed in our patients after liver damage had already progressed. Histologic diagnosis of CR was difficult in all these cases, despite repeated liver biopsy examination. However, all the patients had elevated ALP and γ-GTP levels before jaundice was observed. CR should therefore be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy for hepatitis C. When imaging has excluded large bile duct and/or hepatic artery changes as the

TABLE 2. Risk factors for CR

	CR (n=7)	No CR (n=76)	P
Age at LT (years)	50 (41−67)	56 (36−69)	0.506[a]
Gender, male/female	3/4	44/32	0.352[b]
HCV genotype, 1/non-1	6/1	71/5	0.421[b]
Donor age at LT (years)	46 (28−60)	42 (21−65)	0.857[a]
Donor gender, male/female	4/3	40/36	0.568[b]
Sex mismatch, match/mismatch	0/7	26/50	0.064[b]
ABO mismatch, match/mismatch	5/2	59/17	0.507[b]
Relation to donor, related/nonrelated	3/4	48/28	0.254[b]
HLA-A matched number, 0/1/2/unknown	0/5/2/0	13/44/16/3	0.332[a]
HLA-B matched number, 0/1/2/unknown	2/4/1/0	21/47/5/3	0.778[a]
HLA-DR matched number, 0/1/2/unknown	3/3/1/0	18/47/8/3	0.487[a]
Graft type, left lobe/right lobe	1/6	9/67	0.608[b]
Splenectomy, yes/no	2/5	38/38	0.247[b]
Previous ACR, yes/no	4/3	17/59	0.065[b]
Previous moderate/severe ACR, yes/no	3/4	9/67	0.059[b]
Previous steroid pulse therapy, yes/no	2/5	8/68	0.198[b]
Months from LT to therapy	9.0 (1.8−72.4)	9.1 (2.2−68.8)	0.694[a]
Valuables at initiation of IFN			
Age (years)	55 (41−68)	57 (37−70)	0.599[a]
CNI tacrolimus/cyclosporine	5/1	71/5	0.376[b]
Trough level for tacrolimus (ng/mL)	7.3 (0−7.9)	6.2 (2.6−10.9)	0.641[a]
AST (IU/L)	68 (24−464)	76 (21−331)	0.908[a]
ALT (IU/L)	88 (25−354)	79 (20−392)	0.842[a]
ALP (IU/L)	878 (283−2977)	462 (168−2818)	0.121[a]
γ-GTP (IU/L)	317 (48−1623)	112 (15−1704)	0.051[a]
Bilirubin (mg/dL)	0.8 (0.3−10.4)	0.9 (0.3−4.6)	0.861[a]
Activity grade, A1/A2/A3	4/3/0	50/24/2	0.693[a]
Fibrosis stage, F0/F1/F2/F3	3/3/1/0	4/56/13/3	0.045[a]
Reduction of immunosuppressant during IFN, yes/no	5/2	22/54	0.034[b]
Ribavirin discontinuation during IFN, yes/no	3/4	26/50	0.468[b]
Undetectable HCV RNA during IFN, yes/no	4/3	51/25	0.439[b]

[a] Wilcoxon rank-sum test.
[b] Chi-square test.
Comparison was made between 7 patients with CR and 76 patients without CR despite receiving antiviral therapy for more than 1 year (No CR). Qualitative variables expressed as number. Quantitative variables expressed as median (range).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA, human leukocyte antigen.

potential etiology of abnormal liver function, we believe that cessation of antiviral therapy and initiation of intensive immunosuppressive therapy should be considered, even without histologic confirmation of CR.

Some limitations of this study are its retrospective nature and relatively small sample size. Because the frequency of CR was low, the sample size was not adequate for multivariate analysis.

In conclusion, CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. Reduction of the immunosuppressant dose during antiviral therapy should be avoided and antiviral therapy should not be administered to patients with no or mild fibrosis to prevent antiviral therapy-associated CR. Early CR diagnosis should be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy. In these cases, discontinuation of antiviral therapy and increase in the immunosuppressant dose are recommended when other causes of liver dysfunction are excluded.

