UTTAR PRADESH JOURNAL OF ZOOLOGY

42(17): 62-73, 2021
ISSN: 0256-971X (P)

A SNP STUDY OF HSP GENE AND ITS PROTEIN MODELLING IN LIVER DISEASE CASES FROM NORTH EAST INDIA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Juan Carlos Troiano, Professor, University of Buenos Aires, Argentina.
Reviewers:
(1) Hakim Manzer Alam, Rajasthan University of Veterinary & Animal Sciences, India.
(2) Moses Darpolor, Stillman College, USA.

Received: 05 June 2021
Accepted: 10 August 2021
Published: 24 August 2021

Original Research Article

ABSTRACT

HSP gene polymorphism has been widely studied across the globe and particularly with reference to various liver diseases and HCC. Data pertaining to HSP gene polymorphism is lacking from NE India region and there are lacunae of information at the proteomic and bioinformatics level. NE region of India is known for its high incidence of cancer cases. The current study was designed to study the polymorphism of HSP genes in different liver disease cases from Guwahati, India and to predict 3D structure of the proteins from the studied genes by using different bioinformatics tools as well as calculating different physio-chemical information of those studied proteins. The DNA extraction was done followed by PCR amplification and RFLP. EMBOSS Transeq tool and I- TASSER SERVER were used to model the proteins of interest. The results showed that HSPA1B and HSPA1L polymorphisms are significantly associated with advanced stages of liver diseases. Stable protein 2D and 3D models were successfully proposed in this study. The current study highlights the importance of studying cancer critical gene and the need of bioinformatics softwares to generate data.

Keywords: HSP gene; SNP; HCC; PCR-RFLP; protein modeling; I- TASSER SERVER.

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1. INTRODUCTION

HSPs (heat shock proteins) are a wide group of polypeptide, which are produced at normal levels under usual conditions, but sometimes produced more with regards to cellular stresses, including heat shock, genotoxic agents, nutrient starvation and over expression of oncoproteins etc. [1-4]. Stimulation of heat shock proteins is a serious part of heat shock responses that could be helpful for cell survival with reference to different stress condition [5]. Numerous members of HSPs carry out their activity as molecular chaperons where they stabilize proteins to confirm their correct folding, inhibiting stress-induced protein accumulation or modulating cellular signaling and transcriptional networks [1,6]. The highly expressive HSPs under stress condition are often transcriptionally modulated by a heat shock factor-I or HSF1. With regards to stress conditions, HSF1 is phosphorylated and create homotrimers, then binds to heat shock elements (HSEs) present upstream of HSPs genes and stimulate the transcription of heat shock genes [6-10].

It is seen that the HSPs admit cells to sustain a variety of both exogenous and endogenous factors like heavy metals, oxidants, cytotoxic agents, and other infection etc. [11-13]. HSPs are widely studied in its relation with carcinogenesis. Heat shock protein or HSPs members like Hsp70 and Hsp90 which are known for their immune-modulatory functions, play an important role during cell necrosis to induce any immune response [13,14]. HSPs are mainly known for their excessive association to differentiation, tumor cell expansion and apoptosis [15]. Generally Hsp90 is known for fixing different mutants, some inactive forms of tumor suppressor gene, and some DNA repair proteins like p53 etc. Hsp70 is also known to suppress tumor suppressor proteins like p53 etc. It is also having anti-apoptotic effect and influence on many lysosomal enzymes. There is a great correlation between HSPs and different types of cancer and much clinical evidence has supported it. Study has supported that there is overexpression of Hsp90 in different cancer types like leukemia, lung cancer, breast tumors, and Hodgkin's and non-Hodgkin's B-cell lymphoma. The Hsp70 also show very high progression in gastric cancer, endometrial cancer, osteosarcoma, renal tumors, breast cancer, and leukemia [16-23]. HSP has been studied across the globe and also in India. However, data related to HSP and live diseases from NE India is scanty.

