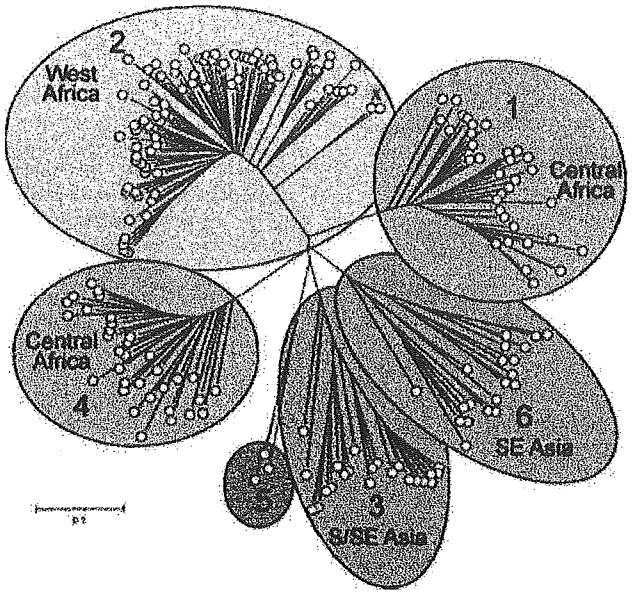

Fig. 2. Evolutionary tree of all available NS5B sequences of HCV. This phylogenetic analysis of the NS5B region of all publicly available nucleotide sequences in the region from 8276 to 8615 (numbered as in the H77 reference sequences, AF009606[63]) demonstrates that HCV variants still fall into 6 distinct genotypes but each contains numerous novel variants discovered in high-diversity areas in sub-Saharan Africa and Southeast Asia. The tree was constructed by neighbor-joining as implemented in the MEGA package,[97] using Jukes-Cantor corrected distances. More divergent members of genotype 2 are indicated with an "x."

pearance of new risk groups and routes of spread,[32,36] such as blood transfusion since the 1940s, the medical use of unsterilized needles for injections and vaccinations, and most specifically to industrialized countries, injecting drug use and the sharing of injection equipment,[32,37-39] has allowed the rapid spread and amplification of "founder" viruses. What we now call subtypes 1a, 1b, 2a, 2b, 3a, and 4a are likely to be the descendants of HCV variants that "seeded" these new, rapidly expanding transmission networks. As discussed later, HCV classification should both recognize the epidemiological associations of these "founder" viruses and incorporate their subtype names into the genotype nomenclature, while acknowledging that such labels are of little or no value in the description of HCV variants in high-diversity areas in sub-Saharan Africa and Southeast Asia.

Recombination. A recent discovery with implications for HCV classification is recombination between genotypes of HCV.[40,41] Homologous recombination in HCV could clearly be facilitated by the overlap in genotype distributions in many parts of the world. It also may be favored by the nature of HCV risk behavior, in which there may be frequent exposures around the time of primary infection (*e.g.*, repeated needle-sharing between several IDUs), and lack of protective immunity from reinfection during chronic HCV infection. Recently, a viable and rapidly spreading recombinant containing structural genes from genotype 2k and non-structural genes from genotype 1b was found in IDUs in St. Petersburg, Russia.[40,41] Inter-subtype (or intra-genotype) recombinants have also been described, such as a 1a/1b recombinant in Peru.[42] The true frequency of recombination may be underestimated because it would be difficult to detect if it occurred between variants of the same subtype. Similarly, it would be difficult to document inter-subtype recombinants where HCV is highly diverse, such as within genotype 2 in West Africa. Finally, although there is a comparative wealth of complete genome sequences of common HCV genotypes, such as 1b, most studies of HCV variability in high diversity areas are based on analysis of single sub-genomic regions, such as NS5B or core/E1, making detection of potential recombination events unlikely.

HCV Genotype Identification. Genotype identification is clinically important because genotypes 1 and 4 are more resistant than genotypes 2 and 3 to the current standard of care, pegylated interferon-α and ribavirin combination therapy.[43] Indeed, most treatment protocols require genotype information to tailor dose and duration of treatment. Genotyping assays are usually based on sequence analysis of an amplified segment of the genome, commonly the 5′untranslated region, because this region is targeted by most diagnostic assays for HCV RNA. Although this region is highly conserved, a well-characterized set of polymorphisms predict genotype and can be conveniently detected by probe hybridization,[44,45] changes in restriction sites[46,47] or by direct sequencing.[48]

For the purposes for which they are normally used (prediction of treatment response and dose scheduling),[43] currently used 5′UTR-based assays are acceptably accurate, with more than 95% concordance with genotypes identified by nucleotide sequencing in NS5B or other coding regions of the genome.[49-55] Several factors, however, preclude their use for definitive genotype identification, for identification of subtypes, and more generally, as an HCV classification tool:

- Although several genotype-specific nucleotide changes in the 5′UTR usually allow each of the 6 main genotypes to be differentiated from each other, there are exceptions. Some genotype 6 variants found in Southeast Asia have 5′UTR sequences identical to those of genotype 1a or 1b.[34-36,56] This illustrates a more general point that, even for genotype identification, the performance of genotyping assays is very much a property of the range of HCV variants tested. The currently used 5′UTR-based assays are unlikely to operate to the published level of accuracy (>95%; see above) in high-diversity areas.

- Even for well-characterized variants of HCV, such as those circulating in Western countries, sequence differences between subtypes may be variable or non-existent in the 5′UTR. For example, a sequence polymorphism at position 243 (numbered as in the H77 reference sequence), frequently used to differentiate subtypes 1a and 1b, is unreliable. In one of the original surveys, 6 (7.5%) of 80 subtype 1a sequences and 1 of 79 1b sequences would have been incorrectly identified on the basis of this polymorphism.[57] A related problem is that although some subtypes may be separately identifiable in the 5′UTR (such as 2a and 2b), others, such as 2c, may not, even though all 3 subtypes are approximately equally divergent from each other elsewhere in the genome (Fig. 1).
- Even relatively short coding regions of the HCV genome provide more definitive information on the genotype or subtype of an HCV variant than the 5′UTR. Although not necessarily required clinically, the nucleotide sequence of a sub-genomic region (including the conserved core gene) allows definitive identification of genotype and generally of the subtype, as well as being able to predict the existence of HCV variants not yet classified.
- For all genotyping assays, whether based on the 5′UTR or elsewhere in the genome, there is an intrinsic assumption that the genotype inferred from 1 region reflects that of the genome as a whole. Although few recombinant forms have been described, the spread of HCV variants such as the 2k/1b recombinant and the generation of further hybrid viruses in multiply exposed individuals would increasingly limit the accuracy of genotyping assays, and importantly for their clinical use, attenuate their predictive value for treatment response.

As should be evident from these points, HCV identification is an activity distinct from HCV classification. Classification provides the framework on which the specificity and accuracy of genotyping assays can be assessed, and for this purpose an agreed and consistent set of classification criteria, and a system of assigning genotype names is required. The following section discusses the issues in HCV classification in which consensus is required, and is followed by a series of classification and nomenclature proposals designed to maintain clarity in this field.

Current Issues to Resolve in HCV Classification

Several problems and uncertainties with current classification schemes for HCV have been identified and cause both inconsistencies with the nomenclature of HCV variants in published papers and difficulties for the organization and retrieval of HCV sequences from the 3 databases. These can be summarized as follows:

Diversity Within Genetic Groups. Although the primary division of HCV variants into 6 genetic groups is evident from phylogenetic analysis (Figs. 1 and 2), it has been increasingly recognized that there is considerably more genetic diversity within groups 2, 3, and 6 than found between the originally classified subtypes 1a and 1b, and 2a, 2b, and 2c.[34] In the past, it had been additionally proposed that more divergent variants within groups 3 and 6 qualify as separate major genotypes of HCV. At the HCV Classification meeting in Santa Fe, genetic group 6 was proposed to be re-designated as "clade 6," its variants retain their proposed genotype designations as genotypes 6, 7, 8, 9, and 11; similarly, "clade 3" should contain variants classified as genotypes 3 and 10.[2] In this scheme, the one-to-one correspondence between genetic group and genotype is lost.