MATERIALS AND METHODS

Patients

A total of 232 patients with HCV-related end-stage liver disease underwent LDLT at Kyoto University Hospital between March 1999 and September 2012. Two patients who received a liver graft from an identical twin were excluded from this study, because they did not require immunosuppression because of genetic identity. Of the remaining 230 patients, 157 patients were followed up for more than 6 months after LDLT in our hospital. Antiviral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and September 2012. They were diagnosed with recurrent hepatitis C after LDLT via serum HCV RNA analysis and histologic evidence. The remaining 32 patients did not receive antiviral therapy for various reasons: serum HCV RNA negative after LDLT (n=4), no histologic hepatitis C recurrence in the follow-up period (n=13),

no fibrosis seen by liver histology (n=8), and ongoing treatment for the other complications (n=7). CR was defined histologically according to the updated International Banff Schema for Liver Allograft Rejection with the following criteria: (a) the presence of bile duct atrophy/pyknosis affecting most of the bile ducts with or without bile duct loss, (b) convincing foam cell obliterative arteriopathy, or (c) bile duct loss affecting more than 50% of the portal tracts (*13*). Patients who were diagnosed with CR based on these diagnostic criteria during or within 6 months after terminating antiviral therapy were examined for antiviral therapy-associated CR. The clinical features of these 7 patients with CR were compared with those of 76 patients who did not have CR despite receiving antiviral therapy for more than 1 year to determine the risk factors for CR.

The study protocol was approved by the ethics committee at Kyoto University and performed in compliance with the Helsinki Declaration.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, 40 patients with recurrent hepatitis C after LDLT received treatment with IFN-α-2b plus ribavirin (*25*). From May 2004 to June 2011, patients received combination therapy with peg-IFN-α-2b plus ribavirin (*26*). Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months. Patients who tested negative for serum HCV RNA for more than 6 months after completing IFN therapy were defined as having achieved SVR. For those who tested positive for serum HCV RNA after 12 months of treatment, therapy was discontinued or switched to maintenance therapy with low-dose peg-IFN (*27*), and patients were classified as having shown no response.

Histologic Assessment

Liver biopsy examination was performed when patients showed abnormal liver function tests, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by two pathologists (H.H. and A.M.-H.) with extensive experience in the pathology of LT. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores (*28*).

Immunosuppression

Tacrolimus with low-dose steroid or MMF was administered to most patients for immunosuppression (*25*). The target whole blood lower level for tacrolimus was 10 to 15 ng/mL during the first 2 weeks, 10 ng/mL thereafter, and 5 to 8 ng/mL starting from the second month. Steroid therapy was initiated at a dose of 10 mg/kg methylprednisolone before graft reperfusion then tapered down from 1 mg/kg per day on days 1 to 3, to 0.5 mg/kg per day on days 4 to 6, and to 0.3 mg/kg per day on day 7. Subsequently, oral prednisolone was continued at 0.3 mg/kg per day until the end of the first month, and this was followed by 0.1 mg/kg per day until the end of the third month. After that, steroid administration was terminated. MMF was initiated at a starting dose of 10 to 15 mg/kg on day 1, which was gradually increased to a target dose of 30 mg/kg, and this was continued for 6 months. Thereafter, MMF administration was terminated. Four patients received cyclosporine microemulsions instead of tacrolimus. MMF and/or prednisolone was administered again to patients who experienced refractory rejection or required reduction of the tacrolimus or cyclosporine dose because of adverse events and then tapered down gradually. Twenty-seven patients who received ABO-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone (*29*).

Virologic Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific primers (*30*). The serum HCV RNA load was evaluated before LDLT, before IFN treatment, once a month during treatment, and 24 weeks after treatment using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor; Roche Molecular Systems, Pleasanton, CA) until April 2008. A real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems) was used alternatively from May 2008.