In the area of computational biology, the protein homology modeling or comparative modelling has become a very important and useful tool which is a templated based modelling process. They are mainly employed to predict protein 3D structure when only sequence data for a protein is available which mainly search for the template sequence in the database based on the similarity between them. By predicting the protein 3D structure, the functions of the protein may also be determined. Overall this computational protein modelling is helpful to researcher over experimental methods like X-ray crystallography and NMR spectroscopy by means of its time consuming and its success rate to all the proteins [24-26]. There are many tools for protein homology modeling of which modeller, SWISS- MODEL, Geno3D, I-TASSER are widely used. All the tools use the same strategy for modeling the structure which is categorized into 4 main steps: identification of target protein, alignment of the sequence, building the model and validating the models.

With the above background the current study was designed to further investigate probable association of HSPA1B and HSPA1L gene a risk factor for development of HCC in liver disease from NE India. Different insilico methods were also used to generate 3D model of polymorphic HSP protein as well as calculating other associated parameters of those proteins which may be helpful for further experimental modelling. The hypothesis for the current study is that the HSPA1B and HSPA1L gene polymorphism is significantly associated as a risk factor in HCC cases from NE India population also and there might be a possible importance of HSP protein modelling using bioinformatics tools that can add to the existing knowledge.

2. MATERIALS AND METHODS

2.1 Collection of Samples and Diagnosis

A total of 200 cases were enrolled from the North east cancer hospital and down town Hospital, and the study was carried out in the CIF and Departmental laboratory of Biotechnology, Assam down town University, Assam, India. Out of all the cases, 100 were healthy individuals without any underlying liver diseases (mostly volunteers) and rest were liver disease cases of different categories enrolled during January 2016 – November 2018. Patients were assessed based on history, LFT, clinical tests, HBV and HCV serology test, blood sugar level, lipid profile and imaging of liver. For the complicated cases PET scan was used. Diagnosis was mainly based on histopathology and cytology findings. HCC cases were confirmed as per 2001 EASL guidelines.

2.2 Extraction, Amplification and Detection of DNA

The DNA was isolated from whole blood which was collected in EDTA vial for all cases. Isolation was

performed using phenol chloroform method and commercial kit method (Wizard Genomic DNA extraction kit, Promega) as well. Gel electrophoresis was performed for the detection of DNA and was observed under E-gel imager (Life Technologies). PCR was performed and the gene was amplified using specific primers under standardized protocol. After amplification they were analyzed on 2% agarose gel.

2.3 RFLP of Amplified DNA

RFLP was performed using NcoI and PstI restriction enzymes in the PCR amplicons. After digestion they were observed in 2% PAGE under E-gel imager (Life Technologies).

2.4 Sequencing of DNA

Representative amplicons were then sent for commercial sequencing. The sequences were analyzed by using Geneious prime software. Also many sequences of HSP gene were collected from databases.

2.5 Homology Modeling of Protein and 3d Modelling of Protein

NCBI BLAST was performed for the nucleotide sequence to check their similarity in the database. Sequences having more than 60% similarity were later used for protein modelling. For the modeling step, the nucleotide sequences found after DNA sequencing were translated using EMBOSS Transeq tool [27,28] and based on the presence of different open reading frames best protein sequence was selected and the proteins were modeled by using I-TASSER SERVER [29,30,31]. The I- TASSER SERVER predicts protein 3d structure and their function based on homology modeling approach which mainly identify protein's structural templates from the protein data bank (PDB) and in the next step it predicts different protein 3D structure. Different protein parameters like molecular weight, extinction coefficient, theoretical isoelectric point (pI), aliphatic index, instability index, and grand average of hydropathicity values (GRAVY) were also calculated for all the proteins using SIB's ProtParam tool (https://web.expasy.org/protparam/) [32].

2.6 Statistical Analysis

Odds ratio (OR) was used to evaluate the degree of association and CI (confidence interval) was calculated for the OR by using Woolf's method. Chi–square test or Fisher's exact test was performed for the comparison of Allele frequencies and different genotypes. The significance level was considered at *P* less than 0.05 where according to the specificities and comparison *P* values were corrected. Epi info was used to calculate the OR and p values.

