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Original articles

Messa a punto di una metodica molecolare *home-made* per la definizione del genotipo virale e caratterizzazione dei profili di resistenza farmacologica in pazienti con epatite B cronica

Evaluation of a "home-made" method to determine viral genotype and characterize mutations conferring drug resistance in chronic hepatitis B patients

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INTRODUCTION

The pathomechanisms and kinetics of the replication of hepatitis B (HBV) are mainly influenced by the capacity of the virus for spontaneous point mutations. These have led to the expansion of 8 major genotypes (A-H) which are characterized by a total genomic difference of at least 8% and also show different geographic distribution [1].

Actually the determination of genotypes is mainly made for epidemiological reasons and the investigation of infection chains as e.g. hospital infections, but it is also of interest for the prediction of the clinical course and management of the infection [2]. Thus patients infected with far eastern genotype B show a more rapid seroconversion of anti-HBe antibodies than those with the Asian genotype C. In Europe a more severe course of infection is observed with the rare genotype F than with the prevalent genotypes D or A. Moreover the polymerase gene of genotype F seems to be genetically rather distant from the HBV vaccine strains, suggesting suboptimal protection [3]. Asian genotypes B and C seem to have a more benign clinical course than genotypes A and D. Additionally most recent data suggest that also the response to interferon treatment is more promising for certain genotypes like type B [4, 5]. This evidence together with the rapid development of drug resistant mutants under treatment highlights the need for molecular typing in the clinical practice. Since the introduction at the end of nineteen's of the negative isomer of the racemic mixture of 2-deoxy-3-thiacytidine as a powerful inhibitor of HBV reproduction as a new drug, numerous point mutations have been reported in resistance against lamivudine, adefovir and entecavir with the primary mutations involving V173L, L180M, M204V/I/S, A181V, N236T, T184G/S, S202I/G, M250V and the secondary F166L, I169T, A181V/T, V214A, Q215S, I233V [6-9].

The scope of this study was to evaluate a sequencing based genotyping method for usability in a local diagnostic laboratory for rapid identification of viral subtypes and the above point mutations conferring drug resistance.

MATERIAL AND METHODS

- *Samples:* 6 reference plasma samples from the Azienda Ospedaliera Santa Maria degli Angeli, Pordenone, Italy, with proven lamivudine resistance were tested in a blinded study together with 18 samples from patients with chronic HBV infection from the local gastroenterology department of the Azienda Sanitaria dell´Alto Adige, in Bolzano, Italy.
- *Molecular methods:* The reference samples were genotyped at the Department of Laboratory Medicine from the Azienda Ospedaliera Santa Maria degli Angeli, Pordenone, using the commercially available TRUGENE® HBV Genotyping Assay (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) according to the described conditions of the manufacturer.
The *home made* test system consisted of nucleic acid extraction of 500-800 µl of plasma in the automated NucliSENS® *easyMAG*™ (BioMerieux SA, 69280-Marcy l'Etoile-France) in a final volume of 55 µl.
Amplification of a 737 bp amplicon (position 254-990 according to the numbering of Okamoto et al.) was performed using the primers HBPr 256 and KL 14 described by Chan et al. and Kidd-Ljunggren et al. [10-12]. 10 µl of the extracted DNA was reacted with 20 pmol of primer's mix and the PCR performed with the SYBR GREEN Master Mix reagent (Applied Biosystems, Foster City, CA) for 47 cycles and an annealing temperature of 50°C. The fragments which were clearly visible on agarose gels were treated with alkaline phosphatase (ExoSAP-IT, GE-Healtcare, Milano, Italia) to inactivate remaining primers, labeled with the BigDye Terminator v 3.1 RR-cycle sequencing kit, purified by gel filtration on columns (*Centri-Sep*, Princeton Separation, Adelphia, NJ, USA) and finally run on the ABI Prism 3130 Genetic Analyzer (reagents and machine from Applied Biosystems, Foster City, CA).
- *Bioinformatics:* The sequences were aligned and genetic relatedness evaluated by neighbor joining and bootstrap analysis with reference sequences which have been deposited in a local database created within the program TraceEdit Pro (from RIDOM Bioinformatics, Münster, Germany). A representative bibliography of relevant genotypes was based on an extended Swedish study of circulating subtypes [13]. Additional selected sequences from GenBank were included and valuable information about new isolates provided by Dr's Helene Norder, Solna, Sweden and Syria Laperche, Paris, France. The analysis of the region showing mutations (aa80 to 275) was also defined with the program <geno2pheno> (http://www.geno2pheno.org).