Statistical Analysis

To evaluate the association between patient characteristics and CR, the characteristics were defined and compared between patients with and without CR. Medians and ranges were determined for continuous variables, and data were analyzed using the Wilcoxon rank-sum test. Categorical variables were expressed as counts, and data were analyzed using the chi-square test. A significance level of $P<0.05$ was considered significant. Statistical analyses were performed using PASW Statistics version 18.0.0 (SPSS, an IBM company).

REFERENCES

1. Berenguer M, Prieto M, San Juan F, et al. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. *Hepatology* 2002; 36: 202.
2. Feray C, Caccamo L, Alexander GJ, et al. European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. *Gastroenterology* 1999; 117: 619.
3. Forman LM, Lewis JD, Berlin JA, et al. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; 122: 889.
4. Gane E. The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. *Liver Transpl* 2003; 9: S28.
5. Prieto M, Berenguer M, Rayon JM, et al. High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. *Hepatology* 1999; 29: 250.
6. Sanchez-Fueyo A, Restrepo JC, Quinto L, et al. Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. *Transplantation* 2002; 73: 56.
7. Velidedeoglu E, Mange KC, Frank A, et al. Factors differentially correlated with the outcome of liver transplantation in HCV+ and HCV- recipients. *Transplantation* 2004; 77: 1834.
8. Gordon FD, Kwo P, Vargas HE. Treatment of hepatitis C in liver transplant recipients. *Liver Transpl* 2009; 15: 126.
9. Terrault NA. Hepatitis C therapy before and after liver transplantation. *Liver Transpl* 2008; 14: S58.
10. Berenguer M. Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. *J Hepatol* 2008; 49: 274.
11. Fernandez I, Ulloa E, Colina F, et al. Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for post-transplant recurrent hepatitis C. *Liver Transpl* 2009; 15: 948.
12. Stanca CM, Fiel MI, Kontorinis N, et al. Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. *Transplantation* 2007; 84: 180.
13. Demetris A, Adams D, Bellamy C, et al. Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An International Panel. *Hepatology* 2000; 31: 792.
14. Demetris AJ. Distinguishing between recurrent primary sclerosing cholangitis and chronic rejection. *Liver Transpl* 2006; 12: S68.
15. O'Grady J. The immunoreactive patient: rejection and autoimmune disease. *Liver Transpl* 2011; 17: S29.
16. O'Leary JG, Kaneku H, Susskind BM, et al. High mean fluorescence intensity donor-specific anti-HLA antibodies associated with chronic rejection postliver transplant. *Am J Transplant* 2011; 11: 1868.
17. Berenguer M, Palau A, Fernandez A, et al. Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2006; 12: 1067.
18. Dousset B, Conti F, Houssin D, et al. Acute vanishing bile duct syndrome after interferon therapy for recurrent HCV infection in liver-transplant recipients. *N Engl J Med* 1994; 330: 1160.
19. Feray C, Samuel D, Gigou M, et al. An open trial of interferon alfa recombinant for hepatitis C after liver transplantation: antiviral effects and risk of rejection. *Hepatology* 1995; 22: 1084.

20. Stravitz RT, Shiffman ML, Sanyal AJ, et al. Effects of interferon treatment on liver histology and allograft rejection in patients with recurrent hepatitis C following liver transplantation. *Liver Transpl* 2004; 10: 850.
21. Selzner N, Guindi M, Renner EL, et al. Immune-mediated complications of the graft in interferon-treated hepatitis C positive liver transplant recipients. *J Hepatol* 2011; 55: 207.
22. Roche B, Sebagh M, Canfora ML, et al. Hepatitis C virus therapy in liver transplant recipients: response predictors, effect on fibrosis progression, and importance of the initial stage of fibrosis. *Liver Transpl* 2008; 14: 1766.
23. Berenguer M, Schuppan D. Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. *J Hepatol* 2013; 58: 1028.
24. Coilly A, Roche B, Samuel D. Current management and perspectives for HCV recurrence after liver transplantation. *Liver Int* 2013; 33: 56.
25. Ueda Y, Takada Y, Haga H, et al. Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. *Transplantation* 2008; 85: 855.
26. Ueda Y, Takada Y, Marusawa H, et al. Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. *Transplantation* 2010; 90: 661.
27. Ueda Y, Marusawa H, Kaido T, et al. Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation. *J Viral Hepat* 2012; 19: 32.
28. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; 24: 289.
29. Raut V, Mori A, Kaido T, et al. Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. *Transplantation* 2012; 93: 99.
30. Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201.

