where n is the number of different species identified, fi is the observed frequency of a particular variant in the quasispecies, and N is the total number of clones analyzed [12,13]. The mean viral complexity in each sample was determined by calculating the total amounts of the Shannon entropy at each nucleotide position divided by the total nucleotide number (e.g., 3215 bases) of each HBV genome sequence.

Nucleotide sequence accession number

All sequence reads have been deposit in DNA Data Bank of Japan Sequence Read Archive (http://www.ddbj.nig.ac.jp/index-e.html) under accession number DRA000435.

Results

Validation of multiplex ultra-deep sequencing of the HBV genome

To differentiate true mutations from sequencing errors in the determined sequences, we first generated viral sequence data from the expression plasmid, pcDNA3-HBV-wt#1, encoding wild-type genotype C HBV genome sequences [28]. For this purpose, we determined the PCR-amplified HBV sequences derived from the expression plasmid using high-fidelity Taq polymerase to take the PCR-induced errors as well as sequencing errors into consideration. Viral sequences determined by the conventional Sanger method were used as reference sequences for aligning the amplicons obtained by ultra-deep sequencing. Three repeated ultra-deep sequencing generated a mean of 77,663 filtered reads, corresponding to a mean coverage of 38,234 fold at each nucleotide site (Table S2). Errors comprised insertions (0.00003%), deletions (0.00135%), and nucleotide mismatches (0.037%). The mean overall error rate was 0.034% (distribution of per-nucleotide error rate ranged from 0 to 0.13%) for the three control experiments, reflecting the error introduced by high-fidelity PCR amplification and by multiplex ultra-deep sequencing that remained after filtering out problematic sequences. We also confirmed that multiplex ultra-deep sequencing with and without the high-fidelity PCR amplification with HBV-specific primer sets showed no significant differences in the error rates on the viral sequence data (mean error rate 0.034% vs 0.043%). Accordingly, we defined the cut-off value in its current platform as 0.3%, a value nearly 1 log above the mean overall error rate.

Next, we performed additional control experiments to verify the detectability of the low abundant mutations that presented at a frequency of less than 0.3%. For this purpose, we introduced expression plasmids with a single-point mutation within that encoding a wild-type viral sequence with a ratio of 1:1000 and assessed the sensitivity and accuracy of quantification using high-fidelity PCR amplification followed by multiplex ultra-deep sequencing in association with the different coverage numbers (Table S3). Repeated control experiments revealed that the threshold for detecting low-abundant mutations at an input ratio of 0.10% among the wild-type sequences ranged between 0.11% and 0.24%, indicating that there was no significant difference in the detection rate or error rates under the different coverage conditions. Based on these results, the accuracy of ultra-deep sequencing in its current platform for detecting low-level viral mutations was considered to be greater than 0.30%.

Viral complexity of the HBV quasispecies in association with clinical status

To clarify HBV quasispecies in association with clinical status, we performed multiplex ultra-deep sequencing and determined the HBV full-genome sequences in the liver and serum with chronic HBV infection. First, we compared the sequences of the viral genome determined in the liver tissue with those in the serum and found no significant differences in the viral population between the liver and serum of the same individual. Indeed, the pattern and distribution of genetic heterogeneity of the viral nucleotide sequences in the liver tissue were similar to those observed in the serum of the same patient (Figure S1), suggesting that a similar pattern of viral heterogeneity was maintained in the liver and serum of patients with chronic HBV infection.

Next, we compared the viral heterogeneity in the liver of chronic-naïve and chronic-NA cases. A mean of 5,962,996 bp nucleotides in chronic-naïve cases and 4,866,783 bp nucleotides in chronic-NA cases were mapped onto the reference sequences, and an overall average coverage depth of 1,855 and 1.514 was achieved for each nucleotide site of the HBV sequences, respectively (Table 2). The frequencies of mutated positions and altered sequence variations detected in each viral genomic region are summarized in Table 2. The overall mutation frequency of the total viral genomic sequences was determined to be 0.87% in chronic-naïve cases and 0.69% in chronic-NA cases. Most genomic changes observed in viral variants were single base substitutions, and the genetic heterogeneity of the viral nucleotide sequences was equally observed throughout the individual viral genetic regions, including the pre-surface (preS), S, pre-core~core (preC-C), and X (Table 2). Consistent with the findings obtained from the viral mutation analyses, the overall viral complexity determined by the Shannon entropy value was 0.047 in chronic-naïve and 0.036 in chronic-NA cases, and the viral complexity was equally observed throughout the individual viral genetic region (Figure 1A). Among chronic-naïve cases, we observed no significant differences in the viral complexity in HBV DNA level, age, or degree of fibrosis (Figure 1B).

High sensitivity of the G1896A pre-C mutant to nucleos(t)ide analogues

Emergence of G1896A mutation in the pre-C region, and A1762T and G1764A mutations in the core-promoter region is well known to be associated with HBe-seroconversion [7–9]. We then evaluated the prevalence of these three mutations in the chronically HBV-infected liver, in association with HBe serologic status and the NA treatment history. In chronic-naive cases, 6 and 8 patients showed the pre- and post- HBeAg seroconversion status, respectively (Table 3). The mean prevalence of the G1896A pre-C mutant in HBeAg-positive cases was lower than that in the HBeAg-negative cases (27.4% and 46.5%, respectively). Importantly, however, 4 of 8 HBeAg-negative cases showed a relatively low prevalence of the G1896A pre-C mutant (Liver #8, #12, #13, #14), and all but one case (Liver #10) showed a high prevalence of the A1762T and G1764A mutations, irrespective of HBe serologic status and NA treatment history (Table 3). These findings suggested that other mutations except G1896A, A1762T and G1764A were also involved in the HBeAg seroconversion status. Notably, liver tissues of all but one (Liver #17) chronic-NA cases showed extremely low levels of the G1896A pre-C mutant (0.0, 0.0, 0.1, and 1.1%), suggesting the high sensitivity of the G1896A pre-C mutant to NA (Table 3).

To confirm the difference of the sensitivity to NA between the wild-type and the G1896A pre-C mutant, we examined the dynamic changes of the relative proportion of the G1896A pre-C mutant in the serum of 14 treatment-naïve patients before and after entecavir administration. Consistent with the findings obtained by ultra-deep sequencing, quantitative real-time PCR revealed that entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in 13 of 14 cases (92.9%)

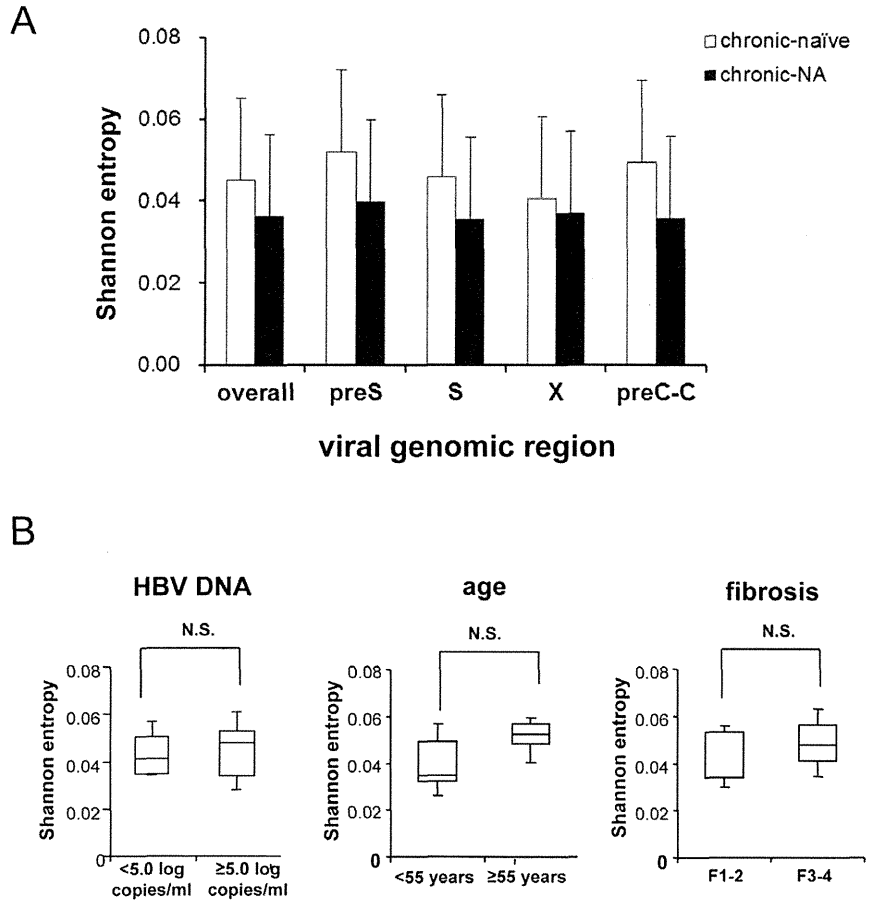

Figure 1. Viral complexity of the HBV quasispecies in association with clinical status. (A) The Shannon entropy values for each viral genomic region were determined in the liver of chronic-naïve and chronic-NA cases. (B) Among the chronic-naïve cases, the Shannon entropy values are shown for patients with serum HBV DNA levels less than 5.0 log copies/ml (<5.0) and greater than 5.0 log copies/ml (≥5.0) (left panel), patients under the age of 55 years (<55) and over the age of 55 (≥55) (middle panel), and patients with low (F1–2) and high (F3–4) liver fibrosis levels (right panel). preS: pre-surface, preC-C: precore~core N.S.: not significant.
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Table 2. The frequency of mutation rate and the Shannon entropy in each viral genome region.

	Liver	
	Chronic-naïve (N = 14)	**Chronic-NA (N = 5)**
Average aligned reads	93,172	76,043
Average aligned nucleotides	5,962,996	4,866,783
Average coverage	1,855	1,514
Mutation rate (%)		
Overall	0.87	0.69
preS	0.92	0.81
S	0.96	0.71
preC-C	1.05	0.72
X	0.63	0.61
Shannon entropy	0.047	0.036

Mutation rate (%): the ratio of total different nucleotides from the reference sequence to total aligned nucleotides.
preS: pre-surface, preC-C: pre-core~core.
doi:10.1371/journal.pone.0035052.t002

irrespective of their HBeAg serostatus, while the G1896A pre-C mutant were detectable in substantial proportion before treatment in all cases (Figure 2A, 2B and 2C; $p = 0.001$). These results further support the findings that HBV clones comprising the G1896A mutation were more sensitive to NA than those with wild-type sequences.

Prevalence of drug-resistant HBV clones in the liver of treatment-naïve patients

Increasing evidence suggests that drug-resistant viral mutants can be detected in the serum of treatment-naïve patients with chronic HBV infection [20,21]. Thus, we next determined the actual prevalence of spontaneously-developed drug-resistant mutants in chronically-infected liver of treatment-naïve patients to evaluate whether NA treatment potentiates the expansion of drug-resistant clones. The drug-resistant mutations examined included two mutations resistant to lamivudine and entecavir, four mutations resistant to entecavir, and three mutations resistant to adefovir [16,17]. Based on the detection rate of the low-level viral clones determined by the control experiments, we identified the drug-resistant mutants present in each specimen at a frequency of more than 0.3% among the total viral clones. Based on these criteria, at least one resistant mutation was detected in the liver of all of the chronic-naïve cases with chronic HBV infection (Table 4).

