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Mutation Pattern of Lamivudine Resistance in Relation to Hepatitis B Genotypes**Hepatitis B Genotypes Differ in their Lamivudine Resistance Associated Mutation Pattern**

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ABSTRACT

1
2 Little is known about differences between individual hepatitis B genotypes and mutation
3 patterns associated with lamivudine resistance. This study analyses the lamivudine associated
4 mutation pattern in relation to the four major HBV-genotypes A to D.
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7 The PubMed database was screened for keywords "HBV OR Hepatitis B", "YMDD", "genotype",
8 and "lamivudine"; all identified publications published till June 2009 were analysed for
9 differences in mutation pattern. To confirm the literature based findings the databases of two
10 reference laboratories in Tübingen, Germany and in Melbourne, Australia were analysed.
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13 Twenty nine studies were identified reporting 827 patients with known hepatitis B genotype
14 who underwent lamivudine treatment and developed resistance mutations. The literature data
15 revealed that genotype A favors the rtM204V mutation unlike the other major genotypes
16 ($p < 0.001$), which corresponds to a significant difference in the mutation pattern of genotypes
17 endemic in Asian countries and those found in the rest of the world. These significant findings of
18 the literature review could be reproduced in the analysis of the databases from Tübingen and
19 Melbourne. Furthermore, the rtL180M mutation is significantly connected to the rtM204V
20 mutation in genotypes A, B, and C, respectively.
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25 It is concluded that there is proof that HBV-genotypes differ in their mutation pattern of
26 lamivudine resistance. Future studies will need to evaluate whether this will translate into
27 genotype specific differences in resistance emergence on either entecavir or telbivudine as
28 these antivirals differ in their mutation profile, rtM204V for entecavir and rtM204I for
29 telbivudine.
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34 Key Words: HBV, hepatitis B, genotype, lamivudine, resistance, YMDD
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Introduction:

Lamivudine was the first approved oral antiviral for the treatment of chronic hepatitis B virus (HBV) infection and remains one of the most frequently used nucleoside analogues, mostly because it is cheaper, though it may not be the most cost effective because of lower efficacy and high resistance rates [Buti et al., 2009]. There are now several other antiviral drugs available for the treatment of chronic HBV infection, most of which are superior to lamivudine because of higher efficacy and lower resistance rates. Thus, where available these more effective antivirals should be used.

The drugs available currently for the treatment of chronic infection can be divided into interferons and antivirals. The latter can be divided into a “lamivudine-like” and an “adefovir-like” group of antivirals. The first group comprises lamivudine, telbivudine, and entecavir, while the later comprises adefovir and tenofovir. Each of the antiviral substances is associated with a distinct mutation pattern which is associated with reduced drug efficacy or drug resistance, respectively [Tillmann, 2007].

The lamivudine-like substances can be characterized by their selection for mutants with a methionine to valine or methionine to isoleucine in the YMDD motif of the HBV-polymerase, named rtM204V or rtM204I, respectively. There was no association between substitution of either valine or isoleucine and outcome when no other treatment option was available [Enomoto et al. 2007]. Likewise, there is no evidence for a role of rtM204I vs. rtM204V concerning subsequent patient’s clinical response when treated with adefovir or tenofovir [Tillmann, 2008]. This aspect, however, has changed with the availability of two other drugs known to select mutations in the YMDD motif, entecavir and telbivudine. The development of entecavir resistance *in vivo* seems to depend on the presence of the rtL180M-rtM204V [Tenney et al. 2004; Walsh et al., 2010]. In contrast, telbivudine mainly selects a rtM204I resistance mutation *in vivo* [Lai et al., 2007; Liaw et al., 2009]. However, studies concerning telbivudine have recruited most of their patients from Asia with genotype B or C dominance [Lai et al., 2007; Hou et al., 2008; Liaw et al., 2009].

There are currently eight HBV genotypes known which are endemic to different regions of the world. Genotype A dominates Northern Europe, but it is also found in North Africa and India. Genotypes B and C are prevalent in the Asian-Pacific area. Genotype D is found in the Mediterranean area, the Middle East, and India. Genotypes E-H have been isolated in samples from West Africa, the Americas, and France. [Norder et al., 2004].

While HBV-genotypes have been demonstrated to be relevant for the efficacy of interferon based therapies, there is no evidence at present that genotypes modify efficacy of nucleos(t)ide based therapies [Wiegand et al., 2008].