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Reactivation from occult HBV carrier status is characterized by low genetic heterogeneity with the wild-type or G1896A variant prevalence

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Background & Aims: Individuals negative for hepatitis B surface antigen (HBsAg) but positive for antibodies to hepatitis B core antigen (anti-HBc) are at risk of hepatitis B virus (HBV) reactivation under immunosuppressive conditions. We investigated clinical features and viral genetics in patients with reactivation from occult HBV infection triggered by chemotherapy or immunosuppressive therapy.

Methods: Clinical courses of 14 individuals originally HBsAg-negative but anti-HBc-positive that experienced HBV reactivation were examined. Ultra-deep sequencing analysis of the entire HBV genome in serum was conducted. Prevalence of the G1896A variant in latently infected livers was determined among 44 healthy individuals that were HBsAg-negative but anti-HBc-positive.

Results: In 14 cases, HBV reactivation occurred during (n = 7) and after (n = 7) termination of immunosuppressive therapy. Ultra-deep sequencing revealed that the genetic heterogeneity of reactivated HBV was significantly lower in patients with reactivation from occult HBV carrier status compared with that in patients from HBsAg carrier status. The reactivated viruses in each case were almost exclusively the wild-type G1896 or G1896A variant. The G1896A variant was detected in 42.9% (6/14) of cases, including two cases with fatal liver failure. The G1896A variant was observed in the liver tissue of 11.4% (5/44) of individuals with occult HBV infection.

Conclusions: Reactivation from occult HBV infection is characterized by low genetic heterogeneity, with the wild-type G1896 or G1896A variant prevalent.

Keywords: G1896A pre-core variant; Genetic heterogeneity; Immunosuppressive therapy; Occult HBV infection; Ultra-deep sequencing.

Received 10 December 2013; received in revised form 7 April 2014; accepted 24 April 2014; available online 4 May 2014

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Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; anti-HBc, antibodies to hepatitis B core antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; pre-C, pre-core; T-bil, total bilirubin.

Introduction

Clinical features and pathophysiology of hepatitis B virus (HBV) infection are determined by the balance between the host immune response and viral replication. Individuals with persistent HBV infection are at risk of viral reactivation when the host immune system is weakened. HBV reactivation can occur in patients positive for hepatitis B surface antigen (HBsAg) in the serum, under immunosuppressive conditions [1–4]. Evidence has revealed that individuals who are HBsAg-negative but positive for antibodies to hepatitis B core antigen (anti-HBc) can also undergo HBV reactivation, commonly referred to as *de novo* hepatitis B infection, in response to chemotherapy or immunosuppression [5,6]. HBV persists in the liver after the disappearance of HBsAg in individuals with previous exposure to the virus, retaining the serological footprint of anti-HBc positivity, with such a status defined as an occult HBV infection [7,8]. Based on viral transmission studies in living-donor liver transplant patients, we previously demonstrated that most healthy individuals with an occult HBV infection were latently infected by the episomal form of HBV, with ongoing viral replication occurring in the liver [9,10]. Subsequently, we encountered an occult HBV patient with leukemia who developed fatal liver failure caused by viral reactivation [11]. Current guidelines issued by the American Association for the Study of Liver Diseases indicate that immunocompromised patients should undergo testing for HBsAg and anti-HBc before receiving chemotherapy or immunosuppressive therapy; antiviral prophylaxis is recommended for HBV carriers at the onset of chemotherapy or immunosuppression [12]. However, the detailed clinical features and viral genetics of reactivation from occult HBV carrier status are not yet fully understood because of the low incidence of viral reactivation in HBsAg-negative immunocompromised individuals. For example, Hui *et al.* examined the frequency of *de novo* HBV hepatitis among

patients with malignant lymphoma [6]. They reported that 3.3% (8/244) of HBsAg-negative patients receiving rituximab-containing chemotherapy developed HBV reactivation. Moreover, HBV reactivation can also occur only infrequently in HBsAg-negative individuals without hematological malignancies under immunosuppressive conditions [13].