Table 3. The prevalence of G1896A mutation in the pre-C region, and A1762T and G1764A mutations in the core-promoter region in the liver of patients chronically infected with HBV.

			Mutation Frequency					
	HBeAg/HBeAb	NA (duration of treatment)	G1896A (Pre C)		A1762T (CP)		G1764A (CP)	
Chronic-naive								
Liver #1	+/−	-	640/1652	(38.7)	1647/1941	(84.9)	1683/1979	(85.0)
Liver #2	+/−	-	9/596	(1.5)	682/687	(99.3)	683/689	(99.1)
Liver #3	+/−	-	273/672	(40.6)	767/769	(99.7)	757/760	(99.6)
Liver #4	+/−	-	204/701	(29.1)	610/625	(97.6)	602/621	(96.9)
Liver #5	+/−	-	27/152	(17.8)	249/250	(99.6)	245/248	(98.8)
Liver #6	+/−	-	228/621	(36.7)	727/729	(99.7)	743/744	(99.9)
Liver #7	−/+	-	740/1193	(62.0)	1908/1913	(99.7)	1888/1913	(98.7)
Liver #8	−/+	-	111/1892	(5.9)	2321/2325	(99.8)	2335/2339	(99.8)
Liver #9	−/+	-	10935/10944	(99.9)	12019/12032	(99.9)	12163/12170	(99.9)
Liver #10	−/+	-	4554/4593	(99.2)	1/5191	(0)	4/5188	(0.1)
Liver #11	−/+	-	811/921	(88.1)	1234/1236	(99.8)	1226/1228	(99.8)
Liver #12	−/+	-	93/1265	(7.4)	1234/1234	(100)	1228/1229	(99.9)
Liver #13	−/+	-	83/877	(9.5)	1465/1529	(95.8)	1485/1549	(95.9)
Liver #14	−/+	-	0/717	(0)	1078/1410	(76.5)	1089/1414	(77.0)
Chronic-NA								
Liver #15	−/+	LAM (156w)	0/390	(0)	441/453	(97.4)	435/448	(97.1)
Liver #16	−/+	ETV (1w)	0/1399	(0)	1624/1632	(99.5)	1625/1630	(99.7)
Liver #17	−/+	LAM (144w)	345/816	(42.3)	988/991	(99.7)	994/994	(100)
Liver #18	−/+	LAM (98w)	2/3963	(0.1)	1015/1188	(85.4)	1190/1194	(99.7)
Liver #19	−/+	LAM (11w)	48/4214	(1.1)	3438/3456	(99.5)	3446/3462	(99.5)

Values in parenthesis show mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
NA: nucleotide analogue, pre C: precore, CP: core promoter, LAM: lamivudine, ETV: entecavir.
doi:10.1371/journal.pone.0035052.t003

The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing in 14 chronic-naïve cases ranged from 0.3% to 30.0%, indicating that the proportion of resistant mutations substantially differed in each case. The most commonly detected mutation was M204VI (9 cases) and M250VI (11 cases), which were resistant to lamivudine and entecavir, and entecavir, respectively. Other mutations resistant to adefovir were detected in 7 (50.0%) and 3 (21.4%) cases at A181TV and N236T, respectively (Table 4).

Nine (64.2%) chronic-naïve cases possessed the M204VI mutants in their liver tissues and the proportion of mutant clones among the totally infected viruses ranged from 0.3% to 1.1% among the M204VI mutant-positive patients. In chronic-NA cases, 4 of 5 (80.0%) liver tissues harbored the M204VI mutants with the proportion among the totally infected viruses ranging from 0.4% to 18.7% (Table 4), while the mean serum HBV DNA was suppressed below 2.6 log copies/ml (Table 1). These results suggest that the mutant HBV clones comprising various drug-resistant mutations could latently exist even in the liver of NA treatment-naïve cases.

Expansion of drug-resistant HBV clones harboring M240VI mutations in response to NA administration

To clarify the risk of latent expansion of drug-resistant mutations due to NA treatment, we next examined the early dynamic changes of the prevalence of M204VI mutants in the serum of treatment-naïve patients in response to entecavir treatment. Ultra-deep sequencing provided a mean 40,791- and 38,823-fold coverage of readings, which were mapped to the M204VI nucleotide position at the YMDD sites of each reference sequence in patients before and after entecavir treatment.

Five of 14 (35.7%) patients harbored the M204VI mutations prior to entecavir treatment. Although the serum HBV DNA levels were significantly reduced in response to entecavir in all cases, the M204VI mutant clones were detected in 9 cases (64.3%) after entecavir administration (Table 5). Notably, one patient (Serum #3) who harbored the M240VI mutant clones at baseline had a relatively large expansion of drug-resistant clones among the total viral population in a time-dependent manner in response to entecavir treatment (Table 5). Similarly, M240VI mutant clones became detectable after entecavir administration in four patients (Serum #1, #7, #12, #13) that harbored no resistant mutants at baseline (Table 5). We found no correlation between the degree of the increase in the relative prevalence of M204VI mutant clones and that of the reduction in serum HBV DNA levels. Although only a limited number of patients exhibited a substantial increase in M204VI mutant clones after administration of anti-viral therapy, our findings might suggest that entecavir treatment latently causes selective survival of drug-resistant mutants in treatment naïve patients with chronic HBV infection.

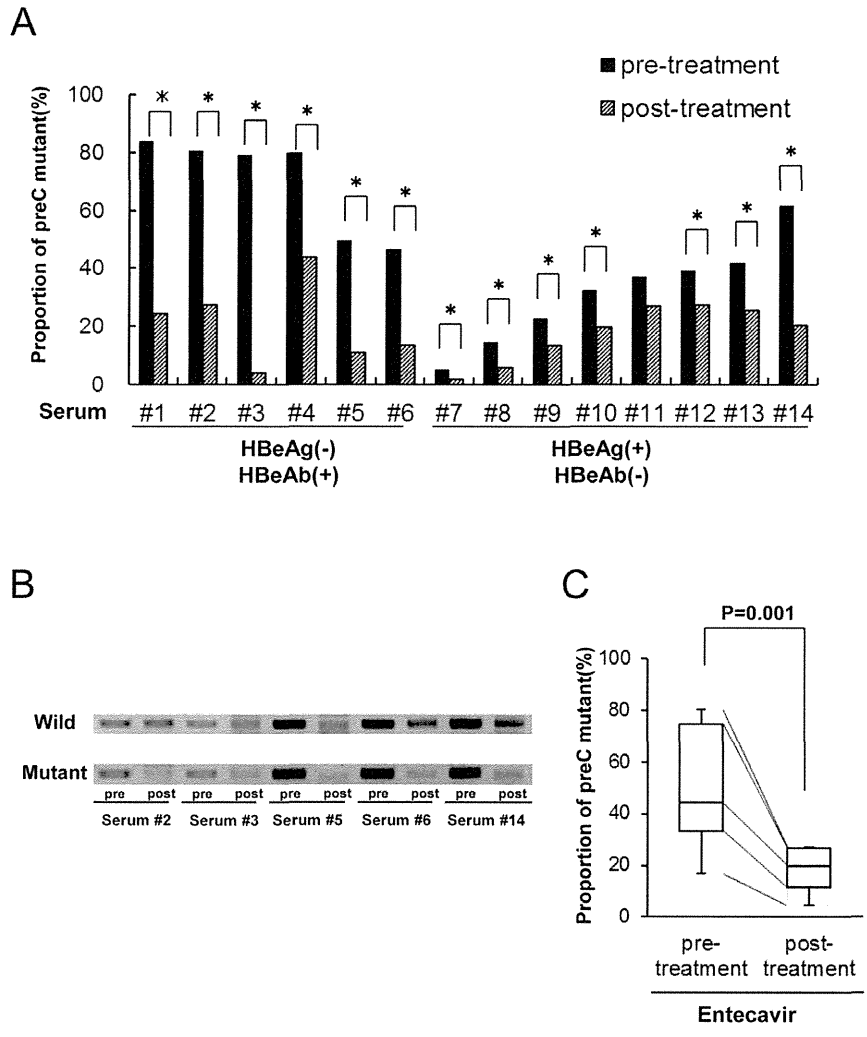

Figure 2. The reduction in the relative proportion of the G1896A pre-C mutant clones after entecavir administration. (A) The relative proportion of the G1896A pre-C mutant was determined in the serum of treatment-naïve patients pre- and post-entecavir administration using quantitative real-time PCR. Serum #1~6 were HBeAg-negative and HBeAb-positive, and Serum #7~14 were HBeAg-positive and HBeAb-negative before treatment. *: $p<0.05$ (B) Semiquantitative PCR analysis was performed using primers specific to the wild-type (upper panel) or G1896A pre-C mutant (lower panel) pre- and post-entecavir administration. A representative result from 5 cases is shown. (C) The relative proportion of the G1896A pre-C mutant was compared in 14 treatment-naïve patients between pre- and post-entecavir administration.
doi:10.1371/journal.pone.0035052.g002

Discussion

Direct population sequencing is the most common method for detecting viral mutations [29]. Conventional sequencing techniques, however, are not efficient for evaluating large amounts of genetic information of the viruses. Newly developed ultra-deep sequencing technology have revolutionized genomic analyses, allowing for studies of the dynamics of viral quasispecies as well as rare genetic variants of the viruses that cannot be detected using standard direct population sequencing techniques [30,31]. The sensitivity of ultra-deep sequencing analysis is primarily limited by errors introduced during PCR amplification and the sequencing reaction, thus it is a challenge to distinguish rare variants from sequencing artifacts. In the present study, we optimized the ultra-deep sequencing with a multiplex-tagging method and reproducibly detected variants within HBV quasispecies that were as rare as 0.3%. Based on this ultra-deep sequencing platform, we determined the abundant genetic heterogeneity of HBV at the intra- and inter-individual levels.

Because of its ability to handle abundant viral genome information, ultra-deep sequencing allowed us to evaluate low-abundant virus variants of patients with chronic HBV infection in detail. It is widely accepted that HBe seroconversion is highly associated with the emergence of G1896A pre-C and/or A1762T and G1764A core promoter mutant clones [7–9]. Unexpectedly, however, our results showed a diverse range of G1896A frequency (0–99.9%) in HBeAg-negative subjects and a high prevalence of core promoter mutations, irrespective of HBe serostatus. Consistent with our observation, previous studies utilizing conventional sequencing methods reported that the frequency of the G1896A pre-C mutant ranged from 12% to 85% [32]. All but one patient (Liver #10) showing a predominance of A1762T and G1764A were infected with genotype C, while patient#10 was infected with genotype B. Because A1762T and G1764A are reported to be significantly more frequent in genotype C [33], the difference in the prevalence of A1762T and G1764A in our study might be a reflection of the viral HBV genotype rather than HBe serostatus. Further investigation of the actual prevalence of these mutations

Table 4. The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing derived from liver tissue.