3. RESULTS AND DISCUSSION

HBV and HCV are well established etiological risk factor for HCC and current study also acknowledges that in accordance with numerous previous studies [33]. However a strong association was missing may be due to the smaller number of HCC cases enrolled in the study. Age above 35 years was found to be a risk factor with an increased risk of almost 2 fold {2.235[0.8276-6.117]} [34] while smoking and consumption of alcohol were also found to be risk factor for development of HCC in the current study with an odds ratio of {2.235[0.8276-6.117]} and {1.926[0.5852-6.339]} respectively [35]. An interesting finding was the involvement of consumption of processed meat which increased the risk by almost 10 fold {10.14 [5.317-19.93]; p <.001} [36]. Also family history with liver disease was also found to enhance the end stage disease by roughly 3 times {**2.688**[0.852-8.9479]} [Table 1].

The results of PCR RFLP at brief can be understood from the RFLP results which show the HSPA1B gene as the product is of 1117 bp (allele A). The restriction enzyme PstI generates products of 936 and 181 (allele B). The RFLP results show the HSPA1L gene as the product is of 878 bp (allele B). The restriction enzyme NcoI generates products of 551 bp and 327bp (allele A) [Diagrams not shown in the text].

Prevalence of AA genotype of HSPA1L gene in majority of the cases both in cases and control was observed compared to AB and BB genotype. When OR and p values were calculated for the allelic distribution, it was found that AA genotype is associated with almost 8-fold increase risk in HCC cases compared to the chronic hepatitis cases as a whole with a significant p value (>.005). Similarly, when HCC group were compared to CHB and CHC cases the risk was also found to be enhanced by 5.5 and 10 fold respectively [Table 2]. For HSPA1B gene, increased risk was observed when HCC was compared against CHC cases (5 fold) [Table 3]. Our study results in this line are in accordance to previous published studies [37, 38]. Understanding HSP70 polymorphism is important as there are many studies across the globe that establishes its association as a risk factor with hepatocellular carcinoma [39]. Also the positive association of HSP has been well documented in other disease like diabetic foot ulcer [40], Obesity, type II diabetes, and metabolic syndrome like hypertension [41] etc.

Table 1. Demographic profile and the risk factors

Characteristics	HCC (20)	CHRONIC HEPATITIS (80)	Controls (100)	OR	*P* value
Etiology					
HBV	10(50%)	40(50%)	-	**1.175** [0.7782-1.776]	0.44
HCV	10(50%)	40(50%)	-		
Sex					
Male	17(85%)	58(72.5%)	52(52%)	1.0 [0.3753-2.664]	>0.99
Female	03(15%)	22(27.5%)	48(48%)	0.4652[0.1241-1.745]	0.2490
Age (years)					
≤35	08(40%)	49(61.25)	80(80%)	0.4218[0.155-1.148]	0.08
≥35	12(60%)	31(38.75%)	20(20%)	**2.371**[0.8711-6.453]	0.08
Smoking habit					
Smoker	08(40%)	48(60%)	49(49%)	-	-
Non smoker	12(60%)	32(40%)	51(51%)	**2.235**[0.8276-6.117]	0.10
Alcohol					
Yes	16(80%)	54(67.5%)	55(55%)	-	--
No	04(20%)	26(32.5%)	45(45%)	**1.926**[0.5852-6.339]	0.2764
Consumption of processed meat					
Yes	16(80%)	22(27.5%)	25(25%)	**10.14** [5.317-19.93]	**<.001**
No	04(20%)	58(72.5%)	75(75%)		
Education					
Lower/illiterate	01(5%)	03(4.12)	02(2%)	**1.351**[0.133-13.72]	0.79
Middle	12(60%)	36(45%)	47(47%)	-	-
High	07(35%)	41(51.25%)	51(51%)	0.5122[0.1851-1.418]	0.193
Family history of disease					
Yes	06(30%)	11(13.75%)	07(7%)	**2.688**[0.852-8.9479]	0.08
No	14(70%)	69(86.25%)	93(93%)	-	-