Various mutations in HBV genomes have important implications for sensitivity to antiviral therapy [14,15], and for the pathophysiology of liver diseases. As an example, acute infection with HBV variants containing point mutations at nucleotide 1896 (G1896A) in the pre-core (pre-C) region represents a high risk for developing acute liver failure (ALF) [16–18]. Similarly, predominant reactivation of G1896A variants is frequently observed in HBsAg-positive carriers who develop fatal viral reactivation under immunosuppressive conditions without antiviral prophylaxis [19]. Recent evidence indicates that reactivation from occult HBV infection is of particular concern because the clinical course and outcome of those patients commonly results in severe liver dysfunction and fatal ALF [6], with most fatal cases predominantly containing G1896A pre-C variants [20]. There are an estimated 3 billion individuals who are positive for anti-HBc worldwide, including 10% of the total population in Europe, 15% in the United States, 20% in Japan, and more than 50% in highly endemic areas such as China and Taiwan [21,22]. However, little is known about the prevalence of HBV infection with G1896A pre-C variants among occult HBV carriers, and how reactivation of G1896A pre-C variants leads to fatal consequences.

We examined HBV reactivation in HBsAg-negative and -positive patients. To clarify characteristics of the viral genome and its association with the pathophysiology of HBV reactivation, we used ultra-deep sequencing. This technique allowed for parallel amplification and detection of the full length of the HBV genome for a large number of sequences [23], and assisted in determining the genetic complexity of reactivated viral clones and the prevalence of G1896A pre-C variants.

Patients and methods

Patients and samples

Between April 2007 and July 2013, there were 1377 patients negative for HBsAg and positive for anti-HBc testing (220 patients with hematologic malignancies, 790 patients with solid tumors, and 367 patients with noncancerous diseases), prior to initiation of chemotherapy or immunosuppressive therapy at Osaka Red Cross Hospital, Hyogo Prefectural Amagasaki Hospital, Kitano Hospital, and Kyoto University Hospital. Among them, a total of 14 patients were diagnosed with HBV reactivation and their serum samples were available for further analyses (Table 1). All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and lacked any risk factors for external viral transmission, as demonstrated by the absence of blood transfusion, drug abuse, sexual contact, or blood contact with a known hepatitis virus carrier. No patients were co-infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus. All patients were longitudinally followed up at 0.5–3-month intervals until analysis (July 2013) or death. ALF was defined as the presence of hepatic encephalopathy and deranged blood coagulation (prothrombin time international normalized ratio >1.5) [24].

Serum samples were obtained at diagnosis of HBV reactivation as demonstrated by the appearance of circulating HBsAg and HBV DNA under immunosuppressive conditions. Serological HBV markers, including HBsAg, antibodies to HBsAg, anti-HBc, hepatitis B e antigen (HBeAg) and antibodies to HBeAg were measured by chemiluminescent enzyme immunoassay (CLEIA; Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) (COBAS Taqman HBV test; Roche, Branchburg, NJ, USA) with a lower detection limit of 2.1 log copies/ml. The level of HBV DNA was retrospectively quantified in eight samples from five patients with reactivation from occult HBV infection.

To examine the genetic heterogeneity and prevalence of G1896A variants, liver tissue was obtained from 45 consecutive healthy donors negative for HBsAg and positive for anti-HBc who underwent hepatectomy for living-donor liver transplantation at Kyoto University from April 1998 to March 2001. Additionally, we examined the reactivated viruses derived from the serum of six patients who had typical serologic characteristics of the inactive HBsAg carrier state before immunosuppressive therapy. These cases were originally HBsAg-positive, while liver function tests were within the normal range before viral reactivation.