	M204V/I		L180M		T184S/A/I/ L/G/C/M		S202C/G/I		I169T	
Drugs	**LAM/ETV**		**LAM/ETV**		**ETV**		**ETV**		**ETV**	
Chronic-naive										
Liver #1	**27/5421**	**(0.5%)**	2/3694	(-)	9/3886	(-)	5/5613	(-)	5/3784	(-)
Liver #2	**35/5344**	**(0.7%)**	0/538	(-)	1/563	(-)	17/6340	(-)	0/512	(-)
Liver #3	**13/1363**	**(1.0%)**	0/304	(-)	1/358	(-)	1/1379	(-)	0/264	(-)
Liver #4	11/5113	(-)	0/556	(-)	**2/547**	**(0.4%)**	11/5133	(-)	0/639	(-)
Liver #5	**2/117**	**(1.1%)**	0/409	(-)	1/380	(-)	1/189	(-)	1/474	(-)
Liver #6	12/8451	(-)	0/309	(-)	0/328	(-)	22/8457	(-)	0/334	(-)
Liver #7	**10/3098**	**(0.3%)**	1/1547	(-)	3/1477	(-)	8/3161	(-)	0/1621	(-)
Liver #8	**13/2442**	**(0.5%)**	1/2378	(-)	6/2312	(-)	1/2564	(-)	1/2507	(-)
Liver #9	**67/13879**	**(0.5%)**	2/5443	(-)	2/5107	(-)	6/13804	(-)	0/5650	(-)
Liver #10	16/7400	(-)	0/3524	(-)	3/3283	(-)	5/7113	(-)	0/3492	(-)
Liver #11	0/412	(-)	1/1328	(-)	**1/295**	**(0.3%)**	0/425	(-)	3/4729	(-)
Liver #12	**4/1098**	**(0.4%)**	1/1389	(-)	0/1272	(-)	2/1102	(-)	0/1544	(-)
Liver #13	**8/2476**	**(0.3%)**	1/2192	(-)	3/2085	(-)	4/2529	(-)	4/5029	(-)
Liver #14	5/3713	(-)	0/2009	(-)	4/1925	(-)	2/3820	(-)	5/3784	(-)
Chronic-NA										
Liver #15	0/339	(-)	0/49	(-)	0/49	(-)	0/338	(-)	0/40	(-)
Liver #16	**28/7278**	**(0.4%)**	0/4403	(-)	6/4053	(-)	14/7556	(-)	6/6084	(-)
Liver #17	**177/945**	**(18.7%)**	0/1059	(-)	0/1009	(-)	0/945	(-)	0/1051	(-)
Liver #18	**13/2655**	**(0.5%)**	0/1239	(-)	0/1185	(-)	**10/2708**	**(0.4%)**	0/1332	(-)
Liver #19	**80/6795**	**(1.2%)**	0/3168	(-)	2/2971	(-)	3/6734	(-)	0/3384	(-)

	M250V/I		A181T/V		N236T		P237H	
Drugs	**ETV**		**ADV**		**ADV**		**ADV**	
Chronic-naive								
Liver #1	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Liver #2	**9/2079**	**(0.4%)**	**2/549**	**(0.4%)**	1/1144	(-)	1/1188	(-)
Liver #3	**10/1699**	**(0.6%)**	**1/298**	**(0.3%)**	3/1636	(-)	1/1666	(-)
Liver #4	**3/388**	**(0.8%)**	**3/549**	**(0.5%)**	0/560	(-)	0/533	(-)
Liver #5	**2/91**	**(2.2%)**	1/409	(-)	0/55	(-)	0/60	(-)
Liver #6	0/214	(-)	**6/305**	**(2.0%)**	**1/294**	**(0.3%)**	0/257	(-)
Liver #7	**7/1289**	**(0.5%)**	4/1531	(-)	**24/2738**	**(0.9%)**	1/2692	(-)
Liver #8	2/1117	(-)	**689/2336**	**(29.5%)**	2/1713	(-)	0/1639	(-)
Liver #9	**27/7325**	**(0.4%)**	**38/5334**	**(0.7%)**	1/6607	(-)	4/6702	(-)
Liver #10	**12/3815**	**(0.3%)**	0/3454	(-)	**13/3245**	**(0.4%)**	2/3272	(-)
Liver #11	**1/199**	**(0.5%)**	1/972	(-)	0/251	(-)	0/251	(-)
Liver #12	**2/672**	**(0.3%)**	**408/1362**	**(30.0%)**	0/598	(-)	0/597	(-)
Liver #13	1/947	(-)	2/2160	(-)	0/1406	(-)	1/1374	(-)
Liver #14	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Chronic-NA								
Liver #15	**1/303**	**(0.3%)**	**2/49**	**(4.1%)**	0/377	(-)	0/384	(-)
Liver #16	1/922	(-)	0/4403	(-)	1/1597	(-)	3/1572	(-)
Liver #17	0/755	(-)	1/1050	(-)	0/698	(-)	**145/698**	**(20.8%)**
Liver #18	1/1464	(-)	2/1206	(-)	0/3156	(-)	0/3107	(-)
Liver #19	8/3834	(-)	**16/3128**	**(0.5%)**	0/3372	(-)	0/3428	(-)

(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
LAM: lamivudine, ADV: adefovir, ETV: entecavir.
doi:10.1371/journal.pone.0035052.t004

Table 5. The prevalence of M204VI mutation at YMDD site in patients before and after entecavir administration.

	Entecavir treatment				
	Before		After		
	Prevalence of the mutated clones		Prevalence of the mutated clones		Period of NA treatment
Serum #3	222/32,238	(0.7%)	2,284/23,791	(9.6%)	2w
Serum #2	401/34,041	(1.2%)	266/25,301	(1.1%)	24w
Serum #5	521/48,723	(1.1%)	245/25,521	(1.0%)	56w
Serum #8	748/65,573	(1.1%)	336/28,702	(1.2%)	48w
Serum #9	312/30,599	(1.0%)	169/14,172	(1.2%)	56w
Serum #1	9/22,843	(-)	2,839/34,162	(8.3%)	8w
Serum #7	26/65,564	(-)	923/66,458	(1.4%)	4w
Serum #12	91/65,616	(-)	258/27,958	(0.9%)	24w
Serum #13	11/23,209	(-)	206/64,747	(0.3%)	32w
Serum #4	3/7,923	(-)	39/65,575	(-)	12w
Serum #6	52/65,582	(-)	77/55,273	(-)	16w
Serum #10	38/22,522	(-)	8/21,053	(-)	8w
Serum #11	47/43,853	(-)	5/16,520	(-)	16w
Serum #14	42/42,784	(-)	40/36,668	(-)	12w

Mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
doi:10.1371/journal.pone.0035052.t005

and the elucidation of other unknown mutations involved in HBe seroconversion are necessary for a better understanding of the underlying mechanisms of HBe seroconversion.

One thing to be noted is that the majority of the chronic-NA cases had extremely low levels of the G1896A pre-C mutant in their liver tissues, even though those cases were serologically positive for anti-HBe and negative for HBeAg. Moreover, entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in the serum of the majority of patients irrespective of their HBeAg serostatus, while the G1896A pre-C mutant clones were detectable in a substantial proportion before treatment in all cases. These findings suggest that the G1896A pre-C mutant have higher sensitivity to NA than the wild-type viruses. Consistent with this hypothesis, several previous studies reported that NA is effective against acute or fulminant hepatitis caused by possible infection with the G1896A pre-C mutant [34,35]. Based on these findings, early administration of NA might be an effective strategy for treating patients with active hepatitis infected predominantly with the G1896A pre-C mutant.

Ultra-deep sequencing has a relatively higher sensitivity than conventional direct population sequencing and is thus useful for detecting drug-resistant mutations not detected by standard sequencing [20,21]. Recently, we revealed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, suggesting a putative risk for the expansion of resistant clones to anti-viral therapy [19]. Here, we demonstrated that various drug-resistant HBV variants are present in a proportion of chronically HBV-infected, NA-naïve patients. Several studies using ultra-deep sequencing provided evidence that naturally-occurring drug-resistant mutations are detectable in treatment-naïve individuals with human immunodeficiency virus-1 infection [30,36,37]. Consistent with the cases of human immunodeficiency virus-1 infection, a few studies detected minor variants resistant to NA in the plasma of treatment-naïve patients with chronic HBV infection [20,21]. It remains unclear, however, whether these minor drug-resistant mutations have clinical significance. Our observation of the relative expansion of viral clones with the M204VI mutation during entecavir therapy in some cases indicates the possibility that preexisting minor mutants might provide resistance against NA through the selection of dominant mutant clones. Future studies with a larger cohort size are required to clarify the clinical implications of the latently existing low-abundant drug-resistant mutations.

The current ultra-deep parallel sequencing technology has limitations in the analyses of viral quasispecies. First, because the massively-parallel ultra-deep sequencing platform is based on a multitude of short reads, it is difficult to evaluate the association between nucleotide sites mapped to different genome regions in a single viral clone. Indeed, potential mutational linkages between the pre-C and reverse transcriptase regions were difficult to elucidate due to the short read length of the shotgun sequencing approach. Second, accurate analysis of highly polymorphic viral clones by ultra-deep sequencing is also difficult because the identification of mutations depends strongly on the mapping to the reference genome sequences.

In conclusion, we demonstrated that the majority of patients positive for anti-HBe and negative for HBeAg lacked the predominant infection of the G1896A pre-C mutant in the presence of NA treatment, suggesting that the G1896A pre-C mutant have increased sensitivity to NA therapy compared with wild-type HBV. We also revealed that drug-resistant mutants are widely present, even in the liver of treatment-naïve HBV-infected patients, suggesting that the preexisting low-abundant mutant clones might provide the opportunity to develop drug resistance against NA through the selection of dominant mutations. Further analyses utilizing both novel and conventional sequencing technologies are necessary to understand the significance and clinical relevance of the viral mutations in the pathophysiology of various clinical settings in association with HBV infection.

Supporting Information

Figure S1 Comparison of the viral complexity between the liver and serum of the same individual. Shannon entropy values throughout the whole viral genome of the liver and serum of the representative two cases are shown. (upper two panels, case #11; lower two panels, case #14). preC-C: pre-core~core, preS: pre-surface, P: polymerase.
(TIF)

Table S1 The oligonucleotide primers for amplifying HBV sequences in each clinical specimen.
(DOCX)

Table S2 Error frequency of Ultra-deep sequencing for the expression plasmid encoding wild-type genotype C HBV genome sequences by the three control experiments.
(DOCX)

Table S3 The sensitivity and accuracy of detecting the low abundant minor clones in association with the different coverage numbers.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NN HM. Performed the experiments: NN HM. Analyzed the data: NN HM YU AN TF ST KS TC. Contributed reagents/materials/analysis tools: NN HM YU YO TK SY SU. Wrote the paper: NN HM YU KT TC.