+ Digits in bold indicates significant values

Table 2. Showing allelic distribution of HSPA1L gene

Groups	N	Genotype distribution (%)			Chi square test(2^0 of freedom)	*P* value	Allele frequencies (%)		OR (95% CI)	*P* value
		AA	AB	BB			A	B		
A.										
I. Control	100	71(71%)	22(22%)	07(7%)	20.41	<**0.001***	164(82%)	36(18%)	0.1174[.0055-0.644]	0.006
II.HCC	20	19(95%)	01(5%)	0(0)	14.56	**<0.001**$^{+}$	39(97.5%)	1(2.5%)	7.869[1.406-167.8]	.0095
III.CH	80	61(76.25%)	11(13.75%)	08(10%)	0.6418	<0.42^{++}	133(83.13%)	27(16.87%)	0.925[0.53-1.603]	0.39
B.										
I. Control	100	71(71%)	22(22%)	07(7%)	20.41	**<0.001***	164(82%)	36(18%)	0.1174[.0055-0.644]	0.006
II.HCC	20	19(95%)	01(5%)	0(0)	10.29	**<0.0013**$^{+}$	39(97.5%)	1(2.5%)	5.515[.8761-124.9]	.0367
III.CHB	40	32(80%)	6(15%)	02(5%)	2.189	<0.13^{++}	70(87.5%)	10(12.5%)	0.6517[0.293-1.36]	0.1314
C.										
I. Control	100	71(71%)	22(22%)	07(7%)	20.41	**<0.001***	164(82%)	36(18%)	0.1174[.0055-0.644]	0.006
II.HCC	20	19(95%)	01(5%)	0(0)	18.01	**<0.0013**$^{+}$	39(97.5%)	1(2.5%)	10.39[1.77-227.4]	
III.CHC	40	29(72.5%)	5(12.5%)	06(15%)	0.099	<0.75^{++}	63(78.75%)	17(21.25%)	1.228[.6321-2.33]	

**: Group I vs Group II; +: Group II vs III; ++: Group I vs Group III*

Distribution of HSPA1L gene

A. Control, hepatocellular carcinoma(HCC), Chronic hepatitis(CH)

B. Control, hepatocellular carcinoma(HCC), Chronic hepatitis B(CHB)

C. Control, hepatocellular carcinoma(HCC), Chronic hepatitis C(CHC)

Table 3. Showing allelic distribution of HSPA1B gene

Groups	N	Genotype distribution (%)			Chi square test(2^0 of freedom)	*P* value	Allele frequencies (%)		OR (95% CI)	*P* value
		AA	AB	BB			A	B		
A.										
I. Control	100	81(81%)	16(16%)	03(3%)	6.494	0.0108*	178 (89%)	22 (11%)	2.016[0.7832-4.86]	0.058
II.HCC	20	13(65%)	06(30%)	1(5%)	0.533	<0.46+	32 (80%)	8 (20%)	2.146[0.9484-5.274]	0.034
III.CH	80	48(60%)	08(10%)	24(30%)	10.6	<0.0011++	104 (65%)	56 (35%)	**4.358**[2.523-7.631]	**<0.001**
B.										
I. Control	100	81(81%)	16(16%)	03(3%)	6.494	0.0108*	178 (89%)	22(11%)	**2.016**[0.7832-4.86]	0.058
II.HCC	20	13(65%)	06(30%)	1(5%)	8.42	<0.003+	32(80%)	8(20%)	0.6402 [0.232- 1.817]	0.1883
III.CHB	40	33(82.5%)	03(7.5%)	04(10%)	0.1355	<0.71++	69(86.25%)	11(13.75%)	**1.289**[0.5739-2.78]	0.2596
C.										
I. Control	100	81(81%)	16(16%)	03(3%)	6.494	0.0108*	178(89%)	22(11%)	**2.016[0.7832-4.86]**	0.058
II.HCC	20	13(65%)	06(30%)	1(5%)	14.59	<**0.0001**+	32(80%)	8(20%)	**5.072[2.123-13.08]**	**<0.001**
III.CHC	40	15(37.5%)	05(12.5%)	20(50%)	38.36	**<0.001**++	35(43.75%)	45(56.25%)	**10.28[**5.541-19.53]	**<0.001**