The purpose of this study was to analyse the pattern of mutation in the YMDD motif associated with lamivudine therapy in relation to HBV-genotypes. Mutation patterns were identified in

1 relation to HBV-genotypes through a systematic literature review and confirmed the
2 observations by molecular genetic analyses of two reference laboratories in Germany and
3 Australia.
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7 **Methods:**

8 **Literature review:**

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10 The Medline database of the U.S. National Library of Medicine (PubMed) was screened for
11 publications using keywords "HBV OR Hepatitis B", "YMDD", "genotype", and "lamivudine". All
12 manuscripts published until June 2009 were considered. Data regarding lamivudine treated
13 patients with mutations in the YMDD motif were extracted and analyzed in regard to mutation
14 pattern on lamivudine.
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18 Sources that did not contain the specific data for the analysis in the categories above were
19 excluded from further analysis.
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22 If information on genotyping was not included in the source, genotype was assumed according
23 to today's knowledge on endemics of HBV-genotypes. I.e. Korea is known to almost exclusively
24 harbor genotype "C" [Kim et al., 2007]. Thus, if no genotype was given, then Korean data was
25 regarded as genotype "C". Genotype was assumed in sources when such assumptions seem
26 justifiable. For countries that are known for the appearance of multiple genotypes such as
27 Canada, the US, and Australia data were not included without genotype specification.
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33 **Mutation analysis from two databases:**

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35 The most recent HBV polymerase/reverse transcriptase (RT) sequences, tested for the presence
36 of drug resistance associated RT mutations, from 143 patients were extracted from the SeqHepB
37 database [Yuen et al., 2007] located at the Victorian Infectious Diseases Reference Laboratory,
38 Australia. Selection criteria were: first-time lamivudine monotherapy during sample collection,
39 and presence of rtL80I/V, rtV173L, rtL180M and/or rtM204I/V in the HBV sequence.
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43 Thirty seven sera of HBV-infected patients with lamivudine resistance were collected at the
44 Department of Molecular Pathology, University Hospital of Tübingen, Germany, for diagnostic
45 purposes and studied in accordance to national ethical principles. YMDD mutations were
46 identified by sequencing of a region spanning the entire YMDD-motive from nt 467 to nt 845
47 (numbering according to GenBank accession no. Z72478). In brief, HBV DNA was extracted from
48 patient serum as described previously [Toan et al., 2006]. The HBV polymerase region was
49 amplified by nested PCR using the following primer pairs: outer primer pair HBV-2003: 5'-
50 GCACCTGTATTCCCATCCC-3' and HBV-2380 5'-GGCCTGTAAACAGGAAGTT-3'; inner primer pair
51 HBV-2046 5'-CCTATGGGAGTGGGCCTCAGT-3' and HBV-2220 5'-
52 CAAAAGAAAATTGGTAACAGCGGTA-3'. Reactions for first and second PCR were at 94°C for 30
53 seconds, 52°C for 30 seconds, and 72°C for 30 seconds followed by a final extension for 10
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1 minutes at 72°C for 35 cycles. Direct DNA sequencing of the PCR products on both strands using
2 primers for nested PCR (HBV-2046 and HBV 2220) was performed as described previously [Song
3 et al., 2003]. The sequences obtained were matched with the National Center for Biotechnology
4 Information GenBank and compared with recently described HBV-prototypes (Accession No. for:
5 HBV-A Z72478; HBV-B D00329; HBV-C X01587; HBV-D V01460; HBV-E X75657; HBV-F X75658;
6 HBV-G AF160501) All samples were analysed in duplicate.
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10 11 **Statistics:**

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13 Data were calculated using Microsoft Excel. The level of association between HBV genotype and
14 lamivudine-resistant patterns of different HBV mutants was evaluated using SPSS (12.0.1, SPSS,
15 Chicago, Illinois, USA). The occurrence of rtM204I and rtM204V was determined as well as co-
16 occurrence of both substitutions in the HBV sequences, and performed statistical analysis on the
17 number of sequences with rtM204I present vs. rtM204I absent and rtM204V present vs.
18 rtM204V absent, with co-occurrence being counted in presence for both. Furthermore, the
19 frequency of co-occurrence of both patterns of substitutions among different genotypes was
20 compared. When available the occurrence of rtL180M substitution was also evaluated. Chi-
21 Square test of independence was used when appropriate.
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26 **Results:**

