JCI insight

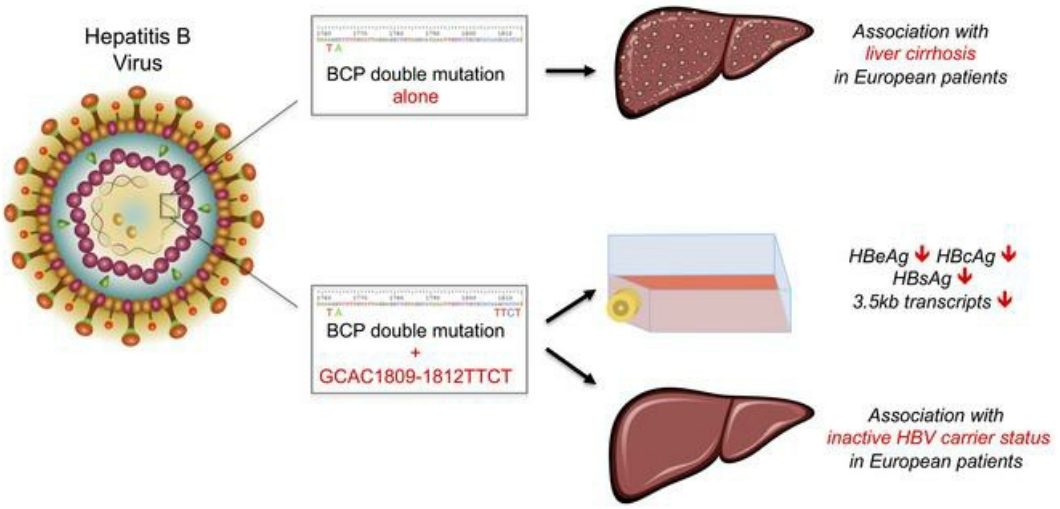
Quadruple mutation GCAC1809-1812TTCT acts as a biomarker in healthy European HBV carriers

Kai-Henrik Peiffer, … , Pietro Lampertico, Eberhard Hildt

JCI Insight. 2020. https://doi.org/10.1172/jci.insight.135833.

Research In-Press Preview Hepatology Virology

Graphical abstract



Quadruple mutation GCAC1809-1812TTCT acts as a biomarker in healthy European HBV carriers

Kai-Henrik Peiffer[1,2], Catrina Spengler[2], Michael Basic[1,2], Bingfu Jiang[2], Lisa Kuhnhenn[1], Wiebke Obermann[3], Tobias Zahn[2], Mirco Glitscher[2], Alessandro Loglio[4], Floriana Facchetti[4], Gert Carra[2], Alica Kubesch[1], Johannes Vermehren[1], Viola Knop[1], Christiana Graf[1], Julia Dietz[1], Fabian Finkelmeier[1], Eva Herrmann[5], Jonel Trebicka[1], Arnold Grünweller[3], Stefan Zeuzem[1], Christoph Sarrazin[1,6], Pietro Lampertico[4]*, Eberhard Hildt[2,7]*

*both authors contributed equally to this work

[1]*University Hospital Frankfurt, Department of Gastroenterology and Hepatology, D-60590 Frankfurt, Germany*

[2]*Paul Ehrlich Institute, Division of Virology, D-63325 Langen, Germany;*

[3]*Institute of Pharmaceutical Chemistry, Philipps-University Marburg, Germany*

[4]*A. M. and A. Migliavacca Center for Liver Disease, Division of Gastroenterology and Hepatology, Fondazione IRCCS Cà Granda Maggiore Hospital, University of Milan, Milan, Italy*

[5]*J.W. Goethe University, Department of Medicine, Institute of Biostatistics and Mathematical Modeling*

[6]*St. Josefs Hospital, Department of Gastroenterology, 65189 Wiesbaden, Germany*

[7]*German Center for Infection Research (DZIF), Gießen-Marburg-Langen, Germany*

Corresponding author:

Dr. Kai-Henrik Peiffer;

Medical Clinic 1; University Hospital Frankfurt

60590 Frankfurt

Phone: +49 69/6301-5122; Fax: +49 69/6301-83112

eMail: kai-henrik.peiffer@kgu.de

E-mail addresses and telephone numbers of all authors:

CS; Catrina.Spengler@pei.de, tel.: +49-6103-772140

MS; Michael.Basic@kgu.de, tel.: +49-69-63015512

BJ; Bingfu.Jiang@pei.de, tel.: +49-6103-772140

LK; lisa.kuhnhenn@mail.de, tel.: +49-69-63015512

WO; wiebkeobermann@staff.uni-marburg.de, tel.: +49 6421-285553

TZ; tobezahn@gmx.de , tel.: +49-6103-772140

MG; mirco.glitscher@pei.de

AL; ale.loglio@gmail.com, tel.: +39-2503-20408

FF; floriana.facchetti@gmail.com, tel.: +39-2503-20408

GC; Gert.Carra@pei.de, tel.: +49-6103-772140

AK; Alica.kubesch@kgu.de, tel.: +49-69-63015512

JV; Johannes.vermehren@kgu.de, tel.: +49-611-4475420

VK; Viola.knop@kgu.de, tel.: +49-69-63015512

CG; Christiana.graf@kgu.de, tel.: +49-69-63015512

JD; julia.dietz@em.uni-frankfurt.de, tel.: +49-69-63015512

FF; fabian.finkelmeier@kgu.de, tel.: +49-69-63015512

EH; Herrmann@Med.Uni-Frankfurt.de, tel.: +49-69-630187694

JT; Jonel.Trebicka@kgu.de, tel.: +49-69-63015512

AG; arnold.gruenweller@staff.uni-marburg.de, tel.: +49 6421-285553

SZ; zeuzem@em.uni-frankfurt.de, tel.: +49-69-63014544

CS; sarrazin@em.uni-frankfurt.de, tel.: +49-611-1771251

PL; pietro.lampertico@unimi.it, tel.: +39-2503-20408

EHildt; Eberhard.Hildt@pei.de, tel.: +49-6103-772140

Number of figures and tables:

2 tables and 6 figures, 2 supplementary figures

Word count:

7871

Abbreviations:

HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; BCP, basal core promoter; HBcAg, Hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBx, Hepatitis B x protein; HBsAg, hepatitis B surface antigen; real-time PCR, real-time polymerase chain reaction; PEI, polyethyleneimine; GT, genotype; WB, Western Blot; MFE, Minimum Free Energy

Statement of Interests:

Financial Support/ Conflicts of interest

The authors have declared that no conflict of interest exists.

ABSTRACT:

In search of new prognostic markers, many mutation analyses of the HBV genome were performed. However, the Kozak sequence preceding precore was covered only infrequently in these analyses. In this study, HBV core promoter/precore region was sequenced in serum samples of European inactive HBV carriers (n=560). Quadruple mutation GCAC1809-1812TTCT was found with a high prevalence of 42% in the Kozak sequence preceding precore among all HBV genotypes. GCAC1809-1812TTCT was strongly associated with coexistence of basal core promoter (BCP) double mutation A1762T/G1764A and lower HBV DNA levels ($p<0.0001$). In vitro GCAC1809-1812TTCT leads to drastically diminished synthesis of pregenomic(pg)RNA, precore mRNA, core, HBsAg and HBeAg. Calculation of the pgRNA secondary structure suggests a destabilization of the pgRNA structure by A1762T/G1764A that is compensated by GCAC1809-1812TTCT. In 125 patients with HBV-related cirrhosis, GCAC1809-1812TTCT was not detected. While a strong association of GCAC1809-1812TTCT with inactive carrier status ($p<0.0001$) was observed, BCP double mutation was strongly correlated with cirrhosis ($p<0.0001$), but this was only observed in absence of GCAC1809-1812TTCT. In conclusion, our data reveal that GCAC1809-1812TTCT is highly prevalent in inactive carriers, and acts as a compensatory mutation for BCP double mutation. GCAC1809-1812TTCT seems to be a biomarker of good prognosis in HBV infection.

Keywords:

HBV**;** TTCT; prognostic marker; BCP; compensatory mutation

INTRODUCTION

Chronic infection with HBV affects approximately 257 million people worldwide and is a major cause for the development of advanced liver disease and hepatocellular carcinoma (HCC) (1). However, the individual risk for disease progression and or HCC development is variable and depends on both viral and host factors. As current antiviral treatment strategies with nucleos(t)ide analogs are usually cost-intensive long-term therapies with potential side effects, patients who will benefit from this treatment have to be cautiously selected (2-4). Furthermore, patients who do not fulfill treatment criteria have to be followed over a long time period as an increased risk for disease progression and HCC development remains. Therefore, many efforts were made to establish reliable prognostic biomarkers.

In addition to HBV DNA levels and quantitative(q) surface antigen (HBsAg) levels as established biomarkers (5-9), several viral polymorphisms and mutations in preS gene, precore gene and basal core promoter (BCP), were extensively studied and were found to be associated with the course of disease and treatment response (10-12). However, although described as prognostic markers they have not been established in daily clinical practice so far. For example, the double mutation A1762T/G1764A is the most common mutation in BCP and was found in some studies to be associated with progression to advanced liver disease and HCC development (11, 13-15). However, although a pronounced prevalence of A1762T/G1764A was observed in patients with advanced liver disease, the frequency of this mutation is also approx. 50% in our cohort (16) of HBV inactive carriers. These data are contradictory and further limit the specificity of A1762T/G1764A as a robust prognostic marker in clinical practice.

The present study aimed to further specify the A1762T/G1764A-related clinical phenotype and to establish additional biomarkers for HBV related prognosis. We analysed the genetic variability of the core promoter and the Kozak sequence preceding precore in serum samples of inactive carriers and of patients with established compensated HBV-related liver cirrhosis. In addition we performed bioinformatical modeling of pregenomic RNA secondary structures and in vitro analyses of A1762T/G1764A and the quadruple point mutation GCAC1809-1812TTCT in hepatoma cells.