**: Group I vs Group II; +: Group II vs III; ++: Group I vs Group III*

Distribution of HSPA1B gene

A. Control, hepatocellular carcinoma(HCC), Chronic hepatitis(CH)

B. Control, hepatocellular carcinoma(HCC), Chronic hepatitis B(CHB)

C. Control, hepatocellular carcinoma(HCC), Chronic hepatitis C(CHC)

Table 4. Showing different ligand binding sites for the proteins

Sl no	Protein name	C - score	Potential ligand binding site	Inference
1	HSP1F	-4.96	L124 and L127.	
2	HSP1R	-3.69	H121 and Q137	
3	HSP3F	-4.33	H22,D56,Y86,I87,H88	
4	HSP3R	-4.35	D61 and L58	
5	HSP5F	-4.67	L73 and F72	
6	HSP5R	-2.66	R39,T40,I43,N44,A47,F75,L76,C77,L79 and S80	
7	HSP6F	-4.61	I15,C37,L39,F51,S53,C65,C66,I125,M127 and F143	
8	HSP6R	-4.59	N146 and E143.	C score is in [-5,2] and C
9	HSP8F	-3.98	A16, L17, S20, and V85	score > -1.5
10	HSP8R	-4.11	A10 and D13	
11	HSP10F	-2.61	L134 and L141	
12	HSP10R	-3.45	I20, C111, P112, H113, L138, F162, I163 and P164.	
13	HSP11F	-3.84	L32, L34, S35, R36, I39, A66, G70, I76, L77, Q82, P108, N111, N115, G118,F119, L153, A157 and N160	
14	HSP11R	-4.38	K102 and E105	
15	HSP12F	-2.73	R87, E90, R91 and H110	
16	HSP12R	-4.18	P62, L65, H69 and R70	

Table 5. Different physiochemical property calculation by Protparam

Sl. no	Protein name	No of amino acids	Formula	Molecular weight	Theoretical pI	Extinction coefficients	Estimated half-life(hour)	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
1	HSP1F	186	$C_{881}H_{1379}N_{239}O_{268}S_{13}$	20023.86	7.46	27095	7.2	62.21	83.82	0.066
2	HSP1R	188	$C_{998}H_{1519}N_{261}O_{268}S_{7}$	21686.05	7.91	38055	5.5	49.05	82.39	-0.415
3	HSP3F	174	$C_{1069}H_{1556}N_{222}O_{242}S_{4}$	21517.70	9.32	73705	1.9	57.72	138.33	0.533
4	HSP3R	240	$C_{1483}H_{2118}N_{288}O_{344}S_{4}$	29613.09	8.85	102935	1.9	40.01	141.42	0.684
5	HSP5F	187	$C_{930}H_{1418}N_{236}O_{257}S_{8}$	20273.40	8.50	32220	20	45.73	91.23	0.224
6	HSP5R	185	$C_{889}H_{1402}N_{254}O_{280}S_{12}$	20513.16	6.06	19075	1.1	56.06	81.14	-0.352
7	HSP6F	182	$C_{873}H_{1299}N_{225}O_{272}S_{12}$	19682.98	4.38	38095	1	58.52	74.95	0.039
8	HSP6R	182	$C_{896}H_{1461}N_{261}O_{257}S_{8}$	20258.53	10.08	21095	4.4	59.59	89.95	-0.362
9	HSP8F	144	$C_{693}H_{1084}N_{198}O_{201}S_{2}$	15469.56	10.28	27500	1.4	68.04	55.76	-0.678
10	HSP8R	98	$C_{477}H_{725}N_{135}O_{150}S_{2}$	10814.94	5.17	23615	20	43.89	58.88	-0.773
11	HSP10F	186	$C_{889}H_{1375}N_{237}O_{251}S_{9}$	19687.67	8.28	30855	4.4	52.88	91.40	0.284
12	HSP10R	184	$C_{1009}H_{1510}N_{270}O_{259}S_{12}$	21951.46	9.66	49765	1.1	52.38	68.37	-0.315
13	HSP11F	185	$C_{953}H_{1471}N_{245}O_{246}S_{14}$	20745.50	8.64	28460	4.4	37.84	99.68	0.301
14	HSP11R	159	$C_{811}H_{1320}N_{230}O_{205}S_{8}$	17829.30	9.85	10345	1.4	44.21	117.04	0.178
15	HSP12F	212	$C_{1092}H_{1682}N_{308}O_{301}S_{13}$	24358.03	8.98	30660	1	67.07	85.99	-0.080
16	HSP12R	185	$C_{953}H_{1500}N_{274}O_{265}S_{6}$	21228.43	9.36	12615	20	43.53	83.73	-0.489