RESULTS

As shown in Table 1 the home made PCR/sequencing protocol correctly identified the HBV genotype as well as the pattern of viral resistance. From the chronic HBV patients at the local gastroenterology department the majority of isolates belonged to genotype D (13/18=72%) followed by genotype A (4/18=22%). One genotype C was detected in a Chinese patient who presumably had acquired the infection in his country of origin (Figure 1).
Analysis of the point mutations in the reference samples (Table 1) revealed identical results with both tests with the majority of mutations affecting M204I/V (position 204, YMDD changed to YIDD or YVDD) and L180M (position 180, change of LLAQ to LMAQ) respectively. This was also the case in the tested samples from the local gastroenterology department where additionally several other locations were found altered (Table 2). If present, both mutations of M204V/I and L180M were detected together in the majority of cases (6 out of 8)

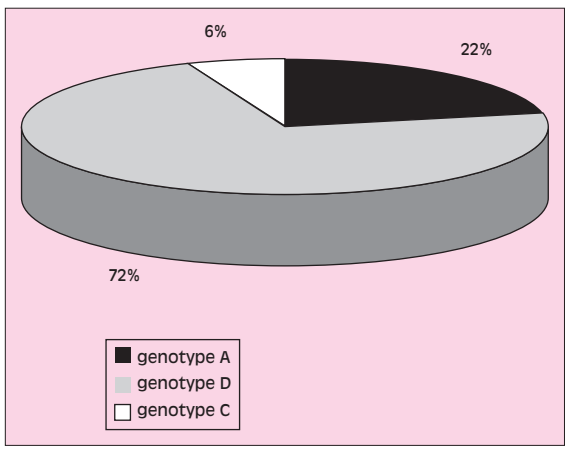

Figure 1 - Distribution of genotypes in HBV isolates in Bolzano.

and also a difference of the substitutions was observed regarding the respective HBV genotypes. Thus M204I mutations were more frequent in genotype D (4 out of 5) whereas M204V seemed to be more prevalent in the tested genotype A isolates (2 out of 3). Mutations affecting G173L (pos 173 GVGL) were not found. In the only genotype C isolate tested a different pattern was found with E263D and I269L (Table 2).

Table 1 - Comparison of the results obtained by the two methods (home-made and commercial test).

Sample identification	*Genotype*		*Mutation analysis*					
	Home-made test	*Commercial test*	*Home-made test*			*Commercial test*		
			Genome position					
			204	*180*	*173*	*204*	*180*	*173*
HBV-19	D	D	YIDD	LMAQ	wt	YIDD	LMAQ	wt
HBV-20	A	A	YIDD	LMAQ	wt	YIDD	LMAQ	wt
HBV-21	D	D	Wt	wt	wt	wt	wt	wt
HBV-22	D	D	Wt	wt	wt	wt	wt	wt
HBV-23	D	D	YIDD	wt	wt	YIDD	wt	wt
HBV-24	A	A	Wt	wt	wt	wt	wt	wt

Table 2 - Genotypes and mutations of viral isolates from the 18 patients of the local Gastroenterology department of the Azienda Sanitaria dell'Alto Adige (home-made test).

Sample identification	*Genotype*	*Mutation analysis*			
		Genome position			
		204	*180*	*173*	*Other*
HBV-1	D	wt	LMAQ	wt	-
HBV-2	D	wt	LMAQ	wt	-
HBV-3	A	wt	wt	wt	-
HBV-4	D	wt	wt	wt	-
HBV-5	A	wt	wt	wt	-
HBV-6	D	wt	wt	wt	-
HBV-7	D	wt	wt	wt	-
HBV-8	D	wt	wt	wt	-
HBV-9	A	YVDD	LMAQ	wt	-
HBV-10	D	YIDD	wt	wt	-
HBV-11	D	YIDD	LMAQ	wt	-
HBV-12	D	wt	wt	wt	-
HBV-13	A	YVDD	LMAQ	wt	L217R, F221Y
HBV-14	D	wt	wt	wt	-
HBV-15	D	wt	wt	wt	S213T
HBV-16	D	wt	wt	wt	K270R, Y257H, L91I
HBV-17	D	YVDD	LMAQ	wt	T184A, M250L
HBV-18	C	wt	wt	wt	E263D, I269L

