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Research Article

Molecular analysis of Hepatitis B virus sub-genotypes and incidence of preS1/preS2 region mutations in HBV-infected Egyptian patients from Mansoura†

Running title: Molecular analysis of Hepatitis B virus in Egypt

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Abstract

Hepatitis B virus (HBV) is one of the major causes of viral hepatitis worldwide. Despite the prevalence of HBV infection in Egypt, few studies have focused on sub-genotyping of the virus. Moreover, no studies are available regarding the mutational analysis of the preS1/preS2 region of the viral genome, or its impact on hepatocellular carcinoma (HCC) development in Egypt. In this study, we have analyzed the sub-genotypes and incidence of mutations in the preS1/preS2 region of HBV present in HBV-infected patients, from Mansoura city (located in the centre of Nile Delta region of Egypt), via partial sequencing of this specific region. Moreover, we have investigated the impact of these mutations on HCC development by measuring serum alpha fetoprotein level and abdominal ultrasound examination of the HBV-infected patients. According to our results, all samples were genotype D in which sub-genotype D1 was predominant. In addition, the results revealed mutations in the preS1/preS2 region, which could result in either immature preS1 protein or completely inhibit the translation of the preS2 protein. However, there was no incidence of HCC development in patients infected with mutated HBV in the preS1/preS2 region. In summary, for the first time our work has proved the predominance of sub-genotype D1 among HBV-infected Egyptian patients in Mansoura city, Nile Delta region, Egypt, and incidence of mutations in the preS1/preS2 region of HBV genome. This current study opens up research opportunities to discuss the impact of HBV mutations on the development of HCC in Egypt. This article is protected by copyright. All rights reserved

Keywords: Egypt; HBV; Mutation; PreS1/PreS2 region; Sub-genotyping

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1. Introduction

Hepatitis B virus (HBV) infection is one of the most dangerous global health problems, that can result in a wide variety of liver diseases, including acute hepatitis, chronic hepatitis, and hepatocellular carcinoma (HCC) [1]. The genome of HBV consists of a relaxed circular, partially double-stranded DNA of approximately 3.2 kbp. It includes four partially overlapping open-reading frames (ORFs): preS/S, preCore/Core, Pol, and X. Multiple in-frame translation start codons are characteristic for the preS/S and preCore/Core regions [1]. The preS/S ORF encodes three different envelope proteins which, according to the size, are termed; large, middle and small. The large, middle and small proteins are encoded by the genes preS1, preS2, and S, respectively [2]. During HBV infection, the preS proteins might have a crucial role in the viral assembly and the attachment to hepatocytes [3-4].

According to the sequence analysis, HBV is divided into ten genotypes; A to J [5]. Some genotypes, such as A, B, C, D and F, have been further classified into several sub-genotypes [6]. Indeed, HBV genotyping and sub-genotyping are crucial tools to understand the course of infection, selection of treatment strategy and the prediction of treatment results [6-7]. Therefore, it is not surprising that several research efforts in the last decade have been directed to discover the different HBV sub-genotyping in several countries [8-11].

During HBV replication, the lack of proofreading ability of the reverse transcriptase leads to an error rate that is 10-fold higher than other DNA virus [12]. This high error rate together with the high rate of viral replication leads to the incidence of high mutations rates [13]. Several studies have investigated the consequences of different HBV mutations, and it was discovered that HCC development is highly associated with mutations in the preS1 and preS2 region [14-16]. Additionally, the preS1 deletion was found to be related to the viral replication increase and disease progression [17]. It is also hypothesized that absence of the middle protein, preS2, may lead to inefficient neutralization of the virus, resulting in more severe HBV infection [18].

In Egypt, HBV genotype D is the most predominant in various regions [19-21]. Although about 2 to 3 million Egyptians are chronically infected with HBV, there are only a few reports focusing on the sub-genotyping of the virus, while there is no information available on the mutational analysis of the preS1/preS2 region [22]. Therefore, we were motivated in this study to determine the sub-genotypes of HBV isolates collected from Mansoura University hospitals located in Mansoura city, Nile delta region, Egypt, and to investigate the probable mutations in the preS1/preS2 region of the viral genome and its impact on HCC development.