Fig. 1. 3D structure of HSP1F

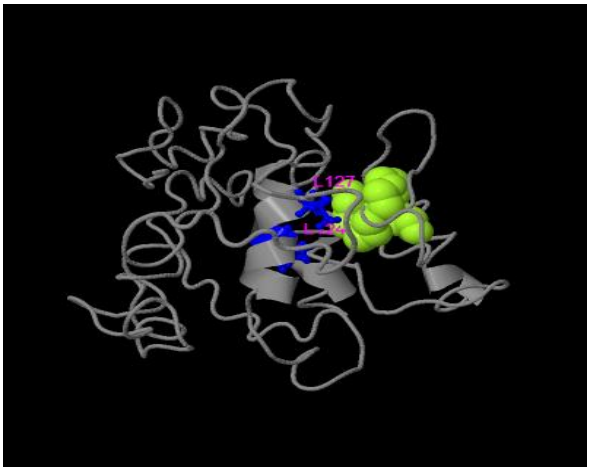

Fig. 2. Showing potential ligand binding sites for HSP1F

Proteins 3D structures were successfully modelled for the entire polymorphic HSP gene in the cases. A total of 16 proteins were modelled and their functions were calculated. Potential ligand binding sites were identified which are summarized in Table 4. Homology modeling of protein can be used recognize minute differences between related proteins whose structure are not solved and thus generating information about protein function and indicating further experimental work for the same [42]. Though they show lots of error in many times still they can be used to model proteins which are not structurally available. The accuracy of the models is always highly depending on the selection of template molecule. In our study the models have shown less RMSD value indicating a good quality 3D model. But the same structure needs to be solved by X-ray crystallography or NMR study.

C score or confidence score was also calculated for all the proteins to see the quality of the predicted model and it was within the permissible limit defined by the I- TASSER server (Table 4) [43]. Potential ligand binding sites were also identified which may be useful for targeting different drug molecule in future.

The physio-chemical property calculation for a protein is very much important which can tell about different properties of protein. In our study the aliphatic index values were high for most of the proteins which conclude the proteins were thermo stable over high temperature. The Grand average of hydropathicity (GRAVY) values calculated were between -2 to +2 which were in its limit (Table 5). Different protein parameters were also calculated and they are described in the Table 5.

4. CONCLUSION

This study reveals the prevalence of AA genotype over AB and BB genotype in both cases and control population of NE region which has got more importance for studying different cancer critical genes. Modeling of the protein will generate some baseline data which may helpful for further research work.

For every protein sequence, I- TASSER server predicted 5 different structures and out of all them the good model was selected based on the C-score, TM-score and RMSD value. For all the modeled protein, C –score was calculated at permissible limit whereas TM –score was found less than 0.5 which indicated the random similarity in the predicted structures as describe in a previous study on gel K and G gene from *Sphingomonas paucimobilis* [44]. There are very less information about insilico modelling of polymorphic HSP protein. A recent study have reported the modelling of HSP70 and HSF1 by using different tools and concluded that the best hit was obtained from I- TASSER server which can screen out 10 best template for modelling of the protein [45]. So the model information which are being generated in this study may help in future research work leading to computer aided drug discovery [46].

ETHICAL APPROVAL

The study was approved by the ethical committee board of Assam down town University, Assam, India (Memo no. adtu/Ethics/PhD Scholar/2016/011) and confronted to the ethical committee guidelines of EASL Helsinki 1975.

ACKNOWLEDGEMENT

The authors acknowledge the laboratory facility provided by Assam Down Town University and NE cancer hospital and down town hospital for providing samples and clinical data.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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