The imposition of an additional tier of variability, however, leads to largely arbitrary classification decisions that compromised the simplicity of the original primary assignment of HCV genetic groups as genotypes. For example, both subtype 3b and the proposed new genotype 10a are both in genetic group 3 but are both highly divergent in sequence from subtype 3a, much more so than other subtypes of genotype 3 (Fig. 1). The decision to classify 10a as a genotype and 3b as a subtype was based on a difference in nucleotide sequence divergence in the coding region of only 3% (23% between 3a and 3b, 26% between 3a and 10a). This is much lower than the 31% to 34% divergence between variants in different genetic groups (such as between 1a and 2a). Divergence between the various proposed genotypes in group 6 is similarly consistently lower (mean, 27%; range, 21%-29%) than between the originally classified genotypes. Genetic group 2 may similarly contain more divergent sequences than the norm for subtypes (marked as "x" in Fig. 2). This might lead to the addition of further, equally arbitrary, genotype designations in a geographical region where otherwise genotype 2 variants are predominant in the population.

Apart from the difficulty in placing this further dividing line between genotype and clade, the resulting classification in a subtype/genotype/clade hierarchy is geographically inconsistent. To many, the scheme has been confusing, because in some cases, a clade contains only 1 genotype and the terms are interchangeable (*e.g.*, genotype 1/clade 1); in others a clade may contain 5 or more genotypes (*e.g.*, clade 6, genotypes 6, 7, 8, 9, and 11). This confusion and lack of consensus has led to continuing nomenclature differences between publications

whenever variants from Southeast Asia and elsewhere are described.

Conflicting Subtype Designations. There are many examples of conflicting nomenclature within currently classified HCV variants. Most of these inconsistencies comprise 2 different subtypes being referred to by the same name, such subtypes "4a" found in Egypt[7] and Zaire.[12] Conversely, the same variant may be described with different subtype designation, such as VAT96, designated as "2k,"[58] and RU169 designated as "2j."[59] These occurrences will have to be resolved in an agreed catalogue of HCV variants, and for retrieval of sequences from the HCV databases.

Recombination. Currently no method exists for classifying recombinant forms of HCV. For database retrieval and for cataloguing the occurrence of recombinant viruses, a nomenclature system that recorded its genotype composition and provided unique identifiers for pattern of breakpoints would be of value. This system is in place for HIV-1 and might be used as a model for HCV.[60] Here, designation of inter-subtype recombinant viruses as (circulating) recombinant forms (RFs) requires detection and complete genome sequences of a recombinant virus from 3 or more independently infected individuals. Each new recombinant should have breakpoints in the same positions in each sequence. Each is then numbered sequentially in order of discovery, with subtype identification letters listed alphabetically to approximately indicate their composition. The HCV recombinant in St. Petersburg[40,41] would therefore be designated as RF 01_1b2k.

Table 1. Confirmed HCV Genotypes/Subtypes

Genotype*	Locus/Isolate(s)†	Accession number(s)	Reference(s)
Genotype 1			
1a	HPCPLYPRE, HPCCGAA	M62321, M67463	67, 68
1b	HPCJCG, HPCHUMR	D90208, M58335	69, 70
1c	HPCCGS, AY051292	D14853, AY051292	71
Genotype 2			
2a	HPCPOLP, JFH-1	D00944, AB047639	72, 73
2b	HPCJ8G, JPUT971017	D10988, AB030907	9, 74
2c	BEBE1	D50409	75
2k	VAT96	AB031663	58
Genotype 3			
3a	HPCEGS, HPCK3A	D17763, D28917	76, 77
3b	HPCFG	D49374	78
3k	HPCJK049E1	D63821	59
Genotype 4			
4a	HCV4APOLY	Y11604	79
Genotype 5			
5a	EUH1480, SA13‡	Y13184, AF064490	80, 81
Genotype 6			
6a	HCV12083, 6a33	Y12083, AY858526	82
6b	Th580	D84262	83
6d	VN235	D84263	83
6g	HPCJK046E2	D63822	59
6h	VN004	D84265	83
6k	VN405	D84264	83

NOTE. Tables 1, 2, and 3 were compiled by a working group of Donald Murphy, Erwin Sablon, and Phillipe Halfon.

*Consensus proposed genotype/subtype names. For instances in which multiple sequences of a HCV genotype are available, two sequences have been listed, prioritized by (1) publication date, or (2) submission date when unpublished.

†Locus (or isolate name, if locus is the same as the accession number).

‡Sequence obtained from acute phase plasma of a chimpanzee experimentally infected with (human-derived) isolate SA13.

Consensus Classification Proposals

Each of these issues in HCV classification was discussed, and the following consensus decisions were made. These are proposals for standardizing the nomenclature of currently described variants of HCV, and the future designation of new subtypes and genotypes as they are discovered.

Division of HCV Into Clades/Genotypes. The primary division of HCV variants remains the 6 genetic groups, irrespective of the hugely increased numbers of subtypes or variants since found within these groups. The consensus acknowledges that different levels of within-group diversity are found between genotypes, and different relationships within them. Nevertheless, varying degrees of diversity are becoming apparent in other genotypes (*e.g.,* among the genotype 2 variants from West Africa), and it is difficult and arbitrary to specify a degree of sequence divergence below which a subtype designation is made, and above which a new genotype is assigned. This difficulty is epitomized by the problems with the classifications of 3b and 10a within genotype 3 (see above).

The following points summarize the recommendations concerning the designation of HCV genotypes:

1. The primary division of HCV will henceforth be based on the 6 genetic groups apparent from Figs. 1, 2, and other published sequence analyses of HCV. Division of HCV variants into the 6 genetic groups of HCV is supported by each of the principal methods of phylogenetic analysis of the core/E1, NS5B, and complete genome sequences (Table 1). These comprise tree-building by: (i) neighbor-joining and unweighted pair group method with arithmetic mean from pairwise distances computed with a variety of substitution models, (ii) parsimony, and (iii) maximum likelihood. For distance-based methods, greater than 70% of trees (actually invariably greater than 90%) support the primary division of HCV variants into the 6 genetic groups, with no consistent support for any higher-level grouping. Consistency between phylogenetic methods is required for the assignment of new genotypes (see specific proposals below).

Table 2. Listing of HCV Variants With Proposed Changes in Genotype Nomenclature

Proposed Designation*	Published Designation	Status†	Isolate‡	Region Sequenced§	Reference(s)
Genotype 2					
2k	2j	C	RU169	NS5B (D86532), 3'UTR (D86532)	83
2j	2i	P	BA047	NS5B (D86530), 3'UTR (D86530)	83
2n	2e	P	NL50	C/E1 (L39309), NS5B (L44602)	84
2o	4f/2f	P	FR4	C/E1 (L38333), NS5B (L38373)	84
2p	2f	P	NL33	C/E1, (L39300), BS5B (L44601)	84
2q	2k	P	BA045	NS5B (D86529), 3'UTR (D86529)	83
Genotype 3					
3k	10a	C	HPCJK049E1	Complete genome (D63821)	59
Genotype 4					
4r	4a	P	Z4	C/E1 (U10236/L16652)	12, 64
			FrSSD120	C/E1 (AJ401097), NS5B (AJ291282)	93
4n	4 alfa	P	1359	C/E1 (AF271874)	65
4o	4 beta	P	2153	C/E1 (AF271882), NS5B (AF271815)	65
Genotype 6					
6c	7d	P	Th846	C/E1 (D37843), NS5B (D37857)	35
6d	7b	C	VN235	Complete genome (D84263)	83
6e	7a	P	VN540	C/E1 (D88474), NS5B (D87361)	34
6f	7e	P	BB7	NS5B (D28541)	96
6f	7c	P	Th271	C/E1 (D37844), NS5B (D37858)	35
6g	11a	C	HPCJK046E	Complete genome (D63822)	59
6h	9a	C	VN004	Complete genome (D84265)	83
6i	9b	P	Th555	C/E1 (D37849), NS5B (D37863)	35
6j	9c	P	Th553	C/E1 (D37848), NS5B (D37862)	35
6k	8b	C	VN405	Complete genome (D84264)	34
6l	8a	P	VN507	C/E1 (D88470), NS5B (D87357)	34

*Proposed new name based on revised criteria for genotype designations.

†Classification status; C: Confirmed; P: Provisional.

‡Example of isolates referred to in associated publications (last column).