RESULTS

GCAC1809-1812TTCT is highly prevalent in inactive carriers and strongly associated with BCP double mutation A1762T/G1764A

To search for additional polymorphisms/mutations associated with the clinical phenotype of BCP double mutation re-analysis of 504 primary sequences (16) and sequencing of additional 56 serum samples of the BCP region of HBsAg carriers from the Albatros study was performed (patients demographics in Table 1). Besides a high prevalence of A1762T/G1764A in 61% (340/560) of the samples, we observed that additional mutations in the Kozak sequence at position nt1809-1812 directly preceding the precore start codon were found in 51% of the samples (283/560; Figure 1A). In 42% of samples (233/560) a quadruple substitution of TTCT instead of GCAC (GCAC1809-1812TTCT) could be identified at this position. In addition in 9% (50/560) non-TTCT mutations (single-, double or triple point mutations) at position nt1809-1812 were detected (Figure 1A). The presence of GCAC1809-1812TTCT was strongly associated with the coexistence of BCP double mutation A1762T/G1764A (Figure 1B). While this mutation was found in samples with A1762T/G1764A in 66%

(226/340), in samples without A1762T/G1764A the GCAC1809-1812TTCT variant was detected only in 3% (7/220; Figure 1B). In contrast, non-TTCT mutations at nt1809-1812 were not associated with the BCP double mutation A1762T/G1764A (Figure 1B).

Association of GCAC1809-1812TTCT with BCP double mutation is independent of the HBV genotype and GCAC1809-1812TTCT is associated with lower HBV DNA levels

To evaluate a possible genotype-dependency the prevalence of GCAC1809-1812TTCT among HBV genotypes (GT) A-E was analysed. GCAC1809-1812TTCT was frequently detected among all analysed genotypes with an overall prevalence of 47% in GTA (76/161), 22% in GTB (8/36), 25% in GTC (5/20), 36% in GTD (107/295) and 77% in GTE (37/48). In the BCP positive subgroup, the GCAC1809-1812TTCT prevalence was higher among all genotypes (Figure 1C). Again a strong association of GCAC1809-1812TTCT with BCP double mutation was found among all genotypes.

To analyse the impact of GCAC1809-1812TTCT on the clinical phenotype the mutation was correlated with HBV DNA and qHBsAg levels. GCAC1809-1812TTCT in combination with BCP double mutation A1762T/G1764A was associated with significant lower HBV DNA levels (2.51 log IU/ml vs. 3.14 log IU/ml; $p < 0.001$) in comparison to A1762T/G1764A without GCAC1809-1812TTCT and in comparison to samples without any of these mutations (Figure 1D). This association was observed for the overall study population as well as for the genotypes A and D individually (for detailed analyses in the genotype see suppl. Figure 1A-E). In contrast, A1762T/G1764A alone without GCAC1809-1812TTCT was not associated with

changes in HBV DNA levels. None of these mutations were associated with changes in qHBsAg levels in the overall study population and among the genotypes (Figure 1E and suppl. Figure 2A-E).

GCAC1809-1812TTCT reduces HBsAg expression in vitro

To further investigate the underlying mechanism that might trigger the correlation of GCAC1809-1812TTCT with the BCP double mutation and lower HBV DNA levels, in vitro experiments were performed. An HBV genome was isolated from a serum sample of one patient infected with HBV genotype A harbouring both A1762T/G1764A and GCAC1809-1812TTCT and this genome was cloned as a 1.2 x mer into pUC vector. Based on this genome a total of eight vectors containing different combinations of BCP double mutation (BCP) and GCAC1809-1812TTCT mutation (TTCT) were synthesized via site-directed mutagenesis (for an overview of the synthesized vectors see Figure 2A). Because of the partially overlapping open reading frames of HBV, both of these mutations appear in the core promoter as well as in the HBx gene. Therefore, we introduced the mutated or wildtype specific sequences in the respective core promoter gene (1), or the HBx gene (2) or both genes (3) independently. In respect of our clinical data, the variants harbouring the GCAC1809-1812TTCT mutation (TTCT1-3) contain in addition the A1762T/G1764A mutation. A construct without these two mutations (BCP0 + TTCT0) was used as a reference.

In hepatoma cells, expressing the two variants harbouring GCAC1809-1812TTCT in the core promoter position (TTCT1 and TTCT3) slightly lower HBsAg levels were detected by HBsAg-specific ELISA (Figure 2B). In addition, HBsAg levels were

slightly lower in the supernatants of cells expressing these two variants (Figure 2C). In contrast no changes in HBsAg expression were observed in cells expressing the genome with GCAC1809-1812TTCT only in the HBx position (TTCT2). In cells expressing the variants with BCP double mutation without GCAC1809-1812TTCT (BCP1-3) no significant changes in HBsAg expression were observed in the ELISA of lysates and supernatants. These data indicate that GCAC1809-1812TTCT leads to a slight reduction of HBsAg when present in the core promoter.

GCAC1809-1812TTCT reduces core expression and HBeAg release in vitro

For core quantification, Western blot (WB), HBcAg-specific ELISA and immunofluorescence analyses were performed. WB analysis using a polyclonal HBcAg/HBeAg-specific serum revealed that almost no core/HBeAg was detected in lysates of cells, which express the variants. The specificity of the blot was proven by using an additional genome containing the precore double mutation G1896A/G1899A, which was used as an additional HBeAg negative control and by using an HBeAg positive wildtype genome as a positive control (Figure 3A). In supernatants, core/HBeAg was detected only in case of the HBeAg positive control via WB analysis (data not shown). By a core-specific ELISA of lysates significant less core was detected in case of the variants harbouring BCP double mutation in the promoter position (BCP1 and BCP3 and all TTCT variants (1-3)). However, while the addition of GCAC1809-1812TTCT in the core promoter position (TTCT1 and TTCT3) led to an even stronger reduction of core, GCAC1809-1812TTCT in the HBx position (TTCT2) did not lead to a further reduction of core (Figure 3B). No significant changes in core amount were observed for the variant containing the BCP double mutation in the HBx position (BCP2). By the core-specific ELISA of supernatants core

was not sufficiently detectable (data not shown). In immunofluorescence analysis using an antibody for assembled core, specific, but only weak core signals were observed in cells expressing these variants (Figure 3D). In line with the Core-specific ELISA slightly lower core signals were obtained for the constructs with the BCP double mutation in the promoter position (BCP1 and BCP3) and even lower signals were observed in cells expressing the constructs with the additional GCAC1809-1812TTCT mutation in the promoter position (TTCT1 and TTCT3).

For analysis of HBeAg release semiquantitative HBeAg-ELISA of supernatants was performed (Figure 3C). Here, we observed that HBeAg levels were moderately, but not significantly decreased in cells expressing genomes with the BCP double mutation A1762T/G1764A located in the promoter region (BCP1 and BCP3). In contrast, a very strong reduction of HBeAg levels nearly to the detection limit was detected in cells expressing the constructs containing the additional GCAC1809-1812TTCT mutation, but again only when this mutation was present in the core promoter position (TTCT1 and TTCT3). To analyse if the GCAC1809-1812TTCT mutant alone also abolishes the release of HBeAg we added another construct in this analysis with GCAC1809-1812TTCT in the promoter and HBx position but without the additional BCP double mutation (BCP0 + TTCT3). Also with this construct, a very strong, but slightly less pronounced, reduction of HBeAg was observed.

GCAC1809-1812TTCT impairs RNA synthesis in vitro

To further investigate if GCAC1809-1812TTCT might influence the synthesis of the different HBV-specific transcripts we performed Northern blot analysis. The Northern blot revealed that all HBV transcripts are detectable in all variants (Figure 4A).

However, besides reduced *HBx*- (0.7kb) and *HBsAg*- (2.1 and 2.4kb) specific transcripts, markedly less 3.5kb RNA transcripts were detected in case of the two variants harbouring GCAC1809-1812TTCT in the core promoter position (TTCT1 and TTCT3). In case of the other constructs all RNA transcripts were detected in almost comparable amounts. In line with the Northern Blot 3.5kb transcripts measured by real-time PCR were also drastically reduced in the TTCT1 and TTCT3 variants (Figure 4B). Here, also a moderate reduction of 3.5kb transcripts was observed for the TTCT2 construct with the mutation in the HBx position. In contrast, in cells expressing BCP double mutation either in the promoter or the HBx position the amount of 3.5kb transcripts was moderately increased. For further differentiation of the 3.5kb transcripts in pregenomic(pg)RNA and precore mRNA also real-time PCR was used. We observed that while GCAC1809-1812TTCT in both positions (TTCT1-3) led to a strong reduction of precore mRNA, a reduction of pgRNA was only observed for the variants with this mutation in the promoter position (Figure 4C,D). In contrast, the amount of pgRNA and precore mRNA was moderately increased by all constructs harbouring only the BCP double mutant.

By real-time PCR analyses of supernatants (Figure 4E) no significant differences were detected among all variants in comparison to the variants without any of these mutations (BCP0-TTCT0). As BCP double mutation was described in some studies to increase pgRNA encapsidation and all of our analysed GCAC1809-1812TTCT constructs (TTCT1-3) harbour in addition the BCP double mutation (BCP 3) we analysed if the addition of GCAC1809-1812TTCT in the BCP variant affects HBV DNA release. Here we observed that when GCAC1809-1812TTCT is present in both positions extracellular HBV DNA levels are slightly, but significantly decreased in comparison to the BCP3 variant (Figure 4F). For further analyses, we included

another construct harbouring the GCAC1809-1812TTCT mutation (BCP0-TTCT3) without the additional BCP double mutation and compared it to the TTCT3 variant. Here, we observed in the GCAC1809-1812TTCT variant without the BCP mutation a significantly diminished released of HBV DNA of approximately two fold in comparison to the variants which harbour the additional BCP double mutation (Figure 4G).