The Kyoto University Ethics Committee approved this study, and written informed consent was obtained from all patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Sequencing

PCR and direct population Sanger sequencing, ultra-deep sequencing of the HBV genome, sequencing data analysis, and statistical analysis are described in the Supplementary materials and methods.

Data deposition

Sequence reads with Genome Analyzer were deposited in the DNA Data Bank of Japan Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.shtml) under accession number DRA001211.

Results

Clinical features and outcomes of reactivation from occult HBV infection after immunosuppression

Baseline clinical and virological characteristics of 14 patients who developed HBV reactivation under immunosuppressive conditions are summarized in Table 1. All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and had no history of liver dysfunction. Pre-reactivation sera from five patients were available for further analysis, and confirmed that serum HBV DNA was undetectable in the repeated high-sensitivity PCR [10]. Among the 14 patients, 12 cases had hematological malignancy and received chemotherapy with steroids (n = 12) and/or rituximab (n = 7), and with (n = 4) or without (n = 8) hematopoietic stem cell transplantation (Table 1). One patient was diagnosed with psoriasis and had single-agent cyclosporine therapy for 4 years. Another patient had colon cancer and underwent surgery followed by S-1 (Tegafur/gimeracil/oteracil; Taiho Pharmaceutical Co., Tokyo, Japan) adjuvant chemotherapy.

The median time between initiation of chemotherapy or immunosuppressive therapy and diagnosis of HBV reactivation was 15.6 months (range: 1.0–57.7 months) (Table 1). Viral reactivation in seven of the 14 cases occurred 9.5 months (median; range: 6.4–39.8 months) after termination of chemotherapy or immunosuppressive therapy, while the remaining seven cases developed HBV reactivation during chemotherapy or immunosuppressive therapy. Median serum alanine aminotransferase (ALT) levels and HBV DNA levels at the time of HBV reactivation were 652 IU/ml [range: 15–2028] and 6.6 log copies/ml [range: 5.0–9.0], respectively (Table 2).

All patients except case #5 were treated with entecavir (ETV) (0.5 mg, once daily) immediately after diagnosis of HBV reactivation to suppress viral activity (Table 2). Representative clinical courses of patients with reactivation from occult HBV infection are shown in Fig. 1. Four of 14 patients (cases #2, #6, #9, and #11) got tested for HBV markers at 1–3 months intervals and started the ETV treatment after HBV DNA appearance (Table 2). The remaining ten patients were diagnosed with HBV reactivation

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Table 1. Clinical characteristics of patients with reactivation from occult HBV and HBsAg carrier status BEFORE viral exacerbation.

Case	Age/ sex	Anti-HBs	Primary disease	Treatment	Use of steroids	HSCT	Period between HBV reactivation and	
							start of treatment (months)	end of treatment (months)
Reactivation from occult HBV carrier status								
#1	48M	+	ML	Fludarabine	+	+	57.7	39.8
#2	25M	-	AML	IDA + AraC	+	+	27.0	19.2
#3	59M	Unknown	Colon cancer	S-1	-	-	3.6	During treatment
#4	61M	Unknown	ML	R-CHASE	+	+	13.8	9.5
#5	64M	-	MM	MP→CAD	+	+	13.6	6.4
#6	72M	-	ML	MTX + AraC →Rituximab	+	-	10.9	During treatment
#7	78M	Unknown	ML	R-CVP	+	-	34.7	34.2
#8	66M	Unknown	MM	MP	+	-	49.1	6.6
#9	61F	-	ML	R-FND	+	-	1.0	During treatment
#10	66M	Unknown	Psoriasis	Cyclosporine	-	-	37.8	During treatment
#11	79F	Unknown	ML	R-CHOP	+	-	3.7	During treatment
#12	81F	-	ML	R-CVP	+	-	11.2	7.6
#13	84F	Unknown	ML	R-CHOP	+	-	17.4	During treatment
#14	87F	+	MM	MP	+	-	23.1	During treatment
							median: 15.6	**median: 9.5**
Reactivation from HBsAg carrier status								
#15	32F	-	Sjögren synd.	PSL	+	-	15.1	During treatment
#16	63F	-	Raynaud's dis.	PSL	+	-	20.4	During treatment
#17	42F	-	Aortitis synd.	PSL	+	-	122.2	During treatment
#18	59M	-	Lung cancer	Chemotherapy[a]	+	-	17.9	During treatment
#19	54M	-	RA	MTX + PSL	+	-	11.5	During treatment
#20	72M	-	RA	Bucillamine	-	-	6.7	During treatment
							median: 16.5	