§Regions sequenced (accession numbers in parentheses), prioritized for (1) complete genome; (2) Core/E1 and NS5B regions; (3) other regions where core/E1 and NS5B regions are not both available.

2. The genetic groups will be termed "genotypes." The previously proposed term "clade" to describe an HCV genotype might be regarded as an alternative, more descriptive term for genotype, and is currently used in the VIIIth ICTV Report.[1] However, for consistency with previous classifications of HCV and current clinical usage, we recommend the use of the term "genotype" for genetic group in HCV sequence databases and publications.
3. Variants of HCV currently designated with genotype numbers above 6 will be renamed according to the genotype group in which they fall, and with the next available subtype designation (Table 2). For example, genotype 10a will be re-classified as 3k, 7a as 6e, and so forth. The proposed changes to the nomenclature are presented in Table 2.
4. The identification of new genotypes will henceforth require demonstration of a consistent independent phylogenetic grouping away from any of the currently classified genotypes of HCV (see later discussion).

Classification and Nomenclature of Previously Described Subtypes of HCV. The group believed the existing nomenclature of HCV genotypes and subtypes provided a valuable framework for ongoing studies of genetic variation. The following points summarize the group's decisions and recommendations for subtype designations:

1. Existing designations where they are consistent will be retained, irrespective of the criteria agreed for the designation of new subtypes (Tables 1 and 3).
2. Variants within genotypes 3 and 6 that have been re-designated as subtypes (see previous section) will be incorporated into the updated list.
3. HCV variants with conflicting names in the literature have been re-designated on consultation with the originating authors (Table 2).

Assignment of New Genotypes of HCV. Further variants of HCV likely will be discovered that merit their assignment as new genotypes, such as the candidate new genotype obtained from Central Africa.[61,62] To ensure their correct classification, it is essential to demonstrate that there is no significant grouping within any of the existing genotypes. This has to be demonstrated by rigorous phylogenetic analysis of a complete sequence of the coding region of the virus. This analysis will additionally

Table 3. Provisionally Assigned HCV Subtypes

		Accession Number(s)*		
	Isolate†	Core/E1	NS5B	Reference(s)
Genotype 1				
1d	HC1-N15, HC1-N16	L39299, L39302	L38377, L38372	84
1e	CAM1078, QC248	L38349(C), AY894555	L38361, AY894553	62, 84
1f	FR2	L38350	L38371	84
1g	2152, 1382	AF271822, AF271820	AF271798, AF271797	65
1h	98CM1521, QC94	AY256790(C), AY434131	AY257087, AY434132	32, 62
1i	FR16, QC77	n.a., AY434119	L48495, AY434120	62, 85
1j	QC2, QC89	AY434158, AY434128	AY434106, AY434129	62
1k	QC68, QC82	AY434112, AY434122	AY434113, AY434123	62
1l	98CM1383, 98CM1427	AY256789(C), AY256792(C)	AY257083, AY257091	32
Genotype 2				
2d	NE92, BN177	L39294, n.a.	L29634, AF037244	66, 86
2e	JK020, JK025	D49745, D49746	D49760, D49761	59
2f	JK081, JK139	D49754, D49757	D49769, D49777	59
2g	MED017	n.a.	X93323	26
2h	MED007	n.a.	X93327	26
2i	FR13, HN4	n.a., X76411/X76415	L48492, L48499	87, 88
2j	BA047, QC106	n.a., AY894528	D86530, AY894526	62, 83
2l	FR15	n.a.	L48494	85, 89
2m	QC76, QC104	AY434116, AY434143	AY434117, AY434144	62
2n	NL50	L39309	L44602	84
2o	FR4	L38333	L38373	84
2p	NL33	L39300	L44601	84
2q	BA045	n.a.	D86529	83
Genotype 3				
3c	NE048	D16612	D14198/D16613	33
3d	NE274	D16620	D14200/D16621	33
3e	NE145	D16618	D16619	33
3f	NE125, PK64	D16614, n.a.	D14203/D16615, L78842	33, 87
3g	IND1751, IND1452	X91423/X91307, X91306(C)	X91417, X91418	90
3h	QC29, SOM1	U33437(C), AF216792/AF216786	AF279120, AF216789	91, 92
3i	IND674, QC100	X91300(C), AY434137	X91422, AY434138	62, 90
Genotype 4				
4b	Z1	U10235/L16677	n.a.	12, 64
4c	Z6, GB358	U10238/L16678, L29606	n.a., L29607	12, 64, 66
4d	DK13, SD006	U10192/L16656, n.a.	n.a., D86537	12, 64, 83
4e	CAM600, GB809	L29589, L29629	L29590, L29626	66
4f	G22, FR12	L29595, L38332	L29593, L38370	66, 84
4g	GB549	L29620	L29621	66
4h	GB438, FrSSD35	L29610, n.a.	L29611, AJ291249	66, 93
4i	CAR4/1205	L36439	L36437	28
4j	CAR1/501	n.a.	L36438	28
4k	B14, FrSSD174	L39282, n.a.	L44597, AJ291294	84, 93
4l	SD002, 2116	n.a., AF271881	D86534, AF271816	65, 83
4m	SD035, 1797	n.a., AF271876	D86543, AF271813	65, 83
4n	1359, QC97	AF271874, AY434134	n.a., AY434135	62, 65
4o	2153, QC59	AF271882, AY434107	AF271815, AY434108	62, 65
4p	FrSSD158, QC139	AJ401099(E), AY434149	AJ291285, AY434150	62, 93
4q	QC86, QC107	AY434125, AY434146	AY434126, AY434147	62
4r	Z4, FrSSD120	U10236/L16652, AJ401097(E)	n.a., AJ291282	12, 64, 93
4t	98CM1458, QC85	AY256808(C), AY706996	AY257072, AY706997	32, 62
Genotype 6				
6c	Th846	D37843	D37857	35
6e	VN540, VN998	D88474, D31971	D87361, D30797	34
6f	Th271, EUTH36	D37844, U31261(C)	D37858, U31276	24, 35
6i	Th555, EUTH100	D37849, L50554(C)	D37863, L50535	35, 94
6j	Th553, EUTH1	D37848, L49473(C)	D37862, L49481	35, 56
6l	VN507, VN531	D88470, D88472	D87357, D87359	34
6m	EUBUR1, B4/92	L49480(C), D63943/D63944	L49484, D28543	56, 95
6n	D86/93, EUTH86	D63945, U31259(C)	D28545, U31275	24, 96
6o	VN4, QC33	L38341, AY894537	L38382, AY894535	62, 84
6p	VN12, QC123	L38340, AY894534	L38380, AY894532	62, 84
6q	QC57, QC176	AY754632, AY754617	AY754633, AY754618	62

*Accession numbers of sequences from the core/E1 and NS5B regions. Where two examples are listed, a comma divides the accession numbers from the two entries; "n.a.": not available; "/": denotes that the core/E1 or NS5B sequences are available from two different accession numbers; (C): only core sequence available; (E): only E1 sequence available.

†Listing of up to two examples of each provisionally assigned HCV subtype prioritized according to (1) availability of complete or near complete core/E1 and NS5B sequences, (2) publication date, (3) GenBank/EMBL/DDBJ submission date. Where possible, the isolate names referred to in associated publications (last column) are listed for ease of reference.

confirm the absence of recombination with sequences from other genotypes.

The following criteria were proposed for identification and designation of a variant of HCV as a new genotype:

1. *Provisional designation.* This requires one complete coding region sequence to be obtained, the demonstration of a separate grouping from other genotypes by phylogenetic analysis, and an absence of recombination. The sequence of a candidate new genotype should be independently analyzed by submission to one of the HCV databases. The sequence will be analyzed by a variety of phylogenetic methods described previously. This will allow the sequence to be assigned with the next available genotype number, and the subtype designation "a," for example, genotype 7a.
2. *Confirmed designation.* This requires coding sequences of 2 or more HCV variants from infections that are not directly linked epidemiologically. The sequences should further demonstrate a lack of grouping with current classified genotypes by the above methods. This further analysis, and any available sequences from subgenomic regions such as core/E1 and NS5B (see later discussion), will provide valuable reference information on the genetic heterogeneity within the newly designated genotype, the existence of subtypes, the geographical origins of the variants, and their likely designation in 5′UTR-based genotyping assays.