Changes in thermodynamic stability of pgRNA secondary structures in the context of BCP- and GCAC1809-1812TTCT-mutations

As described above GCAC1809-1812TTCT is highly prevalent in inactive carriers and is strongly associated with BCP double mutation A1762T/G1764A. Furthermore, this mutation leads to lower HBV DNA levels in vivo and negatively impacts the synthesis of pgRNA and viral proteins in vitro. To analyse if the coexistence of the BCP- and GCAC1809-1812TTCT mutations might influence HBV pgRNA secondary structure, calculation of the thermodynamic stability of nt1730-1930 pgRNA genomic region was performed for these two mutations as recently described (17). We calculated that the RNA secondary structure of the A1762T/G1764A containing pgRNA is thermodynamically less stable with an increase in the Minimum Free Energy (MFE) value of 2,2 kcal/mol compared to the wildtype pgRNA secondary structure (Figure 5A and B). The calculated MFE of the A1762T/G1764A containing pgRNA appeared to be higher (-66,7kcal/mol; GC content of 47%) in comparison to pgRNA lacking this mutation (-68,9kcal/mol; GC content of 48%). After the implementation of GCAC1809-1812TTCT additionally to A1762T/G1764A (Figure 5C), the RNA secondary structure is stabilised by a hairpin structure and the MFE decreases approximately to the wildtype level (-69,1kcal/mol; GC content of 46%).

However, the introduction of GCAC1809-1812TTCT without A1762T/G1764A (Figure 5D) results in a calculated MFE (-69,2kcal/mol; GC content of 47%), which is comparable to the thermodynamic stability of the wildtype sequence. These data suggest that while the introduction of A1762T/G1764A leads to a slight destabilization of the pgRNA secondary structure, GCAC1809-1812TTCT might restore the thermodynamic stability of the pgRNA.

GCAC1809-1812TTCT is absent in patients with compensated HBV-related liver cirrhosis

To analyse the prevalence of GCAC1809-1812TTCT in patients with advanced HBV-related liver disease, sequencing of the BCP region was performed in viraemic serum samples from another cohort of 125 patients with HBV related compensated liver cirrhosis including 24 patients with history/presence of HCC or development of HCC after sampling (patients demographics are summarized in Table 2). As another European cohort, the main genotypes were genotype A and D in this population (10,4% and 74,4%, respectively). Overall, the A1762T/G1764A mutation was found in the vast majority of samples (65%; 81/125). However, while any other mutation (single, double or triple point mutation) in the Kozak sequence nt1809-1812 was detected in 15% (16/125), the quadruple mutation GCAC1809-1812TTCT was not detected in a single sample (0%; 0/125; Figure 6A).

GCAC1809-1812TTCT acts as a biomarker

To examine if GCAC1809-1812TTCT might act as a potential novel biomarker, its

prevalence was compared among the different cohorts. The prevalence of GCAC1809-1812TTCT was significantly higher ($p<0.0001$) in our cohort of inactive carriers in comparison to patients with established compensated liver cirrhosis (Figure 6B). While GCAC1809-1812TTCT in combination with BCP was found in 40% of the inactive carriers this combination was absent in patients with cirrhosis. Additionally, a strong association with inactive carrier status was found when compared to the subgroup of patients with established compensated liver cirrhosis and HCC (Figure 6C).

Next, we asked if the GCAC1809-1812TTCT status might be able to enhance the specificity of BCP double mutation as a prognostic marker for advanced HBV-related liver disease. We observed that the presence of BCP alone without further specification of GCAC1809-1812TTCT status was neither associated with inactive carrier status nor with established compensated liver cirrhosis or HCC (Figure 6B, C). In contrast, the presence of BCP double mutation without coexistence of GCAC1809-1812TTCT was strongly associated with liver cirrhosis and HCC, respectively. While BCP double mutation without GCAC1809-1812TTCT was found only in 20% (114/560) of the inactive carrier patients it was found in the majority of patients with compensated liver cirrhosis (65%, 81/125) and the HCC subgroup (75%, 18/24).

DISCUSSION

Recently, we performed extensive mutation analyses of the core, precore and preS regions in a large European cohort of inactive HBV carriers (16). In the present study, 560 patients from this cohort were analysed for additional core mutations associated with BCP double mutation A1762T/G1764A. As recently described the BCP double

mutation A1762T/G1764A was frequently (61%) found in the overall study population (16, 18). In addition to the frequently described BCP double mutation, we describe in this study to our knowledge for the first time a highly prevalent and prognostic relevant quadruple point mutation (GCAC1809-1812TTCT) in the Kozak sequence directly preceding the precore initiation codon. Located directly between two highly conserved regions, which are involved in the regulation of transcription (nt1770-1808 and nt1813-1849) (19), this mutation was found in 42% in the overall study population and frequently among all major HBV genotypes (A-E). It leads to the substitution of alanine to phenylalanine and proline to serine in the region around the precore initiation codon (nt1808-1817), which is highly conserved in all genotypes (20). In addition, due to the overlapping open reading frames of the HBV genome, this mutation also leads to corresponding changes in the downstream region of the HBx gene. Different double and triple point mutations at nt1809-1812 were described in two studies from South Africa including patients infected with HBV genotype A (14, 20). The double point mutation G1809T/C1812T was observed to reduce HBeAg synthesis in vitro (20). In another German study different point mutations at nt1809-1812 were recently described in patients with HIV/HBV co-infection, HBV blood donors and patients with HBV-related chronic liver disease (21).

In our study, we observed a strong association of GCAC1809-1812TTCT with BCP double mutation A1762T/G1764A in the inactive carrier group. While the majority of samples with the presence of BCP double mutation were also positive for GCAC1809-1812TTCT, this additional mutation was found only infrequently in samples without BCP double mutation. In addition, GCAC1809-1812TTCT but not BCP double mutation is significantly associated with lower HBV DNA levels. Therefore, our recent unexpected observation that BCP double mutation was

associated with lower HBV DNA levels (16) can be explained by the high prevalence of GCAC1809-1812TTCT in patients with BCP double mutation.

The impact of BCP double mutation on molecular virology was studied in several other in vitro and in vivo studies (16, 19, 22-30) with heterogeneous results. While precore RNA was found to be decreased in most studies, mostly minor impact on levels of pgRNA and no significant impact on HBsAg-specific RNAs were observed in most of these in vitro studies. In addition, an increase of pgRNA encapsidation and synthesis of progeny virus was observed by some groups. Interestingly, BCP double mutation was found in both HBeAg positive and HBeAg negative patients, underlining that this mutation does not completely abolish HBeAg, like for example the precore mutation G1896A, which converts TGG to the stop codon TAG (18). In our study, the amount of 3.5kb transcripts was moderately increased in cells expressing BCP double mutation either in the promoter or in the HBx position, which is in line with several publications (19, 28). But interestingly, in addition to the pgRNA also the precore mRNA was moderately increased by all variants harboring the BCP double mutant, including the variant with this mutation in the HBx position (BCP). In contrast to our data, decreased levels of precore mRNA were found in some studies (23, 24, 28). Nevertheless, in other studies, only low or moderate changes in precore mRNA and HBeAg release were observed (25, 29). As demonstrated by Parekh et al. the impact of BCP double mutations and other mutations strongly depend on the genetic background of the isolates (29). In line with other studies, extracellular HBV DNA was not significantly altered (24, 25, 30).

In addition, we analysed the impact of GCAC1809-1812TTCT on viral transcripts, HBsAg/core expression and HBeAg/HBV DNA release. We found that GCAC1809-1812TTCT drastically reduces the amount of both 3.5kb transcripts and to a lesser

extent the level of the other viral transcripts (2.4/2.1/0.7kb). This was only observed when the mutation was present in the core promoter position, but not when it was present in the HBx position, which indicates a promoter-mediated HBx-independent mechanism. The observed reduction of viral transcripts leads to reduced synthesis of HBsAg, core and HBeAg as detected in our in vitro study. However, why these mutations, which are located in the promoter of the core gene, also affect transcription of HBs and HBx RNAs remains unclear. As cccDNA synthesis can also be induced in hepatoma cells (31), one possible explanation might be that due to the reduced transcription of pgRNA, less pgRNA might be available to restore the cccDNA pool, which might subsequently reduce the overall transcriptional activity. In addition, GCAC1809-1812TTC may also affect the structure of the DNA template, which might again reduce transcription of all transcripts. However, further research has to be performed to address this question. Although, we observed that GCAC1809-1812TTCT reduces HBsAg synthesis in vitro interestingly no differences in HBsAg levels were detectable in our patients. This can be explained by several recent reports, including one of our group in which also patient's sera of the Albatros study were analysed. In these studies, it was concluded that integrated DNA has to be considered as a potent source for sufficient HBsAg expression in HBeAg negative patients (32-34). Therefore integrates might also contribute to compensation of an impaired HBsAg secretion in our patients.

Extracellular HBV DNA levels were not significantly changed by GCAC1809-1812TTCT in our in vitro study when compared to our reference genome, although pgRNA levels were strongly decreased and lower serum HBV DNA levels were observed in our patients sera. BCP double mutation was described in some studies to increase pgRNA encapsidation and synthesis of progeny viruses in vitro. As all our

analysed GCAC1809-1812TTCT variants harbour in addition the BCP double mutation a diminishing effect of the GCAC1809-1812TTCT mutation on virion release might be curtained by the additional presence of the BCP double mutation. Indeed, we observed that the presence of GCAC1809-1812TTCT without the BCP double mutation leads to significantly lower levels of extracellular HBV DNA, although this effect is only moderate and other factors have to be considered. In our patients lower pgRNA levels due to GCAC1809-1812TTCT might negatively impact the capacity for restorations of the cccDNA pool (19). This might lead to a reduction of transcriptional active cccDNA over a longer period of time and subsequently to a reduction of serum HBV DNA levels. In addition, the expression of core protein is reduced by GCAC1809-1812TTCT, which is required for virion synthesis.