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2. Materials and Methods

2.1. Patients and serum samples

Blood samples were collected from 38 HBsAg-positive patients in hospitals of Mansoura University, and arbitrarily numbered Egy1 to Egy38. The patients were from Mansoura city and its surrounding villages. This work was done after the approval of the administrative authorities (Research Ethics Committee) in the Faculty of Pharmacy, Mansoura University, Egypt. The participants' rights, subjects and interests, all were well protected in this research. Informed consent was obtained from each participant before the study. For all the investigated samples, data concerning age, sex and antiviral treatment were collected. In total, the study included 20 females and 18 males; aged 21-54 years old. Abdominal ultrasound examination was performed to all HBV-infected patients to detect any incidence of HCC development. Diabetic patients and HBV patients co-infected with hepatitis C virus (HCV) were excluded from this study.

Blood samples (5 ml) were collected by vein puncture from each patient and left for 20-30 minutes to clot at room temperature. The clotted blood samples were centrifuged at 1500 rpm for 10 minutes to separate the serum from the blood cells. The serum samples were then transported on ice and analyzed for its biochemical parameters before being divided into two aliquots and stored at −80 °C. One aliquot was kept for serological analysis, while the other was used for other types of analysis.

2.2. Liver function analysis and serological markers

Isolated sera were used to analyze liver enzymes (ALT and AST), albumin, and bilirubin using the appropriate detection kits according to the manufacturer's manual (ELITech Clinical Systems, France). Serological assays for HBV markers (HBsAg and anti-HBc [IgG and IgM]) were done using a commercial enzyme immunoassay (MiniVidas, France).

Detection of anti-HBc IgM in serum samples was used for the discrimination between acute and chronic HBV infections [23]. Alpha Fetoprotein (AFP) level, a marker for hepatocellular carcinoma [24], was measured in the serum samples of all chronic HBV-infected patients using ELISA technique according to the protocol of the manufacturer (Calbiotech company, USA) via ELx808™ Absorbance Microplate Reader (Biotek).

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2.3. Extraction, detection and quantification of HBV DNA

DNA was extracted from each serum sample (200 µl) using QIAamp DNA mini blood kit (QIAGEN GmbH, Germany), according to the manufacturer's manual, and eluted with 200 µl elution buffer. The purified viral DNA was detected by direct PCR amplification of 439 bp of the preS region using the primers P1 and P2 (Table 1) utilizing DreamTaq DNA polymerase (Thermo Scientific), as described previously [25]. The plasmid HBV 1.3-mer WT replicon was used as a positive control during the PCR and it was a gift from Wang-Shick Ryu [26] (Addgene plasmid # 65459). Moreover, a no template PCR was used as a negative control. All PCR reactions in this study were performed in MultiGene™ Mini Personal Thermal Cycler (Labnet, USA). The PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The size of the fragments was confirmed using 100 bp plus marker (Thermo Scientific).

Viral load was measured using artus HBV RG PCR kit (QIAGEN GmbH, Germany) according to the instructions of the manufacturer. This quantitative analysis has relied on the direct detection of a specific amplicon (134 bp) of the HBV genome, under the fluorescence channel, cycling green of the Rotor-Gene Q real-time PCR cycler (Qiagen).

2.4. Genotyping, sub-genotyping and preS1/preS2 mutational analysis of HBV samples

HBV genotyping was performed as previously described [27] with slight modifications. Two successive rounds of PCR primers were performed using different primers (Table 1). The first round included use of the universal outer primers P1 and S1-2 (1063 bases). In the second round, three mixtures (A, B, and C) of primers were used to determine the genotype of samples. Mixture A was used for genotypes A, B, and C, mixture B was used for genotypes E, and F, while mixture C was specific for genotype D. Mixture A consisted of a universal B2 primer, in addition to the genotype specific primers (BA1R, BB1R, BC1R). Mixture B included the universal B2R together with BE1 and BF1. Finally, mixture C consisted of the universal B2R primer together with BD1. The expected sizes of fragments for the genotypes A, B, C, D, E, F were 68, 281, 122, 119, 167, and 97, respectively. For the detection of genotype D, we used BD1 alone with B2R, instead of being included in mixture B to clearly observe the difference in the sizes of bands.