27 28 **Literature Review:**

29
30 A total number of 72 publications were identified through the indicated search (see supplement
31 table 1). All available publications till June 2009 were included. Twelve publications did not refer
32 to patient related data, lamivudine therapy, or mutations in the YMDD motif at all. 23
33 publications referred to technical methods or did not link genotypes with information on amino
34 acid substitution at amino acid position rtM204. Eight studies reported mutations in the YMDD
35 motif, but did not differentiate the pattern of rtM204V vs. rtM204I.
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39 Ten studies did not present specific information on HBV genotypes, but could be assumed due
40 to almost unique genotype pattern in the respective countries. Seven studies [Paik et al., 2006;
41 Lee et al., 2006; Lee et al., 2005; Joh et al., 2005; Hong et al., 2004; Kim et al., 2003; Gwak et al.,
42 2007] presenting 147 patients originated from Korea and were therefore assumed as genotype
43 C. Two studies presenting twelve patients from Greece and Turkey [Economou et al., 2005; Pai
44 et al., 2005] were assumed as genotype D [Norder et al. 2004]. Ten patients from Poland
45 presented by Gora-Gebka H et al. [2007] were assumed to be genotype A [Deterding et al.,
46 2008].
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50 Thus, 29 studies could be included, which reported on HBV-genotypes and pattern of rtM204I
51 vs. rtM204V substitutions. These 29 studies represented 827 individual patients with either
52 rtM204V or rtM204I substitutions: 110 cases with genotype A, 173 cases with genotype B, 435
53 cases with genotype C, and 109 cases with genotype D.
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1 Differences in the rtM204V/I substitution pattern could be identified. The dominant resistance
2 mutation in genotype A was rtM204V (80.9%), while genotype B, C, and D showed a preference
3 of rtM204I (53.2%, 77.0% and 56.8%, respectively ($p < 0.001$). Co-presence of both rtM204I and
4 rtM204V was significantly more frequently found in genotype C (19.8%) than in any of the other
5 three genotypes ($< 5\%$; see table 1a,b). ($p < 0.001$)
6

7 Genotype B, C, and D all selected preferentially for the rtM204I substitution ($> 50\%$); this was
8 significantly different from genotype A ($p < 0.001$). However, genotype C had a significantly
9 higher prevalence of the rtM204I substitution than genotypes B and D, respectively ($p < 0.001$,
10 see table 1a & b).
11

12 In order to rule out bias caused by the assumption of HBV genotypes in cases where data were
13 not provided, the analysis was repeated without the cases with assumed genotypes. All findings
14 that were significant in the initial analysis remained significant in the smaller analysis (data not
15 shown).
16

17 The rtL180M substitution in relation to the rtM204V or rtM204I pattern and HBV-genotype

18 Only a few studies have reported on the amino acid substitution leucine to methionine at
19 position 180 (rtL180M) [Bozdayi et al., 2003; Kim et al. 2003; Economou et al., 2005; Li et al.,
20 2005; Woo et al., 2007; Bottecchia et al., 2008; Iacomi et al., 2009]. Iacomi et al. [2009] and
21 Bottecchia et al. [2008] reported 28 and 15 HBV-genotype A cases with rtL180M mutations,
22 respectively. Notably, the rtM204V mutation was associated with the rtL180M mutation in all 38
23 cases. In contrast, only one of the five cases described with a rtM204I mutation was associated
24 with the rtL180M mutation ($p < 0.001$, see table 2).
25

26 One study has reported the rtL180M substitution in relation to genotype B [Li et al., 2005]. The
27 rtL180M was present in all 45 cases of rtM204V, while the frequency of rtL180M was only 43.8%
28 (28/64; $p < 0.001$, (see Table 2)) in cases with rtM204I.
29

30 Three studies reported on a total of 38 genotype C cases in relation to YMDD changes and the
31 rtL180M mutation [Kim et al. 2003; Woo et al., 2007; Bottecchia et al., 2008]. Similar to
32 genotypes A and B, all ten cases of rtM204V also presented with a rtL180M change. However,
33 cases of genotypes A and B with the rtM204I substitution were found infrequently with the
34 rtL180M change, whilst the majority of genotype C cases with rtM204I also harbored rtL180M
35 (18/24, 75%). The difference of rtL180M occurrence in relation to rtM204I and M204V was not
36 significantly different within genotype C (see Table 2).
37