Next, we addressed the question of why GCAC1809-1812TTCT was found so frequently in inactive carriers. In one study evidence was gained that core promoter mutations might arise as a result of RNA secondary structural considerations and that BCP double mutation leads to significant changes in the secondary pgRNA structure (17). To investigate whether GCAC1809-1812TTCT might act as a compensatory mutation for BCP double mutation, we conducted analogue bioinformatical modeling of the secondary pgRNA structure of this region. Interestingly, the secondary pgRNA structure including the BCP double mutation was calculated to be less thermodynamically stable in comparison to the wildtype sequence. However, after the additional introduction of GCAC1809-1812TTCT, the thermodynamic stability of the pgRNA secondary structure was restored approximately to the wildtype level. In summary, in the case of the BCP double mutation, the thermodynamic stability of the pgRNA seems to be decreased in favour of an increased replication capacity, which might be beneficial for the virus in stages of low immune pressure for example in the

immune tolerant phase. However, in later stages of the disease when the evolutionary pressure again increases (i.e. in the immune clearance phase) continuous hepatitis activity and hepatic flares might result in declining serum HBV-DNA levels and may eventually lead to HBeAg seroconversion and development of anti-HBe (27). At this point GCAC1809-1812TTCT might offer an evolutionary advantage as a compensatory mutation for BCP as GCAC1809-1812TTCT, in contrast to BCP double mutation, leads to a strong reduction of HBeAg and it enhances the thermodynamic stability of the secondary structure of the pgRNA by a local energy minimum, which impairs unfolding of the RNA and therefore might lead to a lower translation and reverse transcription. These mechanisms of enhanced stability, lower protein synthesis and importantly HBeAg reduction mediated by GCAC1809-1812TTCT may act as an escape mechanism of the virus.

To investigate, whether GCAC1809-1812TTCT might also act as a biomarker with potential prognostic value, we analysed another cohort of 125 patients with compensated HBV-related liver cirrhosis for the existence of this mutation. Importantly, while other than GCAC1809-1812TTCT mutations at nt1809-1812 were present in 15% of the patients, which is in line with another study (21), GCAC1809-1812TTCT was not found in this cohort at all. Therefore GCAC1809-1812TTCT was found to be strongly associated with inactive carrier status and therefore a benign course of the disease in our study. Although described as a marker for advanced HBV-related liver disease, the overall prevalence of BCP double mutation was not found to be associated with the presence of compensated liver cirrhosis or HCC. But very interestingly, the presence of BCP double mutation alone without additional GCAC1809-1812TTCT mutation was found to be strongly associated with established compensated liver cirrhosis and also with HCC.

Therefore, we suggest GCAC1809-1812TTCT mutation as a promising and robust biomarker for a benign course of the disease. In addition, this mutation might also have the potential to increase the specificity of BCP double mutation as a marker for an unfavourable prognosis. Hence, GCAC1809-1812TTCT should be evaluated in more detail in further studies for possible clinical use. As a limitation of our study, mostly patients infected with HBV genotype A and D were included, especially in the cirrhotic cohort, and it is uncertain if the results can be generalized for all HBV genotypes. Therefore, studies with a higher percentage of other HBV genotypes (especially B and C) infected patients are needed. In addition, further studies should include pre-cirrhotic patients with HBV-related hepatitis and more patients with HBV-related HCC to evaluate the impact of GCAC1809-1812TTCT as a biomarker in these subgroups of patients.

In conclusion, GCAC1809-1812TTCT, a quadruple mutation in the Kozak sequence preceding the precore start codon is highly prevalent and strongly associated with BCP double mutation in a large European cohort of inactive carriers. In vitro GCAC1809-1812TTCT leads to drastically diminished HBeAg levels and a diminished replicative activity, which might explain HBeAg negativity and lower HBV DNA levels as observed in inactive carriers. Importantly, GCAC1809-1812TTCT was not found at all in a large cohort of patients with compensated liver cirrhosis suggesting this mutation as a potential biomarker for HBV related disease.

METHODS

Study Populations

In total 560 participants of the German Albatros trial (Clinical.Trial.gov: NCT01090531) with inactive carriers were included in the analysis. For main inclusion and exclusion criteria of the Albatros trial see the supplementary section. The serum or plasma of these patients was prospectively collected and stored at -80 °C. Analyses of HBV DNA viral load and qHBsAg were limited to patients with available HBV DNA and qHBsAg levels determined in clinical routine (n=560; 100% and n=524; 92%, respectively). In addition, serum samples of 125 patients with HBV-related compensated liver cirrhosis including patients with history/presence or development of HCC after sampling were analysed. HBV genotypes were either determined in clinical routine or by direct sequencing of the polymerase region.

HBV DNA Extraction

Viral DNA was extracted from 200 µl of serum using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

Primers

Primers are described in the supplementary section.

Amplification of the HBV BCP region and full-length HBV genome

A fragment of the region encoding the BCP gene (nt1681-2044) was amplified by (semi-)-nested PCR. The detailed conditions are given in the supplementary section. The entire HBV genome was amplified by using primers P1 and P2 modified from Günther et al. (35) and by using the Expand High Fidelity PCR Kit (Roche, Germany).

Direct sequencing of BCP region and full-length genome

The corresponding DNA was subjected to sequencing PCR according to the manufacturer's protocol (BigDyeDeoxy Terminators; Applied Biosystems, USA). The DNA was sequenced on a 3130xl Genetic Analyzer (Applied Biosystems) and a sensitivity level of about 15%-20% was assumed.

Cell culture and cell treatment

The human hepatoma-derived cell line Huh 7 was cultivated as described (36).

Plasmids

HBV genomes containing A1762T/G1764A and GCAC1809-1812TTCT in different positions in the core promoter and HBx genomic region were commercially synthesized (Genscript; for an overview of analysed HBV genomes see Figure 3a). For control of the assays, a genotype A HBeAg negative genome with precore double mutation G1896A/G1899A (16), a genotype A HBeAg positive genome (37) and pUC18 vector were used.

Chemicals, antibodies and enzymes

Chemicals, antibodies and enzymes used for Western blotting and immunofluorescence microscopy are described in the Supplementary section. HBsAg- (Enzygnost, Siemens), HBeAg- (Cusabio) and HBcAg-specific (Cell Biolabs) ELISA were used according to the manufacturer`s protocol.

SDS-Page and Western blot analysis

Protein was extracted using Radioimmunoprecipitation assay buffer (RIPA) followed by a sonication step for 10 seconds. SDS-Page and Western blot analysis was performed according to standard procedures (38).

Northern blot analysis

RNA was isolated using Trizole. Northern blot analysis was performed according to standard procedures (38).

Transfection of cells

For transient transfection, Huh 7 cells were transfected using 6µl/µg DNA polyethyleneimine (PEI) or X-tremeGENE DNA Transfection Reagent with a ratio of 3:1 of the reagent to µg plasmid DNA, (Polysciences, Warrington, PA) according to the manufacturer`s instructions. Cells and supernatants were harvested 48 hours after transfection and stored at -20 °C. Transfection efficiency was determined by immunofluorescence analysis based on the number of positive cells using an HBsAg-specific antibody (HB01, see supplement), as no major differences were notable, no corrections were made.

RNA Isolation and cDNA synthesis

Frozen cells were lysed using the TriFast reagent (Peqlab) and the RNA isolated by phenol/chloroform extraction. Extracted RNA was solubilised in 15 µL DEPC-H_2O and 4 µg RNA were used for subsequent cDNA synthesis. All samples were incubated with DNase I at 37 °C for 1 h and subsequently incubated with a random hexamer primer to begin cDNA synthesis. Samples were incubated at 42 °C with a Mastermix containing 10 mM dNTPs, reverse transcriptase (RT) and corresponding buffer (Thermofisher). Finally, the RT was inactivated at 72 °C for 10 minutes. cDNA samples were diluted 1:10 and directly used for real time PCR.

Indirect immunofluorescence analysis

Immunofluorescence staining was performed as described (39) and analysed using a confocal laser scanning microscope and ZEN 2012 software (Carl Zeiss).

Calculation of pregenomic RNA secondary structures of nt1730-1930 genomic region of HBV

To predict the secondary structures of the pregenomic (pg) RNA regions and for calculation of the thermodynamic stability of the folded RNAs the web service *RNAfold* (40) was used. Nucleotides 1730-1930, as well as the same RNA region containing the respective mutations, were entered and the Minimum Free Energy (MFE) was calculated avoiding isolated base pairs and therefore helices of a length of 1.

Statistical analysis

χ^2 test or Fisher's exact test was used for group comparisons of categorical data and the Wilcoxon-Mann-Whitney U test was used for group comparisons of ordered data as appropriate. Differences within HBV DNA and qHBsAg serum levels were determined using the nonparametric Kruskal-Wallis test with post-hoc Dunn's test. Single non-parametric t-tests were used for analyses of in vitro experiments with two groups and multiple t-test with the Holm-Sidak method were performed to correct for multiple group comparisons and to determine statistical significance for analyses of the in vitro experiments with more than two groups. Outliers were identified using the ROUT method (Q = 1 %). Statistical analysis was done with BiAS for Windows, version 11 (Epsilon, Germany) and GraphPad Prism 8 (GraphPad Software, Inc., USA). P values <0.05 were considered significant.