Sub-genotyping of genotype D samples was carried out as described previously [28]. The fragment (439 bp) of HBV genome located in the preS region between nucleotides 2823 and 80 was amplified directly using the primers P1 and P2 utilizing the Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the

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manufacturer's manual. The PCR amplified products were purified (Qiagen) and sent to Macrogen Inc (Korea) to be sequenced from both directions using the same primers that were utilized in PCR amplification. Sequencing was performed for all samples Egy1-38.

The results of sequencing were aligned with available sequences of similar genomic region in the GenBank using the CLUSTAL W method. Phylogenetic tree was constructed using the neighbour-joining algorithm of MEGA 7 software, with a 1000 Bootstrap replicates. Additionally, the sequenced samples were investigated for possible mutations in the preS1 and preS2 regions after comparison with a reference sequence of HBV genotype D with the accession number AB104709 [20]. In this comparison, the free service of DNA translation tool in ExPASy website was utilized. Moreover, the sequences that showed incidence of mutations were sequenced one more time for further confirmation.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 program (GraphPad Software, Inc.). The results of biochemical markers were represented as mean±SD.

3. Results:

3.1. Detection and quantification of HBV DNA

HBV DNA was detected in all serum samples by PCR amplification of part of the preS region (439 bp) (Fig. 1A). Concerning quantification of HBV DNA, the viral load values ranged from 150-10^6 copies/ml.

3.2. Genotyping and sub-genotyping of HBV samples

To perform PCR-genotypying, we used primers that are specific for genotype D (Table 1); since it is predicted to be the most prevalent genotype in Egypt [19, 29]. According to our results, all samples were confirmed as genotype D (Fig. 1B). Partial sequences of the preS1/preS2 region revealed great similarity to the sub-genotype D1 sequences originated from Siberia, Sudan, Iran and United States. Additionally, the phylogenetic analysis showed the predominance of the sub-genotype D1 in all samples (Fig. 2). The partial sequence of the preS1/preS2 region of the Egy1-38 samples, obtained in this study, were submitted to

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DDBJ/EMBL/GenBank database and assigned with the accession numbers indicated in brackets as shown in Fig 2.

3.3. Mutational analysis of preS1/preS2 region

As demonstrated in Table 2, analysis of the translated fragments resulting from partial sequencing of the preS1/preS2 region showed a premature stop codon in the preS1 gene of one sample (Egy9). Regarding the preS2 region, deletion mutations in the start codon were demonstrated in four samples (Egy3, Egy4, Egy11, Egy19).

3.4. Biochemical differentiation between mutated and nonmutated HBV samples in the preS1/preS2 region

By analysis of anti-HBc IgM, all the samples revealed chronic infection of HBV except for sample Egy1 was indicated as acute infection. Analysis of liver functions and AFP level in serum samples of all HBV-infected patients was performed (Table 3). Sequenced samples of chronic HBV-infected patients were further divided into two groups according to the incidence of mutation. The sample of acute HBV-infected patient (Egy1) was excluded from calculation of the mean of the measured parameters. Previously, it was shown that there is a correlation between mutations in the preS1/preS2 regions and the incidence of HCC [14-16]. Therefore, we analyzed the level of the HCC tumor marker (AFP) and performed abdominal ultrasound examination for HBV-infected patients. According to our results, there was no incidence of abnormal liver functions in all chronic HBV-infected patients. Moreover, the AFP level in the mutated HBV samples was lower than 400 ng/ml and therefore, there was no incidence of HCC development that was confirmed by the ultrasound examination.

4. Discussion

HBV is one of the major threats of public health worldwide [1]. In Egypt, few studies are concerned with sub-genotyping of the virus, and no studies are available for the mutational analysis of the preS1/preS2 region of the viral genome. Our study was conducted on HBV samples collected from Mansoura University hospitals located in Mansoura city, Egypt. Mansoura is located in the centre of Nile Delta region of Egypt and is considered as one of the most prevalent cities of viral hepatitis in Egypt [29]. Indeed, there are previous studies that focused only on analysis of biochemical and serological markers in HBV-infected patients in Mansoura

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rather than genotyping or mutational analysis of the HBV genome itself. These studies included a survey of HBV sole infection [30] or HBV co-infection with HCV [31].