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Original Article

Efficacy and safety of prophylaxis with entecavir and hepatitis B immunoglobulin in preventing hepatitis B recurrence after living-donor liver transplantation

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Aim: Hepatitis B recurrence after liver transplantation can be reduced to less than 10% by combination therapy with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). The aim of this study was to evaluate the efficacy and safety of prophylaxis with entecavir (ETV), which has higher efficacy and lower resistance rates than LAM, combined with HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

Methods: Twenty-six patients who received ETV plus HBIG (ETV group) after LDLT for hepatitis B virus (HBV)-related end-stage liver disease were analyzed by comparing with 63 control patients who had received LAM plus HBIG (LAM group).

Results: The survival rates of the patients treated with ETV plus HBIG was 73% after both 1 and 3 years, and there was no statistical difference between the patients in the ETV group and LAM group. No HBV recurrence was detected during the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 4% at 3 years and 6% at 5 years in the LAM group. No patients had adverse effects related to ETV administration.

Conclusion: ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Key words: entecavir, hepatitis B, liver transplantation, living donor

INTRODUCTION

THE RECURRENCE OF hepatitis B virus (HBV) infection after liver transplantation for HBV-related diseases resulted in poor outcomes before the development of effective prophylaxis with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). Without the prophylaxis, the majority of patients developed recurrent infections due to HBV in the early phases after liver transplantation, and the recurrence resulted in rapidly progressive liver injury, early graft loss and reduced survival.[1–3] The development of prophylaxis dramatically reduced the post-transplant recurrence of hepatitis B and markedly improved prognosis. The most widely used prophylaxis so far has been a combination therapy of LAM and i.v. HBIG.

In the non-transplant setting, the long-term use of LAM resulted in high rates of emergence of resistance to the drug, with rates ranging 14–32% after 1 year and 60–70% after 5 years of treatment. In most cases, the resistance was the result of selection of LAM-resistant mutations in the YMDD motif of the DNA polymerase domain of HBV.[4] Moreover, the emergence of HBV strains with mutations that allow escape from hepatitis B surface antibody (anti-HBs) recognition has been reported in patients vaccinated for HBV,[5,6] in patients with chronic hepatitis B[7,8] and in liver transplant recipients after HBIG administration.[9–11] Therefore, the emergence of LAM resistance and HBIG resistance might

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increase the risk of recurrence during long-term administration of LAM and HBIG, although the rate of HBV recurrence in liver transplant recipients who received prophylaxis with LAM and HBIG for more than 10 years has not been reported to date. At present, several nucleoside analogs are available for the treatment of chronic hepatitis B[4]. Among them, there is entecavir (ETV), a carbocyclic analogue of 2′-deoxyguanosine, which has been shown to have higher efficacy than LAM in patients with chronic hepatitis B. In addition, ETV has a higher genetic barrier to resistance than LAM. The resistance to ETV requires at least three mutations including rtM204V/I, which causes LAM-resistance, rtL180M, and a mutation at one of the following codons: rtT184, rtS202 or rtM250.[4] Therefore, ETV is now used as a first-line therapy in the treatment of chronic hepatitis B worldwide. Data available in the published work suggest that, in transplant recipients, ETV plus HBIG represents a better prophylaxis protocol than LAM plus HBIG for long-term prevention of HBV recurrence after liver transplantation. However, the efficacy and safety of this treatment is largely unknown.

The aim of this study was to evaluate the efficacy and safety of prophylaxis with ETV and HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

METHODS

Patients

WE RETROSPECTIVELY ANALYZED the medical records of 97 patients who underwent LDLT for HBV-related end-stage liver diseases from September 2002 to December 2010. Of these, eight patients were excluded from our study because they had breakthrough hepatitis due to HBV with LAM-resistant mutations and were prescribed LAM plus adefovir before liver transplantation. Accordingly, 89 patients were enrolled in this study.

Prophylaxis with ETV or LAM combined with HBIG

Lamivudine plus HBIG therapy was given to all recipients with HBV-related end-stage liver diseases from September 2002 to November 2006, as reported previously.[12] From December 2006, we changed the protocol for prophylaxis to ETV plus HBIG. ETV at a dose of 0.5 mg/day or LAM at a dose of 100 mg/day was given before transplantation, usually when the patient was referred to the hospital and scheduled for transplantation. Preoperative ETV or LAM prophylaxis was followed by combination with HBIG after transplantation. The first application of HBIG at a dose of 200 IU/kg body mass was administrated i.v. during the anhepatic phase of LDLT, and repeated every day for the first 5 days post-surgery. HBV serological markers were examined at weekly intervals for the first 2 months after the transplant, then at monthly intervals, and 1000 IU of HBIG was periodically administrated to maintain the serum anti-HBs titers at more than 500 IU/L during the first 6 months and 200 IU/L thereafter throughout the follow-up period.[12]

Immunosuppression

Tacrolimus and low-dose steroid therapy were administrated to induce immunosuppression in most patients.[13] Mycophenolate mofetil was administrated to patients who experienced refractory rejection or required reduction of tacrolimus dose due to adverse events. Patients who received ABO blood-type-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone.[14]

Diagnosis of HBV activation

Activation of HBV was diagnosed when hepatitis B surface antigens (HBsAg) and/or HBV DNA became positive in the serum of the patients. After LDLT, HBsAg, anti-HBs and serum HBV DNA were measured at least at 3 monthly intervals. Serological HBV markers, including HBsAg, anti-HBs, hepatitis B core antibody, hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe), were measured by chemiluminescent enzyme immunoassay (Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche, Branchburg, NJ, USA). LAM-resistant YMDD mutant virus was detected by the PCR enzyme-linked mini-sequence assay.[15]

Statistical analysis

Baseline characteristics are shown in Table 1. For continuous variables, medians and ranges are given, and the significance of the data was analyzed with the Wilcoxon rank sum test. For categorical variables, counts are given, and the data were analyzed with the χ^2-test. Survival rates and the rates of patients who showed HBV activation after LDLT were estimated using the Kaplan–Meier method and compared using log–rank tests. $P < 0.05$ was considered significant.

Table 1 Baseline characteristics of 90 patients

	Entecavir + HBIG ($n = 26$)	Lamivudine + HBIG ($n = 63$)	*P*-value
Age (years)	55 (33–68)	53 (26–64)	0.062†
Men/women	19/7	46/17	0.995‡
Primary disease			0.595‡
Acute liver failure	6 (23%)	9 (14%)	
Liver cirrhosis, HCC^-	6 (23%)	20 (32%)	
Liver cirrhosis, HCC^+	14 (54%)	34 (54%)	
HBV markers before LDLT			
$HBsAg^+$	24 (92%)	61 (97%)	0.350‡
$HBeAg^+$	6 (23%)	18 (29%)	0.595‡
HBV DNA before LDLT	<2.6 (<2.6–7.6<)	3.7 (<2.6–7.6<)	0.010†
<2.6 log IU/mL	14 (54%)	19 (30%)	0.024‡
Follow-up period (months)	25.1 (0.2–58.6)	70.6 (0.5–109.2)	<0.001†

Qualitative variables are shown in number; and quantitative variables expressed as median (range).
†Wilcoxon rank sum test.
‡χ^2-Test.
HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living-donor liver transplantation.

RESULTS

Patient characteristics

TWENTY-SIX PATIENTS who received ETV plus HBIG (ETV group) after LDLT for HBV-related end-stage liver disease were included in this study. Baseline characteristics of these patients are listed in Table 1 and compared with those of 63 control recipients who received LAM plus HBIG (LAM group) at our institute already present in our database. The two groups of patients did not differ significantly by age, sex, primary diseases or serological markers for HBV before LDLT. Serum HBV DNA levels before LDLT were significantly lower in the ETV group than in the LAM group. Fourteen of 26 patients (54%) showed less than 2.6 log IU/mL of serum HBV DNA in the ETV group. Median follow-up period was 25.1 months (range, 0.2–58.6) in the ETV group, whereas it was 70.6 months (range, 0.5–109.2) in the LAM group.

Efficacy and safety of prophylaxis with ETV plus HBIG

Survival rates of the patients treated with ETV plus HBIG estimated by Kaplan–Meier analysis was 73% at both 1 and 3 years (Fig. 1a). There was no difference between the ETV group and the LAM group, in which survival rates were 81% at 1 year, 78% at 3 years and 73% at

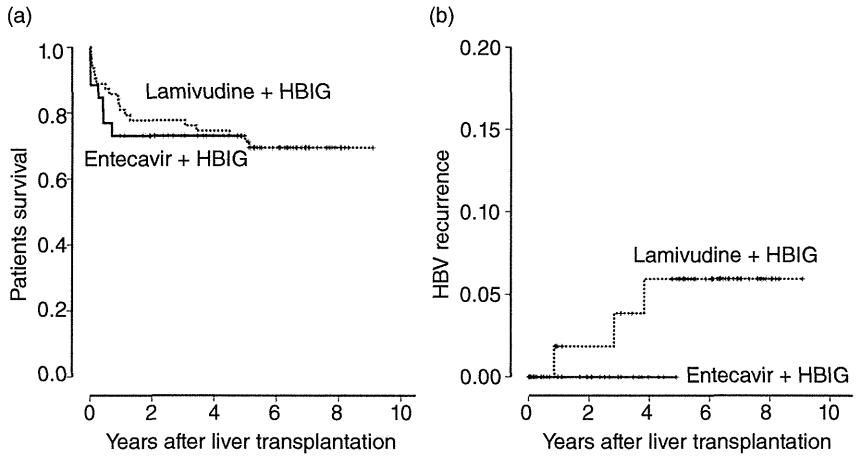

Figure 1 (a) Post-transplantation survival rates and (b) hepatitis B virus (HBV) recurrence after living-donor liver transplantation in HBV positive recipients who received entecavir and hepatitis B immunoglobulin (HBIG) (solid line), or lamivudine and HBIG (dotted line), estimated by Kaplan–Meier method.

5 years. Causes of death in patients in the ETV group were pneumonia ($n = 2$), sepsis ($n = 1$), pulmonary hemorrhage ($n = 1$), cerebral hemorrhage ($n = 1$), graft liver failure ($n = 1$) and multiple organ failure ($n = 1$), none of which were related to ETV. No HBV recurrence was detected in the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 2% at 1 year, 4% at 3 years and 6% at 5 years in the LAM group (Fig. 1b). Three patients in the LAM group had HBV recurrence at 10, 34 and 46 months after LDLT. The emergence of HBV with LAM-resistant mutations in the YMDD motif was confirmed in two of the three patients. HBV mutations of another patient could not be determined because of the low level of serum HBV DNA. As the follow-up period of the ETV group was shorter than that of the LAM group and the HBV recurrence in the LAM group occurred in long-term follow-up after LDLT, the rate of HBV recurrence was not significantly different between the ETV and LAM groups. No patients had adverse events due to ETV administration.