Assignment of New Subtypes of HCV. Different issues apply to the assignment of new subtypes. Some geographical regions contain so much diversity within genotypes that it is of little value to continue classifying them as subtypes. Elsewhere, however, subtype labels have particular epidemiological value and are widely used as genetic markers in studies of past and ongoing virus transmission of HCV in different risk groups.

To recognize this distinction, new subtype designations should only be provided where there is evidence for its spread in particular transmission networks, and where its identification would be of epidemiological value. The simplest method to achieve this distinction is to require evidence of infection with a new proposed subtype of HCV in several independently infected individuals.

The following criteria for assignment of new subtypes were proposed:

Provisional designation. Three or more examples of infection with a new proposed subtype are required for subtype designation. Sequences are required from both the core/E1 region (sequence data available from 90% or more nucleotides corresponding to positions 869 to 1292 in the H77 reference sequences, accession number AF009606)[12,63-65] and the NS5B region (data from 90% or more positions in the region 8276-8615 in H77).[7,8,66] The sequences of primers suitable for amplification of these regions from a wide range of HCV genotypes will be made available on the public databases.

Sequences will be analyzed by a variety of distance-based, parsimony, and maximum likelihood methods, and evidence sought for consistent phylogenetic grouping together and distinctness from other subtypes. Because currently classified subtypes of HCV differ in nucleotide sequence from each other by more than 15%, at least this level of divergence will be expected from other HCV variants within the genotype. However, as described in the Introduction, the existence of separately identifiable subtypes is primarily an epidemiological phenomenon associated with its recent spread. Because subtype designation are primarily epidemiological labels, it is clearly not appropriate or of value to develop formal criteria for their assignment. Indeed, the varying degrees of sequence divergence of variants within different genotypes would make the development of such criteria extremely difficult.

Candidate subtypes will be provisionally assigned with the next available subtype letter for the genotype on submission to one of the HCV databases. Sequences from the 5′UTR will be of value for assessment of their appearance in commonly used genotyping assays but are not required. Single or pairs of variants of HCV that would otherwise be designated as new subtypes by these criteria will not be assigned a subtype letter in the database.

Confirmed designation. One or more complete genome sequences will be required for confirmed designation. This will allow the degree of sequence divergence from other subtypes over the whole genome to be assessed as well as confirming an absence of recombination.

Assignment of Recombinant Forms of HCV. It is important that the classification scheme for HCV genotypes should be able to incorporate HCV recombinants. However, with the current description of only 2 or 3 confirmed or possible recombinants in the literature, it was deemed to be of less immediate importance to classify these formally, and to develop rules for nomenclature. Until review at a subsequent classification meeting, sequences with evidence of recombination will be annotated as such in the databases, with options to include or exclude them from downloads or analyses of sequences.

Interface With HCV Sequence Databases. The HCV sequence databases are in a unique position to support the effort to make the HCV nomenclature more uniform. By assigning geno/subtypes to the sequences that people retrieve and download, they can influence the

commonly used nomenclature of existing sequences, whereas they can have a coordinating role in assigning new geno/subtypes and keeping track of these, especially before journal publication. The databases are also committed to assist in the naming of new geno/subtypes, through helping researchers name proposed new geno/subtypes, by checking existing names for consistency and correcting any inconsistencies that are found, by making it easy for the field to keep track of which geno/subtype names have already been assigned, and by providing tools for genotype or subtype identification and detecting recombinants.

The HCV database websites will provide access to the criteria for assignment of new genotypes and subtypes of HCV developed in this consensus paper, and make HCV researchers, reviewers, and journal editors aware of these guidelines. They will provide the listing of current assigned subtypes and genotypes (based on Tables 1 and 3), but will be automatically updated as sequence data are submitted, showing which designations exist in the databases, but not those that have been given out and not yet published. The distinction between "provisional" and "confirmed" designations will also be implemented in the databases through the provision of a separate field for this category. The genotype name re-assignments in Table 2 will similarly be made available, and the 3 databases will keep in continuous contact to ensure that the nomenclature of currently existing sequences is uniform and free of conflicts.

Summary

This report describes a series of proposals for the classification of HCV variants into genotypes and subtypes. It addresses both current problems with the nomenclature of existing variants, and incorporates our improved understanding of the genetic diversity and epidemiology of HCV into the revised criteria for the designation of new genotypes and subtypes. The consensus meeting provided the opportunity to compile for the first time a full listing of currently described variants of HCV (Tables 1 and 3), and the opportunity to perform the minimum number of genotype and subtype name re-assignments to create consistency in nomenclature (Table 2).

Finally, these proposals serve as a framework for access to the 3 databases, which will follow the revised nomenclature presented here for sequence retrieval, and to use the revised criteria for classification in their coordinating role in the assignment of new genotypes and subtypes; this will be of major value in preventing future inconsistencies in nomenclature.

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Polymerase Domain B Mutation Is Associated with Hepatitis Relapse during Long-Term Lamivudine Therapy for Chronic Hepatitis B

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Key Words
Lamivudine · Breakthrough hepatitis · INNO-LiPA · Hepatitis B virus DNA · Domain B

Abstract
Breakthrough hepatitis remains the major issue in long-term lamivudine therapy for chronic hepatitis B. However, the emergence of drug-resistant hepatitis B virus (HBV) is not always accompanied by a relapse of hepatitis. To elucidate factors predictive of breakthrough hepatitis, 53 patients with genotype C of HBV on long-term lamivudine therapy were analyzed. HBV reappeared during therapy in 19 patients with a cumulative incidence of 15% at 1 year, 34% at 2 years, and 60% at 3 years. Within this group, breakthrough hepatitis developed in 12 patients (63%). A polymerase gene domain B mutation (rt180M) emerged in 13 patients, and domain C mutations (rt204I, rt204V) were found in 19 patients. The rt180M mutation was associated with breakthrough hepatitis ($p < 0.05$) with a positive predictive value of 85% and a negative predictive value of 83%. Patients with the rt180M mutation had higher HBV-DNA levels during viral breakthrough compared to patients with rt180wt ($p < 0.05$). The mutational pattern of rt204 was not associated with breakthrough hepatitis. In conclusion, genotypic assays for the rt180M mutation after viral breakthrough may be useful in predicting the risk of breakthrough hepatitis and in deciding when to initiate alternative or additive nucleoside analogue therapy.

Introduction

Lamivudine, a nucleoside analogue, is now widely used as primary therapy for chronic hepatitis B virus (HBV) infection [1]. The initial clinical response is usually favorable with high rates of HBV suppression, normalization of serum alanine transaminase (ALT) levels, loss of detectable serum HBe antigen, as well as histologic improvement [2–7]. However, short-term therapy cannot completely eliminate the HBV pool in the liver [8], and cessation of therapy usually leads to withdrawal hepatitis [9–11]. Consequently, long-term therapy is required in the majority of patients to maintain the suppressive effects of lamivudine.

Unfortunately, long-term therapy has drawbacks as well. Response rates may gradually decrease due to the emergence of drug-resistant HBV. This resistant virus form is characterized by amino acid mutations in the catalytic domains of the polymerase gene. Two mutations are frequently observed in association with lamivudine resistance: a mutation of methionine to isoleucine or va-

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line in codon 204 of the catalytic domain C, and a mutation of leucine to methionine in codon 180 of the catalytic domain B [12]. The emergence of these mutations leads to the reappearance of HBV-DNA (viral breakthrough) and the relapse of hepatitis (breakthrough hepatitis) [13] at cumulative rates of 14–32% at 1 year and 38–49% at 2 years [2, 4, 5, 14, 15].

However, biochemical changes do not always correlate with drug resistance. In some cases of viral breakthrough during long-term lamivudine therapy, patient ALT levels remain unaffected, and these patients continue to exhibit histologic improvement [12, 15]. Conversely, there are also patients who develop a severe flare-up of their hepatitis or even frank decompensation when viral resistance emerges [6, 16–20]. The risk of hepatitis B relapse thus becomes an important issue in patients undergoing long-term treatment with lamivudine. While various factors such as pretreatment HBV-DNA levels, ALT levels, presence of HBe antigen, and certain genotypes, may be associated with the emergence of resistant viral strains, factors predictive of a patient's clinical outcome have not yet been defined. Given the wide variability in patient responses to viral breakthrough and the potential morbidity and mortality associated with the worst outcomes, identifying pretreatment factors predictive of breakthrough hepatitis could be very relevant to clinical practice.