Study approval

The study was approved by the local ethic committee of University clinic of Frankfurt (97/09) and written informed consent was obtained from all patients. It was not appropriate or possible to involve patients or the public in the design, or conduct, or

reporting, or dissemination of our research. The study was performed in accordance with the provisions of the *Declaration of Helsinki* and good clinical practice guidelines.

Authors contribution:

Guarantor of the article: Kai-Henrik Peiffer

Concept and design: KP, EHildt; acquisition of data: JV, VK, JD, FF, JT, PL, CG, CSarrazin; analysis and interpretation of data; KP, EHildt, CS, MB, BJ, MG, LK, WO, TZ, GC, AK, AG, drafting of the manuscript; KP, CS, MB, AG, EHildt; critical revision of the manuscript for important intellectual content; SZ, CSarrazin, PL; statistical analysis; MB, MG, CS, EH

Acknowledgment:

This work was supported by DZIF (Deutsches Zentrum für Infektionsforschung), TTU Hepatitis (CS and EHildt); DFG-Grant PE 2403/2-1 (KP) and HI 858/12-1 (EH); grant of the DRUID consortium (AG, KP, SZ and EHildt), grant of the Ellen Schairer-Stiftung (KP); grant of the Scolari Stiftung (KP and SZ).

REFERENCES

1. WHO. Hepatitis B Fact sheet, Updated April 2017.
2. European Association for the Study of the Liver. Electronic address eee, and European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol.* 2017.
3. Terrault NA et al. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology.* 2016;63(1):261-83.
4. Sarin SK et al. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int.* 2016;10(1):1-98.
5. Chen CJ et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *Jama.* 2006;295(1):65-73.

6. Iloeje UH, Yang HI, Su J, Jen CL, You SL, and Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology.* 2006;130(3):678-86.
7. Brunetto MR et al. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. *Gastroenterology.* 2010;139(2):483-90.
8. Liu J et al. Serum Levels of Hepatitis B Surface Antigen and DNA Can Predict Inactive Carriers With Low Risk of Disease Progression. *Hepatology.* 2016;64(2):381-9.
9. Tseng TC et al. High levels of hepatitis B surface antigen increase risk of hepatocellular carcinoma in patients with low HBV load. *Gastroenterology.* 2012;142(5):1140-9 e3; quiz e13-4.
10. Fan YF et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. *Hepatology.* 2001;33(1):277-86.
11. Kao JH, Chen PJ, Lai MY, and Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology.* 2003;124(2):327-34.
12. Chen CH et al. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. *J Infect Dis.* 2008;198(11):1634-42.
13. Tseng TC et al. Higher proportion of viral basal core promoter mutant increases the risk of liver cirrhosis in hepatitis B carriers. *Gut.* 2015;64(2):292-302.
14. Baptista M, Kramvis A, and Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. *Hepatology.* 1999;29(3):946-53.
15. Kuang SY. Specific mutations of hepatitis B virus in plasma predict liver cancer development. *Proc Natl Acad Sci.* 2004;101(10):3575-80.
16. Kuhnhenn L et al. Impact of HBV genotype and mutations on HBV DNA and qHBsAg levels in patients with HBeAg-negative chronic HBV infection. *Aliment Pharmacol Ther.* 2018;47(11):1523-35.
17. Kidd AH, and Kidd-Ljunggren K. A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. *Nucleic Acids Res.* 1996;24(17):3295-301.
18. Tong S, and Revill P. Overview of hepatitis B viral replication and genetic variability. *J Hepatol.* 2016;64(1 Suppl):S4-16.
19. Quarleri J. Core promoter: a critical region where the hepatitis B virus makes decisions. *World J Gastroenterol: WJG.* 2014;20(2):425-35.
20. Ahn SH, Kramvis A, Kawai S, Spangenberg HC, Li J, Kimbi G, et al. Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. *Gastroenterology.* 2003;125(5):1370-8.
21. Belyhun Y, Liebert UG, and Maier M. Analysis of HBV basal core promoter/precore gene variability in patients with HBV drug resistance and HIV co-infection in Northwest Ethiopia. *PloS one.* 2018;13(2):e0191970.
22. Baumert TF, Marrone A, Vergalla J, and Liang TJ. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. *J Virol.* 1998;72(8):6785-95.
23. Buckwold VE, Xu Z, Chen M, Yen TS, and Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol.* 1996;70(9):5845-51.

24. Gunther S, Piwon N, and Will H. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653) --> T, A(1762) --> T and G(1764) --> A mutations in the core promoter. *J Gen Virol.* 1998;79 (Pt 2):375-80.
25. Jammeh S, Tavner F, Watson R, Thomas HC, and Karayiannis P. Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. *J Gen Virol.* 2008;89(Pt 4):901-9.
26. Laskus T, Rakela J, Nowicki MJ, and Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. *Gastroenterology.* 1995;109(5):1618-23.
27. Liaw YF. Natural history of chronic hepatitis B virus infection and long-term outcome under treatment. *Liver Int.* 2009;29 Suppl 1:100-7.
28. Moriyama K, Okamoto H, Tsuda F, and Mayumi M. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. *Virology.* 1996;226(2):269-80.
29. Parekh S et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol.* 2003;77(12):6601-12.
30. Sterneck M et al. Functional analysis of HBV genomes from patients with fulminant hepatitis. *Hepatology.* 1998;28(5):1390-7.
31. Wing PA et al. A dual role for SAMHD1 in regulating HBV cccDNA and RT-dependent particle genesis. *Life Sci Alliance.* 2019;2(2).
32. Cornberg M, Wong VW, Locarnini S, Brunetto M, Janssen HL, and Chan HL. The role of quantitative hepatitis B surface antigen revisited. *J Hepatol.* 2017;66(2):398-411.
33. Peiffer KH et al. Divergent preS Sequences in Virion-Associated Hepatitis B Virus Genomes and Subviral HBV Surface Antigen Particles From HBV e Antigen-Negative Patients. *J Infect Dis.* 2018;218(1):114-23.
34. Wooddell CI et al. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. *Sci Transl Med.* 2017;9(409).
35. Gunther S, Li BC, Miska S, Kruger DH, Meisel H, and Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol.* 1995;69(9):5437-44.
36. Blight KJ, McKeating JA, and Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol.* 2002;76(24):13001-14.
37. Peiffer KH et al. Intracellular accumulation of subviral HBsAg particles and diminished Nrf2 activation in HBV genotype G expressing cells lead to an increased ROI level. *J Hepatol.* 2015;62(4):791-8.
38. Asubel F, Brent R, Kingston R, Moore D, Seidman J, and Smith J. *Current protocols of molecular biology.* New York: John Wiley; 2004.
39. Brandenburg B et al. A novel system for efficient gene transfer into primary human hepatocytes via cell-permeable hepatitis B virus-like particle. *Hepatology.* 2005;42(6):1300-9.
40. Gruber AR, Lorenz R, Bernhart SH, Neubock R, and Hofacker IL. The Vienna RNA websuite. *Nucleic Acids Res.* 2008;36(Web Server issue):W70-4.