DISCUSSION

IN THIS STUDY, we demonstrated that ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Two studies of patients receiving a combination of ETV and HBIG after liver transplantation have been previously reported.[16,17] One study demonstrated that 30 recipients who received ETV plus HBIG prophylaxis had no recurrence of HBV and no adverse effect relating to ETV.[17] The other study showed that no HBV recurrence was observed in two recipients with HBV-associated cirrhosis receiving ETV, tenofovir and HBIG.[16] Both studies showed the efficacy and safety of prophylaxis with ETV and HBIG in preventing short-term recurrence of HBV after liver transplantation. The current study confirmed their results for longer follow-up periods. Our results showed that prophylaxis with ETV and HBIG has similar efficacy and safety to that with LAM and HBIG, but did not show any further advantage of ETV compared to LAM treatment. Longer follow up might be needed to reveal the difference of HBV recurrence rate. One characteristic of our present report is that all patients in this study underwent LDLT. Our results suggest that prophylaxis with ETV and HBIG in patients after LDLT has similar efficacy and safety to patients after deceased-donor liver transplantation demonstrated in the previous reports.[16,17] More recently, efficacy of ETV monotherapy in preventing recurrence of HBV for liver transplant recipients with chronic hepatitis B was reported.[18] The study demonstrated that most patients showed disappearance of HBsAg and undetectable serum HBV DNA after liver transplantation without HBIG. Although long-term efficacy of ETV monotherapy needs be confirmed, both our data and previous reports suggest that ETV is an effective and safe antiviral agent in the post-transplant setting.

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REVIEWS AND PERSPECTIVES

REVIEWS IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

Robert F. Schwabe and John W. Wiley, Section Editors

Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation

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Chronic inflammation, regardless of infectious agents, plays important roles in the development of various cancers, particularly in digestive organs, including *Helicobacter pylori*–associated gastric cancer, hepatitis C virus–positive hepatocellular carcinoma, and colitis-associated colon cancers. Cancer development is characterized by stepwise accumulation of genetic and epigenetic alterations of various proto-oncogenes and tumor-suppressor genes. During chronic inflammation, infectious agents such as *H pylori* and hepatitis C virus as well as intrinsic mediators of inflammatory responses, including proinflammatory cytokines and reactive oxygen and nitrogen species, can induce genetic and epigenetic changes, including point mutations, deletions, duplications, recombinations, and methylation of various tumor-related genes through various mechanisms. Furthermore, inflammation also modulates the expressions of microRNAs that influence the production of several tumor-related messenger RNAs or proteins. These molecular events induced by chronic inflammation work in concert to alter important pathways involved in normal cellular function, and hence accelerate inflammation-associated cancer development. Among these, recent studies highlighted an important role of activation-induced cytidine deaminase, a nucleotide-editing enzyme essential for somatic hypermutation and class-switch recombination of the immunoglobulin gene, as a genomic modulator in inflammation-associated cancer development.

Keywords: H pylori; HCV; Mutation Induction; Epigenetics.

Nearly 150 years ago, Rudolf Virchow noted that inflammatory cells are present in tumor tissues and that tumors develop at sites of chronic inflammation; he suggested that chronic inflammation plays important roles in cancer development. Since then, many clinical and epidemiologic studies have confirmed a strong association between inflammation and cancer (Table 1).[1,2] For instance, epidemiologic studies have shown that approximately 10%–15% of cancers were related to chronic infections with viruses, bacteria, or parasites,[3–7] and, moreover, that up to 25% of all cancers were associated with chronic inflammation irrespective of the presence or absence of infection.[5–7]

In inflammation-associated cancer development, in addition to infectious agents such as *Helicobacter pylori* and hepatitis C virus (HCV), many intrinsic mediators of inflammation including proinflammatory cytokines, eicosanoids, growth factors, and reactive oxygen species (ROS) and reactive nitrogen species exert important effects in cancer development through various mechanisms. These include enhancement of cell growth and mobility, induction of angiogenesis, and inhibition of apoptosis. However, a hallmark of cancer development is the stepwise accumulation of various genetic and epigenetic alterations of the genome. Indeed, recent genomewide analysis of human cancer tissues revealed that a single cancer cell generally possesses approximately 100 mutations in coding regions, 10–20 of which are known as driver genes that contribute to cancer development,[8–10] and, moreover, that there are many somatic gene rearrangements, including duplications, deletions, and inversions in human cancer genomes.[11,12] In addition to genetic alterations, recent studies also have shown that chronic inflammation enhances epigenetic changes as represented by DNA methylation.[13] It is estimated that several hundreds to thousands of genes are methylated in a cancer cell,[14] and that

Abbreviations used in this paper: A, adenine; AID, activation-induced cytidine deaminase; C, cytosine; CDKN, cyclin-dependent kinase inhibitor; COX-2, cyclooxygenase 2; DNMT, DNA methyltransferase; DSS, dextran sulfate sodium; EZH2, enhancer of zeste homolog 2; G, guanine; H, histone; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; Let-7, lethal-7; LPS, lipopolysaccharide; miRNA, microRNA; MAPK, mitogen-activated protein kinase; MLH1, mutL homolog 1; mRNA, messenger RNA; MSH, mutS homolog; NF-κB, activation of transcription factor nuclear factor κB; NOS, nitric oxide synthase; PSC, primary sclerosing cholangitis; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; T, thymine; TNF-α, tumor necrosis factor-α; U, uracil.

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Table 1. Inflammation-Associated Cancers in Digestive Organs

Inflammation-associated cancer	Underlying inflammation
Barrett's cancer	Reflux esophagitis
Gastric cancer	*H pylori*–induced chronic gastritis
Colitic cancer	Inflammatory bowel disease
	Celiac disease
HCC	HCV and hepatitis B virus chronic hepatitis
	Primary biliary cirrhosis
Cholangiocarcinoma	Primary sclerosing cholangitis
Pancreatic cancer	Chronic pancreatitis
	Hereditary pancreatitis
Lymphoma	*H pylori*–induced mucosa-associated lymphatic tissue lymphoma
	HCV-associated lymphoma
	Celiac disease–associated lymphoma

aberrant DNA methylation is present even in normal-appearing tissues, being involved in field cancerization.[13,15,16]

Digestive organs are inhabited by many microorganisms and are infiltrated by many immune cells in physiological and pathologic conditions, and thus they are more or less accompanied by certain levels of inflammation. Here, we review mechanisms of how inflammation is involved in cancer development in digestive organs, particularly focusing on the role of chronic inflammation in inducing genetic and epigenetic changes.

Cancers in Digestive Organs Associated With Inflammation

Many cancers arise in digestive organs. Indeed, gastric cancer remains the third leading cause of cancer death in men and the fifth leading cause in women, and colorectal cancer is the third most commonly diagnosed cancer in men and the second most commonly diagnosed in women worldwide.[17] In addition, hepatocellular carcinoma (HCC) is one of the most frequent malignancies and its incidence is increasing not only in an endemic area for the hepatitis virus but also in the United States and other Western countries.[18] Digestive organs cover a large part of the body surface in contact with the outer environment. Accordingly, they are inhabited not only by many microorganisms but also exposed to ingested food or chemical agents, and therefore infiltrated by many immune cells in pathologic as well as normal conditions, supporting the perpetuation of chronic inflammation. Therefore, it is reasonable that many cancers in digestive organs are associated with inflammation.

The best examples of inflammation-associated cancer in human beings are gastric cancer and HCC. Since the discovery of *H pylori* by Warren and Marshall[19] in 1982, it has been well established that *H pylori*–positive patients with chronic gastritis have a significantly higher risk for gastric cancer than *H pylori*–negative subjects,[20] and, moreover, careful investigations have shown more than 95% positivity for *H pylori* infection in gastric cancer patients.[21] On the other hand, hepatitis B virus and HCV infections account for approximately 60% and 33% of the total HCC cases in developing countries and 23% and 20% in developed countries, respectively,[6,22] and the majority of HCCs develop in patients who have chronic hepatitis or cirrhosis. Other inflammation-associated cancers in digestive organs are colitic cancers developed in patients with inflammatory bowel disease (IBD) or celiac disease,[23–25] primary sclerosing cholangitis (PSC)-associated cholangiocarcinoma,[26] primary biliary cirrhosis–associated HCC,[27] and Barrett's cancer developed in patients with reflux esophagitis.[28] In addition, the incidence of pancreatic cancer in patients with chronic pancreatitis is reported to be 4–8 times higher than in the general population,[29] and, more strikingly, the incidence of pancreatic cancer in patients with hereditary pancreatitis is 53 times higher than in the normal population,[30] indicating that chronic pancreatitis is a risk for pancreatic cancer.

In addition to cancers, inflammation is also a risk for developing various lymphomas in digestive organs. These include *H pylori*–induced mucosa-associated lymphatic tissue lymphoma or plasmacytoma,[31,32] HCV-related lymphoma,[33] and lymphoma related to celiac disease.[34]

Mechanisms for Inflammation-Associated Cancer Development

The inflammatory response is coordinated by a large range of mediators, which are released from immune cells, mesenchymal cells, and epithelial cells; these mediators exert various functions in maintaining or resolving inflammation, and at the same time are involved in cancer development. Among the mediators, cytokines play central roles in diversifying the inflammatory process, and interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 are known to be the major cytokines important for inflammation and cancer development.[35–37]

IL-1β and TNF-α act directly on epithelial cells to induce activation of transcription factor nuclear factor-κB (NF-κB), a key transcription factor mediating inflammation and cancer development.[36,37] NF-κB activation not only promotes growth or suppresses apoptosis of epithelial cells but also stimulates the production of growth factors and cytokines such as epidermal growth factor and IL-6, enhances cyclooxygenase (COX)-2 induction, and increases ROS production.[38] The induced COX-2 subsequently has many functions, including enhancement of cell growth and angiogenesis.[39] ROS modifies protein function.[40] IL-6 activates signal transducer and activator of transcription 3 (STAT3) and thereby enhances cell growth and stimulates growth factor production, including the Reg protein.[41] Interestingly, TNF-α and IL-6 often create a positive-feedback loop during cancer development.[42]

At the same time, these cytokines also activate mitogen-activated protein kinase (MAPK) cascades. For instance, TNF-α and IL-6 have been shown to activate the extracellular signal-regulated kinase/MAPK cascade, an impor-

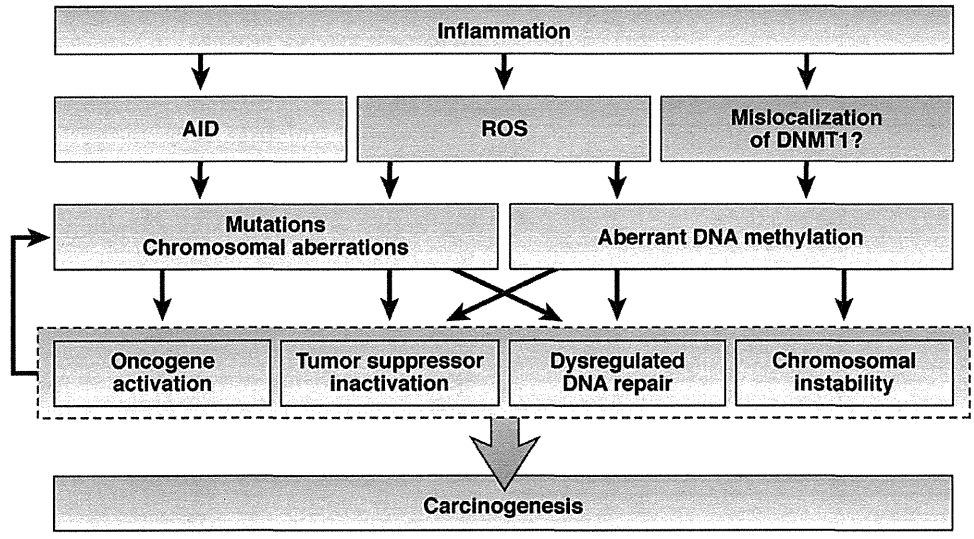

Figure 1. Molecular link between inflammation, genetic and epigenetic alterations, and carcinogenesis. Inflammation contributes to ROS production and transcriptional up-regulation of the DNA mutator enzyme, AID. These 2 factors were capable of inducing somatic mutations and chromosomal aberrations in tumor-related genes. On the other hand, inflammation results in mislocalization of DNMTs, inducing aberrant DNA methylation. The resulting genetic and epigenetic changes, including the activation of oncogenes, inactivation of tumor-suppressor genes, and dysregulation of DNA repair genes, could enhance genetic instability further, finally leading to carcinogenesis.