Finally, when analyzing treatment resistance, it is also important to consider the HBV viral genotype. Viral genotype may have some bearing on patient outcomes with long-term lamivudine therapy. For example, patients infected with genotype A who develop viral breakthrough while on lamivudine tend to have higher HBV-DNA levels than patients who harbor other HBV genotypes such as D. Investigators have recently shown that the pattern of polymerase gene mutation leading to lamivudine resistance may be different for genotype A [21]. A double mutation of methionine to valine in codon 204 and leucine to methionine in codon 180 was prevalent in genotype A, while a methionine to isoleucine mutation in codon 204 occurred more frequently in genotype D. These differences in mutational patterns may be linked to an association between genotype and HBV-DNA levels after viral breakthrough.

Currently, the most prevalent HBV genotype in Japan is genotype C [22]. Genotype C is reported to be associated with a more aggressive clinical course and increased resistance to interferon therapy when compared to genotype B [23–25]. Given the pertinence of genotype in lamivudine resistance, differentiating the clinical significance of the various polymerase gene mutations in genotype C becomes critical.

The aim of the present study is to elucidate factors associated with breakthrough hepatitis during long-term lamivudine therapy in HBV genotype C infections. Mutations in domains B and C of the polymerase gene, core promoter gene and precore gene were analyzed to determine if specific mutational patterns might be associated with different clinical outcomes.

Patients and Methods

Therapeutic Protocol

Fifty-three patients with chronic hepatitis B genotype C who were consecutively started on long-term lamivudine monotherapy between August 1999 and November 2003 at Musashino Red Cross Hospital were analyzed retrospectively. There were 32 males and 21 females; mean age was 48.8 ± 11.8 years. At the start of therapy, all patients had detectable levels of HBV-DNA in their blood by polymerase chain reaction (PCR), as well as elevations in serum ALT levels. All patients were also found not to have either hepatitis C or human immunodeficiency virus antibodies in their blood. No patient received interferon or any other antiviral agents during the study or within 6 months of initiating lamivudine therapy. Patients were treated with a single oral dose of 100 mg of lamivudine every day; median duration of lamivudine therapy was 689 (range 207–1,736) days. All patients remained on lamivudine therapy throughout the course of study except those who developed breakthrough hepatitis. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Quantification of HBV-DNA

Blood samples were obtained at the start of therapy and then once every month during therapy. The serum level of HBV-DNA was determined using transcription-mediated amplification and hybridization protection assays (Fujirebio Inc., Tokyo, Japan) that have a detection range of 3.7–8.7 log genome equivalents (LGE)/ml [26].

Definitions

Viral breakthrough was defined as an elevation of more than 1 LGE/ml of HBV-DNA accompanied by mutations in the polymerase gene on 3 consecutive determinations during monthly testing after a period of HBV-DNA suppression. No case that met this definition experienced a spontaneous decline in HBV-DNA thereafter. Breakthrough hepatitis was defined as a sustained elevation in serum ALT levels on 2 consecutive determinations 2 weeks apart in concert with viral breakthrough.

Analysis of Precore and Core Promoter Mutations

Mutations in the precore and core promoter regions were analyzed at baseline. The A1762T and G1764A mutations in the basic core promoter [27] were detected by a commercially available en-

zyme-linked specific probe assay (Smitest HBV core promoter mutation detection kit, Genome Science Laboratory, Tokyo). The G1896A stop codon mutation in the precore region was detected by an enzyme-linked mini sequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo). Use of both of these assays has been described previously [28].

Analysis of Lamivudine-Resistant HBV

Blood samples at the time of viral breakthrough were analyzed for mutations in the HBV polymerase associated with lamivudine resistance using INNO-LiPA HBV DR analysis (Innogenetics, Inc., Ghent, Belgium) [29, 30]. Briefly, DNA isolated from the serum was amplified by nested PCR and used for hybridization to the LiPA strips. The probes on the INNO-LiPA HBV DR strip cover the amino acids of codon 180 (wild-type leucine (L) and mutant methionine (M)) and codon 204 (wild-type methionine and mutants valine (V) and isoleucine (I)). The amino acid positions on the HBV polymerase gene are numbered for consistency with the newly established standardization of nomenclature for lamivudine-resistance mutations rt180M and rt204V/I (originally designated as L528M or L526M and M552V/I or M550V/I) [31].

Statistical Analysis

For statistical analysis the STAT View software package was used. Categorical data were analyzed using Fisher's exact test. Continuous variables were compared with Student's t test. A Kaplan-Meier estimate and the log-rank test were used to calculate the median time to and the significance of viral breakthrough, as well as the median time to and the significance of breakthrough hepatitis. Cox's proportional hazard model and stepwise logistic regression analysis were used for multivariate analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics, Pretreatment Variables and Clinical Course

Prior to initiation of lamivudine therapy, the mean HBV-DNA level was 7.1 ± 1.1 LGE/ml, and the mean ALT was 215 ± 285 U/l. Of a total of 53 patients infected with genotype C of HBV, 30 patients had detectable HBeAg in their serum (56.6%). A precore stop codon mutation was detected in 21 patients (42%), and core promoter mutations were detected in 44 patients (88%). The median treatment period was 689 (range 207–1,736) days.

During therapy, detectable levels of HBV-DNA fell below 4 LGE/ml in 42 (79.3%) patients. Viral breakthrough occurred in a total of 19 patients; in these patients, the median time to viral breakthrough was 473 (range 224–1,128) days. The cumulative incidence of viral breakthrough was 15% at 1 year, 34% at 2 years, and 60% at 3 years (fig. 1).

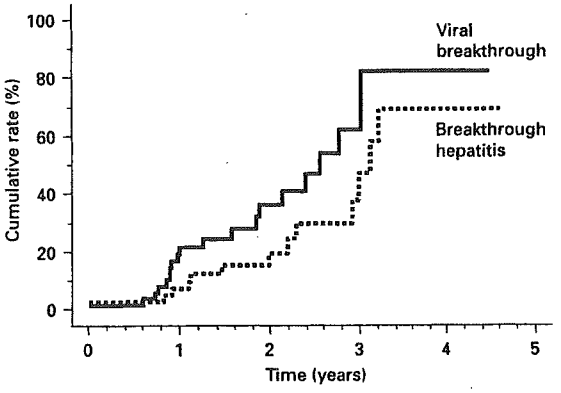

Fig. 1. The cumulative rate of viral breakthrough and breakthrough hepatitis. Kaplan-Meier plot of time to viral breakthrough and time to breakthrough hepatitis in 53 patients treated with lamivudine.

Among those 19 patients who developed viral breakthrough, 12 patients (63%) also developed breakthrough hepatitis. The median time to hepatitis after viral breakthrough was 111 days. The other 7 patients remained in biochemical remission. The cumulative incidence of breakthrough hepatitis was 4% at 1 year, 17% at 2 years, and 45% at 3 years (fig. 1).

Pretreatment variables including age, gender, presence of HBe antigen, HBV-DNA levels, ALT levels, and precore and core promoter mutations were analyzed. These variables were not found to be associated with viral breakthrough or with breakthrough hepatitis (table 1).

Variables at 24 Weeks of Treatment

HBV DNA levels after 24 weeks of lamivudine therapy correlated significantly with eventual viral breakthrough. At week 24 of therapy, HBV-DNA levels were above 4 LGE/ml in 23 patients. Moreover, there was a significant difference in time to viral breakthrough between those whose HBV-DNA levels were above and those whose levels were below 4 LGE/ml after 24 weeks of therapy ($p = 0.007$, Kaplan-Meier log-rank test; fig. 2a). Patients with HBV-DNA levels above 4 LGE/ml had a 3.5-fold higher probability of viral breakthrough compared to the other patients (Cox's proportional hazard model, 95% CI 1.33–9.34, $p = 0.012$). In contrast, the HBV-DNA levels above 4 LGE/ml at week 24 were not associated with breakthrough hepatitis (fig. 2b).