DISCUSSION

Long term suppression of viral replication is critical for the management of HBV infection. Loss of HBeAg or HBsAg with or without seroconversion are decisive regarding the management of antiviral therapy. Repeated monitoring of serum DNA levels is mandatory as an early response is predictive for a sustained response as well as the likelihood of development of viral resistance [14]. Additionally the access to viral resistance testing is desirable as clinical data from Asia suggest that viral resistance might not be detected early enough for efficient countermeasures if testing is based on antigen and subsequent quantitative DNA tests only [15].

Our present PCR/sequencing test is not only suitable for the reliable determination of HBV genotype and the genomic region amplified by the primers specified by Chan et al. and Kidd-Ljunggren et al. but also allows the determination of the most prevalent resistance mutations [11, 12]. In the Bolzano hospital, like in the Pordenone hospital, these consisted mainly of M204V/I and L180M mutations which in the majority being combined mutations. The finding that the M204I mutations were more frequent in the genotype D isolates whereas the M204V seems to be more prevalent in genotype A isolates is in accordance with recently published data about differences of specific point mutations in the different lamivudine resistant HBV genotypes [16].

There were also several other mutations detected with our test, which in combination certainly could also contribute to a drug resistant phenotype. Thus the mutations at positions E263D and I269L found in the only genotype C isolate tested only sporadically have been associated with resistance in the literature [17].

In summary our preliminary study shows the potential of a rapid PCR and sequencing based approach for HBV genotyping and simultaneous detection of the mutations associated with drug resistance in the dominant virus strain. Validated according to consensus recommendations this test can be recommended not only as a confirmatory test for available commercial assays but also for routine laboratory diagnosis [18]. Provided the regular update of the local sequencing database to include newly identified isolates it is a valuable tool also for the detection and characterization also of new subgenotypes and mutation profiles. In connection with national reference laboratories and ongoing international multicenter studies this provides valuable information not only for the local and European epidemiological picture but also for the improvement of diagnostic procedures and therapy.

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Conflict of interest: none to be declared.

Keywords: HBV, genotyping, drug resistance, mutation, sequencing.

SUMMARY

We compared a *home-made* sequencing system to analyze plasma samples from patients with chronic HBV infection with the commercial TRUGENE® HBV Genotyping Assay. A PCR and sequencing protocol based on published primers was applied to detect the viral genotypes as well as the major patterns of point mutations leading to resistance to lamivudine, adefovir and entecavir. For the determination of HBV genotypes the obtained sequences were aligned with a database created within the RIDOM TraceEdit program and publicly available reference sequences. Our results showed perfect correlation with the commercial system, with types D (72%) and A (22%) being the most frequent genotypes. The resistance loci were also reliably detected with mostly combined L180M and M204V/I mutations as the local patterns. M204I mutations were more frequent in genotype D, M204V in genotype A isolates. G173L mutations were not found. The only genotype C isolate tested revealed a different pattern (E263D and I269L). These data speak for the usability of this rapid amplification and sequencing approach for routine genotyping of HBV isolates and simultaneous determination of the drug resistance profile of the dominant viral species.

RIASSUNTO

Un sistema di sequenziamento home made, *utilizzato per analizzare campioni di plasma di pazienti con infezione cronica da HBV, è stato confrontato con il test commerciale TRUGENE® HBV Genotyping Assay.*
Il protocollo, messo a punto per individuare i genotipi virali e i principali pattern *di mutazioni puntiformi in grado di sostenere resistenza a lamivudina, adefovir ed entecavir, ha previsto una reazione di amplificazione e sequenziamento genico basata su* primer *pubblicati. Per la determinazione dei genotipi virali le sequenze ottenute sono state allineate con quelle presenti nel database creato all'interno del programma RIDOM TraceEdit e di riferimento disponibili. I risultati ottenuti hanno dimostrato una perfetta correlazione con il sistema commerciale, indicando più frequenti i genotipi D (72%) e A (22%). Il pattern di resistenza locale più diffuso è stata la combinazione L180M e M204V/I. La mutazione M204I è risultata più frequente nei genotipi D, M204V nei genotipi A isolati. La mutazione G173L non è stata ritrovata. L'unico genotipo C isolato ha evidenziato un* pattern *singolare (E263D e I269L). Questi dati indicano un'effettiva fruibilità routinaria del sistema di amplificazione e sequenziamento adottato per la genotipizzazione degli isolati di HBV e la simultanea determinazione del profilo di farmacoresistenza della specie virale dominante.*