Regarding genotyping and sub-genotyping, our results showed the predominance of genotype D in Mansoura city, Egypt. Globally, genotype D is predominating in the Mediterranean region, the Middle East, and South Asia [28]. Our data of partial sequencing of the preS1/preS2 region were consistent with the PCR-genotyping, as well as the investigated sequences with the specific 33 nucleotides deletions in the preS1 region characteristic for HBV genotype D (the whole genome of HBV genotype D is 3182 bp) [32]. These results were in agreement with previous studies performed in the north eastern of Egypt [19], North Egypt [20], South Egypt [21] and Cairo [22]. However, neither genotype B nor mixed infections (A/D) were detected in any of our cases as was demonstrated previously in Cairo [33]. We regarded that since Cairo is the capital of Egypt, and therefore it is targeted by foreigners (e.g. tourists, international students, etc.), it is expected that the source of the odd cases of non-D HBV genotypes in the study of "Zekri *et. al.*, 2007" [33] is transferred from foreigners to Egyptians.

To our knowledge, no investigation was performed for the sub-genotyping of HBV genotype D in Mansoura. Moreover, only few studies emphasized the sub-genotyping of HBV genotype D in Egypt. Such studies demonstrated that only the sub-genotype D1 was predominating either in north eastern of Egypt [19] or in South Egypt [21]. These findings were in alignment with our results that demonstrated for the first time the prevalence of sub-genotype D1 in Mansoura city, Nile Delta region, Egypt as indicated by phylogenetic analysis.

Regarding the analysis of preS1/preS2 mutations, most of the previous studies focused on its detection in genotypes A, B, and C [34-38]. On the other hand, a few studies have investigated the probable mutations of preS1/preS2 region in genotype D [39]. Moreover, no previous data were available on the mutational analysis of the preS1/preS2 region of HBV genome in Egypt. Therefore, we were interested in studying mutations in this region; particularly it carries one of the most variable sequences of the HBV genome [18]. Both preS1 and preS2 mutations were found to play a role in HCC development; mainly by induction of endoplasmic reticulum stress pathway [14-16]. After investigating the sequence of the 38 samples, we detected mutations that have led to immature preS1 protein (2.6 %), and complete deletion of the preS2 protein (10.5 %). PreS1 mutations mainly include in-frame deletion of nucleotides that led to shortened but expressed preS1 protein [39-40]. Such mutations were found principally in genotypes B and C [39] and to lower extent in genotype D [40]. In our work, we detected a unique substitution mutation to the stop codon (Egy9 isolate) that leads to premature termination of the expression of the preS1 protein (Table 3). To our knowledge, we did not find substitution mutations that

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lead to early termination of the preS1 protein in previous studies. Regarding preS2 mutations, we detected substitution mutations in the initiation codon (4 isolates) that abolishes the translation of the protein (Table 3). Previous studies demonstrated point mutations in the start codon of the preS2 protein, in addition to in-frame deletion mutations [39, 41-42]. Both in-frame deletion mutations in the preS1/preS2 proteins, in addition to the point mutation of the preS2 protein start codon were significantly associated with HCC incidence [39]. However in our study, none of the detected mutations were associated with HCC development. HCC is initially detected in patients with AFP level > 400ng/ml [43-44]. Therefore, there was no incidence of HCC in mutated HBV-infected patients (Table 3) that was confirmed by the negative abdominal ultrasound detection of HCC. However, HCC can be developed in those patients subsequently, so further studies should be performed to follow up the AFP level, liver functions at different time intervals, and advanced imaging techniques, for an early detection of HCC in these patients [45-46]. Also, it should be mentioned that nearly all HCC patients detected in the clinics of Egypt are infected with HCV. Therefore, it is not easy to find HCC patients among chronic HBV infected patients who would help to prove the correlation between HBV mutation and HCC development in HBV-infected patients in Egypt.