tant signaling pathway involved in many processes in carcinogenesis including cell proliferation, migration, and angiogenesis.[43,44] Similarly, IL1-β, TNF-α, and IL-6 all activate c-Jun N-terminal kinase (JNK). Although JNKs are attributed primarily to proapoptotic cell death or tumor suppression in response to inflammation or various stressors,[45] JNK activation, particularly JNK1, by proinflammatory cytokines has been reported to contribute to inflammation-associated cancer development through cell death–induced compensatory proliferation.[45-48] In this regard, an interesting thing to note is that *H pylori* directly activates extracellular signal–regulated kinase/MAPK and JNK in human gastric cells via a type IV secretion system–dependent mechanism.[49,50]

Thus, these mediators of inflammation form a complex of regulatory networks, and appear to work in concert to enhance cancer development. However, for normal cells eventually to be transformed and become cancer cells with clonal expansion, inflammation has to damage cellular DNA, either genetically or epigenetically, leading to permanent alteration within the genome.

Inflammation and Genetic Modulation

Cancer is a genetic disease resulting from stepwise accumulation of genetic and epigenetic alterations that drives the progressive transformation of normal cells into malignant derivatives.[51] Inactivation of tumor-suppressor genes and/or activation of oncogenes caused by somatic mutations, DNA copy number changes, or chromosomal aberrations are widely detectable in human cancer cells. Among them, the tumor suppressor *TP53* gene is one of the most frequent targets for genetic alterations in many human cancers.[52] An important point to note is that *TP53* mutations frequently are present also in noncancerous tissues with chronic inflammation before cancer development. Indeed, multiple genetic changes in the *TP53* gene have been detected in various inflammatory tissues such as IBD,[53,54] Barrett's esophagus,[55] and HCV-associated chronic hepatitis.[56] For example, by analyzing the individual crypt mutation burden across plaques of the dysplasia, it was shown that mutations in *TP53* genes could be identified in the majority of inflamed crypts of patients with ulcerative colitis.[57] Moreover, *TP53* mutations are detectable at the frequencies of 4–15 nucleotides of 10^4 nucleotides in the hepatocytes of the patients with chronic HCV infection.[56] Normal mutation rates cannot account for such abundant genetic changes that accumulate in inflamed epithelial cells, suggesting that certain molecular mechanisms underlie such a large number of genetic alterations. Therefore, to understand the mechanisms of inflammation-associated tumorigenesis, several possible intrinsic mutagens responsible for genetic aberrations in the inflammatory condition have been proposed. Among them, free radicals and intrinsic DNA mutator enzymes appear to be important candidates in the setting of chronic inflammation (Figure 1).

Free radicals refer to any molecular species with one or more unpaired electron(s), including ROS and reactive nitrogen species.[38] Interestingly, increases in *TP53* gene mutations at codons 247 and 248 are paralleled by an enhanced expression of nitric oxide synthase (NOS) in the inflamed lesions of the colonic tissues of patients with ulcerative colitis.[54] HCV infection also induces inducible NOS messenger RNA (mRNA) expression, thereby enhancing NO production, which in turn results in DNA breaks and enhanced mutation frequencies.[58] Moreover, an increased level of NO accelerated spontaneous tumor

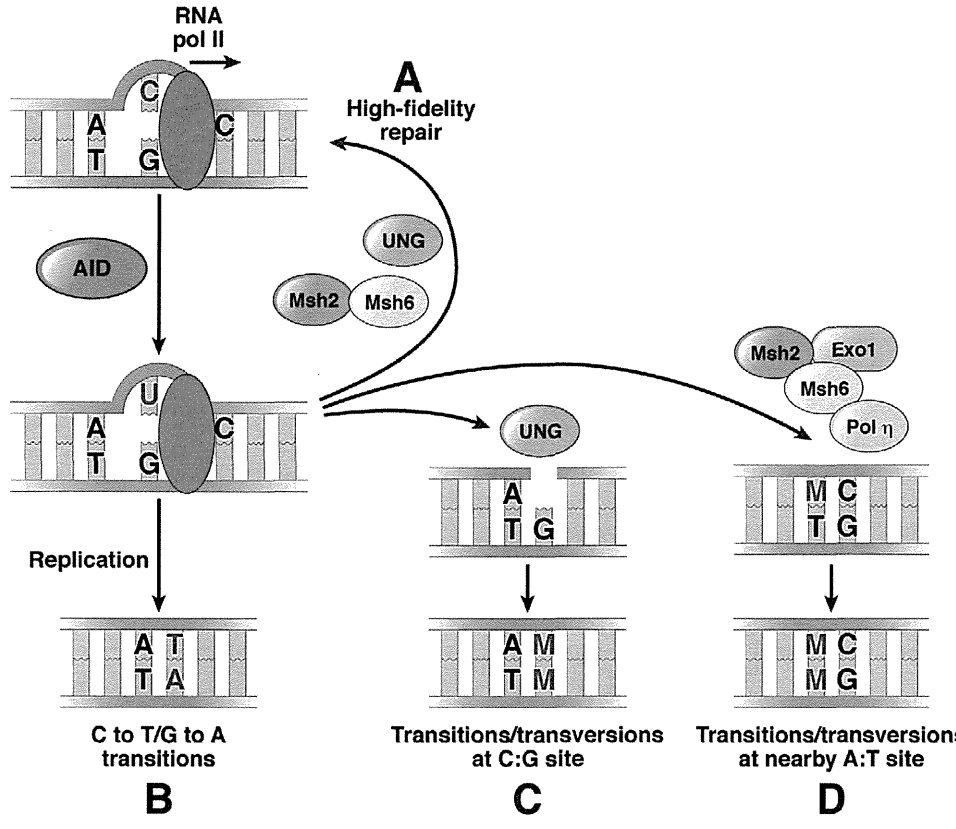

Figure 2. Mechanism of mutation induction by AID activity. AID deaminates cytosine (C), resulting in the generation of a uracil (U), and therefore can transform a DNA C:G pair into a U:G mismatch. (*A*) The AID-generated U:G mismatch can be recognized by uracil-DNA-glycosylase (UNG) or MSH2/MSH6 heterodimer and repaired correctly. (*B*) If DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, (*C*) generation of an abasic site by UNG or (*D*) recognition of the U:G mismatch by the MSH2/MSH6 heterodimer induces any mutations in the AID-generated U:G mismatch or at a nearby A:T site, respectively, in an error-prone manner (indicated as M).

development, mostly lymphomas, in a *Trp53*-deficient mouse model infected with *Cryptosporidium parvum.*[59]

In the inflammatory condition, cellular ROS levels are increased substantially, and nucleic acids exposed to ROS generate various modified bases such as oxidatively altered purines and pyrimidines.[60] These modified nucleic acids could induce the putative DNA damage, including single- or double-stranded DNA breaks, DNA intrastrand adducts, and DNA protein cross-links.[61] In addition, ROS alters the mismatch repair function and allows mutations to accumulate in microsatellite sequences.[62] It has been well recognized that oncogene activation is capable of inducing genomic instability in precancerous lesions as well as cancer cells.[63] In this regard, ROS is also a putative mediator that links excessive activity of oncogene products and DNA damage. For example, oncogene *c-MYC* overexpression results in DNA damage before the S phase in association with ROS induction in normal human fibroblasts.[64] These findings suggested that the cumulative situation of ROS production, a condition of so-called *oxidative stress*, is involved in both the initiation and progression of inflammation-associated cancers through the induction of genetic instability.

Importantly, the typical mutation pattern induced by oxidative stress cannot account for a mutation signature observed in many human cancer tissues, particularly in inflammation-associated cancers. Among the oxidized nucleosides, one of the common products of free radical attack on DNA is 8-hydroxydeoxyguanine, which is considered to be a biomarker of oxidative stress.[65] The typical pattern of nucleotide alterations induced by 8-hydroxydeoxyguanine is guanine (G)/cytosine (C) to thymine (T)/adenine (A) transversions, which have been observed in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) and *TP53* tumor-suppressor gene in lung and liver cancers.[66,67] However, recent genomewide analyses clearly showed that G/C to T/A transversions account for a minor proportion of the total mutations identified in human cancer cells, and instead C/G to T/A transitions are the most prevalent mutation pattern in various cancer tissues, including inflammation-associated cancers.[68] Thus, it appears reasonable to assume that there is an alternative mechanism that accounts for the most frequent mutational pattern, C/G to T/A transitions, detected in many human cancer tissues.

Recently, several human enzymes that are capable of inducing nucleotide alterations have been identified, providing a new avenue for understanding mutagenesis mechanisms.[69] Among them, activation-induced cytidine deaminase (AID) is a well-defined molecule involved in DNA mutations in the human genome. Through its enzymatic activity, AID can deaminate C on target DNA to produce a uracil (U), and therefore turns a DNA C:G pair into a U:G mismatch. When DNA replication starts before recognition by the repair system, a U:G mismatch gives rise to C/G to T/A transition. Alternatively, recognition of a U:G mismatch by uracil-DNA-glycosylase or mutS homolog 2 (MSH2)/mutS homolog 6 (MSH6) heterodimer

induces mutations in the U:G mismatch or at the nearby A:T site (Figure 2). As a result, AID can induce any type of mutations.[70] Under physiological conditions, AID contributes to generating antibody gene diversification in activated B lymphocytes by inducing somatic hypermutation and class switch recombination of immunoglobulin gene.[71] In sharp contrast to the favorable function of AID in the immune system, the role of AID in tumorigenesis through induction of genetic instability was first suggested in hematopoietic malignancies. A number of studies have shown that increased AID expression in various neoplasms of the B lymphocytic lineage was associated with unfavorable mutations and chromosomal translocations.[72,73] For instance, AID has been shown to be responsible for the chromosomal breaks in *c-MYC*, leading to a *c-MYC/IgH* translocation in B-cell lymphoma.[74] Moreover, AID induces *breakpoint cluster region–Abelson murine leukemia viral oncogene homolog 1* mutations leading to imatinib resistance in chronic myeloid leukemia cells.[75] Because the target of AID-mediated genotoxic effects was not restricted to immunoglobulin genes and a variety of other genes also received the AID-mediated mutations in B cells,[70] it was not surprising that aberrant up-regulation of AID induced genetic alterations in various tumor-related genes, leading to the transformation of hematopoietic cells.