REFERENCES

[1] Liaw Y.F., Brunetto M.R., Hadziyannis S. The natural history of chronic HBV infection and geographical differences. *Antivir. Ther.* 15 (Suppl. 3), 25-33, 2010.
[2] Scalia G., Russo R., Palermo I., et al. Clinical, virological and epidemiological aspects of acute B hepatitis in the Catania area (Italy). *Le Infezioni in Medicina* 18 (3), 169-174, 2010.
[3] Tacke F., Amini-Bavil-Olyaee S., Heim A,. Luedde T., Manns M.P., Trautwein C. Acute hepatitis B virus infection by genotype F despite successful vaccination in an immune-competent German patient. *J. Clin. Virol.* 38, 353-357, 2007.
[4] Lin C.L., Kao J.H. The clinical implications of hepatitis B virus genotype: recent advances. *J. Gastroenterol. Hepatol.* 26 (Suppl. 1), 123-130, 2011.
[5] Chen C.H., Lee C.M., Hung C.H., et al. Hepatitis B virus genotype B results in better immediate, late and sustained responses to peginterferon-alfa in hepatitis-B-e-antigen-positive patients. *J. Gastroenterol. Hepatol.* 26, 461-468, 2011.
[6] Scotto G., Palumbo E., Fazio V., Cibelli D.C., Saracino A., Angarano G. Efficacy and tolerability of lamivudine alone versus lamivudine plus alpha-interferon for treatment of chronic active hepatitis B in patients with a precore-mutant variant. *Le Infezioni in Medicina* 14 (3), 145-151, 2006.
[7] Khudyakov Y. Coevolution and HBV drug resistance. *Antivir. Ther.* 15, 505-515, 2010.
[8] Locarnini S.A., Yuen L. Molecular genesis of drug-resistant and vaccine-escape HBV mutants. *Antivir. Ther.* 15, 451-461, 2010.
[9] Felaco F.M., Leone S., Stanzione M. Therapeutic options in chronic HBV infection. *Le Infezioni in Medicina* 15 (3), 149-158, 2007.
[10] Okamoto H., Tsuda F., Sakugawa H., et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.* 69, 2575-2583, 1988.
[11] Chan H.L., Wong M.L., Hui A.Y., Hung L.C., Chan F.K., Sung J.J. Hepatitis B virus genotype C takes a more aggressive disease course than hepatitis B virus genotype B in hepatitis B e antigen-positive patients. *J. Clin. Microbiol.* 41, 1277-1279, 2003.
[12] Kidd-Ljunggren K., Myhre E., Bläckberg J. Clinical and serological variation between patients infected with different Hepatitis B virus genotypes. *J. Clin. Microbiol.* 42, 5837-5841, 2004.
[13] Norder H., Couroucé A.M., Coursaget P., et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47, 289-309, 2004.
[14] Andersson K.L., Chung R.T. Monitoring during and after antiviral therapy for hepatitis B. *Hepatology* 49 (Suppl. 5), S166-S173, 2009.
[15] Chainuvati S., Cheng J., Hou J.L., et al. Patterns of managing chronic hepatitis B treatment-related drug resistance: a survey of physicians in Mainland China, South Korea, Taiwan, and Thailand. *Hepatol. Int.* 3, 453-460, 2009.
[16] Damerow H., Yuen L., Wiegand J., et al. Mutation pattern of lamivudine resistance in relation to hepatitis B genotypes: hepatitis B genotypes differ in their lamivudine resistance associated mutation pattern. *J. Med. Virol.* 82, 1850-1858, 2010.
[17] Chang U.I., Lee Y.C., Wie S.H., et al. Evolution of viral load and changes of polymerase and precore/core promoter sequences in lamivudine-resistant hepatitis B virus during adefovir therapy. *J. Med Virol.* 79, 902-910, 2007.
[18] Rabenau H.F., Kessler H.H., Kortenbusch M., Steinhorst A., Raggam R.B., Berger A. Verification and validation of diagnostic laboratory tests in clinical virology. *J. Clin. Virol.* 40, 93-98, 2007.