Fig. 1

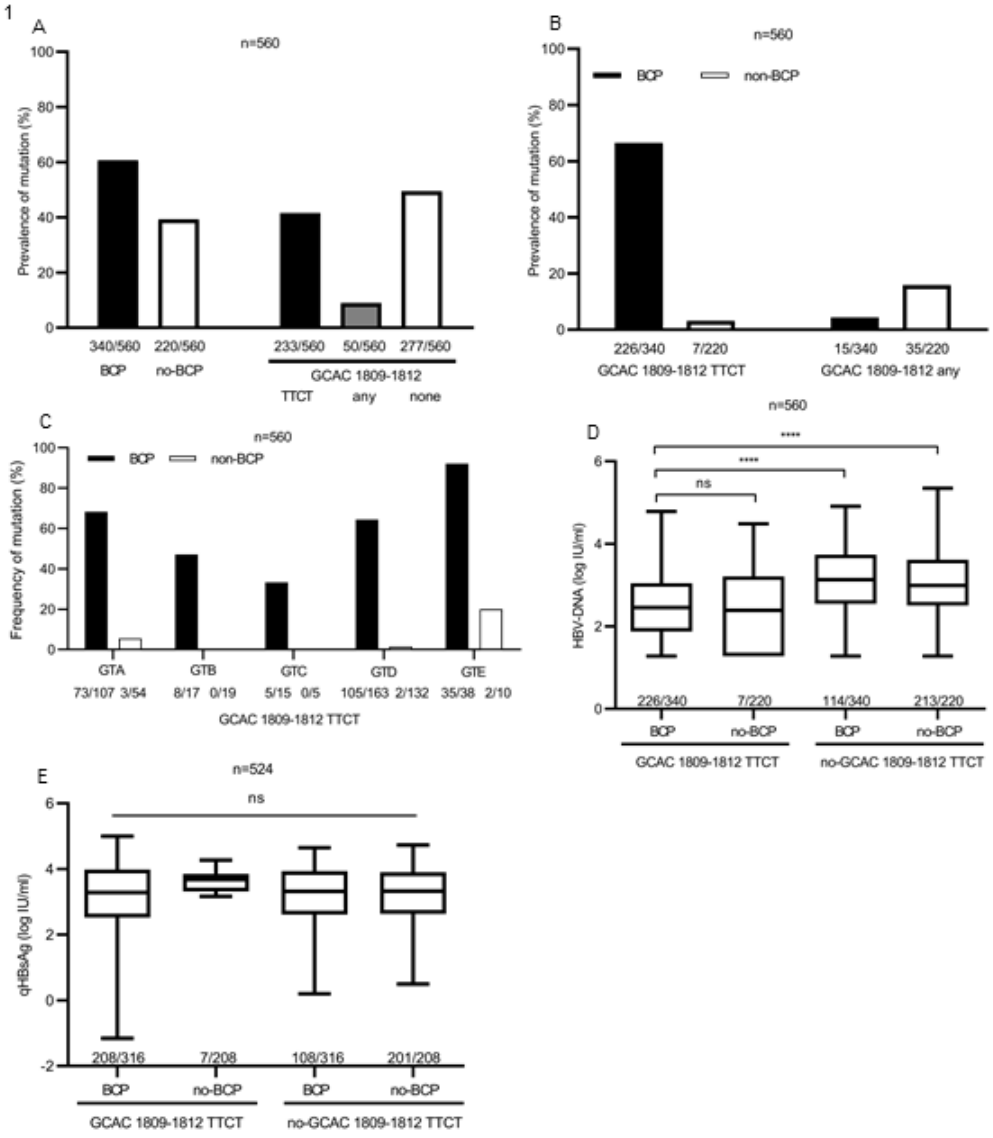
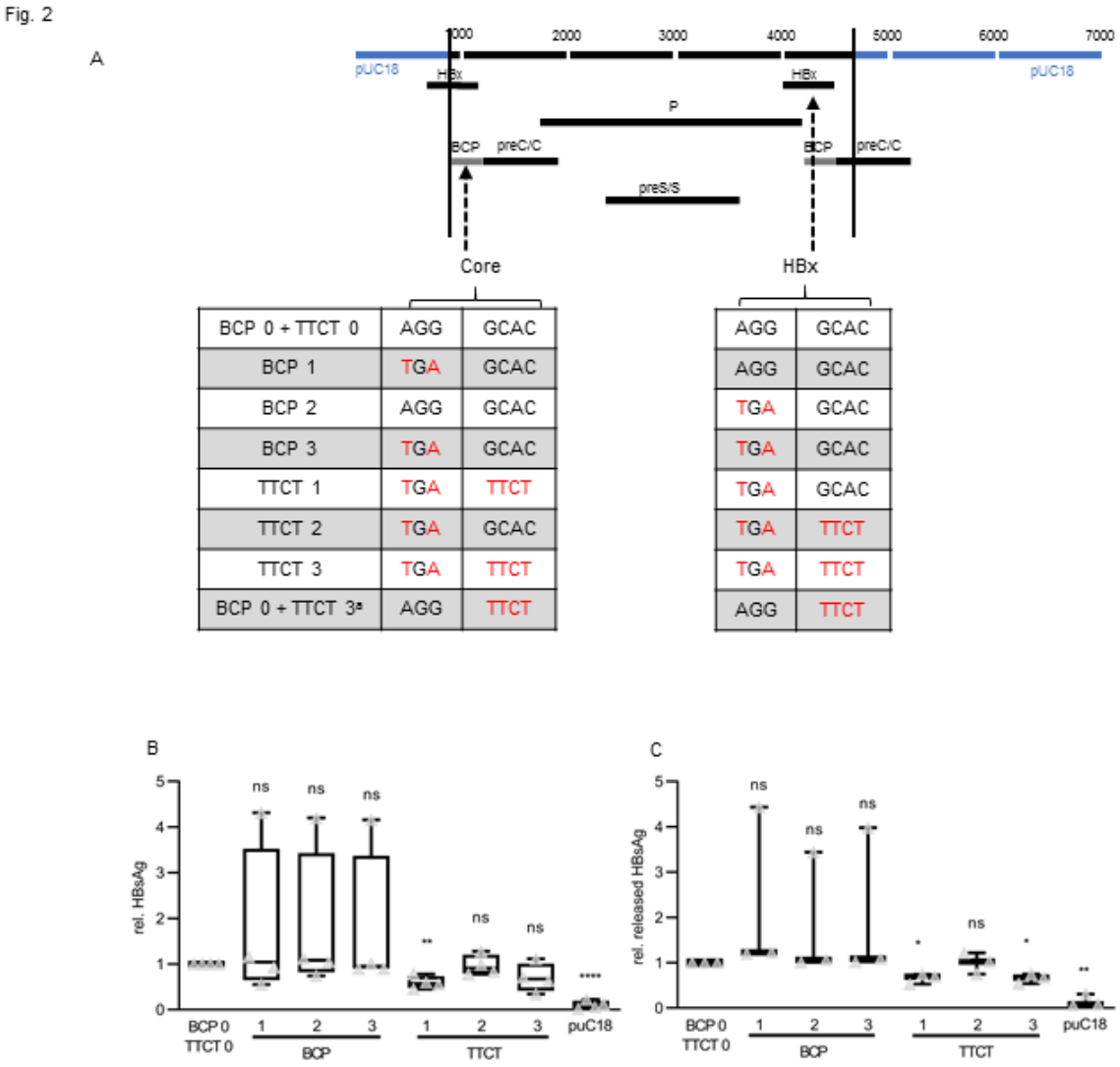


Figure 1: High prevalence of GCAC1809-1812TTCT and association with BCP double mutation A1762T/G1764A and lower HBV DNA levels in HBsAg carriers.

A-C) Prevalence of (A) BCP double mutation A1762T/G1764A and mutations at nt1809-1812, (B) mutations at nt1809-1812 dependent on coexistence of A1762T/G1764A and (C) mutations at nt1809-1812 dependent on coexistence of A1762T/G1764A among different genotypes A-E in sera of 560 patients with a chronic HBV infection (HBsAg carriers) from the Albatros cohort. D-E) Association of A1762T/G1764A and GCAC1809-1812TTCT with (D) HBV DNA levels and (E) qHBsAg levels in inactive carriers from the Albatros cohort; GT, genotypes, Data are

shown as following: median (line inside the box), first and third quartile (upper and lower limit of the box, respectively) and the highest and lowest values are represented by the top and bottom whiskers. A Kruskal-Willis test with post-hoc Dunn's test was performed to determine statistical significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. 2



	Core		HBx	
BCP 0 + TTCT 0	AGG	GCAC	AGG	GCAC
BCP 1	TGA	GCAC	AGG	GCAC
BCP 2	AGG	GCAC	TGA	GCAC
BCP 3	TGA	GCAC	TGA	GCAC
TTCT 1	TGA	TTCT	TGA	GCAC
TTCT 2	TGA	GCAC	TGA	TTCT
TTCT 3	TGA	TTCT	TGA	TTCT
BCP 0 + TTCT 3[a]	AGG	TTCT	AGG	TTCT

Figure 2: Diminished synthesis of HBsAg by GCAC1809-1812TTCT in vitro.

(A) Overview of expressed genotype A genomes based on a 1.2mer isolate from a

patient of the Albatros cohort (TTCT3) with both the A1762T/G1764A (BCP) double mutation and the GCAC1809-1812TTCT (TTCT) quadruple mutation in core promoter and HBx (due to partially overlapping reading frame of HBV). 0=absence of the mutation, 1=mutation only in core promoter, 2=mutation only in HBx, 3=mutation in both core promoter and HBx. In respect to the clinical data, all variants (TTCT1-3) contain in addition the A1762T/G1764A BCP mutation. A construct without these two mutations (BCP0 + TTCT0) was used as a reference. For analysis of extracellular DNA an additional genome harboring GCAC1809-1812TTCT in HBx and core promoter but without the A1762T/G1764A BCP double mutation was used (BCP0/TTCT3): A 1.1mer HBeAg wildtype genome and an HBeAg negative genome harboring a G1896A/G1899A Precore mutation were used as controls. [a] utilized for immunofluorescence, HBeAg Elisa and extracellular HBV DNA. (B, C) HBsAg-specific ELISA of (B) lysates, n=4, and (C) supernatants, n=3. Data are shown as following: median (line inside the box), first and third quartile (upper and lower limit of the box, respectively) and the highest and lowest values are represented by the top and bottom whiskers. Multiple t-test with the Holm-Sidak method were performed to correct for multiple group comparisons and to determine statistical significance, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Fig. 3