38 Three studies reported on genotype D and its relation to the rtL180M and the rtM204V or
39 rtM204I change [Bozdayi et al., 2003; Economou et al., 2005; Iacomi et al., 2009]. 31/35 (88.6%)
40 patients infected with rtM204V also had rtL180M. This was significantly more frequent than in
41 cases with rtM204I (12/41; 29.3%; $p < 0.001$, see Table 2).
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43 The occurrence of rtL180M was significantly less frequently associated with rtM204I in genotype
44 B and genotype D than genotype C ($p = 0.018$, and $p < 0.001$, respectively). Though only one of five
45 genotype A cases infected with rtM204I also had a rtL180M. However, the frequency of rtM204I
46 overall was too low to reach the level of significance (see Table 2).
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1 Additionally Li et al. [2005] presented six rtL180M positive patients without association to a
2 rtM204V or rtM204I change, which could be due to prior famciclovir therapy; Bozdayi et al.
3 [2003] reported the occurrence of rtL180M in co-mutation with the exchange of methionine
4 against serine in position 204 (M204S). Patients from both studies were positive for genotype D.
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8 **Lamivudine resistance associated substitution analysis from two databases:**

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10 The University Hospital of Tübingen and the Victorian Infectious Diseases Reference Laboratory,
11 Melbourne had information on 37 and 143 patients, respectively.
12

13
14 The Australian patients harbored genotype A (n=39), genotype B (n=27), genotype C (n=36), and
15 genotype D (n=34). The remaining seven individuals were positive for genotype F or G and were
16 not incorporated.
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19 The German database mainly had patients infected with genotype A (n=16) and D (n=17). Two
20 cases were infected with genotype E, one with genotype C, and one was not genotyped.
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24 **Analysis of the “Melbourne” Database:**

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27 Analysis of differences in “YMDD” mutation preference revealed a significant difference in the
28 behavior of genotype A and B. As observed from the literature data, genotype A favors the
29 rtM204V substitution (79.5%), while genotype B is associated with rtM204I (75%; $p < 0.001$).
30

31
32 The number of rtM204V positive cases was higher in genotype C than the number of cases with
33 a rtM204I mutation (58.3% versus 44.4%, respectively). Genotype D on the other hand seemed
34 to favor the selection of the rtM204I substitution (67.6% vs. 38.2%) in line with the literature
35 data. However, the findings in genotype C and D were not statistically significant, but showed a
36 trend only ($p = 0.059$ for the difference in rtM204I mutation frequency and $p = 0.102$ for the
37 difference in rtM204V mutation frequency and see table 3a and 3b).
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41 Eighty-seven (64%) of the 137 patients positive for genotype A-D were positive for rtL180M ,
42 thus the rtL180M substitution was associated with the rtM204V change. This finding was
43 significant in all genotypes (see table 4).
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47 **Analysis of the “Tübingen” Database:**

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49 Thirty of the 37 patients studied harbored the rtM204V change, which was associated with
50 rtL180M in 26 of the cases, while only one of seven patients was identified with rtM204I that
51 also harbored rtL180M. This difference was significant despite the small numbers ($p = 0.001$).
52

53
54 The profile of rtM204V change vs. rtM204I change was analysed in relation to HBV genotype.
55 The rtM204V mutation was more dominant in genotype A (93.7%) compared to Genotype D
56 (70.6%). This difference was not statistically significant ($p = 0.1748$, see Table 5).
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Additional substitutions associated with lamivudine resistance mutation pattern in relation to HBV genotypes

The additional substitution rtL80I, rtV173L, and rtA181V/T have been described to occur in relation to lamivudine resistance [Warner and Locarnini 2008; Warner et al., 2007; Delaney et al., 2003].

Melbourne Dataset:

Among the 143 patients, 39 had a rtL80I/V mutation (rtL80I in 21 and rtL80V in 18 cases, respectively), and 21 patients had a rtV173L mutation. There was no difference in distribution of rtL80I vs. rtL80V. Only one patient was found in whom both rtL80I/V and rtV173L ($p=0.005$) were detected. Furthermore, there was a striking difference depending on genotype in relation to both rtL80I/V and rtV173L (s. Table 6).

Tübingen Dataset:

Among the 37 patients analysed, whilst four were found to be infected with rtL80I, four had rtV173L, one had rtA181V and none had rtA181T. There was no significant difference, but rtL80I was more frequent in genotype D (3/17, 17.6%) than genotype A (1/16, 6.25%; not significant [n.s.]) and rtV173L showed the same distribution. However, in none of the cases was rtL80I co-occurring with rtV173L. The rtV173L substitution was associated solely with the selection of rtM204V (4/30, 13.3%), while the rtL80I was present in 1/7 (14.2%) cases with rtM204I and in 3/27 (11.1%; n.s.; s. Table 7) individuals with rtM204V.