As described, activation of NF-κB is induced in response to various inflammatory stimulations, and is deeply involved in multiple processes of cancer initiation and progression.[36] Interestingly, NF-κB is a major transcription factor for AID in B cells that is activated through cluster of differentiation 40-TNF receptor superfamily member 5 ligation by T cells (CD40),[76] suggesting that AID might link NF-κB activation and genetic instability in nonlymphoid cells in the setting of inflammation. In agreement with this hypothesis, AID expression is induced in response to proinflammatory cytokine stimulation via the NF-κB–dependent pathway in various epithelial cells (Figure 3). In hepatocytes, AID expression is induced by TNF-α through the I-κB kinase–dependent NF-κB signaling pathway.[77] Consistent with a previous finding that the HCV core protein triggers the activation of NF-κB in hepatocytes,[78] the HCV core protein itself also up-regulates endogenous AID in cultured hepatocytes.[77] NF-κB–mediated induction of AID expression is not limited to hepatocytes. In human gastric epithelial cells, AID expression is induced by TNF-α stimulation via activation of NF-κB, but is not detected in nonstimulated cells.[79] More interestingly, aberrant AID expression is induced by the infection of a pathogenic *H pylori* strain, the cytotoxin-associated gene pathogenicity island–positive strain that is capable of introducing bacterial virulence factors into the host cells through a type IV secretion system and activating NF-κB, indicating that both bacterial factors introduced into epithelial cells and the inflammatory mediators such as TNF-α and IL-1β induced by *H pylori* infection cooperatively promote aberrant AID expression in *H pylori*–infected gastric mucosal cells. Similar

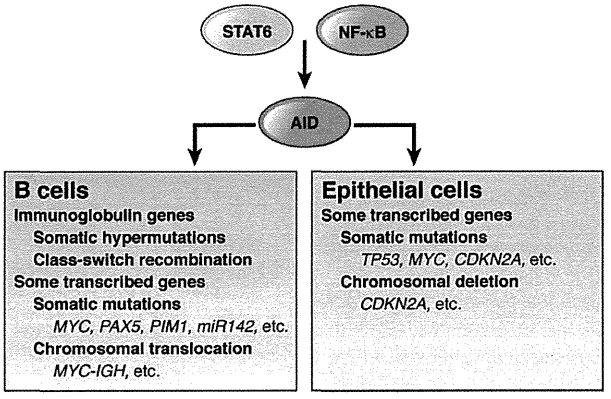

Figure 3. AID exerts both favorable and unfavorable effects. AID is a molecule that is indispensable for the diversification of immunoglobulin genes by inducing both somatic hypermutation and class-switch recombination in activated B lymphocytes. The genotoxic activity of AID, however, can be aimed to trigger the genetic alterations at both the nucleotide and chromosomal levels not only in B lymphocytes but also epithelial cells in the inflammatory conditions.

to hepatocytes and gastric mucosal cells, TNF-α stimulation resulted in up-regulation of endogenous AID in human colonic cells via the I-κB kinase–dependent NF-κB signaling pathway.[80] In addition, IL-4 and IL-13, which are involved in T helper 2 cell–type immune response in IBD, induced aberrant AID expression in a STAT6-dependent manner in human colonic epithelial cells.[80] Of note, IL-4 is known to induce AID also in B cells.[71]

Consistent with the in vitro analyses, aberrant AID expression is widely detectable in not only various inflammation-associated cancer tissues but also in a variety of inflamed epithelial tissues in which tumorigenic risk is high, including chronic hepatitis and cirrhosis caused by HCV infection,[56] chronic gastritis caused by *H pylori* infection,[79] IBD,[80] PSC,[81] and the columnar cell-lined Barrett's esophagus.[82]

The impact of AID expression in nonlymphoid epithelial cells was clarified using both in vivo and in vitro systems with aberrant AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced lymphoma development via the accumulation of somatic mutations in various nonimmunoglobulin genes, including the proto-oncogene *c-Myc*.[83] More importantly, further phenotypic analyses revealed that AID transgenic mice also develop neoplasia in epithelial tissues, including lung, liver, and stomach, accompanied by the emergence of *Trp53* mutations, indicating that aberrant AID expression in epithelial cells can induce genetic instability, leading to cancer development.[83,84] It is widely recognized that the frequently mutated tumor-related genes differ among different cancers. For instance, nucleotide alterations in the *K-RAS* are detectable in almost all human pancreatic cancers,[85] whereas it is relatively low in other human tumors. Similarly, the *c-MYC* is a frequent target for genetic alterations in human lung cancers, whereas its nucleotide alterations are rare in hepatocellular carcinoma.[86] However, the mechanisms underlying the accumulation

of organ-specific genomic changes in oncogenic pathways are not well known. Interestingly, organ-specific changes in mutational profiles were observed in the epithelial tissues of the AID transgenic mice. Indeed, the *c-Myc* gene was mutated frequently in noncancerous tissue of the lung, whereas *K-ras* gene mutations frequently were detectable in gastric cancer developed in AID transgenic mice.[84] Thus, the organ-specific differences in the mutational profiles in AID transgenic mice suggest the possibility that the target preference of AID-induced mutagenesis in different tissues might contribute to the diversity of tissue-specific oncogenic pathways in various epithelial organs.

In vitro analyses using human cultured cells with constitutive AID expression revealed that *TP53* mutations were induced frequently by AID genotoxic activity in hepatocytes, and gastric, colonic, and bile duct epithelial cells.[77,79–81] Similar to the *TP53* gene, the cyclin-dependent kinase inhibitor (*CDKN*)-*2B-CDKN2A* locus was identified as a target for AID-mediated genotoxic activity. The *CDKN2B-CDKN2A* locus encodes the potent suppressor proteins $p16^{INK4a}$, $p15^{INK4b}$, and $p14^{ARF}$, which regulate the activities of the retinoblastoma protein and the TP53 transcription factor. Aberrant AID expression preferentially induces somatic mutations at the *CDKN2B-CDKN2A* locus in gastric epithelial cells and biliary cells.[81,87] Moreover, comparative genomic hybridization analysis clearly showed that constitutive AID activation in cultured gastric epithelial cells caused submicroscopic deletions as represented by copy number losses of various chromosomal loci, especially at the *CDKN2B-CDKN2A* locus at 9p21. Copy number reduction of *Cdkn2b-Cdkn2a* also was seen in the gastric mucosa of AID transgenic mice.[87] In agreement with the preferential deletions at the *CDKN2B-CDKN2A* locus in gastric epithelial cells by AID introduction, AID expression was required for inducing DNA single-strand breaks in the *CDKN2B* gene in leukemia cells,[88] and, furthermore, the deletion of the *CDKN2B-CDKN2A* locus frequently is detectable in AID-expressing lymphoid blast crisis leukemia cells.[75] These findings suggest that AID can induce both mutations and deletions at the same gene locus, and, moreover, that the representative tumor-suppressor genes, *TP53* and *CDKN2B-CDKN2A*, may be common targets for AID-mediated genotoxic effects in various human tissues in the setting of inflammation.

Finally, a recent finding that a deficiency of endogenous AID reduced the incidence of both accumulation of somatic mutations in the *Trp53* gene and the development of colitis-associated colorectal cancers further supports the critical role of AID in inflammation-associated cancer development via its ability to induce genetic alterations in tumor-related genes.[89]

Inflammation and Epigenetic Modulation

Epigenetic modifications are DNA-associated modifications that are inherited upon somatic cell replication, which include DNA methylation and histone modifications.[90] Coordinated changes of epigenetic modifications control development and tissue differentiation, and erasure of epigenetic modifications is involved in reprogramming. In somatic cells, DNA methylation is present in repetitive elements, CpG-sparse regions, and in a very limited number of CpG islands.[91,92] DNA methylation of a CpG island in a promoter region causes silencing of its downstream gene, whether it is a protein-coding gene or a microRNA (miRNA) gene, by forming nucleosomes and thus possibly blocking access of RNA polymerase II to the promoter.[93,94] In contrast, DNA methylation of a gene body often is associated with increased gene expression.[91,95]

Histone modifications denote chemical modifications, such as acetylation, methylation, and ubiquitination of lysine and arginine residues of histones, mainly H3 and H4, but also H2A and H2B.[93] Specific histone modifications, such as acetylation of histones H3 and H4 (H3Ac and H4Ac) and trimethylation of lysine 4 of histone H3 (H3K4me3), are associated with active gene transcription. In contrast, dimethylation and trimethylation of H3 lysine 9 (H3K9me2 and H3K9me3) and trimethylation of H3 lysine 27 (H3K27me3) are associated with gene repression. H3K9me2 represses gene transcription in concert with DNA methylation, whereas H3K27me3 works independently of DNA methylation.[96] Trimethylation of H3 lysine 36 (H3K36me3) is considered to mark exonic regions of active genes. However, the mechanisms of how histone modifications are inherited upon somatic cell replication remains unclear.[97]

In cancer cells, the presence of regional hypermethylation and global hypomethylation has been described.[98,99] Regional hypermethylation refers to aberrant DNA methylation of promoter CpG islands physiologically kept unmethylated.[95,100] If aberrant methylation is induced in a promoter CpG island it consistently induces silencing of its downstream gene.[90] Many tumor-suppressor genes that have promoter CpG islands, such as *CDKN2A*, mutL homolog 1 (*MLH1*), cadherin-1, and RAS-association domain family 1, isoform A, can be inactivated permanently by aberrant DNA methylation as drivers, which have significant roles in cancer development. At the same time, most of the aberrant DNA methylation of promoter CpG islands are considered to be passengers that play no role in carcinogenesis.[14] Several hundreds to thousands of promoter CpG islands are methylated aberrantly in a cancer, and the number is too large for all of them to be drivers. Moreover, most of the genes methylated in cancers are not expressed in normal tissues,[101,102] and such genes are considered not to be involved in carcinogenesis. Global hypomethylation was shown to be causally involved in carcinogenesis by inducing genomic instability.[103] In addition, induction of H3K27me3 is considered to be an alternative mechanism to induce gene silencing,[96] and aberrant H3K27me3 was observed in promoter regions consisting of 200–600 genes.[96,104] Again, the number is very large, and most are expected to be passengers.