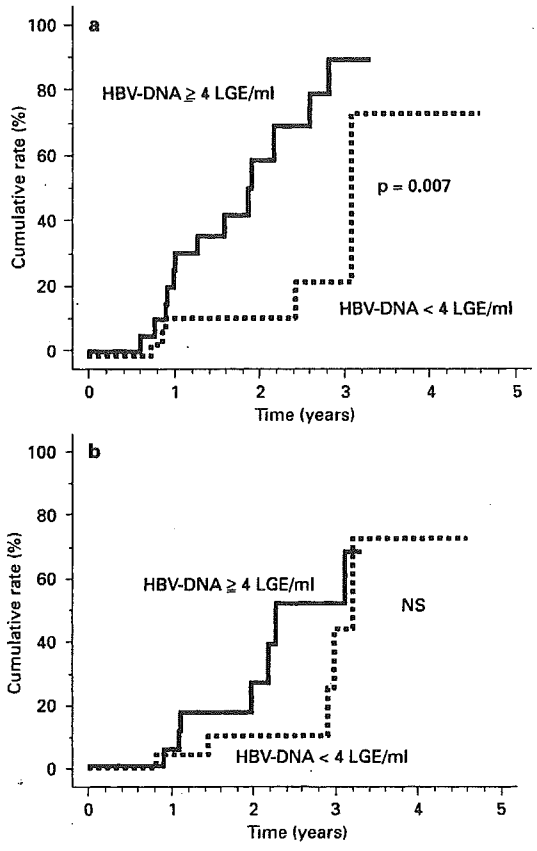

Fig. 2. The cumulative rate of viral breakthrough and breakthrough hepatitis according to HBV-DNA levels after 24 weeks of lamivudine therapy. Patients were divided into 2 groups according to HBV-DNA levels after 24 weeks of lamivudine therapy. Kaplan-Meier plots of time to viral breakthrough (**a**) and time to breakthrough hepatitis (**b**) are shown. Log-rank tests show that higher HBV-DNA levels after 24 weeks of lamivudine therapy are associated with the more rapid development of viral breakthrough ($p = 0.007$) but not with breakthrough hepatitis.

Resistance-Associated Mutations, HBV-DNA Levels after Viral Breakthrough

At the time of viral breakthrough, a polymerase gene mutation in domain B (rt180M) was detected in 13 patients, and a mutation in domain C was detected in 19 patients (rt204I in 10, rt204V in 6 patients and a mixture of rt204I and rt204V in 3 patients). Six patients had a single rt204I mutation; 4 patients had rt180M/rt204I double mutations; 6 patients had rt180M/rt204V double mutations; and 3 patients had rt180M/rt204I/V (mixture) double mutations. The emergence of the rt180M mutation was significantly associated with breakthrough hepatitis: breakthrough hepatitis occurred in 11 of 13 patients with the rt180M mutation, while only 1 of 6 patients with rt180wt (baseline wild-type) developed significant biochemical changes ($p = 0.01$, Fisher's exact test; table 2). The positive predictive value of the presence of rt180M was 85%, and the negative predictive value was

Table 1. Pretreatment variables in association with viral breakthrough and breakthrough hepatitis

	Viral breakthrough		Breakthrough hepatitis	
	no (n = 34)	yes (n = 19)	no (n = 41)	yes (n = 12)
Gender (male/female)	22/12	10/9	16/25	5/7
Age, years	49.8 ± 11.9	47.1 ± 11.8	49.2 ± 11.5	47 ± 13.3
ALT, U/l	255 ± 338	141 ± 128	230 ± 314	159 ± 148
HBcAg positive/negative	17/17	6/13	22/19	8/4
HBV-DNA, LGE/ml	7.0 ± 1.1	7.3 ± 1.1	7.1 ± 1.1	7.2 ± 1.2
CP mutations (M/W)	25/6	19/0	32/6	12/0
PC mutation (M/W)	14/17	7/12	15/23	6/6

HBcAg = Hepatitis Bc antigen; CP = core promoter; PC = precore; M = mutant type; W = wild type. There were no variables that had a statistically significant association with viral breakthrough or with breakthrough hepatitis.

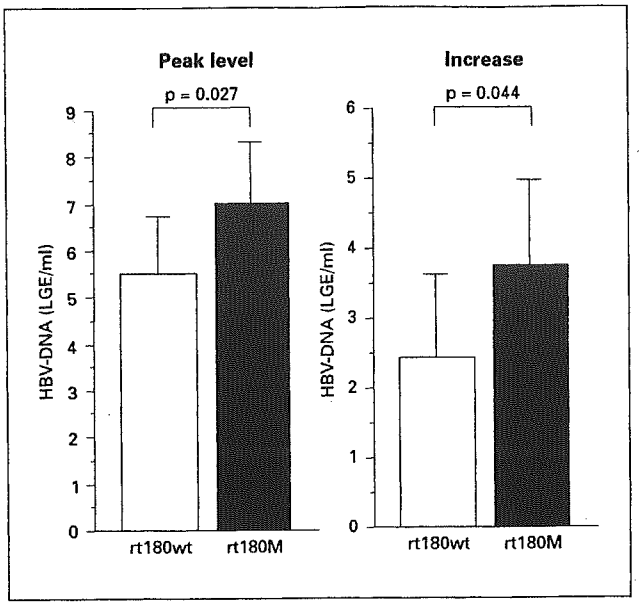

Fig. 3. The association between domain B mutations and HBV-DNA levels after viral breakthrough. After viral breakthrough, patients with the rt180M mutation had higher peak levels of HBV-DNA (p = 0.027) and larger increases of HBV-DNA (p = 0.044) compared to patients with rt180wt.

Table 2. Mutational patterns of the polymerase gene in association with breakthrough hepatitis

Mutational pattern	Breakthrough hepatitis		
	no (n = 7)	yes (n = 12)	p value
rt180M/rt180wt	2/5	11/1	0.01
rt204I/rt204V/rt204V+I	5/2/0	5/4/3	NS
rt204V (yes/no)	2/5	7/5	NS
rt204I (yes/no)	5/2	8/4	NS

NS = Not significant.

83%. Patients with the rt180M mutation had a 27.5-fold higher probability of breakthrough hepatitis compared to those patients with rt180wt (logistic regression analysis, 95% CI 2.00–378.93, p = 0.013). In contrast, mutational patterns of rt204 were not associated with breakthrough hepatitis: only 8 of 13 patients with rt240V mutation and 5 of 10 patients with rt240I developed breakthrough hepatitis (p = 0.35, Fisher's exact test; table 2).

During viral breakthrough, patients with the rt180M mutation had a larger increase in HBV-DNA levels (3.7 ± 1.2 vs. 2.4 ± 1.2 LGE/ml, p = 0.044) and higher peak values of HBV-DNA (7.0 ± 1.3 vs. 5.5 ± 1.2 LGE/ml, p = 0.027) compared to those patients with rt180wt (fig. 3).

Discussion

In this study, we found that the rt180M mutation in domain B of the HBV polymerase gene was significantly associated with breakthrough hepatitis during long-term lamivudine therapy in patients with chronic hepatitis B from genotype C. The positive predictive value of this mutation for breakthrough hepatitis was 85%, and the negative predictive value was 83%. The rt180M mutation was always detected as a double mutation with an rt204 mutation. Moreover, it was linked to higher subsequent levels of HBV-DNA during viral breakthrough compared to similar episodes in patients with a single rt204 mutation. Conversely, the mutational patterns of rt204, namely rt204I or rt204V, were not significantly associated with breakthrough hepatitis. Thus, the development of an rt180M mutation in patients on long-term lamivudine therapy might be a useful predictor for breakthrough hepatitis. As such, the presence of this mutation may also be helpful in deciding whether or not to proceed with alternative or additive nucleoside analogues as salvage therapy.

Lamivudine plays an important role in the treatment of patients with chronic hepatitis B. Short-term treatment is insufficient for clearing the virus [8], while long-term treatment is associated with the development of drug-resistant HBV. These strains of drug-resistant HBV do not always precipitate a relapse of hepatitis [12, 15], but the mechanism and predictive factors for the differing clinical outcomes have not been identified.

Previous studies examined factors such as genotype [21], ALT levels [32–34], HBV-DNA levels prior to therapy [21, 33–35], the degree of decline in HBV-DNA levels during therapy [17, 36, 37], the presence of HBeAg [32, 35, 38], and the presence of core promoter mutations [39]. While all of these factors have been found to be related to the appearance of resistant virus strains, the data are inconsistent, and the ultimate role of these factors remains controversial [36]. Moreover, how these and other factors are related to different clinical outcomes after the reappearance of HBV has not been elucidated. This study attempts to identify those predictive factors for break-

through hepatitis during long-term lamivudine therapy that may have an important impact on clinical outcomes.