Finally, it should be mentioned it was demonstrated previously that the point mutation W4P in the preS1 gene was restricted only in male patients [47]. Although in our work, mutations in either preS1 or preS2 were not limited to sex as indicated in the mutated samples Egy3, Egy4, Egy9, Egy11, and Egy19 (Table 3).

5. Conclusions:

Taken together, our data have proved the predominance of HBV genotype D and sub-genotype D1 among HBV-infected patients in Mansoura city, Nile Delta region, Egypt. Additionally, we detected for the first time mutations in the preS1/preS2 region, but with no incidence of HCC development. We recommend sequencing of the preS1/preS2 region in early diagnosed Egyptian HBV-infected patients. Afterwards, the level of AFP should be followed periodically over a long period of time, particularly in HBV cases mutated in the preS1/preS2 region. Such follow up may support the early detection of HCC incidence. We are looking forward that this work will represent a novel starting point for further research that correlates HCC incidence and HBV genome mutations.

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Conflicts of interests:

The authors declare that they have no competing interests.

Ethical approval:

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institution. This work was done after the approval of the administrative authorities (Research Ethics Committee) in the Faculty of Pharmacy, Mansoura University, Egypt. The participants' rights, subjects and interests, all were well protected in this research. Informed consent was obtained from each participant before the study.

Competing interests:

None

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Table headings

Table 1 Primers used in this study.

Table 2 Types and distribution of mutations in the preS1/preS2 region observed in this study.

Table 3 Analysis of liver functions and AFP levels in the HBV-infected patients.

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Figure legends

Fig. 1. PCR Detection (A) and genotyping (B) of HBV DNA of the samples. (A) Detection of HBV DNA was carried out by PCR amplification of part of the preS region (439 bp). (B) Genotyping of HBV DNA was accomplished by detection of the characteristic band for genotype D at 119 bp. Lane 1: 100 bp plus marker; lanes 2: No template PCR was used as a negative control; lane 3: The standard plasmid (HBV 1.3-mer WT replicon) was used as a positive control; lanes 4-13: Representative 10 samples of the total of 38 samples.

Fig. 2. A rooted phylogenetic tree based on 81 sequences of 431 nucleotides within the PreS1/PreS2 region. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.55415684 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 81 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 332 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Reference sequences are labeled by Genotype_GenBank accession number_Country of origin. Egyptian clinical samples were indicated by either black squares (nonmutated samples) or black triangles (mutated samples).

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Table 1 Primers used in this study.

Primer	Sequence (position, specificity, and polarity)	Reference
P1	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (2823–2845, universal, sense)	[25]
P2	5'-TTCCTGAACTGGAGCCACCA -3' (61-80, universal, antisense)	[25]
S1-2	5'-CGAACCACTGAACAAATGGC-3' (685–704, universal, antisense)	[27]
Mix A		
B2	5'-GGCTCMAGTTCMGGAACAGT-3' (67–86, genotypes A to E specific, sense)	[27]
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (113–134, genotype A specific, antisense)	[27]
BB1R	5'-CAGGTTGGTGAGTGACTGGAGA-3' (324–345, genotype B specific, antisense)	[27]
BC1R	5'-GGTCCTAGGAATCCTGATGTTG-3' (165–186, genotype C specific, antisense)	[27]
Mix B		
BE1	5'-CACCAGAAATCCAGATTGGGACCA-3' (2955–2978, genotype E specific, sense)	[27]
BF1	5'-GYTACGGTCCAGGGTTACCA-3' (3032–3051, genotype F specific, sense)	[27]
B2R	5'-GGAGGCGGATYTGCTGGCAA-3' (3078–3097, genotypes D to F specific, antisense)	[27]
Mix C		
BD1	5'-GCCAACAAGGTAGGAGCT-3' (2979–2996, genotype D specific, sense)	[27]
B2R	5'-GGAGGCGGATYTGCTGGCAA-3' (3078–3097, genotypes D to F specific, antisense)	[27]
An "M" stands for A or C nucleotide, while a "Y" stands for C or T nucleotide.		

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Table 2 Types and distribution of mutations in the preS1/preS2 region observed in this study.