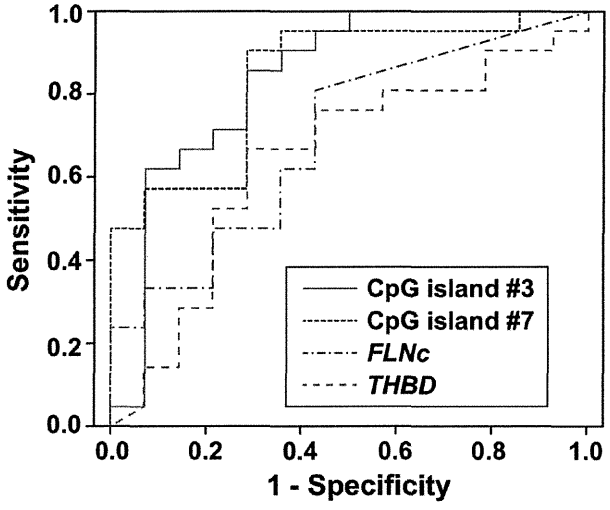

Figure 4. The degree of epigenetic field defects can be assessed using methylation levels of appropriate marker CpG islands, mostly passengers. Receiver-operating characteristic curves were drawn to distinguish gastric mucosae of gastric cancer patients and those of healthy individuals with past infections by *H pylori*. The receiver-operating characteristic curves of newly isolated methylation risk markers, CpG islands #3 and #7, had a much larger area under the curve values than those of 2 previously isolated markers, filamin C (FLNc) and thrombomodulin (THBD), reaching 0.78–0.84. Modified from Nanjo et al.[169]

As inducers of aberrant DNA methylation, aging was first indicated,[105] and chronic inflammation then was suggested by the presence of aberrant DNA methylation of specific tumor-suppressor genes in noncancerous colonic mucosae of patients with IBD.[106,107] Aberrant DNA methylation was present more frequently in liver tissues of patients with HCC than in those with metastatic liver tumors.[108] By measuring methylation levels of passenger genes in gastric mucosae of *H pylori*-infected individuals, a very close association between *H pylori* infection and high methylation levels in gastric mucosa was shown.[15] Aberrant DNA methylation is particularly prominent in chronic inflammation-associated cancers, such as gastric cancer, HCCs, colitic cancer, cholangiocarcinoma, Barrett's cancer, and pancreatic cancer.[13] These findings strongly indicated that the major inducer of aberrant DNA methylation is chronic inflammation.

Levels of aberrant DNA methylation accumulated in normal-appearing tissues correlate with the risk of gastric, colon, breast, and renal cancers.[15,109–112] Such accumulation mainly involves passenger genes and driver genes to some extent, and is considered to form an epigenetic field for cancerization (epigenetic field defect) (Figure 4).[113] Chronic inflammation-associated cancers are known to show multiple events, which can be explained by the presence of a field defect in normal-appearing tissues. Along with the accumulation of genetic alterations, an epigenetic field defect is deeply involved in the development of inflammation-associated cancers. The degree of epigenetic field defects can be measured easily using methylation levels of marker genes,[114] which are passenger genes in most cases and show relatively high methylation levels in predisposed tissues.[113]

Mechanistic studies, including cause and effect of accumulated aberrant DNA methylation and chronic inflammation, were conducted using animal models. When *H pylori*-induced inflammation was suppressed by cyclosporine A in Mongolian gerbils, induction of aberrant DNA methylation markedly was suppressed, although the number of *H pylori* in gastric mucosae was unaffected.[16] This indicated that inflammation, not *H pylori* itself, is critical for induction of aberrant DNA methylation. Expression analysis of inflammation-related genes showed that expression levels of *Il-1b*, *Nos*, *Tnf*, and chemokine (C-X-C motif) ligand 2 correlated with methylation levels in gastric mucosae. *H pylori*-induced inflammation was capable of inducing aberrant DNA methylation, but not repeated induction of acute inflammation by ethanol or a high sodium concentration.[115] *Il-1β*, *Nos2*, and *Tnf* were specifically up-regulated by the *H pylori*-induced inflammation. Notably, in human beings, a polymorphism of the *IL-1B* promoter was associated with not only gastric cancer susceptibility,[35] but also the presence of the CpG island methylation phenotype in gastric cancers.[116]

Another animal model for methylation induction by chronic inflammation is mouse colitis induced by administration of dextran sulfate sodium (DSS).[117] Aberrant DNA methylation of multiple genes occurred in DSS-induced colitis mucosae before induction of colon tumors, showing an epigenetic field.[118] The induction of aberrant DNA methylation was unaffected even in severe combined immunodeficiency mice that lacked T and B cells, suggesting that infiltrated macrophages might be critical for methylation induction. Gene expression analysis in colonic mucosae in wild-type and severe combined immunodeficiency mice showed that expression levels of *Il-1b*, *Nos*, and *Ifn-g* were associated with methylation induction in colonic mucosae. Taken together with the finding in the *H pylori*-infected gerbils, infiltration of macrophages and resulting secretion of Il-1β and Tnf-α, as well as production of active oxygen species, are believed to be involved in induction of aberrant DNA methylation in epithelial cells (Figure 1).

Several in vitro studies have been conducted to examine inflammatory signals that lead to methylation induction in target cells. Treatment of insulinoma or blood cells with IL-1β or a NO donor induced methylation of endogenous genes by increasing activity of DNA methyltransferase(s) (DNMTs).[119] IL-6 induces DNMT1 transcription by increasing its promoter activity and suppressing microRNA (miR)-148a and miR-152, both of which target DNMT1.[120,121] Although some studies suggested that DNA methylation is induced by IL-1β or IL-6, the changes were marginal, possibly because identification of appropriate target CpG islands was difficult and the levels of increase were too small to be detected by ordinary methods. Prostaglandin E2 treatment of cancer cell lines increased DNMT1 and DNMT3B expression, and induced

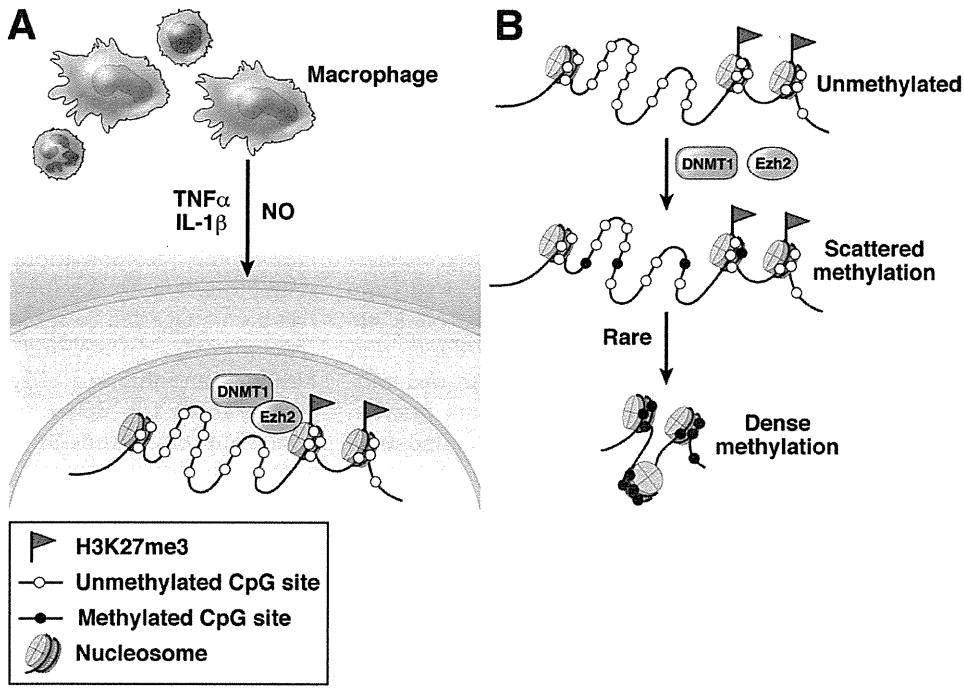

Figure 5. Current model of aberrant DNA methylation induction by chronic inflammation. (*A*) Cytokines, such as IL-1 and TNF-a from macrophages, and oxidative stress, such as NO, are associated with methylation induction in epithelial cells. EZH2 and DNMT1 are reported to be recruited to a promoter CpG island of a damaged gene, and mark it with a flag of H3K27me3. (*B*) Scattered methylation, introduced by DNMT1, leads to dense methylation although the frequency is low.

DNA methylation of specific genes, which also was observed in vivo.[122]

In contrast to in vitro studies, mRNA expression levels of Dnmt1, Dnmt3A, and Dnmt3B were not increased in vivo, such as colonic mucosae with DSS-induced colitis,[16] and human gastric tissues with *H pylori* infection.[123] In line with these in vivo findings, O'Hagan et al[124] recently showed in vitro that oxidative damage recruits complexes containing DNMTs, a histone deacetylase (sirtuin 1), and histone methyltransferase (enhancer of zeste homolog 2 [EZH2]) to damaged chromatin, and induces DNA methylation. They also showed that in Apc^{Min} mice infected with an inflammation-inducing bacterium, Dnmt1 and Ezh2 are recruited to promoter CpG islands of untranscribed or minimally transcribed genes. Promoter CpG islands with H3K27 me3 and without RNA polymerase II are susceptible to DNA methylation induction.[101,102]

Taken together, we can hypothesize a model for aberrant DNA methylation induction in vivo (Figure 5). Inflammatory signals mainly from macrophages, such as IL-1β, TNF-α, and IL-6, and oxidative stress, possibly produced by NO synthase, are likely to recruit a complex with DNMT1 and EZH2 to promoter CpG islands with H3K27 me3 flag and without protection by RNA polymerase II. Because DNA methylation is harmful to a gene, aberrant DNA methylation is likely to be induced only rarely and at scattered CpG sites within a CpG island (seeds of methylation).[123] Most seeds of methylation are erased during cell replication, but can lead to dense methylation of a CpG island at very low frequencies.[125,126] If such dense methylation is induced in a promoter CpG island of a tumor-suppressor gene, the tissue becomes predisposed to carcinogenesis, and forms an epigenetic field defect.

In addition to aberrant DNA methylation of promoter CpG islands, cancer cells are characterized by global DNA hypomethylation as well as aberrant hypomethylation of oncogenes.[99,127] Gastric mucosa infected by *H pylori* displays global hypomethylation.[128] In this regard, it is interesting to note that AID recently was shown to be involved in active DNA demethylation during fetal development.[129] Mechanistically, AID deaminates 5-methyl cytosine to yield T. This T subsequently would be removed by either of the T:G mismatch-specific glycosylases, thymidine DNA glycosylase, or methyl-CpG binding domain protein 4. The resulting abasic site then would be replaced by an unmethylated C via base excision repair processes, resulting in DNA demethylation. Notably, AID participates in active demethylation by 5-methyl cytosine hydroxylase, ten-eleven translocation 1, and subsequent gene expression in the dentate gyrus of adult mouse brain.[130] Thus, whether AID is involved in DNA demethylation during cancer development is an interesting topic for future studies.[131] The fact that AID targets the chromatin marked by H3K4 me3 histone modification,[132] in contrast to preferential DNA methylation at promoter CpG islands with H3K27 me3 histone modification,[101,102] might suggest opposing mechanisms for induction of DNA methylation and demethylation.

Inflammation and miRNA Modulation

miRNAs are short noncoding RNAs that regulate the expression of many target genes post-transcriptionally, and thus are involved in a variety of cellular functions. Recent studies have revealed that miRNAs have important roles in cancer development as either oncogenes or tumor-suppressor genes by regulating various cancer-related proteins or mRNA expressions.[133,134] In-