Exploratory Analysis of changes in the surface antigen (HBsAg) in relation to polymerase changes and HBV genotype

The HBV genome is composed of four overlapping open reading frames (ORF). The polymerase and surface protein ORF's overlap, thus each mutation in the polymerase could potentially influence two codons on the HBsAg. Four polymerase amino acid positions were analysed for this aspect: a.) rtL80 corresponding to sG71 and sY72; b.) rtV173 corresponding to sE/V164 and sW/L165; c.) rtL180 corresponding to sS171 and sW172; and d.) rtM204 corresponding to sI195 and sW196. This information was available in 136 patients.

The polymerase changes at rtL80 and rtL180 are not associated with an amino acid change in the s-gene, whilst the rtV173L change is associated with a sE164D substitution in all cases. The rtV173L mutation was absent in genotype B and so not surprising then, the sE164D change was not observed.

Amino acid substitutions at rtM204 resulted in a more complex pattern of HBsAg changes, which is more dependent on the rtM204 mutation than on the HBV-genotype. The rtM204I does

1 not alter the sI195, while the rtM204V always results in a change to sI195M, which is
2 independent of genotype. sW196 can be changed to a leucine (L), serine (S), or stop codon. The
3 majority of patients with the rtM204I mutation harbour a sW196L mutation, while the majority
4 of rtM204V mutation patient harbour the sW196 wild-type. Other mutations were relatively
5 rare (see table 8).
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10 **Discussion:**

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12 The role of the different mutation patterns had no clinical relevance when lamivudine and
13 adefovir were the only therapeutic options. However, this relevance of the mutation pattern
14 might change with the availability of telbivudine and entecavir. Resistance to these two drugs is
15 associated with "opposite" changes in the YMDD motif: rtM204V is associated with entecavir
16 resistance, and rtM204I is associated with telbivudine resistance [Tenney et al., 2004;
17 Papatheodoridis and Deutsch, 2008; Liaw et al., 2008]. No data on the role of genotype and
18 different mutation pattern concerning response to entecavir and telbivudine are available;
19 further analysis of the studies with entecavir and telbivudine and substitution pattern and
20 frequency are warranted. A possible scenario is that resistance to telbivudine may be delayed in
21 Genotype "A" patients, while resistance to entecavir might be less likely in case of genotype B
22 and C, even after pre-treatment with lamivudine. There was one study which actually suggested
23 worse response to entecavir in the setting of rtM204V vs. rtM204I mutations [Suzuki et al.,
24 2008].
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30 Although lamivudine is considered obsolete according to guidelines from several clinical practice
31 regions [Lok and McMahon 2004], lamivudine remains today's mostly used and cheapest
32 available drug against HBV infection. Lamivudine should not be used but substituted by more
33 effective therapies with higher antiviral activity and lower resistance rates. As discussed above,
34 entecavir and telbivudine have opposite preference in regard to "YMDD" motif pattern.
35 Therefore lamivudine use carries the risk to burn both medications for future use because it
36 selects both mutational patterns – rtM204I and rtM204V - in all major genotypes. This would
37 limit the chance of a successful treatment with entecavir in case of a rtM204V mutation and
38 most likely will impact the chance of responding to telbivudine in case of a rtM204I. As this has
39 not been studied formally in either telbivudine or entecavir, this remains a hypothesis at
40 present.
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45 In addition, the mutation pattern was evaluated independently from the YMDD motif, and also
46 found strong genotype dependency. The Tübingen dataset, however, was too small to confirm
47 the findings from the Melbourne database. The level of significance, however, was different for
48 the different genotypes concerning the rtL80I/V mutation.
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51 This study demonstrates for the first time that there is a significant difference in mutation
52 pattern between the four major genotypes of HBV. The strength of this study is the concordance
53 of RT mutation patterns found in the three different datasets. Nonetheless, the limitation of this
54 study is that it was a retrospective analysis. Likewise, data on HBeAg status and coinfection with
55 other viral pathogens (e.g. HIV or HCV) were not available for the majority of the reviewed
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1 papers, and were therefore also not included for the confirmation part of the study using data
2 from Australia and Germany. Another limitation is that methods to detect changes in the YMDD
3 motif were heterogeneous in the different studies, which lead to differences in the rate that
4 minor species substitutions are detected [Woo et al., 2008]. However, the initial literature based
5 observation was confirmed in the prospective analysis of the genome databases from two
6 reference laboratories.
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8
9 In conclusion there is a difference in the pattern of YMDD mutants between the major HBV-
10 genotypes. Future studies are needed to prove if this difference in mutational pattern will
11 modify frequency of resistance development against telbivudine or entecavir, respectively. Thus,
12 it would be valuable to study resistance emergence in regard to telbivudine and entecavir in
13 relation to HBV genotypes.
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Table 1a: Distribution of YMDD mutations in various genotypes according to literature review