[a]Carboplatin, paclitaxel → docetaxel → gemcitabine, vinorelbine → cisplatin, irinotecan.
AML, acute myeloid leukemia; AraC, cytarabine; dis, disease; CAD, cyclophosphamide, doxorubicin, dexamethasone; F, female; HBsAg, hepatitis B surface antigen; HSCT, hematopoietic stem cell transplantation; IDA, idarubicin; M, male; ML, malignant lymphoma; MM, multiple myeloma; MP, melphalan, prednisolone; MTX, methotrexate; PSL, prednisolone; RA, rheumatoid arthritis; R-CHASE, rituximab, cyclophosphamide, cytosine arabinoside, etoposide, dexamethasone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, doxorubicin, prednisolone; synd, syndrome; R-FND, rituximab, fludarabine, mitoxantrone, dexamethasone.

when they had elevated levels of serum ALT and ETV was given in these cases (except case #5) after the appearance of liver dysfunction. After administering ETV, serum HBV DNA levels decreased in 11 cases (excluding cases #13 and #14), accompanied by reduced serum ALT levels. Nine (69.2%) of these cases showed loss of HBsAg with the appearance of anti-HBs at a median time of 2.9 months (range: 0.6–13.5 months) following the commencement of ETV treatment (Table 2). After confirming stable HBsAg/anti-HBs seroconversion, ETV was stopped in three of nine cases after 15.2 months (mean; range: 6.8–26.8 months). The four cases without HBsAg disappearance included two cases (#6 and #8) with follow-up of <3 months after ETV administration, and two cases (#13 and #14) that developed fatal ALF before complete disappearance of HBsAg. When the latter two were diagnosed with HBV reactivation, liver function had already deteriorated (serum total bilirubin (T-bil) was 8.0 mg/dl for #13 and 2.3 mg/dl for #14) and they died of liver failure 33 (#13) and 16 days (#14) after ETV administration.

Low heterogeneity of the reactivated viruses in patients with reactivation from occult HBV infection

To identify characteristics of viral clones related to HBV reactivation, we determined the entire virus genome sequence using ultra-deep sequencing. We first conducted a control experiment to validate the efficacy and errors in the sequencing platform. We determined two full-length plasmid-derived HBV sequences using expression plasmids encoding wild-type HBV as a template. Sequencing generated 1,229,416 and 2,205,237 filtered reads, corresponding to a mean coverage of 34,026 and 61,504 fold at each nucleotide site. The mean nucleotide mismatch error rate was 0.038% in Control #1 and 0.015% in Control #2, with the distribution of per-nucleotide error rate 0–0.24% and 0–0.16%, respectively; the mean overall error rate was 0.45% and 0.26%, respectively (Supplementary Table 1). This reflected the error introduced by sequencing. We defined the cut-off value in the current platform as 1% to exclude mismatch errors and to detect low-abundance mutations.

We then conducted ultra-deep sequencing on samples from the 14 patients with reactivation from occult HBV infection. A mean of 605,890 reads were mapped onto the reference sequences, and a mean coverage depth of 16,712 bp was achieved for each nucleotide site of HBV sequences (Table 3). The frequency of the overall mismatch mutations, which were nucleotides that did not match to the reference sequences, was 0.015% (15/100,000).

To define the characteristics of the reactivated HBV clones, we compared these clones with those derived from reactivated