We found that pretreatment variables, such as mutations in the core promoter and precore genes, HBV DNA levels, ALT levels, and the presence of HBe antigen, were not associated with viral breakthrough or with breakthrough hepatitis. This finding leads us to believe that identifying high-risk patients prior to initiating lamivudine therapy may not be possible. All cases with breakthrough had mutation in the core promoter, but the difference did not reach statistical significance possibly due to the small number of patients with the wild-type sequence in the core promoter. Further study including a larger number of patients with the wild-type sequence in core promoter may be necessary to elucidate the significance of the mutation in the core promoter in breakthrough.

However, when patients were analyzed after 24 weeks of lamivudine therapy, we found that HBV-DNA levels were significantly associated with the development of viral breakthrough, a finding consistent with previous reports [17, 36, 37]. Additionally, higher rates of spontaneous mutations in the viral genome are likely to be associated with higher replication levels [40], leading to the emergence of resistant HBV. Given these findings, monitoring for HBV-DNA levels at 24 weeks may be useful in targeting patients at higher risk for viral breakthrough but not necessarily for breakthrough hepatitis.

When parameters at the time of viral breakthrough were analyzed, the rt180M mutation was significantly associated with the occurrence of breakthrough hepatitis. Among those we studied the rt180M was the sole maker that was associated with breakthrough hepatitis, therefore close monitoring and detection of this mutation may be useful clinically in the prediction of breakthrough hepatitis.

The mechanism underlying the association of rt180M mutation with breakthrough hepatitis is not well understood. Usually, the rt204V/I mutant virus is less efficient at replication than the rt204wt virus. However, in tissue culture, the mutants with both B and C domain mutations (rt180M and rt204I/V) have higher reverse transcriptase activity and replication capacity than those single C-domain mutants (rt180wt and rt204I/V). Domain C rt204 is located in the conserved YMDD motif of the RNA-dependent RNA polymerase and is involved in nucleotide binding [41], while domain B is involved in template positioning [42]. Since these two domains interact in the molecular model of HBV reverse transcriptase [43], it might be speculated that the rt180M mutation interacts with the rt204 mutations. In essence, in double mutants, the B-domain mutation rt180M may compensate for the replication defective C-domain mutants [44], thus accounting for the higher reverse transcriptase activity and replication capacity compared to single C-domain mutants. These in vitro findings are consistent with our findings that patients with both B and C domain mutants were associated with higher HBV-DNA levels during viral breakthrough than those with single C-domain mutants. It has also been suggested that cellular immune responsiveness to HBV may increase after suppression of viremia when using antiviral therapy such as lamivudine [45]. Thus, when viral breakthrough occurs in patients whose cellular immunity has been relatively restored, a relapse of hepatitis may occur. Additionally, the amino acid position 180 may be a more vulnerable target to the restored immune system, a possibility that needs to be examined by further study.

In conclusion, the results of this study offer potentially important clinical ramifications for patients infected with genotype C hepatitis B who are on long-term lamivudine therapy. The quantification of HBV viremia after 24 weeks of lamivudine therapy may predict patients who are at a higher risk for viral breakthrough. Closer monitoring may thus be warranted in those patients with HBV-DNA levels of >4 LGE/ml. When viral breakthrough does occur, genotypic assays for the rt180M mutation should be performed since this mutation may be predictive of hepatitis relapse.

It is our belief that using both a quantitative assay for HBV viremia at 24 weeks and genotypic assays for polymerase mutant detection after viral breakthrough may serve as an effective means of monitoring long-term lamivudine therapy in chronic hepatitis B genotype C patients. Together, the results from these assays can provide useful information that may influence the decision to initiate early induction of salvage therapy.

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Mutagenic effects of ribavirin and response to interferon/ribavirin combination therapy in chronic hepatitis C☆

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***Background/Aims*: To elucidate whether ribavirin acts as a mutagen in the clinical setting and to clarify the relationship between ribavirin-induced mutations and virological response to combined therapy.**

***Methods*: Thirty-four patients with hepatitis C virus (HCV) genotype 1b received ribavirin monotherapy for 4 weeks, followed by a 24-week course of IFN/ribavirin therapy. HCV mutations during a non-treatment observation period and during subsequent ribavirin monotherapy were determined, and the relationship between mutations and response to subsequent IFN/ribavirin therapy was evaluated.**

***Results*: Serum HCV significantly decreased from 6.90 to 6.56 $\log_{10}$copy/ml in response to ribavirin monotherapy ($P<0.0001$). Nucleotide mutations in the NS5A and NS5B regions occurred during ribavirin monotherapy at a rate of 2.9×10^{-2}/site/year and 1.3×10^{-2}/site/year, respectively, a significantly higher rate than the mutation rates during the prior non-treatment observation period (0.60×10^{-2}/site/year and 0.24×10^{-2}/site/year, $P=0.02$, respectively). Mutation rates in the NS5A region were significantly higher in sustained viral responders (SVRs, $n=10$) than in non-responders (8.8×10^{-2}/site/year vs. 0.38×10^{-2}/site/year, $P=0.0005$, respectively). In the NS5A region, non-synonymous mutations only occurred in SVRs.**

***Conclusions*: Ribavirin may act as a mutagen, and mutations occurring during ribavirin therapy correlate with the virological response to subsequent IFN/ribavirin combination therapy.**

***Keywords*: NS5A; NS5B; ISDR; HCV; HCV dynamics**

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; RdRp, RNA dependent RNA polymerase; PCR, polymerase chain reaction; SVR, sustained viral responder; NR, non-responder; ISDR, interferon sensitivity determining region.

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1. Introduction

Ribavirin, a synthetic guanosine analog, has broad antiviral effects against both DNA and RNA viruses. Although ribavirin monotherapy has minimal efficacy on hepatitis C viral (HCV) eradication [1–3], studies have reported higher sustained response rates following combination therapy with interferon (IFN)-alfa and ribavirin than following IFN-alfa monotherapy [4–7]. Several mechanisms of action of ribavirin have been proposed [8]. In vitro and animal studies, in particular, have demonstrated that the antiviral activity of ribavirin is exerted through its potent mutagenic effects on RNA viruses after being incorporated into newly synthesized genomes by viral RNA-dependent RNA polymerase (RdRp) [9–11]. Still, little information is available regarding the mechanisms responsible for the increased virological efficacy associated with concurrent administration of ribavirin and IFN. No clinical studies to date have determined whether ribavirin induces mutations in the clinical setting nor examined the relationship between the mutagenic effects of ribavirin and viral response to IFN/ribavirin combination therapy.

The present study evaluated a set of patients with chronic hepatitis C. To elucidate whether ribavirin acts as a mutagen in the clinical setting, for each subject the sequential nucleotide mutations occurring during ribavirin monotherapy were compared with mutations occurring in the same patient during the non-treatment observation period immediately preceding the initiation of ribavirin monotherapy as a control. The relationship between mutations observed during ribavirin monotherapy and viral response to subsequent IFN/ribavirin combination therapy was also determined.

2. Methods

2.1. Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital from December 2001 to June 2002, 34 patients of HCV genotype 1b with a high viral load (>100 kcopies/ml by Amplicor-HCV monitor assay; Roche Molecular Diag. Co., Tokyo, Japan) were included in the present study (Table 1). Patients with liver cirrhosis, autoimmune hepatitis, and alcoholic liver injury were excluded from the study. No patient was positive for hepatitis B virus-associated antigen/antibody or anti-human immunodeficiency virus antibody. No patient received immunomodulatory therapy before enrolment in the study. Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration.

2.2. Treatment protocol and study design

Treatment schedule and time points for sequential genetic analysis are described in the upper part of Fig. 1. Following a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks. Ribavirin dosage was 600 mg daily for patients who weighed less than 60 kg and 800 mg daily for patients who weighed between 60 and 80 kg. All patients subsequently received a 24-week course of treatment consisting of intramuscular IFN-alfa 2b (Intron, Schering-Plough, Kenilworth, NJ) at an initial dosage of 6 MU daily in combination plus daily oral ribavirin at the same dosage (600 mg daily or 800 mg daily) as was given during pretreatment monotherapy. After the first 2 weeks of IFN/ribavirin combination therapy, the IFN dosing frequency was reduced to 6 MU three times a week for the remaining 22 weeks.