Mutation	Type and site of mutation	Mutated HBV sample	Accession number
Premature stop codon in the preS1 gene (2850-3182, 1-837)	Substitution mutation T3079A.	Egy9	LC131046
Mutation in the start codon of the preS2 gene (3174-3182, 1-837)	Substitution mutation A3174G, leading to replacement of methionine (ATG), the initiation codon of preS2 protein, with valine (GTG).	Egy3	LC131038
		Egy4	LC131039
		Egy11	LC131049
		Egy19	LC131058
Numbering of nucleotides in the genes preS1, preS2, and those indicating the sites of mutations are according to the Egyptian isolate (genotype D) with accession number AB104709 [20].			

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Table 3 Analysis of liver functions and AFP levels in the HBV-infected patients. Normal values are indicated below the heading of each parameter in the table

Patient number	Sex	Age (years)	Albumin 3.5-5.2 (g/dl)	Total Bilirubin 0.3-1.2 (mg/dl)	ALT 7-40 (U/l)	AST 7-40 (U/l)	AFP 0.5-5 (ng/ml)
Egy1[ǂ]	Male	45	1.9	27.9	231	89	2.1
Egy2	Male	23	3.9	0.7	13	14	4.0
Egy3*	Female	30	3.7	0.9	70	43	4.9
Egy4*	Female	45	4.3	0.8	22	26	5.8
Egy5	Male	37	4.2	0.9	19	16	3.6
Egy6	Male	21	3.3	2.5	51	42	3.2
Egy7	Male	23	2.5	1.0	48	66	5.2
Egy8	Female	54	4.2	0.7	18	27	4.1
Egy9*	Female	52	3.9	0.8	42	45	20.8
Egy10	Female	29	4.8	0.6	36	33	3.3
Egy11*	Male	23	3.9	0.9	42	41	16.2
Egy12	Female	45	3.6	0.7	39	45	3.7
Egy13	Female	46	4.0	0.9	40	35	4.0
Egy14	Male	22	3.5	0.6	38	40	5.2
Egy15	Female	54	3.3	0.8	40	38	2.3
Egy16	Female	46	3.7	0.6	38	40	4.2
Egy17	Male	30	4.2	0.7	32	41	3.3
Egy18	Male	27	3.9	0.8	35	45	3.3
Egy19*	Male	44	4.5	0.85	39	35	5.2
Egy20	Female	32	3.6	1.83	38	41	3.2
Egy21	Female	39	3.9	0.89	37	43	4.5
Egy22	Male	28	2.9	0.9	38	41	4.0
Egy23	Female	21	3.2	0.87	36	39	4.5
Egy24	Male	32	3.2	0.95	39	38	3.7
Egy25	Male	39	3.3	1.02	40	43	3.2
Egy26	Female	47	3.2	0.98	38	41	3.7
Egy27	Female	50	2.8	0.86	39	39	3.2
Egy28	Female	53	2.9	0.7	38	40	3.7
Egy29	Male	42	2.9	0.8	37	41	3.1
Egy30	Male	37	3.2	0.96	39	42	3.5
Egy31	Female	22	3.3	0.8	37	42	2.9
Egy32	Male	28	3.05	0.9	38	41	2.7
Egy33	Female	32	2.98	0.85	37	43	2.9
Egy34	Female	52	3.1	0.87	39	39	2.6
Egy35	Male	46	2.95	0.95	37	38	2.1
Egy36	Female	34	3.1	0.76	38	41	2.2
Egy37	Female	29	3.4	0.85	35	38	2.1
Egy38	Male	31	2.9	0.75	55	51	2.7
			mean±SD	**mean±SD**	**mean±SD**	**mean±SD**	**mean±SD**
Chronic patients (n=37)			3.49±0.54	0.89±0.34	37.76±9.63	39.27±8.57	4.4±3.58
Nonmutated samples (n=32)			3.4±0.52	0.91±0.36	36.94±8.02	39.47±8.79	3.43±0.8
Mutated samples* (n=5)			4.06±0.33	0.85±0.05	43±17.23	38±7.68	10.58±7.42

[ǂ] The acute sample Egy1 was excluded from calculation of the mean of the measured parameters.