	rtM204I (n=515)	rtM204V (n=410)	Mixed (n=98) (rtM204V&rtM204I) *
Genotype A (n=110)	23.6% (26)	80.9% (89)	4.5% (n=5)
Genotype B (n=173)	53.2% (92)	49.7% (86)	2.9% (n=5)
Genotype C (n=435)	77.0% (335)	42,8% (186)	19.8% (n=86)
Genotype D (n=109)	56.8% (62)	45.0% (49)	1.8% (n=2)

Table 1b: Comparison between different genotypes in the literature review. p-values for frequency of the rtM204V, the rtM204I, and the co-presence of both substitutions.

rtM204I	Genotype B	Genotype C	Genotype D
Genotype A	p<0.001	p<0.001	p<0.001
Genotype B		p<0.001	p=0.623
Genotype C	p<0.001		p<0.001

rtM204V	Genotype B	Genotype C	Genotype D
Genotype A	p<0.001	p<0.001	p<0.001
Genotype B		p=0.143	p=0.464
Genotype C	p=0.143		p=0.760

rtM204I&V	Genotype B	Genotype C	Genotype D
Genotype A	p=0.518	p<0.001	p=0.446
Genotype B		p<0.001	p=0.710
Genotype C	p<0.001		p<0.001

* please note, those with mixed infection were counted twice: once for rtM204I and once for rtM20V. Thus, the sum of patients with rtM204I, rtM204I, and mixed rtM204V/I are greater than the total number of patients in each line.

Table 2: rtL180M mutation reported in connection with YMDD motive according to the literature review. P-values reflect the level of significance for the difference for rtL180M occurrence in relation to rtM204V vs. rtM204I for each genotype individually.

Genotype	Number of Studies reporting	Total number of patients	rtM204I without rtL180M	rtL180M +rtM204I	rtM204V without rtL180M	rtL180M +rtM204V	p-values
A	2 [†]	43	4/5 (80%)	1/5 (20%)	0/38 (0%)	38/38 (100%)	p<0.001
B	1 [‡]	109	36/64 (56.2%)	28/64 (43.8%)	0/45 (0%)	45/45 (100%)	p<0.001
C	3 [§]	34*	6/24 (25.0%)	18/24 (75.0%)	0/10 (0%)	10/10 (100%)	p=0.148
D	3 ^{**}	76	29/41 (70.7%)	12/41 (29.3%)	4/35 (11.4%)	31/35 (88.6%)	p<0.001

*Truegene results from Woo et al. [2007].

† [Bottecchia et al., 2008; Iacomi et al., 2009]

‡ [Li et al., 2005]

§ [Kim et al. 2003; Woo et al., 2007; Bottecchia et al., 2008]

** [Bozdayi et al., 2003, Economou et al., 2005; Iacomi et al., 2009]

Table 3a, sequence analysis: Distribution of YMDD-mutations in relation to HBV genotypes in the Melbourne cohort ($p < 0.001$ overall).

	rtM204I (n=69)	rtM204V (n=72)	Mixed (n=4) ^{††}
Genotype A (n=39)	23.1%*(n=9)	79.5%*(n=31)	2.5% (n=1)
Genotype B (n=28)	75%*(n=21)	25%*(n=7)	0% (n=0)
Genotype C (n=36)	44.4% (n=16)	58.3% (n=21)	2.8% (n=1)
Genotype D (n=34)	67.6% (n=23)	38.2% (n=13)	5.9% (n=2)