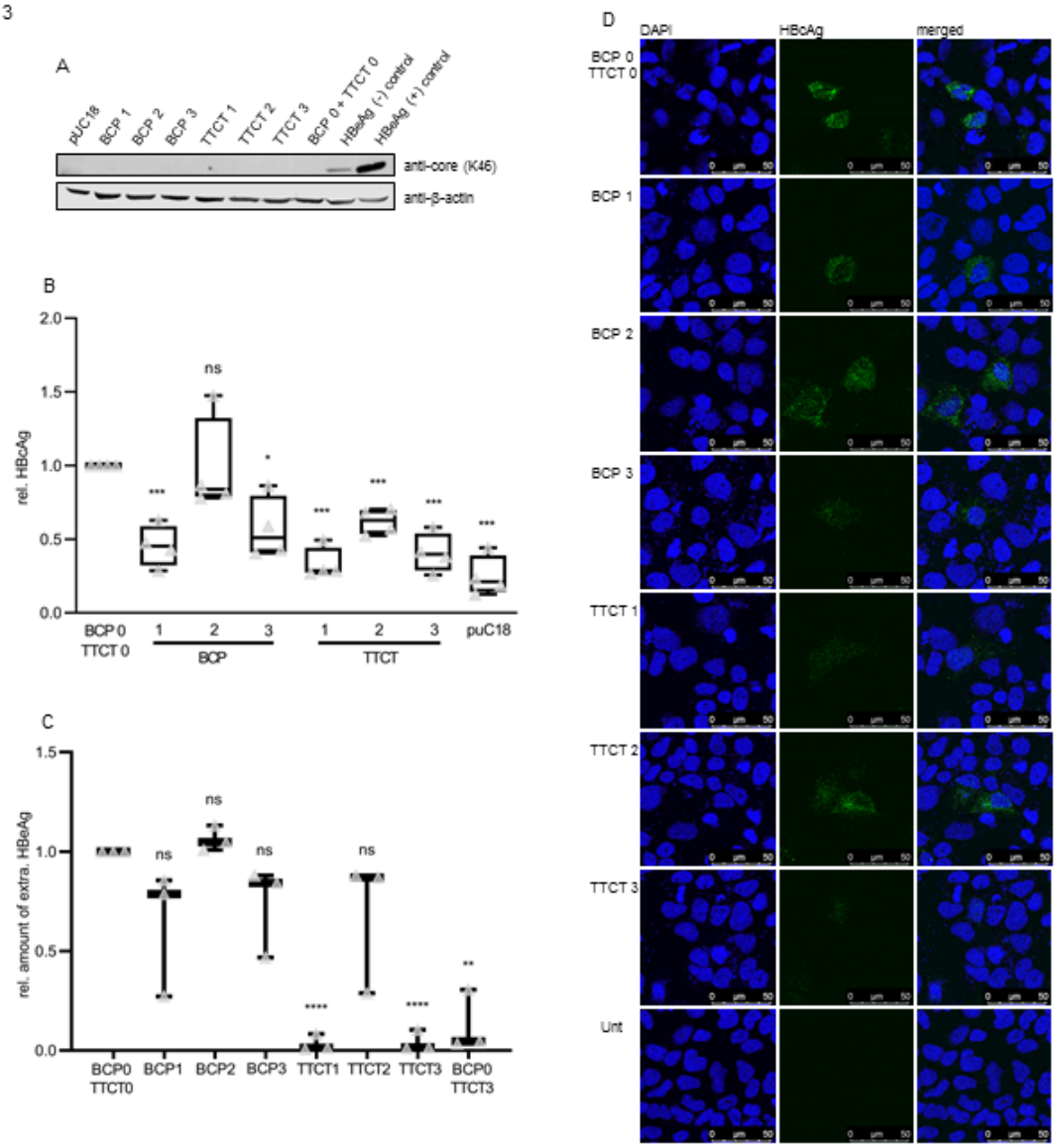


Figure 3: Diminished synthesis of core protein and HBeAg by GCAC1809-1812TTCT in vitro.

(A) Western blot analysis using a core-specific antibody of lysates, as additional control a different HBeAg negative genotype A genome harbouring precore double mutation G1896A/G1899A was used. (B, C) HBcAg- and HBeAg-specific ELISA of lysates and supernatants, (n=4 and n=3, respectively). Data are shown as following: median (line inside the box), first and third quartile (upper and lower limit of the box, respectively) and the highest and lowest values are represented by the top and bottom whiskers. Multiple t-test with the Holm-Sidak method were performed to

correct for multiple group comparisons and to determine statistical significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (D) CLSM analysis of transfected Huh 7 cells stained with the core-specific antibody MAB3120.

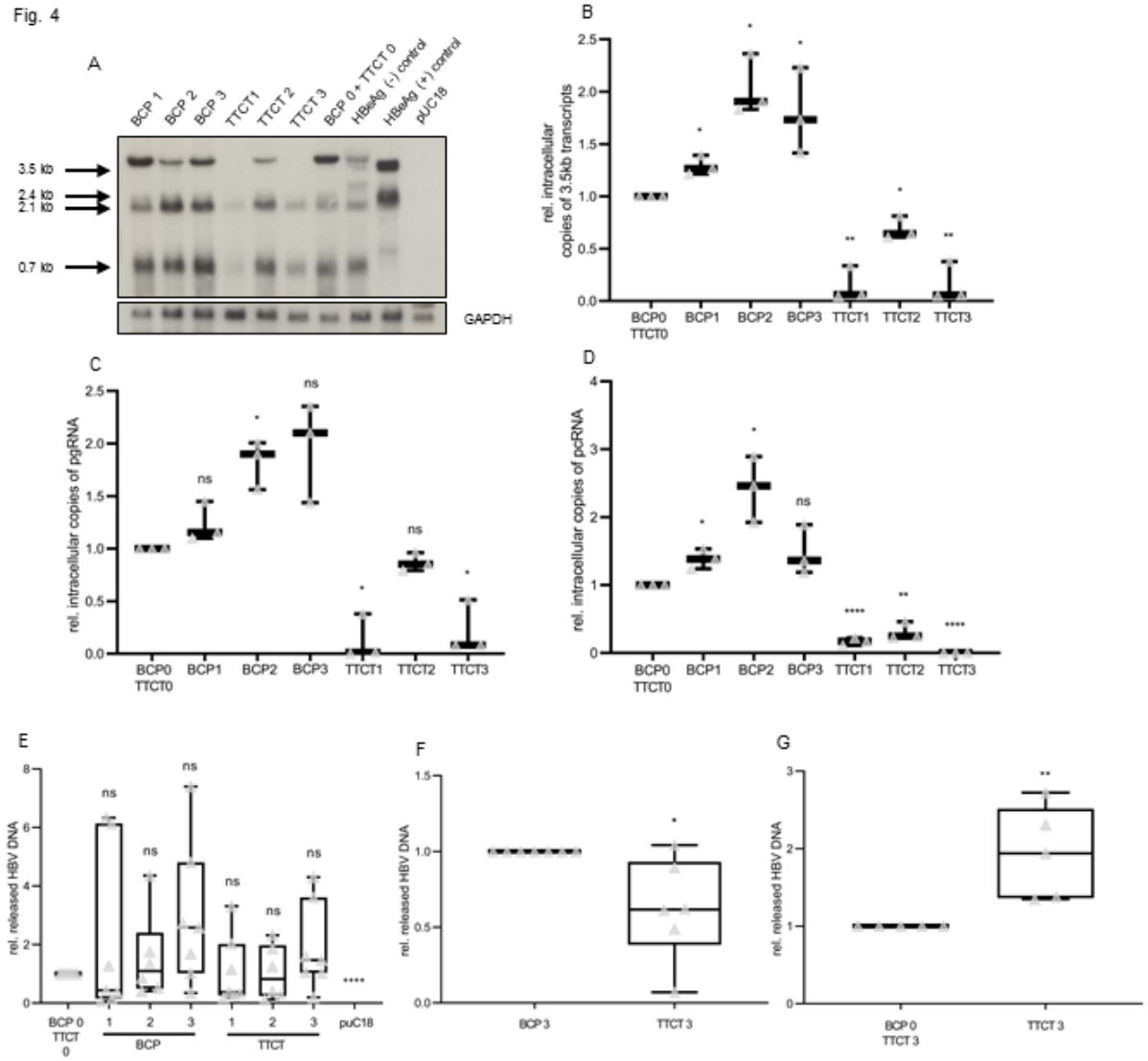


Figure 4: Impaired viral replication by GCAC1809-1812TTCT in vitro

(A) Northern Blot analysis, as additional control an HBeAg negative genotype A genome, harbouring precore double mutation G1896A/G1899A was used; (B-D) real-time PCR analyses of lysates for either total 3.5 kilobase (kb) (B), pregenomic (C) or

precore (D) transcripts; DNA (E-G) real-time PCR analyses of supernatants. For G an additional genome harbouring GCAC1809-1812TTCT in HBx and core promoter but without the A1762T/G1764A BCP double mutation was used (BCP0/TTCT3). Values were normalised to BCP0/TTCT0 (B-E) and represent a total value of n = 3 (B, C, D), n = 7 (E,F) and n = 5 (G) independent experiments. In 4F values of 4E were normalised to BCP3 and in G values were normalised to TTCT3. Data are shown as following (B-G): median (line inside the box), first and third quartile (upper and lower limit of the box, respectively) and the highest and lowest values are represented by the top and bottom whiskers. Multiple t-test with the Holm-Sidak method were performed to correct for multiple group comparisons and to determine statistical significance in 4B-E, a two-tailed student t-test was performed to determine statistical significance in 4F and 4G. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

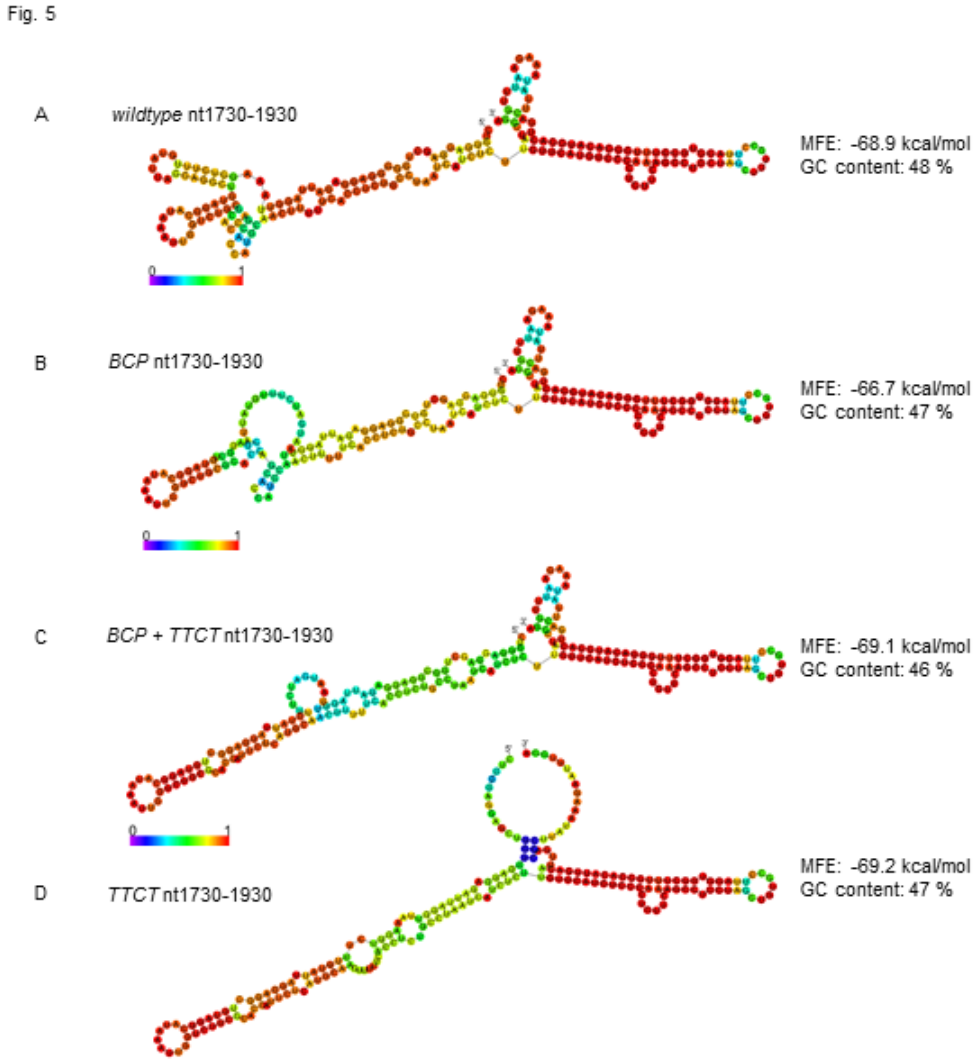


Figure 5: Thermodynamic destabilization of the pgRNA secondary structure by A1762T/G1764A and compensation by GCAC1809-1812TTCT