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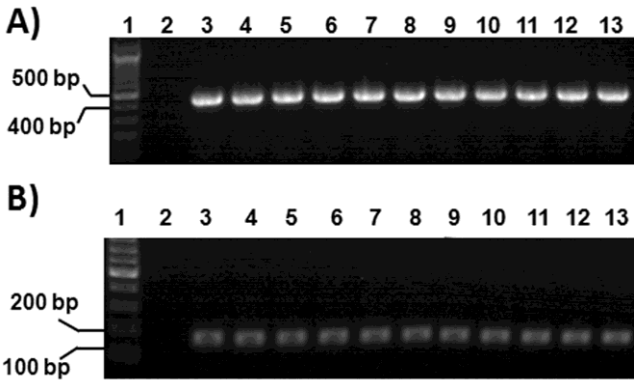

Fig 1

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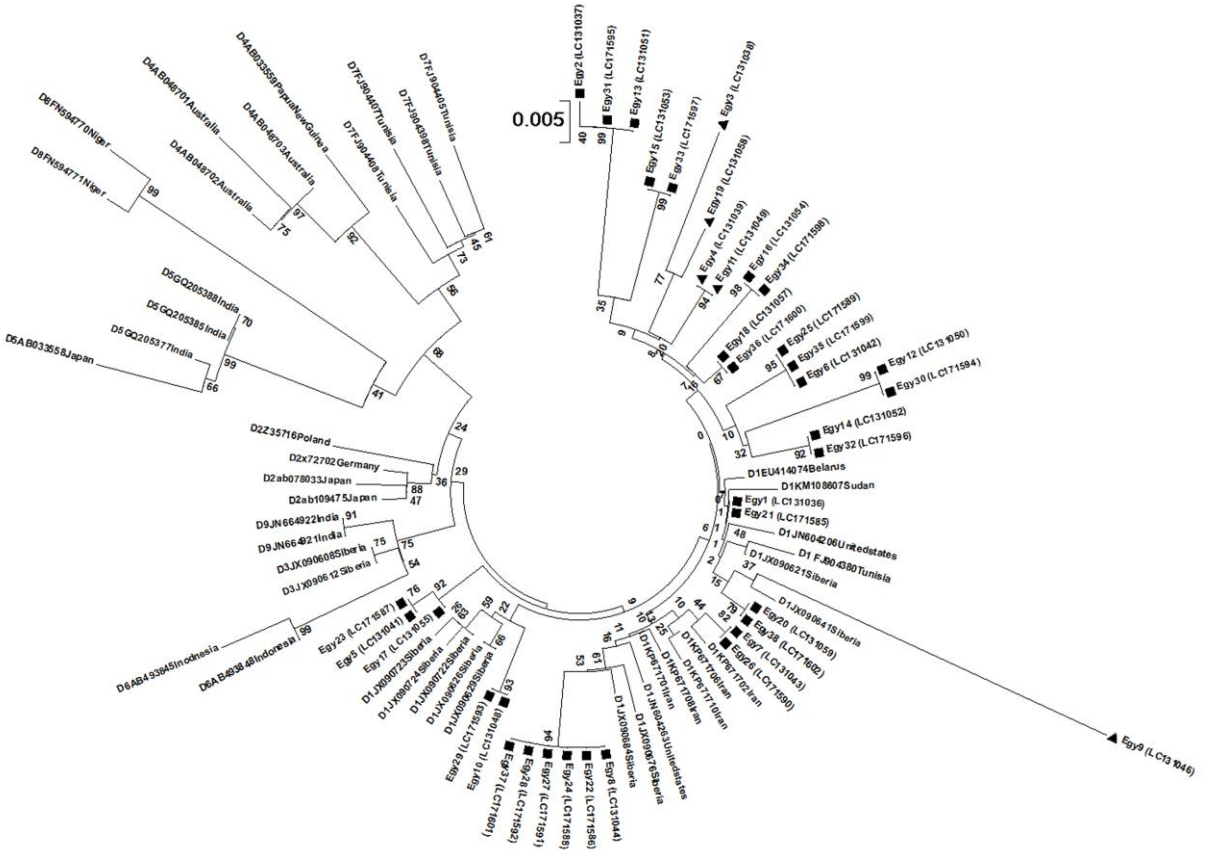

Fig 2

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