Table 3b, sequence analysis: p-values for difference in distribution of YMDD-mutations regarding genotype in the Melbourne cohort

rtM204I	Genotype B	Genotype C	Genotype D
Genotype A	P<0.001	p=0.085	p=0.001
Genotype B		p=0.010	p=0.408
Genotype C	P=0.010		p=0.059

rtM204V	Genotype B	Genotype C	Genotype D
Genotype A	P<0.001	p=0.078	p=0.001
Genotype B		p=0.005	p=0.266
Genotype C	P=0.005		p=0.102

rtM204I&V	Genotype B	Genotype C	Genotype D
Genotype A	P=1	p=1	p=0.595
Genotype B		p=1	p=0.498
Genotype C	P=1		p=0.609

^{††} please note, those with mixed infection were counted twice: once for rtM204I and once for rtM204V. Thus, the sum of patients with rtM204I, rtM204V, and mixed rtM204V/I are greater than the total number of patients in each line.

Table 4: Distribution of rtL180M mutation in relation to genotypes and rtM204I vs. rtM204V.

	rtM204I without L180	rtM204I + rtL180M	rtM204V without rtL180M	rtM204V + rtL180M	rtM204I/V without L180	rtM204I/V + L180
Total (n=143) p<0.001	47/65	18/65	1/74	73/74	0/4	4/4
Genotype A (n=39) p<0.001	7/8	1/8	0	30/30	0/1	1/1
Genotype B (n=28) p<0.001	19/20	1/20	0/6	6/6	0/0	0/0
Genotype C (n=36) p<0.001	10/15	5/15	1/20	19/20	0/1	1/1
Genotype D (n=34) p=0.005	11/21	10/21	0/11	11/11	0	2/2
p-values are comparing occurrence of rtL180M in relation to rtM204I vs. rtM204V						

Table 5, sequence analysis: Distribution of YMDD-mutation in the Tübingen collective: No mixed rtM204I plus rtM204V cases were identified in the Tübingen database

Genotype	rtM204I	rtM204V
Genotype A (n=16)	6.3% (n=1)	93.7% (n=15)
Genotype D (n=17)	29.4% (n=5)	70.6% (n=12)
		P=0.17

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Table 6a: Mutation frequency of rtL80V/I und rtV173L in relation to genotypes within the Melbourne Database and corresponding p-values comparing the four different major genotypes.

	rtL80V/I	rtV173L
Genotype A (n=39)	0 (0%)	8 (20.5%)
Genotype B (n=27)	13 (48.1%)	0 (0%)
Genotype C (n=36)	13 (36.1%)	5 (13.9%)
Genotype D (n=34)	13 (38.2%)	8 (23.5%)

rtL80V/I	Genotype B	Genotype C	Genotype D
Genotype A	p<0.001	p<0.001	p<0.001
Genotype B		p=0.48	p=0.61
Genotype C	p=0.48		p=0.95

rtV173L	Genotype B	Genotype C	Genotype D
Genotype A	p=0.03	p=0.65	p=0.98
Genotype B		p=0.12	p=0.02
Genotype C	p=0.12		0.47

Table 7: Mutation frequency of rtL80V/I und rtV173L in relation to genotypes within the Tübingen Database; no significant differences.

	rtL80V/I	rtV173L
A (n=16)	1 (6.25%)	1 (6.25%)
D (n=17)	3 (17.6%)	3 (17.6%)

For Peer Review

Table 8: sW196 mutation in relation to HBV genotypes and rtM204 mutation.

Amino acid at		HBV-Genotype			
"rt" position rtM204	"sAg" position W196	A (n=8)	B (n=20)	C (n=15)	D (n=21)
rtM204I (n=64)	W196L	6	14	12	18
	W196S		4		2
	W196*	2	1	1	1
	W196L/ W196S			2	
	W196L/ W196*		1		

"rt" position rtM204	"sAg" position W196	A (n=30)	B (n=6)	C (n=20)	D (n=11)
rtM204V (n=67)	W196	29	6	20	11
	W196/ W196L	1			

"rt" position rtM204	"sAg" position W196	A (n=1)	B (n=0)	C (n=1)	D (n=2)
rtM204I/V (n=4)	W196/ W196L	1		1	2

* Stop codon,

cells with a light grey are those with sAg wild type amino acid sAgW196. rtM204V is mostly associated with the wild type amino acid sAgW196, while rtM204I is always associated with a mutated HBsAg amino acid sequence at position 196.

Supplement Table 1: Studies indicated in the PubMed-Search using the search terms “HBV OR Hepatitis B”, “YMDD”, “genotype”, and “lamivudine”

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