A-D: Calculation of the thermodynamic stability of nt1730-1930 pgRNA genomic region genome with the presence of (A) none of the mutations, (B) A1762T/G1764A, (C), A1762T/G1764A and GCAC1809-1812TTCT and (D) GCAC1809-1812TTCT without A1762T/G1764A. The used HBV sequence was derived from an inactive carrier patient with the coexistence of A1762T/G1764A and GCAC1809-1812TTCT. MFE, Minimum Free Energy; GC-content, Guanine-Cytosine-content. Red bars indicate the positions in the sequences where the mutations are localised.

Fig. 6

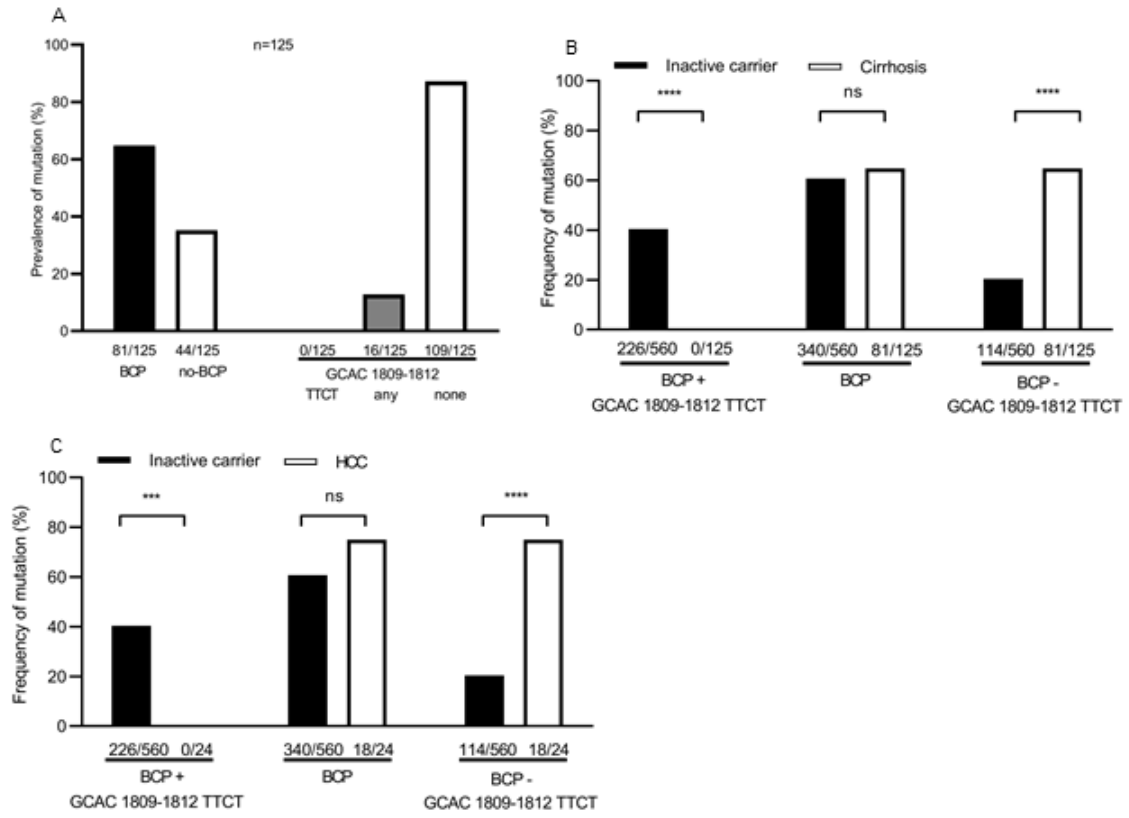


Figure 6: GCAC1809-1812TTCT is absent in patients with cirrhosis and strongly associated with inactive carrier status

(A) Prevalence of BCP double mutation A1762T/G1764A and mutations at nt1809-1812 in patients with liver cirrhosis. B-C) Association of A1762T/G1764A +/- GCAC1809-1812TTCT with (B) the presence of liver cirrhosis and (C) the presence of liver cirrhosis and HCC in comparison to inactive carriers. A χ^2 test was used for group comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table 1: Demographics of patients with inactive carrier status

Parameter	Total, n (%)	GTA, n (%)	GTB, n (%)	GTC, n (%)	GTD, n (%)	GTE, n (%)
n	560 (100)	161 (28.7)	36 (6.4)	20 (3.6)	295 (52.7)	48 (8.6)
Age (years, mean ± SD)	40.7 ± 11.9	45.0 ± 13.1	37.6 ± 9.7	39.0 ± 8.0	39.5 ± 11.4	37.5 ± 10.1
Male gender	228 (41.6)	62 (38.5)	13 (36.1)	3 (15.0)	131 (44.4)	19 (39.6)
Female gender	332 (58.4)	99 (61.5)	23 (63.9)	17 (85.0)	164 (55.6)	29 (60,4)
HBV DNA (mean log IU/ml ± SD)	2.8 ± 0.8	2.7 ± 0.8	3.0 ± 0.7	2.9 ± 0.5	2.9 ± 0.8	2.8 ± 1.0
qHBsAg (mean log IU/ml ± SD) [a]	3.2 ± 1.0	3.6 ± 0.8	2.3 ± 0.9	3.4 ± 0.9	3.0 ± 0.9	3.6 ± 0.7
ALT (mean U/l ± SD)	28.7 ± 14.0	29.3 ± 13.3	23.5 ± 11.7	25.9 ± 10.7	29.4 ± 14.8	27.5 ± 13.1
Ethnicity [b]						
White	404	130	2	1	268	3
Asian	84	9	34	19	21	1
African-American	60	14	0	0	6	40

SD, standard deviation; HBV, hepatitis B virus; qHBsAg, quantitative hepatitis B surface antigen; ALT, alanine transaminase; GTA/B/C/D/E, genotype A/B/C/D/E.
a) qHBsAg was available from 524 patients.
b) Ethnicity was available from 548 patients

Table 2: Demographics of patients with HBV-related liver cirrhosis

Parameter	Total, n (%)	GTA, n (%)	GTB, n (%)	GTC, n (%)	GTD, n (%)	GTF, n (%)
n [a]	125 (100)	13 (10,4)	9 (7,2)	1 (0,8)	93 (74,4)	2 (1,6)
Age (years, mean ± SD)	56.9 ± 10.4	46.9 ± 13.5	62,2 ± 12.4	37.4 ± 0.0	57.3 ± 8.9	54,4 ± 13.9
Male gender	104 (83.2)	11 (84.6)	7 (77.7)	1 (100.0)	76 (81.2)	2 (100.0)
Female gender	21 (16.8)	2 (15.4)	2 (22.3)	0 (0.0)	17 (18.8)	0 (0.0)
HBeAg negative [b]	101 (80.8)	5 (38.5)	7 (77.7)	0 (0.0)	83 (89.3)	0 (0.0)
HBeAg positive [b]	22 (17.6)	8 (61.5)	1 (11.1)	1 (100.0)	9 (9.7)	2 (100.0)
HCC [c]	24 (19.2)	1 (7.7)	2 (22.2)	0 (0.0)	17 (18.3)	0 (0.0)
HCC before sampling	4 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	0 (0.0)
HCC at sampling	4 (16.7)	0 (0.0)	1 (25.0)	0 (0.0)	2 (50)	0 (0.0)
HCC at FU [d]	16 (66.6)	1 (6.3)	1 (6.3)	0 (0.0)	12 (75.0)	0 (0.0)
ALT (mean U/l ± SD)	51.0 ± 130.4	70.0 ± 130.7	38.0 ± 12.8	43.0 ± 0.0	52.0 ± 139.6	100.0 ± 48.1
NUC treatment [e]	47 (37.6)	4 (8.5)	3 (6.4)	0 (0.0)	36 (76.6)	1 (2.1)
Ethnicity [f]						
White	118	13	8	0	90	1
Asian	1	0	0	1	0	0
African-American	1	0	0	0	0	1

SD, Standard deviation; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; ALT, alanine transaminase; GTA/B/C/D/E, genotype A/B/C/D/E; FU, follow-up; NUC, nucleoside analogues.
a) Genotype could not be determined for 7 patients. b) HBeAg status was available from 123 patients. c) Genotype could not be determined for 4 patients. d) Median time until HCC at follow-up in months ± SD: 79 ± 72. e) NUC treatment includes adenofovir dipivoxil, lamivudine, entecavir and tenofovir disoproxil fumarate. [f] Ethnicity was available from 120 patients.