Table 1
Clinical characteristics of the study patients

No. of patients	34
Age (years)	59±8
Gender (M/F)	14/20
Liver histology	
A1/A2/A3	14/15/5
F1/F2/F3	10/15/9
Number of ISDR mutations (0/1)	22/12
Baseline data	
ALT (IU/L)	81±56
Platelet count ($\times 10^3$/ml)	150±52
Viral load (KIU/ml)	572±204
SVR (%)	29.4 (10/34)

Values are expressed as mean±standard deviation.

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined at the following time points: (1) enrolment into the study; (2) end of the non-treatment observation period (immediately before initiation of ribavirin monotherapy); (3) end of the 4-week ribavirin monotherapy (immediately before initiation of IFN/ribavirin combination therapy). For each patient, nucleotide changes between time points 1 and 2 during the non-treatment observation period (mean: 6 months, range: 2–48 months) were used as a control to determine whether mutations observed during the period of ribavirin monotherapy (between time points 2 and 3) represented true effects of ribavirin. Mutation rates for the non-treatment observation period were compared with the mutation rates during the subsequent ribavirin monotherapy period. Moreover, the relationships between observed mutations and the clinical outcome of the subsequent IFN/ribavirin therapy were evaluated.

2.3. Nucleotide sequencing

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined by direct sequencing of polymerase chain reaction (PCR)-amplified DNA, as previously described [12,13]. In brief, after RNA was extracted from sera of the study subjects, NS5A and NS5B regions were amplified by RT-PCR using Taq polymerase, and the sequences corresponding to nucleotides 6703–7320 and 7730–8874 of HCV-J were determined [13]. The sequences of the primers for the NS5A region were the same as described previously [12]. Sequences for the NS5B region, which were amplified with two partially overlapping sets of primers, were as follows: NS5B1-5′ outer set, 5′GGTGAATACCTGGAAATCAAAGAAA3′; NS5B1-3′ outer set, 5′AGAAATGAGTCATCAGAATCATCCT3′; NS5B1-5′ inner set, 5′TGTAAAACGACGGCCAGTATGGGCTTCTCATATGACAC3′; NS5B1-3′ inner set, 5′CAGGAAACAGCTATGACCCATGATGATGTTGCCTAGCC3′: NS5B2-5′ outer set, 5′GCAGAAGAAGGTCACCTTTGACAGA3′; NS5B2-3′ outer set, 5′TCGGGGGCCAAGTCACAACATTGGT3′; NS5B2-5′ inner set, 5′TGTAAAACGACGGCCAGTTTTGACAGACTGCAAGTCCT3′; NS5B2-3′ inner set, 5′CAGG AAACAGCTATGACCTTCTCAGTGACCGTTGAGTC3′. M13-forward and M13-reverse sequences were attached to the 5′-termini of sense and anti-sense nested PCR primers. Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (CN402069, Applied Biosystems, Chiba, Japan), and nucleotide sequences were determined by an automated DNA sequencer Model 373A (Applied Biosystems).

Mutations resulting from ribavirin therapy were defined as the detection of a new nucleotide which was not detected as even a minority population in the prior specimen from that subject. Electropherograms were read by two independent readers without knowledge of the patients' backgrounds and outcomes. A nucleotide detected in the post-ribavirin monotherapy specimen was considered to be a 'new nucleotide' only when both readers could not identify any tracking peak of this nucleotide in a prior specimen.

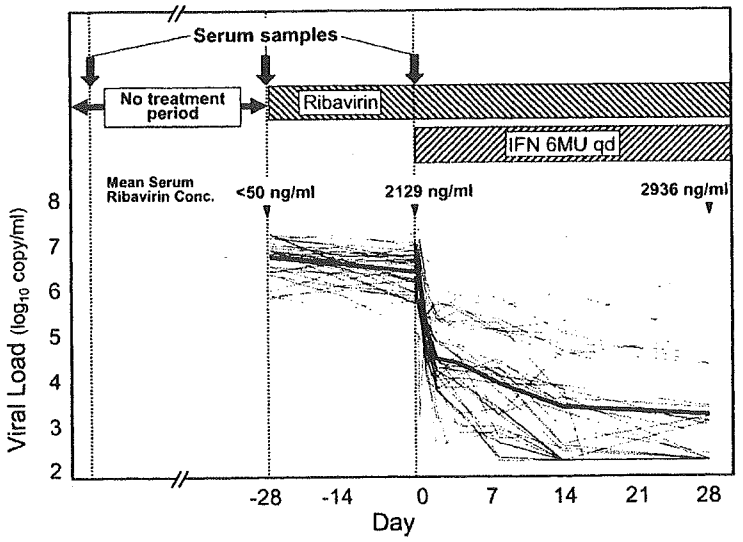

Fig. 1. Treatment schedule and HCV dynamics during ribavirin monotherapy and subsequent IFN/ribavirin combination therapy. After a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks and subsequently received intramuscular IFN-alpha 2b in combination with daily oral ribavirin. For the first 2 weeks of IFN/ribavirin combination therapy, 6 MU of IFN-alpha 2b was given daily; the IFN dosing frequency was then reduced to 6 MU three times a week for the remaining 22 weeks of combination therapy. Serum HCV dynamics of individual patients are shown in dotted lines, and the solid line represents the mean of these values. The nucleotide sequences were serially analyzed at the time points indicated by the arrows. Closed triangles indicate the time points of serum ribavirin concentration measurements.

Peaks less than 10% of the dominant peak were considered to be background signals. To calculate values for *dN* (nonsynonymous substitution), *dS* (synonymous substitution), *dN/dS* ratios, and the number of point mutations, analyses of nucleotide sequences were performed using a software program (MEGA version 2.1.). Separate *dN/dS* ratios were determined for each NS5 region in patients who had nucleotide mutations in the corresponding NS5 regions. To determine the locations of amino acid mutations in the tertiary structure of the NS5B molecule, a crystal structure model of HCV NS5B-RdRp was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom was generated using Rasmol 2.7.2.1. The deduced amino acid sequences of the NS5A and NS5B regions were also compared with a prototype HCV 1b strain, HCV-J [14].

2.4. HCV dynamics in serum

To analyze the effect of ribavirin on viral dynamics, HCV-RNA concentrations were quantified just before and at the end of ribavirin monotherapy, and also at 4, 8, 24, 48, 96, 192, and 336 hours after initiating IFN/ribavirin combination therapy, using real-time detection PCR, as reported previously [15–17]. The detection sensitivity of this assay is approximately 10 copies/ml, and the dynamic range is from 10 copies/ml to more than 1×10^8 copies/ml [17]. For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase by a straight-line fit of the data.

2.5. Definitions of response to therapy

A patient negative for serum HCV-RNA during the first six months following the completion of IFN/ribavirin combination therapy was defined as a sustained viral responder (SVR), and a patient positive for HCV-RNA during this time period was defined as a non-responder (NR).

2.6. Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables were analyzed by the Student's *t*-test for two groups. All tests of significance were two-tailed, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. HCV dynamics

During the 4-week period of ribavirin monotherapy, the mean serum HCV-RNA level significantly decreased from 6.90 to 6.56 log_{10} copy/ml ($P<0.0001$, paired t test) (Fig. 1). Serum HCV dynamics after the start of subsequent IFN/ribavirin combination therapy demonstrated a biphasic kinetic pattern of HCV-RNA decline. The exponential decay slopes for the first phase and the second phase were 2.00 ± 0.77 log_{10}/day and 0.15 ± 0.14 log_{10}/day, respectively.

3.2. The effect of ribavirin on HCV gene mutation and the relationship between mutations and virological response to IFN/ribavirin combination therapy

In a pairwise comparison of the NS5 sequences before and after ribavirin monotherapy, new HCV gene mutations occurring during ribavirin monotherapy were observed in the NS5A region in10 patients and in the NS5B region in 8 patients. Mean gene mutation rates in the NS5A and NS5B regions during ribavirin administration were 2.9×10^{-2}/site/year and 1.3×10^{-2}/site/year, respectively, rates which were significantly higher compared with the rates observed during the prior non-treatment observation periods in